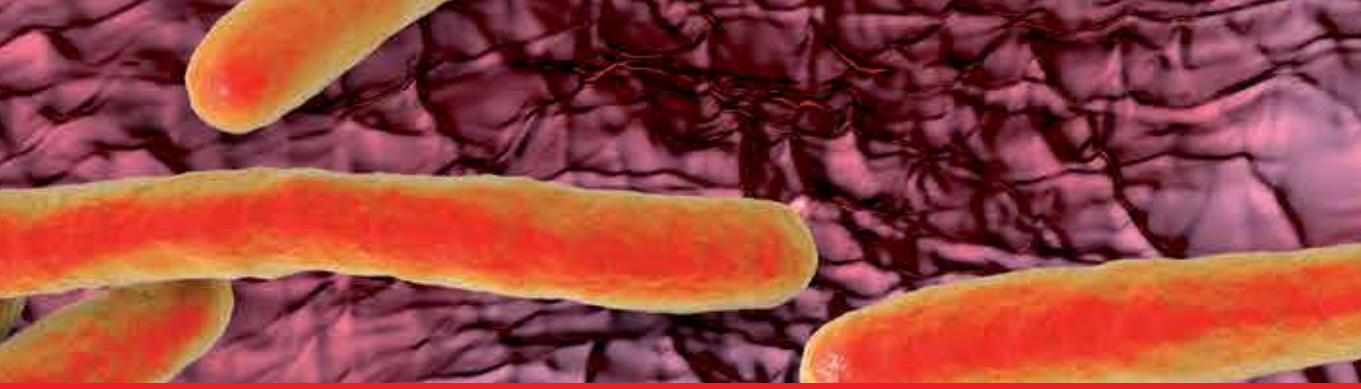

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Understanding Tuberculosis

Deciphering the Secret Life of the Bacilli

Edited by Pere-Joan Cardona

UNDERSTANDING TUBERCULOSIS – DECIPHERING THE SECRET LIFE OF THE BACILLI

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Notice

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Meet the editor

After obtaining his MD at the Universitat Autònoma de Barcelona, Pere-Joan Cardona started an internship in Clinical Microbiology in 1994, in the Hospital Germans Trias i Pujol, where he was familiarized with the problem of TB in its clinical and diagnostic challenges. Invited by Ian Orme at CSU, he was trained on the development of TB experimental models. PhD was obtained in 1999. He became Head of the Experimental Tuberculosis Unit at the Institut Germans Trias i Pujol of Badalona and Assistant Professor of Microbiology at UAB. He has been involved for the last 15 years in the study of the pathophysiology of TB infection. Additionally, he has authored 70 peer-reviewed publications in the field and is responsible for the development of different experimental models in mice, guinea pigs, goats and mini-pigs, and in the development of new drug regimens and vaccines against TB.

Contents

Preface

Mycobacterium tuberculosis has been the paradigm of genetic stability for years, compared to other bacterial families or viruses, which is mainly related to its lack of plasmid content. As recent investigations demonstrate, the bacillus has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of *Mycobium tuberculosis,* together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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Part 1

The "Quiet" Genome

1

Molecular Biomarkers for Ancient Tuberculosis

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1. Introduction

Tuberculosis is an ancient disease. It was recognised and described by Hippocratus (460–390 BCE) and Galen (2nd–3rd century CE) in the western Classical World (Xarchus & Bourandas, 2003), ancient Egypt, India and the Far East (Morse, 1961). The obvious symptoms that attracted attention were the late outcomes of skeletal tuberculosis, where collapsed vertebrae led to scoliosis and Pott's disease, plus the symptoms associated with pulmonary tuberculosis, such as fever, weight loss and haemoptysis (coughing up blood). In the UK, tubercular lesions of the lymph glands (cervical lymphadenitis) were formerly termed scrofula, or the King's evil, and tubercular skin lesions were described as Lupus vulgaris or tuberculous chancre. The palaeopathology of ancient skeletal remains, together with classical and historical reports, demonstrate that tuberculosis occurred in prehistory. However, tuberculosis is still the greatest cause of death from any single infectious disease in the world today, with over one third of the global population infected and an estimated 1.7 million deaths from the disease in 2009 (WHO, 2010). Therefore it is essential to understand the nature of tuberculosis in the past: its distribution, spread and relationship to human society.

The disease is caused by members of a group of very closely related bacteria, termed the *Mycobacterium tuberculosis* complex (MTBC). These are obligate parasites and have the ability to subvert the cell-mediated immune system of the host and to survive and multiply within macrophages. Most human infections are caused by *Mycobacterium tuberculosis* and are usually acquired via the aerosol route from an active case of pulmonary tuberculosis. Infectious aerosols lodge in the alveoli but, in the majority of cases, the bacilli are controlled by the host immune system to form a granuloma and the disease remains latent. Infection can also occur by ingestion – milk or meat from an infected animal can give rise to human zoonotic cases of tuberculosis caused by *Mycobacterium bovis* or other members of the MTBC. In endemic areas, infection takes place in early life and may remain latent throughout a lifetime or become re-activated due to lowered host resistance caused by physical or mental stress, immunosuppression or extreme age (Rustad et al., 2009). Active primary tuberculosis, estimated to occur in 2–5% of cases, normally causes lymphadenitis and subsequent spread via the blood stream can cause meningitis or miliary tuberculosis (Grange & Zumla, 2009). Post-primary tuberculosis is estimated to occur in a similar proportion of people and these

individuals in ancient and historical times would be the recognisable cases of skeletal tuberculosis. Therefore, it is highly significant that these historical cases, diagnosed by skeletal pathology, represent only around 5% of the total number of individuals with the disease.

Because of their very slow growth-rate and clinical significance, the MTBC was one of the first groups of microorganisms to benefit from the introduction of the polymerase chain reaction (PCR) and molecular diagnostics. This led to a an understanding of the epidemiology of tuberculosis (Reed et al., 2009; Smith et al., 2006), the evolution of the MTBC (Brosch et al., 2002; Ernst et al., 2007; Gordon et al., 2009; Gutierrez et al., 2005) and to the realisation that particular lineages of *M. tuberculosis* are associated with the country of origin of their human hosts (Hershberg et al., 2008; Hirsh et al. 2004; Wirth et al., 2008). Total sequencing of the genomes of *M. tuberculosis* (Cole et al., 1998), *M. bovis* (Garnier et al., 2003) and attenuated *M. bovis* Bacille Calmette-Guérin (BCG) vaccine strains (Pan et al., 2011; Seki et al., 2009) has elucidated the relationship between MTBC strain, lineage and pathogenicity. We now understand that the MTBC represents a clonal expansion of pathogenic strains or ecotypes (Smith et al., 2006) each of which is associated with a parallel clonal expansion of their mammalian hosts (Maiden, 2009).

The MTBC is distinct from the large number of environmental mycobacteria, which are generally non-virulent or cause opportunist infections in hosts, especially those with increased susceptibility. A characteristic feature of the mycobacteria is their cell envelope, which contains a high proportion of lipid-rich molecules, such as mycolic acids and phthiocerol dimycocerosate waxes (Minnikin, 1982; Minnikin et al., 2002). These result in a hydrophobic bacterial cell wall with decreased permeability and susceptibility to degradation, that may partially explain the very slow growth rate of the MTBC and persistence of viable organisms after the death of the host (Sterling et al., 2000; Weed & Baggenstoss, 1951). The mycobacteria are members of a taxonomic clade typified by organisms with a high percentage of guanidine and cytosine residues in their DNA. It is believed that the DNA of GC-rich bacteria is structurally more stable than that of other microbes because of the additional hydrogen bond cross-links between the DNA strands.

The wealth of information on the genomics of *M. tuberculosis* strains present in the world today, coupled with our understanding of the co-evolution of *M. tuberculosis* with its human host, has attracted interest in determining the origins and timescale of this relationship. Relevant information can be obtained from archaeology, anthropology and palaeopathology, which provide details on past human populations, societies and the occurrence of infectious diseases. The relative robustness of *M. tuberculosis* biomarkers enables the well-established molecular methods used in diagnostic clinical microbiology to be applied, with appropriate modification, to the study of historical and archaeological remains. Originally the emphasis was on the detection and characterisation of *M. tuberculosis* ancient DNA (aDNA), as this enables the evolution of this group of pathogenic bacteria to be directly investigated by the detection and characterisation of their DNA (Fletcher et al., 2003a, 2000b; Matheson *et al.* 2009; Zink et al., 2001). However, it was soon appreciated that the unique lipid biomarkers found in the MTBC, in addition to enabling the independent verification of aDNA studies (Donoghue et al., 1998, 2010a; Gernaey et al., 2001; Hershkovitz *et al.,* 2008), have the potential to illuminate deep into human prehistory due to their particular stability (Gernaey & Minnikin, 2000; Redman *et al.,* 2009).

2. Ancient DNA (aDNA) from the *M. tuberculosis* complex (MTBC): Background and basics

2.1 DNA degradation and persistence

Within living cells, DNA is subjected to enzymatic repair processes, but this ceases after death. Thereafter, host DNA is rapidly degraded by enzymes derived both from the host and the macro and microbial flora that form part of the natural decay process (Pääbo et al., 2004). As a result of the cumulative changes over time (diagenesis) ancient DNA may develop hydrolytic and oxidative lesions. The breakdown of the N-glycosyl bond between the sugar and the base, in the presence of water, leads to hydrolytic cleavage and DNA fragmentation. Hydrolytic depurination causes a preferential loss of guanine and adenine, whereas the pyrimidines cytosine and thymine are 40-fold more susceptible to hydrolytic deaminisation (O'Rourke et al., 2000). Oxidative damage, especially to pyrimidines, can result in the formation of substances such as hydantoins, that block extension during PCR (Höss et al., 1996). DNA strands may also become chemically cross-linked due to Maillard products (Poinar et al., 1998), formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids (Pääbo et al., 2004). Local environmental conditions have a strong impact on the persistence of aDNA, such as the temperature, the pH at the site, the availability of water and oxygen and fluctuations of all these factors over time (Poinar, 2003). Indeed, these factors outweigh the impact of the chronological age of samples. For example, a 20^0C decrease in temperature reduces base degradation 10- to 25-fold (Höss et al., 1996). Mycobacterial DNA is more robust than that of mammals, but its persistence depends not only upon the local environmental conditions but also the nature of the infection at the time of death of its host. Therefore, *M. tuberculosis* aDNA is often highly localized and DNA extraction protocols may have to be optimized for specimens from different sites.

2.2 Selection of specimens and sampling

In the early days of palaeomicrobiology, the criteria drawn up by researchers working on ancient mammalian DNA were recommended. These included preliminary screening tests, such as using the degree of amino acid racemization (Poinar & Stankiewicz, 1999) or collagen yield (Götherström et al., 2002), as an indication of the extent of DNA preservation. However, it appears that these are not reliable indicators, even of mammalian aDNA (Fernañdez et al, 2009; Kaestle & Horsburgh, 2002). More recently, Ottoni et al. (2009) discovered that there was better recovery of aDNA from animal bones that showed evidence of cooking, concluding that parameters based on protein diagenesis are not always useful for predicting ancient DNA survival. Work on other microbial pathogens, such as *Yersinia pestis* – the cause of bubonic plague – demonstrate that the dental pulp cavity in sound adult teeth is an excellent source of aDNA (Drancourt et al., 1998). It is believed that adsorption to hydroxyapatite increases the stability of aDNA (Götherström et al., 2002; Tuross, 1994) and any microorganisms present in the blood will potentially be present (Donoghue, 2008a).

For the examination of material for tuberculosis, the most common specimens available are bones. Most active cases of tuberculosis present as a lung disease, so ribs are a good source of *M. tuberculosis* DNA. Tuberculous lesions in the ribs arise by extension from spinal

lesions, from haematogenous spread from some remote soft tissue focus, or by direct spread from disease in the lungs, pleura, or chest wall lymphatic system (Mays et al., 2002). Initially, only bones with lesions were examined (Spigelman & Lemma, 1993), but it is now clear that in the majority of ancient cases of tuberculosis there are no lesions, but *M. tuberculosis* aDNA is present due to haematologous spread or by direct contact with infected tissue (Donoghue, 2011; Donoghue et al., 2011). Mummified tissue (Salo et al., 1994), skin (Faerman et al., 1997; Konomi et al., 2002), dental pulp (Faerman et al, 1997; Matheson et al, 2009) and calcified pleura (Donoghue et al., 1998) have also yielded MTBC aDNA.

Unfortunately, many published protocols based on human and animal aDNA research recommend that bones are pre-treated with bleach, ultraviolet light, or the outer bone surface is removed. The aim is thereby to remove surface contamination, but such procedures may inadvertently remove the very *M. tuberculosis* aDNA that is being sought (Donoghue, 2008a).

2.3 MTBC aDNA extraction and detection by conventional PCR

The amount of material examined by different investigators varies greatly (Donoghue et al., 2009), but the subsequent extraction procedures follow a similar pattern. Mineralized tissue is powdered and demineralized, enzymes are used to remove proteins, the samples are disaggregated with agents such as phenol-chloroform or guanidium thiocyanate, and DNA is captured by silica, on to filters or membranes, or simply precipitated with isopropanol. Due to the persistent mycobacterial cell wall, robust techniques such as bead beating, freeze-thaw cycles in liquid nitrogen, and incubation for longer time periods and at temperatures such as 56^0C are often used. The reagent N-phenacylthiazolium bromide may be used to overcome the problem of Maillard products and enable strand separation (Pääbo et al., 2004). Reagents used for DNA capture may need modification to allow for DNA fragments that are <200 base pairs (bp) in length. In poorly preserved samples the fragment length may be less than 100 bp. Experience has shown that aDNA is unstable in aqueous solution so extraction preparations are best stored as dried silica or precipitates and only re-constituted immediately prior to examination. Thereafter, the aDNA extracts should be aliquoted and stored at -80^0C, if possible, so the number of freeze-thaw cycles can be minimized.

DNA amplification by PCR enables targeted and specific recovery of informative genetic loci. Amplification of MTBC aDNA is usually based on MTBC-specific regions of repetitive sequences in the genome of all members of the complex, such as IS*6110* (Eisenach et al, 1990). The sensitivity of conventional PCR can be increased by further amplification of the amplified PCR product via a nested reaction (Taylor et al., 1996). The primers devised by these two primer sets give rise to amplicons of 123 bp and 92 bp, respectively. IS*6110* may have up to 24 copies/cell in *M. tuberculosis* (Tanaka et al., 2000), although a small percentage of strains have no copies at all and *M. bovis* strains have only a single copy per cell. The alternative specific PCR, based on IS*1081* (Taylor et al., 2005), is preferable in most cases, as there are six copies/cell in each member of the MTBC so quantification is possible. Optimization of the PCR is necessary and modification of the PCR reaction mix is recommended for work with aDNA. Inclusion of stabilizers such as bovine serum albumin is often beneficial, probably due to a variety of effects such as masking non-specific binding sites, stabilizing DNA fragments and binding or otherwise inactivating co-purified PCR

inhibitors (Donoghue, 2008a). Hot-start PCR and excess enzyme can also drive the reaction and overcome residual inhibitors. Detection of amplicon has traditionally been based on agarose gel electrophoresis. Detection is also possible by hybridization of labelled amplicons to a membrane, using a dot block technique, for example. However, real-time PCR enables amplified product with an incorporated fluorescent marker or probe to be monitored directly via a computer screen and the methodology facilitates quantification. For the future, *M. tuberculosis* diagnostics is moving towards isothermal and array technology. Microarrays are not ideal for the direct detection of ancient *M. tuberculosis* in crude extracts, due to the extensive fragmentation of target sequences. However, the introduction of new platforms based on surface interactions and nanotechnology offer exciting possibilities for the future.

2.4 Further developments in molecular diagnosis

In recent years, the field of molecular diagnostics of tuberculosis has expanded rapidly and a wide variety of new techniques have been introduced, or are currently being validated for clinical use. This is beginning to have an impact on the specialized field of palaeomicrobiology (Section 3.2 below) and, no doubt, there will be many more studies in the future that will be based on such technology.

2.4.1 Real-time PCR methods of MTBC aDNA detection and quantification

Real-time PCR (RT-PCR) or, more correctly, quantitative PCR (qPCR) enables amplified product with an incorporated fluorescent marker or probe to be monitored directly via a computer screen. The underlying principle of non-specific double-stranded DNA binding dye chemistry is that fluorescent dyes such as SYBR Green intercalate with any double stranded DNA. This enables the progress of the amplification to be followed as it progresses, whereas conventional PCR relies upon the detection of amplicon once the reaction has completed. By use of standards and specific primers, the number of copies of amplicon or the absolute amount of DNA can be quantified. Normally a series of peaks are visible on the computer screen, so on completion a melt analysis is performed and the temperature at which strand separation occurs (T_m) is used to determine the targeted sequence. Greater clarity and specificity is conferred by the use of specific DNA probes, which incorporate a fluorescent reporter that is normally quenched (Nazarenko et al., 1997). The fluorescence is only released once the probe has bound to the specific target sequence.

2.4.2 Other methods of MTBC aDNA detection in liquid systems

Several isothermal target amplification methods, which avoid the use of a thermocycler machine, have been developed in the two past decades (Karami et al., 2011). Loop-mediated isothermal amplification (LAMP) is an isothermal molecular method of DNA amplification that has been successfully implemented in the detection of *M. tuberculosis* in clinical specimens (Notomi et al., 2000; Neonakis et al., 2011). The reaction is driven by outer primers leading to strand displacement DNA synthesis, production of a single-stranded template, further DNA synthesis initiated by additional primers, and hybridization to the other end of the target sequence to produce a loop. In subsequent cycles further strand displacement leads to multiple copies of the target sequence. LAMP has several advantages, such as rapidity, high sensitivity, ease of application and cost-effectiveness.

The change of scale by the use of nanoparticles reduces the need for multiple rounds of DNA amplification. For example, direct examination of clinical samples has successfully demonstrated *M. tuberculosis* DNA after an initial round of PCR, using a colorimetric method based on an *M. tuberculosis* probe linked to gold nanoparticles (Baptista et al., 2006).

2.4.3 Detection of non-amplified DNA

An alternative approach to conventional PCR is to directly detect non-amplified DNA by amplification of the detection system such as labelled probes (Bhatt et al., 1999). However, the technology has now been developed to enable direct detection of sequences in non-amplified genomic DNA by means of various sensors. In one example, a piezoelectric biosensor enables real-time and label-free detection of the hybridization reaction between an immobilized probe and the complementary sequence in solution. The DNA probe is immobilized on the sensing surface (10 MHz quartz crystals), while the complementary sequence is present in the genomic DNA, previously fragmented with restriction enzymes (Minunni et al., 2005). This approach has been developed for the detection of *M. tuberculosis* (Kaewphinit et al., 2010). Another specific DNA detection method uses fluorescent semiconductor quantum dots and magnetic beads for fast detection of mycobacteria without any DNA amplification. Two biotinylated oligonucleotide probes are used to recognize and detect specific complementary mycobacterial target DNA through a sandwich hybridization reaction. Quantum dots conjugated with streptavidin and specific probes are used to produce a fluorescent signal. Magnetic beads, conjugated with streptavidin and a genus-specific probe are used to isolate and concentrate the DNA targets (Gazouli et al., 2010). Surface primer extension reactions may also be used to quantitatively detect unamplified, double-stranded genomic DNA (Martins et al., 2010). This methodology, by eliminating the need for pre-target labeling or amplification procedures, constitutes an alternative for the direct detection of genomic DNA from solution.

2.5 Authentication and precautions

Lists of precautions to take when working with mammalian aDNA have dominated palaeomicrobiology even though the recommendations may not be appropriate. For example, this is a summary of the "top ten list" drawn up by Poinar (2003):

1. a physically isolated work area, preferably a separate building where no genetic work is carried out;
2. PCR control amplifications, including non-template PCRs, multiple DNA and extraction controls;
3. molecular behaviour i.e. an inverse relationship between amount of PCR amplicon (bp) and length of target sequence;
4. quantification – the copy number of DNA should be assessed;
5. reproducibility – results should be repeatable from both the same and different DNA extracts of a specimen;
6. clone – direct sequencing should be confirmed by cloning amplicons and sequencing at least 10 clones to check for damage-induced errors and the ratio of endogenous to exogenous sequences;
7. independent replication – preferably by the independent examination of separate samples of the same specimen in independent laboratories;

8. biochemical preservation – use indirect assessment of the extent of DNA preservation by assessing the amount of diagenic change in other biomolecules, such as amino acids or lipids;
9. associated remains such as those of animals can be used to check for comparable aDNA survival to human DNA;
10. phylogenetic sense – sequences should be compared with others in appropriate databases to ensure authenticity.

Several of these recommendations have been accepted by palaeomicrobiologists, but a few are problematic:

1. The suggestion of a separate building and clean rooms with filtered air supplies may be advisable for work on human aDNA, where every investigator is a potential source of contamination with modern DNA. However, the MTBC has no environmental reservoir, so provided that researchers do not suffer from tuberculosis it is quite possible to perform palaeomicrobiological research by following good microbiological practice, including plentiful negative controls and independent verification (Donoghue et al., 2009).
2. Findings may not be reproducible because aDNA from tubercle bacilli will be localized. Even repeat samples from the same specimen may not yield a positive result in every case. Therefore, an additional criterion for work on a DNA of pathogenic microorganisms is proposed – that samples should be taken from sites appropriate to what is known of the natural history of the infection (Donoghue & Spigelman, 2006).
3. There is no evidence that cloning is necessary for verification of mycobacterial aDNA. Indeed, the opposite is true as work on *Mycobacterium leprae* (Taylor et al., 2006) showed that cloning gave no added value to data obtained by direct sequencing, but did introduce some errors, which were ascribed to *Taq* polymerase error and slipped strand mispairing. Similar conclusions have been reached in a recent study of mammalian aDNA (Winters et al., 2010).
4. Independent replication of aDNA may give discordant results due to localization of pathogen biomarkers within samples (see point (5) above). MTBC-specific lipid biomarkers may be more sensitive and can verify aDNA data without the need for amplification (Section 4 below) even though determined sceptics (Wilbur et al., 2009) may ignore this (Donoghue et al., 2009).
5. Comparison with different host biomolecular markers is discussed above (Section 2.2) and the conclusion is that aDNA can be found even in samples where other biomolecules are damaged.
6. Comparison with the recovery of aDNA from associated faunal remains is inappropriate for MTBC aDNA for at least two reasons. First, mycobacterial DNA appears to be more robust than mammalian DNA (Section 2.1). In addition, faunal remains are often a poorer source of aDNA than associated human remains (Mays et al, 2001), possibly due to treatment of carcasses after death and the absence of burial (Taylor et al., 2010).

3. Palaeomicrobiology of tuberculosis

3.1 Early studies 1993–2002 and initial conclusions

The earliest molecular studies on aDNA of the MTBC demonstrated proof of principle, but also answered historical questions about the occurrence of tuberculosis in the pre-colonial

Far East (Spigelman & Lemma, 1993) and whether tuberculosis occurred in the Americas before Columbus (Salo et al., 1994). During the first decade of such research it was demonstrated that MTBC aDNA could be found in bone and mummified tissue, from body sites in specimens without lesions and of a broad age range, from locations around the world (Donoghue, 2011). Additional methods of examination included pathology, microscopy and radiology. Authentication was provided by the direct detection of MTBC-specific cell wall lipid markers (Donoghue et al., 1998, Gernaey et al., 2001). Use of additional PCR target sites, including *rpoB*, *mtp40*, *oxyR* and spoligotyping – which uses a dot-blot method based on the MTBC Direct Repeat (DR) region (Kamerbeek et al., 1997), enabled confirmation of the principal human pathogen *M. tuberculosis sensu stricto* (Taylor et al., 1999). The oldest confirmed case of tuberculosis was reported in a Pleistocene bison (17,870 BP) from the Natural Trap Cave, Wyoming, USA. A metacarpal showed suggestive pathology and spoligotyping indicated that the infecting organism was a member of the MTBC, but the species was not confirmed at the time (Rothschild et al., 2001).

3.2 Recent findings and increased understanding

The increased understanding arising from total genome sequencing of *M. tuberculosis* (Cole et al., 1998) led to an appreciation that this group of organisms exhibits sequential deletions that can be used to distinguish between strains and lineages. Therefore, molecular typing protocols were developed based on a combination of synonymous single nucleotide polymorphisms (SNPs) in the *katG* codon 463 (*katG*^{463}), *gyrA* codon 95 (*gyrA*^{95}) and deletions (Brosch et al., 2002). The TbD1 deletion was identified as specific to the human pathogen *M. tuberculosis* and a significant marker of "ancestral" and "modern" strains. Therefore, both SNP typing and deletion analysis have been incorporated into MTBC aDNA studies, provided that the DNA preservation was sufficiently good for such single-copy markers to be amplified and detected.

The next decade included population studies and early epidemiological findings. A well-documented group of over 200 naturally mummified individuals from the 18th century was discovered in a church crypt in Vác, Hungary (Fletcher et al., 2003a). DNA preservation was particularly good and there was a high level of both active and presumed latent infections (Donoghue et al., 2011). It was possible to perform molecular fingerprinting and genotyping based on SNPs and to identify the *M. tuberculosis* aDNA as of "modern" strains. These techniques were used to demonstrate that in a small family group each person was infected with a different *M. tuberculosis* strain (Fletcher et al., 2003b). Interim epidemiological data have also been obtained from an on-going study of early Christian Nubians (550–750 and 750–1500 CE) and it is clear that tuberculosis was widespread, although there are no contemporaneous records and the DNA preservation is much less good (Donoghue, 2008b; Spigelman et al., 2005). Meanwhile, Zink, Nerlich and colleagues have produced a series of papers from a long-term study of burials in Thebes-West in ancient Egypt (Zink & Nerlich, 2004; Zink et al., 2003a, 2003b, 2004), spanning the pre-Dynastic period (5500–3100 BCE) to the New Kingdom (1550–1070 BCE). Molecular typing and spoligotyping indicated human *M. tuberculosis* and there was also evidence of another member of the MTBC, *Mycobacterium africanum*. However, no *M. bovis* was found. Indeed, there has only been one reported case of human tuberculosis associated with *M. bovis* aDNA (Taylor et al., 2007). This was found in a small group of pastoralists in south Siberia, dating from approximately 1761 to 2199 years

BP, placing the remains within the Iron Age period. Further work on the same specimens used qPCR to detect, quantify and characterize the *M. bovis* DNA (Murphy et al., 2009).

The use of qPCR with specific fluorescent reporters should enable the detection of highly fragmented aDNA. This was demonstrated by the detection of a 63 bp *IS6110* target sequence specific for the MTBC in a pre-Hispanic (900–1100 CE) adult from the north coast of Peru (Klaus et al., 2010). Both conventional and qPCR were used to examine skeletal material from western Hungary with palaeopathology suggestive of tuberculosis (Évinger et al., 2011). Samples were dated from 800–1200 CE and the qPCR with a specific 75 bp IS*6110* target sequence was positive in six cases including two from the 9th century, whereas conventional PCR was negative. However, conventional PCR with a 113 bp target sequence for IS*1081* was positive in two of these cases plus one other, but a qPCR probe with a 72 bp target sequence was negative, thus demonstrating the lack of consistency when seeking aDNA from microbial pathogens in human tissue.

3.3 Association of tuberculosis with other diseases

3.3.1 Co-infections

There has been no systemic examination of archaeological or historical material for co-infections, but our current understanding is that a pre-existing infection can increase susceptibility to another. A recent historical example is the influenza pandemic of 1918 where a major cause of death is believed to have been secondary bacterial pneumonia (Morens et al., 2008). It is very likely that additional examples will be found.

For example, parallel developments in the molecular detection of *M. tuberculosis* and *M. leprae* aDNA enabled co-infected individuals to be identified. These were cases of lepromatous leprosy with very typical palaeopathology, who were subsequently discovered to have systemic *M. tuberculosis* aDNA in their skeletal remains (Donoghue et al., 2005). An extensive literature search revealed that such co-infections had been reported in historical times prior to the introduction of chemotherapy; the findings led to a hypothesis that tuberculosis might have been a major factor in the elimination of leprosy from Western Europe.

An example of an association of tuberculosis with a parasite infection comes from pre-colonial northern Peru, where Chaga's disease, caused by the protozoan parasite *Trypanosoma cruzii*, was widespread (Aufderheide et al., 2004). Palaeopathology and aDNA analysis demonstrated both Chaga's disease and tuberculosis in the population and one 12 year-old girl from 910-935 BP was shown to have a co-infection (Arriaza et al., 2008). Another such association between tuberculosis and *Leishmania* spp. infection, possibly also linked to nutritional stress, was reported in preliminary data from early Christian Nubia (Spigelman et al., 2005).

3.3.2 Co-morbidity

There are many examples of increased susceptibility to infection associated with poor nutrition, a compromised immune system e.g. in neonates or the elderly, physical or mental stress due to wars and relocation, and underlying other medical conditions. An example of an association of tuberculosis with reduced lung function due to a massive vertebral

deformity, probably developmental, was described by Kustár et al. (2011) in an 18th century mummified lung from Vác, Hungary. Another individual from the same population, a child aged 1.5 – 2.5 years, showed numerous bony lesions throughout the body (Spigelman et al., 2006). A differential diagnosis, based upon the palaeopathology led to the conclusion that this infant suffered from Langerhans' cell histiocytosis (LCH), also referred to as histiocytosis-X. The aetiology and pathogenesis of LCH are still unknown but it is now thought to be a neoplasm, so the finding of tuberculosis in this child is not surprising. The child would have a repressed immune system, due to marrow replacement by the malignant cells, and thus be vulnerable to tuberculosis, which was widespread in this community.

3.4 MTBC lineages, evolution and timescale

The association of *M. tuberculosis* lineage with that of their human host has been convincingly illustrated in modern populations (Section 1). There is low DNA sequence variation in the MTBC and little, if any, horizontal gene exchange, which prevents reacquisition of genomic regions that have been lost. Therefore, deletions and functionally neutral SNPs are ideal markers for inferring deep phylogenies (Donoghue, 2009). The SNPs in the *katG* codon 463 (*katG*^{463}), *gyrA* codon 95 (*gyrA*^{95}) and the TbD1 deletion (Brosch et al., 2002) enable differentiation of three principal genetic groups within the MTBC. It is believed that these organisms have undergone an evolutionary bottleneck, associated with the adoption of a parasitic lifestyle. Thereafter, both host and pathogen have undergone clonal expansion. The timescale during which this has occurred is of interest, not least because of the realization that the evolution of the MTBC appears to be increasing exponentially today and the underlying factors need to be understood.

Although tuberculosis is still the greatest single cause of death caused by a single microbial pathogen, the high proportion of infected persons with latent infection indicates that host and pathogen have co-existed for a considerable length of time (Donoghue, 2009; Rustad et al., 2009). It is believed that the emergence of human pathogens is related to population density and tuberculosis has long been recognized as associated with the development of agriculture and animal domestication during the Neolithic transition. Palaeopathological data alone cannot distinguish between *M. tuberculosis* and *M. bovis* infection, and the earlier belief that human tuberculosis was derived from the animal disease has proved difficult to shift. However, palaeomicrobiology provides convincing evidence that *M. bovis* is rare in past human populations. Direct evidence of infection with human lineages was provided from ancient Egypt by genotyping and deletion analysis (Zink & Nerlich, 2004, Zink et al., 2003a) and spoligotyping (Zink et al., 2003b). Demonstration of the oldest infection with human lineages of the MTBC (Hershkovitz et al., 2008) was based on five different target sequences, and was confirmed by direct detection of *M. tuberculosis*-specific mycolic acid markers (see Section 4.2 below). The population was from a Pre-Pottery Neolithic site in the Eastern Mediterranean, dated around 9000 years ago, with plentiful evidence of animal domestication. It is therefore of special interest that the *M. tuberculosis* lineage is of a TbD1-deleted strain.

Ancestral sequence inference is a process used in bioinformatics to estimate the rate of evolutionary change under different scenarios. Combination with the direct evidence obtained from palaeomicrobiology, enables confirmation of the presence of particular *M. tuberculosis* lineages in the past, which can strengthen and inform existing models. This has

led to a significant extension of the timescale for the evolution of the MTBC. For example, when Brosch et al. (2002) first published their evolutionary model, it was noted that the 18th century Vác mummies were of *M. tuberculosis* of principal genetic groups 2 and 3, thus proving that these had not evolved during recent times. Six years later it was appreciated that the "modern" TbD1-deleted lineages existed 9000 years ago (Hershkovitz et al., 2008). It appears likely that further extension of the timescale will require the introduction of the more sensitive immobilized DNA technologies to enable detection of the highly fragmented material in such ancient samples.

4. Lipid biomarkers for the *M. tuberculosis* complex (MTBC)

4.1 Established lipid biomarkers for MTBC

The cell envelope of *M. tuberculosis* is based on complex macromolecules linked to produce a mycoloyl arabinogalactan-peptidoglycan organelle (Minnikin 1982; Barry et al., 2007). This organelle is the foundation for a characteristic mycobacterial outer membrane based on covalently bound mycolic acids, interacting with a range of unusual free lipids (Minnikin, 1982; Minnikin et al., 2002). This outer membrane has now been visualized directly (Hoffmann et al., 2008; Zuber et al., 2008) and given the label "mycomembrane". Mycolic acids are, therefore, an integral part of mycobacterial cell envelopes, with proven biomarker value, both in classification and identification, due to variations in the individual mycolate types expressed (Butler & Guthertz, 2001; Dobson et al., 1985). The mycolic acids produced by *M. tuberculosis* are composed of five principal types, as illustrated in Fig. 1A, each type having a range of homologues with different chain lengths (Minnikin, 1982; Minnikin & Polgar, 1967a, 1967b; Watanabe et al., 2001, 2002). This general mycolate fingerprint is shared by *M. tuberculosis* and other members of the MTBC, whose best-studied members include *M. bovis, M. africanum* and *Mycobacterium microti*. MTBC mycolic acids are relatively stable biomarkers, which have been detected in archaeological material up to 9,000 years old (Hershkovitz et al., 2008). The use of mycolate biomarkers in the identification of tuberculosis depends on the clear recognition of profiles characteristic of the MTBC; this will be discussed in detail below (Sections 4.2 & 4.3).

The so-called "free" lipids, which associate with the "polysaccharide-bound" mycolic acids to form the outer myco-membrane (Minnikin, 1982; Minnikin et al., 2002), are also a source of diagnostic lipid biomarkers. The mycocerosic and mycolipenic acids (Fig. 1B) are the best-studied examples, the former being components of phthiocerol dimycocerosate waxes and the latter being part of pentaacyl trehalose glycolipids (Minnikin et al., 1983, 1985a, 1985b, 2002). Mycocerosic acids are found in a limited number of mycobacterial species, including *Mycobacterium kansasii, M. leprae* and *Mycobacterium haemophilum* in addition to members of the MTBC; *Mycobacterium marinum* and *Mycobacterium ulcerans* have closely related acids (Minnikin et al., 2002). The distribution of the different mycocerosate types has been defined (Daffé & Lanéelle, 1988; Minnikin et al., 1985a; Minnikin et al., 1993a), with MTBC having a characteristic pattern composed of mainly C_{29}, C_{30} and C_{32} components (Fig. 1B). In contrast, only a single C_{27} mycolipenate (Fig. 1B) is usually encountered and principally in only *M. tuberculosis*. The value of mycocerosic and mycolipenic acid lipid biomarkers in the diagnosis of ancient tuberculosis has been investigated by Redman et al. (2009). An important aspect of identifying lipid biomarkers in archaeological samples is that such lipids are being increasingly implicated as virulence factors in the pathogenesis of *M.*

tuberculosis (Gordon et al. 2009; Neyrolles & Guilhot, 2011); this is a further avenue for research into the evolution of the host/pathogen relationship.

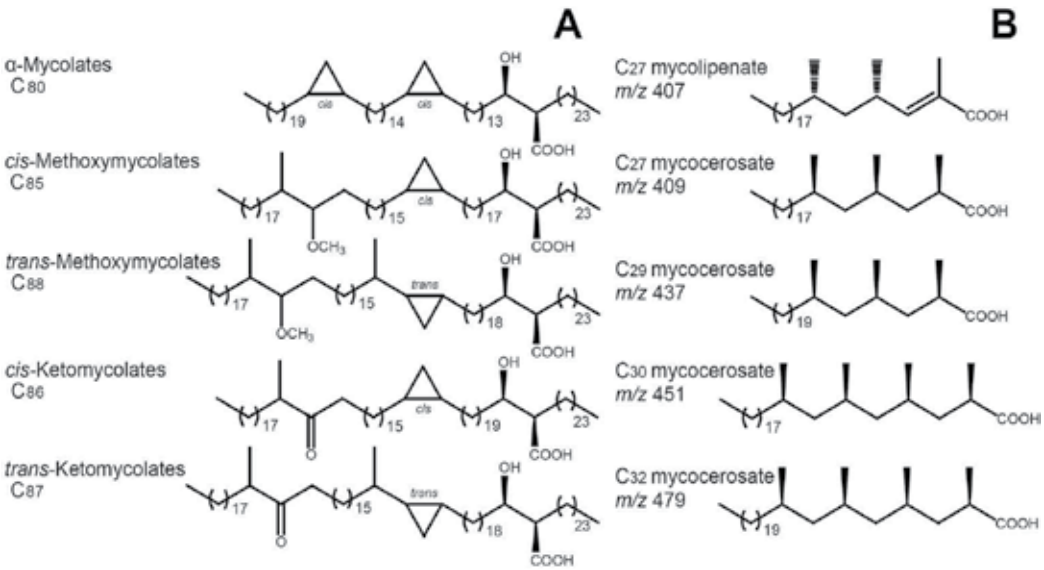

Fig. 1. Structures of selected lipid biomarkers for *M. tuberculosis*. **A.** The main components of each mycolic acid class are shown; each class comprises a limited range of homologous components with different chain lengths. **B.** Mycolipenic and mycocerosic acids; for each component, the ions (*m/z*) monitored on negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS) of pentafluorobenzyl esters of these acids are given.

4.2 HPLC recognition of MTBC mycolic acid patterns in archaeological samples

The mycolic acids from *M. tuberculosis* (Fig. 1A) comprise three principal classes, α-, methoxy- and keto-, which can be separated from each other by simple so-called "normal phase" chromatography. Such separations are readily achieved by thin-layer chromatography (Dobson et al., 1985; Minnikin, 1993) or by high performance liquid chromatography (HPLC) on silica gel media (Minnikin, 1993; Qureshi et al., 1978; Steck et al., 1978). Normal phase profiles of total mycolates, simply showing α-, methoxy- and ketomycolate classes, are not diagnostic for the MTBC, as they are shared with a range of other mycobacterial species (Dobson et al., 1985; Minnikin, 1982; Watanabe et al., 2001, 2002). "Reverse phase" HPLC separates mycolates both according to chain length and polarity (Minnikin, 1993; Qureshi et al., 1978; Steck et al., 1978), and a characteristic "tight envelope" of peaks is produced by members of the MTBC (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008). This diagnostic profile has been found to be sufficient for the routine diagnosis of modern clinical tuberculosis, using computerized comparison to an internal standard (Butler & Guthertz, 2001). It is conceivable, however, that a combination of various factors could produce an envelope of peaks mimicking that

characteristic of tuberculosis. Extra dimensions of information are readily available if reverse phase HPLC is carried out on the individual α-, methoxy- and keto-mycolate classes (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008; Minnikin et al., 1993b). As shown in Fig. 1A, methoxy- and keto-mycolates have sub-classes depending on the presence of the alternative *cis*-cyclopropane or methyl branched *trans*-cyclopropane moieties; these sub-classes are not readily separable by normal phase chromatography (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008; Watanabe et al., 2001).

The first detection of mycolic acid biomarkers in archaeological skeletal material was performed in studies by Gernaey et al., (1998, 1999, 2001, 2002) and extended to calcified pleura (Donoghue et al., 1998). The sensitive HPLC analysis used mycolate methylanthryl esters, previously developed to diagnose modern tuberculosis in human sputum (Minnikin et al., 1993b). The derivatized mycolic acids were isolated by reverse phase HPLC and the total mycolate fraction was collected and analyzed by normal phase HPLC to separate the α-, methoxy- and ketomycolate classes. These individual mycolate classes were then resolved into envelopes of peaks diagnostic for MTBC by further reverse phase HPLC. The value of this effective sequential protocol will be demonstrated below (Fig. 2) for a more recent example (Hershkovitz et al., 2008). The archaeological material investigated by Gernaey et al. (1998, 1999, 2002) was a collection of 19th century skeletons excavated from the site of the old Newcastle upon Tyne Infirmary, UK; there was a good correlation with burial records. The power of combining aDNA and mycolate analyses was first demonstrated for 1,400 year old calcified pleura from Karkur in the Negev desert (Donoghue et al., 1998, 2004). In another combined study, Gernaey et al. (2001) showed that mediaeval skeletons from 1,000 years ago in Addingham, Yorkshire, UK had evidence of tuberculosis. These particular landmark samples were in fact the first in which mycolic acid, or any other, lipid biomarkers had been seen. Some of these early investigations have been reviewed by Gernaey & Minnikin (2000).

The methylanthryl derivatives used in the above pioneering studies were not ideal as their relative instability required that the sequential HPLC analyses must be done quickly, with minimum storage. A systematic exploration of derivatization protocols resulted in the selection of pentafluorobenzyl (PFB) mycolic acid esters, further esterified with pyrenebutyric acid (PBA). Ample justification of this selection was provided by confirmation of the oldest proven case of tuberculosis in ribs from a woman and child from a neolithic pre-pottery settlement at Atlit-Yam in the Eastern Mediterranean, dated at around 9,000 BP (Hershkovitz et al., 2008). The mycolic acid profiles for these extracts are shown in Fig. 2. The immaculate preservation of the mycolic acid biomarkers is illustrated by the remarkable similarity of the total mycolate profiles from the three skeletal extracts with that from authentic *M. tuberculosis* (Fig 2A). It must be remembered that the profiles in Fig. 2A represent a conglomerate of all five classes shown in Fig. 1, but it is a characteristic of MTBC mycolates that they cluster together to give a "tight envelope" of distinct peaks. Subjecting the collected total mycolates to normal phase HPLC (Fig. 2B) shows the proportions of the α-, methoxy- and ketomycolate classes but the methoxy- and keto- components having either *cis*- or *trans*- cyclopropane rings (Fig. 1A) are not separated.

Reverse phase HPLC of the collected α-mycolates (Fig. 3A) gives a profile of simple regularly spaced peaks corresponding to the single class of α-mycolates with two *cis*-cyclopropane rings; the main C_{80} component corresponds to the structure shown in Fig. 1A. The reverse phase HPLC profiles for the methoxymycolates from *M. tuberculosis* and the Atlit-Yam skeletons (Fig. 3B) are particularly informative. The main C_{85} component is the *cis*-methoxymycolate shown in Fig. 1A and the minor C_{88} component (Fig. 3B) is the principal *trans*-methoxymycolate given in Fig. 1A. Again the excellent correlation between the methoxymycolate profiles from standard *M. tuberculosis* and the archaeological samples illustrates the remarkable preservation of these samples. The main C_{87} *trans*-ketomycolate (Fig. 1A) dominates the reverse phase HPLC of the ketomycolates (Fig. 3C); the C_{86} *cis*-ketomycolate (Fig. 1A) is a very minor component (Fig. 3C). The particular mosaic of patterns, shown in Figs. 2 and 3, appears to be very characteristic for *M. tuberculosis sensu stricto*. In continuing unpublished studies, it is becoming apparent that the patterns recorded for *M. bovis* may possibly be distinguished by different proportions of the *cis*- and *trans*-methoxy- and ketomycolates; this is in accord with detailed structural studies (Watanabe et al., 2001, 2002).

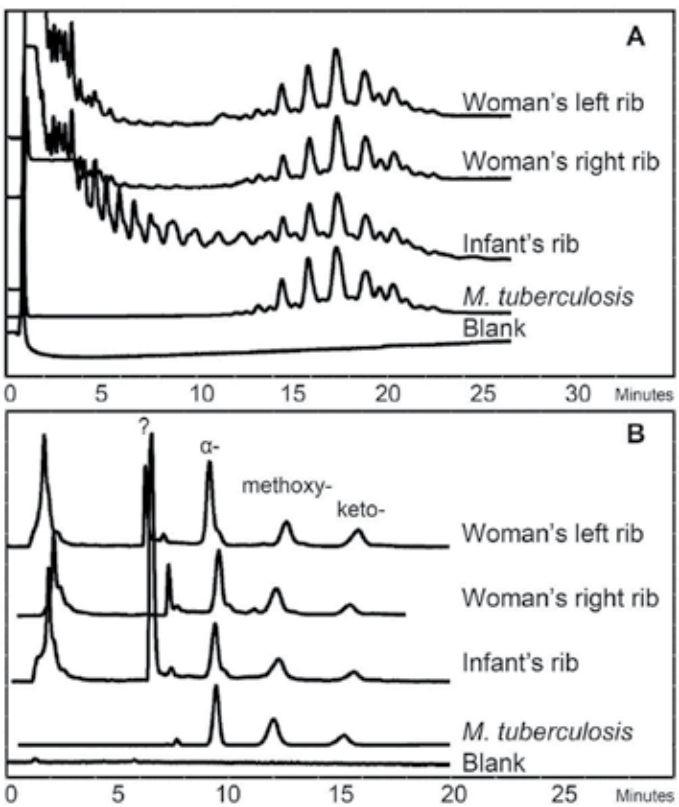

Fig. 2. HPLC of pyrenebutyric acid (PBA) esters of pentafluorobenzyl (PFB) esters of mycolic acids extracted from skeletons from Atlit-Yam and standard *M. tuberculosis*. Reproduced from Hershkovitz et al. (2008). **A.** Reverse phase HPLC of total mycolates. **B.** Normal phase HPLC of total mycolates, collected from 2A.

In addition to providing diagnostic profiles, HPLC allows quantitative data to be recorded. In particular, the relative proportions of the α-, methoxy- and ketomycolate types (Fig. 1A) are readily determined, as exemplified (Table 1) for the mycolates from Atlit-Yam (Hershkovitz et al., 2008). The α-mycolates contribute about half the mixture, with one quarter to one third being methoxymycolates and one tenth to one fifth being ketomycolates (Table 1). This good correlation of mycolate class proportions in the skeletal extracts with the *M. tuberculosis* standard is a clear indicator of good sample preservation. As will be discussed below for another study (Minnikin et al., 2011), degraded samples appear to lose their methoxy- and ketomycolates, particularly the latter. It is also possible to estimate the

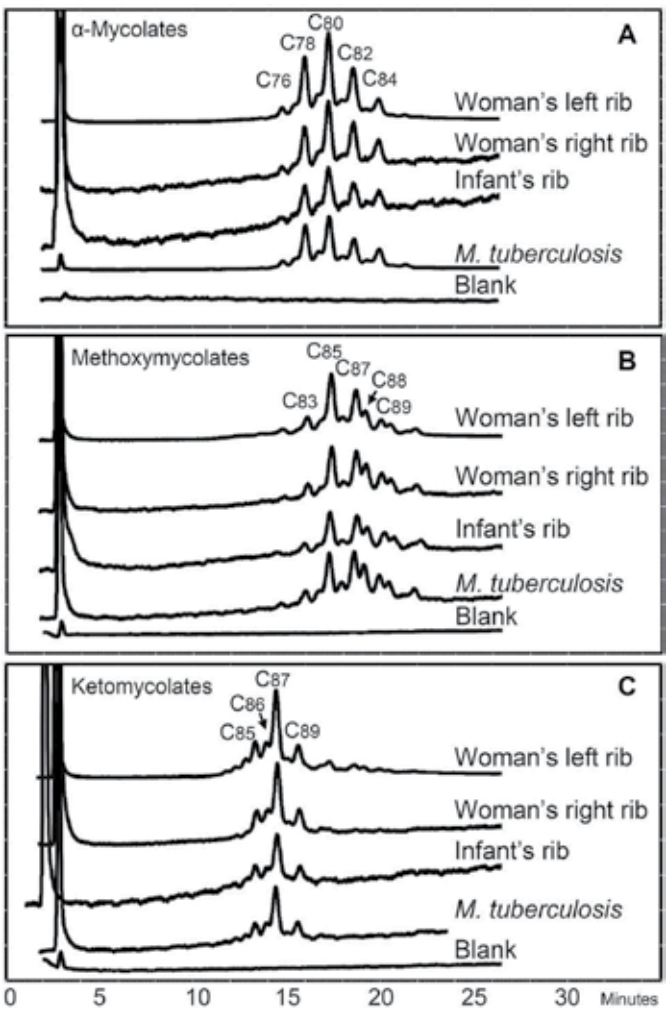

Fig. 3. HPLC of pyrenebutyric acid (PBA) esters of pentafluorobenzyl (PFB) esters of mycolic acids extracted from skeletons from Atlit-Yam and standard *M. tuberculosis*. Reproduced from Hershkovitz et al. (2008). **A, B**, & **C.** Reverse phase HPLC of α-mycolate, methoxymycolate and ketomycolate classes, respectively, collected from the normal phase separation illustrated in Fig. 2B.

absolute amounts of mycolic acids present, as shown (Table 2) for the Atlit-Yam extracts (Hershkovitz et al., 2008). The amount of mycolate in the 635 mg Woman left rib (12.80 μg) is most exceptional, equating to an almost weighable and visible one hundredth of a milligram! This suggests a very heavy tuberculosis infection in the bone from the Woman, as compared to that from the Infant, which is 168 times less; such snapshot comparisons are not statistically valid, however, as it would be necessary to examine a comparable range of bones from each individual several times over.

Mycolate	Woman's left rib	Woman's right rib	Infant's rib	*M. tb.* standard
Alpha-	56.3	52.65	53.2	48.2
Methoxy-	25.2	34.50	32.2	37.4
Keto-	18.5	12.85	14.6	14.4

Table 1. Percentage ratios of alpha-, methoxy- and ketomycolates in skeletons from Atlit-Yam. Reproduced from Hershkovitz et al. (2008).

Bone sample	Bone mass	Mycolate in bone	Mycolate/bone load
Woman's left rib	635 mg	12.80 μg	20.14 μg/g
Woman's right rib	483 mg	1.697 μg	3.51 μg/g
Infant's rib	589 mg	0.073 μg	0.12 μg/g

Table 2. Absolute amounts of mycolic acids in skeletons from Atlit-Yam. Reproduced from Hershkovitz et al. (2008).

Another example of good mycolic acid biomarker preservation is provided by the interesting case of Dr. Granville's mummy (Donoghue et al., 2010a). The subject is the lady Irtyersenu of the 26th Dynasty (*ca.* 2600 BP) from the necropolis of Thebes. Although aDNA from the MTBC was detected, the material was very difficult to work with, possibly due to the embalming method, which appears to have been unusual. In contrast, the mycolic acid HPLC traces were almost as pristine as those from the Atlit-Yam samples (Figs. 2 and 3) (Hershkovitz et al., 2008). These results, in conjunction with aDNA detection, clearly confirmed tuberculosis infection, which may have made a major contribution to the death of Irtyersenu (Donoghue et al., 2010a). However, pristine mycolic acid HPLC traces are by no means the norm in archaeological samples from subjects suspected to have suffered from tuberculosis. In a study aimed to test the possibility of detecting lipid biomarkers in archaeological material, after aDNA has been extracted and analyzed, a range of samples from subjects suspected to have been infected with tuberculosis, leprosy or both were investigated (Minnikin et al., 2011). Evidence of mycolic acids was found in all samples, thereby proving that it was possible to isolate lipid biomarkers from aDNA analysis residues; the hydrophobic lipids were not extracted by the aqueous media used to release aDNA. The total mycolate reverse phase HPLC profiles were complex and the normal phase HPLC traces all had α-mycolates but the methoxy- and ketomycolates were variable to non-existent, excepting two cases where good clear peaks for ketomycolates were recorded. The results in this paper are too complex to summarize concisely. For the 12 cases, suffice it to say that five diagnoses agreed with the aDNA (three tuberculosis, two leprosy), five cases did not correlate clearly and for two extracts the mycolic acids were so degraded that it was impossible to support positive aDNA diagnoses. This instructive study suggests that it is

important to obtain as much biomarker information as possible, particularly where mixed tuberculosis/leprosy cases are a probability. For one particular 7th century skeleton from the Avar period in Hungary, it was possible to recognise mycolic and mycocerosic acids from both *M. tuberculosis* and *M. leprae* (Lee et al., 2012). The quantitative data suggested a predominance of tuberculosis over leprosy in contrast to the bone pathology which indicated only leprosy. The presence of mycolic acids supported an aDNA diagnosis of leprosy in a 1st to 4th century CE skeleton from Uzbekistan (Taylor et al., 2009).

4.3 Mass spectrometry in detection of mycolic acid biomarkers for ancient tuberculosis

Mass spectrometry was a key technique in establishing the essential structures (Fig. 1A) of the mycolic acids of *M. tuberculosis* for the first time (Minnikin & Polgar, 1967a, 1967b; Minnikin, 1982), providing accurate molecular weights. Initially, the individual α-, methoxy- and ketomycolates were analysed separately by the pioneering but rather cumbersome high energy Electron Ionisation (EI) mode of mass spectrometry, which produces complex spectra with characteristic fragmentation patterns. Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) mass spectrometry is a convenient lower energy technique that often gives peaks corresponding to molecular weights augmented by ubiquitous sodium ions ($M + Na^+$). MALDI-TOF mass spectrometry was used to characterize a range of individual mycolate types in a comprehensive study (Watanabe et al., 2001) aimed at determining the precise location of functional groups in mycolic acids (Watanabe et al., 2002). In a parallel study, Laval et al. (2001) demonstrated that MALDI-TOF mass spectrometry of mycolic acid methyl esters can be used to provide a characteristic profile of total mycolic acid composition.

Based on the previous study by Laval et al. (2001), Mark et al. (2010) used MALDI-TOF mass spectrometry to search for tuberculosis mycolic acids in ancient bones. However, the profiles recorded by Mark et al. (2010) did not resemble those expected for mycolic acids, so a response to this paper was published (Minnikin et al., 2010). This response suggested guidelines for the satisfactory recognition of mycolic acid profiles diagnostic for *M. tuberculosis*. Remarkably, the original clearly inadequate conclusions were vigorously defended by Mark et al. (2011), without providing any new convincing data. It is most important, therefore, to demonstrate beyond doubt that the data provided by Mark et al. (2010, 2011) are not evidence for tuberculosis infection in any of the analyzed archaeological samples, whatsoever; the problems raised by these papers are considered in detail below.

The paper of Minnikin et al. (2010) provided a considered in-depth constructive analysis of the inadequacies of the data shown in Mark et al. (2010), so these criticisms will not be repeated in detail. The essence of the problems in both papers published by Mark et al. (2010, 2011) is exemplified by the data shown in Figs. 4 and 5. The profile shown in Fig. 4A (Mark et al., 2010) is suggested to be that of standard *M. tuberculosis* mycolic acids, but it only shows a series of regularly spaced peaks, more suggestive of polymeric material than distinct components of the family of mycolic acids shown in Fig. 1A. Similarly, the profile for an extract of a skeleton from Sükösd-Ságod (grave 19) (Fig. 4B) again showed a regular series of peaks, with limited correspondence between the data in Figs. 4A and 4B. The different classes of mycolic acids (Fig. 1A) occur naturally as groups of peaks with one or

two major components accompanied by several minor homologues (Minnikin & Polgar, 1967a, 1967b; Minnikin, 1982; Watanabe et al., 2001, 2002). Mycolic acids (Fig. 1A) are long-chain fatty acids, with series of homologues whose biosynthetic pathways dictate a general spacing of two methylene groups (-CH_2.CH_2-) amounting to 28 atomic mass units (amu). It is, therefore, totally impossible that the patterns presented in Figs. 4A and 4B, with spacings of 44 amu, can be assigned to homologous series of mycolic acids. Mark et al. (2010) hypothesize that the ion spacings are a result of carbon dioxide (44 amu) loss but this does not correlate with any known properties or published behaviour of any mycolic acids or, indeed, any other fatty acids. As suggested previously (Minnikin et al., 2010), an alternative explanation for the regular 44 amu spacing of the components is that the peaks are derived from a material incorporating polyethylene glycol repeating units. These polymeric polyethylene glycol-based preparations are in widespread industrial use and the risk of their appearance in mass spectra is well-known, as emphasized by, for example, by Keller et al. (2008) and Schiller et al. (2004), the latter reproducing a representative MALDI-TOF mass spectrum. Another general point, which comprehensively disqualifies the profiles in Figs. 4A and 4B, is the undisputed fact that mycolic acids do not have recorded molecular weights greater than 1350 amu (Laval et al., 2001; Watanabe et al., 2001, 2002) so the presence of alien substances is suspected. As noted above, these and other criticisms have been thoroughly aired by Minnikin et al. (2010) in a constructive attempt to establish reliable guidelines for the recognition of mycolic biomarkers in the diagnosis of ancient tuberculosis.

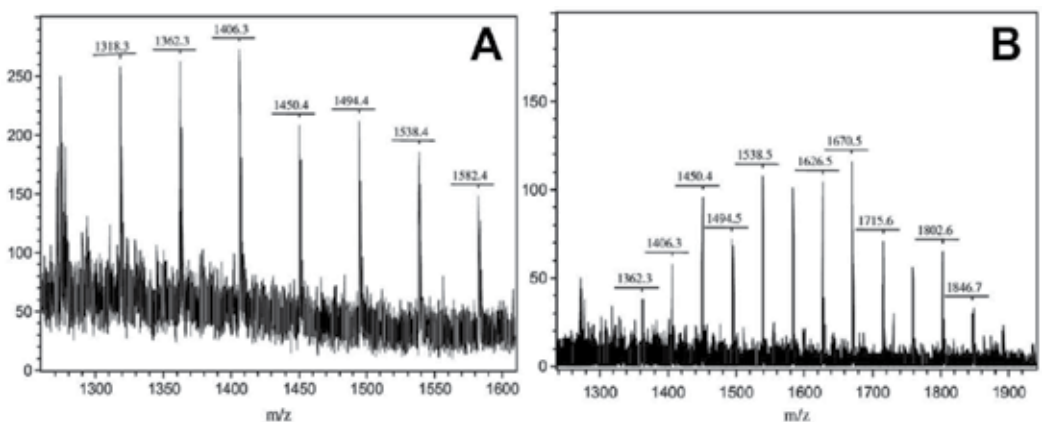

Fig. 4. MALDI-TOF mass spectra, reproduced with permission from Mark et al. (2010). **A**. Mycolic acid standard; Fig. 2 of Mark et al. (2010). **B**. Extract of bone sample from Sükösd-Ságod grave 19; Fig. 3A of Mark et al. (2010).

However, Mark et al. (2011) declined to acknowledge the obvious and indisputable errors and deficiencies, raised by Minnikin et al. (2010), in the original paper of Mark et al. (2010). In a most unsatisfactory manner, Mark et al. (2011) were able to submit their response on 17th December 2009, precisely one calendar month after the receipt of Minnikin et al. (2010) on 19th November 2009. This indisputably proves that unauthorized privileged information had been provided to Mark et al. (2011), well in advance of the due confidential reviewing process for the manuscript of Minnikin et al. (2010). Disregarding the deficiencies and practises in the publication process, attention must be focussed on the data and arguments advanced in the publication of Mark et al. (2011). As shown in Fig. 5A, the mass spectrum of

an authentic sample of *M. tuberculosis* mycolic acids was recorded (Mark et al. 2011) and this is very different from that shown in Fig. 4A, for supposedly the same material (Mark et al., 2010). The mass spectrum of an extract from the skeleton from Sükösd-Ságod (grave 19) (Fig. 5B) is again clearly distinct from the spectrum recorded for the same bones (Fig 4B) by Mark et al. (2010). More disturbingly, the supposedly positive spectrum recorded in Fig. 5B has little resemblance to that of the authentic standard (Fig. 5A). The most significant, but unknown, peak at *m/z* 1361.1 (Fig 5B) is clearly too large in mass to be a mycolic acid, as the

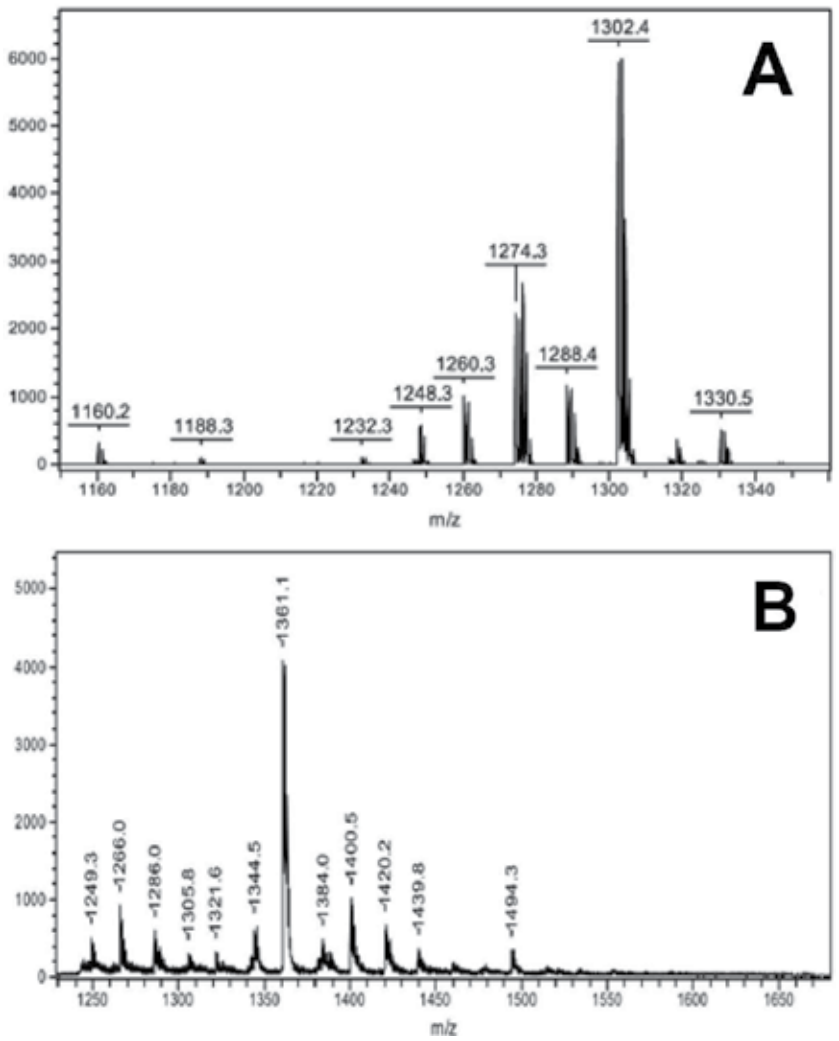

Fig. 5. MALDI-TOF mass spectra, reproduced with permission from Mark et al. (2011). **A**. Mycolic acid standard, provided by David E. Minnikin (University of Birmingham, UK); Fig. 3B of Mark et al. (2011). Peaks at *m/z* 1160 and 1188 are C_{78} and C_{80} α-mycolates, *m/z* 1274 is C_{85} *cis*-methoxymycolate and *m/z* 1302 is C_{87} *trans*-ketomycolates (see Minnikin et al., 2010 for a spectrum of the same sample). **B**. Extract of bone sample from Sükösd-Ságod grave 19; Fig. 4A of Mark et al. (2011).

highest component in the authentic standard is centred around *m/z* 1330 (Fig. 5A). In three other supposedly positive mass spectra, reported by Mark et al. (2011), the unknown, non-mycolic acid, peak at *m/z* 1361 is the main component. Incontrovertibly, the spectrum of the extract of the skeleton from Sükösd-Ságod (grave 19), shown in Fig. 5B, does not provide any evidence for the presence of tuberculosis, providing a double negative diagnosis for the same skeleton as the profile in Fig. 4B was also woefully inadequate.

The science displayed by Mark et al. (2010) has been thoroughly discussed by Minnikin et al. (2010) but it is also necessary, for the record, to draw critical attention to claims made in Mark et al. (2011). In this paper, there is an extensive discussion (page 1112, section 3) about whether it is likely to be possible to detect unmodified mycolic acids in archaeological samples. The key statement is "it would be very surprising if the mass spectra of ancient mycolic acid biomarkers were exactly the same as those of recent standards and clinical samples"; presumably this is aimed at providing some justification for claiming that the spectra exemplified in Figs. 4B and 5B (this Chapter) represent naturally modified mycolic acids. This argument is taken further in the statement "the identification of ancient mycolic acids and their metabolites could only be carried out with accurate and systematic chemical modelling of the mycolic acid post-mortem diagenesis". The implication of this statement is that diagenetic studies are a prerequisite but there are no suggestions about how this might be done. One way to approach this is to make extracts of suspected infected archaeological material and examine them by established objective protocols, such as HPLC, to determine if mycolic acids or their degradation products are recognisable. This is precisely the approach taken by Gernaey et al. (2001), Hershkovitz et al. (2008), Donoghue et al. (2010) and Minnikin et al. (2011); in the latter publication substantial degradation of mycolic acids is clearly recorded but in the others clearly recognisable mycolic acid patterns are documented. In the same paragraph (page 1112, section 3) of Mark et al. (2010), the paper of Donoghue et al. (2010a) is criticised by writing that "several significant differences can be observed on the standard chromatograms and the results of the bone samples". Such small differences are, however, valuable in showing that the extracts of bone samples are not contaminated by material from the standards. To suggest that such small differences might invalidate a diagnosis is contradictory to the arguments rehearsed elsewhere by Mark et al. (2011), which favoured a degree of diagenesis as a positive indication. Another criticism, levelled by Mark et al. (2011), concerns the presence of unknown peaks (labelled '?'), such as that in Figure 4 of Donoghue et al. (2010a); this particular unknown peak is in the same category as those shown in Fig. 2B (this Chapter) for the normal phase HPLC of mycolate derivatives from the Atlit-Yam skeletons (Hershkovitz et al., 2008). This unknown material, labelled '?' in Fig. 2B, represents residual material from the initial reverse phase isolation of the total mycolates (Fig. 2A) and there is no necessity to know its identity. Indeed one purpose of the normal phase HPLC analysis (Fig. 2B) of the total mycolates is simply to remove this contaminating material and obtain purified α-, methoxy- and ketomycolate classes for diagnostic reverse phase HPLC (Figs. 3A-C). Quite incredibly, the whole concept of normal phase chromatography is dismissed by Mark et al. (2011) as "not a 'simple' technique for the accurate separation of the components"; the reference (Neue, U.D., 1997. HPLC Columns. Wiley-VCN, New York.), quoted in support of this opinion, is only a document describing different types of chromatographic columns. However, normal phase chromatography is the bedrock of chemical research, being performed literally thousands of times each day in laboratories worldwide!

Mark et al. (2011) attempt to rationalize the disparate results presented in Mark et al. (2010, 2011) by claiming that choice of MALDI-TOF mass spectral matrices dramatically influences the resulting spectrum. In Mark et al. (2011) (page 1113, section 3) it is stated that "The results with using fullerene as the matrix are incomparable with the mass spectra made by using CHCA or 2,5-DHB". The essential conclusion is that the use of 2,5-DHB (2,5-dihydroxybenzoic acid) results in spectra such as those in Fig. 5, but using fullerene produces the spectra shown in Fig. 4, for supposedly the same samples. The explanation given by Mark et al. (2011) (page 1117, section 3.2) for the spectra in Fig. 5 is "On these spectra the *m/z* 44.01 fragmentation pattern could not be observed, because the fullerene needed much higher laser energy for ionization than the 2,5-DHB matrix, thus the fragmentation was stronger in the first case"; this "first case" refers to the spectra shown in Fig. 4. This hypothesis is linked to the problem of explaining the 44 amu spacing of the peaks in the spectra (Fig. 4) recorded by Mark et al. (2010). According to Mark et al. (2010, 2011) in profiles, such as that shown in Fig. 4A, a peak such as *m/z* 1406.3 should decarboxylate to give *m/z* 1362.3, losing 44 amu (CO_2). How then does *m/z* 1362.3 lose a further 44 amu to produce *m/z* 1318, as it would have already lost its carboxyl group? This is a scientific *non sequitur* unworthy of further consideration. The suggestion by Minnikin et al. (2010) that the sequences of peaks in Fig. 4 are characteristic of contaminating polymers based on polyethylene glycol (PEG) (Keller et al., 2008; Schiller et al., 2004) is a much more plausible explanation. The proposal by Mark et al. (2011) is that an authentic MALDI-TOF spectrum (Fig. 5A) of standard mycolic acids, using 2,5-DHB as matrix, can be changed to that shown in Fig. 4A simply by using C_{60} fullerene as matrix. This is a revolutionary but unlikely proposal that, if it is to be believed, must be substantiated by systematic research.

It is instructive to review the information required, using existing methods, to positively identify a mycolic acid pattern diagnostic for tuberculosis. The use of sequential reverse/normal/reverse phase HPLC analysis (Figs. 2 & 3) produces diagnostic patterns that, if the mycolates are undegraded, correlate well with standard material. Importantly, however, this protocol enables the key C_{80} (C_{78}) α-mycolates (Fig. 3A), C_{85} *cis*-methoxymycolates (Fig. 3B) and C_{87} *trans*-ketomycolates (Fig. 3C) to be recognised. It must be understood that related mycolic acids are present in many mycobacteria, but extensive studies have been carried out (Minnikin et al. 2010; Watanabe et al. 2001, 2002) to establish that the above combination of principal mycolic acid components (Fig. 1A, Fig. 3) is characteristic for *M. tuberculosis*. Any diagnoses, using mass spectrometry or any other technique, must recognise the presence of these characteristic components. In the case of MALDI-TOF mass spectrometry, the key C_{80} (C_{78}) α-mycolates, C_{85} methoxymycolates and C_{87} ketomycolates can be recognised in standard extracts, as shown in Fig. 5A, but this pattern must be recognisable in extracts of archaeological material. The studies of Mark et al. (2010, 2011) claim that recognisable MALDI-TOF mass spectra have been obtained for mycolic acids extracted from archaeological material. Simple visual comparison of the authentic mycolic acid spectrum, shown in Fig. 5A, with the spectra exemplified in Figs. 4B & 5B, or any other spectra published in Mark et al. (2010, 2011), reveals nothing remotely comparable. The bottom line conclusion, therefore, is that Mark et al. (2010, 2011) have not identified *M. tuberculosis* mycolic acids in bone samples and literature reference to these papers must never support the claim that mycolic acids were detected.

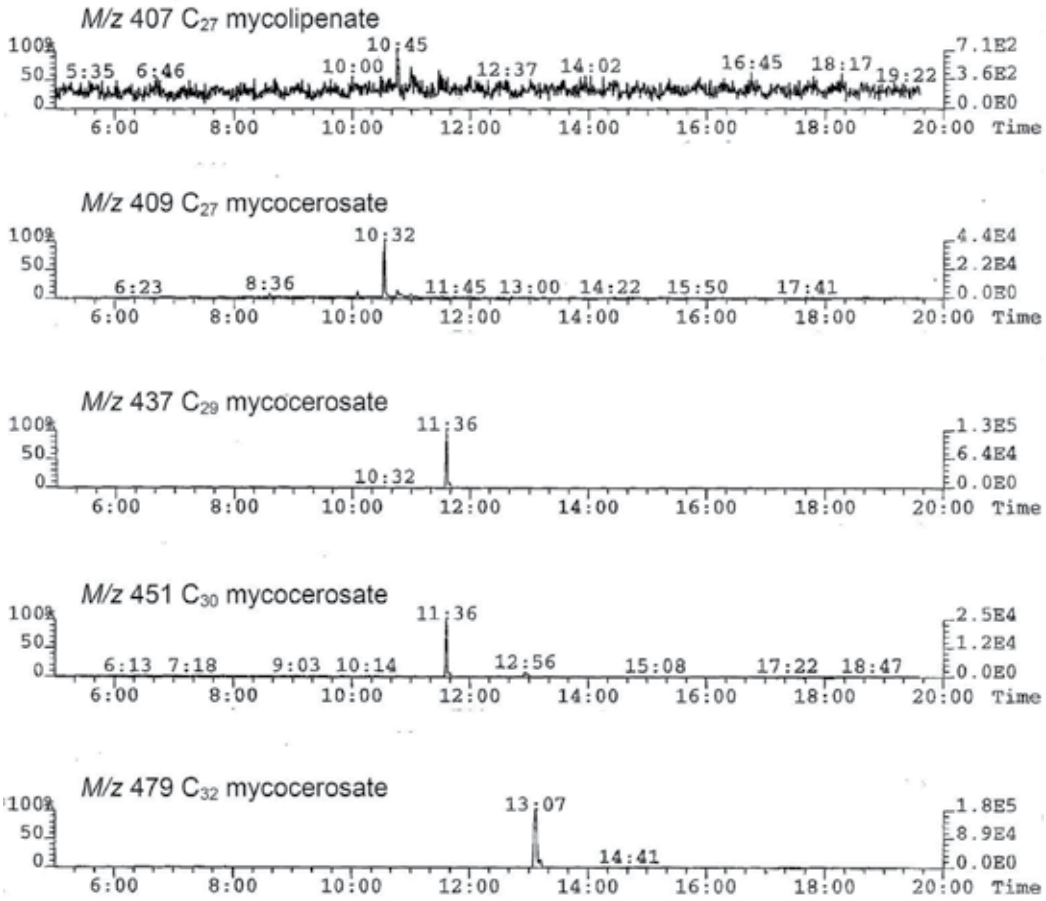

Fig. 6. Selected ion monitoring negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS) of pentafluorobenzyl esters from Coimbra skeleton C8, reproduced with permission from Redman et al. (2009). The C_{27} *M/z* 407 ion is for mycolipenic acid; the remainder are for the indicated C_{27} to C_{32} mycocerosic acids (Fig. 1B).

The detailed analysis given above highlights the serious problem of perpetuating the conclusions in the papers of Mark et al. (2010, 2011). This demonstrates the potential risks and hazards for workers coming into a field of research and attempting to utilize a particular unproven technique to provide quick answers to a particular question. It is imperative that such researchers obtain basic knowledge and carry out the groundwork to enable them to perform effective objective science before producing premature publications. It is apparent, in the broad field of "archaeological science", that some "artistic license" is more than acceptable to some researchers and certain editors of learned journals. In the present scenario, however, where specific chemical biomarkers are being used to diagnose ancient tuberculosis, there is absolutely no latitude in the identification of these distinct well-characterized chemical compounds. They are either positively identified or they are not and well-proven methods must be used along the guidelines advocated by Minnikin et al. (2010). Another most unsatisfactory aspect of the unconstructive publication by Mark et al.

(2011) is that, while the senior authors, on this paper, publically condemned the constructive paper of Minnikin et al. (2010), they were keen to collaborate in the use of HPLC analysis of mycolic acids to support their osteological studies. These collaborative enterprises, performed in good faith, have produced a joint publication (Lee et al., 2012) and joint conference presentations (Donoghue et al., 2010b; Pálfi et al., 2010).

4.4 Mycocerosic and mycolipenic acid biomarkers for tuberculosis

The current integrated strategy for the use of lipid biomarkers in tuberculosis diagnosis involves alkaline hydrolysis followed by conversion of released fatty acids to pentafluorobenzyl esters, which are separated into non-hydroxylated and mycolate fractions (Redman et al., 2009; Hershkovitz et al., 2008). The latter are derivatized and examined by HPLC, as described above but the former are analyzed by negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS), using selected ion monitoring to detect the diagnostic mycocerosic and mycolipenic acids (Minnikin et al., 1993a). The protocol was applied to extracts of 49 skeletons from the 1837–1936 Coimbra Identified Skeletal Collection and an example of a positive tuberculosis diagnosis is shown in Fig. 6 (Redman et al., 2009). The *m/z* 407 peak (10.45 min) corresponds to C_{27} mycolipenate (Fig. 1B) with the others representing C_{27}, C_{29}, C_{30} and C_{32} mycocerosates (Fig. 1B). This pattern is characteristic of the MTBC complex (Minnikin et al., 1993a; Redman et al., 2009), a particularly diagnostic feature being the co-chromatography (11.36 min) of the C_{29} and C_{30} acids (Fig. 6). This phenomenon results from the larger C_{30} acid being relatively more volatile due to the additional methyl branch (Fig. 1B). There was a 72% correlation of the mycocerosic acid profiles with the Coimbra burial records (Redman et al., 2009). Mycolipenic acids were detected only in skeletons, which were positive for mycocerosates but only in one third of these. Mycolipenic acids are components of pentaacyl trehalose glycolipids, which are likely to be degraded more rapidly than the phthiocerol dimycocerosate waxes (Minnikin et al., 2002).

5. Conclusion

After a slow beginning, almost two decades ago, characterization of *M. tuberculosis* aDNA has been systematically developed in a limited number of laboratories. The analysis of aDNA can provide a wealth of information about the particular strain of *M. tuberculosis* diagnosed, but the exact information is governed by the degree of preservation. The fact that aDNA analyses are now firmly established is due partly to technological advances but mainly due to established skills in key laboratories, built up over an extended period. Conclusive results can be obtained by aDNA analyses alone, but confirmatory biomarkers are valuable in completing the overall diagnosis. The tubercle bacillus and related mycobacteria are rich in unusual lipids, which are not produced in mammalian tissues. Building on sensitive methods, previously developed to detect lipid biomarkers for modern tuberculosis, and inspired by the initial aDNA results, mycolic acid biomarkers for tuberculosis were detected for the first time in archaeological bone just over a decade ago. After a flurry of initial studies, time was taken to explore more robust mycolic acid methods and expand the range of lipids to include characteristic mycocerosic and mycolipenic acids. Currently, aDNA and lipid analyses are established as a powerful combination to diagnose both tuberculosis and leprosy and, indeed, co-infections; these analyses can be performed on

the same sample. This is not a static situation, however, as new powerful methods are becoming available for analyses of aDNA and lipids; additionally, the range of lipids available for detection is by no means exhausted.

The availability of established aDNA and biomarker protocols and expertise provides avenues into a range of interlocking research areas. As noted above, evolutionary pathways can be verified for tuberculosis, also providing evidence about virulence as the key lipid biomarkers are implicated in this process. The very existence of apparently intact key lipids in ancient samples is also of basic chemical interest; for example, the mycolic acids from the 9,000 year old Atlit-Yam skeletons probably represent the oldest known cyclopropane rings! It is important also to study leprosy, as the evolutionary processes of these two mycobacterial diseases are fatally intertwined with many clear co-infections. The relative prevalence of tuberculosis and leprosy can give clear indications of the prevailing social environment. In tuberculosis/leprosy co-infections it is becoming possible to estimate the relative bacterial load in particular bones and thereby obtain indications regarding which disease was the immediate cause of death. For each disease and co-infections it will be instructive to examine bones throughout particular individual skeletons in order to gain information about dissemination. Evidence of tuberculosis and leprosy can be quite clear in the osteological record, with characteristic bone lesions and deformations and bone loss in the case of leprosy. Biomarker analysis can help illuminate and reinforce the diagnoses of skilled osteologists and compare bacterial loads in bones with and without disease indication. An appealing aspect of lipid biomarker analyses is the possibility of extracting the lipids with neutral solvents, avoiding any chemical or physical damage to particularly valuable bone samples. It must be borne in mind, however, that biomarker analyses for the diagnosis of ancient mycobacterial disease are currently sophisticated procedures, which cannot be easily attempted without time being spent in accumulating the necessary skills and experience.

The evolution of life on earth is a complex web of competitive and/or symbiotic interactions. Humans, related primates and all mammals are dependent on a symbiosis with many microorganisms, whose cells outnumber those of the host. From the perspective of the microorganism, the mammalian host provides an ecological niche in which it can multiply and evolve to improve its prospects for survival. In some cases, the interaction of microorganisms with mammals is a rapid, pathogenic process resulting in the demise of the host and the infecting agent passes on to a new subject. Early hominids and humans with a hunter/gatherer lifestyle had a low population density, so an alternative relationship with slow-growing organisms such as the pathogenic mycobacteria, emerged. Such pathogens have a long-term relationship with their host, thus enabling persistence of the organism until transmission is possible. This is likely to occur at the extremes of life, when the host immune response is immature or less effective, and at times of physical or mental stress, often associated with war, famine, poverty or social unrest. This latter scenario is typical of the ancient scourges of tuberculosis and leprosy, caused by *M. tuberculosis* and *M. leprae*, respectively.

Several decades ago, it appeared that BCG vaccination and combinations of effective drugs were conquering tuberculosis. However, declining vaccination efficacy, misuse of drug regimens and the rapid spread of HIV/AIDS-related immunodeficiency, together with increased urbanisation and population density, have provided the opportunity for

accelerated evolutionary changes to *M. tuberculosis* and the emergence of highly drug-resistant and readily transmissible strains. Modern genomic approaches are also highlighting the great diversity existing within the inhomogeneous species labelled as *M. tuberculosis*. The developing science of paleogenomics is enabling approximate timelines for the evolution of *M. tuberculosis* to be laid down. The analysis of biomarkers is the prime way to verify the various proposed evolutionary pathways and to provide a direct timescale, rather than one inferred from bioinformatic analysis. In this review the contributions of ancient DNA (aDNA) and lipid biomarker analyses have been elaborated and critically assessed.

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7. References

Arrieza, B.T., Cartmell, L.L., Moragas, C., Nerlich, A.G., Salo, W., Madden, M. & Aufderheide, A.C. (2008). The bioarchaeological value of human mummies without provenience. *Chungara, Revista de Antropología Chilena*, 40, 55–65.

Aufderheide, A.C., Salo, W., Madden, M., Streitz, J., Buikstra, J., Guhle, F., Arrieza, B., Renier, C., Wittmers, Jr., L.E., Fornaciari, G. & Allison, M. (2004). A 9,000-year record of Chagas' disease. *PNAS*, 101, 2034–2039.

Baptista, P.V., Koziol-Montewka, M., Paluch-Oles, J., Doria, G. & Tranco, R. (2006). Gold-nanoparticle-probe-based assay for rapid and direct detection of *Mycobacterium tuberculosis* DNA in clinical samples. *Clin Chem*, 52, 1433–1434.

Barry, C.E., Crick, D.C. & McNeil, M.R. (2007). Targeting the formation of the cell wall core of *M. tuberculosis*. *Infect Disord Drug Targets* 7, 182-202.

Bhatt, R., Scott, B., Whitney, S., Bryan, R.N., Cloney, L. & Lebedev, A. (1999). Detection of nucleic acids by cycling probe technology on magnetic particles: high sensitivity and ease of separation. *Nucleosides and Nucleotides*, 18, 1297–1299.

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D. & Cole, S.T. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *PNAS*, 99, 3684–3689.

Butler, W.R. & Guthertz, L.S. (2001). Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species. *Clin Microbiol Rev*, 14, 704-726.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry III, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393, 537–544.

Daffé, M, & Lanéelle, M.A. (1988). Distribution of phthiocerol diester, phenolic mycosides and related compounds in mycobacteria. *J Gen Microbiol*, 134, 2049–2055.

Dobson, G., Minnikin, D.E., Minnikin, S.M., Parlett, J.H., Goodfellow, M., Ridell, M. & Magnusson, M. (1985). Systematic analyses of complex mycobacterial lipids. In: *Chemical Methods in Bacterial Systematics.* Goodfellow, M. & Minnikin, D. E., pp. 237-265, Academic Press, London, UK.

Donoghue, H.D., Spigelman, M., Zias, J., Gernaey-Child, A.M. & Minnikin D.E. (1998). *Mycobacterium tuberculosis* complex DNA in calcified pleura from remains 1400 years old. *Lett Appl Microbiol,* 27, 265-269.

Donoghue, H.D., Spigelman, M., Zias, J., Gernaey-Child, A.M. & Minnikin D.E. (2004). Demonstration of *Mycobacterium tuberculosis* complex DNA in calcified pleura from human remains excavated in Karkur. In: *Horvat Karkur 'Illit.* Figueras, P., pp. 316–320, Ben-Gurion University of the Negev Press, Beer-Sheva, Israel.

Donoghue, H.D., Marcsik, A., Matheson, C., Vernon, K., Nuorala, E., Molto, J.E., Greenblatt, C.L. & Spigelman, M. (2005). Co-infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in human archaeological samples: a possible explanation for the historical decline of leprosy. *Proc Roy Soc B*, 272, 389–394.

Donoghue, H.D. & Spigelman, M.. (2006). Pathogenic microbial ancient DNA – a problem or an opportunity? *Proc Roy Soc B*, 273, 641–642.

Donoghue, H.D. (2008a). Molecular palaeopathology of human infectious disease, In: *Advances in Human Palaeopathology*, Pinhasi, R. & Mays, S., pp. 147–176, John Wiley & Sons, Ltd, ISBN 978-0-470-03602-0, Chichester, UK.

Donoghue, H.D. (2008b). Paleomicrobiology of tuberculosis, In: *Paleomicrobiology – Past Human Infections*, Raoult, D. & Drancourt, M., pp. 75–97, Springer-Verlag GmbH, ISBN 978-3-540-75854, Berlin & Heidelberg, Germany.

Donoghue, H.D. (2009). Human tuberculosis – an ancient disease, as elucidated by ancient microbial biomolecules. *Microbes and Infection*, 11, 1156–1162.

Donoghue, H.D., Hershkovitz, I., Minnikin, D.E., Besra, G.S., Lee, O.Y.-C, Galili, E., Greenblatt, C.L., Lemma, E., Spigelman, M. & Kahila Bar-Gal, G. (2009). Biomolecular archaeology of ancient tuberculosis: Response to "Deficiencies and challenges in the study of ancient tuberculosis DNA" by Wilbur *et al*. 2009. *J Archaeol Sci*, 36, 2797–2804.

Donoghue, H.D., Lee, O.Y.-C., Minnikin, D.E., Besra, G.S., Taylor, J.H. & Spigelman, M. (2010a). Tuberculosis in Dr Granville's Mummy: a molecular re-examination of the first Egyptian mummy to be scientifically examined and given a medical diagnosis. *Proc Roy Soc B*, 277, 51–56.

Donoghue, H.D., Molnár, E., Pálfi, G., Besra, G.S., Lee, O.Y-C. & Minnikin, D.E. (2010b). Biomolecular and morphological traces of mycobacterial infections in an 8th century anthropological sample: a comparative study. *Final Programme 10th International Conference on Ancient DNA and Related Biomolecules, Munich 10th – 13th October 2010.* http://www.adna2010.com/_medien/_content/file/ADNA_2010_final_Programme.pdf

Donoghue, H.D. (2011). Insights gained from palaeomicrobiology into ancient and modern tuberculosis. *Clin Microbiol Infect*, 17, 821–829.

Donoghue, H.D., Pap, I., Szikossy, I. & Spigelman, M. (2011). Detection and characterization of *Mycobacterium tuberculosis* DNA in 18th century Hungarians with pulmonary and

extra-pulmonary tuberculosis, In: *Yearbook of Mummy Studies 1*, Gill-Frerking, G., Rosendahl, W., Zink, A. & Piombino-Mascali, D., pp. 51–56, Verlag Dr. Friedrich Pfeil, ISBN 978-3-89937-137-6, Münich, Germany.

Drancourt, M., Aboudharam, G., Signoli, M., Dutour, O. & Raoult, D. (1998). Detection of 400-year-old *Yersinia pestis* DNA in human dental pulp: an approach to the diagnosis of ancient septicaemia. *PNAS*, 95, 12637–12640.

Eisenach, K.D., Cave, M.D., Bates, J.H. & Crawford, J.T. (1990). Polymerase chain reaction of a repetitive DNA sequence specific for *Mycobacterium tuberculosis. J Infect Dis*, 161, 977–981.

Ernst, J.D., Trevejo-Nuñez & Banaiee, N. (2007). Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis. *J Clin Invest*, 117, 1738–1745.

Évinger, S., Bernert, Zs., Fóthi, E., Wolff, K., Kővári, I., Marcsik, A., Donoghue, H.D., O'Grady, J., Kiss, K.K. & Hajdu, T. (2011). New skeletal tuberculosis cases in past populations from Western Hungary (Transdanubia). *HOMO – Journal of Comparative Human Biology*, 62, 165–183.

Faerman, M., Jankauskas, R., Gorski, A., Bercovier, H. & Greenblatt, C.L. (1997). Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA analysis. *Ancient Biomolecules*, 1, 205–214.

Fernández, E., Ortiz, J.E., Pérez-Pérez, A., Prats, E., Turbón, D., Torres, T. & Arroyo-Pardo, E. (2009). Aspartic acid recemization variability in ancient human remains: implications in the prediction of ancient DNA recovery. *J Archaeol Sci*, 36, 965–972.

Fletcher, H.A., Donoghue, H.D., Holton, J., Pap, I. & Spigelman, M. (2003a). Widespread occurrence of *Mycobacterium tuberculosis* DNA from 18th–19th century Hungarians. *Am J Phys Anthropol*, 120, 144–152.

Fletcher, H.A., Donoghue, H.D., Taylor, G.M., van der Zanden, A.G.M. & Spigelman, M. (2003b). Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. *Microbiology*, 149, 143–151.

Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V. & Hewinson, R.G. (2003). The complete genome sequence of *Mycobacterium bovis*. *PNAS*, 100, 7877–7882.

Gazouli, M., Liandris, E., Andreadou, M., Sechi, L.A., Masala, S., Paccagnini, D. & Ikonomopolous, J. (2010). Specific detection of unamplified mycobacterial DNA by use of fluorescent semiconductor quantum dots and magnetic beads. *J Clin Microbiol*, 48, 2830–2835.

Gernaey, A.M., Minnikin, D.E., Copley, M.S., Power, J.J., Ahmed, A.M.S., Dixon, R.A., Roberts, C.A., Robertson, J.D., Nolan, J. & Chamberlain, A. (1998). Detecting ancient tuberculosis. *Internet Archaeol,* http://intarch.ac.uk/journal/issue5/gernaey_index.html.

Gernaey, A.M., Minnikin, D.E., Copley, M.S., Ahmed, A.M.S., Robertson, D.J., Nolan, J & Chamberlain,A.T. (1999). Correlation of the occurrence of mycolic acids with tuberculosis in an archaeological population. In: *Tuberculosis Past and Present,* Pálfy, G., Dutour, O., Deák J. & Hutás, I., pp. 275-282, Golden Book Publisher Ltd., Tuberculosis Foundation, Szeged, Hungary.

Gernaey, A.M. & Minnikin, D.E. (2000). Chemical methods in paleopathology. In: *Human Osteology in Archaeology and Forensic Science,* Cox, M. & Mays, S., pp. 239-253, Greenwich Medical Media Ltd., London, UK.

Gernaey, A.M., Minnikin, D.E., Copley, M.S., Dixon, R.A., Middleton, J.C. & Roberts, C.A. (2001). Mycolic acids and ancient DNA confirm an osteological diagnosis of tuberculosis. *Tuberculosis*, 81, 259–265.

Gernaey, A.M., Minnikin, D.E., Copley, M.S., Ahmed, A.M.S. & Chamberlain, A. (2002). A correlation between mycolic acid prevalence and reported tuberculosis frequency suggests a "biomarker" for poverty in ancient populations. In: *Archaeometry 98,* Jerem, E. & Biró, K.T., pp. 27-32, Archaeopress, Oxford, UK.

Gordon, S.V., Bottai, D., Simeone, R., Stinear, T.P. & Brosch, R. (2009). Pathogenicity in the tubercle bacillus: molecular and evolutionary determinants. *BioEssays*, 31, 378–388.

Götherström, A., Collins, M.J., Angerbjörn, A. & Lidén, K. (2002). Bone preservation and DNA amplification. *Archaeometry*, 44, 395–404.

Grange, J.M. & Zumla, A.I. (2009). Tuberculosis, In: *Manson's Tropical Diseases Twenty-second edition,* Cook, C. & Zumla, A.I., pp. 983–1038, Saunders, Elsevier, ISBN978-1-4160-4471-0, Edinburgh, UK.

Gutierrez, M.C., Brisse, S., Brosch, R., Fabre, B., Omaïs, B., Marmiesse, M., Supply, P. & Vincent, V. (2005). *PLoS Pathogens*, 1, e5 (7 pages).

Hershberg, R., Lipatov, M., Small, P.M., Sheffer, H., Niemann, S., Homolka, S., Roach, J.C., Kremer, K., Petrov, D.A., Feldman, M.W. & Gagneux, S. (2008). High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLoS Biology*, 6, e311, (14 pages).

Hershkovitz, I., Donoghue, H.D., Minnikin, D.E., Besra, G.S., Lee, O.Y-C., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Kahila Bar-Gal, G. & Spigelman, M. (2008). Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLoS ONE*, 3, e3426-e3426.

Hirsh, A.E., Tsolaki, A.G., DeReimer, K., Feldman, M.W. & Small, P.M. (2004). Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. *PNAS*, 101, 4871–4876.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J.M. & Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *PNAS,* 105, 3963-3967.

Höss, M., Jaruga, P., Zastawny, T.H., Dizdaroglu, M. & Pääbo, S. (1996). DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Research*, 24, 1304–1307.

Kaestle, F.A. & Horsburgh, K.A. (2002). Ancient DNA in anthropology: methods, applications and ethics. *Yearbook of Physical Anthropology*, 45, 92–130.

Kaewphinit, T., Santiwatanakul, S., Promtmas, C. & Chansiri, K. (2010). Detection of non-amplified *Mycobacterium tuberculosis* genomic DNA using piezoelectric DNA-based biosensors. *Sensors*, 10, 1846–1858.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M. & van Embden, J. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol*, 35, 907–914.

Karami, A., Gill, P., Motamedi, M.H. & Saghafinia, M. (2011). A review of the current isothermal amplification techniques: Applications, advantages and disadvantages. *Journal of Global Infectious Diseases*, 3, 293-302. Retrieved from < http://www.jgid.org/text.asp?2011/3/3/293/83538>

Keller, B.O., Sui, J., Young, A.B. & Whittal, R.M. (2008). Interferences and contaminants encountered in modern mass spectrometry. *Anal Chim Acta*, 627, 71-81.

Klaus, H.D., Wilbur, A.K., Temple, D.H., Buikstra, J.E., Stone, A.C., Fernandez, M., Wester, C. & Tam, M.E. (2010). Tuberculosis on the north coast of Peru: skeletal and molecular paleopathology of late pre-Hispanic and post contact mycobacterial disease. *J Archaeol Sci*, 37, 2587–2597.

Konomi, N., Lebwohl, E., Mowbray, K., Tattersall, I. & Zhang, D. (2002). Detection of mycobacterial DNA in Andean mummies. *J Clin Microbiol*, 40, 4738–4740.

Kustár, Á., Pap, I., Végvári, Z., Kristóf, L.A., Pálfi, G., Karlinger, K., Kovács, B. & Szikossy, I. (2011). Use of 3D virtual reconstruction for pathological investigation and facial reconstruction of an 18th century mummified nun from Hungary, In: *Yearbook of Mummy Studies 1*, Gill-Frerking, G., Rosendahl, W., Zink, A. & Piombino-Mascali, D., pp. 83–93, Verlag Dr. Friedrich Pfeil, ISBN 978-3-89937-137-6, Münich, Germany.

Laval, F., Lanéelle, M-A, Déon, C., Monsarrat, B. & Daffé, M. (2001). Accurate molecular mass detemination of mycolic acids by MALDI-TOF mass spectrometry. *Anal Chem*, 73, 4537-4544.

Lee, O.Y-C., Bull, I.D., Molnár, E., Marcsik, A., Pálfi, G., Donoghue, H.D., Besra, G.S. & Minnikin, D.E. (2012). Integrated strategies for the use of lipid biomarkers in the diagnosis of ancient mycobacterial disease. In: *Proceedings of the 12th Annual Conference of the British Association for Biological Anthropology and Osteoarchaeology*. Mitchell, P.D. & Buckberry J., Archaeopress, Oxford, UK, In press.

Maiden, M.C.J. (2009). Putting leprosy on the map. *Nature Genetics*, 41, 1264–1266.

Mark, L., Patonai, Z., Vaczy, A., Lorand, T. & Marcsik, A. (2010). High-throughput mass spectrometric analysis of 1400-year-old mycolic acids as biomarkers for ancient tuberculosis infection. *J Archaeol Sci*, 37, 302–305.

Mark, L., Gulyas-Fekete, G., Marcsik, A., Molnár, E., & Pálfi, G. (2011). Analysis of ancient mycolic acids by using MALDI TOF MS: response to "Essentials in the use of mycolic acid biomarkers for tuberculosis detection" by Minnikin *et al.*, 2010. *J Archaeol Sci*, 38, 1111-1118.

Martins, S.A.M., Prazeres, D.M.F., Fonseca, L.P. & Monteiro, G.A. (2010). Quantitation of non-amplified genomic DNA by bead-based hybridization and template mediated extension coupled to alkaline phosphatase signal amplification. *Biotechnology Letters*, 32, 229–234.

Matheson, C.D., Vernon, K.K., Lahti, A., Fratpietro, R., Spigelman, M., Gibson, S., Greenblatt, C. & Donoghue, H.D. (2009). Molecular exploration of the first-century *Tomb of the Shroud* in Akeldama, Jerusalem. *PLoS ONE*, 4, e8319 (13 pages).

Mays, S.A., Taylor, G.M., Legge, A.J., Young, D.B. & Turner-Walker, G. (2001). Paleopathological and biomolecular study of tuberculosis in a medieval skeletal collection from England. *Am J Phys Anthropol*, 114, 298–311.

Mays, S., Fysh, E. & Taylor, G.M. (2002). Investigation of the link between visceral surface rib lesions and tuberculosis in a Medieval skeletal series from England using ancient DNA. *Am J Phys Anthropol*, 119, 27–36.

Minnikin, D.E. & Polgar, N. (1967a). Mycolic acids from human and avian tubercle bacilli. *Chemical Communications*, 916–918.

Minnikin, D.E. & Polgar, N. (1967b). The methoxymycolic and ketomycolic acids from human tubercle bacilli. *Chemical Communications*, 1172-1174.

Minnikin, D.E. (1982). Lipids: Complex lipids, their chemistry, biosynthesis and role. In: *The Biology of Mycobacteria.* Ratledge, C. & Stanford, J., pp. 95-184, Academic Press, London, UK.

Minnikin, D.E., Dobson G. & Hutchinson, I.G. (1983). Characterization of phthiocerol dimycocerosates from *Mycobacterium tuberculosis. Biochim Biophys Acta,* 753, 445-449.

Minnikin, D.E., Dobson, G., Goodfellow, M., Magnusson, M. & Ridell, M. (l985a). Distribution of some mycobacterial waxes based on the phthiocerol family. *J Gen Microbiol* 131, 1375-1381.

Minnikin, D.E., Dobson, G., Sesardic, D. & Ridell, M. (1985b). Mycolipenates and mycolipanolates of trehalose from *Mycobacterium tuberculosis. J Gen Microbiol,* 131, 1369-1374.

Minnikin, D.E. (1993). Mycolic acids. In: *CRC Handbook of Chromatography: Analysis of Lipids*. Mukherjee, K.D. & Weber N., pp. 339-348, CRC Press, Boca Raton, Florida, USA.

Minnikin, D,E., Besra, G.S., Bolton, R.C., Datta, A.K., Mallet, A.I., Sharif, A., Stanford, J.L., Ridell, M. & Magnusson M.. (1993a). Identification of the leprosy bacillus and related mycobacteria by analysis of mycocerosate profiles. *Ann Soc Belg Méd Trop* 73 (Suppl. 1), 25-34.

Minnikin, D.E., Bolton, R.C. Hartmann, S. Besra, G.S., Jenkins, P.A., Mallet, A.I., Wilkins, E., Lawson A.M. & Ridell., M. (1993b). An integrated procedure for the direct detection of characteristic lipids in tuberculosis patients. *Ann Soc Belg Méd Trop,* 73 (Suppl. 1), 13-24.

Minnikin, D. E., Kremer, L., Dover, L. G. & Besra, G. S. (2002). The methyl-branched fortifications of *Mycobacterium tuberculosis*. *Chem Biol,* 9, 545-553.

Minnikin, D.E., Lee, O.Y.-C., Pitts, M., Baird, M.S. & Besra, G.S. (2010). Essentials in the use of mycolic acid biomarkers for tuberculosis detection: response to "High-throughput mass spectrometric analysis of 1400-year-old mycolic acids as biomarkers for ancient tuberculosis infection" by Mark et al., 2010. *J Archaeol Sci*, 37, 2407-2412.

Minnikin, D.E., Besra, G.S., Lee, O.Y-C., Spigelman, M. & Donoghue, H.D. (2011). The interplay of DNA and lipid biomarkers in the detection of tuberculosis and leprosy in mummies and other skeletal remains, In: *Yearbook of Mummy Studies 1*, Gill-Frerking, G., Rosendahl, W., Zink, A. & Piombino-Mascali, D., pp. 109-114, Verlag Dr. Friedrich Pfeil, ISBN 978-3-89937-137-6, Münich, Germany.

Minunni, M., Tombelliu, S., Fonti, J., Spiriti, M.M., Mascini, M., Bogani, P. & Buiatti, M. (2005). Detection of fragmented genomic DNA by CR-free piezoelectric sensing using a denaturation approach. *J Am Chem Soc,* 127, 7966–7967.

Morens, D.M., Taubenberger, J.K. & Fauci, A.S. (2008). Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis*, 198, 962–970.

Morse, D. (1961). Prehistoric tuberculosis in America. *Am Rev Respir Dis,* 83, 489–504.

Murphy, E., Chistov, Y.K., Hopkins, R., Rutland, P. & Taylor, G.M. (2009). Tuberculosis among Iron Age individuals from Tyva, South Siberia: palaeopathological and biomolecular findings. *J Archaeol Sci*, 36, 2029–2038.

Nazarenko, I.A., Bhatnagar, S.K., & Hohman, R.J. (1997). A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Research*, 25, 2516–2521.

Neonakis, I.K., Spandidos, D.A. & Petinaki, E. (2011). Use of loop-mediated isothermal amplification of DNA for the rapid detection of *Mycobacterium tuberculosis* in clinical specimens. *Eur J Clin Microbiol Infect Dis*, 30, 937–942.

Neyrolles, O. & Guilhot, C. (2011). Recent advances in deciphering the contribution of *Mycobacterium tuberculosis* lipids to pathogenesis. *Tuberculosis* 91 187-195.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28, e63 (7 pages).

O'Rourke, D.H., Hayes, M.G. & Carlyle, S.W. (2000). Ancient DNA studies in physical anthropology. *Ann Rev Anthropol*, 29, 217–242.

Ottoni, C., Koon, H.E.C., Collins, M.J., Penkman, K.E.H., Rickards, O. & Craig, O.E. (2009). Preservation of ancient DNA in thermally damaged archaeological bone. *Naturwissenschaften*, 96, 267–278.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L. & Hofreiter, M. (2004). Genetic analyses from ancient DNA. *Ann Rev Gen*, 38, 645–679.

Pálfi, G., Molnár, E., Pap, I., Besra, G.S., Lee, O.Y-C., Minnikin, D.E. & Donoghue, H.D. (2010). A medieval *Mycobacterium tuberculosis - M. leprae* coinfection from Hungary: the coincidence of biomolecular and osteological findings. *Final Programme 10th International Conference on Ancient DNA and Related Biomolecules, Munich 10th - 13th October 2010.*
http://www.adna2010.com/_medien/_content/file/ADNA_2010_final_Programme.pdf

Pan, Y., Yang, X., Duan, J., Lu, N., Leung, A.S., Tran, V., Hu, Y., Wu, N., Liu, D., Wang, Z., Yu, X., Chen, C., Zhang, Y., Wan, K., Liu, J. & Zhu, B. (2011). Whole-genome sequences of four *Mycobacterium bovis* BCG vaccine strains. *J Bacteriol*, 193, 3152–3153.

Poinar, H.N. (2003). The top 10 list: criteria of authenticity for DNA from ancient and forensic samples. *Progress in Forensic Genetics 9 Book Series: International Congress Series*, 1239, 575–579.

Poinar, H.N. & Stankiewicz, A. (1999). Protein preservation and DNA retrieval from ancient tissues. *PNAS*, 96, 8426–8431.

Poinar, H.N., Hofreiter, M., Spaulding, W.G., Martin, P.S., Stankiewicz, A., Bland, H., Evershed, R.P., Possnert, G. & Pääbo, S. (1998). Molecular coproscopy: dung and diet of the extinct Ground Sloth *Nothrotheriops shastensis. Science*, 281, 402–406.

Qureshi, N., Takayama, K., Jordi, H.C., Schnoes, H.K., 1978. Characterization of the purified components of a new homologous series of α-mycolic acids from *Mycobacterium tuberculosis* H37Ra. *J Biol Chem,* 253, 5411-5417.

Redman, J.E., Shaw, M.J., Mallet, A.I., Santos, A.L., Roberts, C.A., Gernaey, A.M. & Minnikin, D.E. (2009). Mycocerosic acid biomarkers for the diagnosis of tuberculosis in the Coimbra Skeletal Collection. *Tuberculosis*, 89, 267–277.

Reed, M.B., Pichler, V.K., McIntosh, F., Mattia, A., Fallow, A., Masala, S., Domenech, P., Zwerling, A., Thibert, L., Menzies, D., Schwartzman, K. & Behr, M.A. (2009). Major *Mycobacterium tuberculosis* lineages associate with patient country of origin. *J Clin Microbiol,* 47, 1119–1128.

Rothschild, B.M., Martin, L.D., Lev, G., Bercovier, H., Bar-Gal, G.K., Greenblatt, C., Donoghue, H., Spigelman, M. & Brittain, D. (2001) *Mycobacterium tuberculosis*-complex DNA from an extinct bison dated 17,000 years BP. *Clin Infect Dis,* 33, 305–311.

Rustad, T.R., Sherrid, A.M., Minch, K.J. & Sherman, D.R. (2009). Hypoxia: a window into *Mycobacterium tuberculosis* latency. *Cellular Microbiology*, 11, 1151–1159.

Salo, W., Aufderheide, A.C., Buikstra, J. & Holcomb, T.A. (1994). Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. *PNAS,* 91, 2091–2094.

Seki, M., Honda, I., Fujita, I., Yano, I., Yamamoto, S. & Koyama,A. (2009). Whole genome sequence analysis of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Tokyo 172: a comparative study of BCG vaccine substrains. *Vaccine*, 27, 1710–1716.

Smith, N.H., Kremer, K., Inwald, J., Dale, J., Driscoll, J.R., Gordon, S.V., van Soolingen, D., Hewinson, R.G. & Smith, J.M. (2006). Ecotypes of the *Mycobacterium tuberculosis* complex. *J Theoret Biol*, 239, 220–225.

Smith, N.H., Hewinson, R.G., Kremer, K., Brosch, R. & Gordon, S.V. (2009). Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis. Nature Reviews Microbiology*, 7, 537–544.

Spigelman, M. & Lemma, E. (1993). The use of the polymerase chain reaction (PCR) to detect *Mycobacterium tuberculosis* in ancient skeletons. *Int J Osteoarchaeol*, 3, 137–143.

Spigelman, M., Greenblatt, C., Vernon, K., Zylber, M., Sheridan, S., Van Gerven, D., Shaheem, Z., Hansraj, F. & Donoghue, H. (2005). Preliminary findings on the paleomicrobiological study of 400 naturally mummified human remains from upper Nubia. *Journal of Biological Research (Rubbettino-Soveria Mannelli)*, 80, 91–95.

Spigelman, M., Pap, I. & Donoghue, H.D. (2006). A death from Langerhans cell histiocytosis and tuberculosis in 18th Century Hungary - what palaeopathology can tell us today. *Leukemia,* 20, 740-742.

Schiller, J., Süß, R., Arnhold, J., Fuchs, B., Leßig, J., Müller, M., Petković, M., Spalteholz, H., Zschörnig, O. & Arnold, K. (2004). Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. *Prog Lipid Res*, 43, 449-488.

Steck, P.A., Schwartz, B.A., Rosendahl, M.S. & Gray, G.R., 1978. Mycolic acids. A reinvestigation. *J Biol Chem,* 253, 5625-5629.

Sterling, T.R., Pope, D.S., Bishai, W.R., Harrington, S., Gershon, R.R. & Chaisson, R.E. (2000) Transmission of *Mycobacterium tuberculosis* from a cadaver to an embalmer. *New England Journal of Medicine*, 342, 246–248.

Tanaka, M.M., Small, P.M., Salamon, H. & Feldman, M.W. (2000). The dynamics of repeated elements: applications to the epidemiology of tuberculosis. *PNAS*, 97, 3532–3537.

Taylor, G.M., Crossey, M., Saldanha, J. & Waldron, T. (1996). DNA from *Mycobacterium tuberculosis* identified in mediaeval human skeletal remains using polymerase chain reaction. *J Archaeol Sci*, 23, 789–798.

Taylor, G.M., Goyal, M., Legge, A.J., Shaw, R.J. & Young, D. (1999). Genotypic analysis of *Mycobacterium tuberculosis* from medieval human remains. *Microbiology*, 145, 899–904.

Taylor, G.M., Young, D.B. & Mays, S.A. (2005). Genotypic analysis of the earliest known prehistoric case of tuberculosis in Britain. *J Clin Microbiol*, 43, 2236–2240.

Taylor, G.M., Watson, C.L., Bouwman, A.S., Lockwood, D.N.J. & Mays, S.A. (2006). Variable nucleotide tandem repeat (VNTR) typing of two paleopathological cases of lepromatous leprosy from Mediaeval England. *J Archaeol Sci*, 33, 1569-1579.

Taylor, G.M., Murphy, E., Hopkins, R., Rutland, P. & Chistov, Y. (2007). First report of *Mycobacterium bovis* DNA in human remains from the Iron Age. *Microbiology*, 153, 1243–1249.

Taylor, G.M., Blau, S., Mays, S., Monot, M., Lee, O.Y.-C., Minnikin, D.E., Besra, G.S., Cole S.T. & Rutland, P. (2009). *Mycobacterium leprae* genotype amplified from an archaeological case of lepromatous leprosy in Central Asia. *J Archaeol Sci,* 36, 2408-2414.

Taylor, G.M., Mays, S.A. & Huggett, J.F. (2010). Ancient DNA (aDNA) studies of man and microbes: general similarities, specific differences. *Int J Osteoarchaeol*, 20, 747–751.

Tuross, N. (1994). The biochemistry of ancient DNA in bone. *Experientia*, 50, 530–535.

Watanabe, M., Aoyagi, Y., Ridell, M. & Minnikin, D.E. (2001). Separation and characterization of individual mycolic acids in representative mycobacteria. *Microbiology*, 147, 1825-1837.

Watanabe, M., Aoyagi, Y., Mitome, H., Fujita, T., Naoki, H., Ridell, M. & Minnikin, D.E. (2002). Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids. *Microbiology,* 148, 1881-1902.

Weed, L.A. & Baggenstoss, A.H. (1951). The isolation of pathogens from tissues of embalmed human bodies. *Am J Clin Pathol*, 21, 1114-1120.

Wilbur, A.K., Bouwman, A.S., Stone, A.C., Roberts, C.A., Pfister, L.-A., Buikstra, J.E. & Brown, T.A. (2009) Deficiencies and challenges in the study of ancient tuberculosis DNA. *J Archaeol Sci*, 36, 1990–1997.

Winters, M., Barta, J.L., Monroe, C. & Kemp, B.M. (2010). To clone or not to clone: method analysis for retrieving consensus sequences in ancient DNA samples. *PLoS ONE*, 6, e21247 (8 pages).

Wirth, T., Hildebrand, F., Allix-Béguec, C., Wölbeling, F., Kubica, T., Kremer, K., van Soolingen, D., Rüsch-Gerdes, S., Locht, C., Brisse, S., Meyer, A., Supply, P. & Niemann, S. (2008). Origin, spread and demography of the *Mycobacterium tuberculosis* complex. *PLoS Pathogens*, 4, e1000160 (10 pages).

World Health Organisation (2010). Tuberculosis. Fact sheet 104. Accessed 1st August 2011. Available from: <http://www.who.int/mediacentre/factsheets/fs104/en/>

Xarchus, K.C. & Bourandas, J. (2003). Injuries and diseases of the spine in the ancient times. *Spine*, 28, 1481–1484.

Zink, A., Haas, C.J., Reischl, U., Szeimies, U. & Nerlich, A.G. (2001). Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *J Med Microbiol*, 50, 355–366.

Zink, A.R., Grabner, W., Reischl, U., Wolf, H. & Nerlich, A.G. (2003a). Molecular study on human tuberculosis in three geographically distinct and time delineated populations from ancient Egypt. *Epidemiol Infect*, 130, 239–249.

Zink, A.R., Sola, C., Reischl, U., Grabner, W., Rastogi, N., Wolf, H. & Nerlich, A.G. (2003b). Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping. *J Clin Microbiol*, 41, 359–367.

Zink, A.R. & Nerlich, A.G. (2004). Molecular strain identification of the *Mycobacterium tuberculosis* complex in archival tissue samples. *J Clin Pathol*, 57, 1185–1192.

Zink, A.R., Sola, C., Reischl, U., Grabner, W., Rastogi, N., Wolf, H. & Nerlich, A.G. (2004). Molecular identification and characterization of *Mycobacterium tuberculosis* complex in ancient Egyptian mummies. *Int J Osteoarchaeol*, 14, 404–413.

Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G. & Daffé, M. (2008). Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *J Bacteriol,* 190, 5672-5680.

2

Genomic Variability of *Mycobacterium tuberculosis*

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1. Introduction

Genomic variability provides the basis for adaptation and evolution and constitutes a fascinating aspect of the metabolically and phylogenetically diverse microbial world. Variability in bacteria has been extensively studied both because it enables inferring evolutionary relationships and because it plays an important role in host-pathogen interactions. Microbiologists, who have long struggled with species classification, have gained a more recent appreciation of the level of genetic diversity in microorganisms that has led to new awareness of what may constitute a bacterial "species" (Doolittle & Zhaxybayeva, 2009). In the clinical setting, genomic variability can represent a significant barrier to treatment. Many pathogens can acquire mutations or foreign genetic material through horizontal gene transfer (HGT) in response to the selective pressure imposed by the host immune system and by chemotherapy (Hawkey & Jones, 2009, Sampson, 2011), resulting in strains that are difficult to eradicate in hospitals as well as during long-term infection. Understanding the extent of genomic variability and its effects on disease in the case of pathogens that display genetic homogeneity and low variability, as is the case for the causative agent of tuberculosis, *Mycobacterium tuberculosis*, is particularly fascinating. The success of *M. tuberculosis* is intimately tied to the infectious process and its interaction with the human host, which is believed to have resulted from a long process of co-evolution (Donoghue, 2009, Gutierrez *et al.*, 2005). As a result, *M. tuberculosis* is capable of subverting the immune response and persisting as a latent form within an individual and for millennia within the human population.

Despite the availability of chemotherapy and the continued efforts to control the disease, tuberculosis continues to be one of the top ten causes of morbidity and mortality worldwide, with approximately 9 million cases per year, according to the World Health Organization (Lawn & Zumla, 2011). In spite of the growing interest and continued efforts, there are still significant gaps in our knowledge regarding both the pathogen and its interaction with the host that hamper control strategies. The appearance and spread of multi-drug (MDR) as well as extensively drug resistant (XDR) strains of *M. tuberculosis* represent a growing threat worldwide and underscore the importance of effective diagnosis and treatment. Given the

burden to public health and the complexity of the disease, an effective control of tuberculosis must involve diverse approaches and will require a better understanding of the host as well as of the environmental and bacterial factors that govern disease outcome.

M. tuberculosis belongs to the *Mycobacterium tuberculosis* Complex (MTBC), a group of slow-growing mycobacteria that are closely related at the DNA level and share identical 16S rRNA gene sequences but that differ in terms of phenotypes and host preference (Brosch *et al.*, 2001, Sreevatsan *et al.*, 1997). The MTBC includes the human-adapted strains *M. tuberculosis*, *Mycobacterium africanum* and *Mycobacterium canneti*, being *M. cannetti* the most divergent within the MTBC complex (Gutierrez et al., 2005). The MTBC also includes animal-adapted strains. *M. bovis* has a wider host range and is the main cause of tuberculosis in other animal species. *M. microti* is a pathogen of rodents, *M. pinnipedii* causes disease in sea lions and seals, *M. caprae* is a pathogen of goats and, recently, "*M. mungi*" was isolated from mongoose (Alexander *et al.*, 2010, Mostowy & Behr, 2005). The high similarity at the DNA level suggests that this group could have resulted from a bottleneck event that led to the expansion of a successful clone that then gave rise to different host-adapted ecotypes of the same species (Smith *et al.*, 2006).

Understanding the differences that underlie the biology and evolution of the MTBC has been the focus of considerable work (Smith *et al.*, 2009, Comas & Gagneux, 2009). Members of the MTBC have a highly clonal population structure where recent events of HGT are essentially absent (Supply et al., 2003, Gutierrez et al., 2005). This contrasts with many other microorganisms where horizontally acquired genetic material can play important roles in acquisition of novel virulence determinants and properties such as antibiotic resistance and the capacity to exploit different environmental niches. Recent surveys using MTBC strains that are more representative of global isolates, as well as advances in genome sequence analysis, have indicated, however, that there is more variation than previously anticipated and that this variation can be used to both distinguish isolates as well as to trace phylogenetic lineages (Hershberg *et al.*, 2008).

A greater knowledge of the diversity present in *M. tuberculosis* and MTBC strains can also lead to deeper understanding of the biological consequences associated with strain variability. The variation in circulating *M. tuberculosis* isolates has been critical for identification of strains, outbreaks and changes within the population. It has also in some cases been associated with phenotypic properties that are relevant in terms of the disease, such as transmission potential, immunological response and manifestation of the disease (Nicol & Wilkinson, 2008). However, the link between genotypic and phenotypic properties is not necessarily evident given the complexity of the host-pathogen interaction and the effect of environmental factors. In this context, a deeper understanding of the population structure and dynamics of new clonal lineages, with mutations that contribute to a particular lineage's success, can provide great insight regarding the appearance and spread of strain variants relevant to public health and to the control, treatment and eradication of tuberculosis.

This chapter will provide an overview of recent studies regarding genetic variability in *M. tuberculosis*. This will include a brief description of the importance of variability for the study of the evolution of the MTBC. Also, we will address the mechanisms of genomic variation in a pathogen characterized by genetic homogeneity and inappreciable HGT by

illustrating how genomic variability can emerge as a consequence of mutations that result in Single Nucleotide Polymorphisms (SNPs) and Large Sequence Polymorphisms (LSPs), namely insertions and deletions. We will then discuss the importance of variability in disease outcome.

2. Genetic diversity and phylogeny of *M. tuberculosis*

The availability of the complete *M. tuberculosis* genome sequence (Cole *et al.*, 1998) opened new ways to conduct studies and to understand the evolution of the closely related MTBC strains. By using Bacterial Artificial Chromosomes (BAC) libraries it was shown that seven loci were deleted in *M. bovis* with respect to *M. tuberculosis,* reinforcing previous studies indicating that these strains probably originated from a common ancestor (Gordon *et al.*, 1999, Sreevatsan et al., 1997). This was more fully appreciated by comparative genomics studies (Brosch *et al.*, 2002) that also divided the *M. tuberculosis* strains into "ancient" and "modern" based on a deletion known as TbD1 in the modern strains. Several molecular markers have been developed to type strains and infer phylogenetic relationships. Some of these are considered more useful for epidemiological studies, such as transmission, re-infection and/or reactivation, while others are considered more robust phylogenetic markers that can help to decipher the evolution of *M. tuberculosis*. The methods used for epidemiology include restriction fragment length polymorphism (RFLP) of IS*6110* sites (van Embden *et al.*, 1993, van Soolingen *et al.*, 1993), spoligotyping to identify unique spacers within the Clustered Regulatory Short Palindromic Repeats (CRISPR) or Direct Repeat (DR) region (van Embden *et al.*, 2000, Brudey *et al.*, 2006, Kamerbeek *et al.*, 1997), and the identification of Variable Number of Tandem Repeats-Mycobacterial Interspersed Repetitive Units (MIRUs-VNTR) that are strain-specific repeats of short DNA sequences at different positions of the chromosome (Supply et al., 2003). Molecular markers that provide more robust phylogenetic information and have helped to shape the evolutionary scenario of *M. tuberculosis* include LSP, SNPs and Multilocus Sequence Analysis (MLSA) (Filliol *et al.*, 2006, Gagneux *et al.*, 2006, Gutacker *et al.*, 2006, Comas *et al.*, 2009) (Figure 1). Although it has been argued that the use of RFLPs, spoligotyping and VNTR markers is highly prone to convergent evolution and thus to homoplasies (i.e., the same spoligotyping can be observed in strains belonging to different lineages), recent studies show that, at least for the main lineages, this does not seem to be the case (Kato-Maeda *et al.*, 2011). However, more studies are required to clarify this issue.

Based on our current view of its evolutionary history, *M. tuberculosis* can be divided into six phylogeographical lineages, which have been adapted to their local human populations (Figure 1). The use of different molecular makers, such as spoligotyping, LSPs and SNPs, can also classify the global population of MTB into comparable groups. For instance, Lineage 1 (Indo-Oceanic lineage) corresponds to the East African-Indian (EAI) family; Lineage 2 (East Asian Lineage) corresponds to the Beijing family; Lineage 3 or East African-Indian corresponds to the Central Asia (CAS) family; Lineage 4 is the Euro American Lineage that includes the Haarlem, LAM, X, T, S and Tuscany families; Lineage 5 (West African Lineage 1) and Lineage 6 (West African Lineage 2) correspond to AFRI 2 and AFRI 1, respectively, by spoligotyping (Sola *et al.*, 2001, Brudey et al., 2006). Based on the evidence accumulated from these studies, it has been suggested that *M. tuberculosis* evolved as a human pathogen in Africa, which is also the continent where all main *M. tuberculosis*

lineages have been isolated (Hershberg et al., 2008). Moreover, the "ancient" lineages described by Brosh (2002) are present in West Africa and the spread of the "modern" lineages are associated with the human migration out of the African continent (Wirth *et al.*, 2008, Hershberg et al., 2008).

Phylogenetic studies have also shown that clinical strains of *M. tuberculosis* are more genetically variable than originally expected (Hirsh *et al.*, 2004, Gagneux et al., 2006, Hershberg et al., 2008). Moreover, genetic variability can be translated into phenotypic differences, such as transmission capacity, virulence and pathogenicity that can have epidemiological consequences and affect the outcome of the disease. Although there are few studies showing a clear association between lineage and transmission capacity it is now clear that Lineage 2 (Beijing family) *M. tuberculosis* has spread globally more than any other lineage (Parwati *et al.*, 2010). The use of spoligotyping to type paraffin-embedded strains obtained from tuberculosis patients in different time periods has also shown an increase of this genotype over time in Africa. Its isolation from children, which is a measure of recent transmission, increased from 13% in 2000 to 33% in 2003 in South Africa (Cowley *et al.*, 2008). The capacity of the Beijing genotype to spread more than other lineages is not completely understood but it has been suggested that factors contributing to its expansion could involve the selective pressure imposed by BCG vaccination and drug treatment (Parwati et al., 2010).

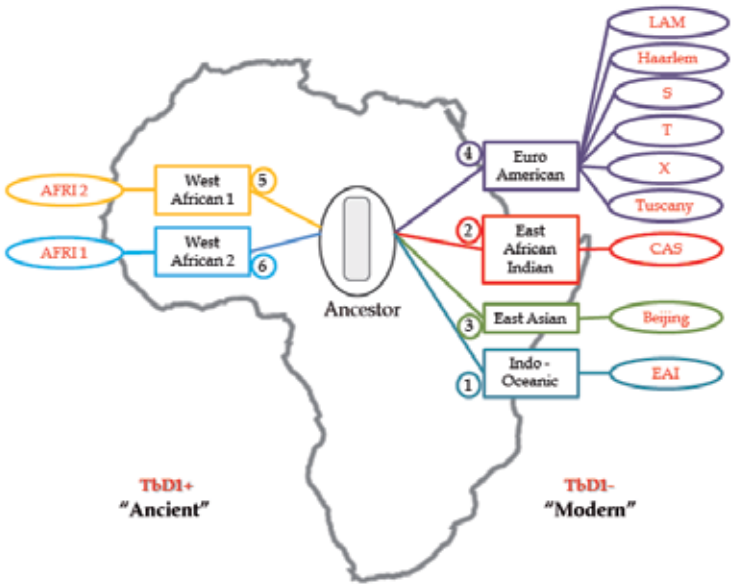

Fig. 1. Schematic representation of the phylogeography of *M. tuberculosis*. squares indicate the 6 main Lineages and circles are representative of the spoligotype families

The successful transmission of particular strains is not limited to the Beijing genotype. In a recent study, where guinea pigs were exposed to air from a HIV-tuberculosis ward, one non-Beijing strain was shown to be responsible for most of the secondary infections observed (Escombe *et al.*, 2008). In addition to transmission capacity, it is also currently accepted that genetically different *M. tuberculosis* strains produce markedly different

immuno-pathological events and affect disease manifestation. For example, in a study conducted in Vietnamese patients, a clear association between the Euro American Lineages of *M. tuberculosis* and pulmonary rather than meningeal tuberculosis was observed, suggesting these strains are less capable of extra-pulmonary dissemination than other strains in the study population (Caws *et al.*, 2008). In a study using a cohort of patients and household contacts in Gambia, both *M. africanum* and *M. tuberculosis* were equally transmitted to the household contacts but *M. tuberculosis* Beijing strains were most likely to progress to disease (de Jong *et al.*, 2008). Another source of evidence came from a recent study associating Lineages 1, 5 and 6, with a higher pro-inflammatory cytokine response when compared with the modern Lineages 2, 3 and 4 (Portevin *et al.*, 2011).

3. SNPs in *M. tuberculosis*

Genetic diversity within bacterial species is usually generated by mutations and by the exchange of genetic material. The process of HGT is thought to be an important driver of bacterial evolution in both pathogenic and non-pathogenic bacteria (Becq *et al.*, 2007). Horizontally transferred genes can be acquired in clusters known as genomic islands or pathogenicity islands that can be identified by characteristics that distinguish them from the host genome, such as GC content, flanking nucleotide repeats and insertion elements. In the case of *M. tuberculosis*, there is evidence of ancient gene transfer events that could have taken place in a progenitor tubercle bacilli pool before the clonal expansion that gave rise to the MTBC (Gutierrez et al., 2005). One of these events involved the Rv0986-8 virulence operon (Rosas-Magallanes *et al.*, 2006) that could have originated from genetic exchange between an environmental bacillus ancestor and other bacterial species (Nicol & Wilkinson, 2008). In the absence of recent events of HGT, modern *M. tuberculosis* lineages evolve essentially by mutations that alter its genome, resulting in SNPs and LSPs, such as deletions and insertions, the latter mainly mediated by transposition of the IS*6110* insertion element.

Although allelic variation in MTBC organisms is quite restricted when compared with other pathogenic bacteria (Sreevatsan et al., 1997), there is a growing recognition that there is substantial genetic diversity among isolates. At the level of SNPs changes can be either synonymous (sSNP) or non-synonymous (nsSNP) and this diversity has been undeniably useful for typing and defining evolutionary relationships among strains. SNPs provide many advantages for the analysis of phylogenetic relationships among microorganisms, especially among closely related clonal organisms such as the MTBC. Initial descriptions of the *M. tuberculosis* population structure involved analysis of SNPs in the *katG* and *gyrA* genes and defined three major genetic groups (Sreevatsan et al., 1997). Later surveys have extended this strategy to include more than 100 sSNPs identified in 112 *M. tuberculosis* isolates (Gutacker et al., 2002). In more recent work using 159 sSNPs identified by whole-genome comparison of sequenced strains, it was possible to classify 212 isolates into 56 haplotypes that grouped strains into six *M. tuberculosis* SNP Cluster Groups (SCG) and one SCG that grouped all the *M. bovis* strains (Filliol et al., 2006). A re-evaluation of the SNP phylogeny was obtained by using *de novo* sequencing of 89 randomly distributed genes in 108 global strains (Comas et al., 2009). This study suggested that initial classification could be done using a subset of discriminatory SNPs and then, if further molecular characterization were needed, a MIRU-VNTR typing technique could be applied to differentiate individual strains. However, the choice of discriminatory SNPs is not an easy

task. For example, SNPs comparison in 32 fully sequenced strains that caused an outbreak in a community in Canada, allowed the identification of two co-circulating "lineages" with the same MIRU-VNTR profile (Gardy *et al.*, 2011), which would not have been evident if only the discriminatory SNPs used previously had been included. The study allowed tracking the transmission and demonstrated the power of coupling comparative genomics with social epidemiological studies.

Genetic variation at the SNP level can also have profound implications in strain fitness and disease outcome. One such case applies to the Esx protein family that has been implicated in host-pathogen interactions. To survey genetic diversity in the Esx family, and its potential for antigenic variation, all *esx* genes were sequenced from 108 clinical isolates of *M. tuberculosis* belonging to different clades. The SNP distribution affecting Esx proteins indicated high genetic variability and a total of 109 unique SNPs, 59 of which were non-synonymous. Some of the resultant amino acid substitutions affected known Esx epitopes likely to result in immune variation, thus revealing a dynamic *esx* gene family (Vasilyeva *et al.*, 2009).

Another important area of research focuses on variability associated with specific phenotypes of clinical importance, such as antibiotic resistance. In *M. tuberculosis,* resistance to antibiotics results essentially from mutations, such as SNPs, that can be acquired during treatment and can spread within the population. The mutations conferring antibiotic resistance can have a variable effect on strain fitness and bacteria can develop compensatory mechanisms to recover fitness capacity (Borrell & Gagneux, 2011). Isoniazid (INH) resistance in *M. tuberculosis* is associated with mutations in the genes *katG*, *inhA* and *ahpC*. Most identified mutations map to *katG*, which encodes the catalase-peroxidase required to activate INH (Ramaswamy & Musser, 1998) and to protect *M. tuberculosis* from the oxidative free radicals in the macrophage. Thus *M. tuberculosis* INH resistant strains are less virulent (Pym et al, 2002). However, the katG^{S315T} mutation, the most common mutation for INH resistance (Sandgren *et al.*, 2009), results in reduced INH activation while maintaining KatG activity and virulence in mice (Pym *et al.*, 2002) suggesting compensatory evolution as has been suggested in other bacteria (Maisnier-Patin & Andersson, 2004). If compensatory evolution occurs in MDR and XDR strains it will have deep impacts in the control of tuberculosis (Borrell & Gagneux, 2011), an area that must be further investigated.

The identification of SNPs associated with resistance has also indicated the existence of multiple gene determinants for resistance, not all of which have been fully identified. Streptomycin (Sm) resistance, for example, is associated in the majority of cases with mutations in *rpsL* and *rrs* (Sreevatsan et al., 1997). However, 27% of Sm-resistant strains lack mutations in these genes. There is evidence that in some cases mutations in *gidB,* a gene coding for a 7-methylguanosine (m7G) methyltransferase specific for 16S rDNA, are associated with low level of Sm resistance (Donoghue, 2011). However, some susceptible strains also contain such mutations, thus requiring sequence analysis of more *M. tuberculosis* clinical isolates to better understand the role of *gid*B gene mutations in Sm resistance.

A longstanding question in tuberculosis has been the precise mechanisms by which mycobacteria can acquire resistant mutations, especially during latent infections. The mutation rates that confer antibiotic resistance have been determined *in vitro*, yet the slow growth and different metabolic states of *M. tuberculosis* during infection make it difficult to

assess the *in vivo* rates. This was achieved in a recent report, however, using whole genome sequencing and identification of SNPs generated during different disease states in macaque monkeys (Ford *et al.*, 2011). Similar mutation rates were observed during latency and during active disease, and these were also consistent with *in vitro* rates. Based on these results and on the types of SNPs observed, it was suggested that *M. tuberculosis* can acquire mutations during latency and that these mutations are the result of oxidative DNA damage rather than errors in replication. This could be explained by increased oxidative damage during latency, as a result of the immune response, or by diminished DNA repair in metabolically quiescent bacilli (Ford *et al.*, 2011).

The identification of SNPs in *M. tuberculosis* has provided important insight regarding genetic variability and evolution of this pathogen. SNPs can also impact strain fitness, as is evident by the acquisition of antibiotic resistance markers. It remains to be seen if many of the identified SNPs have an effect on the biology of *M. tuberculosis* and the host-pathogen interaction. Whole genome sequencing will undoubtedly allow more extensive SNP identification and analysis on a genome-wide scale. As more sequence data becomes available, comparative genomics studies may help to identify markers that can contribute to our understanding of the molecular mechanisms underlying phenotypes such as drug resistance and persistence.

4. Large Sequence Polymorphisms

LSPs can include both insertions and deletions (indels) and have been identified as one of the main sources of genomic variability in *M. tuberculosis*. The effect of LSPs can vary and may provide insights into the biology of *M. tuberculosis* strains. Large deletions have been shown to group closely related strains and have been associated with phylogeographical lineages, suggesting that a deletion event is specific to a particular lineage (Tsolaki *et al.*, 2004). Some LSPs occur rarely in the population and could have arisen from random genomic events and then become associated with a particular phylogenetic lineage (Alland *et al.*, 2007). In contrast, other LSPs are present in multiple strains from different lineages, as a result of selective pressure, and are not necessarily associated with particular groups (Alland et al., 2007).

Soon after completing the genome sequence of the laboratory strain H37Rv (Cole et al., 1998), the clinical isolate, strain CDC1551 that had caused an outbreak in the United States, was sequenced (Fleischmann *et al.*, 2002). A whole genome comparative study carried out using these two genomic sequences identified 1,075 SNPs and 86 LSPs larger than 10 bp. The analysis of these LSPs using a panel of 169 clinical isolates, showed that clinical strains were genetically more variable than expected from a clonal bacterial population (Fleischmann et al., 2002).

The continued advances in methods for high-throughput nucleic acid sequencing now allow more rapid generation of sequence data and thus access to information from a growing number of sequenced clinical *M. tuberculosis* genomes. Up to now, there are more than 200 on-going sequencing projects of *M. tuberculosis* strains with different characteristics, such as strains with epidemic potential and strains characterized by multidrug resistance, as well as isolates obtained before and after a passage through an immunocompetent animal model, among others (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). This information,

together with new bioinformatics algorithms will be an invaluable resource for probing these genomes in an effort to further understand the evolution, epidemiology, emergence of drug resistance and phenotypic variability associated with tuberculosis disease.

4.1 Comparative genomics to assess variability

With the growing number of sequenced strains becoming available, the comparison of complete genomes from different clinical isolates of *M. tuberculosis* becomes an attractive and powerful tool to explore genotypic similarities and differences. This approach can also provide important insights regarding the genotype-phenotype relationship in *M. tuberculosis,* and can therefore contribute to the development of control measures for tuberculosis. In this respect, we carried out whole genome comparisons of six fully sequenced *M. tuberculosis* strains, four clinical isolates and two laboratory strains which showed high synteny, as expected, and no large rearrangements (Cubillos-Ruiz *et al*., 2008), except for a large inversion seen in the KZN strain that could be due to sequencing errors and incomplete data (Figure 2). Most of the 1,428 LSPs identified were indels involving 120 genes that affected primarily 1) mobile genetic elements such as insertion sequences and prophages, 2) non-coding regions, and 3) the PE/PPE family of genes. The LSPs identified in this work differed among strains, were distributed along the entire genome and were used to identify strain-specific insertions and deletions. When fitted to an exponential decay function these data indicated a tendency towards accumulation of more deletions than insertions, consistent with the notion of genome decay in *M. tuberculosis* (Cubillos-Ruiz et al., 2008). One other remarkable finding was that laboratory strains contained less strain specific polymorphisms than the clinical isolates, suggesting that the selective pressure imposed by the human immune system could be driving variability. The existence of strain-specific polymorphisms also opened the possibility that specific indels could be associated with particular lineages and thus could also be used as markers for strain typing and surveillance.

Taking into account the growing evidence of the phylogeographical origin of *M. tuberculosis* (Gagneux et al., 2006, Wirth et al., 2008), we speculated that the strain-specific polymorphisms could be common to strains of a particular lineage rather than being an exclusive property of one particular isolate. To test this hypothesis, we evaluated strain-specific indels and previously identified SNPs associated with strains of the Haarlem lineage using a large panel of well-characterized *M. tuberculosis* strains (Olano et al, 2008, Cubillos-Ruiz et al., 2008). Six large deletions, two specific IS*6110* insertions and two SNPs were significantly associated with the Haarlem family and thus proposed as genomic signatures of this lineage (Cubillos-Ruiz *et al*., 2010). These results were completely congruent with spoligotyping and with RFLP data, as well as with the new assignation of a URAL family instead of the Haarlem 4 sublineage (Abadia *et al*., 2010). One particularly interesting result was the identification of deletions that affected previously proposed drug targets. These include the gene Rv1354c, which encodes a diguanylate cyclase (DGC) enzyme involved in regulating the levels of c-di-GMP, a bacterial second messenger implicated in survival and adaptation to different environmental conditions (Gupta *et al*., 2010), and gene Rv2275 that codes for a cytochrome P450, Cyp121 (McLean & Munro, 2008). Both of these genes were deleted in the Haarlem strains analyzed, indicating that they would not be adequate targets for antimicrobials. Although this particular study was limited to Haarlem strains, it raises the possibility that other lineage-specific genomic differences might impact treatment and

control. More studies will be needed to address this issue and to verify the presence of specific gene targets.

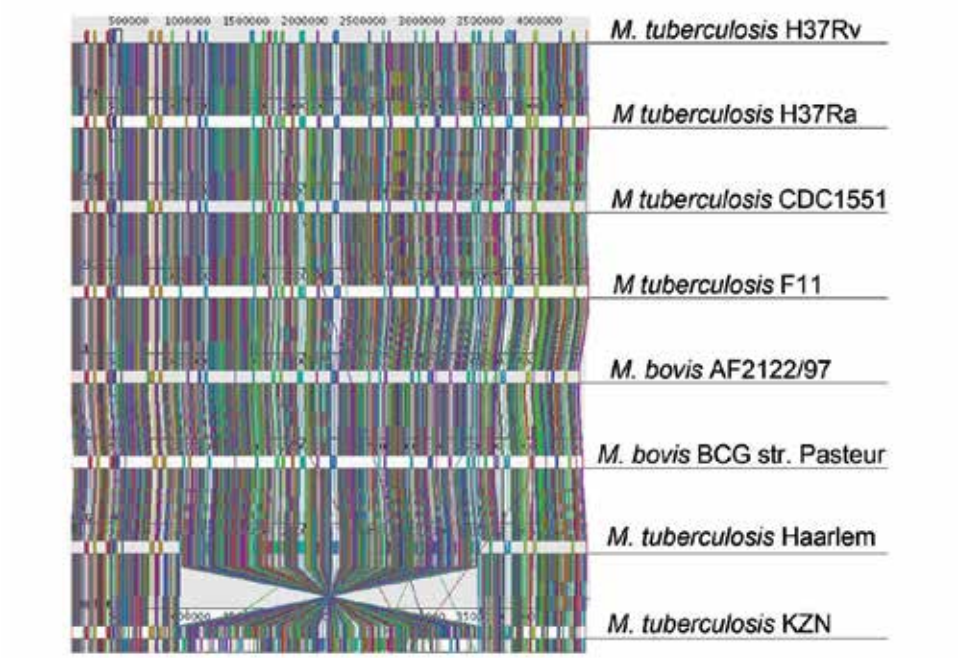

Fig. 2. Whole genome alignment generated with the MAUVE software, showing synteny and, in the case of strain KZN, the presence of a genomic inversion.

5. Insertions

The presence of insertion elements in different bacteria has been well appreciated for some time, especially because of the impact they can have on the host genome (Siguier *et al.*, 2006). Insertion elements can not only re-shape the genome but can also cause mutations and alter gene expression. In the case of pathogens such as *M. tuberculosis* the presence of insertion elements can generate genotypic variation and mediate changes that can affect gene function. This variability can therefore alter properties such as strain fitness and transmissibility and even play a role in the evolution of *M. tuberculosis*.

The insertion elements present in the *M. tuberculosis* genome were described in detail upon completion of the *M. tuberculosis* H37Rv whole genome sequence (Gordon et al., 1999). *M. tuberculosis* harbors four main insertion elements, IS*6110*, IS*1081*, IS*1547* and the IS-like element, all of them present in multiple copies. The best studied of these is the 1.36Kb IS*6110*, originally described by Thierry et al. in 1990 (Thierry *et al.*, 1990), which belongs to the group of IS*3* elements and is characterized by having two partially overlapping open reading frames that allow production of a transposase by translational frameshifting (McEvoy *et al.*, 2007). It also has 28 bp imperfect terminal inverted repeats and generates 3- to 4 bp direct repeats upon insertion (McAdam *et al.*, 1990, Thierry *et al.*, 1990, Mendiola *et al.*, 1992). The IS*6110* element is present exclusively in strains of the MTBC that can harbor

from zero to 25 copies per genome (Brosch *et al.*, 2000). For this reason and due to its high degree of copy number and insertion site variation, IS*6110*- RFLP has been widely used for epidemiological purposes and is considered the "gold standard" to study the transmission dynamics of *M. tuberculosi*s (van Embden *et al.*, 1993, Small *et al.*, 1994, Safi *et al.*, 1997). The discriminatory power of the IS*6110*-RFLP method depends on there being sufficient variation and copy number to differentiate between unlinked isolates while allowing identification of specimens that are related (Wall *et al.*, 1999). Thus IS*6110*-RFLP is used to distinguish between epidemiological events but its use as marker for strain evolution is still under debate (McEvoy et al., 2007).

The consequence of IS*6110* transposition can differ depending on the position of integration, with phenotypic outcomes ranging from lethality to its bacterial host due to gene inactivation to possible benefits. There are four mutational events that can be generated by IS*6110* transposition: 1) Integration in intragenic regions; 2) Alteration of IS*6110* flanking regions; 3) Recombination/gene deletion; 4) Alteration of IS*6110* promoter activity (McEvoy et al., 2007). Intragenic insertions interrupt open reading frames and can inactivate genes; this is the most frequently described event in certain clinical isolates. For these interruptions to be observed they must occur in genes that are dispensable for survival of the bacterium or are redundant in function. These insertion events can also alter immune recognition or virulence properties, as has been suggested for insertions in members of the PPE gene family or in the phospholipase C gene region (McEvoy *et al.*, 2009a, Vera-Cabrera *et al.*, 2001). It has also been observed that the regions flanking an IS*6110* insertion contain additional mutations, suggesting that this element can have a disruptive effect on the DNA region of insertion that results in mutations (Warren *et al.*, 2000).

Insertion elements can mediate deletions, as has also been shown for *M. tuberculosis,* where gene deletion can occur by homologous recombination between two flanking copies of IS*6110*. For example, deletion of the *plcA* gene in clinical *M. tuberculosis* strains displays a decrease capacity to cause pulmonary cavitation, clearly showing the phenotypic effects of transposition in a clinical setting (Kato-Maeda *et al.*, 2001a). Not all the insertions described in clinical isolates have deleterious or silent effects on the mycobacterial cell; some studies have reported that IS*6110* can up-regulate expression of downstream genes from an outward-directed promoter at its 3' end, conferring selective advantages. In particular, an insertion found within the *phoP* promoter region in an MDR *M.bovis* strain, which had produced outbreaks in the United States and Spain (Rivero *et al.*, 2001), was shown to increase *phoP* expression 10-fold in *M. smegmatis* and was proposed to be responsible for the high transmissibility levels of the original *M. bovis* isolate (Soto *et al.*, 2004).

Given the high variability of IS*6110* elements in the genomes of MTBC strains and the possible consequences of insertion on strain phenotype, there has been an interest in identifying the precise insertion locations in *M. tuberculosis* clinical isolates. Different methodologies developed, based on PCR, sequencing and cloning, have suggested that the IS*6110* element inserts preferentially into non-coding regions (Otal *et al.*, 2008, Warren et al., 2000, Thorne *et al.*, 2011, Kim *et al.*, 2010, McEvoy *et al.*, 2009b, Wall et al., 1999). This can be explained by the fact that insertions in functional genes essential for strain growth, maintenance and pathogen integrity would be harmful to the cell and thus, not maintained in the population. Preferential insertion loci or hotspots have also been identified, some of

which include the phospholipase C region (Vera-Cabrera et al., 2001), members of the PPE gene family (McEvoy et al., 2009a), the *dnaA - dnaN* intergenic region (Turcios *et al*., 2009), the RD724 gene (Kim *et al*., 2010) and insertion into the IS*1547* element (Fang *et al*., 1999). PPE genes are considered to be important antigens during the host-pathogen interaction and have been proposed to play a role in evasion of the immune response (Sampson, 2011). Thus variability in the PPE genes generated through IS*6110* transposition could help to evade the immune system during infection and confer advantage to strains. In contrast to these hotspot regions, some loci where integration is rare or not observed have also been identified and these represent sites where *in vivo* transposition events can be harmful to strain fitness and growth (Yesilkaya *et al*., 2005).

We recently developed a novel high-throughput method using next-generation sequencing to identify the flanking regions of the IS*6110* insertion element in over 500 *M. tuberculosis* isolates mainly from Latin-America and Europe. In this study we identified previously reported hotspot regions of insertion as well as novel sites (Table 1) (Reyes et al., submitted).

Locus	Gene ID	Description	# strains	# independent sites	Hotspots
MT3426:MT3427 (RvD5)	MT3426: moaA-3	Probable conserved membrane protein	195	1	A
Rv0403c	mmpS1		225	6	A*, H
Rv0835:Rv0836c	lpqQ: Rv0836c		227	5	A
Rv1754c	-	Conserved hypothetical protein	394	4	A*
Rv2336	-	Hypothetical protein	188	2	A*
Rv2814c-Rv2815c	-	IS6110, transposase	485	2	A*
Rv3113	-	Possible phosphatase	218	2	A

Table 1. Hotspot insertion sites in *M. tuberculosis* identified by high-throughput sequencing (Reyes et al., submitted). Hotspots (H) or Ancestral (A) insertions for a given lineage; A* indicates an ancestral insertion in a locus in that more than one lineage.

The copy number of IS*6110* elements in the genomes of circulating *M. tuberculosis* strains can be highly variable and is ultimately limited by the deleterious effects of IS*6110* transposition (McEvoy et al., 2007). Although most *M. tuberculosis* isolates have multiple copies of the IS*6110* element, the presence of a copy in the DR region of the MTBC strains suggests that this could be an ancestral insertion site. It has also been observed that some successful *M. tuberculosis* strains tend to have a high copy number of the IS*6110* element and that this might correlate with phenotypic properties (Alonso *et al*., 2011). In a recent report, a Beijing family strain considered to have a high transmissibility rate was found to have 19 copies of the IS*6110* element, four of which were shown to up-regulate downstream gene expression. One of these was in the gene Rv2179c, which is normally expressed inside macrophages, suggesting that this gene could influence the infectious process and that the strain's high degree of transmissibility could be due to the up-regulation caused by the IS*6110* insertion (Alonso *et al*., 2011). However, some clinical strains and MTBC members with a low number

of copies of the IS*6110* are also epidemiologically successful. In general, though, there is still insufficient information regarding the factors that influence the frequency of transposition, such as the genomic context of the insertion element within a particular strain background. The variation in the number of IS*6110* elements among *M. tuberculosis* isolates also raises the possibility that copy number is the result of the evolution of particular lineages as strains cope with IS*6110* transposition and its resulting genetic variability, and in some cases even selecting for phenotypically favorable events, while keeping genome integrity and avoiding deleterious effects.

6. Implication of variability on disease control

From the pool of individuals that come in contact with and are infected with *M. tuberculosis*, only about 10% will develop disease. The manifestation of the disease in these individuals, however, can vary greatly from a self-limited infection in the lungs to extra-pulmonary and disseminated cases (Nicol & Wilkinson, 2008). The outcome of infection must therefore be influenced both by host factors that may predispose to infection, and to genetic variation in the tubercle bacillus itself. Several host factors have been associated with risk for disease, such as malnutrition, vitamin D deficiency, NRAMP1 polymorphisms, diabetes and co-infection with HIV (Malik & Godfrey-Faussett, 2005). A recent study analysing the impact of pathogen variability in recombinant congenic mice indicated that host control of the infection varied depending on the infecting strain and the stage of infection. The dynamic response to disease suggests that in addition to host genetic determinants, the pathogen background also influences the outcome of infection (Di Pietrantonio *et al.*, 2010). Studies with both laboratory and clinical strains have also suggested a correlation between strain genotype and the infectious process. This correlation, however, has been difficult to resolve in great part due to the difficulty associated with working with this slow-growing pathogen and to problems associated with extrapolation from animal models (Nicol & Wilkinson, 2008). The integration of genomics and epidemiological data has been able to link some cases of genetic variability with strain phenotypic characteristics. By analysing deletions in clinical isolates it was suggested, for example, that strains causing cavitary disease had fewer deletions, indicating that the accumulation of mutations affected pathogenesis (Kato-Maeda *et al.*, 2001b). Mutations that altered the PE_PGRS33 protein, which may be involved in cell-cell interactions and antigenic variation, have also been connected with clustering and pathogenesis and thus with clinical and epidemiological characteristics of *M. tuberculosis* isolates (Talarico *et al.*, 2007). Similarly, an analysis of the genetic variation at the *plcD* locus indicated that variability in this region was possibly associated with pathogenesis and disease manifestation (Yang *et al.*, 2005). Studies involving strains that cause pulmonary and extra-pulmonary infections have also indicated that extra-respiratory strains were more efficient at infecting macrophages and could also have higher infectivity *in vivo* (Garcia de Viedma et al., 2005).

The growing consensus that the main MTBC lineages are associated with geographic origin suggests co-evolution of lineages with their hosts and thus adaptation that must involve events of strain variation. More recent evidence of the restricted geographical niche of certain lineages came from an Ibero-America MDR *M. tuberculosis* survey showing that circulation of Latin American MDR strains was restricted to particular areas and also that transnational transmission was scarce (Ritacco *et al.*, 2011). The Beijing lineage, one of the most extensively studied families, has been responsible for several epidemic outbreaks and

in some cases has been associated with multidrug resistance (Hanekom *et al.*, 2011). Its capacity to spread within a population is evident from epidemiological studies and emphasizes the possibility that certain strain properties could contribute to this lineage's expansion in the population (Nicol & Wilkinson, 2008). The increased virulence of these isolates was associated with the production of a phenolic glycolipid (PGL) that affects the host immune response, and which is absent in many other *M. tuberculosis* families (Ordway *et al.*, 2007, Hanekom et al., 2011). More recent work suggests that although PGL can contribute to *M. tuberculosis* virulence, it probably requires additional bacterial factors (Sinsimer *et al.*, 2008). Other examples stem from studies of strains that have caused outbreaks, such as strains CDC1551 and HN878, the latter also a member of the Beijing family. In these and other studied cases, it appears that some of the effects observed have to do with the capacity of these strains to induce variable inflammatory responses (Coscolla & Gagneux, 2010). Despite these studies, many of the clinical outcomes associated with strain variability still need to be further examined, particularly in other *M. tuberculosis* lineages before precise genotypic variability can be associated with phenotypic differences.

The emergence and spread of drug-resistant strains is particularly disturbing and provides additional examples where strain variability can have a profound effect on disease outcome. One particularly alarming case was the epidemic caused by an XDR strain in the KwaZulu-Natal region of South Africa that resulted in high mortality, causing the death of 52 of the 53 patients co-infected with HIV in the course of 16 days (Gandhi *et al.*, 2006). To understand more about the dynamics of appearance and dispersion of this highly virulent KZN strain, whole genome sequence analysis was carried out for XDR, MDR and drug sensitive KZN strains. The results indicated that the outbreak was most probably due to clonal expansion of a single strain and that a particular strain genetic background did not necessarily contribute to acquisition of antibiotic resistance (Ioerger *et al.*, 2009). Further work will be needed to better understand this strain's virulence and transmissibility in the community.

Part of the success of *M. tuberculosis* as a human pathogen is due to its capacity to be efficiently transmitted between hosts and to persist for long periods of time despite the host's immune response. A recent study involving whole genome sequencing of 21 strains from the six main *M. tuberculosis* lineages indicated that human T cell epitopes had very little sequence variation and were highly conserved relative to the rest of the genome. It was suggested that these antigens, contrary to expectations, might be under purifying selection and be benefitting from host immune recognition (Comas *et al.*, 2010). This differs from the classical view of immune evasion due to the selective pressure imposed by the immune response and may indicate that new approaches should be considered for vaccine development and control of *M. tuberculosis*.

The genetic variability evident in strains of the MTBC bears relevance to control of tuberculosis since treatment must work against all circulating strains. Rapid and accessible diagnostics for both *M. tuberculosis* and drug resistant isolates are still required, as is the availability of a vaccine that can be universally effective, given the variable efficacy of the currently used BCG vaccine. There are now more that 10 vaccines under phase I trial and the hope is that in the near future at least one of these will prove to be safe and protective by containing *M. tuberculosis* and preventing reactivation. However, future strategies will need to address the need to prevent or eradicate latent infections, especially in view of additional factors affecting disease and the host immune response, such as co-infection with HIV

(Kaufmann, 2010). New and alternative drugs are also required to shorten the current duration of chemotherapy, to act against persistent bacilli and to counteract the spread of drug-resistant strains that frustrate global eradication programs. Due to renewed efforts in recent years, several novel drugs have been identified and are under clinical evaluation or being developed, many of which involve novel targets and mechanisms (Coxon & Dover, 2011). The discovery of novel drugs has involved different approaches that include the use of genomics to identify targets, whole-cell screening and re-engineering of known chemical molecules (Koul *et al.*, 2011). Given the observed strain variability it is nonetheless possible that some of these drugs might vary in efficiency in different strain backgrounds, as was made evident for DGC and Cyp121 in the Haarlem lineage (Cubillos-Ruiz et al., 2010). Thus the heterogeneity among different strains and lineages, as well as of the host-pathogen interaction, must be taken into account when developing novel diagnostics and therapeutic strategies. Extensive analysis of circulating *M. tuberculosis* populations will be required to address the efficacy of treatment and vaccination in different genetic backgrounds. The advent of novel massive sequencing techniques to generate genomic data for multiple strains will undoubtedly allow examination of whole genomes and make such analyses more feasible (Lin & Ottenhoff, 2008).

7. Concluding remarks

Over the last decades there has been a substantial increase in our understanding of the molecular bases of *M. tuberculosis* biology and its interaction with the host. However, the clinical and epidemiology consequences of *M. tuberculosis* infection are still poorly understood. Despite the restricted variability and clonality of the MTBC population, various studies make evident that circulating strains vary in terms of their genomic makeup and differ with respect to virulence and immunogenicity properties. The differences observed in the interactions between pathogen and host and in disease manifestation indicate that variation must play a role in disease and in clinical outcome, even though the extent of the impact of this strain diversity is still unclear (Coscolla & Gagneux, 2010). Thus, the precise role of bacterial factors and the importance of strain diversity in pathogenicity and tuberculosis disease remain elusive, partly due to the complex interplay between host and pathogen that is compounded by additional environmental factors. The strain-to-strain variation also has important consequences for the development of efficient control strategies. The development of new diagnostics tools, drugs and vaccines must somehow incorporate analysis of the differences that characterize host responses and strains, highlighting the importance of continued studies regarding the genetic makeup of circulating strains. The use of modern genetic and molecular tools, including the availability of massive sequencing techniques, can contribute significantly to our understanding of *M. tuberculosis* variability and its possible association with biological properties. Only by realizing the need to incorporate this added level of complexity to the study of tuberculosis, will we be able to tackle the intricacies of this disease and achieve an adequate level of control on a global scale.

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9. References

Abadia, E., J. Zhang, T. dos Vultos, V. Ritacco, K. Kremer, E. Aktas, T. Matsumoto, G. Refregier, D. van Soolingen, B. Gicquel & C. Sola, (2010) Resolving lineage assignation on *Mycobacterium tuberculosis* clinical isolates classified by spoligotyping with a new high-throughput 3R SNPs based method. *Infect Genet Evol* 10: 1066-1074.

Alexander, K. A., P. N. Laver, A. L. Michel, M. Williams, P. D. van Helden, R. M. Warren & N. C. Gey van Pittius, (2010) Novel *Mycobacterium tuberculosis* complex pathogen, M. mungi. *Emerg Infect Dis* 16: 1296-1299.

Alonso, H., J. I. Aguilo, S. Samper, J. A. Caminero, M. I. Campos-Herrero, B. Gicquel, R. Brosch, C. Martin & I. Otal, (2011) Deciphering the role of IS6110 in a highly transmissible *Mycobacterium tuberculosis* Beijing strain, GC1237. *Tuberculosis (Edinb)* 91: 117-126.

Alland, D., D. W. Lacher, M. H. Hazbon, A. S. Motiwala, W. Qi, R. D. Fleischmann & T. S. Whittam, (2007) Role of large sequence polymorphisms (LSPs) in generating genomic diversity among clinical isolates of *Mycobacterium tuberculosis* and the utility of LSPs in phylogenetic analysis. *J Clin Microbiol* 45: 39-46.

Becq, J., M. C. Gutierrez, V. Rosas-Magallanes, J. Rauzier, B. Gicquel, O. Neyrolles & P. Deschavanne, (2007) Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli. *Mol Biol Evol* 24: 1861-1871.

Borrell, S. & S. Gagneux, (2011) Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect* 17: 815-820.

Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen & S. T. Cole, (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci U S A* 99: 3684-3689.

Brosch, R., S. V. Gordon, A. Pym, K. Eiglmeier, T. Garnier & S. T. Cole, (2000) Comparative genomics of the mycobacteria. *Int J Med Microbiol* 290: 143-152.

Brosch, R., A. S. Pym, S. V. Gordon & S. T. Cole, (2001) The evolution of mycobacterial pathogenicity: clues from comparative genomics. *Trends Microbiol* 9: 452-458.

Brudey, K., I. Filliol, S. Ferdinand, V. Guernier, P. Duval, B. Maubert, C. Sola & N. Rastogi, (2006) Long-term population-based genotyping study of *Mycobacterium tuberculosis* complex isolates in the French departments of the Americas. *J Clin Microbiol* 44: 183-191.

Caws, M., G. Thwaites, S. Dunstan, T. R. Hawn, N. T. Lan, N. T. Thuong, K. Stepniewska, M. N. Huyen, N. D. Bang, T. H. Loc, S. Gagneux, D. van Soolingen, K. Kremer, M. van der Sande, P. Small, P. T. Anh, N. T. Chinh, H. T. Quy, N. T. Duyen, D. Q. Tho, N. T. Hieu, E. Torok, T. T. Hien, N. H. Dung, N. T. Nhu, P. M. Duy, N. van Vinh Chau & J. Farrar, (2008) The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*. *PLoS Pathog* 4: e1000034

Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin,

S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead & B. G. Barrell, (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537-544.

Comas, I., J. Chakravartti, P. M. Small, J. Galagan, S. Niemann, K. Kremer, J. D. Ernst & S. Gagneux, (2010) Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet* 42: 498-503.

Comas, I. & S. Gagneux, (2009) The past and future of tuberculosis research. *PLoS Pathog* 5: e1000600.

Comas, I., S. Homolka, S. Niemann & S. Gagneux, (2009) Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. *PLoS One* 4: e7815.

Coscolla, M. & S. Gagneux, (2010) Does M. tuberculosis genomic diversity explain disease diversity? *Drug Discov Today Dis Mech* 7: e43-e59.

Cowley, D., D. Govender, B. February, M. Wolfe, L. Steyn, J. Evans, R. J. Wilkinson & M. P. Nicol, (2008) Recent and rapid emergence of W-Beijing strains of *Mycobacterium tuberculosis* in Cape Town, South Africa. *Clin Infect Dis* 47: 1252-1259.

Coxon, G. D. & L. G. Dover, (2011) Current Status and Research Strategies in Tuberculosis Drug Development. *J Med Chem*.

Cubillos-Ruiz, A., J. Morales & M. M. Zambrano, (2008) Analysis of the genetic variation in *Mycobacterium tuberculosis* strains by multiple genome alignments. *BMC Res Notes* 1: 110.

Cubillos-Ruiz, A., A. Sandoval, V. Ritacco, B. Lopez, J. Robledo, N. Correa, I. Hernandez-Neuta, M. M. Zambrano & P. Del Portillo, (2010) Genomic signatures of the haarlem lineage of *Mycobacterium tuberculosis*: implications of strain genetic variation in drug and vaccine development. *J Clin Microbiol* 48: 3614-3623.

de Jong, B. C., P. C. Hill, A. Aiken, T. Awine, M. Antonio, I. M. Adetifa, D. J. Jackson-Sillah, A. Fox, K. Deriemer, S. Gagneux, M. W. Borgdorff, K. P. McAdam, T. Corrah, P. M. Small & R. A. Adegbola, (2008) Progression to active tuberculosis, but not transmission, varies by *Mycobacterium tuberculosis* lineage in The Gambia. *J Infect Dis* 198: 1037-1043.

Di Pietrantonio, T., C. Hernandez, M. Girard, A. Verville, M. Orlova, A. Belley, M. A. Behr, J. C. Loredo-Osti & E. Schurr, (2010) Strain-specific differences in the genetic control of two closely related mycobacteria. *PLoS Pathog* 6: e1001169.

Donoghue, H. D., (2009) Human tuberculosis--an ancient disease, as elucidated by ancient microbial biomolecules. *Microbes Infect* 11: 1156-1162.

Donoghue, H. D., (2011) Insights gained from palaeomicrobiology into ancient and modern tuberculosis. *Clin Microbiol Infect* 17: 821-829.

Doolittle, W. F. & O. Zhaxybayeva, (2009) On the origin of prokaryotic species. *Genome Res* 19: 744-756.

Escombe, A. R., D. A. Moore, R. H. Gilman, W. Pan, M. Navincopa, E. Ticona, C. Martinez, L. Caviedes, P. Sheen, A. Gonzalez, C. J. Noakes, J. S. Friedland & C. A. Evans, (2008) The infectiousness of tuberculosis patients coinfected with HIV. *PLoS Med* 5: e188.

Fang, Z., C. Doig, N. Morrison, B. Watt & K. J. Forbes, (1999) Characterization of IS1547, a new member of the IS900 family in the *Mycobacterium tuberculosis* complex, and its association with IS6110. *J Bacteriol* 181: 1021-1024.

Filliol, I., A. S. Motiwala, M. Cavatore, W. Qi, M. H. Hazbon, M. Bobadilla del Valle, J. Fyfe, L. Garcia-Garcia, N. Rastogi, C. Sola, T. Zozio, M. I. Guerrero, C. I. Leon, J. Crabtree, S. Angiuoli, K. D. Eisenach, R. Durmaz, M. L. Joloba, A. Rendon, J. Sifuentes-Osornio, A. Ponce de Leon, M. D. Cave, R. Fleischmann, T. S. Whittam & D. Alland, (2006) Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *J Bacteriol* 188: 759-772.

Fleischmann, R. D., D. Alland, J. A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J. F. Kolonay, W. C. Nelson, L. A. Umayam, M. Ermolaeva, S. L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, W. R. Jacobs Jr, Jr., J. C. Venter & C. M. Fraser, (2002) Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J Bacteriol* 184: 5479-5490.

Ford, C. B., P. L. Lin, M. R. Chase, R. R. Shah, O. Iartchouk, J. Galagan, N. Mohaideen, T. R. Ioerger, J. C. Sacchettini, M. Lipsitch, J. L. Flynn & S. M. Fortune, (2011) Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat Genet* 43: 482-486.

Gagneux, S., K. DeRiemer, T. Van, M. Kato-Maeda, B. C. de Jong, S. Narayanan, M. Nicol, S. Niemann, K. Kremer, M. C. Gutierrez, M. Hilty, P. C. Hopewell & P. M. Small, (2006) Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 103: 2869-2873.

Gandhi, N. R., A. Moll, A. W. Sturm, R. Pawinski, T. Govender, U. Lalloo, K. Zeller, J. Andrews & G. Friedland, (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 368: 1575-1580.

Garcia de Viedma, D., G. Lorenzo, P. J. Cardona, N. A. Rodriguez, S. Gordillo, M. J. Serrano & E. Bouza, (2005) Association between the infectivity of *Mycobacterium tuberculosis* strains and their efficiency for extrarespiratory infection. *J Infect Dis* 192: 2059-2065.

Gardy, J. L., J. C. Johnston, S. J. Ho Sui, V. J. Cook, L. Shah, E. Brodkin, S. Rempel, R. Moore, Y. Zhao, R. Holt, R. Varhol, I. Birol, M. Lem, M. K. Sharma, K. Elwood, S. J. Jones, F. S. Brinkman, R. C. Brunham & P. Tang, (2011) Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. *N Engl J Med* 364: 730-739.

Gordon, S. V., B. Heym, J. Parkhill, B. Barrell & S. T. Cole, (1999) New insertion sequences and a novel repeated sequence in the genome of *Mycobacterium tuberculosis* H37Rv. *Microbiology* 145 (Pt 4): 881-892.

Gupta, K., P. Kumar & D. Chatterji, (2010) Identification, activity and disulfide connectivity of C-di-GMP regulating proteins in *Mycobacterium tuberculosis*. *PLoS One* 5: e15072.

Gutacker, M. M., B. Mathema, H. Soini, E. Shashkina, B. N. Kreiswirth, E. A. Graviss & J. M. Musser, (2006) Single-nucleotide polymorphism-based population genetic analysis of *Mycobacterium tuberculosis* strains from 4 geographic sites. *J Infect Dis* 193: 121-128.

Gutacker, M. M., J. C. Smoot, C. A. Migliaccio, S. M. Ricklefs, S. Hua, D. V. Cousins, E. A. Graviss, E. Shashkina, B. N. Kreiswirth & J. M. Musser, (2002) Genome-wide analysis of synonymous single nucleotide polymorphisms in *Mycobacterium tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics* 162: 1533-1543.

Gutierrez, M. C., S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply & V. Vincent, (2005) Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathog* 1: e5.

Hanekom, M., N. C. Gey van Pittius, C. McEvoy, T. C. Victor, P. D. Van Helden & R. M. Warren, (2011) *Mycobacterium tuberculosis* Beijing genotype: A template for success. *Tuberculosis (Edinb)*.

Hawkey, P. M. & A. M. Jones, (2009) The changing epidemiology of resistance. *J Antimicrob Chemother* 64 Suppl 1: i3-10.

Hershberg, R., M. Lipatov, P. M. Small, H. Sheffer, S. Niemann, S. Homolka, J. C. Roach, K. Kremer, D. A. Petrov, M. W. Feldman & S. Gagneux, (2008) High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLoS Biol* 6: e311.

Hirsh, A. E., A. G. Tsolaki, K. DeRiemer, M. W. Feldman & P. M. Small, (2004) Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. *Proc Natl Acad Sci U S A* 101: 4871-4876.

Inoue, J., P. C. Donoghue & Z. Yang, (2010) The impact of the representation of fossil calibrations on Bayesian estimation of species divergence times. *Syst Biol* 59: 74-89.

Ioerger, T. R., S. Koo, E. G. No, X. Chen, M. H. Larsen, W. R. Jacobs, Jr., M. Pillay, A. W. Sturm & J. C. Sacchettini, (2009) Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. *PLoS One* 4: e7778.

Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal & J. van Embden, (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 35: 907-914.

Kato-Maeda, M., P. J. Bifani, B. N. Kreiswirth & P. M. Small, (2001a) The nature and consequence of genetic variability within *Mycobacterium tuberculosis*. *J Clin Invest* 107: 533-537.

Kato-Maeda, M., S. Gagneux, L. L. Flores, E. Y. Kim, P. M. Small, E. P. Desmond & P. C. Hopewell, (2011) Strain classification of *Mycobacterium tuberculosis*: congruence between large sequence polymorphisms and spoligotypes. *Int J Tuberc Lung Dis* 15: 131-133.

Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat & P. M. Small, (2001b) Comparing genomes within the species *Mycobacterium tuberculosis*. *Genome Res* 11: 547-554.

Kaufmann, S. H., (2010) Future vaccination strategies against tuberculosis: thinking outside the box. *Immunity* 33: 567-577.

Kim, E. Y., P. Nahid, P. C. Hopewell & M. Kato-Maeda, (2010) Novel hot spot of IS*6110* insertion in *Mycobacterium tuberculosis*. *J Clin Microbiol* 48: 1422-1424.

Koul, A., E. Arnoult, N. Lounis, J. Guillemont & K. Andries, (2011) The challenge of new drug discovery for tuberculosis. *Nature* 469: 483-490.

Lawn, S. D. & A. I. Zumla, (2011) Tuberculosis. *Lancet* 378: 57-72.

Lin, M. Y. & T. H. Ottenhoff, (2008) Host-pathogen interactions in latent *Mycobacterium tuberculosis* infection: identification of new targets for tuberculosis intervention. *Endocr Metab Immune Disord Drug Targets* 8: 15-29.

Maisnier-Patin, S. & D. I. Andersson, (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res Microbiol* 155: 360-369.

Malik, A. N. & P. Godfrey-Faussett, (2005) Effects of genetic variability of *Mycobacterium tuberculosis* strains on the presentation of disease. *Lancet Infect Dis* 5: 174-183.

McAdam, R. A., P. W. Hermans, D. van Soolingen, Z. F. Zainuddin, D. Catty, J. D. van Embden & J. W. Dale, (1990) Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Mol Microbiol* 4: 1607-1613.

McEvoy, C. R., A. A. Falmer, N. C. Gey van Pittius, T. C. Victor, P. D. van Helden & R. M. Warren, (2007) The role of IS*6110* in the evolution of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 87: 393-404.

McEvoy, C. R., P. D. van Helden, R. M. Warren & N. C. Gey van Pittius, (2009a) Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis* PPE38 gene region. *BMC Evol Biol* 9: 237.

McEvoy, C. R., R. M. Warren, P. D. van Helden & N. C. Gey van Pittius, (2009b) Multiple, independent, identical IS*6110* insertions in *Mycobacterium tuberculosis* PPE genes. *Tuberculosis (Edinb)* 89: 439-442.

McLean, K. J. & A. W. Munro, (2008) Structural biology and biochemistry of cytochrome P450 systems in *Mycobacterium tuberculosis*. *Drug Metab Rev* 40: 427-446.

Mendiola, M. V., C. Martin, I. Otal & B. Gicquel, (1992) Analysis of the regions responsible for IS*6110* RFLP in a single *Mycobacterium tuberculosis* strain. *Res Microbiol* 143: 767-772.

Mostowy, S. & M. A. Behr, (2005) The origin and evolution of *Mycobacterium tuberculosis*. *Clin Chest Med* 26: 207-216, v-vi.

Nicol, M. P. & R. J. Wilkinson, (2008) The clinical consequences of strain diversity in *Mycobacterium tuberculosis*. *Trans R Soc Trop Med Hyg* 102: 955-965.

Olano, J., B. Lopez, A. Reyes, M. P. Lemos, N. Correa, P. Del Portillo, L. Barrera, J. Robledo, V. Ritacco, and M. M. Zambrano. 2007. Mutations in DNA repair genes are associated with the Haarlem lineage of *Mycobacterium tuberculosis* independently of their antibiotic resistance. Tuberculosis (Edinb) 87:502-8.

Ordway, D., M. Henao-Tamayo, M. Harton, G. Palanisamy, J. Troudt, C. Shanley, R. J. Basaraba & I. M. Orme, (2007) The hypervirulent *Mycobacterium tuberculosis* strain HN878 induces a potent TH1 response followed by rapid down-regulation. *J Immunol* 179: 522-531.

Otal, I., A. B. Gomez, K. Kremer, P. de Haas, M. J. Garcia, C. Martin & D. van Soolingen, (2008) Mapping of IS*6110* insertion sites in Mycobacterium bovis isolates in relation to adaptation from the animal to human host. *Vet Microbiol* 129: 333-341.

Parwati, I., R. van Crevel & D. van Soolingen, (2010) Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains. *Lancet Infect Dis* 10: 103-111.

Portevin, D., S. Gagneux, I. Comas & D. Young, (2011) Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. *PLoS Pathog* 7: e1001307.

Pym, A. S., B. Saint-Joanis & S. T. Cole, (2002) Effect of katG mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect Immun* 70: 4955-4960.

Ramaswamy, S. & J. M. Musser, (1998) Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 79: 3-29.

Ritacco, V., M. J. Iglesias, L. Ferrazoli, J. Monteserin, E. R. Dalla Costa, A. Cebollada, N. Morcillo, J. Robledo, J. H. de Waard, P. Araya, L. Aristimuno, R. Diaz, P. Gavin, B. Imperiale, V. Simonsen, E. M. Zapata, M. S. Jimenez, M. L. Rossetti, C. Martin, L. Barrera & S. Samper, (2011) Conspicuous multidrug-resistant *Mycobacterium tuberculosis* cluster strains do not trespass country borders in Latin America and Spain. *Infect Genet Evol*.

Rivero, A., M. Marquez, J. Santos, A. Pinedo, M. A. Sanchez, A. Esteve, S. Samper & C. Martin, (2001) High rate of tuberculosis reinfection during a nosocomial outbreak of multidrug-resistant tuberculosis caused by Mycobacterium bovis strain B. *Clin Infect Dis* 32: 159-161.

Rosas-Magallanes, V., P. Deschavanne, L. Quintana-Murci, R. Brosch, B. Gicquel & O. Neyrolles, (2006) Horizontal transfer of a virulence operon to the ancestor of *Mycobacterium tuberculosis*. *Mol Biol Evol* 23: 1129-1135.

Safi, H., J. Aznar & J. C. Palomares, (1997) Molecular epidemiology of *Mycobacterium tuberculosis* strains isolated during a 3-year period (1993 to 1995) in Seville, Spain. *J Clin Microbiol* 35: 2472-2476.

Sampson, S. L., (2011) Mycobacterial PE/PPE proteins at the host-pathogen interface. *Clin Dev Immunol* 2011: 497203.

Sandgren, A., M. Strong, P. Muthukrishnan, B. K. Weiner, G. M. Church & M. B. Murray, (2009) Tuberculosis drug resistance mutation database. *PLoS Med* 6: e2.

Siguier, P., J. Filee & M. Chandler, (2006) Insertion sequences in prokaryotic genomes. *Curr Opin Microbiol* 9: 526-531.

Sinsimer, D., G. Huet, C. Manca, L. Tsenova, M. S. Koo, N. Kurepina, B. Kana, B. Mathema, S. A. Marras, B. N. Kreiswirth, C. Guilhot & G. Kaplan, (2008) The phenolic glycolipid of *Mycobacterium tuberculosis* differentially modulates the early host cytokine response but does not in itself confer hypervirulence. *Infect Immun* 76: 3027-3036.

Small, P. M., P. C. Hopewell, S. P. Singh, A. Paz, J. Parsonnet, D. C. Ruston, G. F. Schecter, C. L. Daley & G. K. Schoolnik, (1994) The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods. *N Engl J Med* 330: 1703-1709.

Smith, N. H., R. G. Hewinson, K. Kremer, R. Brosch & S. V. Gordon, (2009) Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*. *Nat Rev Microbiol* 7: 537-544.

Smith, N. H., K. Kremer, J. Inwald, J. Dale, J. R. Driscoll, S. V. Gordon, D. van Soolingen, R. G. Hewinson & J. M. Smith, (2006) Ecotypes of the *Mycobacterium tuberculosis* complex. *J Theor Biol* 239: 220-225.

Sola, C., I. Filliol, M. C. Gutierrez, I. Mokrousov, V. Vincent & N. Rastogi, (2001) Spoligotype database of *Mycobacterium tuberculosis*: biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. *Emerg Infect Dis* 7: 390-396.

Soto, C. Y., M. C. Menendez, E. Perez, S. Samper, A. B. Gomez, M. J. Garcia & C. Martin, (2004) IS*6110* mediates increased transcription of the phoP virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. *J Clin Microbiol* 42: 212-219.

Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam & J. M. Musser, (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci U S A* 94: 9869-9874.

Supply, P., R. M. Warren, A. L. Banuls, S. Lesjean, G. D. Van Der Spuy, L. A. Lewis, M. Tibayrenc, P. D. Van Helden & C. Locht, (2003) Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. *Mol Microbiol* 47: 529-538.

Talarico, S., M. D. Cave, B. Foxman, C. F. Marrs, L. Zhang, J. H. Bates & Z. Yang, (2007) Association of *Mycobacterium tuberculosis* PE PGRS33 polymorphism with clinical and epidemiological characteristics. *Tuberculosis (Edinb)* 87: 338-346.

Thierry, D., M. D. Cave, K. D. Eisenach, J. T. Crawford, J. H. Bates, B. Gicquel & J. L. Guesdon, (1990) IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res* 18: 188.

Thorne, N., S. Borrell, J. Evans, J. Magee, D. Garcia de Viedma, C. Bishop, J. Gonzalez-Martin, S. Gharbia & C. Arnold, (2011) IS*6110*-based global phylogeny of *Mycobacterium tuberculosis*. *Infect Genet Evol* 11: 132-138.

Tsolaki, A. G., A. E. Hirsh, K. DeRiemer, J. A. Enciso, M. Z. Wong, M. Hannan, Y. O. Goguet de la Salmoniere, K. Aman, M. Kato-Maeda & P. M. Small, (2004) Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. *Proc Natl Acad Sci U S A* 101: 4865-4870.

Turcios, L., Y. Casart, I. Florez, J. de Waard & L. Salazar, (2009) Characterization of IS*6110* insertions in the dnaA-dnaN intergenic region of *Mycobacterium tuberculosis* clinical isolates. *Clin Microbiol Infect* 15: 200-203.

van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick & et al., (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 31: 406-409.

van Embden, J. D., T. van Gorkom, K. Kremer, R. Jansen, B. A. van Der Zeijst & L. M. Schouls, (2000) Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. *J Bacteriol* 182: 2393-2401.

van Soolingen, D., P. E. de Haas, P. W. Hermans, P. M. Groenen & J. D. van Embden, (1993) Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J Clin Microbiol* 31: 1987-1995.

Vasilyeva, S. V., E. Unur, R. M. Walczak, E. P. Donoghue, A. G. Rinzler & J. R. Reynolds, (2009) Color purity in polymer electrochromic window devices on indium-tin oxide and single-walled carbon nanotube electrodes. *ACS Appl Mater Interfaces* 1: 2288-2297.

Vera-Cabrera, L., M. A. Hernandez-Vera, O. Welsh, W. M. Johnson & J. Castro-Garza, (2001) Phospholipase region of *Mycobacterium tuberculosis* is a preferential locus for IS*6110* transposition. *J Clin Microbiol* 39: 3499-3504.

Wall, S., K. Ghanekar, J. McFadden & J. W. Dale, (1999) Context-sensitive transposition of IS*6110* in mycobacteria. *Microbiology* 145 (Pt 11): 3169-3176.

Warren, R. M., S. L. Sampson, M. Richardson, G. D. Van Der Spuy, C. J. Lombard, T. C. Victor & P. D. van Helden, (2000) Mapping of IS*6110* flanking regions in clinical isolates of *Mycobacterium tuberculosis* demonstrates genome plasticity. *Mol Microbiol* 37: 1405-1416.

Wirth, T., F. Hildebrand, C. Allix-Beguec, F. Wolbeling, T. Kubica, K. Kremer, D. van Soolingen, S. Rusch-Gerdes, C. Locht, S. Brisse, A. Meyer, P. Supply & S. Niemann, (2008) Origin, spread and demography of the *Mycobacterium tuberculosis* complex. *PLoS Pathog* 4: e1000160.

Yang, Z., D. Yang, Y. Kong, L. Zhang, C. F. Marrs, B. Foxman, J. H. Bates, F. Wilson & M. D. Cave, (2005) Clinical relevance of *Mycobacterium tuberculosis* plcD gene mutations. *Am J Respir Crit Care Med* 171: 1436-1442.

Yesilkaya, H., J. W. Dale, N. J. Strachan & K. J. Forbes, (2005) Natural transposon mutagenesis of clinical isolates of *Mycobacterium tuberculosis*: how many genes does a pathogen need? *J Bacteriol* 187: 6726-6732.

3

IS*6110* the Double-Edged Passenger

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1. Introduction

1.1 Insertion sequences in *Mycobacterium tuberculosis* complex

Insertion sequence (IS) is a short DNA mobile genetic element coding for proteins involved in the transposition activity, which allows it to spread within the genome. ISs are widely distributed in prokaryotes and can be grouped into different families established by Mahillon & Chandler (1998) based on structural characteristics and transposase similarities.

In the genus *Mycobacterium* have been located and identified more than 46 ISs from different species, mostly on the basis of sequence similarities (Brosch et al., 2000). In the genome of the members of the *Mycobacterium tuberculosis* complex (MTBC) has been possible to find dispersed IS elements that could be included in various of the following families attending to their characteristics: IS*3*, IS*5*, IS*21*, IS*30*, IS*110*, IS*256*; IS*1535*, IS*L3* and other IS-*like* elements (Gordon et al., 1999, Table 1).

The ISs can induce duplications, deletions, and rearrangements in the bacteria genome, all of them essentials changes for the genome plasticity of the members of MTBC (Mahillon & Chandler, 1998). Not all of the ISs described in *M. tuberculosis* are active and have the availability of transpose from one site to another in the genome, some of the elements are defective copies. Furthermore, some of them have a limited host range (Brosch et al., 2000).

The Table 1 shows the ISs described in *M. tuberculosis* that will be briefly presented below.

1.2 ISs families

The IS*3* family represents an extensive set of insertion elements in bacteria. The features that characterize this family are their length between 1200 and 1600 bp, and their inverted repeats (IRs) between 20 and 40 bp long, as well as the presence of two overlapping open reading frames (ORFs: *orf*A and *orf*B) (Mahillon & Chandler, 1998; McAdam et al., 2000). After the insertion, a duplication of 3 or 4 bp occurs at the insertion point (Mendiola et al., 1992).

Members IS*1540*, IS*1604*, IS*1556/990* and IS*6110* belong to this family in the MTBC. The most representative member of this family is IS*6110*, one of the insertion element most abundant and best characterized in the MTBC. Copies of this IS can be found at 16 positions in the genome of *M. tuberculosis* H37Rv providing an important epidemiological tool (Small & van Embden, 1994).

Other elements of this family, namely IS*1540*, IS*1604* and IS*1556/990*, have missing the IRs and Direct Repeats **(**DRs) or contain mutations in *orf*B making them supposedly inactive and non-functional (Dziadek et al., 1998; McAdam et al., 2000).

Family	**ISs (n^er copies / lenght)**	**Source**
IS*3*	IS*6110* (16 / 1361 bp); IS*1540* (1 / 1164 bp); IS*1604* (1 / 1410 bp); IS*1556/990* (1 / 1346 bp)	Thierry et al., 1990a; Cole et al., 1998; Gordon et al., 1999; Dziadek et al., 1998.
IS*5*	IS*1560* (1+1′ / 1567 bp); IS-like (2 / 968 bp)	Cole et al., 1998; Mariani et al., 1993
IS*21*	IS*1532* (1 / 2609 bp); IS*1533* (1 / 2212 bp); IS*1534* (1 / 2129 bp)	Gordon et al., 1999
IS*30*	IS*1603* (1 / 1327 bp)	Cole et al., 1998
IS*110*	IS*1558* (1+1′ / 1212 bp); IS*1607* (1 / 1227 bp); IS*1608′* (2′ / 1031 bp); IS*1547* (2 / 1351 bp)	Dziadek et al., 2000; Cole et al., 1998; Fang et al., 1999a
IS*256*	IS*1081* (6 / 1324 bp); IS*1552′* (1′ / 844 bp); IS*1553* (1 / 1398 bp); IS*1554* (1 / 1435 bp)	Collins et al., 1991; Cole et al., 1998
IS*605*	IS*1535* (1 / 2322 bp); IS*1536* (1 / 1391 bp); IS*1537* (1 / 1889 bp); IS*1538* (1 / 2055 bp); IS*1539* (1 / 2057 bp); IS*1602* (1 / 2052 bp); IS*1605′* (1′ / 287 bp)	Gordon et al., 1999; Cole et al., 1998
IS*L3*	IS*1555′* (1′ / 398 bp); IS*1557* (2+1′ / 1451 bp); IS*1561′* (1′ / 1319 bp); IS*1606′* (1′ / 330 bp)	Cole et al., 1998; Gordon et al., 1999
Unknown	IS*1556* (1 / 1468 bp)	Cole et al., 1998

Table 1. ISs present in *M. tuberculosis* H37Rv. ‘ Defective copy of IS, putatively inactivated.

Members of the IS*21* family are among the largest bacterial IS elements, with sizes between 2 and 2.5 Kb length. Their IRs are variable. These elements encode two proteins for the transposition (IStA and IStB). Duplication of 4 or 5 bp occurs after transposition at the insertion point. The transposases coded by IS*1532*, IS*1533* and IS*1534* shows homology to the elements of IS*21* family. These elements posses end IRs of 48, 54 and 49 bp respectively and internal DRs (Mahillon & Chandler, 1998). All of them are absent from 40% of *M. tuberculosis* clinical isolates, as well as from *M. bovis* and *M. bovis* BCG Pasteur (Gordon et al., 1999).

The IS*110* family, exhibits unusual features for bacterial ISs, they have not IRs and DRs (McAdam et al., 2000) and may be differentiated in two groups.

The first group, in *M. tuberculosis*, included IS*1608'* and IS*1547,* unlike to other elements, this group have a target sequence: $CATN_{(6\text{-}9)}$(T,C)CCTT. The IS*1547* is one of the members that was detected only in members of the MTBC and seems to be an IS*6110* preferential site for insertion (Fang et al., 1999a). The second group includes IS*1558* and IS*1607,* they have imperfect IRs or lack of them (McAdam et al., 2000). Some copies of these IS elements, in the *M. tuberculosis* genome, are defective as was the case of IS*1558*' and IS*1608*'.

The IS*256* family is, probably, the largest family of ISs in mycobacteria with more than 25% of the known ISs. Their members have been divided in two groups attending to the structural organization (Guilhot et al., 1999). One of the groups comprises the members of MTBC, such as: IS*1081*, IS*1552'*, IS*1553* and IS*1554*.

IS*1081*, the main member of IS*256* family in the MTBC, was found in the genome of *M. bovis*. It is 1324 bp long with 15 bp IR ends and contains a large ORF. There are six copies of IS*1081* in the genome of *M. tuberculosis* H37Rv (Collins et al., 1991). Other elements of IS*256* family, are IS*1552'*, IS*1553* and IS*1554*. They have a single ORF coding for a protein of 281, 409 and 439 aminoacids. It is speculated that IS*1552'* was transferred from *Rhodococcus* into *M. tuberculosis*. In *M. tuberculosis* H37Rv the IS*1552*' is defective.

The IS*5* family is a very heterogeneus group of ISs, with lenghts range between 850 to 1640 bp. Two different ISs of this family have been described in MTBC: IS*1560* and IS-like (Cole et al., 1998; Mariani et al., 1993). One of the two copies of IS*1560* appears to be defective and probably is non-functional in the *M. tuberculosis* H37Rv.

The members of the IS*30* family have a single open reading frame, IRs 20-30 bp, and DRs 2-3 bp long created after insertion (Mahillon & Chandler, 1998). IS603, an insertion sequence 1327 bp lenght and present in a single copy in the *M. tuberculosis*, genome belongs to this family (Table 1) with IRs 63 bp long and DRs have not been detected (McAdam et al., 2000).

M. tuberculosis contains seven members of the IS*605* family (IS*1535*, IS*1536*, IS*1537*, IS*1538*, IS*1539*, IS*1602* and IS*1605'*) in its genome (Gordon & Supply, 2005). These ISs present two overlapping ORFs (Gordon et al., 1999; McAdam et al., 2000).

To the IS*L3* family belongs some defective copies of ISs present in *M. tuberculosis*: IS*1555*', IS*1561*' and IS*1606*' and IS*1557*. The IS*1561*' element is absent from some clinical strains of *M. tuberculosis* (Gordon et al., 1999) and *M. microti* (Gordon & Supply, 2005).

2. Structural organization and function of the IS*6110*

IS*6110* was initially named IS*986*. It is a genomic insertion element of 1361 bp long and shows 28 bp imperfect IRs, and duplications of 3 or 4 bp next to the insertion site. It has two overlapping ORFs (*orf*A and *orf*B) coding for a transposase, showing similarities with elements of the IS*3* family of prokaryotes (Accesion No.: X17348, M29899; Fig. 1).

The IS*6110* was found to be specific of mycobacteria belonging to the MTBC (Thierry et al., 1990a) and it was considered as the main target of the first reference genotyping tool, due to the high degree of polymorphism observed comparing strains of the MTBC (see part 3.2; Otal et al., 1991), turning into an important factor involved in the evolution of the *M. tuberculosis* genome. The sequences of IS*6110* and IS*986*/IS*987* identified in MTBC were practically identical and considered the same IS (Thierry et al., 1990b; McAdam et al., 1990).

Recently, Sankar and cols (2011b) have been suggested variations into the sequence of IS*6110* from different strains of *M. tuberculosis*, which could have implications in its usefulness as target of PCR detection.

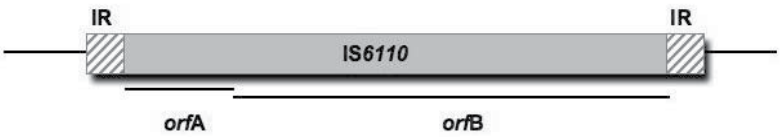

Fig. 1. Structural organization of IS*6110*

IS*6110* does not have a known target or consensus sequence, it has been found within ORFs and intergenic regions (see part 4.1). It may be present up to 25 copies per genome in *M. tuberculosis* (Brosch et al., 2000), only a few number of strains have no copies of this IS (see part 3.1.2).

Many functions have been shown by the IS*6110*: (i) activation of genes during infection (Safi et al., 2004) (ii) participation in the evolution as an epidemiological marker (van Embden et al., 1993) (iii) activation of downstream genes with an activity promoter orientation-dependent (Soto et al., 2004). Finally, it has been suggested that the presence of IS*6110* in *M. bovis* could participate in the adaptation of this bacteria to a particular host, animal or human (Otal et al., 2008). Several of these features are being reviewed herein.

3. The heads: Usefulness of IS*6110*

Soon after the discovery of IS*6110* as a specific element in MTBC, its usefulness as diagnostic tool was explored. Subsequently, at the beginning of the nineties it was demonstrated that two strains isolated of different episodes of a patient had the same IS*6110*-RFLP pattern, in turn, a high degree of polymorphism was observed between strains isolated from different patients (Otal et al., 1991). The fact that IS*6110* varies in copy number and location in the bacterial genomes, along with its stability over time showed their usefulness in genotyping of the MTBC. This IS has been successfully used throughout the world for identifying and characterize members of this complex.

3.1 IS*6110* in the detection of members of the MTB complex

TB is a major public health problem in humans affecting many countries and large numbers of people. There are many reasons to explain the global relevance of this disease, including poverty, the limited vaccine efficacy and the persistence of the pathogen itself. One crucial factor is the difficulty in diagnosis TB. Currently, the main impediment is the lack of adequately sensitive, specificity and rapid tests. Culture and smear microscopy are probably the most common tools used worldwide for confirming the identification of TB in clinical samples. But culture is time consuming and smear microscopy is not specific enough. This has led to its gradual replacement in the developed world by more sensible, specific and rapid methods, such as PCR.

Because of the increased accessibility and convenience of PCR-based detection techniques, these are suitable to replace conventional culture methods. Since bacterial growth is not required, PCR can give results rapidly in as short a period as 1 day. Further PCR modifications, as nested–PCR or multiplex-PCR, can be used to improve results. Over the years, a significant improvement of PCR technologies has been achieved with the development of real-time PCR for the detection of target genes of *M. tuberculosis* in clinical specimens. The main advantages of real-time PCR are a shortened turnaround time, automation of the amplification and product detection and a decreased in the risk of cross-contamination (Espy et al., 2006).

3.1.1 Advantages of IS*6110* as target of the MTBC

To obtain species-specific pathogen identification and detection in clinical samples, specific primers have been designed and tested using PCR-based methods, targeting different genomic sequences of *M. tuberculosis*. These have included IS*6110*, *hsp65*, TRC4 and *mpt40* (Bannalikar et al., 2006; Narayanan et al, 2001; Savekoul et al., 2006; Tumwasorn et al., 1996; Wei et al., 1999). Among these, the most widely investigated has been the IS*6110* being reported as a specific sequence of MTBC (Brisson-Noel et al. 1991; Eisenach, 1994; Sankar et al., 2011a). IS*6110* is an ideal target for PCR. IS*6110* is usually a multi-copy element and randomly distributed throughout the genome. The presence of multiple copies improves the sensitivity of the PCR amplification (Mathema et al., 2006; Sankar et al., 2011a).

Different oligonucleotides derived from that sequence have been successfully used to detect *M. tuberculosis* in all type of clinical specimens. Table 2 summarizes a list of the primers more frequently used in the literature. A problem found was that authors give different names to the same primers. The primers IS1 and IS2 (Eisenach et al., 1990) are the most frequently used, these oligonucleotides amplified a final product of 123 bp from 759 to 881 nucleotide position of IS*6110* (Table 2).

A search in the databases PubMed since 1991 using "IS*6110*" and "diagnostic" as keywords, allowed the identification of 138 papers that showed how IS*6110* could be a useful tool in diagnostic of TB. In 105 of these papers the diagnostic is based on PCR. Up to 5 of the 11 works published during the seven first months of 2011 applied the real time PCR technique in tuberculosis diagnostic using IS*6110* as target sequence.

In most of the cases the authors applied in-house PCR methods and compared results to other methods. Some authors concluded that IS*6110*-based PCR could be used routinely in clinical laboratories for rapid detection of *M. tuberculosis*, in sputum samples allowing early diagnosis and treatment (Ereqat et al., 2011). Evaluation of in-house PCR showed that variability in sensitivity and specificity is high (Cho et al., 2007).

The usefulness of IS*6110* in the detection and identification of MTBC in clinical samples has been demonstrated in many studies, either detecting IS*6110* as single target (Sankar et al., 2010a; Gupta et al., 2010; Inoue et al., 2011) or together to other specific targets (Sankar et al., 2010b; Leung et al., 2011). Multiplex PCR assay can be used for the simultaneous detection of other coinfections in clinical samples (Boondireke et al., 2010).

Additionally, in some cases, the location of IS*6110* specific to one strain can be used. PCR with primers targeting IS*6110* and the flanking region allowed identify and differentiate that

particular strain. This approach can be a useful tool in diagnosis and epidemiological studies.

Name / Target region	Sequence (5´-3´)	Method	Reference
MTB-F / 724-740	GCCGGATCAGCGATCGT	Real Time PCR	Leung et al., 2011
MTB-R / 608 - 629	GCAAAGTGTGGCTAACCCTGAA		
MTB-P / 651 - 666	TTCGACGGTGCATCTG		
/1062 - 1077	CCGAGGCAGGCATCCA	Real Time PCR	Lemaitre et al., 2004
/1112 - 1132	GATCGTCTCGGCTAGTGCATT		
/1095 -1111	TCGGAAGCTCCTATGAC		
TB1 / 104 - 123	GTGCGGATGGTGGCAGAGAT	Nested PCR	Boondireke et al., 2010
TB4 / 387 -406	CCTGATGATCGGCGATGAAC		
TB2 /132 - 152	AGCACGATTCGGAGTGGGCA		
TB3 / 255 - 273	TCAGCGGATTCTTCGGTCG		
INS1 / 631-650	CGTGAGGGCATCGAGGTGGC	PCR	Hermans et al., 1990
INS2 / 856 - 875	CGTAGGCGTCGGTGACAAA		
ISI / 762-781	CCTGCGAGCGTAGGCGTCGG	PCR	Eisenach et al., 1990
IS2 / 854-883	CTCGTCCAGCGCCGCTTCGG		
TB130-F / 710 -729	CAAAGCCCGCAGGACCACGA	Real Time PCR	Inoue et al., 2011
TB130-R / 817-839	TGCCCAGGTCGACACATAGGTGA		
TB130-P / 742-761	CCACAGCCCGTCCCGCCGAT		
/ 367-392	CCGGCCAGCACGCTAATTAACGGTTC	Nested PCR	Cheng et al., 2004
/ 769- 746	TGTGGCCGGATCAGCGATCGTGGT		
/ 455-472	CTGCACACAGCTGACCGA		
/ 670-652	CGTTCGACGGTGCATCTG		

Table 2. List of primers successfully used in IS*6110*-PCR for the detection of MTBC.

3.1.2 Disadvantages of IS*6110* as target of the MTBC

However its wide applicability, targeting IS*6110* may not be by itself sensitive enough to diagnose 100% of the cases. Studies in India documented that 41% of *M. tuberculosis* isolates harboured a single copy of IS*6110* and 1% with no copy (Narayanan et al., 2002). In these situations the use of other targets for PCR in addition to IS*6110* for the detection of TB can be of help (Narayanan et al., 2001; Das et al., 1995; Chauhan et al., 2007; Kusum et al., 2011). A

good approach could be a multiplex real-time PCR targeting IS*6110* and another target, as for example to use multiplex PCR using *hsp*65, protein B or MPB64 genes as targets.

As for DNA detection, another problem using IS*6110*-PCR is that it can detect non-viable mycobacteria for patients with earlier culture-positive specimens that had become culture negative following anti tuberculosis drug therapy (Causse et al., 2011).

M. bovis strains usually contain one to five copies of IS*6110* (Otal et al., 2008), making the use of this IS less advantageous for the detection of this bacteria. The use of an immunomagnetic separation capture followed by PCR based on IS*6110* showed a detection threshold corresponding from 10 CFU in PBS to 1000 CFU for *M. bovis* in infected bovine fresh tissues, providing a sensitive, rapid and specific technique for the diagnosis of bovine tuberculosis (Garbaccio et al., 2010).

On the other hand, Sankar *et al*. analysed the sequence diversity of IS*6110* by using *in silico* approach. They found that IS*6110* insertion sequences harboured variations in its sequence and there are divergences within the copies of one strain. They collected a list of primers from those successfully used in the conventional PCR for the diagnosis of TB, but the reported data showed variation in the sensitivity and specificity for different regions of IS*6110*. All these data suggest that care must be taken when designing specific primers for IS*6110* detection. The authors recommended develop multiplex PCR assays targeting more than one region of the genome of *M. tuberculosis* (Sankar et al., 2011a).

Indeed, the IS*6110* is still a favourite target sequence in the diagnosis of TB. Recently, a high sensitivity and specificity has been reported for the GeneXpert system, a real-time PCR assay that simultaneously detects both MTBC and rifampin resistance. However the accuracy of the Xpert MTB/RIF test for the detection of *M. tuberculosis* complex in paucibacillary samples was found to be lower than that of an in-house IS*6110* real time PCR routinely used since 2004 (Armand et al., 2011).

3.2 Typing of members of the MTBC

DNA fingerprinting of *M. tuberculosis,* based on the variability in both the number and the genomic position of IS*6110*, was standardised in 1993 to generate fingerprints, which permit comparison of the results obtained by different laboratories (van Embden et al., 1993). Such standardization has facilitated investigations into the international transmission of tuberculosis and has allowed to identify specific strains with unique properties such as high infectivity, virulence or drug resistance. Although other techniques based on this insertion sequence and other repetitive elements were described, IS*6110*-RFLP demonstrated the best discriminatory power and reproducibility and was accepted as the gold standard method for *M. tuberculosis* genotype (Kremer et al., 1999). Up to now it is the best-validated genotyping method, however, the requirement of growth culture and the poor discrimination found among the low copy number of IS*6110* strains (LCS), have led to search a better method, based on PCR, discriminative enough to be used on epidemiology.

The application of IS*6110* as molecular tool has given a different global vision on TB. IS*6110*-RFLP has shown to be of great value in, among others: distinguish between recent transmission and reactivation, reinfection, mixed infections, studies of outbreaks, confirmation or rule out laboratory errors. It has also been useful to identify some strains

that may differ in transmission, suggesting that more virulent strains could show different pathogenesis and epidemiological characteristics. The establishment of Databases of the RFLP patterns has allowed to analyse the risk factors for tuberculosis and to detect the prevalent strains and/or the most transmitted strains, among the studied populations.

3.2.1 Recent transmission & population studies

The relatively higher rate of IS transposition on genomes compared to that of mutations in structural genes and other loci has elicited strong interest in the applications of ISs as genetic markers to study bacterial population genetics and phylogeny, especially for species with conserved genomes, as is the case of IS*6110* for *M. tuberculosis* (Fang et al., 2001). At the beginning of the nineties it was demonstrated the utility of IS*6110* in epidemiology (Otal et al., 1991). On the basis of IS*6110*-RFLP, recent transmission of TB has been associated to those patients whose isolates presented the same RFLP pattern or were included in a "cluster". The use of IS*6110*-RFLP analysis in population studies has considerably advanced our knowledge of the epidemiology of *M. tuberculosis*. Above all, large population studies have led to better understand how transmission occurs in the population. One study carried out in The Netherlands concluded that a short time span between the first two patients in a cluster was the strongest predictor for large cluster episodes (Kik et al., 2008). In this regard, after two population studies carried out in Zaragoza, Spain, along three years each, a change in patterns' transmission of TB was detected (López-Calleja et al., 2007). One susceptible strain designed as "MTZ" caused a susceptible outbreak involving more than one hundred inhabitants (18% of the TB cases). This kind of studies have made possible the detection and characterization of specific *M. tuberculosis* epidemic strains (Lopez-Calleja et al., 2009).

Recent studies indicate that multidrug-resistant *M. tuberculosis* has emerged in many countries for the past few years, without the concomitant development of health systems able to provide adequate treatment. MDR and XDR strains can be transmitted among the population (Bifani et al., 1996; Samper et al., 1997; Samper et al., 2005). It is known that the pattern of IS*6110*-RFLP does not usually change after acquisition of resistances of the strain, nevertheless, the complementary characterization of the genes conferring the resistance helps in contact tracing (Gavin et al., 2009). More recently, drug-resistance and molecular epidemiology of TB in the Murmansk region was investigated in a 2-year population-based surveillance of the civilian population. The study showed that MDR-TB strains were actively transmitted in the northern Russia (Mäkinen et al., 2011). In Ukraine, where increase of TB cases is maintained, the number of drug-resistant isolates was reported to be growing steadily, and transmission of drug-resistant isolates seems to contribute to the spread of resistant TB (Dymova et al., 2011). The MDR-TB genotyping databases allow the comparison of *M. tuberculosis* strains to improve the application of appropriate public health actions at a national level and, ideally, it should be extended across country borders (Bifani et al., 2001; Gavin et al., 2011; Ritacco et al., 2011).

The current population studies have been essential not only to gain a better understanding of how to implement effective TB control measures but also to analyse the importance of immigration. In Germany, the dynamics of TB transmission between TB high-prevalence immigrant and TB low-prevalence local populations confirm that there is no significant TB transmission from high to low-prevalence population. This could be probably due to the good performance of TB screening programmes, to low degree of mixing high to low

populations or by a combination of both (Barniol et al., 2009). One study was carried out to evaluate the origins of the resistant isolates in Finland, a country with a low incidence of TB. They have raised worries concerning the risk of disease in near-frontier contacts and they conclude that it is very probable that cases of MDR in Finland are mostly caught abroad (Vasankari et al., 2011).

Several studies illustrate the situation in the highest TB incidence areas, such as two areas of India (Shanmugam et al., 2011; Purwar et al., 2011) or Uganda (Asiimwe et al., 2009). Other study that gives an overview of the distribution of genotypes of *M. tuberculosis* in Korea, found that drug resistance phenotypes were more strongly associated with Beijing family (see part 4.1.3). The Beijing genotype strains are also a major cause of TB (75% of MDR-TB) in the Aral Sea region, they are also strongly associated with drug resistance, independent of previous TB treatment and may be strongly contributing to the transmission of MDR-TB (Cox et al., 2005). In a population-based study carried out in rural China, the association between the Beijing family showed that a specific IS*6110*-RFLP and MIRU genotype 223325173533 were associated with MDR and with increased transmissibility (Hu et al., 2011).

3.2.2 Recurrent tuberculosis: Relapse or reinfection?

The frequency and determinants of exogenous reinfection and of endogenous reactivation of TB in patients previously treated are poorly understood. The importance of reinfection as a cause for recurrence of TB is unclear and has potential public-health implications. Different studies have used IS*6110* genotyping to answer this question. The possibility of genotyping the isolates from initial and recurrent disease episodes allows to differentiate an episode of reinfection from that of relapse of TB.

At this respect, differences are shown depending on the incidences of TB and of the HIV status of the patients. In Spain, a country with a low incidence rate of TB, two studies on this issue were conducted. In the Gran Canaria Island, 2.4% of the cases had recurrent TB in a 5 years-period. Up to 44% of them corresponded to exogenous reinfection proved by IS*6110* genotypes (Caminero et al., 2001). In a second study conducted in Madrid extended twelve years, up to 3.1% of the patients had a second episode of TB. Only one recurrent case showed different genotypes, suggesting exogenous re-infection. Re-infection is possible among people in low-risk areas, but the rates are lower than those occurring in high-risk areas (Cacho et al., 2007). On the other hand, in countries with high incidence as India, most of the recurrences after successful treatment of TB are due to exogenous reinfection in HIV-infected persons, in contrast to endogenous reactivation in HIV-uninfected persons. Strategies for prevention and treatment of TB infection must take these findings into consideration (Narayanan et al., 2010). Conversely, one study carried out in Karinga Malawi, concluded that HIV increases the rate of recurrent TB by increasing the rate of reinfection disease (Crampin et al., 2010). Other authors reviewed different studies on recurrence and argued that, apart from extreme situations, the problem of recurrence due to reinfection has few implications for TB-control programmes (Lambert et al., 2003).

3.2.3 Limits of IS*6110* as epidemiological tool

A common dilemma of the different markers used for typing tuberculosis, including IS*6110*, is how to interpret the variability of the patterns. If two *M. tuberculosis* isolates from 2

different patients present the same genotype, transmission may have occurred between them. However, once transmission has occurred, the genotypes may change, resulting in divergent fingerprints. The advantage of IS*6110* as marker is that the clock of change of the IS*6110* patterns was determined in serial isolates; the half-life was extrapolated to be 3.2 years. These changes were predicted more common for persons with extrapulmonary disease and for those who had both pulmonary and extrapulmonary isolates. This fact supported the use of IS*6110* typing in epidemiologic studies of recent transmission of TB (de Boer et al., 1999). The results of a study carried out to estimate the recent transmission based on IS*6110*-RFLP suggested that the interpretation of the recent transmission index, and the resulting necessary public health interventions, will vary according to how researchers account for spontaneous mutation when estimating transmission from the genotyping data (Benedetti et al., 2010).

In spite of all the studies carried out with this genomic element, some limitations have been found. Besides the technical difficulties that IS*6110* typing presents for some laboratories (the long time that the mycobacteria requires to growth, the equipment and the software required for the analysis), this method have also demonstrated difficulties for differentiating LCS, including *M. bovis* strains and is unable to identify strains with cero copies. Some studies have solved this problem by applying a second technique for these cases (Thong-On et al., 2010). Other studies with high prevalence of strains with LCS do not recommend this technique in their settings (Asgharzadeh et al., 2011). Mixed infections represent another limitation, which could be underestimated using IS*6110*-RFLP and could be confused with exogenous reinfection (Shamputa et al., 2006). The mixed tuberculosis infection suspected as a result of the IS*6110*-RFLP method could be clearly identified by MIRU-VNTR typing, which is more sensitive for the detection of multiple *M. tuberculosis* strains (Allix et al., 2004).

4. The tails: Risks of IS*6110*

Understanding the changes that occur in genomes among isolates of *M. tuberculosis* would give insights into their corresponding differences causing disease.

Many mechanisms can be related to changes in the bacterial genomes, being those mediated by ISs one of the most relevant and better studied (Galas & Chandler, 1989). According to general data, it was considered that among 5 to 15% of spontaneous mutations in the bacterial genomes were due to changes in the ISs locations.

The more common mechanism used by IS to move along genomes is transposition following the enzymatic activity of their encoded transposases, this transposition could lead to the generation of 3-4bp direct repeats (DR) immediately flanking the IS sequences, as it occurs to IS*6110* (Thierry et al., 1990b). Recombination is also another mechanism participating in the changes of the location of ISs along the genomes. All those mechanisms lead to IS mediated gene rearrangements, inversions, deletions etc in the bacterial genomes.

ISs could have also some polar effect on the flanking genes, particularly on downstream genes. It has been demonstrated the occurrence of gene activation due to the presence of out-warding promoters within the elements as well as the formation of new promoters upon insertion (Galas & Chandler, 1989).

All those changes could be a risky to the bacteria's genomes integrity, being the carriage of mobile IS either a potential enemy with deadly influence on the bacterial fitness or a helpful ally contributing to the improvement of that fitness. Our current knowledge on how the IS*6110*-mediated mutations influence in the genome plasticity of the *M. tuberculosis* genome will be reviewed herewith.

4.1 Moving along the genome

The numerous studies published on IS*6110*-RFLP with epidemiological purposes showed a high level of variability in the locations of this IS along the *M. tuberculosis* genome (see part 3.2). On the basis of those results the rate of transposition of IS*6110* was estimated to be about 18% over a period of 5-6 years. However it seems evident that the events of transposition are related to changes in the environment in which the bacteria are involved. It was suggested that transpositional events occur following mutational burst instead of following a constant mutation rate; this can explain the observation that changes in RFLP patterns would occurred more frequently during transmission and before diagnosis (soon after the bacilli enter inside the host) or after relapses or any other main event during the course of the infection (Schürch et al., 2010). In agreement to this consideration, two rather different half-life times were calculated for the IS*6110*-RFLP patterns stability in serial patient's isolates: 0.6 and 10.7 years; this most probably be due to changes in the patient's management or to the course of the infection in the different settings compared (Schürch et al., 2010).

Independently of why, how or when its transpositions occurred, IS*6110* mediates genome plasticity of members of the MTBC, and that plasticity is ongoing both under controlled environment *in vitro* and during infection *in vivo* (Fang et al., 1999b).

To confirm the last assertion, some papers described changes in the RFLP pattern during infection. This is showing that microevolution of the bacilli mediated by IS could occur not only during transmission between patients but also during the course of the disease in a single patient (Al-Hajoj et al., 2010). Besides, the comparison of the whole-genomes of six different H37Rv strains, collected from several laboratories, showed that multiple IS*6110* transposition events have occurred in the genome even under *in vitro* "controlled" environments (Ioerger et al., 2010).

4.1.1 How to identify the IS*6110* insertion sites

Since late nineties, several methods have been applied to identify and sequence the loci in which the IS was integrated inside the genome. The methods applied for the identification and sequence of the flaking-regions included cloning of the agarose-excised hybridizing bands (Beggs et al., 2000); reverse dot blot assay (Steinlein & Crawford, 2001); whole-genome microarrays (Kivi et al., 2002), ligation-mediated PCR (Otal et al., 2008) and construction of BACs libraries (Alonso et al., 2011) among others. All these procedures are usually cumbersome and show difficulties to detect all the insertions present, particularly in those strains carrying high IS*6110* copy number.

The development of high throughput whole-genome sequencing procedures has allowed the overcome of some of those difficulties, however this procedure is so far not of general

applicability. Whole-genome sequences of tens of MTBC strains are currently finished or at several degrees of accomplishment, however, that number could not compete with the thousand IS*6110*-RFLP patterns already registered at the available data-bases.

New technologies are being currently under development aiming to determine the IS*6110* insertional sites of a high number of *M. tuberculosis* isolates by using high-throughput methodologies, such as the Masive-Insertion Site sequencing (IS-seq) (Sandoval et al., ESM-2010). Such a kind of procedures will surely help to unravel the IS flanking region sequences in a more feasible manner.

4.1.2 Where IS*6110* can be inserted

The identification of the sites of insertion, and its relationships with the phenotype of the corresponding strain, could allow to have insights into the biological meaning of the genes targeted. The identification of those sites showed that this element could interrupt coding regions as well as be located in non-coding sequences (Fang et al., 1999a). The interruption of coding regions can be seen as a sort of natural knock-out mutation of the target gene. On the other hand, the insertion of the element in non-coding regions would have secondary consequences, such as the increasing or decreasing of the expression of the neighbouring genes (McEvoy et al., 2007).

The high variation detected in the RFLP pattern comparing multiple *M. tuberculosis* isolates showed apparent lack of preferential location of the IS in the bacterial genome. However, one of the first conclussions made evident was that the insertion was not fully at random.

Hermans and co-workers showed the first *hot-spot* integrative region in the genome described for this element, known as D*irect Repeat* (DR) locus (Hermans et al., 1991). With minor exceptions, all members of the MTBC carry a copy of the IS*6110* integrated in that locus, and that characteristic has been exploited for the development of a widely applied typing procedure called Spoligotyping (see part 3.2). Later on, another *hot-spot* site of integration was described, namely the *insertional preferential locus* (*ipl*) (Fang et al., 1997). It was shown that this corresponded to the ORF of the virulent reference strain Rv0797 that encodes for another insertion sequence, IS*1547* (see part 1.2). These preferential integration sites, are characterized by the occurrence of insertion in more than one site close each other (Sampson et al., 2001). The list of preferential sites for the insertion of this element identified at the moment rose to about half a dozen and most probably will be increased (McEvoy et al., 2009).

Appart of the identification of preferential loci for the IS*6110* insertions, the location of the insertions along the genome was not equally organized. After the complete genome sequence of the reference virulent strain, namely H37Rv, the IS*6110* was found to be inserted more often in some genome regions, on the contrary, other regions lacked in the presence of this IS (Cole et al., 1998). Up to near the 800 first kbp from the origin replication fail in carrying copies of IS*6110* in the strain H37Rv. Besides, IS*6110* was otherwise located more or less randomly along the rest of the genome. The conclusion was that this part of the genome could be more abundant in essential genes. This result was also seen when studies of other strains were accomplished.

Comparison of the IS*6110*-RFLP pattern to the corresponding list of insertion loci showed that RFLP has limited level of discriminative power. Thus, the finding of more insertion loci

than RFLP bands is not a rare event (Beggs et al., 2000; Warren et al., 2000; Alonso et al., 2011). This result is more evident in those strain carrying high copy number of the IS.

The influence that the insertion could have in the content of active/non active genes was considered that could give insights into the number of genes required for infection, being thus a source of information to detect which were the genes or gene content essential for virulence (McEvoy et al., 2007).

Some works were devoted to compare the insertion loci of virulent with those of avirulent strains. The attenuated vaccine strain *M. bovis* BCG has major differences on the content of IS*6110* compared to the virulent strain *M. tuberculosis* H37Rv: one and 16 copies respectively. However the IS*6110* copy number per genome not appears to be related to the attenuation of the bacilli (see part 5). In fact, the avirulent strain H37Ra has a supplementary copy compared to its parenteral strain the virulent H37Rv. Comparison of H37Rv and H37Ra genomes showed two main differences among them mediated by the insertion of IS*6110*. However these changes have not a clear role in the attenuation of the avirulent strain (Brosh et al., 1999).

Comparison of several BCG strains showed differences among them in relation to IS*6110*. The "ancestral" BCG (for example, BCG tokio) carries two copies of the IS sited in the DR region and upstream the two component system *pho*P-*pho*R (see part 4.2). This last copy was lost in the "modern" BCG (for example, BCG pasteur) that has a single copy inserted in the preferential loci mentioned, namely DR region (Brosh et al., 2007).

Identification of essential genes could be also possible through the detection of those never carrying inserted ISs, following the assessment that those mutations could be deleterious for the bacteria. An *in silico* study, based on previous experimental data, estimated that the *M. tuberculosis* genome contains 35% of essential genes (Lamichhane et al., 2003). Even though the data on genome loci with insertion identifies transposition/recombination events either in coding or in non-coding regions, generally speaking, there has been detected higher number of insertion loci inside coding region. However, the non-coding sequences represent only 10% of the genome suitable to host IS. Therefore the proportion of insertions inside non-coding region is actually higher compared to the proportion of insertions inside coding regions (Table 3). This could represent a sort of "ORF-preserving" behaviour of the genome variability mediated by IS*6110* transposition. This is consistent with the suggestion of a greater selection against intra-genic insertion in *M. tuberculosis* during infection *in vivo* than when grown *in vitro* (Yesilkaya et al., 2005).

In a study conducted over 161 clinical isolates of *M. tuberculosis*, the insertion sites of the IS*6110* were determined (Yesilkaya et al., 2005). Only 100 ORF were affected by insertion, and was considered by the authors that represented a global low number of non-essential genes. In conclusion most of the genes in *M. tuberculosis* might play important role for infection and transmission.

From the data obtained thus far, a high proportion of the *IS6110* coding-targeted genes correspond to the functional category containing PE-PPE group of genes (see references in Table 3). These genes are very characteristics of the MTBC members and are considered related to the antigen variability of the bacilli (McEvoy et al., 2009).

Reference	Isolates studied	Sites identified	Coding (%)	Non coding (%)	Comments
Beggs et al., 2000 Alonso et al., 2011	3	32	13 (0.33)	19 (4.3)	*M. tuberculosis* Beijing family
Warren et al., 2000 Sampson et al., 2001	34	97	57 (1.44)	33 (7.5)	Identified 13 preferential loci
Kivi et al., 2002	8	41	28 (0.7)	13 (2.9)	(a)
Yesilkaya et al., 2005	161	818	491 (0.12)	327 (0.74)	
Otal et al., 2008	7	12	6 (0.15)	6 (1.36)	*M. bovis*

Table 3. Number of IS*6110* inserted sites recorded from the literature. Percentages were approximate considering that 90% and 10% of the genome corresponded respectively to coding and non-coding sequences.
(a) With the exception of the direct repeat loci, all low copy number strains analyzed in this study have IS*6110* inserted exclusively inside coding regions.

As previously mentioned, the hallmark that identifies the transposition of IS*6110* is the presence of 3-4bp direct repeats immediately flanking the IS sequence. The current availability of annotated whole genome sequences of members of the MTBC allow to differentiate, for each of the IS copy, if the insertion was due to transposition or recombination mechanisms. According to the data derived from 81 insertions in 10 of those members, transposition is the more frequent mechanism used by this IS to be inserted into the MTBC genome regardless the number of copies carried by the genome or the target sequence (insertion into coding or no-coding regions) (Table 4). In all cases, the insertion in the direct repeat loci has been as consequence of a transposition event.

MTBC	copies of IS*6110*	3-4bp	no bp repeat
M. tuberculosis H37Rv	16	12	4
M. tuberculosis H37Ra	17	13	4
M. tuberculosis CDC1551	4	4	0
M. tuberculosis KZN	14	9	5
M. tuberculosis F11	17	15	2
M. africanum	7	6	1
„M. canettii“	2	1	1
M. bovis	1	1	0
M. bovis BCG Pasteur	1	1	0
M. bovis BCG Tokio	2	2	0
TOTAL	81	64 (79%)	17 (21%)

Table 4. Data collected from whole genome sequences of the corresponding strains (http://www.ncbi.nlm.nih.gov/). For each genome, the number of copies of the IS*6110* per genome was indicated as well as how many carry or not the 3-4bp direct repeat sequence.

4.1.3 IS*6110* in the genome of the Beijing family

Efforts were addressed on the study of clinical isolates particularly relevant under microbiological, clinical or epidemiological aspects. This was the case of members of the Beijing family.

Since the first description in 1995, the *M. tuberculosis* Beijing strain becames a main health problem worldwide. It was responsible of one of the most important outbreaks due to multidrug-resistant strain in the USA during the early nineties (Moss et al., 1997). The *M. tuberculosis* Beijing identifies a family that includes highly transmissible drug resistant and drug susceptible strains, being currently responsible of about one third of the global TB cases (Alonso et al., 2011).

The members of the Beijing family usually are high copy number strains (HCS) of IS*6110* (between 15-25 copies per genome) suggesting the relevance of this element in the variability of their genomes. Supporting this possibility, sublineages of this family were identified to carry an important genome duplication that involves up to 8% of the genome (corresponding to more than 300 genes). Copies of IS*6110* were identified flanking that duplication, thus suggesting the occurrence of homologous recombination event mediated by this IS (Domenech et al., 2010).

The insertion sites of IS*6110* of two drug resistant Beijing strains (W and 210) were determined (Beggs et al., 2000). These strains shared up to 17 insertion sites. Several features related to IS*6110* characterize this family, such as the presence of one copy in the *ori*C region, the deletion of the right-site DR spacers (from spacers 1 to 34) and similar RFLP multiband pattern profile (Hanekom et al., 2011).

Recently, in a study undertaken in the laboratory of one of the authors (Alonso et al., 2011) the insertion sites of another Beijing strain were determined and compared to those from strains W and 210. A higher proportion of insertion in non-coding region was found including one locus with putative promoter-influence activity (see part 4.2). Nine loci common to all three Beijing strains, including the *ori*C, were also identified.

The presence of the IS in *ori*C, the region that control the replication of the genome, is expected to have some influence on the synchronization of the bacterial cell division (Casart et al., 2008). This site is currently considered a preferential locus, and multiple transposition events were described in several clinical isolates from patients in Caracas (Venezuela) (Turcios et al., 2009). Both the infection in the animal model as well as the *in vitro* growth rate were further analyzed for those clinical strains, and compared to strains lacking in IS at the *ori*C region (Casart et al., 2008). The presence of IS*6110* in the origin of replication enlarge the bacilli and causes slow growth rate *in vitro*; besides the IS apparently causes attenuation in the animal model.

4.2 Switching *on* and *off* genes

To date, the data collected on *M. tuberculosis* confirm that its genome is highly conserved. This result raises the possibility that differences among isolates be more likely found through the study of regulatory and/or metabolic pathways. Taken into consideration the previous assertion, we should not forget that a big proportion of the ORFs identified in the tubercle bacilli are of unknown function. Nevertheless, following the previous statement, the insertion of IS*6110* outside ORFs even though saves the bacilli of direct knockout of one/several gene, could putatively have important consequences for gene expression and then influence in the metabolic activity of the bacilli.

IS insertion could interfere both the initiation and the termination of gene expression providing it inserted up- or down-stream the gene coding sequence. It is considered that the

influence of the IS on the downstream genes is related to the distance among the gene and the 3'-end of the IS. Thus, a promoter influence is possible within the range of 31 to 300bp of distance among them.

This could be due to a polar effect of the IS and also due to the presence of an outward promoter that was identified close to the 3'-end of IS*6110* (Safi et al., 2004; Soto et al., 2004).

The promoter carried by IS*6110* has the relevance of being activated inside monocytes (Safi et al., 2004) and its activity was demonstrated in several genes not only in the strain H37Rv but also in other clinical strains including Beijing strains (Safi et al., 2004). Remarkably that promoter activity has been demonstrated by the upregulation of the main two-component system of this bacterium, namely *pho*P/*pho*R (Soto et al., 2004).

In this context, it is noteworthy the presence of the IS inserted between *dna*A-*dna*N proteins that control the genome replication. This insertion was identified in many strains including several belonging to the Beijing family (see part 4.1.3). Moreover the IS could be inserted in both directions in this region (Turcios et al., 2009; Casart et al., 2008) having thus putatively a variable influence on the bacterial cell division.

Reference	bacteria/strain	ORFs (genes)
Soto et al., 2004	*M. bovis*	Rv0757 (*pho*P/*pho*R)
Safi et al., 2004	*M. tuberculosis*	Rv0797 and Rv3327 (IS*1547*) Rv1468c (PE-PGRS) Rv2166c, Rv2478c and Rv3188 (CHP*) Rv2288 (FAD-cofactor containing domain)
Turcios et al., 2009	*M. tuberculosis*	Rv0002 (*dna*N)
McEvoy et al., 2009	*M. tuberculosis*	Rv2352c, Rv2353c and Rv2354c (PPE38, PPE39 and PPE40 respectivily)
Alonso et al., 2011	*M. tuberculosis*/GC1237	Rv1468c (PE-PGRS) Rv2179c (CHP*) Rv3427c (IS*1532*)

* CHP: Conserved Hypothetical Protein.

Table 5. Identified genes located under the putative influence of the IS*6110* promoter activity. Changes in the gene expression were demonstrated in some of the cases.

Much effort should be used to complete the record of the loci in which IS*6110* was inserted. That knowledge will much help to our understanding of the mechanisms used by the tubercle bacilli to cause Tuberculosis so successfully.

5. The IS*6110* content: How many are the best number?

A high variability in number of IS*6110* is observed amongst the different strains of the MTBC. While *M. bovis* usually has a unique copy, *M. tuberculosis* varies from zero to twenty five. In any case, it is difficult to answer the question: What is the best number for the bacteria?

5.1 *M. tuberculosis* low copy number strains (LCS) & high copy number strains (HCS)

M. tuberculosis strains with less than six copies of IS*6110* are usually referred as low copy number strains (LCS) in the literature. A few clinical investigations reported the presence of LCS in regions as India, Vietnam or Tanzania. (Barlow et al., 2001; Sankar et al., 2011a). The 66% of the *M. tuberculosis* strains isolated in Tiruvallur, South India, presented a single copy of the IS*6110* or LCS (Shanmugam et al., 2011). In Kanpur district, , North India, the 17% of the *M. tuberculosis* isolates were LCS (Purwar et al., 2011). High copy number *M. tuberculosis* strains (HCS), with six or more copies of IS*6110,* were reported by a greater number of papers. One study from Brazil, reported that 93.6% of *M. tuberculosis* strains had at least six copies ranging from 1 to 18 (Suffys et al., 2000). In San Francisco, of 1,326 isolates investigated, 90% had six o more copies and only two isolates had no copies of IS*6110* (Yang et al., 1998).

A majority (96.2%) of the 183 strains fingerprinted from Kampala were HCS. These strains were isolated from patients with known HIV sero-status. The number of IS*6110* copies ranged from 1 to 20 and the frequency of occurrence of IS*6110* bands was similar between the two serogroups. The most prevalent pattern observed had 14 copies of IS*6110* with the same distribution comparing HIV seropositive and HIV seronegative patients (Asiimwe et al., 2009).

Chauhan et al analyzed 308 isolates of *M. tuberculosis* from different parts of India and 56 per cent of the isolates showed HCS of IS*6110.* At the regional level, there was not much difference in the IS*6110* copy numbers of isolates from different parts of that country (Chauhan et al., 2007).

A long term population based study analysing 1759 clinical strains from the state of Alabama showed that 65% corresponded with HCS. The results of this study demonstrated that clustering cases is clearly associated with different social factors and risk behaviors but not with high or low number of copies of the IS*6110* (Kempf et al., 2005).

5.2 Are there any clinical properties associated to LCS or HCS?

After revision of the literature looking for the origin of outbreaks including MDR cases, it was evident that both LCS and HCS were involved in outbreaks at similar proportion. Some examples of large outbreaks in population studies showing different copy number strains are listed in Table 6.

The Beijing family is one of the lineages with the highest number of copies of IS*6110* (see part 4.1.3). There are controversies among the behaviour of the Beijing lineage. On the one hand, a Beijing strain named GC1237 has been responsible of epidemic outbreaks since its appeared in the community in 1993 (Caminero et al., 2001), on the other hand, one study conducted in Cape Town (South Africa) found no significant association between the *M. tuberculosis* genotype and transmissibility within the household (Marais et al., 2009). Besides, there are outbreaks reported caused by LCS, as was the extensive transmission of *M. tuberculosis* in a rural population with minimal risk factors for TB. This strain was designated as CDC 1551 and the fingerprint showed only 4 copies of the IS*6110* (Valway et al., 1998).

Because in most population-based studies the proportion of cases with isolates that have five or fewer copies of IS*6110* is low, the impact of these cases in the study of the overall transmission of tuberculosis in a community will be low.

	Strain name	Family	N^{er} copies IS6110	Reference
Susceptible strains				
Zaragoza	MTZ	U	12	Lopez-Calleja et al., 2009
The Netherlands	Harlingen Type	Haarlem	12	Kiers et al., 1997
Canarias	GC1237	Beijing	19	Caminero, et al., 2001
Greenland	GC2		10	Søborg, et al., 2001
New York	CDC1551		4	Kelley et al., 1999
MDR / XDR strains				
South- Africa	KNZ	LAM	13	Streicher et al. 2011
Argentina	M	Haarlem	8	Ritacco et al., 1997
Spain	MBZ	Bovis	2	Samper et al., 2007
New York	W	Beijing	23	Bifani et al., 1996

Table 6. *M. tuberculosis* complex strains causing large outbreaks.

5.3 LCS *versus* HCS and IS*6110* location

Several reports have strongly suggested that the severity and clinical manifestations of tuberculosis depend on the immunogenicity and pathogenicity of the infecting *M. tuberculosis* strain. In this regard the IS*6110* sequence varies in number and position within the genome generating a high level of DNA polymorphism among strains.

The location of IS*6110* in *M. bovis* isolates from endogenous reactivation cases from elderly people were studied in comparison to the bovine *M. bovis* strains, concluding that the presence of more copies in human strains could be related to the adaptation from the animal to the human host (Otal et al., 2008).

In addition to the DR locus, Fomukong et al. detected a highly preferred site of insertion of IS*6110*, namely "DK1", in *M. tuberculosis* strains with low copy number. However, the prevalence of this site decreases in HCS, suggesting a separate lineage for the HCS and the LCS (Fomukong et al., 1997). This contrasted with the *M. bovis* strains analysed without copy inserted at the same genomic position that *M tuberculosis* strains (Otal et al., 2008). This agreed with the idea that LCS of *M. tuberculosis* and *M. bovis* evolved separately after the progenitor acquired IS*6110* at the DR region. According to Fomoukong *et al* (1997), among the different Beijing strains analysed until now, no IS*6110* has been detected in the DK1 locus (data not shown).

Molecular epidemiological data support the observation that the copy-number of IS*6110* in members of the MTBC may change over time. Factors affecting this rate may include the nature and duration of disease in a host and the opportunity to go through different host environments during the transmission cycle.

IS*6110* has been also checked as a tool to analyze the evolution of members of the MTBC. Transposition may have influence on the evolution of the strains, thus the parental strains should carry low copy number and the descendant, more evolved, would carry high copy number. One example that theoretically support that consideration is the Beijing family, members of this family are IS*6110* high copy-number and have shown high prevalence and high transmissibility (Mc Evoy et al., 2007). These characteristics could be seen as selective advantages of bacteria to its main purpose: infect humans (Hanekom et al., 2011). However the previous statement was theoretically possible, the presence of preferential sites, together to the presence of forbidden sites makes the study of IS*6110* variation in the genomes useless as evolutionary tool (Kivi et al., 2002).

6. References

Al-Hajoj, S.A.; Akkerman, O.; Parwati, I.; al-Gamdi, S.; Rahim, Z.; van Soolingen, D.; van Ingen, J.; Supply, P. & van der Zanden, A.G. (2010). Microevolution of *Mycobacterium tuberculosis* in a tuberculosis patient. *Journal of Clinical Microbiology*. Vol.48, No.10, (October 2010), pp. 3813-3816, ISSN 0095-1137.

Allix, C.; Supply, P. & Fauville-Dufaux, M. (2004). Utility of fast mycobacterial interspersed repetitive unit-variable number tandem repeat genotyping in clinical mycobacteriological analysis. *Clinical Infectious Diseases, Vol.*39, No.6, (September 2004), pp.783-789, ISSN 1058-4838, online ISSN 1537-6591.

Alonso, H.; Aguilo, J.I.; Samper, S.; Caminero, J.A.; Campos-Herrero, M.I.; Gicquel, B.; Brosch, R.; Martín, C. & Otal, I. (2011). Deciphering the role of *IS6110* in a highly transmissible *Mycobacterium tuberculosis* Beijing strain, GC1237. *Tuberculosis* (Edinb). Vol.91, No.2, (March 2011), pp. 117-126, ISSN 1472-9792.

Armand S, Vanhuls P, Delcroix G, Courcol R & Lemaitre N. (2011)..Comparison of the Xpert MTB/RIF test with an IS*6110*-TaqMan real-time PCR assay for direct detection of *Mycobacterium tuberculosis* in respiratory and nonrespiratory specimens. *Journal of Clinical Microbiology*. Vol.49, No.5, (May 2011), pp. 1772-1776, ISSN 0095-1137

Asgharzadeh, M.; Kafil, HS.; Roudsary, AA.; Hanifi, GR. (2011). Tuberculosis transmission in Northwest of Iran: using MIRU-VNTR, ETR-VNTR and IS*6110*-RFLP methods. *Infection, Genetics and Evolution*. Vol.11, No.1, pp. 124-131, (January 2011), ISSN: 1567-1348.

Asiimwe, BB.; Joloba, ML.; Ghebremichael, S.; Koivula, T.; Kateete, DP.; Katabazi, FA.; Pennhag, A.; Petersson R. & Kallenius, G. (2009). DNA restriction fragment length polymorphism analysis of *M. tuberculosis* isolates from HIV-seropositive and HIV-seronegative patients in Kampala, Uganda. *BMC Infectious Diseases*. Vol.9, No.12, (February 2009), ISSN 1471-2334.

Bannalikar AS, Verma R. (2006). Detection of *Mycobacterium avium* & *M. tuberculosis* from human sputum cultures by PCR-RFLP analysis of *hsp65* gene & *pncA* PCR *Indian Journal Medical Research*., Vol.123, No.2, (February2006), pp 165 -172, ISSN 0971-5916

Barlow RE, Gascoyne-Binzi DM, Gillespie SH, Dickens A, Qamer S & Hawkey PM.(2001) Comparison of variable number tandem repeat and IS*6110*-restriction fragment length polymorphism analyses for discrimination of high- and low-copy-number IS*6110 Mycobacterium tuberculosis* isolates. *Journal of Clinical Microbiology*. Vol.39, No.7, (July 2001) pp. 2453-2457, ISSN 0095-1137

Barniol, J.; Niemann, S.; Louis, VR.; Brodhun, B.; Dreweck, C.; Richter, E.; Becher, H.; Haas, W. & Junghanss, T. (2009). Transmission dynamics of pulmonary tuberculosis between autochthonous and immigrant sub-populations. *BMC Infectious Diseases*. Vol.9, No.197, (December 2009), ISSN 1471-2334.

Beggs, M.L.; Eisenach, K.D. & Cave, M.D. (2000). Mapping of IS*6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*. Vol.38, No.8 (August, 2000), pp. 2923-2928, ISSN 0095-1137.

Benedetti, A.; Menzies, D.; Behr, MA.; Schwartzman, K. & Jin, Y. (2010). How close is close enough? Exploring matching criteria in the estimation of recent transmission of tuberculosis. *American Journal of Epidemiology* Vol.172, No.3, (August 2010), pp. 318-326, ISSN 0002-9262.

Bifani, P.J.; Plikaytis, B.B.; Kapur, V.; Stockbauer, K.; Pan, X.; Lutfey, M.L.; Moghazeh, S.L.; Eisner, W.; Daniel, T.M.; Kaplan, M.H.; Crawford, J.T.; Musser, J.M. & Kreiswirth, B.N. (1996). Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *JAMA*, Vol.275, No.6, (February 1996), pp. 452-457, ISSN: 0098-7484.

Bifani, P.J.; Mathema, B.; Campo, M.; Moghazeh, S.; Nivin, B.; Shashkina, E.; Driscoll, J.; Munsiff, S.S.; Frothingham, R. & Kreiswirth, B.N. (2001). Molecular identification of streptomycin monoresistant Mycobacterium tuberculosis related to multidrug-resistant W strain. *Emerging Infectious Diseases*. Vol.7, No.5, (September-October 2001), pp. 842-848, ISSN: 1080-6059.

Boondireke, S.; Mungthin, M.; Tan-ariya, P.; Boonyongsunchai, P.; Naaglor, T.; Wattanathum, A., Treewatchareekorn, S. & Leelayoova S. (2010). Evaluation of sensitivity of multiplex PCR for detection of *Mycobacterium tuberculosis* and *Pneumocystis jirovecii* in clinical samples. *Journal of Clinical Microbiology*. Vol.48, No.9 (September, 2010), pp. 3165-3168, ISSN 0095-1137.

Brisson-Noel A, Aznar C, Chureau C, Nguyen S, Pierre C, Bartoli M, Bonete R, Pialoux G, Gicquel B, Garrigue G. (1991) Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. *Lancet*. Vol. 338, No. 8763 (August, 1991), pp. 364-366, ISSN 0140-6736.

Brosch, R.; Philipp, W.J.; Stavropoulos, E.; Colston, M.J.; Cole, S.T. & Gordon, S.V. (1999). Genomic analysis reveals variation between *Mycobacterium tuberculosis* H37Rv and the attenuated *M. tuberculosis* H37Ra strain. *Infection and Immunity*. Vol. 67, No. 11 (November, 1999), pp. 5768-5774, Erratum in: Infect Immun 2000 Jan;68(1):427. ISSN 0019-9567.

Brosch, R.; Gordon, S.V.; Eiglmeier, K.; Garnier, T.; Tekaia, F.; Yeramian, E. & Cole, S.T. (2000). Genomics, biology, and evolution of the *Mycobacterium tuberculosis* complex. In: *Molecular Genetics of Mycobacteria*, Hatfull, G. F. & Jacobs, W. R. Jr (Eds), pp. 19-36, American Society for Microbiology Press, ISBN 1-55581-191-4, Washington DC, USA.

Brosch, R.; Gordon, S.V.; Garnier, T.; Eiglmeier, K.; Frigui, W.; Valenti, P.; Dos Santos, S.; Duthoy, S.; Lacroix, C.; Garcia-Pelayo, C.; Inwald, J.K.; Golby, P.; Garcia, J.N.; Hewinson, R.G.; Behr, M.A.; Quail, M.A.; Churcher, C.; Barrell, B.G.; Parkhill, J. & Cole, S.T. (2007) Genome plasticity of BCG and impact on vaccine efficacy. *Proc Natl Acad Sci U S A.* Vol. 104, No. 13, (March 2007), pp. 5596-5601, ISSN 0027-8424.

Cacho, J.; Pérez Meixeira, A.; Cano, I.; Soria, T.; Ramos Martos, A.; Sánchez Concheiro, M.; Samper, S.; Gavin, P. & Martín, C. (2007). Recurrent tuberculosis from 1992 to 2004 in a metropolitan area. *European Respiratory Journal*. Vol.30, No.2, (August 2007), pp. 333-337, ISSN: 0903-1936.

Caminero, J.A., Pena, M.J., Campos-Herrero, M.I., Rodríguez, J.C., García, I., Cabrera, P., Lafoz, C., Samper, S., Takiff, H., Afonso, O., Pavón, J.M., Torres, M.J., van Soolingen, D., Enarson, D.A. & Martin, C. (2001). Epidemiological evidence of the spread of a *Mycobacterium tuberculosis* strain of the Beijing genotype on Gran Canaria Island. A*merican Journal of Respiratory and Critical Care Medicine,* Vol. 164, No.7, (October 2001), pp. 1165-1170, ISSN: 1073-449X.

Casart, Y.; Turcios, L.; Florez, I.; Jaspe, R.; Guerrero, E.; de Waard, J.; Aguilar, D.; Hérnandez-Pando, R. & Salazar, L. (2008). *IS6110* in *ori*C affects the morphology and growth of *Mycobacterium tuberculosis* and attenuates virulence in mice. *Tuberculosis* (Edinb). Vol. 88, No. 6, (November 2008), pp. 545-552, ISSN 1472-9792.

Causse M, Ruiz P, Gutierrez-Aroca JB & Casal M.(2011). Comparison of two molecular methods for rapid diagnosis of extrapulmonary tuberculosis. *Journal of Clinical Microbiology*. Vol.49, No. 8, (August 1990) pp. 3065-3067, ISSN 0095-1137.

Chauhan DS, Sharma VD, Parashar D, Chauhan A, Singh D, Singh HB, Das R, Aggarwal BM, Malhotra B, Jain A, Sharma M, Kataria VK, Aggarwal JK, Hanif M, Shahani A & Katoch VM. (2007) Molecular typing of *Mycobacterium tuberculosis* isolates from different parts of India based on IS*6110* element polymorphism using RFLP analysis. *Indian Journal Medical Research*. Vol. 125, No.4, (April 2007), pp. 577-581, ISSN 0971-5916.

Cheng VC, Yam WC, Hung IF, Woo PC, Lau SK, Tang BS &Yuen KY. (2004). Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. *Journal of Clinical Pathology*. Vol. 57, No. 3, (September 2004), pp. 281-285, ISSN 1472-4146.

Cho SN. (2007). Current issues on molecular and immunological diagnosis of tuberculosis. *Yonsei Medical Journal*. Vol. 48, No. 3, (June 2007), pp. 347-359, ISSN 0513-5796.

Cole, S. T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S. V.; Eiglmeier, K.; Gas, S.; Barry, C. E. 3rd; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M. A.; Rajandream, M. A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J. E.; Taylor, K.; Whitehead, S.; Barrell, B. G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, Vol. 393, No. 6685, (June 1998), pp. 537-544, ISSN 0028-0836.

Collins, D. M. & Stephens, D. M. (1991). Identification of an insertion sequence, IS*1081*, in *Mycobacterium bovis*. *FEMS Microbiology Letters*, Vol. 67, No. 1, (September 1991), pp. 11-15, ISSN 1574-6968.

Cox, HS.; Kubica, T.; Doshetov, D.; Kebede, Y.; Rüsch-Gerdess, S. & Niemann, S (2005). The Beijing genotype and drug resistant tuberculosis in the Aral Sea region of Central Asia. *Respiratory Research*. Vol.8, No.6, pp. 134. (November 2005), ISSN: 1465-9921.

Crampin, AC.; Mwaungulu, JN.; Mwaungulu, FD.; Mwafulirwa, DT.; Munthali, K.; Floyd, S.; Fine, PE.; Glynn, JR. (2010). Recurrent TB: relapse or reinfection? The effect of

HIV in a general population cohort in Malawi. *AIDS* Vol. 24, No.3, (January 2010), pp. 417-426, ISSN: 1473-5571.

Das S, Paramasivan CN, Lowrie DB, Prabhakar R, Narayanan PR. (1995). IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. *Tubercle Lung Diseases*. Vol. 76, No. 6, (December 1995), pp. 550-554, ISSN 0962-8479.

de Boer, AS.; Borgdorff, MW.; de Haas, PE.; Nagelkerke, NJ.; van Embden, JD. & van Soolingen, D. (1999). Analysis of rate of change of *IS6110* RFLP patterns of Mycobacterium tuberculosis based on serial patient isolates. *The Journal of Infectious Diseases*. Vol.180, No.4, (October 1999), pp. 1238-1244, ISSN 0022-1899.

Domenech, P.; Kolly, G.S.; Leon-Solis, L.; Fallow, A. & Reed, M.B. (2010). Massive gene duplication event among clinical isolates of the *Mycobacterium tuberculosis* W/Beijing family. *Journal of Bacteriology*. Vol. 192, No. 18, (September 2010), pp. 4562-4570, ISSN 0021-9193.

Dymova, M.A.; Liashenko, O.O.; Poteiko, P.I.; Krutko, V.S.; Khrapov, EA. & Filipenko, M.L. (2011). Genetic variation of *Mycobacterium tuberculosis* circulating in Kharkiv Oblast, Ukraine. *BMC Infectious Diseases* Vol. 11, No.77, (March 2011), ISSN 1471-2334.

Dziadek, J.; Sajduda, A.; Dale, J. W. & McFadden, J. J. (1998). IS*990*, a new species-specific insertion-sequence-related element of the *Mycobacterium tuberculosis* complex. *Microbiology*, Vol. 144, No. 12, (December 1998), pp. 3407-3412, ISSN 1350-0872.

Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford. (1990). Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *Journal of Infectious Disease*. Vol. 161, No. 5, (May 1990), pp. 977-981, Online ISSN 1537-6613 - Print ISSN 0022-1899.

Eisenach KD. (1994). Use of an insertion sequence for laboratory diagnosis and epidemiologic studies of tuberculosis. *Annals Emergency Medicine*. Vol. 24, No. 3, (September 1994), pp. 450-453, ISSN 0196-0644.

Ereqat S, Bar-Gal GK, Nasereddin A, Said S, Greenblatt CL, Azmi K, Qaddomi SE, Spigelman M, Ramlawi A, Abdeen Z. (2011). Pulmonary tuberculosis in the West Bank, Palestinian Authority: molecular diagnostic approach. *Tropical Medicine International Health.* Vol. 16, No. 3, (March 2011) pp. 360-367, ISSN 1360-2276.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF. (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinincal Microbiology Reviews*. Vol. 19, No. 1, (January 2006), pp. 165-256, ISSN 0893-8512.

Fang, Z. & Forbes, K. J. (1997). A *Mycobacterium tuberculosis* IS*6110* preferential locus (*ipl*) for insertion into the genome. *Journal of Clinical Microbiology*, Vol. 35, No. 2, (February 1997), pp. 479-481, ISSN 0095-1137.

Fang, Z.; Doig, C.; Morrison, N.; Watt, B. & Forbes, K. J. (1999a). Characterization of IS*1547*, a new member of the IS*900* family in the *Mycobacterium tuberculosis* complex, and its association with IS*6110*. *Journal of Bacteriology*, Vol. 181, No. 3, (February 1999), pp. 1021-1024, ISSN 0021-9193.

Fang, Z.; Doig, C.; Kenna, D.T.; Smittipat, N.; Palittapongarnpim, P.; Watt, B. & Forbes, K. J. (1999b). *IS6110*-mediated deletions of wild-type chromosomes of *Mycobacterium*

tuberculosis. Journal of Bacteriology, Vol. 181, No. 3, (February 1999), pp. 1014-20, ISSN 0021-9193.

Fang, Z., Kenna, D. T.; Doig, C.; Smittipat, D. N.; Palittapongarnpim, P.; Watt, B. & Forbes K.J. (2001). Molecular Evidence for Independent Occurrence of *IS6110* Insertions at the Same Sites of the Genome of *Mycobacterium tuberculosis* in Different Clinical Isolates. *Journal of Bacteriology*, Vol.183, No.18 (September 2001), pp. 5279 – 5284, ISSN: 0021-9193.

Fomukong N, Beggs M, el Hajj H, Templeton G, Eisenach K & Cave MD. (1997) Differences in the prevalence of IS*6110* insertion sites in *Mycobacterium tuberculosis* strains: low and high copy number of IS*6110*. *Tubercle and Lung Disease*. Vol. 78, No. 2, (1998), pp. 109-116, ISSN 0962-8479.

Galas, D.J. & Chandler, M. (1989). In: *Mobile DNA*, Berg, D.E. & Howe, M.M. (Eds), pp.(109-162). American Society for Microbiology Press, ISBN 1-55581-191-4, Washington DC, USA.

Garbaccio SG, Cataldi AA. (2010). Evaluation of an immunomagnetic capture method followed by PCR to detect *Mycobacterium bovis* in tissue samples from cattle. *Revista Argentina Microbiologia*. Vol. 42, No. 4, (October-December 2010), pp. 247-253, ISSN 0325-7541.

Gavín, P.; Iglesias, MJ.; Jiménez, MS.; Herrera-Leon, L.; Rodríguez-Valín, E.; Rastogi, N.; March, J.; González-Palacios, R.; Palenque, E.; Ayarza, R.; Hurra, E.; Campos-Herrero, I.; Vitoria, MA.; Lezcano, MA.; Revillo, MJ.; Martin, C. & Samper, S (2009). Multidrug-resistant *Mycobacterium tuberculosis* strain from Equatorial Guinea detected in Spain. *Emerging Infectious Diseases*. Vol. 15, No.11, (November 2009), pp. 1858-1860, ISSN: 1080-6059.

Gavín, P.; Iglesias, MJ.; Jiménez, MS.; Rodríguez-Valín, E.; Ibarz, D.; Lezcano, MA.; Revillo, MJ.; Martín, C. & Samper, S; on behalf of The Spanish Working Group on MDR-TB (2011). Long-term molecular surveillance of multidrug-resistant tuberculosis in Spain. *Infection, Genetics and Evolution*. Vol.27, (May 2011) ISSN: 1567-1348.

Gordon, S. V.; Heym, B.; Parkhill, J.; Barrell, B. & Cole, S. T. (1999). New insertion sequences and a novel repeated sequence in the genome of *Mycobacterium tuberculosis* H37Rv. *Microbiology*, Vol. 145, No 4, (April 1999), pp. 881-892, ISSN 1350-0872..

Gordon, S. V. & Supply, P. (2005). Molecular epidemiology of tuberculosis. In: *Tuberculosis and the Tubercle Bacillus,* S. T. Cole, K.D. Eisenach, D. N. MacMurray & W. R. Jacobs Jr (Eds.), pp. 191-202. American Society for Microbiology Press, ISBN 1-55581-295-3, Washington, DC, USA.

Gupta N, Sharma K, Barwad A, Sharma M, Rajwanshi A, Dutta P, Sharma A. (2010). Thyroid tuberculosis - role of PCR in diagnosis of a rare entity. *Cytopathology*, (November 2010) doi: 10.1111/j.1365-2303.2010.00830.

Guilhot, C.; Jackson, M. & Gicquel, B. (1999). Mobile Genetic Elements and Plasmids: Tools for Genetic Studies. In: *Mycobacteria: Molecular Biology and Virulence*, Ratledge, C. & Dale, J. (Eds), pp. 17-37. Blackwell Publishing Ltd., Print ISBN: 9780632053049, Oxford, UK.

Hanekom, M.; Gey van Pittius, N.C.; McEvoy, C.; Victor, T.C.; Van Helden, P.D. & Warren, R.M. (2011). *Mycobacterium tuberculosis* Beijing genotype: A template for success. *Tuberculosis* (Edinb). (August 2011) ISSN 1472-9792.

Hermans PW, van Soolingen D, Dale JW, Schuitema AR, McAdam RA, Catty D, vanEmbden JD. (1990). Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *Journal of Clinical Microbiology*. Vol. 28, No. 9, (September 1990) pp. 2051-2058, ISSN 0095-1137.

Hermans, P. W.; van Soolingen, D.; Bik, E. M.; Haas, P. E. de; Dale, J. W. & van Embden, J. D. (1991). Insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infection and Immunity*, Vol. 59, No. 8, (August 1991), pp. 2695-2705, ISSN 0019-9567.

Hu, Y.; Ma, X.; Graviss, EA.; Wang, W.; Jiang, W. & Xu, B. (2011). A major subgroup of Beijing family *Mycobacterium tuberculosis* is associated with multidrug resistance and increased transmissibility. *Epidemiology and Infection*. Vol.139, No.1, (January 2011), pp. 130-138, ISSN: 0950-2688.

Inoue M, Tang WY, Wee SY, Barkham T. (2011).Audit and improve! Evaluation of a real-time probe-based PCR assay with internal control for the direct detection of *Mycobacterium tuberculosis complex*. *European Journal of Clinical Microbiology and Infectious Diseases*. Vol. 30, No. 1, (January 2011), pp. 131-135, ISSN 0732-8893.

Ioerger, T.R.; Feng, Y.; Ganesula, K.; Chen, X.; Dobos, K.M.; Fortune, S.; Jacobs, W.R. Jr.; Mizrahi, V.; Parish, T.; Rubin, E.; Sassetti, C. & Sacchettini, J.C. (2010). Variation among genome sequences of H37Rv strains of *Mycobacterium tuberculosis* from multiple laboratories. *Journal of Bacteriology*. Vol. 192, No. 14, (July 2010), pp. 3645-3653, ISSN 0021-9193.

Kelley, CL. & Collins, FM. (1999) Growth of a highly virulent strain of *Mycobacterium tuberculosis* in mice of differing susceptibility to tuberculous challenge. *Tuberculosis and Lung Diseases,* Vol. 79, No. 6, (December 1999), pp. 367-370, ISSN 1472-9792.

Kempf MC, Dunlap NE, Lok KH, Benjamin WH Jr, Keenan NB & Kimerling M. (2005). Long-term molecular analysis of tuberculosis strains in alabama, a state characterized by a largely indigenous, low-risk population. *Journal of Clinical Microbiology*. Vol. 43, No. 2, (February 2005), pp. 870-878, ISSN 0095-1137

Kiers, A., Drost, A.P., van Soolingen, D.& Veen, J. (1997).Use of DNA fingerprinting in international source case finding during a large outbreak of tuberculosis in The Netherlands. *International Journal of Tuberculosis and Lung Diseases*. Vol. 1, No. 3, (June 1997), pp. 239-245, ISSN 1027-3719.

Kik, SV.; Verver, S.; van Soolingen, D.; de Haas, P.E.; Cobelens, F.G.; Kremer, K.; van Deutekom, H. & Borgdorff, M.W. (2008). Tuberculosis outbreaks predicted by characteristics of first patients in a DNA fingerprint cluster. A*merican Journal of Respiratory and Critical Care Medicine,* Vol. 178, No.1, (July 2008), pp. 96-104, ISSN 1073-449X.

Kivi, M.; Liu, X.; Raychaudhuri, S.; Alatman, R.B. & Small, P.M. (2002). Determining the genomic locations of repetitive DNA sequences with a whole-genome microarray: *IS6110* in *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*. Vol. 40, No. 6, (June 2002), pp. 2192-2198, ISSN 0095-1137.

Kremer K, van Soolingen D.; Frothingham, R.; Haas, W.H.; Hermans, P.W.; Martín, C.; Palittapongarnpim, P.; Plikaytis, B.B.; Riley, L.W.; Yakrus, M.A.; Musser, JM. & van Embden J.D. (1999). Comparison of methods based on different molecular epidemiological markers for typing of Mycobacterium tuberculosis complex

strains: interlaboratory study of discriminatory power and reproducibility. *Journal of Clinical Microbiology,*Vol.37, No.8, (August 1999), pp. 2607-2618, ISSN: 0095-1137.

Kusum S, Aman S, Pallab R, Kumar SS, Manish M, Sudesh P, Subhash V & Meera S. (2011) Multiplex PCR for rapid diagnosis of tuberculous meningitis. *Journal Neurology*. (March 2011), doi 10.1007/s00415-011-6010-4, Print ISSN 0340-5354.

Lambert, ML.; Hasker, E.; van Deun, A.; Roberfroid, D.; Boelaert, M. & van der Stuyft, P. (2003). Recurrence in tuberculosis: relapse or reinfection? *The Lancet Infectious Diseases*. Vol.3, No.5, (May 2003), pp. 282-287, ISSN 1473-3099.

Lamichhane, G.; Zignol, M.; Blades, N.J.; Geiman, D.E.; Dougherty, A.; Grosset, J.; Broman, K.W. & Bishai, W.R. (2003). A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. Vol. 100, No. 12, (June 2003), pp. 7213-7218, ISSN 0227-8424.

Lemaitre N, Armand S, Vachee A, Capilliez O, Dumoulin C & Courcol R. (2004). Comparison of the real-time PCR method and the Gen-Probe amplified *Mycobacterium tuberculosis* direct test for detection of *Mycobacterium tuberculosis* in pulmonary and nonpulmonary specimens. *Journal of Clinical Microbiology*. Vol. 42, No.9, (September 2004), pp. 4307-4309, ISSN 0095-1137.

Leung ET, Zheng L, Wong RY, Chan EW, Au TK, Chan RC, Lui G& Lee N, Ip M. (2011). Rapid and Simultaneous Detection of *Mycobacterium tuberculosis complex* and Beijing/WGenotype in Sputum by an Optimized DNA Extraction Protocol and a Novel Multiplex Real-Time PCR. *Journal of Clinical Microbiology*. Vol. 49, No.7, (Jul y2011) pp. 2509-2515, ISSN 0095-1137.

López-Calleja, AI.; Lezcano, MA.; Vitoria, MA.; Iglesias, MJ.; Cebollada, A.; Lafoz, C.; Gavin, P.; Aristimuño, L.; Revillo, MJ.; Martin, C. & Samper, S (2007). Genotyping of Mycobacterium tuberculosis over two periods: a changing scenario for tuberculosis transmission. *The International Journal of Tuberculosis and Lung Disease*. Vol. 11, No.10, (October 2007), pp. 1080-1086, ISSN 1027-3719.

López-Calleja, AI.; Gavín, P.; Lezcano, MA.; Vitoria, MA.; Iglesias, MJ.; Guimbao, J.; Lázaro, MA.; Rastogi, N.; Revillo, MJ.; Martín, C. & Samper, S (2009). Unsuspected and extensive transmission of a drug-susceptible *Mycobacterium tuberculosis* strain. *BMC Pulmonary Medicine* Vol. 9; No. 3, (January 2009), ISSN 1471-2466.

Mahillon, J. & Chandler, M. (1998). Insertion Sequences. *Microbiology and Molecular Biology Reviews*, Vol. 62, No. 3, (September 1998), pp. 725-774, ISSN 1092-2172.

Mäkinen, J.; Marjamäki, M.; Haanperä-Heikkinen, M.; Marttila, H.; Endourova, LB.; Presnova, SE.; Mathys, V.; Bifani, P.; Ruohonen, R.; Viljanen, MK. & Soini, H. (2011). Extremely high prevalence of multidrug resistant tuberculosis in Murmansk, Russia: a population-based study. *European Journal of Clinical Microbiology & Infectious Diseases*. DOI 10.1007/s10096-011-1200-7, (March 2011), ISSN 0934-9723.

Marais BJ, Hesseling AC, Schaaf HS, Gie RP, van Helden PD, Warren RM. (2009) *Mycobacterium tuberculosis* transmission is not related to household genotype in a setting of high endemicity. *Journal of Clinical Microbiology.* Vol. 47, No. 5, (May 2009), pp. 1338-1343, Print ISSN 0095-1137.

Mariani, F.; Piccolella, E.; Colizzi, V.; Rappuoli, R. & Gross, R. (1993). Characterization of an IS-like element from *Mycobacterium tuberculosis. Journal of General Microbiology*, Vol. 139, No. 8, (August 1993), pp. 1767-1772, ISSN 1350-0872.

Mathema B, Kurepina NE, Bifani PJ & Kreiswirth BN. (2006). Molecular epidemiology of tuberculosis: current insights. *Clinical Microbiology Reviews*. Vol. 19, No 4, (October 2006), pp. 658-685, ISSN 0893-8512.

McAdam, R. A.; M. Hermans, P. W.; Van Soolingen, D.; Zainuddin, Z. F.; Catty, D.; van Embden, J. D. A. & Dale, J. W. (1990). Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS*3* family. *Molecular Microbiology*, Vol. 4, No. 9, (September 1990), pp. 1607- 1613, ISSN 1365-2958.

McAdam, R. A.; Quan, S. & Guilhot, C. (2000). Mycobacterial Tansposons and their applications. In: *Molecular Genetics of Mycobacteria*, Hatfull, G. F. & Jacobs, W. R. Jr (Eds), pp. 69−84, American Society for Microbiology Press, ISBN 1-55581-191-4, Washington DC, USA.

McEvoy, C.R.; Falmer, A.A.; Gey van Pittius, N.C.; Victor, T.C.; van Helden, P.D. & Warren, R.M. (2007). The role of IS*6110* in the evolution of *Mycobacterium tuberculosis*. *Tuberculosis* (Edinb). Vol. 87, No. 5, (September 2007), pp. 393-404. ISSN 1472-9792.

McEvoy, C.R.; van Helden, P.D.; Warren, R.M. & Gey van Pittius, N.C. (2009). Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis* PPE38 gene region. BMC Evolutionary Biology. Vol. 9, (September 2009), pp. 237, ISSN 1471-2148

Mendiola, M. V.; Martín, C.; Otal, I. & Gicquel, B. (1992). Analysis of the regions responsible for IS*6110* RFLP in a single *Mycobacterium tuberculosis* strain. *Research in Microbiology*, Vol. 143, No. 8, (October 1992), pp. 767-772, ISSN 0923-2508.

Moss, A.R.; Alland, D.; Telzak, E.; Hewlett, D. Jr.; Sharp, V.; Chiliade, P.; LaBombardi, V.; Kabus, D.; Hanna, B.; Palumbo, L.; Brudney, K.; Weltman, A.; Stoeckle, K.; Chirgwin, K.; Simberkoff, M.; Moghazeh, S.; Eisner, W.; Lutfey, M. & Kreiswirth, B. (1997). A city-wide outbreak of a multiple-drug-resistant strain of *Mycobacterium tuberculosis* in New York. *International Journal of Tuberculosis and Lung Diseases*. Vol. 1, No. 2, (April 1997), pp. 115-121, ISSN 1027-3719.

Narayanan S, Das S, Garg R, Hari L, Rao VB, Frieden TR & Narayanan PR. (2002). Molecular epidemiology of tuberculosis in a rural area of high prevalence in South India: implications for disease control and prevention. *Journal Clinical Microbiology*. Vol. 40, No. 12, (December 2002) pp. 4785-4788, ISSN 0095-1137

Narayanan S, Parandaman V, Narayanan PR, Venkatesan P, Girish C, Mahadevan S & Rajajee S. (2001). Evaluation of PCR using TRC(4) and IS*6110* primers in detection of tuberculous meningitis. *Journal Clinical Microbiology*. Vol. 39, No. 5, (May 2001) pp. 2006-2008, ISSN0095-1137.

Narayanan, S.; Swaminathan, S.; Supply, P.; Shanmugam, S.; Narendran, G.; Hari, L.; Ramachandran, R.; Locht, C.; Jawahar, MS.; Narayanan, PR. (2010). Impact of HIV infection on the recurrence of tuberculosis in South India. *The Journal of Infectious Diseases*. Vol.201, No.5, (March 2010), pp. 691-703, ISSN 0022-1899.

Otal, I.; Martín, C.; Vincent-Lévy-Frebault, V.; Thierry, D. & Gicquel, B. (1991). Restriction fragment length polymorphism analysis using IS*6110* as an epidemiological marker in tuberculosis. *Journal of Clinical Microbiology,* Vol.29, No.6, (June 1991), pp. 1252-1254. Print ISSN: 0095-1137. Online ISSN: 1098-660X.

Otal, I.; Gomez, A. B.; Kremer, K.; de Haas, P.; García, M. J.; Martín, C. & van Soolingen, D. (2008) Mapping of IS*6110* insertion sites in *Mycobacterium bovis* isolates in relation

to adaptation from the animal to human host. *Veterinary Microbiology*, Vol. 129, No. 3-4, (June 2008), pp. 333-341, ISSN 0378-1135.

Purwar, S.; Chaudhari, S.; Katoch, VM.; Sampath, A.; Sharma, P.; Upadhyay, P. & Chauhan, DS. (2011). Determination of drug susceptibility patterns and genotypes of *Mycobacterium tuberculosis* isolates from Kanpur district, North India. *Infection, Genetics and Evolution*. Vol.11,No.2, (March 2011), pp. 469-475, ISSN: 1567-1348.

Ritacco, V., Di Lonardo M., Reniero A., Ambroggi M., Barrera L., Dambrosi A., López B., Isola N. & Kantor, I.N. (1997). Nosocomial spread of Human Immunodeficiency Virus-related multidrug-resistant tuberculosis in Buenos Aires, *Journal Infection Diseases,* Vol. 176, No. 3, (September 1997), pp. 637-642, Print ISSN 0022-1899.

Ritacco, V.; Iglesias, MJ.; Ferrazoli, L.; Monteserin, J.; Dalla, Costa ER.; Cebollada, A.; Morcillo, N.; Robledo, J.; de Waard, JH.; Araya, P.; Aristimuño, L.; Díaz, R.; Gavin P.; Imperiale, B.; Simonsen, V.; Zapata, EM.; Jiménez, MS.; Rossetti, ML.; Martin C.; Barrera L. & Samper, S (2011). Conspicuous multidrug-resistant *Mycobacterium tuberculosis* cluster strains do not trespass country borders in Latin America and Spain. *Infection, Genetics and Evolution*, (June 2011), ISSN 1567-1348.

Safi, H.; Barnes, P. F.; Lakey, D. L.; Shams, H.; Samten, B.; Vankayalapati, R. & Howard, S. T. (2004). IS*6110* functions as a mobile, monocyte-activated promoter in *Mycobacterium tuberculosis*. *Molecular Microbiology*, Vol. 52, No. 4, (May 2004), pp. 999–1012, ISSN 1365-2958.

Samper, S.; Martín, C.; Pinedo, A.; Rivero, A.; Blázquez, J.; Baquero, F.; van Soolingen, D. & van Embden, J. (1997). Transmission between HIV-infected patients of multidrug-resistant tuberculosis caused by *Mycobacterium bovis*. *AIDS* Vol. 11, No.10, (August 1997), pp. 1237-1242, ISSN 1473-5571.

Samper, S.; Iglesias, MJ.; Rabanaque, MJ.; Gómez, LI.; Lafoz, MC.; Jiménez, MS.; Ortega, A.; Lezcano, MA.; Van Soolingen, D.; Martín, C. & The Spanish Working Group on MDR-TB. (2005). Systematic molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* complex isolates from Spain. *Journal of Clinical Microbiology,* Vol.43, No.3, (March 2005), pp. 1220-1227, ISSN 0095-1137.

Samper, S. & Martín, C. (2007). Spread of extensively drug-resistant tuberculosis. *Emerging Infectious Diseases*. Vol. 13, No.4, (April 2007), pp. (647-648), ISSN: 1080-6059.

Sampson, S.; Warren, R.; Richardson, M.; van der Spuy, G. & van Helden, P. (2001). IS*6110* insertions in *Mycobacterium tuberculosis*: predominantly into coding regions. *Journal of Clinical Microbiology*, Vol.39, No. 9, (September 2001), pp. 3423-3424. ISSN 0095-1137.

Sandoval, A.; Reyes, A.; Cubillos, A.; Ritacco, V.; Lopez, B.; Robledo, J.; Samper , S.; Martin, C.; Garcia, M.J.; Zambrano, M.M.; Mitra, R. & del Portillo, P. (2010). Deciphering the biological meaning of the IS*6110* insertion element. *31st Annual Congress of the European Society of Mycobacteriology*. Bled, Slovenia, 4-7 July, 2010.

Sankar S, Balakrishnan B, Nandagopal B, Thangaraju K & Natarajan S. (2010a). Comparative evaluation of nested PCR and conventional smear methods for the detection of *Mycobacterium tuberculosis* in sputum samples. *Molecular Diagnosis and Therapy*. Vol. 14, No. 4, (August 2010), pp. 223-227, ISSN 1177-1062.

Sankar S, Balakrishnan B, Nandagopal B, Thangaraju K & Natarajan S. (2010b). Comparative evaluation of two polymerase chain reactions targeting different genomic regions

to detect *Mycobacterium tuberculosis* in sputum. *Indian Journal of Medical Microbiology*, Vol. 28, No. 4, (December 2010), pp. 303-307, ISSN 0255-0857.

Sankar S, Ramamurthy M, Nandagopal B & Sridharan G. (2011a). An appraisal of PCR-based technology in the detection of *Mycobacterium tuberculosis*. *Molecular Diagnostic Therapy*. Vol. 15, No. 1, (February 2011) pp. 1-11, ISSN 1177-1062.

Sankar, S.; Kuppanan, S.; Balakrishnan, B. & Nandagopal, B. (2011b). Analysis of sequence diversity among IS*6110* sequence of *Mycobacterium tuberculosis*: possible implications for PCR based detection. *Bioinformation*, Vol. 6, No. 7, (June 2011), pp. 283-285, ISSN 0973-2063.

Savelkoul PH, Catsburg A, Mulder S, Oostendorp L, Schirm J, Wilke H, van der Zanden AG & Noordhoek GT. (2006). Detection of *Mycobacterium tuberculosis complex* with Real Time PCR: comparison of different primer-probe sets based on the *IS6110* element. *Journal of Microbiological Methods*. Vol. 66, No 1, (July 2006), pp. 177-180, ISSN 0167-7012.

Schürch, A.C.; Kremer, K.; Kiers, A.; Daviena, O.; Boeree, M.J.; Siezen, R.J.; Smith, N.H. & van Soolingen, D. (2010). The tempo and mode of molecular evolution of *Mycobacterium tuberculosis* at patient-to-patient scale. *Infection, Genetics and Evolution*, Vol. 10, No.1, (January 2010), pp. 108–114, ISSN 1567-1348.

Shamputa, IC.; Jugheli, L.; Sadradze, N.; Willery, E.; Portaels, F.; Supply, P. & Rigouts, L. (2006). Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. *Respiratory Research* Vol. 7, No. 99, (July 2006), ISSN 1465-9921.

Shanmugam, S; Selvakumar, N. & Narayanan, S. (2011). Drug resistance among different genotypes of *Mycobacterium tuberculosis* isolated from patients from Tiruvallur, South India. *Infection, Genetics and Evolution*. Vol.11, No.5, (July 2011), pp. 980-986, ISSN 1567-1348.

Small, P. M. & van Embden, J. D. A. (1994). Molecular epidemiology of tuberculosis. In: *Tuberculosis: Pathogenesis, protection and Control,* B. Bloom (Ed.), pp. (569-582). American Society for Microbiology Press, ISBN 978-1-55581-072-6, Washington, DC, USA.

Søborg, C., Søborg, B., Pouelsen, S., Pallisgaard, G., Thybo, S. & Bauer, J. (2001). Doubling of the tuberculosis incidence in Greenland over an 8-year period (1990-1997). *International Journal of Tuberculosis and Lung Diseases*. Vol. 5, No. 3, (March 2001), pp. 257-265, ISSN 1027-3719.

Soto, C. Y.; Menéndez, M. C.; Pérez, E.; Samper, S.; Gómez, A. B.; García, M. J. & Martín, C. (2004) IS*6110* mediates increased transcription of the phoP virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. *Journal of Clinical Microbiology*, Vol. 42, No. 1, (January 2004), pp. 212-219, ISSN 0095-1137.

Steinlein, L.M. & Crawford, J.T. (2001). Reverse dot blot assay (insertion site typing) for precise detection of sites of *IS6110* insertion in the *Mycobacterium tuberculosis* genome. Journal of Clinical Microbiology. Vol. 39, No. 3, (March 2001), pp. 871-878, ISSN 0095-9567.

Streicher, et al. 2011 Molecular epidemiological perspective of the emergence and treatment of MDRTB in South Africa. (2011). Infection, Gen In Press

Suffys PN, Ivens de Araujo ME, Rossetti ML, Zahab A, Barroso EW, Barreto AM, Campos E, van Soolingen D, Kremer K, Heersma H & Degrave WM. (2000). Usefulness of

IS*6110*-restriction fragment length polymorphism typing of Brazilian strains of *Mycobacterium tuberculosis* and comparison with an international fingerprint database. *Research Microbiology*. Vol. 151, No. 5, (Jun 2000), pp. 343-351, ISSN 0923-2508.

Thierry, D.; Cave, M.; Eisenach, K.; Crawford, J. T.; Bates, J. H.; Gicquel, B. & Guesdon, J. L. (1990a). IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Research*, Vol. 18, No. 1, (January 1990), pp. 188, ISSN 0305-1048.

Thierry, D.; Brisson-Noel, A.; Vincent-Levy-Frebault, V.; Nguyen, S.; Guesdon, J. L. & Gicquel, B. (1990b). Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. *Journal of Clinical Microbiology*, Vol. 28, No. 12, (December 1990), pp. 2668–2673, ISSN 0095-1137.

Thong-On, A.; Smittipat, N.; Juthayothin, T.; Yanai, H.; Yamada, N.; Yorsangsukkamol, J.; Chaiprasert, A.; Rienthong, D.; Billamas, P. & Palittapongarnpim, P (2010). Variable-number tandem repeats typing of *M. tuberculosis* isolates with low copy numbers of IS*6110* in Thailand. *Tuberculosis*. Vol.90, No.1, (January 2010), pp. 9-15, ISSN 1472-9792.

Tumwasorn S, Kwanlertjit S, Mokmued S & Charoenlap P. (1996).Comparison of DNA targets for amplification by polymerase chain reaction for detection of *Mycobacterium tuberculosis* in sputum. *Journal Medical Association of Thailand*. Vol. 79, No. 1, (December 1996), pp. 113-118, ISSN 0125-2208.

Turcios, L.; Casart, Y.; Florez, I.; de Waard, J. & Salazar, L. (2009). Characterization of IS*6110* insertions in the dnaA-dnaN intergenic region of *Mycobacterium tuberculosis* clinical isolates. *Clinical Microbiology and Infection*. Vol. 15, No. 2, (February 2009), pp. 200-203, ISSN 1198-743X.

Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, Jones JS, Westmoreland H, Onorato IM. (1998). An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. *The New England Journal of Mededicine*. Vol. 338, No. 10, (March 1998), pp. 677-6778, ISSN 0028-4793.

van Embden, J. D. A.; Cave, M. D.; Crawford, J. T.; Dale, J. W.; Eisenach, K. D.; Gicquel, B.; Hermans, P.; Martin, C.; McAdam, R.; Shinnick, T. M. & Small, P.M. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *Journal of Clinical Microbiology*, Vol. 31, No. 2, (February 1993), pp. 406–409, ISSN 0095-1137.

Vasankari, T.; Soini, H.; Liippo, K. & Ruutu, P. (2011). MDR in Finland - still rare despite the situation in our neighboring countries. *The Clinical Respiratory Journal*. May 6. doi: 10.1111/j.1752-699X.2011.00242.x. (June 2011), ISSN: 1752-698.

Warren, R.M.; Sampson, S.L.; Richardson, M.; Van Der Spuy, G.D.; Lombard, C.J.; Victor, T.C. & van Helden, P.D. (2000). Mapping of IS*6110* flanking regions in clinical isolates of *Mycobacterium tuberculosis* demonstrates genome plasticity. *Molecular Microbiology*. Vol. 37, No. 6, (September 2000), pp. 1405-1416, ISSN 0950-382X.

Wei CY, Lee CN, Chu CH, Hwang J & Lee CP. (1999). Determination of the sensitivity and specificity of PCR assays using different target dnas for the detection of *Mycobacterium tuberculosis*. *Kaohsiung Journal Medical Science*. Vol. 15, No. 7, (Jul y1999), pp. (396-405) ISSN 1607-551X.

Yang Z, Barnes PF, Chaves F, Eisenach KD, Weis SE, Bates JH & Cave MD. (1998). Diversity of DNA fingerprints of *Mycobacterium tuberculosis* isolates in the United States.

Journal of Clinical Microbiology. Vol. 36, No. 4, (April 1998) pp. 1003-1007, ISSN 0095-1137.

Yesilkaya, H.; Dale, J. W.; Strachan, N. J. & Forbes, K. J. (2005). Natural transposon mutagenesis of clinical isolates of *Mycobacterium tuberculosis*: how many genes does a pathogen need? *Journal of Bacteriology*, Vol. 187, No. 19, (October 2005), pp. 6726-6732, ISSN 0021-9193.

4

Response of Mycobacterial Species to an Acidic Environment

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1. Introduction

Bacteria must be able to respond to a host of environmental stresses. The ability is vital if bacteria are to withstand insults both of an external location as well as during infection of an animal host. Many investigators study these responses individually in order to better understand pathogenic processes. Desiccation, response to UV light, heat, and cold stress can be encountered in the external environment. Low oxygen tension, heat stress, oxidative stress, nitrosidative stress, and acidic stress are all environments that can be encountered upon infection *in vivo* of a host. This review focuses on acidity as a stress which can be important in mycobacterial pathogenesis. Acidity can be found in such environments as acidic water and soil and can affect mycobacteria in an animal host. Mycobacteria encounter acidic stress at sites of inflammation and within phagosomes of macrophages. Exposure to acidic stress in the external environment may prime mycobacteria to upregulate genes involved in pathogenesis. Upregulation in response to acidic stress may prime mycobacteria to be more resistant to other stresses and to be more able to persist *in vivo*.

2. Environmental mycobacteria

In contrast to *Mycobacterium tuberculosis* which is found exclusively in a human or animal host, environmental mycobacteria are found in the environment. Contamination of water or other environmental locations can then lead to infection. Environmental mycobacteria are in general not transmitted person to person but instead environment to person. Therefore the environmental milieu is important in priming mycobacteria for survival. If acidity is encountered in the host upon infection of a human, those genes that aid in resistance in the environment are already upregulated. Upon being engulfed by macrophages acid primed mycobacteria are already somewhat resistant to that *in vivo* environment.

Mycobacterium avium has been found to be isolated from acidic swamp waters in the Southeastern United States (Kirschner et al; 1992). *M. avium* isolates grew at pH levels as low as 4.0 and grew at acidic pHs just as well as at neutral pH (Kirschner et al; 1999). Mycobacteria have also been isolated from brook waters draining from acidic coniferous forests in Finland. Furthermore, growth seems to be negatively correlated with pH. As pH lowers there is a propensity to isolate mycobacteria (Livanainen et al; 1999). Thus low pH

may favor isolation of mycobacteria from environmental samples. In fact *Mycobacterium parascrofulaceum* has been isolated from acidic hot springs with a pH as low as 3.0 in Yellowstone National Park. (Santos et al; 2007). In addition *Mycobacterium montefiorense* was isolated from soils at pH 2.0 (Uyttebroek et al; 2007). It seems that mycobacteria that grow at neutral and low pH behave differently from other environmental bacteria that grow at neutral pH but poorly at acidic pH. Thus environmental mycobacteria seem to have exploited an environmental niche that other bacteria have a difficult time occupying. Mycobacteria such as *Mycobacterium avium* as well as others are common contaminants of municipal water supplies where they serve as a reservoir to infect human beings. In fact in areas where there is a prevalence of mycobacteria in acidic waters, there also is an increased prevalence of infections due to these environmental mycobacteria. It is tempting to speculate that environmental mycobacteria present in acidic conditions are more resistant to acidic environments in a human or animal host. In addition those mycobacteria which are exclusively human pathogens may have evolved from ancestral mycobacteria that already demonstrated an exquisite ability to withstand acidic stress. Thus it is no wonder that mycobacteria within the *Mycobacterium tuberculosis* complex have evolved to occupy and resist the partially acidic environment of the phagosome of alveolar macrophages.

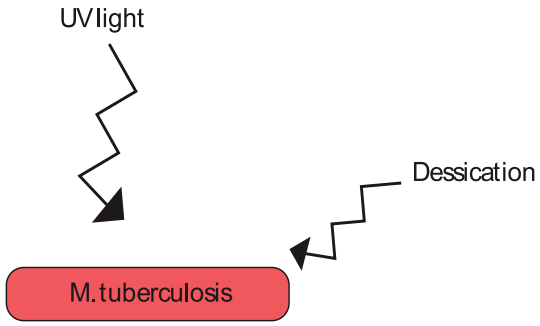
Mycobacteria may bear an intrinsic ability to resist environmental stresses. The cell wall and outer membrane help to protect environmental mycobacteria from acidic stress. Mycobacteria contain a thick cell wall surrounding an inner membrane. The cell wall is partially composed of a peptidoglycan-arabinogalactan polymer and is quite thick. In addition they contain an outer membrane that is somewhat different from what is found in gram negative organisms. The mycobacterial outer membrane is composed of mycolic acids in addition to other components and the lipid structure may serve to protect mycobacteria from environmental stresses (Hoffman et al; 2008, Neiderweis et al; 2010). It has been proposed that the mycobacterial cell wall and outer membrane rich in lipids act as an effective barrier against the entry of protons (Mechnikoff, 1905). In support of the idea that the waxy lipid rich cell envelope inhibits the toxic effects of acidic stress, many acid susceptible mutants are within genes that are predicted to have cell wall functions or are involved in lipid metabolism (Fisher et al; 2002, Saviola et al; 2002, Vandal et al; 2009).

While mycobacteria may have an intrinsic ability to resist acidic stress, they also bear inducible responses to acidic stress. One of these inducible systems is the acid tolerance system (ATR) where prior exposure to acidic stress confers protection upon subsequent exposure with more extreme acidic stress. Mycobacteria possess a blunted acid tolerance system as it confers only 2-3 fold protection if the mycobacteria are first exposed to pH 5.0 and then challenged with pH 3.0 (O'Brien et al; 1996). This is much lower than what is found in enteric bacteria that exhibit a 1,000 to 10,000 fold protection against acidic stress if they are conditioned prior with a mild acidity. Thus while mycobacteria possess an acid tolerance response it is much less protective than in gram negative organisms.

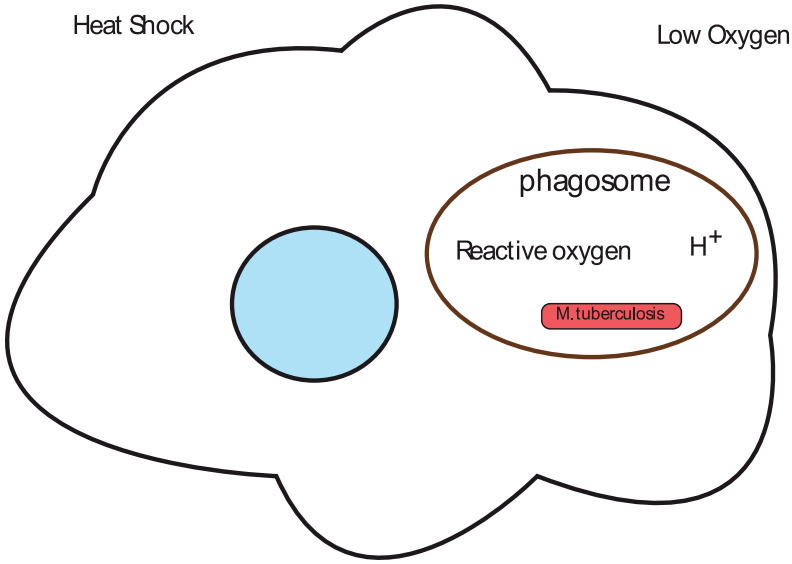
Environmental mycobacteria respond to acidic stress by upregulating a variety of genes presumably necessary to resist this stress. In addition there are examples of a number of genes that when mutated result in mycobacteria that are more sensitive to acidic stress. Investigations into the stressome of *Mycobacterium avium subsp. paratuberculosis* revealed that 195 genes are upregulated at acidic pH (Wu et al; 2007). *M. avium subsp. paratuberculosis* is the causative agent of Johne's disease and has been implicated in

Crohn's disease in humans. This mycobacterium is exposed to acidic pH in the alimentary tract of cattle and is also exposed to acidity within the phagosomes of macrophages of which it is an intracellular parasite. In addition *M. avium subsp. paratuberculosis* may encounter acidity within sites of inflammation. The large number of genes in *M. avium subsp. paratuberculosis* that are upregulated in response to acidic stress indicates that this is an important environmental condition *in vivo*. Of the 195 genes upregulated by acidic stress, 6 of the genes are common between heat shock and acidic stress. In addition general stress response genes *htpX*, *clpX*, and *relA* were upregulated. This implies that there is a common stress response pathway that includes both acidity and heat shock. Genes involved in mycolic acid synthesis were upregulated at acidic pH, as well as mycobactin metabolism and a protein kinase (*pknB*). Genes involved in mycobacterial cell entry (*mce1* and *mce4*) and a transcriptional regulator *kpnE* were also upregulated. Many genes were also downregulated and these outnumbered the genes which were upregulated by acidic stress. Perhaps acidic pH is a toxic environment that serves to down regulate transcription in general. This makes those genes that are upregulated functionally even greater in their responsiveness. Deletion mutants in genes *aceAB*, *mbtH2* and *prrA* that were positively regulated by acidic stress were evaluated for growth in mice. Consistent with these genes being important in pathogenesis, infection with these strains resulted in a 1-2 log lower level of colonization of the liver compared to wild type *M. avium subsp. paratuberculosis*.

Mycobacterium smegmatis is a bacterium originally isolated from human smegma (Rose et al; 2009). It has been the cause of opportunistic infections and catheter related infections (Brown et al; 1999, Newton et al; 1993). *M. smegmatis* may encounter acidity at various locations on the surface of the human body including the skin. If this bacterium is successful in invading a human host, it will invariably be engulfed by macrophages where it will encounter acidity within the phagosomes of macrophages. In addition phagosomes of macrophages which contain *M. smegmatis* may have a lower pH than phagosomes of macrophages that have more virulent *M. tuberculosis*. This is presumably because *M. smegmatis* does not inhibit phagosome maturation to the same level that *M. tuberculosis* does. Studying proteomic responses during acidic stress in *M. smegmatis*, 52 proteins were found to be increased in abundance (Roxas and Li, 2009). Some of these proteins were predicted to be transmembrane proteins, involved in transporter activity, or fatty acid metabolism. Fatty acid metabolism could increase cell wall thickness or increase energy storage. *Acr*, encoding a protein typically associated with low oxygen tension was also induced in *M. smegmatis* as was *devSR* encoding a two component system also thought to respond to low oxygen tension. Mutants of *M. smegmatis* that could not grow in the presence of a protonophore at acidic pH were also identified using transposon mutagenesis (Tran et al; 2005). Genes disrupted include those predicted to be involved in phosphonate phosphite assimilation, lipid biogenesis, and methionine biosynthesis. A mutant in the homologue of the *M. tuberculosis lipF* gene was identified which may be involved in cell wall synthesis or energy homeostasis. Extracytoplasmic sigma factors SigE and SigF (Wu et al; 1997, Gebhard et al; 2008) are required by *M. smegmatis* for survival during acidic stress, indicating a global response to acidic stress. In addition a porin gene, *mspA*, is down regulated at acidic pH. Perhaps this may limit the influx of protons which may be toxic to the cell (Hillman et al; 2007).



a) Stresses encountered in the external environment.



b) Stresses encountered in a host macrophage or externally in the host body.

Fig. 1. Stresses encountered by *M. tuberculosis*. One of these stresses is acidity encountered in the human macrophage.

3. Acidic environments *in vivo* encountered by *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is exposed to a variety of acidic conditions within the human body. These can include the classical location within the phagosome of an alveolar macrophage. It may also include the centers of caseating granulomas which can be quite acidic as has been found to be true in a rabbit model of tuberculosis (Dannenberg, 2006). *M. tuberculosis* is expelled from an individual with active tuberculosis as droplet nuclei to be inhaled by a contact. Once inside the lungs of the newly infected individual the bacilli are engulphed by alveolar macrophages and encounter the internal environment of the phagosome. When macrophages phagocytose latex beads or less virulent bacteria the

phagosomes mature, lower their internal pH to as much as 4.5, and fuse with lysosomes to form a phagolysosome. *M. tuberculosis* however appears to modulate the phagosome and inhibit the maturation process (Armstrong and Hart, 1971, Sturgill-Koszycki et al; 1994, Huynh and Grinstein, 2007). This seems to occur partly by exclusion of the proton ATPase from phagosomes containing *M. tuberculosis*. *M. tuberculosis* also seems to freeze the phagosome prior to fusion with the lysosome. The internal pH lowers to approximately 5.5 and can rebound to pH 6.5. Activation of macrophages by INF-γ results in activation of the macrophage and seems to overcome the block in phagosomal maturation. Phagosomes containing *M. tuberculosis* within macrophages stimulated with INF-γ can reduce their pH to 4.5 or lower (Schaible et al;1998, Via et al, 1998,MacMicking et al; 2003, Ehrt and Schnappinger, 2009). For this reason immune status is so important in determining susceptibility in developing an *M. tuberculosis* active infection. Thus the immune response to *M. tuberculosis* can be imperative in controlling replication of the bacilli. Likewise the ability to survive and thrive within the phagosome of an activated macrophage is vitally dependent on the bacilli's ability to respond to acidic stress. It is interesting to note that interfering with the drop in acidity abrogates the upregulation of approximately 80% of the genes which are normally upregulated in *M. tuberculosis* within the macrophage (Rohde et al; 2007). This is more evidence that acidity is one of the key environmental stresses and signals that *M. tuberculosis* encounters *in vivo*.

4. *Mycobacterium tuberculosis* responses to acidic stress

M. tuberculosis responds to the acidic environment in a number of ways. While *M. tuberculosis* may be able to grow at a slightly acidic pH it does not seem to replicate below pH 5.5(Chapman and Bernard, 1962; Portales and Pattyn, 1982). It is unknown if *M. tuberculosis* has an acid tolerance system as is found in *M. smegmatis* (O'Brien et al; 1996). The cell wall and outer membrane are likely to provide a robust intrinsic resistance. In addition a number of genes have been found to be upregulated by acidic stress. This constitutes an adaptive response to the environment that may be very necessary for survival. In addition some genes have been identified that when mutated result in dramatic acid sensitivity, however those genes are expressed constitutively.

pH may be the main signal to which mycobacterial pathogens respond. By interfering with the development of acidity within phagosomes with the addition of concanamycin A, researchers could assess the effect on mycobacterial transcription. Treatment with concanamycin A abrogates much of the transcriptional response. Prevention of the normal drop in pH to 6.4 and maintaining neutral phagosomal pH resulted in 80% of the genes normally upregulated in macrophages failing to be upregulated (Rohde et al; 2007). Thus this points to the importance of acidity in defining the phagosomal environment.

The promoter of the *lipF* gene is known to be upregulated by acidic stress at pH 6.4 to as low as pH 3.6 (Richter and Saviola, 2009). This promoter region from *M. tuberculosis* is uppregulated in fully virulent *M. tuberculosis* as well as in the environmental mycobacterium *M. smegmatis* (Saviola et al; 2002). Thus this shows that in at least this particular case environmental mycobacteria and *M. tuberculosis* complex mycobacteria can respond in a similar way to acidity. Interruption of the DNA region between the *lipF* promoter and the gene resulted in a bacterium much more attenuated for growth in mouse lungs and macrophages indicating *lipF* is important in pathogenesis (Camacho et al; 1999).

M. tuberculosis genes upregulated by acidic conditions found in phagosomes were analysed by microarray analysis and revealed a number of genes which were upregulated at 15 and 30 minutes. These include the *mymA* operon which appears to be under the control of VirS an AraC/XylS family transcription factor (Fisher et al; 2002). When the *mymA* operon was deleted it was revealed to be important for cell wall ultrastructure. The operon was also important for persistence in the spleens of guinea pigs (Singh; 2005). In separate studies the global regulators SigH and SigB were induced by acidic stress implying that these sigma factors are important in responding to acidity (Rohde et al; 2007). *AprABC* is also upregulated at acidic pH and encodes a likely RNA or DNA binding protein. *AprAB*C is expressed in macrophages at acidic pH and is dependent on the two component system *phoP/R* (Abramovitch et al; 2011). Another gene *ompATb* was induced at pH 5.5 and a mutant was attenuated in mice for growth (Raynaud et al; 2002). *OmpATb* encodes a porin that functions specifically at low pH and has been shown to export ammonia. Therefore in the closed environment of the phagosome this porin presumably senses acidic stress and responds by pumping ammonia into the environment in order to lower and neutralize the surrounding acidity (Song et al; 2011). The two component system Rv0902c and Rv903c are thought to regulate *ompATb.*

A transposon mutagenesis study was done to identify *M. tuberculosis* mutants sensitive to acidic pH (Vandal et al; 2008, Vandal et al; 2009). 21 genes were identified of which 15 were shown to be involved in cell wall functions bolstering the idea that the cell envelope is important in protection from acidic stress. When the 21 genes were rescreened it was revealed that only 2 retained acid sensitivity in phosphate citrate buffer. Possibly the tween-80 added to disperse the mycobacteria and to discourage clumping had hydrolyzed into oleic acid. Fatty acids can be toxic to mycobacteria and other bacteria especially at acidic pH and the 19 genes with transposon insertions that are sensitive to acidity in the presence of tween-80 may confer resistance to this manner of stress. Two genes that retained acid sensitivity in phosphate citrate buffer were *Rv2136c* involved in peptidoglycan synthesis and *Rv3671c* a serine protease that may modify the mycobacterial envelope (Biswass et al; 2010). When these two genes were disrupted by transposon mutagenesis, the mycobacteria were unable to maintain their intracellular pH. In addition the *Rv2136c* transposon mutant was hypersusceptible to cell wall stresses such as exposure to sodium dodecyl sulphate (SDS) (Vandal et al; 2009). This is similar to what is seen with a mutation in the *mymA* operon (Singh et al; 2005). Interestingly *Rv3671c* is constitutively expressed showing that stress inducibity may not be the best indicator that a gene is necessary to withstand the stress. A mutation in the *M. tuberculosis* gene *mgtC* caused attenuation in macrophages. Impaired in growth in lungs and spleens of mice, this gene seems to be important for pathogenesis *in vivo*. Additionally, *mgtC* mutants could not grow *in vitro* in low magnesium and mildly acidic pH. A mutant in *Salmonella mgtC* had a similar phenotype. These data indicate that phagosomes are a location bearing low pH and low Mg2+ concentrations (Buchmeir et al; 2000).

Nitrate respiration seems to protect hypoxic *M. tuberculosis* against acid stress. Microaerophilic and hypoxic mycobacteria are more sensitive to acid stress. Exogenouus nitrate appeared to protect under these conditions as it seemed to serve as a terminal electron acceptor (Tan et al; 2010).

5. PhoP/PhoR and acidity

Two component systems are present in many bacterial species. These systems are composed of a sensor kinase that is capable of transmitting a signal from outside the bacterial cell to the internal regions of the cell. The signal may be acidity, low oxygen tension, or some other signal. Once the sensor kinase responds to the signal it then phosphorylates its target the transcriptional regulator, which can activate or repress transcription. Thus these two component systems are capable of sensing an external stimulus and converting it into differential gene regulation. Many genes may lie downstream of the response regulator, thus constituting a manner to coordinate the regulation of many genes to affect the appropriate response to environmental challenges.

In *M. tuberculosis* there are many two component systems. PhoP/R is one of these systems that has been analyzed extensively for its impact on the pathogenesis of this microorganism and is annotated to be involved in phosphate metabolism because of its similarity to a homologue in *Bacillus subtilus*, though it is unlikely to have this predicted function (Perez et al; 2001). PhoP is a response regulator and a member of the OmpR family of transcriptional regulators. PhoP phosphorylation modulates DNA binding by increasing its binding affinity and this occurs via protein protein contacts between phoP protein monomers (Sinha et al; 2008). However these studies were executed with *phoP* cloned and overexpressed from *M. tuberculosis* H37Ra an attenuated strain of the tubercle bacilli. In a different study PhoP from fully virulent *M. tuberculosis* H37Rv but not H37Ra was capable of binding to its own promoter (Chesne-Seck et; 2008). Mutation within the DNA binding domain of PhoP from *M. tuberculosis* H37Ra resulted in diminished DNA binding. PhoP recognizes direct repeat sequences of 9 base pairs in length and can act to repress its own promoter region (Gupta et al; 2006). This is consistent with what is found with other transcriptional regulators in other species. These response regulators are usually present at a low basal level to avoid spurious binding to non-canonical sites that may occur at higher concentrations. Once a critical level of transcriptional regulator protein is available to bind to its DNA binding site it may repress its own synthesis. When transcriptional regulator levels drop due to protein turn over, the repressor site becomes unoccupied, and more transcriptional regulator is synthesized. This is what occurs with the *Escherichia coli* arabinose responsive repressor/activator AraC (Saviola et al; 1998, Lobell and Schleif, 1990), thus ensuring a constant level of transcriptional regulator present within the bacterial cells ready to respond to external stimuli. It seems logical that PhoP would act as a repressor of itself.

The attenuated strain *M. tuberculosis* H37Ra was developed at the turn of the 20th century from a clinical strain *M. tuberculosis* H37 by passaging serially on artificial laboratory media (Steenken et al; 1934). In addition to being attenuated it was revealed that *M. tuberculosis* H37Ra contains many changes within its genome in comparison to its fully virulent counterpart *M. tuberculosis* H37Rv. Looking at the transcriptome many more genes were disregulated than would have been predicted by just looking at polymorphisms in the genome indicating that global transcriptional regulators have been affected. If PhoP as part of a two component system is disregulated this has the ability to affect many 10s or 100s of genes. Indeed *phoP* is mutated within *M. tuberculosis* H37Ra within the DNA binding domain encoding region of the gene replacing a serine with a leucine (Chesne-Seck et al; 2008).

In support of the idea that the *phoP* mutation in *M. tuberculosis* H37Ra is responsible for many of the transcriptional changes compared to *M. tuberculosis* H37Rv, comparison of *M. tuberculosis* H37Ra with a *phoP* deletion strain of fully virulent *M. tuberculosis* H37Rv showed that many genes downregulated in one were also downregulated in the other (Lee et al; 2008). Disruption of *pho*P from virulent *M. tuberculosis* results in a mutant bacterium that cannot grow in macrophages or mice and cannot grow at low Mg^{2+} (Walters et al; 2006). Complementation of *M. tuberculosis* H37Ra with *phoP* from the virulent *M. tuberculosis* H37Rv partially restored the ability of the attenuated strain to replicate in macrophages. Growth restriction of *M. tuberculosis* deleted in *phoP* can be partially overcome by adding Mg^{2+} back into the test system. It is possible that PhoP/R mutations result in cell envelope defects which as a consequence require Mg^{2+} to overcome this deficiency. In fact PhoP positively regulates *pks* 2,3,4 and *mls3* that encode enzymes for production of sulphatides, diacyltrehalose, and polyacyltrehalose which can be found in the cell envelope (Walters et al; 2006). When *pks* 2, 3, and 4 were disrupted in *M. tuberculosis* H37Rv no dramatic effect was seen in pathogenesis of the microorganism. Thus pathogenesis likely lies in other loci controlled by PhoP.

PhoP controls the expression of a variety of genes involved in lipid metabolism, respiration, initial hypoxic response, enduring hypoxic response, response to stress, and genes within the RD1 genomic region known to be deleted in *M. bovis* BCG (Gonzalo-Asensio et al; 2008). Interestingly many of the genes known to be controlled by PhoP are also upregulated by acidic stress. These genes can be upregulated by acidic pH at 6.5 or 5.5, pHs likely encountered in the phagosome of macrophages. Many of these genes under the PhoP regulon and responsive to acidic stress are within the category of controlling lipid metabolism including *pks2*, *pks3*, and *pks4* (Gonzalo-Asensio et al; 2008, Rohde et al; 2007) . *LipF* is one of the genes whose promoter had previously been shown to be upregulated by acidic stress (Saviola et al; 2001). Other genes regulated by PhoP and acidic stress such as *nark1* are involved in respiration while *Rv2390c* is involved in the enduring hypoxic response. *WhiB6* is a transcription factor upregulated by acidic stress and is controlled by PhoP (Rohde et al; 2007). In addition, PhoP controls many of the genes that are expressed upon interaction with macrophages similar to what is found in *Salmonella* PhoP (Perez et al; 2001). It is intriguing to think that PhoP not only regulates genes needed for virulence, but is also sensing acidic stress as one of its signals via the sensor kinase PhoR.

While some *Mycobacterium bovis* Bacille Calmette Guerin (BCG) vaccine strains are mutated in *phoR*, they are generally thought to be missing the RD1 region of their genome compared to fully virulent *M. bovis*. The RD1 region contains some key genes that are required for virulence including *esat-6* and *cfp-10* (Behr et al; 1999, Pym et al; 2002). This indicates that the RD1 region is important in pathogenesis; *M. tuberculosis* H37Ra, however contains the intact RD1 region (Mostowy et al; 2004). PhoP is known to control *esat-6* secretion which is a major antigen recognized by T-cells (Frigui et al; 2008). *M. tuberculosis* H37Ra mutated in *phoP* and reconstituted with *phoP* from H37Rv restablished *esat-6* secretion and recognition of this antigen by T-cells in an infected animal host indicating that Esat-6 secretion lies downstream of PhoP control (Frigui et al; 2008).

The ESX-1 system controls the secretion of a variety of proteins and this secretion system has been designated type VII secretion system (Abdallah et al; 2007). The secreted proteins are the 6 kDa early secreted antigenic target (Esat-6) and the 10 kDa culture filtrate protein

CFP-10. These two proteins form a heterodimer. Interestingly Esat-6 dissociates from CFP-10 at acidic pH and is capable of lysing membranes. *M. tuberculosis* has been observed extraphagosomally in the cytoplasm of macrophages. Mutations in *esx-1* gene result in *M. tuberculosis* bacilli that do not escape from the phagosome into the cytoplasm. This implies that Esat-6 is involved in a response to acidity (Simeone et al; 2009). However PhoP may not directly control secretion of Esat-6 via Esx-1. A secreted transcription factor EspR seems to controls the transcriptional upregulation of Rv3616c which enhances the activity of the secretory Esx-1 apparatus. (Raghavan et al; 2008). When secretory apparatus activity is high, EspR is exported from the mycobacterial cell, leading to decreased transcriptional activity and decreased secretion by Esx-1. Thus this is a feedback loop for this type VII secretion system and PhoP seems not to regulate EspR itself.

PhoP mutants have been analyzed for their efficacy as vaccine strains in animal models. A *phoP* mutation in *M. tuberculosis* resulted in more attenuation compared to *M. bovis* BCG in mice and guinea pigs. In addition the *phoP* mutant induced increased immunity and a *phoP* mutant was superior to *M. bovis* BCG as a vaccine in guinea pig model (Martin et al; 2006, Aguilar et al; 2006). Disrupted *phoP* mutant were attenuated in a Balb-C mouse model of progressive pulmonary TB in immunocompetant mice. There were few small granulomas and no pneumonic lesions. The mutant stimulated longer lasting cellular immunity (Aguilar et al; 2006). It was also attenuated in SCID mice and more attenuated than *M. bovis* BCG at a 10X higher infectious dose (Martin et al; 2006). Therefore immunocompetant patients may be better able to use a *phoP* mutant as a vaccine strain than *M. bovis* BCG which can cause disease in susceptible individuals. This is especially important in sub-Saharan Africa where the Human Immunodeficiency Virus (HIV) epidemic has created an increased number of tuberculosis cases and where much of the population is routinely vaccinated to prevent occurrence of neonatal meningitis. A *M. tuberculosis* strain deficient in *phoP* and less virulent than many BCG strains, may be adept at preventing neonatal meningitis but less likely to cause overt disease in the immunocopromised.

6. Timing of responses to acidity

There seems to be a difference in the timing of upregulation of genes involved in response to acidic stress. At 15-30 minutes a number of genes are upregulated and this seems to constitute an immediate response. This early response may be controlled by VirsS at least for the *mymA* operon as previously described (Singh et al; 2005). The *lipF* promoter part of the PhoP regulon, failed to be identified as upregulated by acidic stress at 15-30 minutes (Fisher et al; 2002). However, this promoter is upregulated after 1.5 hour, with maximum regulation occurring after 24 hours (Saviola et al; 2002). At 2 hours 24 of 44 known PhoP genes are upregulated in macrophages (Rohde et al; 2007). An even later response occurs that results in the accumulation of triacylglycerol that *M. tuberculosis* may need for long term survival under stress. Tgs1 is implicated in triacylglycerol storage and when this gene is deleted the mutant fails to accumulate triacylglycerol within mycobacterial cells. Tgs1 was not upregulated at 15 minutes by acidic shock (Fisher et al; 2002). It was however upregulated upon extended acidic conditions of 3 weeks duration (Sirakova et al; 2006, Low et al; 2009, Deb et al; 2009). Thus there are varying time frames for the response to acidic conditions. There may not be 3 discrete times, but a multitude of response times. Distinct time frames of response to acidity may correlate with transcriptional regulators which

control acid responsive genes. The kinetics of the response will be different for each regulator and as a consequence depend on the exact nature of the signal that the regulator senses. Signals may be a direct or indirect consequence of acidic damage. These questions will be important when considering acid responsive genes as drug targets. Inhibition of a gene expressed early may block establishment of infection but be ineffective during chronic infection. Likewise a drug against a gene upregulated at 3 weeks may be effective against a chronic infection but be unable to prevent establishment of infection.

7. Conclusions

Acidity is a condition that is encountered by both environmental mycobacteria and mycobacteria of the *Mycobacterium tuberculosis* complex. Mycobacteria are hypothesized to possess a large degree of innate resistance to acidic stress as well as an incredible ability to make adaptive changes to withstand acidic environments. This is certainly true for environmental mycobacteria which can survive in extreme conditions. It is interesting to speculate that pathogenic mycobacteria have evolved from environmental mycobacteria and maintain some intrinsic acid resistance as well as a rich and varied ability to respond adaptively to acidic stress. These pathogenic mycobacteria for the most part will never encounter the external environment, yet they are well suited to withstand the hostile environment within the phagosome of macrophages as well as the acidic centers of caseating granulomas. Drug targets against genes necessary to survive acidic stress may be developed to eliminate *M. tuberculosis* present within acidic environments including conditions that favor dormancy.

8. References

Abdallah A, Gey van Pittius N, Champion P, Cox J, Luirink J, Vandenbroucke-Grauls C, Appelmelk B, and Bitter W. 2007. Type VII secretion-mycobacteria show the way. Nature Reviews in Microbiology. 5: 883-891.

Abramovitch RB, Rohde KH, Hsu F, and Russell. 2011. aprABC: a *Mycobacterium tuberculosis* complex-specific locus that modulated pH-driven adaptation to the macrophage phagososme. Molecular Microbiology. 80(3):678-694.

Aguilar D, Infante E, Martin C, Gormly E, Gicquel B, and Hernandez Pando R. 2006. Immunological responses and protective immunity against tuberculosis conferred by vaccination of Balb/C mice with the attenuated *Mycobacteria tuberculosis* (*phoP*) SO2 strain. Clinical and Experimental Immunology. 147:330-338.

Armstrong JA, and Hart D. 1971. Response of cultured macrophages to Mycobacterium *tuberculosis* with observations on fusion of lysosomes with phagosomes. Journal of Experimental Medicine. 134(3): 713-740.

Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, and Small PM. 1999. Comparative genomics of BCG vaccines by whole genome DNA microarray. Science 284:1520-1523.

Biswas T, Small J, Vandal O, Odaira T, Deng H, Ehrt S, and Tsodikov OV. 2010. Structural insight into serine protease Rv3671c that protects *M. tuberculosis* from oxidative and acidic stress. Structure. 18(10): 13353-1363.

Bland CS, Ireland JM, Lozano E, Alvarez ME, and Primm TP. 2005. Mycobacterial ecology of the Rio Grande. Applied and Environmental Microbiology. 71(10):5719-5727.

Brown BA, Springer VA, Steingrube RW, Wilson GE, Pfyffer MJ, Garcia MC, Menendez B, Rodriguez-Salgado KC, Jost Jr SH, Chiu SH, Onyi GO, Bottger EC, and Wallace RJ. 1999. *Mycobacterium wolinski* sp nov. and *Mycobacterium goodii* sp. nov. Two New Rapidly Growing Species Related to *Mycobacterium smegmatis* and Associated with Human Wound Infections: a Cooperative Study from the International Working Group on Mycobacterial Taxonomy . International Journal of Systematic Bacteriology. 49:143-151.

Buchmeier N, Blanc-Potard A, Ehrt S, Piddington D, Riley L,and Groisman EA.2000. A parallel intraphagosomal survival strategy shared by *Mycobacterium tuberculosis* and *Salmonella enterica*. Molecular Microbiology. 35(6):1375-1382.

Camacho LR, Ensergueix D, Perez E, Gicquel B, Guilhot C. 1991 Identification of a virulence cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Molecular Microbiology. 32:257-267.

Chapman JS and Bernard JS. The tolerances of unclassified mycobacteria. I. Limits of pH tolerance. American Review of Respiratory Disease. 86:582-583.

Chesne-Seck M, Barilone N, Boudou F, Asensio JG, Kolattukudy PE, Martin C, Cole ST, Gicquel B, Gopaul DN, and Jackson M. 2008. A point mutation in the two-component regulator PhoP-PhoR accounts for the absence of polyketide-derived acyltrehaloses but not that of phthiocerol dimycocerates in *Mycobacterium tuberculosis* H37Ra.Journal of Bacteriology. 190(4):1329-1334.

Dannenberg AM (2006) Pathogenesis of Human Pulmonary Tuberculosis: Insights from the Rabbit Model. ASM Press, Washington, DC.

Deb C, Lee CM, Dubey VS, Daniel J, Abomoelak B, Sirakova S, Pawar S, Rogers L, Kolattukudy PE. 2009. Plos One 4(6):e6077.

Ehrt S, and Schnappinger D. 2009. Mycobacterial survival strategies in the phagosome: defence against host stresses. Cellular Microbiology. 11(8):1170-1178.

Fisher MA, Plikayatis BB,and Scinnick TM. 2002. Microarray of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagososmes. Journal of Bacteriology. 184(14): 4025-4032.

Frigui W, Bottai D, Majlessi L, Monot M, Josselin E, Brodin P, Garnier T, Gicquel B, Martin C, Leclerc C, Cole ST, and Brosch R. 2008. Control of M. tuberculosis Esat-6 secretion and specific T cell recognition by PhoP. PLOS Pathogens. 4:e22.

Gonzalo-Asensio J, Mostowy S, Harders-Westerveen J, Huygen K, Hernandez-Pando R, Thole J, Behr M, Gicquel B, and Martin C. 2008. PhoP: a missing piece in the intricate puzzle of *Mycobacterium tuberculosis* virulence. Plos One. 3(10):e3496.

Gupta S, Sinha A, and Sarkar D. 2006. Transcriptional autoregulation by *Mycobacterium tuberculosis* PhoP involves recognition of novel direct repeat sequences in the regulatory region of the promoter. FEBS Letters. 580:5328-5338.

Hoffmann C, Leis A, Niederweis M, Plitzko JM, and Engelhardt H. 2008. structure. Proceedings of the National Academy of Science.105(10):3963-7.

Huynh KK, and Grinstein S. 2007. Regulation of vacuolar pH and its modulation by some microbial species. Micobiology and molecular biology reviews. 71(3):452-462.

Kim S, Lee B, Shin SJ, Kim H, and Park J. 2008. Differentially expressed genes in *Mycobacterium tuberculosis* H37Rv under mild acidic and hypoxic conditions. Journal of medical Microbiology. 57:1473-1480.

Kirschner RA, Parker BC and Fakinham JO. 1992. Epidemiology of infection by nontuberculous mycobacteria. X. *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* in acid brown-water swamps of the Southeastern United States and their association with environmental variables. American Review of Respiratory Disease. 1145:271-275.

Krischner RA, Parker BC, and Falkinham JO. 1999. Humic and fluvic acids stimulate growth of *Mycobacterium avium*. FEMS Microbiology Ecology. 30:327-332.

Lee JS, Krause R, Schreiber J, Mollenkobf H, Kowall J, Stein R, Jeon B, Kwak J, Song M, Patron JP, Jorg S, Roh K, Cho S, and Kaufman S. 2008. Mutation in the transcriptional regulator PhoP contributes to avirulence of *Mycobacterium tuberculosis* H37Ra strain. Cell Host and Microbe 3:97-103.

Livanainen E, Martikainen PJ, Vaananen P, and Katila ML. 1999. Environmental factors affecting the occurrence of mycobacteria in brook sediments. Journal of Applied Microbiology. 86:673-681.

Lobell R, and Schleif R. 1990. DNA looping and unlooping by AraC Protein. Science. 250: 528-532.

Low KL, Rao PSS, Shui G, Bendt AK, Pathe K, Dick T, and Wenk MR. 2009. Triacylglycerol utilization is required for regrowth of in vitro hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. Journal of Bacteriology. 191(16): 5037-5043.

MackMicking JD, Taylor GA, and McKinney JD. 2003. Immune control of tuberculosis in IFN-□-inducible LRG 47. Science. 302:654-659.

Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, Soto CY, Clark SO, Hatch GJ, Aguilar D, Ausina V, and Gicquel B. 2006. The live attenuated *M. tuberculosis phoP* strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine. 24:3408-3419.

Metchnikoff E. 1905 Immunity to infective disease, p. 182. Cambridge University Press, Cambridge, United Kingdom.

Mostowy S, Cleto C, Sherman DR, and Behr MA. 2004. The *Mycobacterium tuberculosis* complex transcriptome of attenuation. Tuberculosis. 84:197-204.

Newton JA, Weiss PJ, Bowler WA, and Oldfield EC. 1993. Soft-tissue Infection due to *Mycobacterium smegmatis*: Report of Two Cases. Clinical Infectious Disease.16:531-533.

Niederweis M, Danilchanka O, Huff J, Hoffmann C, and Engelhardt H. 2010. proteins. Trends Microbiol.18(3):109-16.

O'Brien LM, Gordon SV, Roberts IS, and Andrew PW. 1996. Response of *Mycobacterium smegmatis* to acid stress. FEMS Microbiology Letters. 139:11-17.

Perez E, Samper S, Bordas Y, Guilhot C, Gicquel B, and Martin C. 2001. An essential role for *phoP* in *Mycobacterium tuberculosis* virulence. Molecular Micrbiology. 41(1):179-187.

Portales F, and Pattyn SR. 1982. Growth of mycobacteria in relation to pH of the medium. Annals of Microbiology. 133:213-221.

Pym AS, Brodin P, Brosch R, Huerre M, and Cole ST. 2002. Loss of RD1 region contributed to the attenuation of the live tuberculosis vaccine *Mycobacterium bovis* BCG and *Mycobacterium microti*. Molecular Microbiology. 46: 709-717.

Raghavan S, Manzanillo P, Chan K, Dovey K, and Cox JS. 2008. Secreted transcription factor controls *Mycobacterium tuberculosis* virulence. Nature. 454:717-721.

Raynaud C, Papavinasasundaram KG, Speight RA, Springer B, Sander P, Bottger EC, Colston MJ, and Draper P. 2002. The functions of OMpATb a pore- forming protein of *Mycobacterium tuberculosis*. Molecular Microbiology. 46(1):191-201.

Rhode KH, Abramovitch RB, and Russell DG. 2007. *Mycobacterium tuberculosis* invasion of macrophages: linking bacterial gene expression to environmental cues. Cell Host and Microbe. 2:352-364.

Richter L, and Saviola B. 2009. The *lipF* promoter of *Mycobacterium tuberculosis* is upregulated specifically by acidic pH but not by other stress conditions. Microbiological Research. 164(2):228-32 (2009)

Rohde K, Yates RM, Purdy GE, and Russell DG. 2007. *Mycobacterium tuberculosis* and the environment within the phagosome. Immunological Reviews. 219: 37-54.

Rose L, Kaufmann S, and Daugelat S. 2004. Involvement of *Mycobacterium smegmatis* undecaprenyl phosphokinase in biofilm and smegma formation. Microbes and Infection. 6:965-971.

Roxas B, and Li Q. 2009. Acid stress response of a mycobacterial proteome: insights from gene ontology analysis. International Journal of Clinical Medicine. 2:309-328.

Ryndak M, Wang S, and Smith I. 2008. PhoP, a key player in *Mycobacterium tuberculosis* virulence. Trends in Microbiology. 16(11):528-534.

Santos R, Fernandes J, Fernandes N, Oliveira F, and Cadete M. 2007. *Mycobacterium parascrofulaceum* in acidic hot springs in Yellowstone National Park. Applied Environmental Microbiology. 73(15) 5071-5073.

Saviola B, Seabold R, and Schleif R. 1998 Arm-domain interactions in AraC. Journal of Molecular Biology. 278: 539-548.

Saviola B, Woolwine S, and Bishai W. 2002. Isolation of acid-inducible genes of *Mycobacterium tuberculosis* with the use of recombinase based in vivo expression technology. Infection and Immunity. 71(3): 1379-1388.

Schaible RH, Sturgill-Koszycki S, Schlesinger PH, and Russell DG. 1998. Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. Journal of Immunology. 160: 1290-1296.

Schnappinger D, Ehrt S, Voskuil MI, Liu Y, mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, and Schoolnik GK. 2003. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. The Journal of Experimental Medicine. 198(5):693-704.

Simeone R, Bottai D, and Brosch R. 2009. ESX/type VII secretion systems and their role in host-pathogen interaction. Current Opinion in Microbiology. 12:4-10.

Singh A, Gupta R, Vishwakarma RA, Narayanan PR, Paramasivan CN, Ramanathan VD, and Tyagi AK. 2005. Requirement of *mymA* operon for appropriate cell wall ultrastructure and persistence of *Mycobacterium tuberculosis* in the spleens of guinea pigs. Journal of Bacteriology. 187(12):4173-4186.

Sinha A, Gupta S, Bhutani S, Pathak A, and Sarkar D. 2008. PhoP-PhoP interaction at adjacent PhoP binding sites is influenced by protein phosphorylation. Journal of Bacteriology. 190(4): 1317-1328.

Sirakova TD, Dubey VS, Deb C, Daniiel J, Korotkova TA, Abomoelak B, and Kolattukudy PE. 2006. Identification of a diacylglycerol acyltransferase gene involved in accumulation of triacyglycerol in Mycobacterium tuberculosis under stress. Microbiology. 152: 2717-2725.

Song H, Huff J, Janik K, Walter K, Keller C, Ehlers S, Bossman SH, and Niederweis M. 2011 Expression of the *ompATb* operon accelerates ammonia secretion and adaptation of *Mycobacterium tuberculosis* to acidic environments, Molecular Microbiology. 80(4): 900-18.

Streenken W, Oatwat WH, and Petroff SA. 1934. Biological studies of the tubercle bacillus. III. Dissociation and pathogenicity of the R and S variants of the human tubercle bacillus (H37). Journal of Experimental Medicine. 60:515-540.

Sturgill-Koszycki S, Schlesinger PH, Cjakraborty P, Haddix PL, Collins HL, and Fok AK. 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263:678-681.

Tan MP, Sequeira P, Lin WW, Phong WY, Cliff P, Ng SH, Lee BH, Canacho L, Schnappinger D, Ehrt S, Dick T, Pethe K, and Alonso S. 2010. Nitrate respiration protect hypoxic *Mycobacterium tuberculosis* against acid- and reactive nitrogen species. PlosOne. 5(10): e13356.

Tran SL, Rao M, Simmers C, Gebhard S, Olsson K, and Cook GM. 2005. Mutant of *Mycobacterium smegmatis* unable to grow at acidic pH in the presence of the protonophore carbonyl cyanide m-chlorophenylhydrazone. Microbiology. 151:665-672.

Uyttebroek M, Vermeir S, Wattiau P, Ryngaert A, and Springael D. 2007.Characterization of cultures enriched from acidic polycyclic aromatic hydrocarbon-contaminated soil for growth on pyrene at low pH. Applied and Environmental Microbiology. 73(10):3159-3164.

Vandal OH, Nathan CF, and Ehrt S. 2009. Acid resistance in *Mycobacterium tuberculosis*. Journal of Bacteriology. 191(15):4714-4721.

Vandal OH, Pierini LM, Schnappinger D, Nathan CF, and Ehrt S. 2008. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. Nature Medicine. 14(8): 849-854.

Vandal OH, Roberts JA, Odaira T, Schnappinger D, Nathan C, and Ehrt S. 2009. Acid-suceptibility mutants of *Mycobacterium tuberculosis* share hypersusceptibility to cell wall and oxidative stress and to the host environment. Journal of Bacteriology. 191(2): 625-631.

Via LE, Fratti RA, McFalone M, pagan-Ramos E, Deretic D, and Deretic V. 1998. Effects of cytokines on mycobacterial phagosome maturation. Journal of Cell Science. 111:897-905.

Walters SB, Dubnau E, Irina K, Laval F, Daffe M, and Smith I. 2006. The *Mycobacterrium tuberculosis* PhoPR two-component system regulates genes essential for virulence and complex lipid biogenesis. Molecular Microbiology. 60(2):312-330.

Wu C, Schmoller SK, Shin SJ, and Talaat AM. 2007. Defining the stressome of *Mycobacterium avium subsp paratuberculosis* in vitro and in naturally infected cows. Journal of Bacteriology. 189(21):7877-7886.

5

Mycobacterium Tuberculosis Signaling via c-AMP

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1. Introduction

1.1 Cyclic Adenosine Monophosphate (cAMP) metabolism in mycobacteria

1.1.1 General aspects of Adenylyl Cyclases and their presence in *M. tuberculosis* genomes

Adelynate cyclases (ACs), which catalyze synthesis of cAMP from ATP and yield pyrophosphate as a by-product, can be classified into four different classes according to their common features: Class I cyclases, related to enterobacterial adenylate cyclases; Class II, toxic adenylate cyclases isolated from bacterial pathogens; Class III, a large and probably ancient class that comprises cyclases from both eukaryotes and prokaryotes and is strongly related to guanylate cyclases; and Class IV, with mainly one example that differs entirely from all other classes (McCue *et al.*, 2000).

In class I ACs (the enterobacterial type) no long stretch of hydrophobic amino acid residues is present to explain the membrane-bound localization of the adenylate cyclases. In all cases, the proteins are very rich in cysteine residues, an uncommon feature for proteins located in the cytoplasm or at the cytoplasmic border of the membrane. They are also rich in histidine residues, which could indicate that metal ions take part in the folding and/or activity of the polypeptide chain (Mock *et al.*, 1991).

Class II ACs (the calmodulin-activated toxic class) is represented by *Bordetella pertussis* adenylate cyclase. It is synthesized as a large bifunctional polypeptide chain of 1706 amino acid residues. The N-terminal segment of the protein (400 residues) alone displays calmodulin-activated adenylate cyclase activity, whereas the rest of the molecule is responsible for hemolytic activity and for transporting the toxin. After attempts to isolate other members of this class, several examples of similar proteins have now been discovered in *Bacillus anthracis*, *Pseudomonas aeruginosa*, and in Ye*rsinia* species. Comparison of the catalytic regions of the *B. pertussis* and *B. anthracis* adenylate cyclases identified four conserved regions that are involved in catalysis, calmodulin binding and activation. The first region comprises a sequence, Gly-XXXX-Gly(Ala)-Lys-Ser, similar to the nucleotide-binding motif found in many ATP- or GTP-binding proteins. Analysis of the region conserved between the *B. anthracis* and *B. pertussis* enzymes, indicates that these proteins

may form a catalytic center from the cooperation of two halves. The function of calmodulin may be to trigger the appropriate conformational change necessary to form an active catalytic center (Drum *et al.*, 2002).

Class III ACs (the "universal" class**)** form a very diverse collection of enzymes in eubacteria**.** They comprise two domains: the catalytic domain is carboxy-terminal and the regulatory domain is likely an ion transporter in one case and the phosphorylated moiety of a two-component regulatory system in another. Most mammalian ACs are monomeric integral membrane proteins that are catalytically active as pseudoheterodimers (Sunahara *et al.*, 1996),while prokaryotes and lower eukaryotes produce both soluble and membrane-bound nucleotidyl cyclases of variant domain compositions functioning as homodimers (Guo *et al.*, 2001). In general, class III ACs are the most widespread class of cAMP-generating enzymes, and they are further subdivided into four subclasses: IIIa-IIId. Dimerization is required for all class III ACs in order to be active, given the substrate-binding sites are formed at the dimer interface (Abdel Motaal *et al.*, 2006). Class III adenylyl and guanylyl cyclases are proteins with a central four stranded anti-parallel β-sheet structurally similar to the palm domain of DNA polymerases, and α-helices on either side (Shenoy & Visweswariah, 2006b). So far, all Cyclase Homology Domain (CHDs) proteins operate as dimers with mostly two catalytic centers positioned at the dimer interface, where catalysis is based on six highly conserved residues. Two aspartate residues coordinate two metal cofactors (Mg2+ or Mn2+), an asparagine and an arginine stabilize the transition-state and a lysine-aspartate couple specifies ATP as a substrate. Several mycobacterial ACs gene products were early annotated as putative cyclases, but have now been characterized biochemically and structurally.

Class IV ACs was assigned to *Aeromonas hydrophila*, which synthesizes a very small cyclase of 193 residues. This class IV cyclase has an optimal temperature for activity of 65°C and is at least ten times more active than the class I adenylate cyclase in the same organism (Sismeiro *et al.*, 1998). No function has yet been discovered for this protein. Currently, it has been found only in various isolates of *A. hydrophila* and in *Y. pestis.*

Most prokaryotes contain a single adenylate cyclase (AC, e.g. *E. coli*) and some contain none (e.g., *Bacillus* species); however, 16 or 17 genes have been identified as ACs in mycobacteria, and in particular *M. tuberculosis*. AC enzymes require the presence of conserved metal-, substrate-binding (either ATP or GTP) amino acid residues and transition-state stabilizing amino acid residues in their catalytic site (Shenoy & Visweswariah, 2006b).

The genome of *M. tuberculosis* H37Rv encodes 16 class III cyclases while the CDC1551 strain has 17 cyclases. Using the 16 H37Rv class III ACs genes as query, we found they posses different numbers of orthologous genes in the up-to-date sequenced mycobacterial genomes available at MycoDB (http://xbase.bham.ac.uk/mycodb/, Table 1). This suggests that some of these cyclases have more conserved roles than others. Several mycobacterial gene products initially classified as cyclases have now been biochemically and structurally characterized as ACs. In mycobacteria, they retain similar catalytic properties to the mammalian enzymes, in terms of the requirement for divalent cations, and dimerization as a prerequisite to generate the catalytic site.

Bioinformatics analysis has predicted subcellular localization of mycobacterial ACs (Shenoy *et al.*, 2004). Nevertheless, experimental studies have identified proteins like Rv0386 and Rv1358 in mycobacterial cell wall and membranes, despite the absence of

hydrophobic, predicted transmembrane domains (Agarwal, 2009). This suggests that a necessary improvement to prediction software must emphasize these particularities of mycobacteria.

Gene name	Orthologous (No.)
Rv0386	7
Rv0805	7
RV0891c	4
Rv1264	13
Rv1318c	14
Rv1319c	14
Rv1320c	14
Rv1358	7
Rv1359	3
Rv1625c	11
Rv1647	16
Rv1900c	10
Rv2212	6
Rv2564	3
Rv2565	3
Rv3645	16

Table 1. Orthologous to H37Rv AC-domain containing genes present in Mycobacteria

As mycobacteria lack G-proteins, it is unknown how bacterial adenylate cyclase AC activities are modulated. However, polyphosphates isolated from *M. bovis* BCG were potent inhibitors of Rv1625c, Rv1264, and Rv3645, and the mechanism of inhibition proposed is that polyphosphates possibly obstructs the catalytic fold of ACs (Guo et al., 2001).

1.2 cAMP producing (Adenylyl cyclases) and degrading (Phosphodiesterase) mycobacterial enzymes

Six *M. tuberculosis* AC-domain containing proteins (Rv0891c, Rv1264, Rv1359, Rv1647, Rv2212 and Rv1625c) contain just a cyclase domain. Rv1647 and Rv1625c are phylogenetically distant and biochemically distinct AC-domain containing proteins (Shenoy & Visweswariah, 2006a). The remaining AC-domain containing proteins have additional domains that presumably allow them to respond to multiple signals, regulate their activity in response to environmental conditions, and/or expand their repertoire with effector function capability. Five of these multidomain AC-domain containing proteins (Rv1318c, Rv1319c, Rv1320c, Rv2435c and Rv3645c) are membrane-associated and contain HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and phosphatases) domains. HAMP domains are often associated with two-component signal transduction pathways and connect the sensing of extracellular environmental signals with

responding intracellular signalling domains. Mycobacterial ACs with HAMP domains have six transmembrane regions, followed by the HAMP domain and a C-terminal AC domain. Modulators of *M. tuberculosis* AC activity include pH and fatty acids, which were seen to enhance the pH sensitivity of the holoenzyme, and CO_2 levels, and could be signals present during *M. tuberculosis* host infection (Abdel Motaal et al., 2006, Bai *et al*., 2011, Barba *et al*., 2010).

An N-terminal autoregulatory domain in Rv1264 is a pH-response element that inhibits cyclase activity above pH 6.0. Additional AC-domain containing proteins (Rv0386, Rv1358 and Rv2488c) posses both ATPase and helix– turn–helix (HTH) domains. Rv1900c contains an αβ-hydrolase domain (Barba et al., 2010, Bai et al., 2011). Rv0386 is the first AC-domain containing protein as a representative of the family of putative DNA-binding domain-containing cyclases in mycobacteria (Castro *et al*., 2005). Rv0386 has guanylyl cyclase activity that is 20% of its adenylyl cyclase activity.

Rv1625c adenylyl cyclase is particularly unusual, in that it is catalytically active when expressed in mammalian cells or in *E. coli*. The Rv1264 holoenzyme shows higher AC activity at acidic pH (pH 6.0). Similarly, the Rv1264 AC is activated only at low pH (pH 5.5) whereas Rv1647 is active only at high pH (pH 8.5). The localization of Rv1647 in the cell wall and membrane fractions of *M. tuberculosis* might enable the bacteria to sense and respond to extracellular pH shifts. Because *M. tuberculosis* actively avoids phagosomal acidification, sensing of pH and bicarbonate and/or CO_2 could be crucial signaling events in *M. tuberculosis* pathogenesis (Shenoy & Visweswariah, 2006a). In fact, cAMP production in pathogenic mycobacteria increased when the pH of their growth medium was shifted from pH 6.7 to pH 5.5 (Gazdik *et al*., 2009), thus suggesting activation of the acid-responsive ACs.

In *M. tuberculosis*, Rv0998 was recently shown to regulate protein lysine acetylation in a cAMP-responsive manner (Nambi *et al*., 2010), where acetylation is enhanced in the presence of either cAMP or cGMP. Recently, it was shown that a cAMP-dependent protein acetyltransferase inactivates ACs through acetylation of a single, specific lysine residue, and Rv1151c is a NAD+-dependent deacetylase that reactivates ACs. This acetylation/deacetylation system in mycobacteria is likely to sense the extracellular environment through cAMP levels and also key intracellular metabolites, including NAD+ and AcCoA, since cAMP, AcCoA, and NAD+ are required for acetylation and deacetylation, respectively (Xu *et al*., 2011).

Adenylyl cyclase Rv2212 from *M. tuberculosis* has a domain composition identical to the pH-sensing isoform Rv1264, an N-terminal regulatory domain and a C-terminal catalytic domain. The maximal velocity of Rv2212 was the highest of all 10 mycobacterial cyclases investigated to date. Unsaturated fatty acids strongly stimulated Rv2212c activity by increasing substrate affinity (Findeisen *et al*., 2007). In addition, fatty acids greatly enhanced the pH sensitivity of the holoenzyme, thus converting Rv2212c to a pH sensor adenylyl cyclase (Abdel Motaal et al., 2006).

To date, the only identifiable cAMP phosphodiesterase (PDE) in the genome of *M. tuberculosis* H37Rv is the one encoded by the *Rv0805* gene. This enzyme is a dimeric Fe(3+)-Mn(2+) binuclear PDE, where metals coordinated at the catalytic site contribute to dimerization and thus play an additional structural role apart from their involvement in

catalysis (Shenoy *et al.*, 2007). As this gene is found only in pathogenic mycobacteria, Rv0805 may therefore play a key role in the pathogenicity of mycobacteria, not only by hydrolyzing bacterial cAMP, but also by indicating as a protein that can alter cell wall functioning (Podobnik *et al.*, 2009).

2. Participation of cyclic adenosine 3′,5′-monophosphate (cAMP) in mycobacterial gene regulation

Cyclic adenosine 3′,5′-monophosphate (cAMP) is one of the most important second messengers used in bacteria and it has been characterized principally in *Escherichia coli*. However its signaling role in *M. tuberculosis* is beginning to emerge. A large number of AC genes are present in *Mycobacterium tuberculosis* in comparison with other microorganims (Cha *et al.*, 2010, Klengel *et al.*, 2005, Shenoy et al., 2004), and also that *Mycobacterium* just have one class III cNMP phosphodiesterase (Rv0805) that has been identified in *M. tuberculosis* (McCue *et al.*, 2000, Shenoy et al., 2007) with a modest ability to efficiently hydrolyse 3′,5′-cAMP and that responds to H_2O_2 *in vitro* (Bai et al., 2011, Barba et al., 2010). cAMP may serve as both an extra and intracellular signaling molecule in mycobacteria (Agarwal, 2009). cAMP levels are modulated by stress conditions in *M. smegmatis*, where it has been found in concentrations between 10 μM and 1 mM, suggesting that cAMP participates in signalling events within the bacterial cell, mediating its action by downstream effectors (Dass *et al.*, 2008).

It appears that Mycobacteria need to maintain a steady level of cytoplasmatic cAMP in many conditions. For example, it has been shown that cytoplasmic cAMP levels in *E. coli* are reduced three- to fourfold when the carbon source is ~0.2% glucose rather than glycerol (Bai et al., 2011). In contrast, a recent study showed no significant change in the cytoplasmic cAMP levels of *M. bovis* BCG incubated with 0.2% glucose (Bai *et al.*, 2009), or carbon-starved bacteria (Dass et al., 2008). cAMP levels decrease in both fast- and slow-growing mycobacteria in response to very high levels of glucose (2%) (Bai et al., 2011). cAMP levels have been suggested to be high in *Mycobacterium* cells, exceeding up to 100-fold levels found in other bacteria (Nambi et al., 2010, Stapleton *et al.*, 2010, Shenoy & Visweswariah, 2006b, Rickman *et al.*, 2005). However, it is difficult to make a comparison between studies, due to cAMP variations presents in the conditions tested, as well as the different normalization and reporting methods used.

2.1 CRP and cNMP binding proteins

In silico studies predict 10 cNMP binding proteins that encompass a wide range of potential effector functions, suggesting a more complex role for cAMP signalling, probably important during host infection. From these 10 cNMP binding proteins, 7 (Rv0073, Rv0104, Rv2434c, Rv2564, Rv2565, Rv3239c and Rv3728) contain an assortment of putative functional domains, including those associated with transport functions and esterase activities (McCue et al., 2000, Shenoy & Visweswariah, 2006a). Only three cNMP binding proteins have been functionally characterized to date (Bai et al., 2011). Two of these proteins, referred to as CRP (*Rv3676*, for Catabolite Represor Protein) and Cmr (*Rv1675c*, for cAMP and macrophage regulator), contain a HTH DNA binding domains, and belong to the CRP-FNR family of transcription factors (McCue et al., 2000). The third protein from this group, is encoded by

Rv0998 in *M. tuberculosis*, and regulates lysine acetylation in mycobacterial proteins in a cAMP-responsive manner (Nambi et al., 2010). The biological effects of this acetylation are not yet defined.

CRP (cAMP receptor protein) in *Escherichia coli*, is one of the best-studied prokaryotic transcription factor. Currently, a total of 378 target promoters on the *Escherichia coli* genome are proposed to be under the control of cAMP-bound CRP, using a SELEX approach (Shimada *et al.*, 2011). Some of the CRP regulon genes include those encoding the transporters and the catabolic enzymes of glucose (Perrenoud & Sauer, 2005) and non-glucose sugars (Wickstrum *et al.*, 2010), virulence genes (Espert *et al.*, 2011), motility genes (Hollands *et al.*, 2010), GMP synthesis (Husnain *et al.*, 2009), anaerobic growth and nitrate reductases genes (Stewart *et al.*, 2009). To date, in *E. coli*, 2 differents ways in which CRP leads the sigma 70 promoter trancription have been described: in the Class I promoters, CRP binds upstream of the promoter −35 element, at a site centered at position −61.5 or further upstream, and an activating region (AR1) in the downstream subunit of the CRP dimer makes contact with the C-terminal domain of one of the two RNA polymerase α subunits (αCTD). In class II promoters, CRP binds at a target that overlaps the promoter −35 element and is usually centered at position −41.5. AR1 in the upstream subunit of the CRP dimer interacts with αCTD, while a second activating region (AR2) in the downstream subunit interacts with the N-terminal domain of one of the two RNA polymerase α subunits (αNTD)(Hollands et al., 2010).

In a similar manner to the CRP protein from *E. coli*, the *M. tuberculosis* CRP (Rickman *et al.*, 2005) is the best-studied example of a protein implicated in cAMP-mediated signaling. In *M. tuberculosis* CRP is encoded by the gene *Rv3676* and it is homologous to *E. coli* CRP. In *E. coli*, it has been shown that CRP regulates expression of genes required to control metabolism, as well as growth under hypoxic and nutrient-deprived conditions. Like *E. coli* CRP, *M. tuberculosis* Rv3676 possesses an N-terminal cAMP-binding domain and a C-terminal DNA-binding domain. The crystal structures of *M. tuberculosis* CRP at 2.2 and 2.0 A resolution of cAMP-bound (Reddy *et al.*, 2009) and the apo-form (Gallagher *et al.*, 2009), have been reported. Conformational changes required for DNA-binding do not take place in the absence of the second messenger (Reddy et al., 2009). In fact, as opposed to *E. coli* CRP where cAMP binding follows a cooperative mechanism, c-AMP binding sites are independent, and DNA-binding activity is not as enhanced with *M. tuberculosis* Rv3676 (Stapleton *et al.*, 2009). The CRP orthologous in *M. bovis* BCG (CRP_{BCG}) is a fully functional transcription factor, since CRP_{BCG} overcame the virulence deficiency of an *M. tuberculosis crp* mutant (Hunt *et al.*, 2008). Although both of them have a similar ability to bind cAMP and DNA, CRP_{BCG}'s DNA binding affinity is approximately twice that of *Mycobacterium tuberculosis* CRP (CRP_{Mt}) (Bai *et al.*, 2007, Hunt et al., 2008). Interestingly, CRP_{BCG} differs from CRP_{Mt} in just two amino acid residues (L47P and E178K).

The other predicted cAMP-dependent transcriptional regulator, Cmr, was found to negatively regulate the expression of five proteins (GroEL2, Rv2971, PE_PGRS6a, Mdh and Rv1265) (Gazdik & McDonough, 2005). The upstream regions of three of these genes (*mdh*, *groEL2* and *Rv1265*) bound specifically to Cmr in electrophoretic mobility shift assays, consistent with direct regulation of these genes by Cmr. Expression of three of these genes was found to be regulated within macrophages, and this regulation was mediated by Cmr in both *M. tuberculosis* and *M. bovis* BCG (Gazdik et al., 2009). Despite

the importance of Cmr for the cAMP- dependent regulation of these genes, Cmr has not been shown to directly bind cAMP *in vitro*, and cAMP did not affect Cmr's binding to any of their promoter sequences (Gazdik et al., 2009). The mechanism by which Cmr responds to cAMP levels has yet to be discovered, and it is possible that a second factor plays a facilitating role.

2.2 CRP_{Mt} regulon

The cAMP binding transcriptional regulator encoded by *Rv3676* in *M. tuberculosis* has been strongly associated with growth during mycobacterial life cycle. Its deletion resulted in impaired growth in macrophage cell lines and in mice (Hunt et al., 2008, Rickman et al., 2005). Using an exponential enrichment (SELEX) approach, Bai et al. defined the CRP_{Mt}´s palindromic binding motif (C/TGTGANNNNNNT CACG/A) based on 58 predicted binding sites from the *M. tuberculosis* genome, using a combination of *E. coli* CRP binding sites and *M. tuberculosis* DNA sequences recovered by affinity capture using CRP_{Mt} to seed the computational analyses (Bai *et al*., 2005). Akhter et al. used the positional Shannon relative entropy method to predict 19 new putative binding sites for *M. tuberculosis* (Rv3676) CRP, in addition to the 73 sites previously predicted by Bai et al. (Akhter *et al*., 2008, Bai et al., 2005). These additional sites resulted from a difference where Akhter et al. used only the information available from the *M. tuberculosis* CRP-regulon instead of adding up the one available from the *E.coli* CRP-regulon. According to Akhter et al., the *M. tuberculosis* CRP-regulon comprises genes required for critical functions like: (i) cell-wall biogenesis, (ii) central metabolism pathways, (iii) putative regulatory elements controlling cAMP signaling, and (iv) antibiotic resistance (Akhter et al., 2008) (Table 2).

Experimental validation of these predictions has only been completed for Bai et al. model (Bai et al., 2005). Their research showed that mutation of nucleotides G2 or C15 from the palindromic binding motif, abolished CRP_{Mt}´s binding. Both positions are conserved in all predicted binding sites. They also evaluated CRP_{Mt} and CRP_{BCG} binding to seven putative CRP_{Mt} sites, located within intergenic regions, where 6 out of these 7 were found to be functional *in vitro* and *in vivo* (Bai et al., 2007). This increases confidence in the prediction algorithm that was used to identify CRP_{Mt} binding sites, although at this point it is possible to suggest that CRP_{Mt} regulon requires further refinement. On the other hand, it has been shown that *rpfA* is directly activated by CRP_{Mt} (Rickman et al., 2005). The resuscitation-promoting factor (Rpf) is a growth factor that stimulates the growth of aged *M. tuberculosis* cultures, and members of this family are thought to play a role in reactivation of dormant *M. tuberculosis* (Mukamolova *et al*., 2002). Regulation of *rpfA* by CRP_{Mt} suggests that CRP_{Mt} plays a role in persistence and/or reactivation of tuberculosis, but this is only one of many biological functions that may be regulated by CRP_{Mt} in *M. tuberculosis*.

Expression of *M. tuberculosis whiB1*, a member of the Wbl (WhiB-like) family, is controlled positively and negatively by CRP_{Mt} (Stapleton et al., 2010; Agarwal et al., 2006). A CRP_{Mt} binding site ($CRP_{Mt}1$) was detected in *whiB1*'s upstream regulatory region (Smith *et al*., 2010) (Rickman et al., 2005). Reporter assays with native and mutated promoter sequences indicated that transcription from the native, but not the mutant, promoter was affected by cAMP levels via the direct binding with CRP_{Mt} (Agarwal *et al*., 2006). Other evidences indicate that a second CRP_{Mt} binding site ($CRP_{Mt}2$) in the *whiB1* promoter altered CRP_{Mt}'s

Associated function	Gene	Predicted function
Cell-wall biogenesis	*Rv0993*	*galU*, uridine diphosphate-glucose pyrophosphorylase
	Rv3031	Members of the family of enzymes transferring activated sugars
	Rv3032	
	Rv0643c	*mma3*, methoxy mycolic acid synthase
	Rv0904c	AccD3, a putative acetyl CoA carboxylase carboxyl transferase, which catalyzes the initial steps of fatty acid and mycolic acid biosynthesis.
Central metabolism pathways	*Rv2918*	*glnD*, uridyl transferase
	Rv0992c	CHP with putative 5-formyltetrahydrofolate cyclo-ligase
	Rv0520	Methyl transferase believed to be involved in ubiquinone pathway
	Rv3113	Phosphatase
	Rv3114	Nucleoside deaminase
	Rv3505	FadE27, protein possibly involved in regulating probable acyl-CoA dehydrogenase
	Rv3617	EphA, putative epoxide hydrolase
Putative regulatory elements controlling cAMP signaling	*Rv0104*	Hypothetical protein probably implied in cAMP mediated signaling in *M. tuberculosis*
	Rv0103c	Probable cation transporter
	Rv3645	Membrane linked adenylyl cyclase
Antibiotic resistance	*Rv0906*	
	Rv0907	Hypothetical proteins belonging to the β-lactamase family
	Rv0905	*echA6*, enoyl-CoA hydratase
	Rv0908	CtpE, methyl-accepting chemotaxis protein

Table 2. *Mycobaterium* genes belonging to CRP regulon and their associated function

regulatory effect inhibiting *whiB1* expression (Stapleton et al., 2010). Interestingly, only $CRP1_{Mt}$ enhances *whiB1* expression, while CRP_{Mt} 2 alone represses *whiB1* expression. Dnase I footprinting assays allowed to determine the presence at −58.5 of an activating CRP_{Mt} binding site (CRP_{Mt} 1), which matches in seven of the eight nucleotides from the proposed CRP_{Mt} consensus (NGTGNNANNNNNCACA), and also it overlapped with the repressing CRP_{Mt} binding site (CRP_{Mt} 2) centred at −37.5. This second site has a poorer match to the consensus (six of the eight defined bases are matched)(Stapleton et al., 2010, Rickman et al., 2005). CRP_{Mt} 1 site was occupied before the CRP_{Mt} 2 site by a titration test done with increasing concentrations of CRP_{Mt}. It is worth noting that although it was shown that cAMP enhanced binding of recombinant CRP_{Mt} to target DNA, this enhancement was not equivalent to that observed for *E. coli* CRP, where DNA-binding affinity is enhanced by several orders of magnitude in the presence of 0.1 mM cAMP, allowing specific DNA binding at nanomolar concentrations. For CRP_{Mt}, a much less significant enhancement of DNA binding was observed, and higher concentrations of cAMP compared with *E.coli* CRP were required (Stapleton et al., 2010).

On the other hand, out of 92 genes found and predicted by bioinformatics to be regulated by CRP in *M. tuberculosis*, only 18 [including *echA6*, *ctpE*, *accD3*, *sucC*, *sucD*, *glnD, fadE9*, *fbpC1* (Ag85C) and *galU*] have conserved orthologous in *M. leprae*, *M. avium* subsp. *paratuberculosis*, and *M. smegmatis* (Akhter et al., 2008). Incorporation of a plasmid harboring and expressing *M. tuberculosis* Rv3676 in an *M. tuberculosis* strain in which *Rv3676* was absent, induced differential expression of 27 genes when compared to the same mutant harboring the BCG gene orthologous to Rv3676 (Hunt et al., 2008). This constitutes evidence that differences in gene regulatory sequences or the regulators among species exist, and it could explain the number of genes that do not respond in a similar manner. Further characterization of the *M. tuberculosis* CRP regulon as well as analysis of what physiological conditions regulate activation/inactivation are required.

3. Participation of cAMP in mycobacterial pathogenic processes

Little is known about the role of cAMP in mycobacteria, although it is found in both pathogenic and non-pathogenic species. Ingestion of live *M. microti* or *M. bovis* BCG (but not *M. lepraemurium*) increased macrophage intracellular cAMP levels, whereas no change occurred in cells engulfing dead bacilli, latex beads or colloidal gold (Lowrie *et al.*, 1979). The rise in cAMP levels appears directly related to mycobacterial capacity to interfere with phagolysosome formation, evidence suggesting that these microorganisms modify cAMP-dependent signalling pathways as a manner to control virulence and infection (Lowrie *et al.*, 1975, Lowrie et al., 1979). Elevated cAMP levels were correlated with reduced phagolysosome fusion during mycobacterial infection of macrophages (Kalamidas *et al.*, 2006). Increased cAMP levels inside phagocytes were shown to negatively modulate actin-dependent processes, including chemotactic movement and phagocytosis. Macrophage passage was found to have a stimulatory effect on cAMP production by mycobacteria, as cAMP levels within macrophage-passaged mycobacteria were ~50-fold higher than cAMP levels within bacteria incubated in the tissue culture medium alone (Bai et al., 2009).

Evidences exist that cAMP regulates gene expression in mycobacteria during bacterial growth *in vitro* (Stapleton et al., 2010, Dass et al., 2008), and during macrophage infection (Rickman et al., 2005) where some studies identified Cmr as a transcription factor that regulates cAIGs (cAMP-iduced genes) within macrophages, and suggest that multiple factors affect cAMP-associated gene regulation in tuberculosis-complex mycobacteria (Gazdik et al., 2009). Even during phagocytosis, expression may be down regulated in response to high cAMP or NO levels inside the macrophage environment, providing a mechanism to integrate the transcriptional response to two important signals associated with infection (Smith *et al.*, 2010). Generally, increases in cAMP levels compromise the bactericidal activity of the host immune system.

It is likely that each cyclase is associated with a distinct signaling pathway. It is expected that specific cyclases are activated to modify cAMP levels in response to different physiological conditions as for example hypoxia, intramacrophage enviroment or pH changes. cAMP receptor protein (CRP) Rv3676 was found to exist as dimmer and exhibited cAMP binding in a concentration-dependent manner and could bind specifically to the putative CRP/FNR nucleotide sequence elements in response to hypoxia (Akhter et al., 2008). The protein itself is composed of three distinct regions of the polypeptide: a large N-terminal domain that binds cAMP, a long α-helix (termed the C-helix) that mediates most of

Gene	Induction	Repression
Rv0386	Palmitic acid Tetracyclin	7H9 medium shaking Streptomyicin Purified surfactant lipids
Rv0891c	Oleic acid	DETA/NO
Rv1120c	Pulmonary surfactant	Non-replicative persistence
Rv1264	Carbonyl cyanide chlorophenylhydrazone	Palmitic acid DETA/NO
Rv1318c	Oligopeptide permease (Rv3662c-Rv3665c) mutant Palmitic acid	Pulmonary surfactant protein A (human) Acetate
Rv1319c	Hypoxia	Arachidonic acid Hydrogen peroxide Tioridazine Nicotinamide
Rv1320c	Acetate	
Rv1359	Econazole Macrophages Iron	Arachidonic acid Palmitic acid
Rv1625c	Clofazimine + S-nitrosoglutathione Arachidonic acid Thioridazine	Palmitic acid Oleic acid
Rv1647	Linoleic acid Palmitic acid Oleic acid	Streptomyicin Arachidonic acid DETA/NO
Rv1900c	Non-replicative persistence PA 824 Palmitic acid	Tetracycline S-nitrosogluthatione + Chlorpromazine Acetate
Rv2212	Tetracycline	5-chloro-pyrazinamide
Rv2345	Starvation	Thioridazine DETA/NO
Rv2488c	Arachidonic acid Acetate	Tetracycline Palmitic acid Hydrogen peroxide
Rv3645	Oleic acid	Oligopeptide permease (*Rv3662c-Rv3665c*) mutant Acetate Ceramide

Table 3. Growth conditions afecting expression of mycobacterial cyclase domain containing genes

the inter monomer interactions, and a small C-terminal DNA-binding domain. It is also capable of binding to two, three, or four cAMP molecules, but the specificity of recognition sequentially diminishes beyond two cAMP molecules bound to CRP, although the physiological importance of this molecular interactions are not yet known (Stapleton et al., 2010). Data available at Tuberculosis Database (http://www.tbdb.org/, Table 3) might help suggesting particular conditions where each AC gene may be required.

Currently, few studies have addressed the participation of cAMP signalling in *M. tuberculosis* pathogenesis *in vivo*. Rickman *et al.* (Rickman et al., 2005) found that a mutant in *Rv3676* (CRP) had diminished bacterial burden in lung and spleen after intravenous infection of BALB/c mice, compared to wild type bacteria. On the other hand, Agarwal *et al.* (Agarwal *et al.*, 2009) found that a *Rv0386 M. tuberculosis* mutant was affected in its capacity to replicate in BALB/c or C57BL/6 mice lungs, following aerosol infection. Neither publication mentioned the capacity of either strain to kill mice.

In addition to determining bacterial burden in infected lungs Agarwal et al. (Agarwal et al., 2009) demonstrated that cAMP produced by *M. tuberculosis* Rv0386 during J774.16 macrophage infection was a substrate for protein kinase A, in order to control the amount of phosphorylated CREB (CREB-P), by using specific chemical inhibitors of selected signalling transduction pathways. They found CREB-P induced TNF-α production, and led to an unregulated host inflammatory response, which favoured bacterial survival. To date, this is the first indication of how adenylyl cyclase action helps sustaining an infection by pathogenic mycobacteria.

4. References

Abdel Motaal, A., I. Tews, J. E. Schultz & J. U. Linder, (2006) Fatty acid regulation of adenylyl cyclase Rv2212 from Mycobacterium tuberculosis H37Rv. *FEBS J* 273: 4219-4228.

Agarwal, N., & W.R. Bishai, (2009) cAMP signaling in Mycobacterium tuberculosis. *Indian J Exp Biol* 47: 393-400.

Agarwal, N., G. Lamichhane, R. Gupta, S. Nolan & W. R. Bishai, (2009) Cyclic AMP intoxication of macrophages by a Mycobacterium tuberculosis adenylate cyclase. *Nature* 460: 98-102.

Agarwal, N., T. R. Raghunand & W. R. Bishai, (2006) Regulation of the expression of whiB1 in Mycobacterium tuberculosis: role of cAMP receptor protein. *Microbiology* 152: 2749-2756.

Akhter, Y., S. Yellaboina, A. Farhana, A. Ranjan, N. Ahmed & S. E. Hasnain, (2008) Genome scale portrait of cAMP-receptor protein (CRP) regulons in mycobacteria points to their role in pathogenesis. *Gene* 407: 148-158.

Bai, G., M. A. Gazdik, D. D. Schaak & K. A. McDonough, (2007) The Mycobacterium bovis BCG cyclic AMP receptor-like protein is a functional DNA binding protein in vitro and in vivo, but its activity differs from that of its M. tuberculosis ortholog, Rv3676. *Infect Immun* 75: 5509-5517.

Bai, G., G. S. Knapp & K. A. McDonough, (2011) Cyclic AMP signalling in mycobacteria: redirecting the conversation with a common currency. *Cell Microbiol* 13: 349-358.

Bai, G., L. A. McCue & K. A. McDonough, (2005) Characterization of Mycobacterium tuberculosis Rv3676 (CRPMt), a cyclic AMP receptor protein-like DNA binding protein. *J Bacteriol* 187: 7795-7804.

Bai, G., D. D. Schaak & K. A. McDonough, (2009) cAMP levels within Mycobacterium tuberculosis and Mycobacterium bovis BCG increase upon infection of macrophages. *FEMS Immunol Med Microbiol* 55: 68-73.

Barba, J., A. H. Alvarez & M. A. Flores-Valdez, (2010) Modulation of cAMP metabolism in Mycobacterium tuberculosis and its effect on host infection. *Tuberculosis (Edinb)* 90: 208-212.

Castro, L. I., C. Hermsen, J. E. Schultz & J. U. Linder, (2005) Adenylyl cyclase Rv0386 from Mycobacterium tuberculosis H37Rv uses a novel mode for substrate selection. *FEBS J* 272: 3085-3092.

Cha, P. H., S. Y. Park, M. W. Moon, B. Subhadra, T. K. Oh, E. Kim, J. F. Kim & J. K. Lee, (2010) Characterization of an adenylate cyclase gene (cyaB) deletion mutant of Corynebacterium glutamicum ATCC 13032 (vol 85, pg 1061, 2010). *Appl Microbiol Biot* 86: 395-395.

Dass, B. K., R. Sharma, A. R. Shenoy, R. Mattoo & S. S. Visweswariah, (2008) Cyclic AMP in mycobacteria: characterization and functional role of the Rv1647 ortholog in Mycobacterium smegmatis. *J Bacteriol* 190: 3824-3834.

Drum, C. L., S. Z. Yan, J. Bard, Y. Q. Shen, D. Lu, S. Soelaiman, Z. Grabarek, A. Bohm & W. J. Tang, (2002) Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. *Nature* 415: 396-402.

Espert, S. M., E. A. Elsinghorst & G. P. Munson, (2011) The tib adherence locus of enterotoxigenic Escherichia coli is regulated by cyclic AMP receptor protein. *J Bacteriol* 193: 1369-1376.

Findeisen, F., J. U. Linder, A. Schultz, J. E. Schultz, B. Brugger, F. Wieland, I. Sinning & I. Tews, (2007) The structure of the regulatory domain of the adenylyl cyclase Rv1264 from Mycobacterium tuberculosis with bound oleic acid. *J Mol Biol* 369: 1282-1295.

Gallagher, D. T., N. Smith, S. K. Kim, H. Robinson & P. T. Reddy, (2009) Profound asymmetry in the structure of the cAMP-free cAMP Receptor Protein (CRP) from Mycobacterium tuberculosis. *J Biol Chem* 284: 8228-8232.

Gazdik, M. A., G. Bai, Y. Wu & K. A. McDonough, (2009) Rv1675c (cmr) regulates intramacrophage and cyclic AMP-induced gene expression in Mycobacterium tuberculosis-complex mycobacteria. *Mol Microbiol* 71: 434-448.

Gazdik, M. A. & K. A. McDonough, (2005) Identification of cyclic AMP-regulated genes in Mycobacterium tuberculosis complex bacteria under low-oxygen conditions. *J Bacteriol* 187: 2681-2692.

Guo, Y. L., T. Seebacher, U. Kurz, J. U. Linder & J. E. Schultz, (2001) Adenylyl cyclase Rv1625c of Mycobacterium tuberculosis: a progenitor of mammalian adenylyl cyclases. *EMBO J* 20: 3667-3675.

Hollands, K., D. J. Lee, G. S. Lloyd & S. J. Busby, (2010) Activation of sigma 28-dependent transcription in Escherichia coli by the cyclic AMP receptor protein requires an unusual promoter organization. *Mol Microbiol* 75: 1098-1111.

Hunt, D. M., J. W. Saldanha, J. F. Brennan, P. Benjamin, M. Strom, J. A. Cole, C. L. Spreadbury & R. S. Buxton, (2008) Single nucleotide polymorphisms that cause structural changes in the cyclic AMP receptor protein transcriptional regulator of

the tuberculosis vaccine strain Mycobacterium bovis BCG alter global gene expression without attenuating growth. *Infect Immun* 76: 2227-2234.

Husnain, S. I., S. J. Busby & M. S. Thomas, (2009) Downregulation of the Escherichia coli guaB promoter by upstream-bound cyclic AMP receptor protein. *J Bacteriol* 191: 6094-6104.

Kalamidas, S. A., M. P. Kuehnel, P. Peyron, V. Rybin, S. Rauch, O. B. Kotoulas, M. Houslay, B. A. Hemmings, M. G. Gutierrez, E. Anes & G. Griffiths, (2006) cAMP synthesis and degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria. *J Cell Sci* 119: 3686-3694.

Klengel, T., W. J. Liang, J. Chaloupka, C. Ruoff, K. Schroppel, J. R. Naglik, S. E. Eckert, E. G. Mogensen, K. Haynes, M. F. Tuite, L. R. Levin, J. Buck & F. A. Muhlschlegel, (2005) Fungal adenylyl cyclase integrates CO2 sensing with cAMP signaling and virulence. *Curr Biol* 15: 2021-2026.

Lowrie, D. B., V. R. Aber & P. S. Jackett, (1979) Phagosome-lysosome fusion and cyclic adenosine 3':5'-monophosphate in macrophages infected with Mycobacterium microti, Mycobacterium bovis BCG or Mycobacterium lepraemurium. *J Gen Microbiol* 110: 431-441.

Lowrie, D. B., P. S. Jackett & N. A. Ratcliffe, (1975) Mycobacterium microti may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes. *Nature* 254: 600-602.

McCue, L. A., K. A. McDonough & C. E. Lawrence, (2000) Functional classification of cNMP-binding proteins and nucleotide cyclases with implications for novel regulatory pathways in Mycobacterium tuberculosis. *Genome Res* 10: 204-219.

Mock, M., M. Crasnier, E. Duflot, V. Dumay & A. Danchin, (1991) Structural and functional relationships between Pasteurella multocida and enterobacterial adenylate cyclases. *J Bacteriol* 173: 6265-6269.

Mukamolova, G. V., O. A. Turapov, D. I. Young, A. S. Kaprelyants, D. B. Kell & M. Young, (2002) A family of autocrine growth factors in Mycobacterium tuberculosis. *Mol Microbiol* 46: 623-635.

Nambi, S., N. Basu & S. S. Visweswariah, (2010) cAMP-regulated protein lysine acetylases in mycobacteria. *J Biol Chem* 285: 24313-24323.

Perrenoud, A. & U. Sauer, (2005) Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in Escherichia coli. *J Bacteriol* 187: 3171-3179.

Podobnik, M., R. Tyagi, N. Matange, U. Dermol, A. K. Gupta, R. Mattoo, K. Seshadri & S. S. Visweswariah, (2009) A mycobacterial cyclic AMP phosphodiesterase that moonlights as a modifier of cell wall permeability. *J Biol Chem* 284: 32846-32857.

Reddy, M. C., S. K. Palaninathan, J. B. Bruning, C. Thurman, D. Smith & J. C. Sacchettini, (2009) Structural insights into the mechanism of the allosteric transitions of the mycobacterium tuberculosis cAMP receptor protein. *J Biol Chem*.

Rickman, L., C. Scott, D. M. Hunt, T. Hutchinson, M. C. Menendez, R. Whalan, J. Hinds, M. J. Colston, J. Green & R. S. Buxton, (2005) A member of the cAMP receptor protein family of transcription regulators in Mycobacterium tuberculosis is required for virulence in mice and controls transcription of the rpfA gene coding for a resuscitation promoting factor. *Mol Microbiol* 56: 1274-1286.

Shenoy, A. R., M. Capuder, P. Draskovic, D. Lamba, S. S. Visweswariah & M. Podobnik, (2007) Structural and biochemical analysis of the Rv0805 cyclic nucleotide phosphodiesterase from Mycobacterium tuberculosis. *J Mol Biol* 365: 211-225.

Shenoy, A. R., K. Sivakumar, A. Krupa, N. Srinivasan & S. S. Visweswariah, (2004) A Survey of Nucleotide Cyclases in Actinobacteria: Unique Domain Organization and Expansion of the Class III Cyclase Family in Mycobacterium tuberculosis. *Comp Funct Genomics* 5: 17-38.

Shenoy, A. R. & S. S. Visweswariah, (2006a) Mycobacterial adenylyl cyclases: biochemical diversity and structural plasticity. *FEBS Lett* 580: 3344-3352.

Shenoy, A. R. & S. S. Visweswariah, (2006b) New messages from old messengers: cAMP and mycobacteria. *Trends Microbiol* 14: 543-550.

Shimada, T., N. Fujita, K. Yamamoto & A. Ishihama, (2011) Novel Roles of cAMP Receptor Protein (CRP) in Regulation of Transport and Metabolism of Carbon Sources. *PLoS ONE* 6: e20081.

Sismeiro, O., P. Trotot, F. Biville, C. Vivares & A. Danchin, (1998) Aeromonas hydrophila adenylyl cyclase 2: a new class of adenylyl cyclases with thermophilic properties and sequence similarities to proteins from hyperthermophilic archaebacteria. *J Bacteriol* 180: 3339-3344.

Smith, L. J., M. R. Stapleton, G. J. Fullstone, J. C. Crack, A. J. Thomson, N. E. Le Brun, D. M. Hunt, E. Harvey, S. Adinolfi, R. S. Buxton & J. Green, (2010) Mycobacterium tuberculosis WhiB1 is an essential DNA-binding protein with a nitric oxide-sensitive iron-sulfur cluster. *Biochem J* 432: 417-427.

Stapleton, M., I. Haq, D. M. Hunt, K. B. Arnvig, P. J. Artymiuk, R. S. Buxton & J. Green, (2009) Mycobacterium tuberculosis cAMP receptor protein (Rv3676) differs from the Escherichia coli paradigm in its cAMP-binding, DNA-binding and transcription activation properties. *J Biol Chem*.

Stapleton, M., I. Haq, D. M. Hunt, K. B. Arnvig, P. J. Artymiuk, R. S. Buxton & J. Green, (2010) Mycobacterium tuberculosis cAMP receptor protein (Rv3676) differs from the Escherichia coli paradigm in its cAMP binding and DNA binding properties and transcription activation properties. *J Biol Chem* 285: 7016-7027.

Stewart, V., P. J. Bledsoe, L. L. Chen & A. Cai, (2009) Catabolite repression control of napF (periplasmic nitrate reductase) operon expression in Escherichia coli K-12. *J Bacteriol* 191: 996-1005.

Sunahara, R. K., C. W. Dessauer & A. G. Gilman, (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol* 36: 461-480.

Wickstrum, J. R., J. M. Skredenske, V. Balasubramaniam, K. Jones & S. M. Egan, (2010) The AraC/XylS family activator RhaS negatively autoregulates rhaSR expression by preventing cyclic AMP receptor protein activation. *J Bacteriol* 192: 225-232.

Xu, H., S. S. Hegde & J. S. Blanchard, (2011) Reversible Acetylation and Inactivation of Mycobacterium tuberculosis Acetyl-CoA Synthetase Is Dependent on cAMP. *Biochemistry* 50: 5883-5892.

6

Mycobacterium tuberculosis RD-1 Secreted Antigens as Protective and Risk Factors for Tuberculosis

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1. Introduction

Mycobacterium tuberculosis (Mtb) infects about 8 million people every year and causes death of about 2-3 million (Raviglione, 2003). In recent times, there has been a wider spread of tuberculosis, mainly due to emergence of multi drug resistance (MDR) bacilli and enhanced susceptibility to the disease by patients infected with human immunodeficiency virus (HIV) (Elliot et al., 1995; Chintu and Mwinga, 1999). Transmission of the infection by Mtb bacilli is air borne and occurs through inhalation of aerosol containing the bacilli exhaled by coughing, sneezing or spitting by patients suffering from pulmonary tuberculosis. The inhaled bacilli are engulfed by the alveolar macrophages, where the bacilli are able to persist successfully in a latent or proliferating state. This persistence is achieved by modulation of several intracellular signaling pathways in order to create a suitable environment for the bacilli. The interplay of mycobacteria with host signaling pathways is a complex and dynamic process that is not clearly understood. Mtb secretes several molecules that modulate the signaling pathways (Koul et al., 2004). Most of these molecules commonly target macrophages, which helps the bacilli to evade innate immune response and propagate throughout the system (Rosenberger and Finlay, 2003).

The proteins secreted by Mtb have gained attention in recent years as putative vaccine and diagnostic candidates (Harboe et al., 1996; Colangeli et al., 2000). But there have been recent reports about their role in modulation of macrophage signaling pathways leading to compromise of macrophage functions (Trajkovic et al., 2002; Pym et al., 2003; Guinn et al., 2004). Thus the secretory proteins can act as risk or virulent factors too. This notion is also supported by the fact that only live but not dead bacilli can down regulate macrophage functions (Malik et al., 2001). In this chapter, we focus our discussion on the role of the

proteins secreted by region of difference-1 (RD-1), the region that is deleted in all the strains of *M. bovis* BCG.

2. Genetic architecture of Region of Difference-1 (RD-1)

Comparative genome analysis using DNA microarray, bacterial artificial chromosomes (BAC) and the subtractive hybridization between virulent and attenuated strains of Mtb complex and *M. bovis* BCG identified several regions of difference (RD) (Behr et al., 1999; Gordon et al., 1999; Mahairas et al., 1996). A gene segment of 9.5kb that encompasses nine open reading frames (ORF) of Rv3871-Rv3879c is present in virulent strains of Mtb, and which is deleted consistently in all the strains of *M. bovis* BCG (Cole et al., 1998). This region was designated as RD-1. Two of these ORFs, Rv3874 and Rv3875, encode 10-kDa culture filtrate protein (CFP-10) and 6-kDa early secreted antigenic target (ESAT-6) protein respectively. Interestingly, deletion of the RD-1 fragments from Mtb causes loss of its virulence, while introduction of the RD-1 locus into *M. bovis* BCG or *M. microti* resulted in increased virulence and survival properties (Behr, 2002; Pym et al., 2002; Lewis et al., 2003; Demangel et al., 2005). This review will focus on the role of these two proteins in modulation of the macrophage signaling pathways and macrophage functions for the bacteria to persist for longer time. We also discuss about the potential role of these proteins as vaccine candidates owing to their high immunogenicity.

3. Structural biology of CFP-10 and ESAT-6

The ORFs Rv3874 and Rv3875 encoding CFP10 and ESAT6 respectively are cotranscribed into a single RNA product (Cole et al., 1998). Nuclear magnetic resonance (NMR) spectroscopy showed that CFP10 exhibits very little secondary structure and consists mostly of random coils, which are unstructured. The ESAT6, on the other hand has 75% secondary structure in the form of α-helices (Renshaw et al., 2002). One interesting study showed that CFP10 and ESAT6 forms a tight 1:1 complex where both proteins adopt more stable and folded configuration than the native moieties (Renshaw et al., 2002; Lightbody et al., 2004). The complex formation between the two proteins is hydrophobic in nature and led to a significant increase in the helical content of the two proteins. Inside the core of the complex, helix-turn-helix motifs of the two proteins form a quad-helix bundle (Renshaw et al., 2005). Within the complex, the flexible C-terminus of CFP-10 is involved in binding to the cell surface. This was confirmed by the fact that deletion 87 amino acids at C-terminus of CFP-10 inhibited the binding of complex to the cell surface, while deletion of the same in ESAT-6 had no effect (Renshaw et al., 2005). The complex formation between the two proteins was reversible and the complex broke down to individual proteins at 53.4^0C. The complex was also shown to be more stable to proteolytic digestion by trypsin (Meher at al., 2006). The enhanced stability to proteolytic digestion caused lower T cell activation compared to ESAT-6 alone (Marei et al., 2005). ESAT-6 protein was also found to have auto-proteolytic activity; it can self cleave-off six amino acids at the C-terminus which are responsible for its binding to the cell surface. A mutant ESAT-6 lacking these six amino acids was unable to bind to cell surface (Pathak et al., 2007).

4. Use of CFP10 and ESAT6 as tools for diagnosis and vaccine

CFP-10 and ESAT-6 were identified in a screen to identify the proteins present in culture filtrates of Mtb and *M. bovis BCG*, which could induce T cell mediated response. (Andersen et al., 1991a; Andersen et al., 1991b; Andersen et al., 1994; Weldingh et al., 1998; Weldingh et al., 1999). The screen yielded six low molecular weight antigens *viz*. Rv3871, Rv3872, Rv3873, CFP-10, ESAT-6 and Rv3878. These antigens when expressed and purified as recombinant proteins gave strong humoral response in tuberculous guinea pigs while only two antigens i.e. CFP-10 and ESAT-6 showed strong delayed type hypersensitivity (DTH) reaction in the guinea pigs (Weldingh et al., 1999). CFP10 and ESAT6 are potent T cell antigens and induce strong T cell response. In mice infected with Mtb, CFP-10 specific T cells were observed at quite early stage of infection in lungs. These T cells were activated by CFP-10 epitopes and were recruited in large numbers (Kamath et al., 2004). This resulted in production of large amounts of interferon-γ (IFN-γ). Recombinant CFP-10 has also been shown to be a potent T cell antigen, inducing T cell proliferation and IFN-γ production in peripheral blood mononuclear cells in about 70% of purified protein derivative (PPD) positive asymptomatic individuals. CFP-10 was also shown to induce delayed type hypersensitivity (DTH) in Mtb infected guinea pigs but not in *M. bovis* BCG infected guinea pigs (Colangeli et al., 2000). ESAT-6 is also a RD-1 antigen inducing robust levels of IFN-γ by T cells in early stages of *M. tuberculosis* infection (Porsa et al., 2006; Ravn et al., 1999; Skjot et al., 2000; de Jong et al., 2006). Two different T cell epitopes were observed in mice, which were recognized by different MHCII molecules under different circumstances (Dietrich et al., 2005).

5. Macrophage subversion by CFP10 and ESAT6

Despite their well-known role as T cell antigens, CFP10 and ESAT6 modulate several pathways inside the macrophage, thereby creating a suitable environment to persist inside the host cell. Studies from our lab have shown that CFP-10 and ESAT-6 downregulates the production of reactive oxygen species (ROS) inside the macrophages; which in turn dampens the NF-κB transactivation property (Ganguly et al., 2008a, Ganguly et al., 2008b). The inhibition of ROS production was greater with the CFP10:ESAT6 complex compared to the individual proteins. Most of the effects of these proteins seem to be mediated by Toll-like receptors (TLR). Analysis of global phospho-proteome in CFP-10 treated J774.1 macrophages showed that CFP-10 caused de-phosphorylation of a large number of macrophage proteins (Basu et al., 2006; Basu et al., 2009). The de-phsophorylation occurs due to increase in activity of membrane tyrosine phosphatases SHP-1 and SHP-2 (Src homology domain proteins). The increased phosphatase activity is due to reduction in production of ROS inside the macrophages. The ROS production in macrophages occurs through NADPH oxidase pathway. These observations suggest that upon binding of CFP-10 and ESAT-6 to macrophage surface, Mtb is able to reduce the burst of ROS inside the cell which contributes to bactericidal activity. Thus it might be one of the survival strategies of the bacilli. Mtb contains several enzymes to deal with the ROS/oxidative burst like catalase, peroxidase (Kat) (Sherman et

al., 1995; Manca et al., 1999; Ng et al., 2004) as well as superoxide dismutases Sod A and Sod C (Piddington et al., 2001; Zhang et al., 1991). ESAT-6 was also found to inhibit mitogen activated kinase/extracellular signal regulated kinases 1/2 (MAPK/ERK1/2). This occurs due to some phosphatase activity in the nucleus which dephosphorylates ERK1/2. This resulted in reduction in lipopolysaccharide (LPS) induced expression of transcription factor c-myc (Ganguly et al., 2007). ESAT-6 also reduced the LPS-induced expression of several genes like *IL-1β*, *Bax*, *Icam-1* and *tnfr-1*. Recent studies have shown that ESAT-6 binds to toll-like receptor-2 (TLR2) on the macrophage surface; and the six amino acids at the C-terminus of the protein are critical for its TLR2 binding (Pathak et al., 2007). This binding caused inactivation of transcription factors interferon regulatory factors (IRF) and NF-κB. Recent observations from our lab have shown that ESAT-6 down-regulates IFN-γ inducible expression of type I and type IV isomers of MHC class II transactivator (CIITA) in macrophages (Kumar et al., 2011). Interestingly, the downregulation of type I CIITA was independent of TLR-2 while the effect on type IV CIITA was mediated through TLR-2. This suggests that ESAT-6 may bind to other TLRs or some other receptor on macrophages. Another study has shown that ESAT-6 was able to induce apoptosis in human monocytic cell line THP-1 through activation of caspases (Derrick et al., 2007). It was also shown that ESAT-6 could induce pore formation on the surface of some cell types.

Apart from macrophages, CFP-10 and ESAT-6 also modulate functions in dendritic cells (Natarajan et al., 2011; Trajkovic et al., 2004). Studies have shown that CFP-10 induced differentiation of bone marrow cells into dendritic cells (DC) and this involved activation of NF-κB (Latchumanan et al., 2002). CFP-10 also induced maturation of DCs, which caused downregulation of pro-inflammatory cytokines like interleukin-2 (IL-2) and IFN-γ (Natarajan et al., 2003). The CFP-10-differentiated and CFP-10-matured DCs when cultured with the Mtb whole-cell-extract primed T cells, showed reduced levels of pro-inflammatory cytokines IL-12p40 and IFN-γ along with elevated levels of anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β) (Balkhi et al., 2004). Therefore CFP-10 primes DCs to have reduced efficacy to eliminate Mtb. CFP-10 also reduced ROS production during differentiation of DCs compared to the positive stimulator granulocyte macrophage colony-an stimulating factor (GM-CSF). This downregulation of ROS resulted in increased survival of *M. bovis* BCG in these DCs (Sinha et al., 2006). The CFP-10 differentiated DCs also had reduced levels of chemokines RANTES and IP-10 upon infection by mycobacteria (Salam et al., 2008).

6. Virulence linked to CFP10/ESAT6 secretion

CFP-10 and ESAT-6 proteins are secreted without any secretory signal sequence outside the host cell. The secretory apparatus that helps in the secretion of these two proteins have been characterized recently (Guinn et al., 2004; Fortune et al., 2005; Brodin et al., 2006). The CFP10/ESAT6 secretion system is an active process involving several genes, some of them being ATP hydrolyzing chaperones (Stanley et al., 2003; Converse and Cox, 2005; Stanley et al., 2007). Mutations in these genes caused significant reduction in virulence of Mtb in mouse model (Stanley et al., 2003; Majlessi et al., 2005). This

attenuation could be due to the inhibition of CFP10/ESAT6 secretion, which further highlights the role of these two proteins in modulation of macrophage function. A study has shown that one of the components of ESX-1 secretion system, Rv3871 binds to the C-terminus of CFP-10, and this facilitates the secretion of both CFP-10 and ESAT-6. Mutations at the C-terminus resulted in loss of binding to CFP-10 and impaired secretion of the two proteins (Champion et al., 2006). This suggests that CFP-10 and ESAT-6 might be secreted in the form of heterodimeric 1:1 complex out of the cell. The ESX-1 system has four paralogues in Mtb and some of them have been shown to be essential for in-vitro growth of the bacilli (Simeone et al., 2009). In the ESX-1 system, Rv3868, Rv3869, Rv3870, Rv3871 and Rv3877 have been shown to be essential for CFP-10/ESAT-6 secretion while loss of Rv3865 and partial loss of Rv3866 did not affect protein secretion, rather it caused attenuation of the bacilli (Brodin et al., 2006). Thus Rv3865/3866 might be some virulence factor that does not control ESAT-6 secretion. Studies with mutant bacilli showed that ESX-1 system is required for the induction of type I IFN induction that in turn contributes to the spread of the bacilli (Stanley et al., 2007). In *Mycobacterium marinum*, the CFP-10/ESAT-6 secretion manipulates the phagosome-lysosome fusion. Mutations in this secretion system results in enhanced phagosome-lysosome fusion and reduced survival of mycobacteria (Tan et al., 2006; Majlessi et al., 2005; Champion et al., 2006; Xu et al., 2007; Lee et al., 2001). Analyses of deletion mutants of ESAT-6 have identified the key amino acids in complex formation, virulence and secretion (Brodin et al., 2005). The Trp-X-Gly motif on ESAT-6 is involved in complex formation with CFP-10, virulence and induction of specific T cell responses whereas mutations in the six amino acids at the C-terminus had no effect on secretion but caused attenuation. At acidic pH (as normally found in phagosomes), ESAT-6 dissociated from its complexing partner CFP-10 and bound to liposomes, which caused lysis of the liposomes (de Jonge et al., 2007). This could be a mechanism for the Mtb to escape degradation within the phagosome. In dendritic cells, Mtb translocated from phagolysosome to cytoplasm, which is dependent upon the CFP-10/ESAT-6 secretion (van der Wel et al., 2007). This translocation resulted in the death of the host cell.

7. Conclusions

The interaction between mycobacteria and the host macrophage or dendritic cells is very complex and dependent on multiple factors. In this review, we have focused mainly on the modulating activities of CFP10/ESAT6, the molecules which are being evaluated as vaccine candidates, indicating that they may act like double-edged sword generating a favorable response from the host immune system. Apart from CFP-10/ESAT-6, several other protein antigens are also being reported which modulate the macrophage response to Mtb. Further studies are undergoing in our lab to elucidate the finer mechanisms by which these proteins function.

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9. References

Andersen, P. (1994). Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infection and Immunity 62, 2536-2544.

Andersen, P., Askgaard, D., Ljungqvist, L., Bennedsen, J. & Heron, I. (1991a). Proteins released from *Mycobacterium tuberculosis* during growth. Infection and Immunity 59, 1905-1910.

Andersen, P., Askgaard, D., Ljungqvist, L., Bentzon, M.W. & Heron, I. (1991b). T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. Infection and Immunity 59, 1558-1563.

Balkhi, M.Y., Sinha, A. & Natarajan, K. (2004). Dominance of CD86, transforming growth factor- beta 1, and interleukin-10 in *Mycobacterium tuberculosis* secretory antigen-activated dendritic cells regulates T helper 1 responses to mycobacterial antigens. The Journal of Infectious Diseases 189, 1598-1609.

Basu, S.K., Kumar, D., Singh, D.K., Ganguly, N., Siddiqui, Z., Rao, K.V. & Sharma, P. (2006). *Mycobacterium tuberculosis* secreted antigen (MTSA-10) modulates macrophage function by redox regulation of phosphatases. The FEBS Journal 273, 5517-5534.

Behr, M.A. (2002). BCG--different strains, different vaccines? The Lancet Infectious Diseases 2, 86-92.

Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S. & Small, P.M. (1999). Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284, 1520-1523.

Berthet, F.X., Rasmussen, P.B., Rosenkrands, I., Andersen, P. & Gicquel, B. (1998). A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 144 (Pt 11), 3195-3203.

Brodin, P., de Jonge, M.I., Majlessi, L., Leclerc, C., Nilges, M., Cole, S.T. & Brosch, R. (2005). Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis*, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. The Journal of Biological Chemistry 280, 33953-33959.

Brodin, P., Majlessi, L., Marsollier, L., de Jonge, M.I., Bottai, D., Demangel, C., Hinds, J., Neyrolles, O., Butcher, P.D., Leclerc, C., Cole, S.T. & Brosch, R. (2006). Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. Infection and Immunity 74, 88-98.

Brodin, P., Rosenkrands, I., Andersen, P., Cole, S.T. & Brosch, R. (2004). ESAT-6 proteins: protective antigens and virulence factors? Trends in Microbiology 12, 500-508.

Champion, P.A., Stanley, S.A., Champion, M.M., Brown, E.J. & Cox, J.S. (2006). C-terminal signal sequence promotes virulence factor secretion in *Mycobacterium tuberculosis*. Science 313, 1632-1636.

Chintu, C. & Mwinga, A. (1999). An African perspective on the threat of tuberculosis and HIV/AIDS--can despair be turned to hope? Lancet 353, 997.

Colangeli, R., Spencer, J.S., Bifani, P., Williams, A., Lyashchenko, K., Keen, M.A., Hill, P.J., Belisle, J. & Gennaro, M.L. (2000). MTSA-10, the product of the Rv3874 gene of

Mycobacterium tuberculosis, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. Infection and Immunity 68, 990-993.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 537-544.

Converse, S.E. & Cox, J.S. (2005). A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*. Journal of Bacteriology 187, 1238-1245.

de Jong, B.C., Hill, P.C., Brookes, R.H., Gagneux, S., Jeffries, D.J., Otu, J.K., Donkor, S.A., Fox, A., McAdam, K.P., Small, P.M. & Adegbola, R.A. (2006). *Mycobacterium africanum* elicits an attenuated T cell response to early secreted antigenic target, 6 kDa, in patients with tuberculosis and their household contacts. The Journal of Infectious Diseases 193, 1279-1286.

de Jonge, M.I., Pehau-Arnaudet, G., Fretz, M.M., Romain, F., Bottai, D., Brodin, P., Honore, N., Marchal, G., Jiskoot, W., England, P., Cole, S.T. & Brosch, R. (2007). ESAT-6 from *Mycobacterium tuberculosis* dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. Journal of Bacteriology 189, 6028-6034.

Demangel, C., Garnier, T., Rosenkrands, I. & Cole, S.T. (2005). Differential effects of prior exposure to environmental mycobacteria on vaccination with *Mycobacterium bovis* BCG or a recombinant BCG strain expressing RD1 antigens. Infection and Immunity 73, 2190-2196.

Derrick, S.C. & Morris, S.L. (2007). The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression. Cellular Microbiology 9, 1547-1555.

Dietrich, J., Aagaard, C., Leah, R., Olsen, A.W., Stryhn, A., Doherty, T.M. & Andersen, P. (2005). Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. Journal of Immunology 174, 6332-6339.

Elliot, A.M., Halwindii, B., Hayes, R., Luo, N., Mwinga, A.G., Tembo, G., Machiels, L., Steenbergen, G., Pobee, J.O., Nunn, P.P., et al. (1995). The impact of human immunodeficiency virus on response to treatment and recurrence rate in patients treated for tuberculosis: two-year follow-up of a cohort in Lusaka, Zambia. J Trop Med Hyg. 98, 9-21.

Fortune, S.M., Jaeger, A., Sarracino, D.A., Chase, M.R., Sassetti, C.M.,Sherman, D.R., Bloom, B.R. & Rubin, E.J. (2005). Mutually dependent secretion of proteins required for mycobacterial virulence. Proceedings of the National Academy of Sciences of the United States of America 102, 10676-10681.

Ganguly, N., Giang, P.H., Basu, S.K., Mir, F.A., Siddiqui, I. & Sharma, P. (2007). *Mycobacterium tuberculosis* 6-kDa early secreted antigenic target (ESAT-6) protein downregulates lipopolysaccharide induced c-myc expression by modulating the extracellular signal regulated kinases 1/2. BMC Immunology 8, 24.

Ganguly, N., Giang, P.H., Gupta, C., Basu, S.K., Siddiqui, I., Salunke, D.M. & Sharma, P. (2008a). *Mycobacterium tuberculosis* secretory proteins CFP-10, ESAT-6 and the CFP10:ESAT6 complex inhibit lipopolysaccharide-induced NF-kappaB transactivation by downregulation of reactive oxidative species (ROS) production. Immunology and Cell Biology 86, 98-106.

Ganguly, N., Siddiqui, I. & Sharma, P. (2008b). Role of *M. tuberculosis* RD-1 region encoded secretory proteins in protective response and virulence. Tuberculosis 88, 510-517.

Gordon, S.V., Brosch, R., Billault, A., Garnier, T., Eiglmeier, K. & Cole, S.T. (1999). Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Molecular Microbiology 32, 643-655.

Guinn, K.M., Hickey, M.J., Mathur, S.K., Zakel, K.L., Grotzke, J.E., Lewinsohn, D.M., Smith, S. & Sherman, D.R. (2004). Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. Molecular Microbiology 51, 359-370.

Harboe, M., Oettinger, T., Wiker, H.G., Rosenkrands, I. & Andersen, P. (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infection and Immunity 64, 16-22.

Kamath, A.B., Woodworth, J., Xiong, X., Taylor, C., Weng, Y. & Behar, S.M. (2004). Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after *Mycobacterium tuberculosis* infection. The Journal of Experimental Medicine 200, 1479-1489.

Khan, I.H., Ravindran, R., Yee, J., Ziman, M., Lewinsohn, D.M., Gennaro, M.L., Flynn, J.L., Goulding, C.W., DeRiemer, K., Lerche, N.W. & Luciw, P.A. (2008). Profiling antibodies to *Mycobacterium tuberculosis* by multiplex microbead suspension arrays for serodiagnosis of tuberculosis. Clinical and Vaccine Immunology 15, 433-438.

Koul, A., Herget, T., Klebl, B.& Ullrich, A. (2004). Interplay between mycobacteria and host signalling pathways. Nature reviews. Microbiology 2, 189-202.

Kumar, P., Agarwal, R., Siddiqui, I., Vora, H., Das, G. & Sharma, P. (2011). ESAT6 differentially inhibits IFN-gamma-inducible class II transactivator isoforms in both a TLR2-dependent and -independent manner. Immunology and Cell Biology advance online publication 14 June 2011; doi: 10.1038/icb.2011.54

Latchumanan, V.K., Balkhi, M.Y., Sinha, A., Singh, B., Sharma, P. & Natarajan, K. (2005). Regulation of immune responses to *Mycobacterium tuberculosis* secretory antigens by dendritic cells. Tuberculosis 85, 377-383.

Latchumanan, V.K., Singh, B., Sharma, P. & Natarajan, K. (2002). *Mycobacterium tuberculosis* antigens induce the differentiation of dendritic cells from bone marrow. Journal of Immunology 169, 6856-6864.

Lee, V.T. & Schneewind, O. (2001). Protein secretion and the pathogenesis of bacterial infections. Genes & Development 15, 1725-1752.

Lewis, K.N., Liao, R., Guinn, K.M., Hickey, M.J., Smith, S., Behr, M.A. & Sherman, D.R. (2003). Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. The Journal of Infectious Diseases 187, 117-123.

Lightbody, K.L., Renshaw, P.S., Collins, M.L., Wright, R.L., Hunt, D.M., Gordon, S.V., Hewinson, R.G., Buxton, R.S., Williamson, R.A. & Carr, M.D. (2004). Characterisation of complex formation between members of the *Mycobacterium tuberculosis* complex CFP-10/ESAT-6 protein family: towards an understanding of the rules governing complex formation and thereby functional flexibility. FEMS Microbiology Letters 238, 255-262.

Mahairas, G.G., Sabo, P.J., Hickey, M.J., Singh, D.C. & Stover, C.K. (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent M. bovis. The Journal of Bacteriology 178, 1274-1282.

Majlessi, L., Brodin, P., Brosch, R., Rojas, M.J., Khun, H., Huerre, M., Cole, S.T. & Leclerc, C. (2005). Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. Journal of Immunology 174, 3570-3579.

Malik, Z.A., Iyer, S.S. & Kusner, D.J. (2001). *Mycobacterium tuberculosis* phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. Journal of Immunology 166, 3392-3401.

Manca, C., Paul, S., Barry, C.E., 3rd, Freedman, V.H.& Kaplan, G. (1999). *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro. Infection and Immunity 67, 74-79.

Marei, A., Ghaemmaghami, A., Renshaw, P., Wiselka, M., Barer, M., Carr, M. & Ziegler-Heitbrock, L. (2005). Superior T cell activation by ESAT-6 as compared with the ESAT-6-CFP-10 complex. International Immunology 17, 1439-1446.

Meher, A.K., Bal, N.C., Chary, K.V. & Arora, A. (2006). *Mycobacterium tuberculosis* H37Rv ESAT-6-CFP-10 complex formation confers thermodynamic and biochemical stability. The FEBS Journal 273, 1445-1462.

Natarajan, K., Kundu, M., Sharma, P. & Basu, J. (2011). Innate immune responses to *M. tuberculosis* infection. Tuberculosis (2011), doi:10.1016/j.tube.2011.04.003.

Natarajan, K., Latchumanan, V.K., Singh, B., Singh, S. & Sharma, P. (2003). Down-regulation of T helper 1 responses to mycobacterial antigens due to maturation of dendritic cells by 10-kDa mycobacterium tuberculosis secretory antigen. The Journal of Infectious Diseases 187, 914-928.

Ng, V.H., Cox, J.S., Sousa, A.O., MacMicking, J.D. & McKinney, J.D. (2004). Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Molecular Microbiology 52, 1291-1302.

Pathak, S.K., Basu, S., Basu, K.K., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T., Kundu, M. & Basu, J. (2007). Direct extracellular interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2 inhibits TLR signaling in macrophages. Nature Immunology 8, 610-618.

Piddington, D.L., Fang, F.C., Laessig, T., Cooper, A.M., Orme, I.M. & Buchmeier, N.A. (2001). Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infection and Immunity 69, 4980-4987.

Porsa, E., Cheng, L., Seale, M.M., Delclos, G.L., Ma, X., Reich, R., Musser, J.M. & Graviss, E.A. (2006). Comparison of a new ESAT-6/CFP-10 peptide-based gamma interferon assay and a tuberculin skin test for tuberculosis screening in a moderate-risk population. Clinical and Vaccine Immunology 13, 53-58.

Pym, A.S., Brodin, P., Brosch, R., Huerre, M. & Cole, S.T. (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. Molecular Microbiology 46, 709-717.

Pym, A.S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K.E., Marchal, G., Leclerc, C. & Cole, S.T. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nature Medicine 9, 533-539.

Raviglione, M.C. (2003). The TB epidemic from 1992 to 2002. Tuberculosis 83, 4-14.

Ravn, P., Demissie, A., Eguale, T., Wondwosson, H., Lein, D., Amoudy, H.A., Mustafa, A.S., Jensen, A.K., Holm, A., Rosenkrands, I., Oftung, F., Olobo, J., von Reyn, F. & Andersen, P. (1999). Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. The Journal of Infectious Diseases 179, 637-645.

Renshaw, P.S., Lightbody, K.L., Veverka, V., Muskett, F.W., Kelly, G., Frenkiel, T.A., Gordon, S.V., Hewinson, R.G., Burke, B., Norman, J., Williamson, R.A. & Carr, M.D. (2005). Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. The EMBO Journal 24, 2491-2498.

Renshaw, P.S., Panagiotidou, P., Whelan, A., Gordon, S.V., Hewinson, R.G., Williamson, R.A. & Carr, M.D. (2002). Conclusive evidence that the major T-cell antigens of the *Mycobacterium tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6*CFP-10 complex. Implications for pathogenesis and virulence. The Journal of Biological Chemistry 277, 21598-21603.

Rosenberger, C.M. & Finlay, B.B. (2003). Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Nature reviews. Molecular Cell Biology 4, 385-396.

Salam, N., Gupta, S., Sharma, S., Pahujani, S., Sinha, A., Saxena, R.K. & Natarajan, K. (2008). Protective immunity to *Mycobacterium tuberculosis* infection by chemokine and cytokine conditioned CFP-10 differentiated dendritic cells. PloS One 3, e2869.

Sherman, D.R., Sabo, P.J., Hickey, M.J., Arain, T.M., Mahairas, G.G., Yuan, Y., Barry, C.E., 3rd, & Stover, C.K. (1995). Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proceedings of the National Academy of Sciences of the United States of America 92, 6625-6629.

Simeone, R., Bottai, D. & Brosch, R. (2009). ESX/type VII secretion systems and their role in host-pathogen interaction. Current Opinion in Microbiology 12, 4-10.

Singh, A., Mai, D., Kumar, A. & Steyn, A.J. (2006). Dissecting virulence pathways of *Mycobacterium tuberculosis* through protein-protein association. Proceedings of the National Academy of Sciences of the United States of America 103, 11346-11351.

Sinha, A., Singh, A., Satchidanandam, V. & Natarajan, K. (2006). Impaired generation of reactive oxygen species during differentiation of dendritic cells (DCs) by *Mycobacterium tuberculosis* secretory antigen (MTSA) and subsequent activation of MTSA-DCs by mycobacteria results in increased intracellular survival. Journal of Immunology 177, 468-478.

Skjot, R.L., Oettinger, T., Rosenkrands, I., Ravn, P., Brock, I., Jacobsen, S. & Andersen, P. (2000). Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. Infection and Immunity 68, 214-220.

Stanley, S.A., Johndrow, J.E., Manzanillo, P. & Cox, J.S. (2007). The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. Journal of Immunology 178, 3143-3152.

Stanley, S.A., Raghavan, S., Hwang, W.W. & Cox, J.S. (2003). Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. Proceedings of the National Academy of Sciences of the United States of America 100, 13001-13006.

Tan, T., Lee, W.L., Alexander, D.C., Grinstein, S. & Liu, J. (2006). The ESAT-6/CFP-10 secretion system of *Mycobacterium marinum* modulates phagosome maturation. Cellular Microbiology 8, 1417-1429.

Trajkovic, V., Natarajan, K. & Sharma, P. (2004). Immunomodulatory action of mycobacterial secretory proteins. Microbes and Infection 6, 513-519.

Trajkovic, V., Singh, G., Singh, B., Singh, S., and Sharma, P. (2002). Effect of Mycobacterium tuberculosis-specific 10-kiloDaltn antigen on macrophage release of tumour necrosis factor-alpha and nitric oxide. Infection & Immunity 70, 6558-6566.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M. & Peters, P.J. (2007). *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 129, 1287-1298.

Weldingh, K. & Andersen, P. (1999). Immunological evaluation of novel*Mycobacterium tuberculosis* culture filtrate proteins. FEMS Immunology and Medical Microbiology 23, 159-164.

Weldingh, K., Rosenkrands, I., Jacobsen, S., Rasmussen, P.B., Elhay, M.J. & Andersen, P. (1998). Two-dimensional electrophoresis for analysis of *Mycobacterium tuberculosis* culture filtrate and purification and characterization of six novel proteins. Infection and Immunity 66, 3492-3500.

Xu, J., Laine, O., Masciocchi, M., Manoranjan, J., Smith, J., Du, S.J., Edwards, N., Zhu, X., Fenselau, C. & Gao, L.Y. (2007). A unique Mycobacterium ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. Molecular Microbiology 66, 787-800.

Zhang, Y., Lathigra, R., Garbe, T., Catty, D. & Young, D. (1991). Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis.* Molecular Microbiology 5, 381-391.

7

Mycobacterium tuberculosis Transcriptome *In Vivo* Studies – A Key to Understand the Pathogen Adaptation Mechanism

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1. Introduction

The infectious diseases caused by intracellular bacterial pathogens, such as *M. tuberculosis*, are among the most important problems in public health worldwide. The development of an infectious process depends on intricate interactions between the host defence systems and the specific systems regulating mycobacterial gene expression. Changes in expression in response to host defence are a necessary condition for the *M. tuberculosis* survival and functioning. Tracking these changes makes possible to analyze the biochemical cascades that are triggered in response to host defence mechanisms and to find the targets for designing new therapeutics and monitoring bacterial infections; in addition, these results are useful for both theoretical (for example, dynamics of the pathogen transcriptome changes during long-term persistence in the host) and applied (for example, the study of the bacterial response to various therapeutic interventions) research.

The completion of the *M. tuberculosis* genome sequence in 1998 (Cole et al., 1998) marked the beginning of the so called post genome era, the main characteristic of which are large scale studies of genome functional activity. The information on the bacterial genome organization allowed to construct macro- and microarrays containing fragments of a majority of ORFs, which enabled analysis of the pathogen transcription profile variations under different conditions. It's no wonder that the first study of the *M. tuberculosis* transcriptome using microarray technology was carried out in the first year after the publication of the genome sequence (Wilson et al., 1999). In as little as 5 years, there have been published many reports with the results of using microarrays for *in vitro* mycobacterial transcriptome analysis (for review, see (Butcher, 2004; Kendall et al., 2004)).

However, the *in vivo* analysis of mycobacterial gene expression during infection process, which is of special scientific interest, is rather complicated, and this can explain a relatively small number of such works. Experiments with analysis of a pathogen transcriptome *in vivo* are determined by the choice of: (1) an experimental model of infection; (2) a method of pathogen RNA isolation; (3) a method of analysis of RNA or cDNA enriched in bacterial transcripts. The brief features of these steps are given in the review.

2. RNA isolation and bacterial RNA/cDNA enrichment

The widely used scheme of an intracellular bacterial transcriptome analysis is illustrated by Fig. 1.

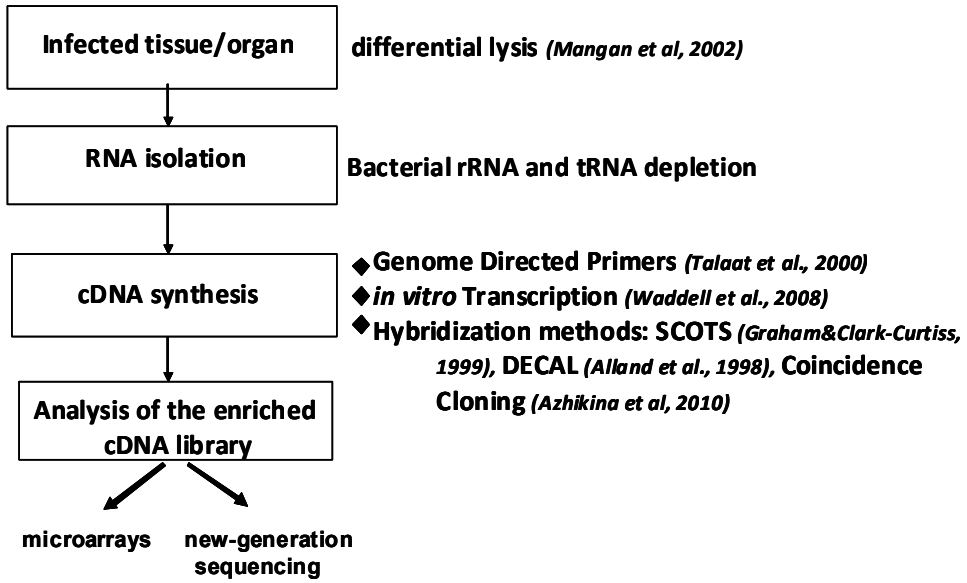

Fig. 1. Stages of intracellular transcriptome analysis (left) and main approaches used to enrich the cDNA library by bacterial transcripts (right).

The methods of RNA isolation and enrichment are described in details in our recent review (Skvortsov & Azhikina, 2010). Here we would like to emphasize that until the present time, practically all ways of preparing cDNA libraries have lead to the further microarray hybridization analysis. Hybridization-based microarray techniques are widely used in transcriptome studies, but development of novel high-throughput DNA sequencing methods (reviewed in (Shendure & Ji, 2008)) obviously demonstrated such microarrays limitations as high background levels owing to cross-hybridization, problems of rare transcript detection, and expression quantification (Shendure, 2008).

We proposed a new method for the evaluation of sequences of bacterial pathogens specifically transcribed in infected tissues (Azhikina et al., 2010). It is based upon the coincidence cloning approach that allows isolation of representative bacterial cDNA pools from infected organs. Co-denaturation and co-renaturation of the excess of bacterial genomic DNA with the cDNA transcribed from total RNA of the infected tissue enabled selective isolation of the bacterial cDNA fraction from the sample, and a single round of coincidence cloning resulted in >1000-fold enrichment of bacterial transcripts. The enriched cDNA library is suitable for high-throughput sequencing analysis, which is less biased and more reliable than other methods including microarrays.

3. Experimental models of infection

Experimental models of tuberculosis used for whole transcriptome studies *in vivo* are very diverse. The most frequently used models and examples of their applications are described below.

3.1 Host phagocytes (mouse and human)

The first work with a whole genome description of *M. tuberculosis* gene expression is that of Schnappinger et al. (Schnappinger et al., 2003). Activated by INF-γ and non-activated mouse macrophages were used in this work as a model system. Rachman et al. characterized the *M. tuberculosis* genes with enhanced expression in activated and inactivated mouse macrophages both relative to each other and to mycobacteria *in vitro* (Rachman et al., 2006). Rohde et al. studied changes in the *M. tuberculosis* transcriptome at the initial stages of infection of mouse macrophages and demonstrated a dynamic enhancement in the expression level of some genes during the first 24 hours post infection (Rohde et al., 2007).

Cappelli et al. were the first to characterize the *M. tuberculosis* whole genome gene expression *in vivo* in human macrophages (Cappelli et al., 2006). Fontan et al. analyzed transcriptomes of *M. tuberculosis* from macrophages of the THP-1 cell line in 4 and 24 hours post infection (Fontan et al., 2008). In the work of Tailleux et al., the authors performed the first comparative analysis of gene expression in *M. tuberculosis* from infected macrophages and dendrite cells (Tailleux et al., 2008). An *in vivo* transcriptome comparison of two differently virulent *M. tuberculosis* strains (H37Rv and H37Ra) was first done by Li et al. (Li et al., 2010).

By the present time, the most extensive study of mycobacterial gene expression *in vivo* is that by Homolka et al. (Homolka et al., 2010). The authors performed a comparative analysis of expression profiles for 3 clinical isolates of *M. africanum*, 12 clinical isolates of *M. tuberculosis*, and two reference laboratory strains (*M. tuberculosis* H37Rv and CDC1551) in activated and non-activated mouse macrophages. This work resulted in the isolation of gene groups whose expression changes irrespective of mycobacterial strain and/or the activation status of the host macrophage.

3.2 Models of *M. tuberculosis* infection in laboratory animals

In 2004, Talaat et al. first performed an analysis of whole genome *M. tuberculosis* gene expression under natural conditions in a living organism (mouse) (Talaat et al., 2004). They studied changes in the pathogen transcriptome composition at different time intervals post infection (7, 14, 21 or 28 days) and for different host genotypes (immunocompetent Balb/c mice and immunodeficient SCID mice). In 2007, Talaat et al. published a paper devoted to the analysis of *M. tuberculosis* gene expression in the lungs of Balb/c mice at later stages of the infection process (Talaat et al., 2007). In 2010, researchers of our group used a new approach to the enrichment of bacterial cDNA for analysis of *M. tuberculosis* gene expression in lung tissues of infected mice (Azhikina et al., 2010). The data obtained by us on quantitative and qualitative composition of the bacterial transcriptome were in good agreement with similar data of the Talaat's group (Talaat et al., 2007).

3.3 Examination of the *M. tuberculosis* transcriptome in human tissues

The report of Rachman et al. published in 2006 is thus far the only work in which *M. tuberculosis* gene expression was studied directly in the human lungs (surgical samples) (Rachman et al., 2006). In this work, the pathogen's whole genome gene expression profiles from granuloma, pericavitary lung tissue and morphologically normal lung tissue were obtained

4. Intracellular *M. tuberculosis* transcriptome

The data obtained by different authors in *in vivo* systems allow to single out a number of the main transcriptome features characteristic of mycobacteria persisting in macrophages. Those are primarily changes in expression of the genes involved in pathogen adaptation, as well as of the genes encoding different factors of immune response. In macrophages, mycobacteria are localized within phagosomes which makes a barrier for the immune system components, but, at the same time, complicates the access of the pathogen to nutrients and microelements. Changes in *M. tuberculosis* gene expression are aimed primarily at forming of the environment able to *in vivo* maintain the functional activity of mycobacteria.

4.1 Lipid metabolism

Lipid metabolism is a key process for *M. tuberculosis* which is directly or indirectly confirmed by the presence in the genome of a considerable number of lipid metabolism genes, indispensability of some genes as follows from data of transposon mutagenesis (Sassetti et al., 2003), decrease or lack of virulence observed in *M. tuberculosis* strains mutant for the genes of this functional category (Neyrolles & Guilhot, 2011), and by other factors.

One of the most characteristic changes in the expression of the genes involved in lipid metabolism is activation of the expression of the genes from clusters *fadA*, *fadB*, *fadD*, *fadE* and *echA* (Azhikina et al., 2010; Munoz-Elias & McKinney, 2006; Schnappinger et al., 2003; Tailleux et al., 2008). These genes encode enzymes involved in β-oxidation of fatty acids and the catabolism of cholesterol. The final products of their activity are acetyl-CoA and propionyl-CoA, the participants of the methylcitrate and tricarboxylic acids cycles.

Enhanced expression of the isocitrate lyase gene *icl1* is also related to the methylcitrate cycle. Isocitrate lyase is a key enzyme of the glyoxylate cycle that is activated when the main carbon source are fatty acids. During this process, activated acetate (acetyl-CoA) is being stepwise converted into malate through the stage of glyoxylic acid formation. Malate can be converted into piruvate by the enzymatic activity of the *pckA* gene product, whose enhanced expression was also observed *in vivo* in mycobacteria (Marrero et al., 2010; Schnappinger et al., 2003; Tailleux et al., 2008). Apart from the maintenance of the glyoxylate bypass, the activity of isocitrate lyase is also needed to utilize cytotoxic propionyl-CoA accumulated during the life cycle of mycobacteria (Savvi et al., 2008). This utilization is possible due to the ability of isocitrate lyase to function as 2-methylisocytrate lyase that facilitates the conversion of propionyl-CoA into succinate. Propionyl-CoA can be also metabolized by a conversion into methylmalonyl-CoA and then into succinate, or included into certain components of the cell wall, such as phthiocerol dimycocerosate (PDIM) or sulfolipid-1 (SL-1) (Russell et al., 2010).

Among other genes of lipid metabolism expressed predominantly *in vivo*, there are *desA* genes encoding desaturases of fatty acids (Homolka et al., 2010; Li et al., 2010; Rachman et al., 2006; Schnappinger et al., 2003). The *papA* and *pks* genes, whose protein products are needed for synthesis of polyketides as components of the *M. tuberculosis* cell wall (Bhatt et al., 2007; Hatzios et al., 2009; Sirakova et al., 2001), are practically always transcribed in experiments. However, the level of their transcription *in vivo* varies, possibly reflecting variations in the lipid metabolism depending upon specific conditions (Azhikina et al., 2010; Homolka et al., 2010; Rohde et al., 2007; Tailleux et al., 2008). Interestingly, transcription of these genes is decreased in the avirulent *M. bovis* BCG and *M. tuberculosis* H37Ra strains as compared with the virulent *M. tuberculosis* H37Rv strain (Li et al., 2010; Rohde et al., 2007).

4.2 Energy metabolism: Cell respiration

According to data obtained in studies of *in vivo M. tuberculosis* gene expression, energy metabolism of mycobacteria is undergoing a significant transformation during infection process. A characteristic of this transformation is a gradual decrease in the level of the type I NADH dehydrogenase (*nuoA-N*) gene expression and increase in expression of the nitrate reductase gene cluster *narGHJI* and of the *narK2* gene, the product of which is a nitrate transporter protein (Azhikina et al., 2010; Schnappinger et al., 2003; Tailleux et al., 2008). Such a metabolic shift most probably suggests that ETC is being reoriented to the using of nitrate electrons as a finite acceptor. Also, in most cases, aa3 type cytochrome c oxidase (*ctaBECD*) and cytochrome c reductase (*qcrCAB*) gene expression is downregulated (Garton et al., 2008; Schnappinger et al., 2003; Shi et al., 2005).

4.3 Protein biosynthesis and cell growth

Decreased expression of ribosomal protein genes (*rpl*, *rpm*, *rps*) indicates a reduced need for the synthesis of new proteins. Usually this phenomenon occurs in conditions non optimal for the pathogen (dendrite cells, activated macrophages) and correlates with decreased expression level of the ATP-synthase (*atpA-H*) gene and slowdown of *M. tuberculosis* replication (Homolka et al., 2010; Tailleux et al., 2008).

4.4 Defense mechanisms, DNA replication

The compartment (early phagolysosome) of *M. tuberculosis* residing at persisting in macrophages represents a rather non-aggressive environment with practically no hydrolytic activity and pH 6.2-6.4. Nevertheless, the mycobacteria are under the influence of many stress factors like active forms of oxygen and nitrogen or the apoptotic death of the host cell. The effect of stress factors induces an upregulation of genes of the DNA repair and recombination (*dinF/G*) systems (Rachman et al., 2006; Schnappinger et al., 2003; Talaat et al., 2004), as well as chaperon genes (*groES*, *groEL1/2*, *dnaJ/K*, *hspX*) (Fontan et al., 2008; Garton et al., 2008; Homolka et al., 2010; Karakousis et al., 2004; Rohde et al., 2007; Tailleux et al., 2008). Certain data indicate that this effect is not a specific reaction on intracellular conditions, but part of adaptive response to stress (Boshoff et al., 2004; Waddell et al., 2004).

4.5 Cell wall, membrane, and transport

The cell wall and inner plasma membrane are components of the complex cell envelope of mycobacteria. The inner plasma membrane contains a lot of transport systems. Some genes of these systems were observed to be upregulated, among them the *irtA/B* genes encoding carboxymycobactin transporters (Li et al., 2010; Schnappinger et al., 2003; Tailleux et al., 2008), the *sugI*, *Rv2040c* and *Rv2041c* genes of carbohydrate transporters (Azhikina et al., 2010; Schnappinger et al., 2003; Tailleux et al., 2008), and the *narK2* gene of the nitrate transporter protein (Azhikina et al., 2010; Garton et al., 2008; Tailleux et al., 2008; Talaat et al., 2007).

Enhanced expression of the genes of siderophore-synthesis enzymes, mycobactin and carboxymycobactin (*mbtA-J*, *mbtL-N*), could be explained by limited access to iron (Azhikina et al., 2010; Schnappinger et al., 2003; Tailleux et al., 2008).

4.6 Factors of *Mycobacterium tuberculosis* virulence

The *M. tuberculosis* virulence depends upon various metabolic processes that provide for successful infection process. The pathogen survival in host cells directly depends on avoiding host defence mechanisms and getting nutrient substances from host tissues for the existence and reproduction. A considerable damage of host tissues and organs at later stages of infection is also needed as it facilitates dissemination of the infection. We will consider the factors that directly affect the host organism in order to suppress host defence mechanisms. One of such immune response modulators is the ESAT-6 protein (Sorensen et al., 1995) encoded by the *esxA* gene and secreted by the type VII secretion system (T7SS) ESX-1 (Abdallah et al., 2007; Simeone et al., 2009). ESAT-6 is one of the immune modulation key factors suggested to be involved in lysis of phagolysosome membranes and macrophage outer membranes thus facilitating spread of mycobacteria from one host cell to another (de Jonge et al., 2007; Kinhikar et al., 2010). The *in vivo* expression of the *esx* cluster genes is under constant control and can be both decreased and increased depending on the conditions (Fontan et al., 2008; Rohde et al., 2007; Schnappinger et al., 2003; Tailleux et al., 2008).

One more important system of immune response modulation is the expression of the PE/PPE family proteins that possess immunogenic properties (Bottai & Brosch, 2009; Sampson, 2011; Voskuil et al., 2004). The level of their expression is also dependent upon specific conditions (Azhikina et al., 2010; Fontan et al., 2008; Schnappinger et al., 2003; Tailleux et al., 2008; Voskuil et al., 2004).

4.7 Transcription regulation

The adaptation to changing conditions of the environment is mainly underlain by the activity of signal and regulatory systems of the bacterium, and therefore by changing expression of transcription regulatory systems' genes. Among genes of the regulatory systems, 13 genes of sigma factors (Manganelli et al., 2004; Rodrigue et al., 2006) are of special interest. Their differential expression was repeatedly observed in *in vivo* experiments. For instance, *sigH* was upregulated in artificial granulomas in mice and in human macrophages (Karakousis et al., 2004), whereas *sigB* and *sigE* were also upregulated

in mouse phagosomes (Rohde et al., 2007), as well as in artificial mouse granulomas and in the mouse lung, respectively (Karakousis et al., 2004; Talaat et al., 2004).

Other transcription regulatory genes whose upregulation was observed in *in vivo* experiments are *whiB3* (Fontan et al., 2008; Rohde et al., 2007), *ethR*, *ideR*, *kstR* and *relA* (Fontan et al., 2008; Schnappinger et al., 2003; Tailleux et al., 2008). In addition, *M. tuberculosis* has 12 two-component regulatory systems (Tucker et al., 2007). Two of them, *phoPR* (Gonzalo-Asensio et al., 2008) and *dosRS* (*devRS*) (Park et al., 2003), were studied more thoroughly than others. It was shown that functional activity of the phoPR system supports the *M. tuberculosis* virulence by regulating the metabolism of complex lipids and the work of the ESX secretion systems (Gonzalo-Asensio et al., 2008). The positive transcription regulator phoP was observed to induce genes under its control in a low-acid (pH 6.4) environment of mouse macrophage phagosomes (Rohde et al., 2007). Not less important is the two component regulatory system dosRS responsible for expression regulation of about 50 genes (Park et al., 2003). Enhanced expression of these genes was repeatedly observed in the course of mycobacterial infection of macrophages (Fontan et al., 2008; Rohde et al., 2007; Schnappinger et al., 2003; Tailleux et al., 2008). Moreover, *dosRS* regulon genes were expressed practically in all other conditions: in *M. tuberculosis* from artificial mouse granulomas, samples of mouse lung tissues and surgical samples of human lung, as well as in samples of sputum and in some experiments *in vitro* (Garton et al., 2008; Homolka et al., 2010; Karakousis et al., 2004; Li et al., 2010; Talaat et al., 2007; Timm et al., 2003). The functional role of this regulon is still not quite clear, but its activity was suggested to be important for *M. tuberculosis* adaptation to variations in the redox status during the infection process (Bacon et al., 2004; Bacon & Marsh, 2007; Rustad et al., 2009).

5. Profiling of *Mycobacterium tuberculosis* gene expression during infection in genetically different mouse models

We have carried out a comparative study of *M. tuberculosis* transcriptomes in order to reveal the features of expression profiles that correlate with progressing disease, and also to understand the difference between efficient and defective defence mechanisms at the level of bacterial gene expression. To this end, at different stages of the infection process, we performed a comparative quantitative and qualitative analysis of the sequences transcribed during infection of mice sensitive (inefficient immune response) and resistant (efficient response) to these bacteria (Skvortsov et al., 2010).

We have compared transcriptomes of *M. tuberculosis* H37Rv in infected mice of two lineages, I/StSnEgYCit (I/St) and C57BL/6JCit (B6). These lineages have been earlier described in detail (Kondratieva et al., 2010), and the B6 lineage was shown to be more resistant to the infection by *M. tuberculosis* than I/St. In particular, the infection process in B6 mice was less aggressive, and the infected mice had a longer survival.

Female mice of both lineages were aerogenically infected with *M. tuberculosis* bacteria. In 4 and 6 weeks post infection, the infected mice were killed, and total lung RNA isolated. Samples of total RNA from lung tissues of I/St and B6 mice were used to synthesize cDNA enriched in fragments of bacterial cDNA using coincidence cloning procedure (Azhikina et al., 2010). As a result, three cDNA libraries were obtained, which represented transcriptomes of *M. tuberculosis* from lung tissues of I/St mice on week 6 post infection

(CC6(SUS)) and from lung tissues of B6 mice on weeks 4 and 6 post infection (CC4(RES) and CC6(RES), respectively). The libraries were analyzed using the 454 pyrosequencing technology. A general scheme of the experiment is shown in Fig. 2, and general characteristics of the libraries analyzed are presented in Table 1. In total, sequences of 190031 cDNA fragments were determined: 73410 from CC4(RES), 75655 from CC4(SUS), and 40966 from CC6(RES).

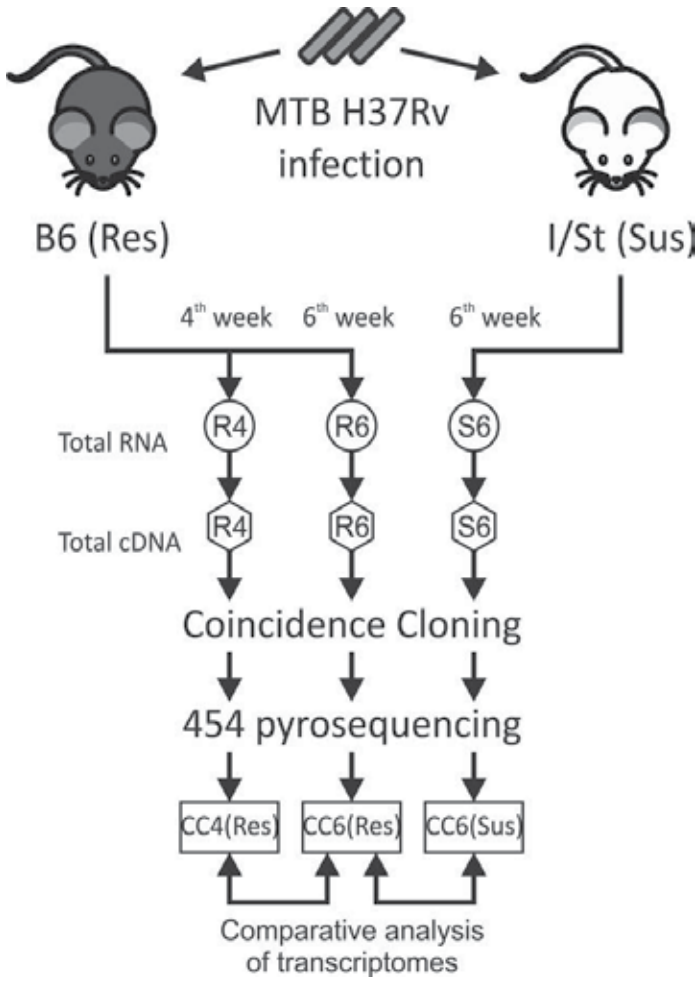

Fig. 2. Scheme of the transcriptomes comparison experiment. Res – genetically resistant mice, Sus – genetically susceptible mice, CC – library enriched with bacterial cDNA

Using a standalone BLASTn algorithm, the nucleotide sequences obtained were mapped to the genome sequence of *M. tuberculosis* H37Rv (GenBank version: AL123456.2). The mapping revealed that the CC4(RES), CC6(SUS) and CC6(RES) samples contained 14990 (20.42%), 43618 (57.65%) and 34234 (83.57%) *M. tuberculosis* sequences, respectively.

The results obtained demonstrate that the technology used allowed us to considerably enrich the cDNA samples with bacterial sequences.

Library	CC4(RES)	CC6(SUS)	CC6(RES)
Total sequences read	73410	75655	40966
M. tuberculosis-specific (unique) sequences	14990	43618	34234
M. tuberculosis-specific (unique) sequences, % of total sequences read	20,4	57,7	83,6
The number of *M. tuberculosis* genes expressed (at least one reading)	1012	1353	1940
Genes expressed, % of the total number of genes in the library	25,2	33,7	48,3

Table 1. Results of sequencing and mapping of the CC4(RES), CC6(RES) and CC6(SUS) libraries

Of 4012 *M. tuberculosis* genes and 7 pseudogenes, in the CC4(RES) sample 1012 (25.2%), in CC4(SUS) - 1353 (33.7%), and in CC6(RES) – 1940 (48.3%) genes were expressed. 1428 (35,5%) genes were not expressed in either of the samples, whereas 469 (11.7%) genes were expressed in each sample.

The distribution of the expressed genes between functional categories is shown in Fig. 3. Mobile elements (insertion sequences and phages) were excluded from the analysis.

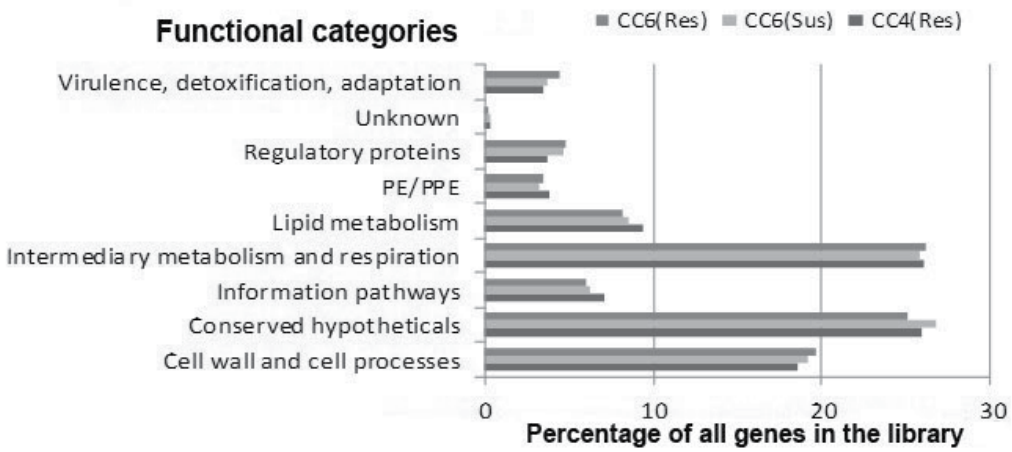

Fig. 3. Assignment to biochemical categories of the *M. tuberculosis* H37Rv genes expressed in samples of lung tissue of B6 mice on weeks 4 and 6 post infection and of I/St mice on week 6 post infection (CC4(RES), CC6(RES) and CC6(SUS) libraries, respectively)

5.1 Genes whose expression is enhanced during infection

The comparison of *M. tuberculosis* transcriptomes during infection in a genetically stable mouse lineage (CC6(RES) vs CC4(RES)) and in genetically different mice (CC6(RES) vs CC6(SUS)), described above, was aimed at the search of genes whose expression is enhanced in the course of infection, specifically in B6 mice on week 6 as compared with

other time points. The comparison of CC6(RES) vs CC4(RES) allowed to reveal 226 genes upregulated in infected tissues of B6 mice. A similar comparison of CC6(RES) vs CC6(SUS) revealed 253 genes upregulated in the CC6(RES) sample (Fig. 4). We concentrated our search on *M. tuberculosis* genes:

1. with expression upregulated only in one of genetically different hosts;
2. with expression upregulated irrespective of genetic features of the host organism. These genes represent a kind of basic set of genes responsible for a universal compensatory reaction of *M. tuberculosis* to unfavourable conditions of the environment. They are termed CUG –Commonly Upregulated Genes.

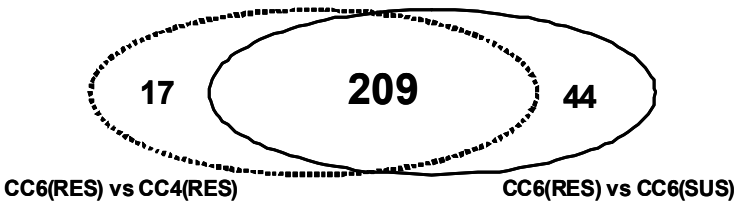

Fig. 4. Venn diagram illustrating the number of upregulated mycobacterial genes in comparisons CC6(RES) vs CC4(RES) (dotted) and CC6(RES) vs CC6(SUS) (solid)

5.1.1 Genes upregulated only in one of genetically different hosts

We found 17 genes with enhanced expression in CC6(RES) vs CC4(RES) and 44 genes in CC6(RES) vs CC6(SUS). Such a statistics probably reflects the fact that in the first case bacterial genes are expressed in one and the same microenvironment, whereas in the second case microenvironments are different resulting in a greater number of genes upregulated.

Genes, the expression of which is enhanced in the CC6(RES) sample only relative to CC4(RES), mostly belong to categories of cell wall and cell processes, intermediary metabolism and respiration, and lipid metabolism. The protein products of 12 out of 17 genes were detected in a fraction of cell membrane or cell wall where they mainly fulfil transport and defence functions. For example, the *embA* gene codes for indolylacetylinositol arabinosyltransferase EmbA involved in the synthesis of arabinan, and mutations in this gene cause resistance to ethambutol. Also, the *Rv3273* gene encodes carbonate dehydratase that participates in sulfate transport (TubercuList, http://tuberculist.epfl.ch). In the analysis performed, we failed to detect metabolic pathways specifically activated at later infection times as compared to early stages.

Comparing the bacterial transcriptomes within different hosts we found greater variety of biochemical pathways. An increase in energy exchange is reflected in enhanced expression of the genes of three NADH-dehydrogenase subunits (*nuoH*, *nuoI*, *nuoL*), as well as in increased activity of the tricarbonic acid cycle and in upregulation of the *Rv1916* gene. The *Rv1916* gene is the second part of the *aceA* (*icl2*) gene which in *M. tuberculosis* H37Rv is divided into two modules, *Rv1915* and *Rv1916* (*aceAa* и *aceAb*), each of which can be expressed independently. Among other important differences, one can highlight enhanced expression of genes, the products of which are responsible for lipid and amino acid metabolism and catabolism (*lipV*, *lipF*, *Rv2531c*), and genes of DNA repair enzymes (*recO*, *recB*).

Such a picture is quite predictable as the microenvironment in a resistant host is a hostile habitat which can explain the need in more active repair systems. Increased gene expression of lipolytic enzymes (*lipF*, *lipV*, *plcA*), enzymes of the tricarbonic acid cycle and *aceAb* may suggest a forced usage of lipids as the source of energy and carbon.

5.1.2 CUGs – genes needed for *M. tuberculosis* adaptation to different host defense mechanisms

We have revealed 209 genes upregulated in both comparisons. According to the results of transposon mutagenesis, the products of 44 out of these genes are essential in *M. tuberculosis* (Sassetti et al., 2003), the list of these genes is given in Table 2. *Rv3569c*, *Rv3537*, and *Rv3563* were earlier shown to be essential for survival in mouse macrophages (TubercuList, http://tuberculist.epfl.ch)

A bit less than one third of the 209 genes belong to the conserved hypothetical (59 genes) and unknown (2 genes) categories. In spite of unknown functions, the genes of these categories may be considered potential therapeutic targets, since their low homology to genes of other microorganisms suggests that they are characteristic just of mycobacteria or even *M. tuberculosis*.

The function of the PE/PPE family proteins is not quite clear, but they are suggested to be needed for antigenic variability in mycobacteria (Karboul et al., 2008). Nevertheless, the *Rv0152c* and *Rv0355c* genes had a high expression level in the CC6(RES) sample, and they were also expressed in the CC4(RES) and CC4(SUS) samples. *Rv3135* encodes a protein essential in *M. tuberculosis* H37Rv that may suggest some additional functions beyond antigenic variability

One more feature of CUGs is an increased activity of amino acid metabolism pathways. It is not clear if the stimulation of this metabolism enzyme expression is due to the absence of available amino acids (and the necessity of their synthesis) or their availability (and the possibility to use them). Poor nutrient conditions of the environment are supported by a high level gene expression of various systems of acquisition and accumulation of nutrients, e.g. such as phosphate (*pstS1*) or iron (*irtA*, *mbtC*, *mbtE*, *mbtF*). A shortage of phosphate is indicated by enhanced expression of the *senX3* gene, a sensor component of the senX3 regX3 two-component system that activates the so called stringent response under phosphorus deficiency. The expression of lipid metabolism genes (fadD, fadE, lipU, *lipJ*) suggests a switch to using lipids as a major source of energy and carbon. Enhanced expression of the *narH* and *narK3* genes implies a switch to anaerobic respiration characteristic of latent infection. Finally, the *secA2* gene is also worth mentioning. This gene codes for translocase SecA2 which is a component of the *M. tuberculosis* secondary transport system Sec that provides for, in particular, secretion of superoxide dismutase SodA and catalase katG. A live vaccine based on an *M. tuberculosis* mutant for the *secA2* gene (Hinchey et al.,2011) showed high efficiency and safety in animal trials. Summarizing, it can be said that CUGs reflect characteristic features of infection in a mouse model. An exception is increased expression of the *atpF* and *atpH* genes, although, according to some reports, their expression decreases in the course of infection as energy demand of the pathogen goes down upon transition to the state of latent infection.

Gene	Name	Function	Description
Rv0015c	*pknA*	RP	transmembrane serine/threonine-protein kinase A
Rv0020c	*fhaA*	IMaR	aspartate aminotransferase
Rv0072	*Rv0072*	IMaR	diaminopimelate epimerase
Rv0089	*Rv0089*	CWaCP	Possible integral membrane efflux protein
Rv0145	*Rv0145*	LM	Possible acyl-CoA dehydrogenase Fad19
Rv0261c	*narK3*	CWaCP	Probable integral membrane nitrite extrusion protein narK3 (nitrite facilitator)
Rv0332	*Rv0332*	CH	Conserved hypothetical protein
Rv0465c	*Rv0465c*	RP	Probable transcriptional regulatory protein
Rv0509	*hemA*	IMaR	Probable glutamyl-trna reductase hemA
Rv0525	*Rv0525*	CH	hypothetical protein
Rv0526	*Rv0526*	IMaR	Possible thioredoxin protein
Rv0558	*menH*	IMaR	ubiquinone/menaquinone biosynthesis methyltransferase
Rv0645c	*mmaA1*	LM	Methoxy mycolic acid synthase 1 mmaa1 (methyl mycolic acid synthase 1) (mmA1) (hydroxy mycolic acid synthase)
Rv0684	*fusA1*	IP	elongation factor EF-2
Rv0712	*Rv0712*	CH	Conserved hypothetical protein
Rv0998	*Rv0998*	CH	Conserved hypothetical protein
Rv1026	*Rv1026*	VDA	hypothetical protein
Rv1106c	*Rv1106c*	IMaR	3-beta-hydroxysteroid dehydrogenase
Rv1186c	*Rv1186c*	CH	hypothetical protein
Rv1202	*dapE*	IMaR	succinyl-diaminopimelate desuccinylase
Rv1232c	*Rv1232c*	CH	hypothetical protein
Rv1306	*atpF*	IMaR	ATP synthase subunit B
Rv1307	*atpH*	IMaR	Probable ATP synthase delta chain
Rv1348	*irtA*	CWaCP	Probable iron-regulated transporter irtA
Rv1514c	*Rv1514c*	CH	Conserved hypothetical protein
Rv1629	*polA*	IP	DNA polymerase I
Rv1663	*pks17*	LM	Probable polyketide synthase pks17
Rv1712	*Cmk*	IMaR	cytidylate kinase
Rv1828	*Rv1828*	CH	hypothetical protein
Rv2382c	*mbtC*	LM	Polyketide synthase MTBC
Rv2476c	*Gdh*	IMaR	probable NAD-dependent glutamate dehydrogenase
Rv2499c	*Rv2499c*	IMaR	Possible oxidase regulatory-related protein
Rv2727c	*miaA*	IMaR	tRNA delta(2)-isopentenylpyrophosphate transferase
Rv2934	*ppsD*	LM	Phenolpthiocerol synthesis type-I polyketide synthase ppsD
Rv3001c	*ilvC*	IMaR	ketol-acid reductoisomerase
Rv3135	*PPE50*	PE/PPE	PPE family protein
Rv3285	*accA3*	LM	Probable bifunctional protein acetyl-/propionyl-coenzyme A carboxylase (alpha chain)

Gene	Name	Function	Description
Rv3411c	*guaB2*	IMaR	inositol-5-monophosphate dehydrogenase
Rv3515c	*fadD19*	LM	Probable fatty-acid-coA ligase fadD19 (fatty-acid-coa synthetase) (fatty-acid-coa synthase)
Rv3593	*lpqF*	CWaCP	Probable conserved lipoprotein lpqF
Rv3627c	*Rv3627c*	CH	hypothetical protein
Rv3658c	*Rv3658c*	CWaCP	Probable conserved transmembrane protein
Rv3793	*embC*	CWaCP	Integral membrane indolylacetylinositol arabinosyltransferase
Rv3799c	*accD4*	LM	Probable propionyl-CoA carboxylase beta chain 4

RP – regulatory proteins; IMAP – intermediate metabolism and respiration; CWaCP – cell wall and cell processes; LM – lipid metabolism; IP – information pathway; VDA – virulence, detoxification, adaptation; PE/PPE – PE/PPE protein families; CH – conserved hypotheticals

Table 2. CUGs, found as essential according (Sassetti et al., 2003)

6. Conclusion

Infectious diseases caused by intracellular pathogenic bacteria represent a significant challenge to health care. The course of the infection depends not only on the protective mechanisms, but also on the specific expression of bacterial genes. Altered expression as a response to the immune reaction of the host organism is critical for the survival and functioning of pathogenic bacteria. Understanding of *M. tuberculosis* transcriptional responses to different stimuli and aggressiveness of the environment gives the opportunity to describe the adaptation mechanisms necessary for bacterial successful survival and colonization of the host.

M. tuberculosis transcription profiling obtained in different conditions allows to define the core set of adaptive genes (we called it "commonly upregulated genes"), which characterize different phases of *M. tuberculosis* intracellular life – from primary infection through latency to reactivation. The expression of these genes can be considered as a universal reaction of mycobacteria to various stress factors of the environment. Accumulation and analysis of such data is the surest way to proceeding and developing effective approaches towards diagnostics and treatment of tuberculosis.

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8. References

Abdallah, A. M., Gey van Pittius, N. C., Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., & Bitter, W. (2007). Type VII

secretion-mycobacteria show the way. *Nat Rev Microbiol*, Vol.5, No.11, (November 2009), pp.883-891, ISSN 1740-1526

Alland, D., Kramnik, I., Weisbrod, T. R., Otsubo, L., Cerny, R., Miller, L. P., Jacobs, W. R., Jr., & Bloom, B. R. (1998). Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A*, Vol.95, No.22, (October 1998), pp.13227-13232, ISSN 0027-8424

Azhikina, T., Skvortsov, T., Radaeva, T., Mardanov, A., Ravin, N., Apt, A., & Sverdlov, E. (2010). A new technique for obtaining whole pathogen transncriptome from infected host tissues. *BioTechniques*, Vol.48, No.2, (February 2010), pp.139–144, ISSN 0736-6205

Bacon, J., James, B. W., Wernisch, L., Williams, A., Morley, K. A., Hatch, G. J., Mangan, J. A., Hinds, J., Stoker, N. G., Butcher, P. D., & Marsh, P. D. (2004). The influence of reduced oxygen availability on pathogenicity and gene expression in Mycobacterium tuberculosis. *Tuberculosis (Edinb)*, Vol.84, No.3-4, (June 2004), pp.205-217, ISSN 1472-9792

Bacon, J., & Marsh, P. D. (2007). Transcriptional responses of Mycobacterium tuberculosis exposed to adverse conditions in vitro. *Curr Mol Med*, Vol.7, No.3, (May 2007), pp.277-286, ISSN 1566-5240

Bhatt, K., Gurcha, S. S., Bhatt, A., Besra, G. S., & Jacobs, W. R., Jr. (2007). Two polyketide-synthase-associated acyltransferases are required for sulfolipid biosynthesis in Mycobacterium tuberculosis. *Microbiology*, Vol.153, No.Pt 2, (February 2007), pp.513-520, ISSN 1350-0872

Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., & Barry, C. E., 3rd (2004). The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism: novel insights into drug mechanisms of action. *J Biol Chem*, Vol.279, No.38, (September 2004), pp.40174-40184, ISSN 0021-9258

Bottai, D., & Brosch, R. (2009). Mycobacterial PE, PPE and ESX clusters: novel insights into the secretion of these most unusual protein families. *Mol Microbiol*, Vol.73, No.3, (August 2009), pp.325-328, ISSN 1365-2958

Butcher, P. D. (2004). Microarrays for Mycobacterium tuberculosis. *Tuberculosis (Edinb)*, Vol.84, No.3-4, (April 2004), pp.131-137, ISSN 1472-9792

Cappelli, G., Volpe, E., Grassi, M., Liseo, B., Colizzi, V., & Mariani, F. (2006). Profiling of Mycobacterium tuberculosis gene expression during human macrophage infection: upregulation of the alternative sigma factor G, a group of transcriptional regulators, and proteins with unknown function. *Res Microbiol*, Vol.157, No.5, (June 2006), pp.445-455, ISSN 0923-2508

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., & Barrell, B. G. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, Vol.393, No.6685, (June 1998), pp.537-544, ISSN 0028-0836

de Jonge, M. I., Pehau-Arnaudet, G., Fretz, M. M., Romain, F., Bottai, D., Brodin, P., Honore, N., Marchal, G., Jiskoot, W., England, P., Cole, S. T., & Brosch, R. (2007). ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. *J Bacteriol*, Vol.189, No.16, (August 2007), pp.6028-6034, ISSN 0021-9193

Fontan, P., Aris, V., Ghanny, S., Soteropoulos, P., & Smith, I. (2008). Global transcriptional profile of Mycobacterium tuberculosis during THP-1 human macrophage infection. *Infect Immun*, Vol.76, No.2, (February 2008), pp.717-725, ISSN 1098-5522

Garton, N. J., Waddell, S. J., Sherratt, A. L., Lee, S. M., Smith, R. J., Senner, C., Hinds, J., Rajakumar, K., Adegbola, R. A., Besra, G. S., Butcher, P. D., & Barer, M. R. (2008). Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med*, Vol.5, No.4, (April 2008), pp.e75, ISSN 1549-1676

Gonzalo-Asensio, J., Mostowy, S., Harders-Westerveen, J., Huygen, K., Hernandez-Pando, R., Thole, J., Behr, M., Gicquel, B., & Martin, C. (2008). PhoP: a missing piece in the intricate puzzle of Mycobacterium tuberculosis virulence. *PLoS One*, Vol.3, No.10, (October 2008), pp.e3496, ISSN 1932-6203

Graham, J. E., & Clark-Curtiss, J. E. (1999). Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc Natl Acad Sci U S A*, Vol.96, No.20, (September 1999), pp.11554-11559, ISSN 1091-6490

Hatzios, S. K., Schelle, M. W., Holsclaw, C. M., Behrens, C. R., Botyanszki, Z., Lin, F. L., Carlson, B. L., Kumar, P., Leary, J. A., & Bertozzi, C. R. (2009). PapA3 is an acyltransferase required for polyacyltrehalose biosynthesis in Mycobacterium tuberculosis. *J Biol Chem*, Vol.284, No.19, (May 2009), pp.12745-12751, ISSN 0021-9258

Hinchey, J., Jeon, B. Y., Alley, H., Chen, B., Goldberg, M., Derrick, S., Morris, S., Jacobs, W. R., Jr., Porcelli, S. A., & Lee, S. (2011). Lysine auxotrophy combined with deletion of the SecA2 gene results in a safe and highly immunogenic candidate live attenuated vaccine for tuberculosis. *PLoS One*, Vol.6, No.1, (January 2011), pp.e15857, ISSN 1932-6203

Homolka, S., Niemann, S., Russell, D. G., & Rohde, K. H. (2010). Functional Genetic Diversity among Mycobacterium tuberculosis Complex Clinical Isolates: Delineation of Conserved Core and Lineage-Specific Transcriptomes during Intracellular Survival. *PLoS Pathog*, Vol.6, No.7, (July 2010), pp.e1000988, ISSN 1553-7366

Karakousis, P. C., Yoshimatsu, T., Lamichhane, G., Woolwine, S. C., Nuermberger, E. L., Grosset, J., & Bishai, W. R. (2004). Dormancy phenotype displayed by extracellular Mycobacterium tuberculosis within artificial granulomas in mice. *J Exp Med*, Vol.200, No.5, (September 2004), pp.647-657, ISSN 0022-1007

Karboul, A., Mazza, A., Gey van Pittius, N. C., Ho, J. L., Brousseau, R., & Mardassi, H. (2008). Frequent homologous recombination events in Mycobacterium tuberculosis PE/PPE multigene families: potential role in antigenic variability. *J Bacteriol*, Vol.190, No.23, (December 2008), pp.7838-7846, ISSN 1098-5530

Kendall, S. L., Rison, S. C., Movahedzadeh, F., Frita, R., & Stoker, N. G. (2004). What do microarrays really tell us about *M. tuberculosis*? *Trends Microbiol*, Vol.12, No.12, (December 2004), pp.537-544, ISSN 0966-842X

Kinhikar, A. G., Verma, I., Chandra, D., Singh, K. K., Weldingh, K., Andersen, P., Hsu, T., Jacobs, W. R., Jr., & Laal, S. (2010). Potential role for ESAT6 in dissemination of *M. tuberculosis* via human lung epithelial cells. *Mol Microbiol*, Vol.75, No.1, (January 2010), pp.92-106, ISSN 1365-2958

Kondratieva, E., Logunova, N., Majorov, K., Averbakh, M., & Apt, A. (2010). Host genetics in granuloma formation: human-like lung pathology in mice with reciprocal genetic susceptibility to *M. tuberculosis* and M. avium. *PLoS One*, Vol.5, No.5, (May 2010), pp.e10515, ISSN 1932-6203

Li, A. H., Waddell, S. J., Hinds, J., Malloff, C. A., Bains, M., Hancock, R. E., Lam, W. L., Butcher, P. D., & Stokes, R. W. (2010). Contrasting transcriptional responses of a virulent and an attenuated strain of Mycobacterium tuberculosis infecting macrophages. *PLoS One*, Vol.5, No.6, (June 2010), pp.e11066, ISSN 1932-6203

Mangan, A., Monahan, I., & Butcher, P. (2002). Gene expression during host-pathogen interactions: approaches to bacterial mRNA extraction and labelling for microarray analysis, In: *Methods in Microbiology*, B. Wren & N. Dorrell, (Eds.), Vol.33, pp.137-151, ISBN 978-0-12-521533-6

Manganelli, R., Provvedi, R., Rodrigue, S., Beaucher, J., Gaudreau, L., & Smith, I. (2004). Sigma factors and global gene regulation in Mycobacterium tuberculosis. *J Bacteriol*, Vol.186, No.4, (February 2004), pp.895-902, ISSN 0021-9193

Marrero, J., Rhee, K. Y., Schnappinger, D., Pethe, K., & Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for Mycobacterium tuberculosis to establish and maintain infection. *Proc Natl Acad Sci U S A*, Vol.107, No.21, (May 2010), pp.9819-9824, ISSN 1091-6490

Munoz-Elias, E. J., & McKinney, J. D. (2006). Carbon metabolism of intracellular bacteria. *Cell Microbiol*, Vol.8, No.1, (January 2006), pp.10-22, ISSN 1462-5814

Neyrolles, O., & Guilhot, C. (2011). Recent advances in deciphering the contribution of Mycobacterium tuberculosis lipids to pathogenesis. *Tuberculosis (Edinb)*, Vol.91, No.3, (May 2011), pp.187-195, ISSN 1873-281X

Park, H. D., Guinn, K. M., Harrell, M. I., Liao, R., Voskuil, M. I., Tompa, M., Schoolnik, G. K., & Sherman, D. R. (2003). Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. *Mol Microbiol*, Vol.48, No.3, (May 2003), pp.833-843, ISSN 0950-382X

Rachman, H., Strong, M., Ulrichs, T., Grode, L., Schuchhardt, J., Mollenkopf, H., Kosmiadi, G. A., Eisenberg, D., & Kaufmann, S. H. (2006). Unique transcriptome signature of Mycobacterium tuberculosis in pulmonary tuberculosis. *Infect Immun*, Vol.74, No.2, (February 2006), pp.1233-1242, ISSN 0019-9567

Rodrigue, S., Provvedi, R., Jacques, P. E., Gaudreau, L., & Manganelli, R. (2006). The sigma factors of Mycobacterium tuberculosis. *FEMS Microbiol Rev*, Vol.30, No.6, (November 2006), pp.926-941, ISSN 0168-6445

Rohde, K. H., Abramovitch, R. B., & Russell, D. G. (2007). Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe*, Vol.2, No.5, (November 2007), pp.352-634, ISSN 1934-6069

Russell, D. G., VanderVen, B. C., Lee, W., Abramovitch, R. B., Kim, M. J., Homolka, S., Niemann, S., & Rohde, K. H. (2010). Mycobacterium tuberculosis wears what it eats. *Cell Host Microbe*, Vol.8, No.1, (July 2010), pp.68-76, ISSN 1934-6069

Rustad, T. R., Sherrid, A. M., Minch, K. J., & Sherman, D. R. (2009). Hypoxia: a window into Mycobacterium tuberculosis latency. *Cell Microbiol*, Vol.11, No.8, (August 2009), pp.1151-1159, ISSN 1462-5822

Sampson, S. L. (2011). Mycobacterial PE/PPE proteins at the host-pathogen interface. *Clin Dev Immunol,* Vol.2011, (January 2011), pp.497203, ISSN 1740-2530

Sassetti, C. M., Boyd, D. H., & Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol*, Vol.48, No.1, (April 2003), pp.77-84, ISSN 1365-2958

Savvi, S., Warner, D. F., Kana, B. D., McKinney, J. D., Mizrahi, V., & Dawes, S. S. (2008). Functional characterization of a vitamin B12-dependent methylmalonyl pathway in Mycobacterium tuberculosis: implications for propionate metabolism during growth on fatty acids. *J Bacteriol*, Vol.190, No.11, (June 2008), pp.3886-3895, ISSN 1098-5530

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C., & Schoolnik, G. K. (2003). Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med*, Vol.198, No.5, (September 2003), pp.693-704, ISSN 0022-1007

Shendure, J. (2008). The beginning of the end for microarrays? Nat Methods, Vol.5, No.7, (July 2008), pp.585-587, ISSN 1548-7105

Shendure, J., & Ji, H. (2008). Next-generation DNA sequencing. *Nat Biotechnol*, Vol.26, No.10, (October 2008), pp.1135-1145, ISSN 1546-1696

Shi, L., Sohaskey, C. D., Kana, B. D., Dawes, S., North, R. J., Mizrahi, V., & Gennaro, M. L. (2005). Changes in energy metabolism of Mycobacterium tuberculosis in mouse lung and under in vitro conditions affecting aerobic respiration. *Proc Natl Acad Sci U S A,* Vol.102, No.43, (October 2005), pp.15629-15634, ISSN 0027-8424

Simeone, R., Bottai, D., & Brosch, R. (2009). ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol*, Vol.12, No.1, (February 2009), pp.4-10, ISSN 1879-0364

Sirakova, T. D., Thirumala, A. K., Dubey, V. S., Sprecher, H., & Kolattukudy, P. E. (2001). The Mycobacterium tuberculosis pks2 gene encodes the synthase for the hepta- and octamethyl-branched fatty acids required for sulfolipid synthesis. *J Biol Chem*, Vol.276, No.20, (May 2001), pp.16833-16839, ISSN 0021-9258

Skvortsov, T. A., & Azhikina, T. L. (2010). A review of the transcriptome analysis of bacterial pathogens *in vivo*: Problems and solutions. *Russian Journal of Bioorganic Chemistry*, Vol.36, No.5, (September 2010), pp.550-559, ISSN 1068-1620

Skvortsov, T., Ignatov, D., Apt, A., & Azhikina, T. (2010). Profiling of *Mycobacterium tuberculosis* gene expression during infection in genetically different mouse models, *Proceedings of the 35th FEBS Congress*, ISSN 1742-464X, Gothenburg, Sweden, 26 June-1 July 2010

Sorensen, A. L., Nagai, S., Houen, G., Andersen, P., & Andersen, A. B. (1995). Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis. *Infect Immun*, Vol.63, No.5, (May 1995), pp.1710-1717, ISSN 0019-9567

Tailleux, L., Waddell, S. J., Pelizzola, M., Mortellaro, A., Withers, M., Tanne, A., Castagnoli, P. R., Gicquel, B., Stoker, N. G., Butcher, P. D., Foti, M., & Neyrolles, O. (2008).

Probing host pathogen cross-talk by transcriptional profiling of both Mycobacterium tuberculosis and infected human dendritic cells and macrophages. *PLoS One*, Vol.3, No.1, (January 2008), pp.e1403, ISSN 1932-6203

Talaat, A. M., Hunter, P., & Johnston, S. A. (2000). Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis. *Nat Biotechnol*, Vol.18, No.6, (June 2000), pp.679-682, ISSN 1087-0156

Talaat, A. M., Lyons, R., Howard, S. T., & Johnston, S. A. (2004). The temporal expression profile of Mycobacterium tuberculosis infection in mice. *Proc Natl Acad Sci U S A*, Vol.101, No.13, (March 2004), pp.4602-4607, ISSN 0027-8424

Talaat, A. M., Ward, S. K., Wu, C. W., Rondon, E., Tavano, C., Bannantine, J. P., Lyons, R., & Johnston, S. A. (2007). Mycobacterial bacilli are metabolically active during chronic tuberculosis in murine lungs: insights from genome-wide transcriptional profiling. *J Bacteriol*, Vol.189, No.11, (June 2007), pp.4265-4274, ISSN 0021-9193

Timm, J., Post, F. A., Bekker, L. G., Walther, G. B., Wainwright, H. C., Manganelli, R., Chan, W. T., Tsenova, L., Gold, B., Smith, I., Kaplan, G., & McKinney, J. D. (2003). Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc Natl Acad Sci U S A*, Vol.100, No.24, (November 2003), pp.14321-14326, ISSN 0027-8424

Tucker, P. A., Nowak, E., & Morth, J. P. (2007). Two-component systems of Mycobacterium tuberculosis: structure-based approaches. *Methods Enzymol*, Vol.423, (June 2007), pp.479-501, ISSN 0076-6879

Voskuil, M. I., Schnappinger, D., Rutherford, R., Liu, Y., & Schoolnik, G. K. (2004). Regulation of the Mycobacterium tuberculosis PE/PPE genes. *Tuberculosis (Edinb)*, Vol.84, No.3-4, (June 2004), pp.256-262, ISSN 1472-9792

Waddell, S. J., Stabler, R. A., Laing, K., Kremer, L., Reynolds, R. C., & Besra, G. S. (2004). The use of microarray analysis to determine the gene expression profiles of Mycobacterium tuberculosis in response to anti-bacterial compounds. *Tuberculosis (Edinb)*, Vol.84, No.3-4, (June 2004), pp.263-274, ISSN 1472-9792

Waddell, S. J., Laing, K., Senner, C., & Butcher, P. D. (2008). Microarray analysis of defined Mycobacterium tuberculosis populations using RNA amplification strategies. *BMC Genomics*, Vol.9, (February 2008), pp.94, ISSN 1471-2164

Wilson, M., DeRisi, J., Kristensen, H. H., Imboden, P., Rane, S., Brown, P. O., & Schoolnik, G. K. (1999). Exploring drug-induced alterations in gene expression in Mycobacterium tuberculosis by microarray hybridization. *Proc Natl Acad Sci U S A*, Vol.96, No.22, (October 1999), pp.12833-12838, ISSN 0027-8424

Part 2

Resisting an Agressive Environment

8

Morphological Characterization of *Mycobacterium tuberculosis*

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1. Introduction

It is more than 100 years since the first Mycobacterium was isolated by Hansen (1874). That was leprosy bacillus, *Mycobacterium leprae,* which even today is still resisting all attempts to cultivate it in the laboratory. The tubercle bacillus, *M. tuberculosis was* discovered eight years later by Robert Koch (1882). The Koch discovery was confirmed by more efficient staining models of Ehrlich (1887) and Ziehl- Neelsen (1883). Under Light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from coccobacilli to long rods. The dimensions of the bacilli have been reported to be 1-10 μm in length (usually 3-5 μm), and 0.2 -0.6 μm width. The possibility of morphological variations in tubercle bacilli was suggested by few investigators like Malassez and Vignal (1883), Nocard and Roux (1887), Metschnikoff (1888), Lubarsch(1899), Fischel(1893), and Vera and Rettger (1939). They showed under unfavorable conditions, i.e., a limited food supply, or oxygen deprivation, *Mycobacterium* assumed a swollen appearance without forming the vacuolar or globoid bodies (Vera and Rettger, 1939). These early reports were based on stained preparations and were subjected of severe criticism (Porter and Yegian, 1945). Today with advances in microscopic technique i.e., transmission electron microscope (TEM), scanning electron (SEM) and atomic force microcopy (AFM), almost all of investigators have been agreed that the Koch bacillus does not always manifest itself in the classical rod shape (figure 1). They become shorter in older cultures, filametous within macrophages and ovoid during starvation (Young *et al*., 2005; Farnia *et al*., 2010; Shleeva *et al*., 2011) and they may produce buds (Chauhan *et a*l., 2006) and branches in extensively drug resistance strains (XDR-TB) (Velayati *et al* 2010; Farnia *et al* 2010). In the following parts the underlying mechanisms that may help the bacilli to change its morphology was highlighted.

2. The role of cell wall in shape maintenance

The cell wall of mycobacterium is characterized by a unique structure which is caused by partly distinct chemical compositions in comparison with the cell wall of other bacteria

(Koike and Takeya, 1961; Imaeda and Ogura, 1963; Imaeda *et al*., 1969). These variations are thought to be advantageous in stressful conditions of osmotic shock or desiccation as well as contributing to their considerable resistance to many drugs (Jarlier and Nikaido, 1990). The *Mycobacterial* cell wall, in principal, consists of an inner layer and an outer layer that surround the plasma membrane (Hett and Rubin, 2008). The outer compartment consists of both lipids and proteins (Draper, 1971, 1998; Draper *et al*., 1998; Brennan and Nikaido, 1995; Brennan, 2003). The inner compartment consists of peptidoglycan (PG), arabinogalactan (AG), and mycolic acid (MA) covalently linked together to form a complex known as MA-AG-PG complex that extends from the plasma membrane outward in layers, starting with PG and ending with MAs. The Peptidoglycan, which forms the "backbone' of the cell wall skeleton,was first studied by Misaki *et al* (1966). It belongs to a family of structures possessed by almost all bacteria and blue-green algae but by no other type of living organism (Schleifer and Kandler, 1977); its presence in mycobacteria provides conclusive evidence that they are not, as was once believed, some sort of intermediate stage between bacteria and fungi. The peptidoglycon is made of peptides and glycan strands. The long glycan strand typically consists of repeating N-acetylglucosamines (NAGs) linked to N-acetylmuramic acid (NAM). These strands are cross linked by peptides bound to the lactyl group on NAMs from different glycan strands. These peptide chains normally consist of L-alanyl-D-*iso*-glutaminyl-*meso*-diaminopimelic acid (DAP) from one strand linked to the terminal D-alanine residue from L-alanyl-D-*iso*-glutaminyl-*meso*- DAP-D-alanine from a different strand (Kotani *et al*., 1970; Wietzerbin *et al*., 1974). This highly cross-linked glycan meshwork of PG that surrounds bacteria is the primary agent that maintains bacterial shape. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and unusual high number of cross-links. Indeed, the degree of peptidoglycon cross linking in the cell wall of *M. tuberculosis* is 70-80%, whereas that in *E. coli* is 20-30%. (Matsuhashi, 1994; nVollmer and Holtje,2004). PG isolated from *E. Coli* retains its rod –like shape even in the absence of all other material (Weidel *et al*., 1960; Weidel and Pelzer, 1964), confirming its role in shape maintenance. Also, treatment of bacteria with lysozyme which degrades PG, results in rod shaped cells becoming round spheroplasts (Lederberg, 1956). Spheroplasts, or round bacteria lacking PG, can be formed in *M. smegmatis* through degradation of PG. Upon transfer to growth media, the spherical bacteria are able to regenerate wild-type rod -shaped cells (Udou *et al*., 1982). This occurs through elongation of bacteria that then branch, septate and fragment. These data argue that shape and size are not simply governed by existing PG, but there must be some genetic heritable determinant also.

3. Peptidoglycan synthesize

Little is known about the biosynthesis of the peptidoglycan of *M. tuberculosis.* However, it is generally assumed to be similar to that of *E. coli* (van Heijenoort, 1998). Generally, peptidoglycan synthesis occurs in four sequential steps. First, inside the cytoplasm, soluble substrates are activated and peptidoglycon units are build. Glucosamine is enzymatically converted into MurNAc and then energetically activated by a reaction with uridine triphosphate (UTP) to produce uridine diphosphate –N-acetylmuramic acid (UDP-MurNAc) (De Smet *et al*., 1999). Second, at cytoplasmic membrane, the units UDP-MurNAc pentapeptide is attached to the bactoprenol " conveyor belt", through a pyrophosphate link

with the release of uridine monophosphate (UMP)(Crick *et al*., 2001; Yuan *et al.,* 2007). Third the bactoprenol molecule translocates the disaccharide pentapeptide precursor to the outside of the cell. The GlcNAc-MurNAc disaccharide is then attached to a peptidoglycan chain using pyrophosphate link between itself and the bactoprenol as energy to drive the reaction. The pyrophosphobactoprenol is converted back to a phosphobactoprenol and recycled. Fourth, outside the cell but near the membrane surface, peptide chains from adjacent glycan chains are cross-linked to each other by a peptide bond exchange (transpeptidation) between the free amine of the amino acid in the third position of the pentapepide (e.g., lysine) or the N-terminus of the attached pentaglycine chain and the D-alanine at the fourth position of the other peptide chain, releasing the terminal D-alanine of the precursor (Wietzerbin *et al*., 1974; Ghuysen, 1991).

4. Control of peptidoglycan synthesis

Enzymes involved in remodeling PG can be grouped as either biosynthetic or hydrolytic. Biosynthetic enzymes include transglycosylase and transpeptidase domains, often found on a single, bifuntional protein. Hydrolytic enzymes include muramidase, glucosaminidase, lytic transglycosylase, amidase, endopeptidase and carboxypeptidase (Young, 2003; Cabeen and Jacobs-Wagner, 2005). The reaction of these enzymes may be antagonistic, or they may physically interact to form complexes capable of breaking bonds to generate openings for new monomers, while also forming bonds necessary to unit PG strands. The production of these enzymes should be regulated, otherwise the bacterial cell wall would be degraded and the bacteria would be lyses. There are several ways to governorate these enzymes; one method is through formation of complexes with other proteins (Hett and Rubin, 2008).These proteins could suppress the activity of the enzyme, or they could be enzymes themselves with antagonistic reactions that join rather than degrade PG. Another possibility is that the enzymes are sequestered from their substrate until they needed. A third method could be that the appropriate substrate is not made available until cleavage of it is required.

5. The role of PG in cell shape regulation

The PG –synthesizing enzyme organize into complexes that likely contributes to the resulting shape. Various models have been proposed which explain how this organization affects the bacterial shape. The two –competing sites" model (TCS) for peptidoglycan assembly advocates that, in bacterial rods, the shape depends on the activity of two biochemical reactions (sites) which occur in the terminal stages of peptidoglycan synthesis; one site is responsible for lateral wall elongation , and the other is responsible for septum formation (Lleo *et al*., 1990; Alaedini and Day, 1999). The two sites compete with each other in such a way that the lateral wall is not extended during septum formation and *vice versa* (Lleo *et al*., 1990; Satta *et al* .,1994). The actual shape of the bacteria is thus determined by the balance between the two competing reactions, correct balance leading to normal rods; abnormal prevalence of the site for lateral wall elongation leads to long rods or filaments, whereas prevalence of the site for septum formation leads to formation of coccobacilli or cocci (Lleo *et al*., 1990). The other bacteria carry only one site for peptidoglycan assembly which can form only septa and can grow only as cocci. Another model is "three –for –one " predict the insertion of PG along a track, using an existing strand of PG as a template

(Holtje, 1998). This result in doubling the length in one direction, but since following a strand, no additional length is added in the direction perpendicular to the strand. Thus width would stay constant. Another theory as to how cells maintain a constant width posits that the poles are capped with a type of PG that prevents rapid turnover or insertion of new PG (De Pedro *et al*., 1997). Thus, the caps would restrict the width of the bacterium

6. How the shape remain constant

Uniform cell shapes are favored by the need to segregate the chromosome and cytoplasmic material between daughter cells (Errington *et al*., 2003). The regular shape would seem to be the best way to ensure each daughter, because a symmetrical cell can be halved accurately by mechanisms that measure length or volume (Helmstetter *et al*., 1990; Young, 2006). In an irregular cell, misplaced septation might leave one cell with both chromosomes or with more than its fair share of other components. Therefore, once a particular shape is adapted bacteria have a vested interest in keeping it (Stewart, 2005). The major incentive for doing so is to maintain a consistent relationship between cytoplasmic volume and surface area so that cell cycle events can be coordinated properly. This is visualized by considering the septation event that creates two daughter cells (Harry, 2001; Errington *et al*., 2003). The septum is formed through the in-ward growth of cytoplamic membrane and cell wall material that invaginates from opposing directions at the central plane of the cell. In such case, the concentration of essential division proteins will not change, but the surface area over which they must act will be greater in the sphere. The amounts of these proteins, if optimized for dimensions of a rod , might not be sufficient to initiate or complete normal septation and division in a coccus (Young, 2006). Thus limited concentrations of division proteins will dictate that the cell maintain a specific and constant diameter. To do this, bacteria must coordinate events spatially and temporally. Recently it was shown that the divisome will assemble at midcell, before chromosomes partitioned. The divisome consists of a set of 10 to 15 proteins that are required to the middle of the cell and are responsible for generating the septum that divides two daughter cells (Margolin, 2006; Buddelmeijer and Beckwith, 2002). This is accomplished by synthesizing septal PG, constricting the cell wall to eventually close off the cytoplasmic compartments of each daughter cell, and finally hydrolyzing part of the PG that holds two together in order to physically separate the cells. These divisome proteins (FtsA, FtsB, FtsE, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsX, FtsZ, Zip A, AmiC and EnvC) encoded in different bacterial genomes and have different function (Di Lallo *et al*., 2003; Karimova *et al*., 2005; Vicente and Rico, 2006). The FtsZ is the first protein to assemble at midcell (Bi and Lutkenhaus, 1992). Its formation of a ring around the cell, just under the plasma membrane, gives the assembled divisome the name Z ring. This sub cellular organelle, a functional analog of the contractile ring used in cytokinesis of many eukaryotic cells, is thought to form the scaffold for recruitment of the other key cell division proteins. In *E. coli*, successful cell division depends on a constant and critical concentration of Ftsz combined with proper proportions of Z-ring stabilizing and destabilizing proteins. Significantly, small changes in the concentrations of FtsZ or other essential division proteins disrupt cell growth. Thus, division is inhibited if FtsZ is under produced, extra divisions occur if the protein is overproduced and no division occurs if FtsZ levels are adequate but FtsZ/FtsA ratio is incorrect (Errington *et al*., 2003; Maki *et al*., 2000; Chauhan *et al*., 2006).

7. Shape variation

The tubercle bacillus is a prototrophic (i.e., it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e., it uses already synthesized organic compounds as a source of carbon and energy), metabolically flexible bacterium(Edson, 1951; Ramakrishnan *et al*., 1972; Niederweis, 2008). The success of tubercle bacilli as a pathogen can be attributed to its extraordinary capacity to adapt to environmental changes throughout the course of infection. Generally, the nutritional quality and physical conditions will determine the temporary lifestyle of bacillus. These changes include: nutrient deprivation, hypoxia, temperature, PH, salinity and various exogenous stress conditions (Vera and Rettger, 1939; Smeulders *et al*., 1999; Honer *et al*., 2001; Young et *al*, 2005; Anuchin *et al*., 2009; Velayati *et al,* 2009; Farnia *et al*., 2010; Singh *et al*., 2010; Shleeva *et al.,* 2002, 2010). Unfortunately, in most of cases we do not know if shape *per se* is beneficial, because few experiments have addressed the question. Knowledge of the physiology of *M. tuberculosis* during this process has been limited by the slow growth of the bacterium in the laboratory and other technical problems such as cell aggregation. Recent advances in microscopy techniques have revealed adaptive changes in size and shape of bacilli under

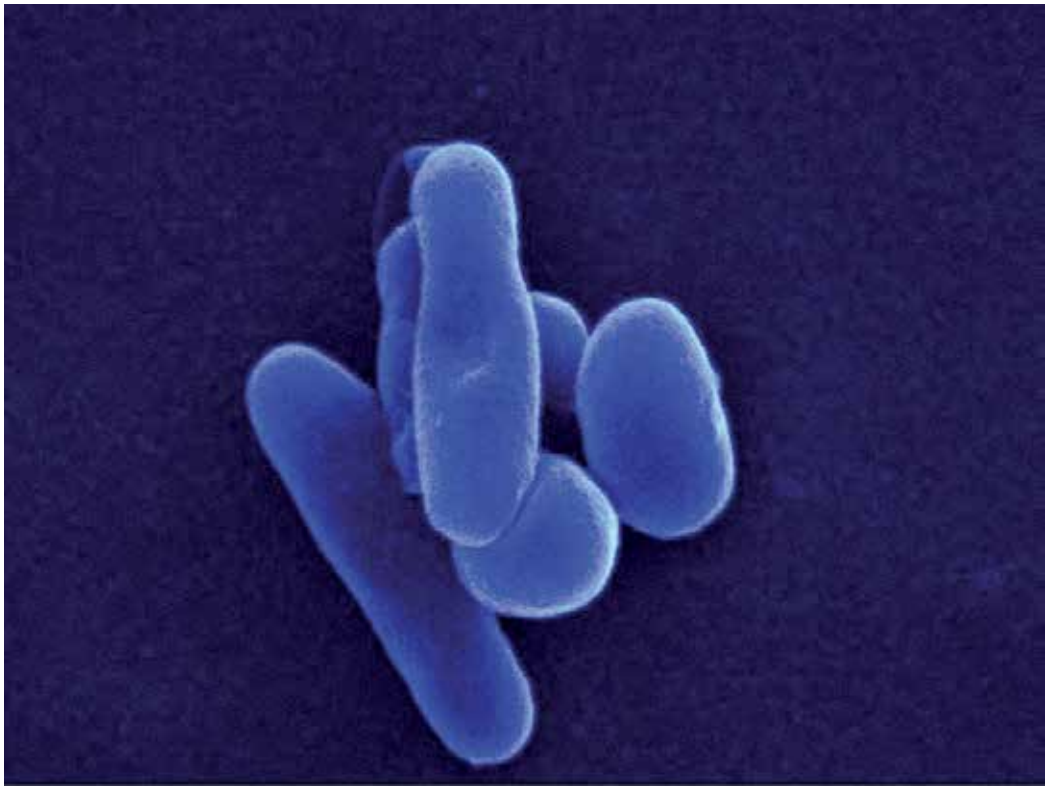

Fig. 1. Scanning electron microscope shows shape variation in *M. tuberculosis* at exponential phase of growth.

stress conditions (Velayati *et al*., 2009,2011; Farnia *et al*., 2010). Briefly, the reported morphological variation in *M. tuberculosis* are classified into two categories; those which frequently seen at exponential phase of growth that is rod, V, Y-shape, branched or buds, and those that are seen occasionally under stress or environmental conditions which are round, oval , ultra-virus, spore like, and cell wall defiant or L-forms.

8. Shape variations during active or exponential phase of growth

The most classical form of tubercle bacilli is a slender rod shape that seen in stained smears. They have smooth, homogenous cytoplasm with clear-cut and well-define outlines. The first electron microscope images of the tubercle bacilli were obtained in 1939 in the laboratories of the Technische Hochschule, Berlin.

von Borries and E. Ruska (1939) published electron micrographs of the avian strain of tubercle bacilli magnified 26,000 times. The cytoplasm of these bacilli contained dark bodies of different sizes. Later on, Lembke and Ruska (1940), culture the bacilli on petragnani medium and observed up to eight large bodies inside the cytoplasm of bacilli. Rosenblatt, Fullam and Gessler (1942) in their studies of tubercle bacilli in the electron microscope, confirmed many earlier observation and added some new data, particularly concerning the internal structure of bacilli. The bacilli varied in size. The size of the strain H_{37} sub-cultured at Columbia University varied from 4.3µ X 0.4µ to 1.0µ X 0.2µ. The cell wall was always present (sometimes it was as thick as 0.03µ) and contained granules. The internal structure showed dense nuclear masses within the granular cytoplasm. The density of the cytoplasm varied; it contained many granules and vacuoles of different sizes. Later on it became clear that the cytoplasm of young cells is dense, the basic dyes stain it deeply and uniformly, and it contains vacuoles and hyper chromic bodies. The cell protoplast was seen surrounded by a 0.023µ thick and ductile cell wall. The cytoplasm itself was covered with a thin cytoplasmic membrane which closely adhered to the cell wall (Rosenblatt *et al*., 1942; Knaysi *et al*., 1950; Werner, 1951; Draper, 1982). In rod like bacilli, the process of cell division resembles that of most grams –positive bacteria (figure, 2). In the equatorial zone of the cell, on the inner side of the cell wall, a double cell plate was formed. The growth of this plate proceeded till the mother cell wall was divided into two daughter cells. The separation of newly formed cells occurred between these plates, which then covered the poles of the right and left cells. Before the cytoplasm divided, the division of cellular bodies was observed (Edwards, 1970; Nishiura *et al*, 1970; Dhal, 2004).

The other types of cell shape (V or Y - shape bacilli) occurs in lower frequency (Dahl, 2004; Farnia *et al* 2010). The V-shape bacilli are caused by snapping post-fission movements (Krulwich and Pate, 1971). The term "snapping division" was first described by Kurth (1898) and has been reported by many other investigators. Upon completion of cell division, one or both of the two daughter cells suddenly swing around, bringing their distal ends closer together while still remaining attached by a small region at their proximal ends. The exact mechanism responsible for snapping postfission movements is not clear. Bisset (1955) claimed that all so-called postfission movements were nothing but artifacts due to mechanical stress on the dividing cells (e.g., cells growing between solid agar and a cover slip) and would not occur if the same cells were grown in liquid cultures. Sguros (1957)

suggested that V-forms resulted from "germ tube extrusions" from each of a pair of attached arthrospores and were not due to postfission movements. More studies have demonstrated that snapping division or V-forms could arise by any of three methods: (I) germination of adjacent coccoid elements, (ii) subpolar germination (budding) of rods, and (iii) snapping postfission movements (Starr and Khan, 1962). In mycobacterium, during septum formation the plasma membrane and inner cell wall grow inward but the outer cell wall layer remains intact. Upon completion of septum formation with a cross-wall, the inner layer may continue to grow and thus exert pressure upon the outer cell wall layer. The outer layer eventually ruptures first on one side of the cell, and the two daughter cells bend in on the side where the outer layer is still intact forming a "V-form (Dahl,2004; Farnia *et al*,2010; Malhotra *et al*., 2010)

Mycobacterium is known to form a "Y-shaped "cells with branches more interior to the cells and of greater length figure 3. Brieger *et al* in 1954, was among the first scientist who demonstrate the branching in the reproductive cycle of *M. avium*. He showed that young culture of bacilli when first transplanted to fresh medium it consists mainly of short coccoid rods. These elongate into filaments (8-10μ) which continue to divide and grow during a phase of filamentous proliferation. The filaments usually have two fully

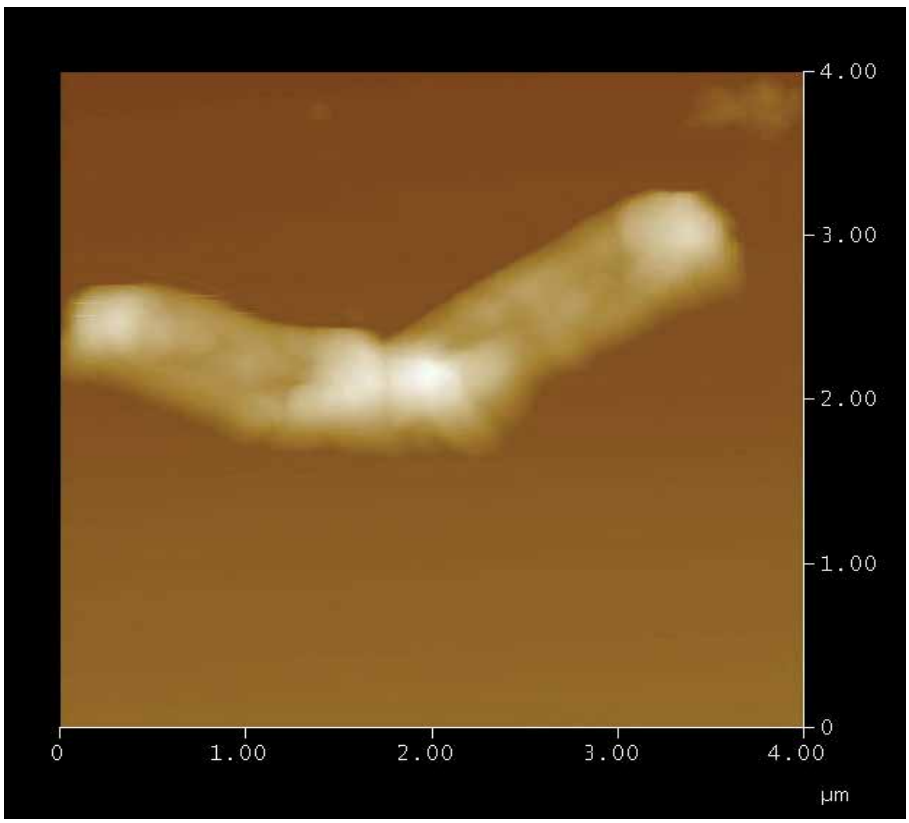

Fig. 2. Atomic force microscopy shows the V-shape *M. tuberculosis* during exponential phase of growth

developed dense bodies in polar positions and in some organisms a number of smaller are also seen scattered among the cellular units and apparently associated with them. The final stage in the reproductive cycle led to a massive production of small rods. At this phase the filaments suddenly break down into masses of short rods which elongate to form the new generation and the cycle is complete. Under electron microscope, it was seen that the filaments were quite separate, and there was no true branching and that the mycelia appearance was produced because the filaments often remained stuck together. In another study, Mizuguchi Y *et al* (1985) showed β-Lactam antibiotics at low concentration induced filamentous cells in the *M. avium-intracellular* complex. Although, the mechanisms of induction of filamentous cells appeared to be different according to the drugs used. Ampicillin induces filaments by inhibiting the septation in a manner similar to its effect on *E. coli,* whereas cephazolin induces filaments but does not inhibit septation. In *M. tuberculosis,* branches were first seen as a small bud that does not grow to any appreciable size before breaking off as a separate cell. Few studies suggested that *M. tuberculosis* grows from the ends of bacilli and not along the length of the cylinder as seen in other well-characterized rod shape bacteria (Thanky *et al*., 2007). This might be true for susceptible isolates, but recently Farnia *et al* (2010) showed that in highly drug resistance strains i.e., XDR-TB and Totally or Extremely drug resistant isolates (TDR or XXDR-TB), branches produce along the cylinder. In fact, about 20 -24% of cells in XDR and XXDR-TB bacilli were dividing by branching, respectively.

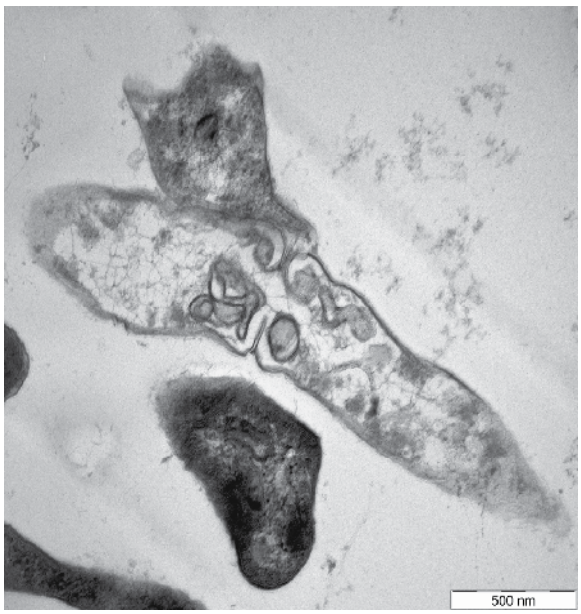

Fig. 3. Transmission Electron Microscopy shows Y-Shape *M. tuberculosis* at exponential phase of growth

9. Cell shapes during dormancy or under limited conditions

The morphological variations in tubercle bacilli become evident when the culture medium was poor. These changes were first reported by Koch himself. In his paper on the "discovery

of the cause of tuberculosis", he described that "under certain conditions, some bacilli contain several spores, in most cases there are two to four of them; oval in form, they are distributed, in uniform intervals, along the axis of the bacilli(1882). Following Koch discovery, Malassez and Vignal (1883) had described, the small "coccoid bodies " which cause tuberculosis infection and named them as cell wall deficient forms (CWD-forms) of tuberculosis. Later on, Spengler (1903, 1905), were among the first scientist who could demonstrated that in older cultures and frequently in sputa, apparently in response to adverse environmental conditions, the smooth cell takes on a fragmented appearance. Much (1907) was able to reproduce granules in the inside of the bacilli as well as scattered around them. These granules, according to Much, cannot be stained by the Ziehl- Neelsen technique but may generate new tubercle bacilli. Later on 1909, Fontes revealed how he had applied double staining to the bacilli, namely Ziehl- Neelson's carbolfuchsin staining and the Gram treatment. In this way he tried to differentiate the pathogenic tubercle bacilli, containing Much granules, from the apathogenic ones without these granules. In 1910, Fontes described the multiplication through division of these granules in the inside of a cell and on its outside and applied the term "virus" to this formation. Fontes described the application to the tubercule bacillus of the well –known method of separating the virus from the substrate by filtering the material through a bacterial filter. He inoculated a guinea pig with the filtered caseous material and transplanted the organs of this animal into a fresh one. When after five months of observation the animal was killed, the autopsy revealed the infiltration of round cells, granules, and occasional acid-fast bacilli in the lymph nodes and the lungs. After years of oblivion, the early works of Fontes were rediscovered by Vandremer (1923). He repeated the Fontes filtration experiments and confirmed the development of acid –fast bacilli on media and in animals inoculated with these filtrates. Calmette (1926) advanced the theory on the role of the the tuberculosis " ultra-virus" in the development of certain forms of the diseases. However, Negre *et al* (1933) denied the existence of filterable forms of the mycobacteria. Few years later, Vera and Rettger (1939) studied four strains of *M. tuberculosis*(hominis), "Koch", 607, 75 and H_{37} in micro-culture by Hill hanging block technique. This method was employed to permit observation of individual cells and their progeny over long periods of time using lucida drawings camera. They could demonstrate various forms which have been described in the literature at one time or another. When they cut off air supply, different variants developed very soon. The bacilli swelled slightly, the cytoplasm become less clear and smooth. The swelling commonly occurred at the ends of cells, so the clubs and dumbbell shapes were formed; cells often became spoon shaped. These swollen structures became increasingly refractive and more sharply delimited, until finally there was a definite superficial resemblance to spores. At the similar time, the ability of the tubercle bacillus to survive environmental hardship in culture was documented by Corper and Cohn in a study published in 1933. In another study, McCune and other colleagues (1965, 1966), showed the capacity of tubercle bacilli to survive in mouse tissue after sterilization. In this model, out bred mice were infected intravenously with 105 colony-forming units of the $H_{37}R_v$ strain of *M. tuberculosis*. They were immediately treated for a period of 12 weeks with the antimycobacterial drugs isonizid (INH) and pyrazinamide (PZA). For 4-6 week period after withdrawal of therapy, the mice showed no evidence of cultivable tubercle bacilli (sterile state). But, 12 weeks after INH and PZA treatment was withdrawn, one-third of the mice developed full-blown active TB, with nearly two-thirds

displaying disease after 24 weeks. Csillag (1962, 1963, and 1964) considered Mycobacteria as dimorphic organisms in the same sense as are some pathogenic fungi, for instance, *Histoplasma capsulatum*. The usual acid fast form of the mycobacteria was termed 'form I' and the form which was not acid fast was termed 'form 2'. When form 2 grown in digest broth, form 2 strains produced cocci which continued to multiply by binary fission and bud formation (Csillag, 1964).

These forms were not produced by mycobacteria grown in rich media such as nutrient broth; Martin's digest broth, yeast extract and Lab-Lemo beef extract. One year later, Stewart-Tull (1965) isolated two forms of mycobacteria and mycococci from *M. phlei* .Nyka W in 1963, described them as "chromophobic tubercle bacilli" in the lungs of patients treated by drugs in association with surgery. This organism morphologically were similar to the acid- fast bacilli, but do not stain with either carbolfuchsin or the counter stains when applied by the classic Ziehl-Neelsen technique or with any other aniline dye. In continuation of his work, he submitted the culture of *M. tuberculosis*, *M. kansasii*, and *M. phlei* to starvation. As a result they lost first their acid fastness, but in this chromophobic state, they survived for at least 2 years, and after that time, produced cultures of acid fast bacilli when transferred onto nutrient media. Since these *in-vitro* bacilli could recover their original biological properties, it was concluded that those bacilli in the lung could also become reactivated and cause a relapse of the disease. Some scientists regard the filterable forms of mycobacteria as being analogous to the so –called L-forms of the other bacterial genera as they also pass through filters (Thacore and Willett, 1963). Some other scientists believe that development of the L-form is a mutation process, while development of the filterable forms is an adaptation of the micro-organisms to enable them to multiply in unfavorable (Imaeda, 1974; Mattman, 1970: Ratnam and Chandrasekhar, 1976). In this regards, Takahashi (1979), reported that tubercle bacilli in caseous lesions seems to be non acid fast, gram negative granules which may revert into acid fast rods, when the caseous lesion begins to liquefy and form tuberculous cavity. Similarly, khomenko and colleagues (1987) showed ultra-fine forms of *M. tuberculosis* in the walls of open cavities in the lungs of experimental animals by electron microscopy. These invisible forms of *M. tuberculosis* are able to revert to the typical bacterial forms. The initial stage of this process is accompanied by the formation of coccoid forms of mycobacteria that can be detected when material is inoculated on to semi-synthetic medium with 10% plasma and by microscopy of the sediment. Lawrence Wayne (1994) postulated that bacilli recovered from granulomatous lesions had adapted to a relatively oxygen starved environment so that they would be unable to grow in an aerated culture and therefore, may be non-cultivable by traditional culture methods (Wayne and Hayes, 1996). In the Wayne model, cultures of the bacterium are subjected to gradual self-generated oxygen depletion by incubation in sealed stirred tubes. Upon the slow shift of aerobic growing *M. tuberculosis* to anaerobic conditions, the culture is able to adapt and survive anaerobiosis by shifting down to a state of nonreplicating persistence. Wayne L showed two phase of growth in mycobacterium under limited oxygen; initially when the level of drops and the turbidity increased in culture tubes (NRP-1) and in anaerobic phase when there is no oxygen and no division (NRP-2). Wayne model was a break through in understanding what may happen to tubercule bacilli in necrotic material (Wayne and Lin, 1982). Although, Kaprelyants *et al* (1993) did not consider the bacilli

obtained by Wayne and Sramek (1994) as dormant because they maintained a high viability and developed sensitivity to metronidazole when anaerobic, thus indicating active metabolism. Therefore, from large accumulated data that found in literature, it become clear that *M. tuberculosis* can adapt rapidly to changing environment inside and outside the host (Parrish *et al*., 1998; Cardona, 2009; Rustad *et al*., 2009). These capacities will allow the tubercle bacilli to survive for long time in a dormant state in the lung tissue. Recently, Peyron *et al* (2008) developed an *in vitro* model of human tuberculosis granulomas. In this model granuloma-specific cell types and their modulation by tubercle bacilli were characterized. More recently, the complete morphological changes that occurs in tubercle bacilli under hypoxic conditions viewed under AFM (every 90 days for 48 months) (Velayati *et al*., 2011). The morphological adaptation classified into two categories; First was temporary adaptation (from 1 to 18 months of latency) in which cells undergoing thickening of cell wall (20.5±1.8 nm versus 15.2±1.8 nm, $P<0.05$), formation of ovoid cells by "folding phenomena"(65-70%), size reduction (0.8± 0.1 µm versus 2.5±0.5 µm), and budding type of cell division (20-25%) (figure 4).

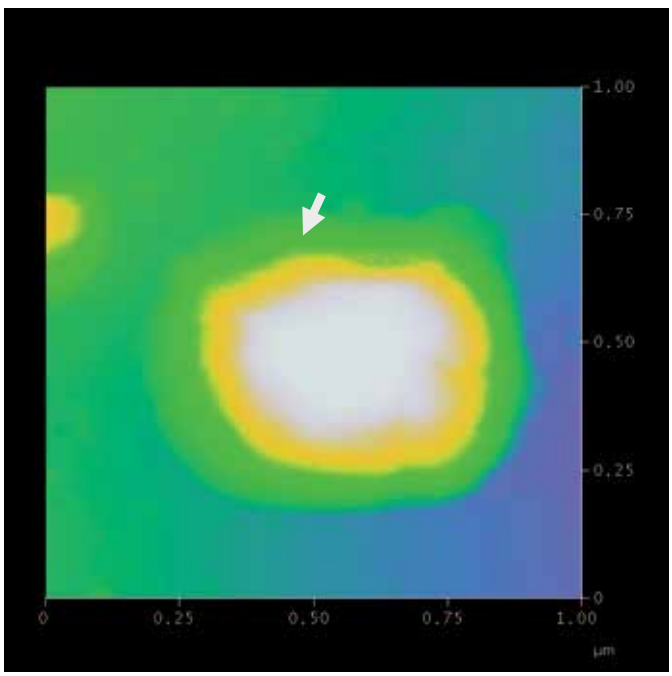

Fig. 4. Atomic force Microscopy shows *M. tuberculosis* under 8 months hypoxic condition. The bacilli becomes round and developed a thickened cell-walls (shows by arrows)

A second feature include changes that accompany development of specialized cells (from 18 to 48 months of latency) i.e., production of spore like cells (0.5 ± 0.2 µm) and their progeny (filterable non -acid fast forms; 150 to 300 µm in size figure 5). Using AFM, they could demonstrate that the filterable non-acid fast forms of bacilli are produced from spore –like cells. These cells were metabolically active and increased their number by symmetrical typing of division and could be stain by gram staining. Inoculation of these cells could induce active tuberculosis in mice. Although, it is important to determine how closely the

in vitro models correlate to the state of *M. tuberculosis* during latent infection. But, if these models are predictive of human disease, the information they provide in combination with advances in animal models, imaging and analysis will substantially aid in the development of drugs capable of killing tubercle bacilli in altered metabolically states, and possibly shortening the course of TB therapy.

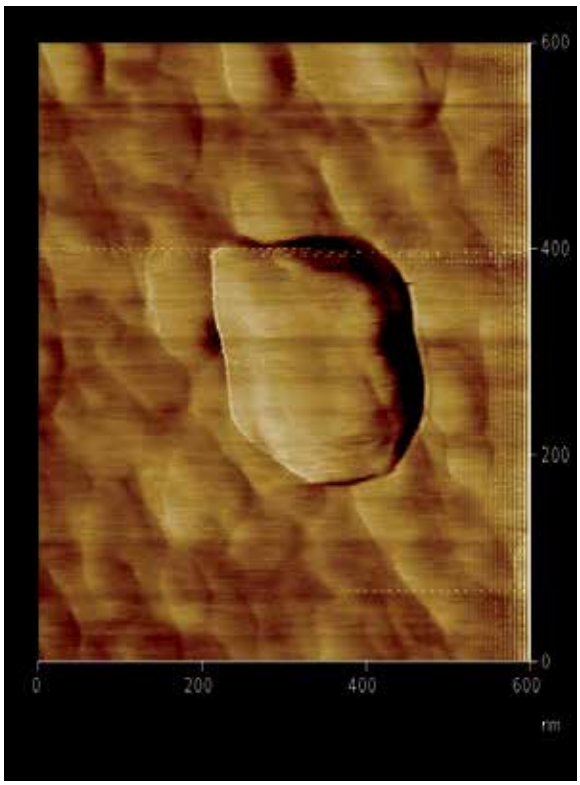

Fig. 5. Atomic force microscopy shows the Latent TB bacilli, after 48 months of latency (Velayati *et al*., 2011).

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11. References

Alaedini, A., and Day, R.A. 1999.Identification of two penicillin-binding multienzyme complexes in Haemophilus influenza. Biochem.Biophys.Res.Commun.264:191-195.

Anuchin, A.M., Mulyukin, A.L., Suzina, N.E., Duda, V.I., El-Registan, G.I., and A.S.Kaprelyants. 2009.Dormant forms of Mycobacterium smegmatis with distinct morphology. Microbiol.155:1071-1079

Bi, E., and J.Lutkenhaus. 1992. Isolation and characterization of ftsZ alleles that affect septal morphology. J. bacteriol. 174:5414-5423.

Bisset, K.A.1955. The cytology and life history of bacteria, 2nd ed. The Williams &Wikins Co., Baltimore.

Brieger, E.M., Cosslett, V.E., and Glauert, A.M. 1954. Reproductive changes in Avian tubercle bacilli studied with electron microscope. J. Gen. Microbiol. 10:294-303.

Brennan, P.J. 2003. Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis(Edinburgh) 83:91-97.

Brennan, P.J., and H. Nikaido. 1995. The envelope of mycobacteria. Annu. Rev. Biochem.64:29-63.

Borries, B.von, and Ruska, E. VolÖufige Mitteilung uber Fortchritte im Bau und in der Leistung des Ubermikroskopes(228), 1938, 17, 99.

Buddelmeijer, N., and J. Beckwith. 2002.Assembly of cell division proteins at the *E.Coli* cell center. Curr. Opin. Microbiol. 5:553-557.

Cabeen, M.T., and Jacobs-Wagner, C. 2005. Bacterial cell shape. Nat.Rev.Microbiol. 3:601-610.

Calmette, A.1924. Existe-t-il dans la nature ou peut-on créer artificiellement des forms saprophytiques du bacilli de Koch qui soient capable de se transformer en bacilles tuberculeux virulent. 5, 716.

Cardona, P.J. 2009. A dynamic re-infection hypothesis of latent tuberculosis infection. Infection. 37:80-86.

Ratnan, S., and S.Chandrasekhar. 1976. The pathogenicity of spheroplasts of *Mycobacterium tuberculosis*. Amer. Rev. Respir. Dis. 114: 549-54

Chauhan, A., Madiraju, M.V., Fol, M., Lofton, H., Maloney, E., Reynolds, R., and M. Rajagopaln. 2006. Mycobacterium tuberculosis cells growing in macrophages are filamentous and deficient in FtsZ rings. J bacterial. 188:1856-65

Crick, D.C., Mahapatra, S., and P.J.Brennan. 2001. Biosynthesis of the arabinogalactan – peptidoglycan complex of Mycobacterium tuberculosis. Glycobiol.11: 107-118.

Cook, G. M., Berney, M., Gebhard, S., Heinemann, M., Cox, R.A., Danilchanka, O., and M. Niederweis. 2009. Physiology of mycobacteria. Advan. Microbial physiol. 55:82-110.

Corper, H.J., and M.L.Cohn.1933. The viability and virulence of old cultures of tubercule bacilli:studies on twelve-year broth cultures maintained at incubator temperature .Ann. Rev.Tuberc.28:856-874.

Csillag, A. 1962. Development of form 2 mycobacterium on autoclaved Lowenstein-jensen medium.Tubercle.43:439-433.

Csillag, A. 1963.Cellular morphology of form 2 mycobacteria on slide culture . J. Gen.Microbiol, 30:21-27.

Csillag, A. 1964. The mycococcus form of mycobacteria. J. Gen.Microbiol. 34:341-352.

Dahl, J.L. 2004. Electron microscopy analysis of Mycobacterium tuberculosis cell division. FEMS Microbiol.lett.240:15-20.

Di Lallo, G., Fagioli, M., Barionovi, D., Ghelardini, P., and L, Paolozzi. 2003. Use of a two – hybrid assay to study the assembly of a complex multicomponent protein machinery :bacteria septosome differentiation .Microbiol. 149:3353-3359.

De Pedro, M.A., Quintela, J.C., Holtje, J.V., and H. Schwarz. 1997. Murein segregation in Escherichia coli. J. Bacteriol. 179:2823-2834.

DeSmet, K.A., Kempsell, K.E., Gallagher, A., Duncan, K., and.D.B.Young. 1999. Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from *Mycobacterium tuberculosis*. Microbiol. 145:3177-3184.

Draper, P. 1971. The walls of Mycobacterium lepraemurium: chemistry and ultrastructure. J.Gen. Microbiol .69:313-324.

Draper, P., Kandler, O., and A.Darbre. 1987. Peptidoglycan and arabinogalactan of Mycobacterium leprae. J.Gen. Microbiol 133:1187-1194.

Draper, P. 1998. The outer parts of the mycobacterial envelop as permeability barriers. Front Biosci. 3:D1253-D1261.

Edson, N.L. 1951. The intermediary metabolism of the mycobacteria. Bacteriol Rev. 15:147-182

Edwards, R. P. 1970. Electron –microscope illustrations of division in Mycobacterium leprae. J. Med. Microbiol. 3:493-499.

Ehrlich P. 1887. Uber die Methylenblaureaktion der lebenden Nervensubstanz. 6:214.

Errington, J., Daniel, R.A., and D.J. Scheffers. 2003. Cytokinesis in bacteria. Microbiolo. Mol. Biol. Rev. 67:52-65.

Farnia P, Masjedi MR, Farnia P, Merza MA, Tabarsi P, Zhavnerko GK, Ibrahim TA, Kuan HO, Ghanavei J, Farnia P, Ranjbar R, Poleschuyk NN, Titov LP, Owlia P, Kazampour M, Setare M, Sheikolslami M, Migliori GB, Velayati AA. 2010. Growth and cell –division in extensive (XDR) and extremely drug resistany (XXDR) tuberculosis strains: transmission and atomic force observation. Int. J. Clin. Exp. Med . 3:320-326

Fischel, F. 1893. Zur Morphologie und Biologie des Tuberkelbacillus.Klin. Wochschr. 30:989-993.

Fontes, A.1909. Estudos sobre a tuberculoze.1:51.

Fontes, A. 1910. Bemerkungen über die Tuberkulose infktion und ihr virus.Portuguese-German text.Memorias Do Institute Oswal-do Cruz, Rio de Janiero.2:141.

Ghuysen, J.M. 1991. Serine beta-lactamases and penicillin–binding proteins .Annu. Rev. Microbiol. 45:37-67.

Harry, E.J., and P.J.Lewis.2003. Early targeting of Min proteins to the cell poles in germinated spores of Bacillus subtilis: evidence for division apparatus-independent recruitment of Min proteins to the division site. Mol.Microbiol.47:37-48.

Hett, E.C and E.JRubin. 2008. Bacterial growth and cell division: a mycobacterial Perspective.Microbiol.Mol.Biol.Rev.72:126-156.

Holtje, J.V. 1998. Growth of the stress –bearing and shape –maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. 62:181-203.

Helmstetter, C.E., and A.C. Leonard. 1990. Involvement of cell shape in the replication and segregation of chromosomes in *Escherichia coli*.Res.Microbiol.141:30-39.

Honer, Z.U., Bentrup, K., and D.G. Russell. 2001. Mycobacterial persistence : adaptation to a changing environment. Trends. Microbiol. 9:597-605.

Imaeda, T., Kanetsuna, F., Rieber, M., Galindo, B., and I.M. Cesari. 1962. Ultrastructural characteristics of mycobacterial growth. J. Med. Microiol. 2:181-186

Imaeda, T., and M. Ogura. 1963. Formation of intracytoplasmic membrane system of mycobacteria related to cell division. J. Bacteriol. 85:150-163.

Imaeda, T.1974.Ultrastruture of L-phase variants isolated from a culture of *Mycobacterium phlei*. J.Med.Microbiol.8:389-405.

Jarlier, V., and H.Nikaido. 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium cheloni*. J Bacteriol. 172:, 1418-1423.

Karimova, G., Dautin, N, and D. Ladant. 2005. Interaction network among Escherichia coli membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. J. Bacteriol. 187:2233-2243

Kaprelyants, A.S., Gottschal, J.C., and D.B, Kell.1993. Dormancy in non-sporulation bacteria. FEMS Microbio Rev.10:271-285.

Knaysi, G., Hillier.J., and C. Fabricant. 1950. The cytology of an avian strain of mycobacterium tuberculosis studied with the electron and light microscope. 60:423

Khomenko, A.G. 1987. The variability of Mycobacterium tuberculosis in patients wth cavitary pulmonary tuberculosis in the course of chemotherapy.Tubercle.68:243-253.

Khomenko, A.G., Fadeeva, N.I., and V. I. Golyshevskaya. 1983. Morphological and biochemical changes in *M.tuberculosis* during chemotherapy. Problems of Tuberculosis, 7: 48-53

Koch, R. 1882. Die Aetiologie der Tuberculose. Berlin Klinische Wochenzeitscrift 19:221-226

Koike, M., and K. Takeya. 1961. Fine structure of intra-cytoplasmic organelles of mycobacteria. Journal of Biophysical and Biochemical Cytology.9:597-608.

Kotani, S., Yanagida, I., Kato, K, and T.Matsuda. 1970. Studies on peptides, glycopeptides and antigenic polysaccharide-glycopeptide complexes isolated from an L-11 enzyme lysate of the cell walls of Mycobacterium tuberculosis strain H_{37}RV. Biken Journal. 13:249-275.

Krulwich, T. A, and J. L Pate. 1971. Ultrastructural explanation for snapping postfission movements in Arthrobacter crystallopoietes. J. Bacteriol. 105:408-412.

Kurth, H. 1898.über die Diagnose des Diphtheriebacillus unter Berücksichtigung abweichender Culturformen desselben. Z.Hyg. Infektionskr. Med.Mikrobiol. Immunol.Virol, 28:409-439

Lederberg, J. 1956. Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. USA. 42:574-577.

Lembke, A., and H.Ruska. 1940. Vergleichende mikroskopische und übermikroskopische Beobachtungen an den Erregern der Tuberkulose. 19:217

Lieo, M. M., Canepari, P., and G, Satta. 1990.Bacterial cell shape regulation :testing of additional predictions unique to the two-competing-sites model for peptidoglycan assembly and isolation of conditional rod-shaped mutants from some wild-type cocci.J.bacteriol.172:3758-3771.

Lubarsch, O. 1899. Zur Kenntniss der Strahlenpilze. Z.Hyg.Infektionskrankh.31:187-220.

Mc Cune, R., and R.Tompsett. 1956. Fate of Mycobacterium tuberculosis in mouse tissue as determined by the microbial enumeration technique. J .Exp. med .104:737-762.

McCune, R.M., Feldmann, F.M, Lambert, H.P., andW.McDermott.1966.Microbiol persistence .I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J.Exp.Med. 123:445-468

Malassez, L., and W, Vignal. 1883. Sur le micro-organism de la tuberculose zooleique .Arch . Physiol. norm. path III, 4:81-105.

Malassez, L., and Vignal, W.1883. Sur le micro-organisme de la tuberculose zoogleique.Arch.Physiol.norm.path., III, 4, 81-105

Malhotra, S., Bhatia, N.K., Kaushal, M., Kaur, N., and A.Chauhan.2010. Pleomorphic appearance in Mycobacterium tuberculosis. J. pub. Health. Epid. 2:11-12

Maki, N., Gestwicki, J. E., Lake, E. M., Kiessling, L. L., and J. Adler. 2000. Motility and chemotaxix of filamentous cells of *Escherichia coli.* J.Bacteriol. 182:4337-4342.

Margolin, W. 2006. Bacterial division: another way to box in the ring. Curr. Biol. 16: R881-R884.

Matsuhashi, M. 1994. Utilization of lipid –linked precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation, P55-72 .In J.M. Ghuysen and R. Hakenbeck (ed). Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.

Mattman, L.H.1970. Cell wall deficient forms of Mycobacteria.Ann.N.Y.Acad.Sc.174:852.

Metschnikoff, E. 1888. Uber die phagocytare Rolle der Tuberkelriesenzellen. Virchow's Arch .Path. Anat. 113:63-94.

Misaki, A., Yukawa, S., Tsuchiya, K., and T, Yamasaki. 1966. Studies on cell walls of Mycobacteria.1. Chemical and biological properties of the cell walls and the mucopeptide of BCG. J. Biochem. Japan 59:388-396.

Mizughchi, Y., Ogawa, M., and T.Udou. 1985. Morohological changes induced by B-Lactam antibiotics in Mycobacterium avium –intracellular complex. Antimicro. Agents . Chem. 27:541-547.

Much, H.1907. über die granuläre, nach Ziehl nicht färbbare Forms des Tuberkulosevirus. 8:, 85.

Niederweis, M. Nutrient acquisition by mycobacteria. 2008.Microbiol .154:679-692.

Nishiura, M., Izuumi, S., Mori, T., Takeo, K., and T.Nonaka.1977. Freeze-etching study of human and murine leprosy bacilli. Inter.J.Lepro.45:248-254.

Neelsen F.1883. Ein casuistischer Beitrag zur Lehre von der Tuberculose. 28:497.

Negre, L., Valtis, J., and F.Van Deinse.1933.Sur les caracteres biologiques des bacilles acido-resistant issus des elements filtrables du virus tuberuleux.Comptes Rendus de la Societe de la Biologie.112:122.

Nocard, M.E., and E, Roux. 1887. Surla culture du bacilli de la tuberculose. Ann.inst.Pasteur, 1:19-29.

Nyka, W.1963. Studies on mycobacterium tuberculosis in lesions of the human lung.A new method of staining tubercle bacilli in tissue sections.Amer.Rev.Resp.Dis.88:670-679.

Nyka, W. 1974. Studies on the effect of starvation on Mycobacteria. Infect.Immun. 9:843-850.

Parrish, N.M., Dick, J.D., and W.R.Bishai. 1998. Mechanisms of latency in Mycobacterium tuberculosis. Trends. Microbiol. 6:107-112

Peyron, M., Vaubourgeix, J., Poquet, Y., Levillain, F., Botanch, C., Bardou, F., Daffe, M., Emile, J.F., Marchou, B., Cardona, P.J., de Chastellier, C., and A.Frèdèric.2008. Foamy

Macrophages from tuberculosis patients granulomas constitute a nutrient-rich reservoir for M.tuberculosis persistence.PLOS Pathogens. 4:1-14.

Porter, K.R and Yegian, D. 1945.Some artifacts encountered in stained preparations of tubercle bacilli .J.Bacteriol.50:563-574.

Ramakrishnan, T., Murthy, P.S., and K.P.Gopinathan. 1972. Intermediary metabolism of mycobacteria. Bacteriol Rev. 36:65-108.

Rosenblatt, M.B., Fullam, E.F., and A.E.Gessler. 1942.Studies of Mycobacteria with electron microscope.Am.Rev.Tuberc.46:587.

Rustad, T.R., Sherrid, A.M., Minch, K, J., and D. R. Sherman.2009. Hypoxia: a window into *Mycobacterium tuberculosis* latency. Cell.Microbiol.11:1151-1159.

Satta, G., Fontana, R., and P.Canepari. 1994. The two –competing site (TCS) model for cell shape regulation in bacteria : the envelope as an integration point for the regulatory circuits of essential physiological events, Adv. Microb. Physiol. 36:181-245.

Schleifer, K.H., and O.Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407-477

Singh, B., Ghosh, J., Islam, N, M., Dasgupta, S., and L.Kirsebom.2010.Growth, cell division and sporulation in mycobacteria. Antonie van Leeuwenhoek.98:165-177.

Shleeva, M.O., Bagramyan, K., Telkov, M.V., Mukamolova, G.V., Young, M., Kell, D.B., and A.S.Karprelyants.2002. Formation and resuscitation of 'non-culturable' cells of Rhodococcus rhodochrous and Mycobacterium tuberculosis in prolonged stationary phase. Microbiol. 148:1581-1591.

Shleeva, M.O., Kudykina, Y.K., Vostroknutova, G.N., Suzina N.E., Mulyukin, A. L., and, A.S.Kaprelyants. 2011. Dormant ovoid cells of M. tuberculosis are formed in response to gradual external acidification. Tubercle . 91:146

Smeulders, M.J., Keer, J., Speight, R.A., and H.D.Williams.1999.Adaptation of mycobacterium smegmatis to stationary phase. J.Bacteriol.181:270-283.

Sguros, P.L.1957.New approach to the mode of formation of classical morphological configurations by certain coryneform bacteria. J.Bacteriol.74:707-709.

Spengler, C. 1903. Tuberkelbacillenzüchtung aus Bakteriengemischen und Formaldehydde sinfection. 42:93

Spengler, C.1905. Zur Formaldehyd-AbtÖtung und Züchtung der Tuberkel-und anderer säure-festen Bacillen.51:335

Starr, M. P., and D.A.Khan. 1962. On the origin of V-forms in *Arthrobacter atrocyaneus.* Arch.Mikrobiol. 42:289-298.

Stewart, G. C. 2005. Taking shape: control of bacterial cell wall biosynthesis. Mol. Microbiol. 57:1171-1181.

Stewart-Tull, D.E.S.1965. Occurrence of dimorphic forms of Mycobacterium phlei. Nature, London 208:603-605.

Takahashi, S.L. 1979. Phase growth of mycobaclena.II.Consideration on the survival of tubercle bacillus in the caseous lesion. Kekkaku. 54:231-236.

Takeya, K., Mori, R., Tokunaga, T., Koike, M., and K.Hisaysune. 1961.Further studies on the paired fibrous structure of mycobacterial cell wall. J. Biophys.& Biochem. Cytol. 9:496-501.

Thacore, H., and H.P.Willett. 1963. Formation of spheroplasts of *M. tuberculosis* by lysozyme treatment .Proceedings of the Society for Experiment Biology and Medicine.114:43-47.

Thanky, N.R., Young, D.B., B.D. 2007.and Robertson. Unusual features of the cell cycle in Mycobacteria: Polar –restricted growth and the snapping –model of cell division. Tubercle. 87:231-236.

Udou, T., Ogawa, and Y.Mizuguchi.1982. Spheroplast formation of Mycobacterium Smegmatis and morphological aspects of their reversion to the bacillary form. J. Bacteriol.151:1035-1039.

Vaudremer, A. 1923.Formers filtrants des bacilles tuberculeux. 89:80

van Heijenoort, J. 1996. Murein synthesis.In Neidhardt, F.C(ed), Escherichia coli and Salmonella; cellular and molecular biology, ASM Press, Washington, D.C., pp1025-1034.

Velayati, A.A., Farnia, P., Masjedi, M.R., Ibrahim, T.A., Tabarsi, P., Haroun, R.Z., Kuan, H.O., Ghanavi, J., Farnia, P., and M.Varahram. 2009. Totally drug-resistant tuberculosis strains: evidence of adaptation at the cellular level. Eur Respir J. 34:1202-1203.

Velayati, A.A., Farnia, P., Ibrahim, T.A., Haroun, R.Z., Kuan, H.O., Ghanavi, J., Farnia, P., Kabarei, A.N., Tabarsi, P., Omar, A.R., Varahram, M., and Masjedi, M.R.2009. Differences in cell wall thickness between resistant and nonresistant strains of

Mycobacterium tuberculosis: using transmission electron microscopy. Chemo. 55:303-7

Velayati, A.A., Farnia, P., Merza. M.A., Zhavnerko, G.K, Tabarsi, P., Titov, L.P, . Ghanavei, J., Farnia, P., Setare, M., Poleschuyk, N.N., Owlia, P., Sheikolslami, M., Ranjbar, R., Masjedi, M.R. 2010. New insight into extremely drug-resistant tuberculosis: using atomic force microscopy. Eur .Respir. J. 36: 1490-3.

Velayati, A.A., Farnia, P., Masjedi, M.R., Zhavnerko, G.K., Merza, M.A., Ghanavei, J., Tabarsi, P., Farnia, P., Poleschuyk, N.N., and G.Ignatyev.2011. Sequential adaptation in latent tuberculosis bacilli: observation by atomic force microscopy (AFM). Int J Clin Exp Med 2011;4(in press).

Vera, H.D., and L.F. Rettger.1940. Morphological variations of the tubercle bacillus and certain recently isolated soil acid fasts with emphasis on filterability. J.Bacteriol.39:659-687.

Vicente, M., and A.I.Rico. 2006. The order of the ring: assembly of *Escherichia coli* cell division components .Mol.Microbiol.61:5-

Vollmer, W., and J.V. Holtje .2004.The architecture of the murein (peptidoglycan) in gram – negative bacteria :vertical scaffold or horizontal layer(s). J.Bacteriol.186:5978-5987.

Wayne, L.G.1994. Dormancy of Mycobacterium tuberculosis and latency of disease. Eur.J.Clin.Microbiol. Infect. Dis. 13:908-914.

Wayne, L.G., and L.G.Hayes.1996. An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence . Infect. Immun. 64:2062-2069.

Wayne, L.G., andH.A.Sramek.1994.Metronidazole is bactericidal to dormant cells of Mycobacterium tuberculosis. Antimicrob .Agents.Chemother.38:2054-2058.

Wietzerbin, J., Das, B.C., Petit, J.F., Lederer, E., Leyh-Bouille, M., and J.M.Ghuysen.1974. Occurrence of D-alanyl(D)-meso-diaminopimelic acid and meso-diaminopimely-meso-diaminopimelic acid interpeptide linkages in the peptidoglycan of mycobacteria. Biochem.13:3471-3476.

Weidel, W., Frank, H., and H.H.Martin. 1960.The rigid layer of the cell wall of *Escherichia coli* strain. J .Gen. Microbiol. 22:158-166.

Weidel, W., and H, Pelzer.1964.Bagshaped macromolecules – a new outlook on bacterial cell walls. Adv.Enzmol.Relat.Areas Mol.Biol. 26:193-232.

Werner, G.H. 1951. La cytologie des bacilles tuberculex etudièe en relation avec leurs caractèe de virulence 59:1043

Young, K.D. 2003. Bacterial shape. Mol. Microbiol. 49:571-580

Young, M., Mukamolova, G.V., and A.S. Kaprelyants. 2005. Mycobacterial dormancy and its relation to persistence .In :parish T, editor .Mycobacterial molecular biology. Norwich: Horizon Scientific Press: 265-320

Young, K.D. 2006. The selective value of bacterial shape. Microbiol . Mol. Biol.Rev. 70 :660-703.

Young, K.D. 2006.The selective value of bacterial shape. Microbiol.Mol.Biol.Rev.70:660-703

Yuan, Y., Barrett, D., Zhang, Y., Kahne, D., Silz, P., and S, Walker. 2007.Crystal structure Of a peptidoglycan glycosyltransferase suggests a model for processive glycan chain synthesis.Proc.Natl.Acad.Sci.USA.104;5348-5353.

Ziehl, F. 1882. Zur Farbung des Tuberkelbacillus. 8:451.

9

Mycobacterial Strains of Different Virulence Trigger Dissimilar Patterns of Immune System Activation *In Vivo*

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1. Introduction

Tuberculosis (TB), one of the major world health problems, is a chronic infection caused by members of the Mycobacterium tuberculosis complex (MTC). In 2009, tuberculosis (TB) caused 1.7 million deaths and 9.4 million new cases. Although recent efforts to improve TB prevention, diagnosis and treatment have contributed to a 35% decrease in the death rate, the emergence of mycobacterial strains with highly virulent phenotypes combined with pandemic HIV infections has added new challenges to control TB.

Host-pathogen interactions during experimental pulmonary tuberculosis have been studied using laboratory mycobacterial strains of well defined, relatively homogeneous virulence. These studies have contributed to uncover immune evasion mechanisms evolved by mycobacteria, and their role to establishing chronic infections. Despite the successful models of experimental tuberculosis and the high homology among MTC strains, the immune mechanisms and the mycobacterial characteristics that cause the remarkable varying degrees of clinical virulence remain barely studied. Although previous reports partially described differences in immunopathogenesis and bacterial growth (R. Chacon-Salinas et al., 2005; J. Dormans et al., 2004; B. Lopez et al., 2003), the effects of different MTC strains both on airways DC and on T cell activation have not been assessed, especially in vivo.

Broadly, mycobacterium of intermediate virulence (e.g. M. tuberculosis H37Rv) seems to reduce DC migration to the mediastinal lymph nodes (A. J. Wolf et al., 2007) which could be associated with a delayed onset of specific effector T cell responses (G. S. Garcia-Romo et al., 2004; R. J. North & Y. J. Jung, 2004; A. J. Wolf et al., 2008), thus allowing early (during the first 4 weeks of infection) exponential Mtb replication. Around 30 days post-infection,

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mycobacterial replication rate is diminished (B. J. Rogerson et al., 2006) while diverse immune evasion mechanisms avoid bacterial killing by T cell-activated macrophages (J. A. Armstrong & P. D. Hart, 1971) and cytotoxic CD8+ T cells (E. M. Weerdenburg et al., 2010). The analysis of these evasion mechanisms used by MTC strains, however, have barely been comparatively assessed (L. Quintero-Macias et al., 2011).

To evaluate the in vivo differences in host-pathogen interaction across the wide range of virulence among MTC strains we used three mycobacterial strains as representative of low (*Mycobacterium canettii*), intermediate (*Mycobacterium tuberculosis* H37Rv), and high (*Mycobacterium beijing*) virulence degrees. The recently defined *Mycobacterium tuberculosis* Beijing (M. Beijing) strains are associated with high virulence and multidrug resistance (I. Parwati et al., 2010), and cause in mice a quick increase of cellular infiltrate with high numbers of colony forming units in the lungs (J. Dormans et al., 2004). Conversely, smooth-type *Mycobacterium tuberculosis* Canettii (M. canettii) strains rarely cause TB in humans and in the experimental mouse model show low cellular infiltrate with limited chronic infection (M. Fabre et al., 2010). Interestingly, our previous results assessing the mechanisms causing the difference in virulence showed an inverse correlation between strain virulence and in vivo cytotoxic responses, as well as higher bacterial burden in the lungs of M. Beijing infected mice (L. Quintero-Macias et al., 2011).

We decided to assess a profile of dendritic cell maturation and T cell exhaustion in vivo during pulmonary infection with these three mycobacterial strains. Since MTC strains are intracellular pathogens, T cells have an important role in mediating cytolysis of infected cells and to induce activation of other immune cells (Y. He et al., 2001; S. Inoue et al., 2005; E. A. Murphy et al., 2001; S. C. Oliveira et al., 2002). For intracellular pathogens like MTb, one way to subvert T cell responses could well be by altering activation/maturation of DCs. Importantly, DCs play an important role both in inducing effector cytotoxic T cells in vivo as well as in Ag-surveillance of mucosal surfaces and in the uptake and transport of mycobacterial bacilli to the lung draining lymphoid tissue, the mediastinal lymph nodes (MedLN)(G. S. Garcia-Romo et al., 2004; A. Pedroza-Gonzalez et al., 2004; A. J. Wolf et al., 2007).

We aimed to describe our recent findings regarding the differential stimulation of DCs and T cells by MTC strains with different virulence. We consider that increasing the research on the differences among MTC strains pathogen-host interactions in vivo might help to better understand, among other things, the underlying limitations of anti-TB vaccines.

2. Materials and methods

2.1 In vivo effects of different mycobacterial lysates over the subsequent activation of DCs

Separate groups of 3-5 BALB/c female mice were intratracheally primed with each lysate (40μg/mice in 40μL) from the three strains used in our experiments (lyCan: M. canettii lysate; lyH37: Mtb H37Rv lysate; lyBei: M. Beijing lysate). Five hours after the mycobacterial lysate, mice were given an intranasal (i.n.) challenge with LPS to assess in DCs the potential effects of prior mycobacterial lysate treatment. Control groups included a) mice primed-boosted both with LPS, or b) mice treated with endotoxin-free saline solution before the LPS challenge, c) mice treated only with endotoxin-free saline solution without further LPS.

Then, 10 hours after LPS challenge, a time which is around the peak of lung DC activation induced by LPS alone, we obtained cell suspensions from lung, BAL (Bronchio-Alveolar Lavage) and MedLN to assess CD86 expression in (Gr1-, MHC-II hi, CD11c+) DCs. Mycobacterial lysates were prepared by one of us (I. Estrada-Garcia, ENCB-IPN).

2.2 Experimental model of airways-induced pulmonary tuberculosis in mice

M. tuberculosis strains were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with OADC (Difco Laboratories). After 1 month of culture, mycobacteria were harvested, adjusted to $2.5x10^5$ bacteria in 100µl sterile endotoxin-free saline solution, aliquoted, and maintained at -70°C until used. Before use, bacteria were stained with fluorescein diacetate (InvitroGen, F1303) and viable bacteria (Kvach, J. T. and Veras, J. R. 1982) (green fluorescence) were counted with an epifluorescence microscope and adjusted to the infective dose. We used the murine model of intra-tracheal infection as described previously (Hernandez-Pando, R. 1996), with some modifications.

Briefly, 3-5 male BALB/c mice from 6-8 weeks of age were anaesthetized with sevoflurane, and 100 µl isotonic sterile endotoxin-free saline solution with 2.5×10^5 viable bacilli were inoculated intra-tracheally. Control animals were inoculated only with isotonic, sterile endotoxin-free saline solution without bacilli. Animals were then maintained in cages fitted with microisolators in a P-3 biosecurity level facility. The protocol was institutionally approved according to ethical norms for use of animals in experimentation. Following infection, at least three to five mice per group were euthanized at every time point selected for the various analysis.

2.3 Staining of cell suspensions for flow cytometry analysis

Monoclonal antibodies used for phenotypic analysis of DC and T cells were anti-CD3-FITC (BD Pharmingen 553062), anti-CD4-PerCP (BD Pharmingen 553052), anti-CD8a-APC (BD Pharmingen 553035), PD-1-PE (BD Pharmingen 551892), anti-CD11c-APC (BD Pharmingen 550261), anti-CD40-PE (BD Pharmingen 553791), anti-MHCII-FITC (BD Pharmingen 553623), anti-Ly6c-A700 (e-biosciences 56-5981-32), and Streptavidin-conjugated with PerCP fluorochrome (SAV-PerCP, BD Pharmingen 554064), biotinylated anti-CD103 (R&D Systems BAF1990). Cell suspensions were prepared by disgregating the organs using a 70µm cell strainer (BD Falcon 352350) and the piston of a 3mL Syringe (BD 309585). Spleen, Lungs, BAL and Mediastinal lymph nodes cell suspensions were washed, incubated 10 min at 4°C with Power Block reagent (Biogenex, HK085-5K) to block Fc receptors, washed, and stained with fluorochrome-coupled mAbs for 15 min at 4°C. Cells were centrifuged and resuspended in FACS buffer. 10^6 and 10^5 live MHC-II high or CD3+ cell cells were acquired respectively. Data was acquired in a Dako Cyan Flow Cytometer and analyzed with FlowJo Software 7.2 (Tree Star, Inc., San Carlos, CA).

3. Results

3.1 Lysates of highly virulent mycobacteria decrease activation of BAL DC in vivo

To test whether mycobacterial components differentially affected the activation patterns of lung DCs, we intra-tracheally treated separate groups of mice with different mycobacterial

lysates prepared from each mycobacterial strain. After five hours mice were challenged with intranasal LPS to determine the subsequent DC activation. CD86 expression on DCs from bronchio-alveolar lavage (BAL), lung and MedLN was assessed after 10 hours of LPS challenge.

Compared to all control groups, M. tuberculosis H37Rv and M. Beijing lysates reduced the LPS-triggered activation of BAL and lung DCs, whereas M. Canettii lysate showed no difference. Interestingly, in these two groups of mice (figure 1, top and middle panels), the reduced activation is observed regarding both the percentage of CD86+ DCs (left Y axis) and the intensity of expression of CD86 (MFI, right Y axis). In the lungs, slight differences were observed only in mice treated with lyBei. In the MedLN, all lysates increased the percentage of CD86+DCs when compared to mice that received two doses of LPS (figure 1, bottom panel, left Y axis).

Our results suggest that the one factor contributing to the different virulence observed among MTC strains lies in how DCs respond to mycobacterial components. These slight but relevant differences are clearly seen in the activation patterns of BAL and lung DCs. Although it remains uncertain whether the patterns observed with lyH37 or lyBei lysates are product of increased DC migration from the BAL to the lungs to the MedLN (which could be associated with the virulence-dependent exacerbated lung infiltrate characteristic of chronic tuberculosis) or a gradient of DC inhibition (which could be associated with the early inhibition of specific T cell responses).

3.2 The CD103+ dendritic cell subset is preferentially activated during virulent Mtb infection

To assess whether infection with different MTC strains induced divergent patterns of DC activation we evaluated the expression of CD40 on lung, MedLN and spleen DCs. CD40 in DCs is a crucial coestimulatory molecule required for naive T cell activation and –especially- for appropriate induction of cytotoxic T cells (P. Bjorck et al., 1997; G. Grouard et al., 1996; A. M. Moodycliffe et al., 2000), in lung, MedLN and spleen DCs. We compared CD40 expression between uninfected mice and mice infected with M. canettii, M. tuberculosis H37Rv, or M. Beijing at chronic infection (60 days after infection). Also, we analyzed two DC subsets distinguished by the expression of the CD103 molecule. In particular the lung CD103+ DC subset, as this is associated with proinflammatory responses and CD8+ T cell activation during intracellular pathogen infections (G. T. Belz et al., 2004; M. L. del Rio et al., 2010; T. S. Kim & T. J. Braciale, 2009).

We found that the infection with virulent mycobacterial strains (Mtb. H37Rv and M. Beijing) mainly activated lung CD103+ DCs (figure 2). In contrast, lung CD103+ DCs of mice infected with M. canettii had the lowest expression of CD40 (figure 2, top panel white bar). Interestingly, in M. Beijing infected mice, MedLN CD103+ DCs showed reduced expression of CD40 (figure 2, middle panel), even below the expression of the control group (uninfected mice). In the spleen, CD40 expression in both DC subsets from infected mice was below levels observed in uninfected mice (figure 2, bottom panel), but showed a direct correlation with virulence (M. Beijing infected mice had the highest expression of CD40 in spleen DCs).

These results indicate that mycobacteria of different virulence induce each a different activation pattern in the regional DCs. M. Beijing infection induced CD103+ DC activation

in the lungs with moderate inhibition in the MedLN (the regional, draining lymph node) and spleen (systemic response). Mtb H37Rv induced the activation of CD103+ DC in both lungs and MedLN while inhibiting CD40 expression in the spleen; and M. canettii reduced CD40 expression in CD103+ DCs in the lungs and spleen, with a slight increase in the MedLN.

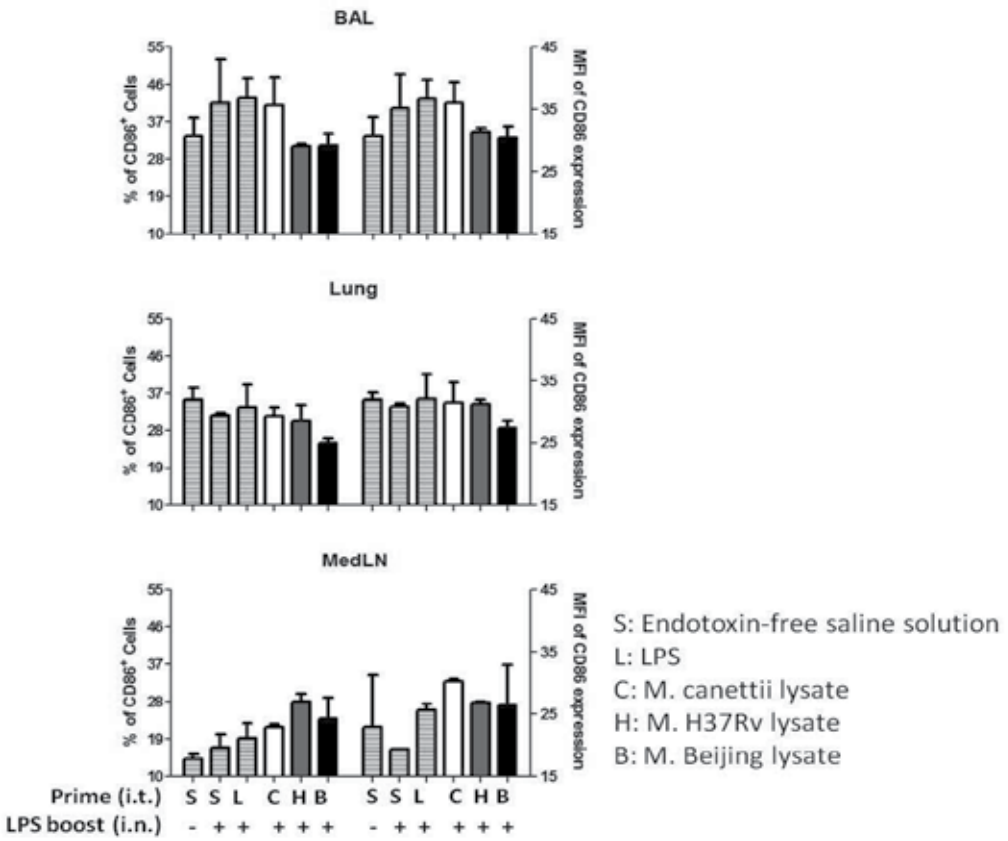

Fig. 1. Prior inoculation of mycobacterial lysate from virulent strains reduces subsequent activation of airways DCs.

Prior inoculation of mycobacterial lysate from virulent strains reduces subsequent activation of airways DCs. Groups of 3-5 BALB/c mice received intra-tracheal (i.t.) lysates prior to intra-nasal (i.n.) LPS stimulation. After 10 hours of LPS inoculation CD86 expression on DCs was determined and compared to control groups (striped bars). Bronchio-alveolar lavage (BAL) and lung DCs from mice that received M. Beijing lysate (black bars) showed a reduction in CD86 expression (mean fluorescence intensity (MFI) and percentage of positive cells). Mtb. H37Rv lysate (gray bars) induced a reduction of CD86 only in BAL DCs while M. canettii lysate (white bars) did not induced a reduction in BAL and lung DCs and neither increased CD86 expression in Spleen DCs.

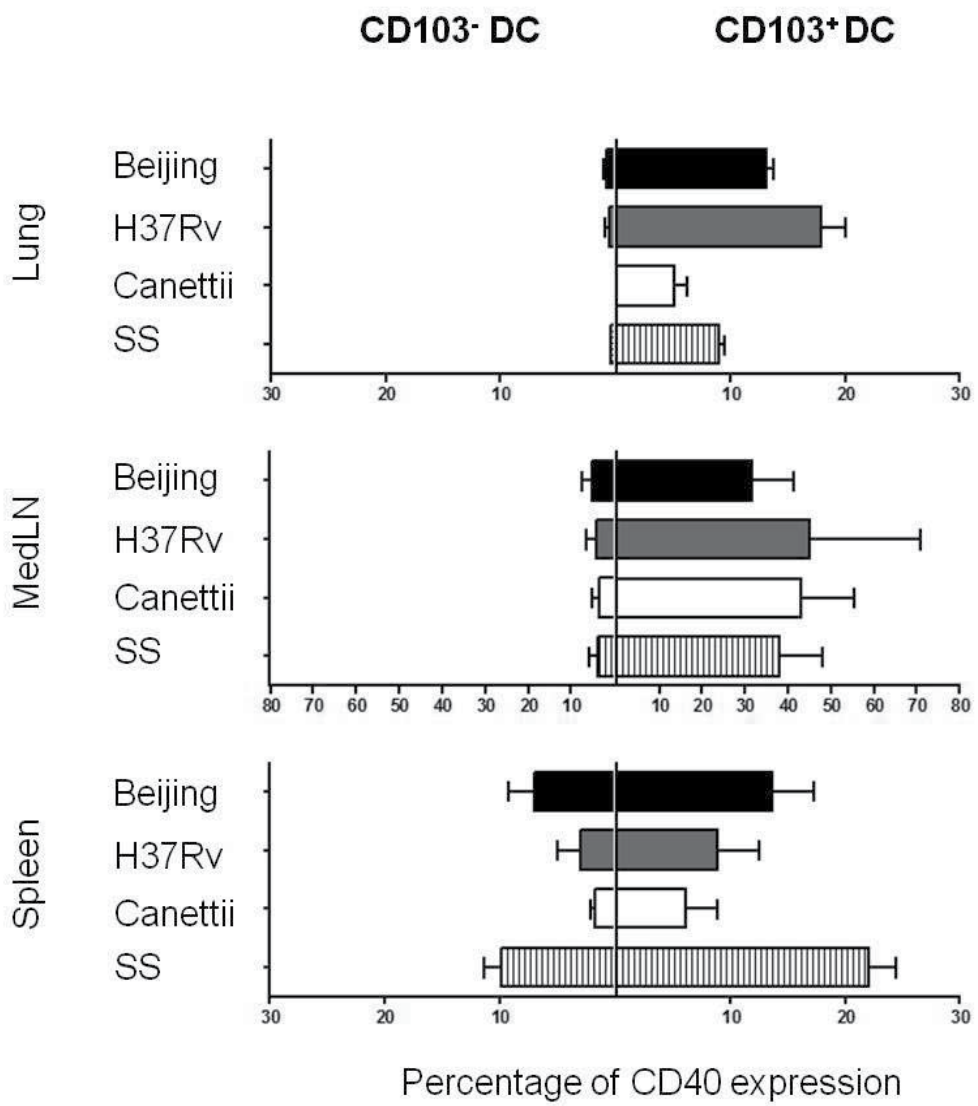

Fig. 2. CD40 expression in CD103+ and CD103- DC subsets during chronic infection with different mycobacterial strains.

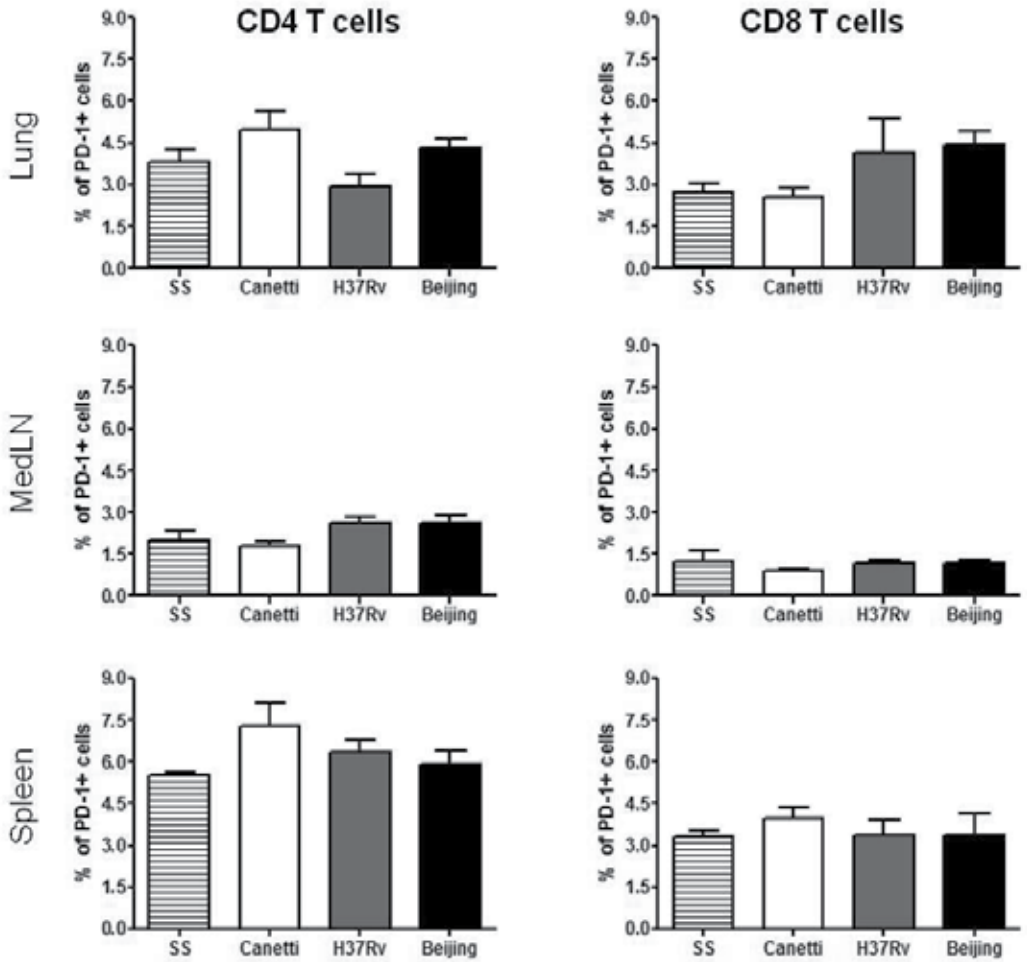

Fig. 3. PD-1 expression in CD4+ and CD8+ T cells during chronic infection with different mycobacterial strains.

3.3 Increased expression of PD-1 on lung CD4+ and CD8+ T cells is observed only during M. Beijing chronic infection

Since T cell expression of the PD-1 molecule has been shown associated in vivo to T cell exhaustion during chronic intracellular infections, we assessed the expression of this molecule during the infection with these three different mycobacteria. T cells showed different patterns of PD-1 expression among groups of infected mice. In M. canettii infected mice only PD-1+ CD4+ T cells are increased in lungs (figure 3, top panel-left plot), in M. tuberculosis H37Rv infection only PD-1+ CD8+ T cells are increased (figure 3, top panel-right plot), and in M. Beijing infected mice both T cell subsets showed increased PD-1 expression (figure 3, top panel). Interestingly, strain virulence and PD-1 expression in both T cell subsets showed an inverse correlation in the spleen, although slight differences in CD8+ PD-1+ percentage were observed among groups (figure 3, bottom panel). Seemingly, none of the strains induced overt changes in PD-1 expression on T cells, although there is a tendency for virulent mycobacteria to increase PD-1 expression on lung CD8+ T cells while M. canettii infection affects CD4+ T cells.

Groups of 3-5 BALB/c mice were intra-tracheally infected with different mycobacterium strains. At 60 days after infection CD40 expression on CD103+ and CD103- DCs was determined. In all groups CD103+ DCs had the highest expression. Mtb. H37Rv (gray bars) and M. Beijing (black bars) increased CD40 expression on lung CD103+ DCs. In MedLN CD103+ DCs, M. Beijing infection reduced CD40 expression while Mtb. H37Rv increased it. In contrast, M. canettii (white bars) infection reduced the percentage of CD103+ CD40+ DC in the lungs with a slight increase in MedLN. In the spleen and compared to uninfected mice (striped bars), all infected mice had reduced expression of CD40 in both CD103+ and CD103- DCs. In infected mice, for both DC subsets in the spleen, CD40 expression followed a direct correlation with virulence (Beijing>H37Rv>Canettii).

T cells expression of the exhaustion-associated marker PD-1 was analyzed at 60 days after infection with different mycobacteria. Overall, slight differences were observed when compared to uninfected mice (stripped bars). Mice infected with M. canettii (white bars) showed a tendency to increase PD-1 expression on lung and spleen CD4+ T cells and on spleen CD8+ T cells. Mice infected with Mtb. H37Rv (gray bars) or M. Beijing (black bars) showed no differences in PD-1 expression on both CD4+ and CD8+ T cells from the spleen or MedLN. In the lungs the two virulent mycobacteria increased PD-1 expression on CD8+ T cells.

4. Discussion

Tuberculosis is a major health problem worldwide, causing around 2 million deaths yearly. Although more than 100 years of research have led to significant improvement in disease control (in 2009, WHO reported a 35% drop in the death rate), it has also revealed a complex landscape of intricate interactions between mycobacteria and host immune system. The relative recent appearance and description of highly virulent strains in combination with the high incidence of tuberculosis in immuno-compromised patients entitles for a deeper understanding of how the immune system reacts to mycobacteria with a broader virulence spectrum.

Most of the current knowledge comes from studying murine models of pulmonary infection with strains of intermediate virulence (e.g. M. tuberculosis H37Rv). In the early phase of infection, mycobacteria are recognized and internalized by resident phagocytic cells like alveolar macrophages and pulmonary dendritic cells. Within these cells, mycobacterium bacilli can escape degradation and start replication. Concomitantly, it appears that lung DC crucial role in migration and activating specific T cells in the MedLN is inhibited. During the chronic phase mycobacteria apparently avoid killing associated with apoptosis of infected cells and remain confined inside granulomas.

Infection with the highly virulent Mycobacterium tuberculosis Beijing (M. Beijing) causes a quick increase of cellular infiltrate with high numbers of colony forming units in the lungs (D. Aguilar et al., 2010; B. Marquina-Castillo et al., 2009). Conversely, infection with smooth-type M. Canettii strains rarely cause TB in humans, and in the experimental mouse model, M. canettii strains show low cellular infiltrate with limited lung bacterial burden during chronic infection (M. Fabre et al., 2010). Importantly, among these three strains, virulence showed a direct correlation with inhibition of in vivo cytotoxicity (L. Quintero-Macias et al., 2011).

In the present study we tried to further define in vivo the virulence differences by assessing the potential effects upon DCs. Regarding DC activation, we observed an apparent differential recognition of M. canettii components by the DCs. When mice were treated with M. canettii lysate, BAL and lung DCs expressed similar levels of CD86 after LPS stimulation in vivo. During infection, lung and spleen CD103+ DCs showed less CD40 expression as compared to the other mycobacterial infection and to uninfected mice. Unlike M. canetti, both Mtb H37Rv and M. Beijing components reduced DC activation in BAL, and during infection, increased CD40 expression in lung DCs. Conceivably, virulent mycobacteria might induce a strong activation of BAL DCs causing the migration towards lung parenchyma and MedLN. The differences observed between Mtb H37Rv and M. Beijing infection suggest a probable scenario where Mtb H37Rv induces DC migration to MedLN whereas M. Beijing prevents MedLN recruitment while increasing systemic distribution.

Homeostatic mechanisms during chronic inflammatory responses on mucosal surfaces tend to increase and bias T cell differentiation to anti-inflammatory and regulatory phenotypes. PD-1 expression on T cells is associated with T cell exhaustion during chronic intracellular infections. Our results showed only slight variations in PD-1 expression during chronic infection. Of note, Mtb H37Rv and M. Beijing infections induced similar increase in the percentage of CD3+CD8+PD1+ lung T cells, while M. canettii infection increased PD-1 expression on lung and spleen CD3+CD4+ T cells. Although PD-1 expression had small variations compared to uninfected mice, a tendency of virulent mycobateria to induce CD8+ PD-1+ T cells was observed and might relate to decreased in vivo cytotoxicity (L. Quintero-Macias et al., 2011).

Several mycobacterial components have been associated to immune system subversion. RD-1-encoded secreted proteins (e.g. ESAT-6) mediate macrophage inhibition by TLR2 recognition and have the potential to form pores in membranes probably facilitating bacterial escape from phagosomes. RD-1 region is associated with virulence since is absent in attenuated M. bovis BCG. However the three strains used in our experiments

carry the RD-1 region (T. A. Halse et al., 2011), suggesting that their differing degrees of virulence might not be directly related to this genomic region. Recent publications have shown the involvement of other RD-1 proteins (EspF, EspG (D. Bottai et al., 2011)) or other genomic regions (RD-2 also lost in attenuated M. bovis BCG (R. A. Kozak et al., 2011)) in virulence.

Mycobacterial cell wall also contributes to virulence. The high content of heterogeneous lipids is a hallmark of mycobacteria. Lipoarabinomanans and lipomanans are two of the major lipidic components that can reduce the activation and cytokine secretion of macrophages. Although limited evidence suggest that cell wall lipids are key in immune response subversion (L. Quintero-Macias et al., 2010; L. M. Rocha-Ramirez et al., 2008), strain-specific lipid characterization during infection represents a challenge yet to be fully engaged. Among the strains used in our experiments, clear differences on the lipid synthesis have only been described for M. Beijing (G. Huet et al., 2009; M. B. Reed et al., 2007).

On the other hand, a decade of intense research on the mucosal immune system provides an outlook of similar complexity with the interaction of an increasing number of cell types within highly specialized microenvironments. In the case of tuberculosis is necessary to consider that the interaction of at least four histological compartments (alveolar space, lung parenchyma, draining lymph nodes, blood) is affected by the balance between mycobacterial virulence and host resistance. Apparently, virulent mycobacteria would preferentially target lung CD103+ DCs and thus avoid activation of the CD103- DCs. Further research is required to determine whether this constitutes an active evasion mechanism and to clarify the role of lung CD103+ DCs in the induction of Mtb-specific T cells or regulatory T cells.

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6. References

Aguilar, D., Hanekom, M., Mata, D., Gey van Pittius, N. C., van Helden, P. D., Warren, R. M.&Hernandez-Pando, R. (2010). Mycobacterium tuberculosis strains with the Beijing genotype demonstrate variability in virulence associated with transmission. *Tuberculosis*, Vol. 90, No. 5, (Sep), pp. (319-325). 1873-281X (Electronic) 1472-9792 (Linking)

Armstrong, J. A.&Hart, P. D. (1971). Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J.Exp.Med.*, Vol. 134, No. 3 Pt 1, pp. (713-740).

Belz, G. T., Smith, C. M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F. R.&Heath, W. R. (2004). Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 101, No. 23, (Jun 8), pp. (8670-8675). 0027-8424 (Print) 0027-8424 (Linking)

Bjorck, P., Banchereau, J.&Flores-Romo, L. (1997). CD40 ligation counteracts Fas-induced apoptosis of human dendritic cells. *International immunology*, Vol. 9, No. 3, (Mar), pp. (365-372). 0953-8178 (Print) 0953-8178 (Linking)

Bottai, D., Majlessi, L., Simeone, R., Frigui, W., Laurent, C., Lenormand, P., Chen, J., Rosenkrands, I., Huerre, M., Leclerc, C., Cole, S. T.&Brosch, R. (2011). ESAT-6 secretion-independent impact of ESX-1 genes espF and espG1 on virulence of Mycobacterium tuberculosis. *The Journal of infectious diseases*, Vol. 203, No. 8, (Apr 15), pp. (1155-1164). 1537-6613 (Electronic) 0022-1899 (Linking)

Chacon-Salinas, R., Serafin-Lopez, J., Ramos-Payan, R., Mendez-Aragon, P., Hernandez-Pando, R., van, S. D., Flores-Romo, L., Estrada-Parra, S.&Estrada-Garcia, I. (2005). Differential pattern of cytokine expression by macrophages infected in vitro with different Mycobacterium tuberculosis genotypes. *Clin.Exp.Immunol.*, Vol. 140, No. 3, pp. (443-449).

del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I.&Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. *Immunol.Rev.*, Vol. 234, No. 1, pp. (268-281).

Dormans, J., Burger, M., Aguilar, D., Hernandez-Pando, R., Kremer, K., Roholl, P., Arend, S. M.&van, S. D. (2004). Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different Mycobacterium tuberculosis genotypes in a BALB/c mouse model. *Clin.Exp.Immunol.*, Vol. 137, No. 3, pp. (460-468).

Fabre, M., Hauck, Y., Soler, C., Koeck, J. L., van, I. J., van, S. D., Vergnaud, G.&Pourcel, C. (2010). Molecular characteristics of "Mycobacterium canettii" the smooth Mycobacterium tuberculosis bacilli. *Infect.Genet.Evol.*, Vol. No. pp.

Garcia-Romo, G. S., Pedroza-Gonzalez, A., Aguilar-Leon, D., Orozco-Estevez, H., Lambrecht, B. N., Estrada-Garcia, I., Flores-Romo, L.&Hernandez-Pando, R. (2004). Airways infection with virulent Mycobacterium tuberculosis delays the influx of dendritic cells and the expression of costimulatory molecules in mediastinal lymph nodes. *Immunology*, Vol. 112, No. 4, pp. (661-668).

Grouard, G., Durand, I., Filgueira, L., Banchereau, J.&Liu, Y. J. (1996). Dendritic cells capable of stimulating T cells in germinal centres. *Nature*, Vol. 384, No. 6607, (Nov 28), pp. (364-367). 0028-0836 (Print) 0028-0836 (Linking)

Halse, T. A., Escuyer, V. E.&Musser, K. A. (2011). Evaluation of a Single-Tube Multiplex Real-Time PCR for Differentiation of Members of the Mycobacterium tuberculosis Complex in Clinical Specimens. *Journal of clinical microbiology*, Vol. 49, No. 7, (Jul), pp. (2562-2567). 1098-660X (Electronic) 0095-1137 (Linking)

He, Y., Vemulapalli, R., Zeytun, A.&Schurig, G. G. (2001). Induction of specific cytotoxic lymphocytes in mice vaccinated with Brucella abortus RB51. *Infection and immunity*, Vol. 69, No. 9, (Sep), pp. (5502-5508). 0019-9567 (Print) 0019-9567 (Linking)

Huet, G., Constant, P., Malaga, W., Laneelle, M. A., Kremer, K., van Soolingen, D., Daffe, M.&Guilhot, C. (2009). A lipid profile typifies the Beijing strains of Mycobacterium tuberculosis: identification of a mutation responsible for a modification of the structures of phthiocerol dimycocerosates and phenolic glycolipids. *The Journal of biological chemistry*, Vol. 284, No. 40, (Oct 2), pp. (27101-27113). 1083-351X (Electronic) 0021-9258 (Linking)

Inoue, S., Golding, B.&Scott, D. (2005). Programming of CTL with heat-killed Brucella abortus and antigen allows soluble antigen alone to generate effective secondary CTL. *Vaccine*, Vol. 23, No. 14, (Feb 25), pp. (1730-1738). 0264-410X (Print) 0264-410X (Linking)

Kim, T. S.&Braciale, T. J. (2009). Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS One*, Vol. 4, No. 1, pp. (e4204). 1932-6203 (Electronic) 1932-6203 (Linking)

Kozak, R. A., Alexander, D. C., Liao, R., Sherman, D. R.&Behr, M. A. (2011). Region of difference 2 contributes to virulence of Mycobacterium tuberculosis. *Infection and immunity*, Vol. 79, No. 1, (Jan), pp. (59-66). 1098-5522 (Electronic) 0019-9567 (Linking)

Lopez, B., Aguilar, D., Orozco, H., Burger, M., Espitia, C., Ritacco, V., Barrera, L., Kremer, K., Hernandez-Pando, R., Huygen, K.&van, S. D. (2003). A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. *Clin.Exp.Immunol.*, Vol. 133, No. 1, pp. (30-37).

Marquina-Castillo, B., Garcia-Garcia, L., Ponce-de-Leon, A., Jimenez-Corona, M. E., Bobadilla-Del Valle, M., Cano-Arellano, B., Canizales-Quintero, S., Martinez-Gamboa, A., Kato-Maeda, M., Robertson, B., Young, D., Small, P., Schoolnik, G., Sifuentes-Osornio, J.&Hernandez-Pando, R. (2009). Virulence, immunopathology and transmissibility of selected strains of Mycobacterium tuberculosis in a murine model. *Immunology*, Vol. 128, No. 1, (Sep), pp. (123-133). 1365-2567 (Electronic) 0019-2805 (Linking)

Moodycliffe, A. M., Shreedhar, V., Ullrich, S. E., Walterscheid, J., Bucana, C., Kripke, M. L.&Flores-Romo, L. (2000). CD40-CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. *The Journal of experimental medicine*, Vol. 191, No. 11, (Jun 5), pp. (2011-2020). 0022-1007 (Print) 0022-1007 (Linking)

Murphy, E. A., Sathiyaseelan, J., Parent, M. A., Zou, B.&Baldwin, C. L. (2001). Interferon-gamma is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunology*, Vol. 103, No. 4, (Aug), pp. (511-518). 0019-2805 (Print) 0019-2805 (Linking)

North, R. J.&Jung, Y. J. (2004). Immunity to tuberculosis. *Annu.Rev.Immunol.*, Vol. 22, No. pp. (599-623).

Oliveira, S. C., Soeurt, N.&Splitter, G. (2002). Molecular and cellular interactions between Brucella abortus antigens and host immune responses. *Veterinary microbiology*, Vol. 90, No. 1-4, (Dec 20), pp. (417-424). 0378-1135 (Print) 0378-1135 (Linking)

Parwati, I., van, C. R.&van, S. D. (2010). Possible underlying mechanisms for successful emergence of the Mycobacterium tuberculosis Beijing genotype strains. *Lancet Infect.Dis.*, Vol. 10, No. 2, pp. (103-111).

Pedroza-Gonzalez, A., Garcia-Romo, G. S., Aguilar-Leon, D., Calderon-Amador, J., Hurtado-Ortiz, R., Orozco-Estevez, H., Lambrecht, B. N., Estrada-Garcia, I., Hernandez-Pando, R.&Flores-Romo, L. (2004). In situ analysis of lung antigen-presenting cells during murine pulmonary infection with virulent Mycobacterium tuberculosis. *Int.J.Exp.Pathol.*, Vol. 85, No. 3, pp. (135-145).

Quintero-Macias, L., Santos-Mendoza, T., Donis-Maturano, L., Silva-Sanchez, A., Aguilar, D., Orozco, H., Gicquel, B., Estrada-Garcia, I., Flores-Romo, L.&Hernandez-Pando, R. (2010). T-cell responses and in vivo cytotoxicity in the target organ and the regional lymphoid tissue during airborne infection with the virulent Mycobacterium tuberculosis MT103 and its lipid mutant fadD26. *Scandinavian journal of immunology*, Vol. 71, No. 1, (Jan), pp. (20-28). 1365-3083 (Electronic) 0300-9475 (Linking)

Quintero-Macias, L., Silva-Sanchez, A., Valderrabano-Ortiz, E., Munguia-Fuentes, R., Aguilar-Leon, D., Hernandez-Pando, R.&Flores-Romo, L. (2011). Reduced in vivo Cytotoxicity and Increased Mycobacterial Burden Are Associated with Virulent Mycobacterium tuberculosis Strains During Lung Infection. *Immunological investigations*, Vol. No. (Jun 2), pp. 1532-4311 (Electronic) 0882-0139 (Linking)

Reed, M. B., Gagneux, S., Deriemer, K., Small, P. M.&Barry, C. E., 3rd. (2007). The W-Beijing lineage of Mycobacterium tuberculosis overproduces triglycerides and has the DosR dormancy regulon constitutively upregulated. *Journal of bacteriology*, Vol. 189, No. 7, (Apr), pp. (2583-2589). 0021-9193 (Print) 0021-9193 (Linking)

Rocha-Ramirez, L. M., Estrada-Garcia, I., Lopez-Marin, L. M., Segura-Salinas, E., Mendez-Aragon, P., Van Soolingen, D., Torres-Gonzalez, R., Chacon-Salinas, R., Estrada-Parra, S., Maldonado-Bernal, C., Lopez-Macias, C.&Isibasi, A. (2008). Mycobacterium tuberculosis lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. *Tuberculosis*, Vol. 88, No. 3, (May), pp. (212-220). 1472-9792 (Print) 1472-9792 (Linking)

Rogerson, B. J., Jung, Y. J., Lacourse, R., Ryan, L., Enright, N.&North, R. J. (2006). Expression levels of Mycobacterium tuberculosis antigen-encoding genes versus production levels of antigen-specific T cells during stationary level lung infection in mice. *Immunology*, Vol. 118, No. 2, pp. (195-201).

Weerdenburg, E. M., Peters, P. J.&van der Wel, N. N. (2010). How do mycobacteria activate CD8+ T cells? *Trends Microbiol.*, Vol. 18, No. 1, pp. (1-10).

Wolf, A. J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K.&Ernst, J. D. (2008). Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J.Exp.Med.*, Vol. 205, No. 1, pp. (105-115).

Wolf, A. J., Linas, B., Trevejo-Nunez, G. J., Kincaid, E., Tamura, T., Takatsu, K.&Ernst, J. D. (2007). Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J.Immunol.*, Vol. 179, No. 4, pp. (2509-2519).

10

Biofilms of *Mycobacterium tuberculosis*: New Perspectives of an Old Pathogen

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1. Introduction

1.1 Persistence of the pathogen is the hallmark of TB pathogenesis

Based on a randomized clinical trial conducted by British Medical Council between 1972 and 1974, the World Health Organization (WHO) and other government agencies implemented a short-course multi-drug regimen for tuberculosis – a disease caused by the infection of *Mycobacterium tuberculosis (BMC, 1972, Fox et al., 1999)*. The regimen is made of three antibiotics, isoniazid, rifampicin and pyrazinamide administered over a period of six months. The extended therapy is essential for sterilizing a small subpopulation of bacilli that presumably acquire phenotypic tolerance to antibiotics (Saltini, 2006, Jindani et al., 2003).

Four decades later, WHO estimates that about 2 billion people in the world still remain asymptomatically infected with *M. tuberculosis*, approximately 5-10% of these visit clinics with symptoms of active tuberculosis, and 1.7 million die of the infection every year (Dye *et al.*, 2009). Moreover, one third of the mortality in HIV-infected patient occurs due to co-infection of *M. tuberculosis*, often with a very high frequency of multi-drug resistant strains (Harrington, 2010, Aaron *et al.*, 2004). It is thus clear that while the existing anti-TB drug regimen has been able to reduce the mortality rate, it has been inadequate in reducing the global burden of the disease. A forward approach towards TB-control must include two critical capabilities: a) to predict and prevent the conversion of asymptomatic infection to active TB, and b) to develop a shorter and more effective therapeutic regimen for active disease. Accomplishing these goals have been difficult because of our limited understanding of the mechanisms employed by *M. tuberculosis* to persist against the challenges of competent host immune system and antibiotics.

Although persistence mechanisms of *M. tuberculosis* in the host remain largely unclear, persistence of most, if not all, microbial species is facilitated by growth and existence in surface-associated and organized communities – called biofilms (Costerton *et al.*, 1999, Fux *et al.*, 2005, Hall-Stoodley *et al.*, 2004, Blankenship & Mitchell, 2006, Branda *et al.*, 2005). Several mycobacterial species including *M. tuberculosis* are now known to spontaneously grow *in vitro* as biofilms that harbor drug tolerant bacilli. This raises questions as to whether biofilms

could also be an *in vivo* persistence mechanism of *M. tuberculosis*. In this chapter we will discuss why it is reasonable to pay serious heed to this question, and what approaches can be used to test this hypothesis.

2. A historical perspective of studies on *M. tuberculosis* persistence

Early glimpses of the unique adaptability of *M. tuberculosis* appeared in two landmark studies conducted in the early 20th Century. First, Corper and Cohn observed that 24 out of 56 *in vitro* cultures of human and bovine isolates contained culturable tubercle bacilli even after 12 years of incubation in sealed containers (Corper & Cohn, 1933). This *in vitro* study revealed the characteristic persistence of *M. tuberculosis* in bacteriostatic condition. Concurrent with this *in vitro* study, Opie and Aronson reported the presence of virulent *M. tuberculosis* bacilli in about 26% of lesions resected from individuals dying of causes unrelated to TB (Opie & Aronson, 1927). While this study demonstrated asymptomatic infection of *M. tuberculosis*, it also opened up questions as to how bacilli are able to evade the immune system and suppress inflammation. In subsequent follow-up studies it appeared that the bacilli were unexpectedly present in uninvolved tissues instead of the presumed primary lesions (Feldman & Baggenstoss, 1939). These *in vivo* studies thus raised speculations that a competent immune system is capable of clearing the bacilli at the primary lesions, but the bacilli could have used escape mechanism to survive at secondary sites in presumably a non-replicating state.

The persistent nature of *M. tuberculosis* re-occupied the spotlight of tuberculosis research during the early phase of antibiotic-era around mid-20th Century. In several bacteriological studies on resected lesions from antibiotic treated individuals the bacilli could be microscopically observed even though individuals had converted to sputum negative (Loring & Vandiviere, 1956, Loring *et al.*, 1955, Vandiviere *et al.*, 1956, 1953). Interestingly, these bacilli in many instances were non-culturable and often associated with resolved lesions, thus raising a debate whether these were dead, or viable but non-culturable bacilli. The idea of viable but non-culturable bacilli seemed more convincing after McDermott and colleagues demonstrated reactivation of TB in mice upon termination of chemotherapy that was sufficient to reduce viability to undetectable levels (McCune *et al.*, 1966). It was, however, not clear in this study as to how and where the viable bacilli persisted, but the correlation between non-culturable bacilli and closed hypoxic lesions (Vandiviere et al., 1956, Haapanen *et al.*, 1959) support the idea that closed lesions could possibly be the primary site of non-replicating persisters which developed in the bacteriostatic environment of the lesions.

Several attempts have been made to investigate the physiology of non-replicating persisters using *in vitro* models such as hypoxic and nutritionally starved cultures. These studies subsequently led to identification of genetic components responsive to these conditions-most notably *isocitrate lyase* (*icl*) of the glyoxalate shunt pathway and the two-component regulatory system *dosR-dosS (Park et al., 2003, Hobby & Lenert, 1957, Wayne & Hayes, 1996, Wayne & Lin, 1982, Saini et al., 2004)*. While the mutation in *icl* impairs the persistence of bacilli in a mice model (McKinney *et al.*, 2000), the phenotype of *dosR-dosS* mutants in animal models have yielded conflicting results (Rustad *et al.*, 2008, Parish *et al.*, 2003, Malhotra *et al.*, 2004).

3. Changing paradigms of *M. tuberculosis* persistence

Despite the demonstration of a non-replicative and physiologically tolerant state of *M. tuberculosis in vitro* as well as the presence of hypoxic environment in granulomas (Via *et al.*, 2008), the hypothesis that the persisters in latent infection and chemotherapy are exclusively the non-replicating subpopulation residing in the bacteriostatic condition of closed lesions remains untested (Gomez & McKinney, 2004, Parrish *et al.*, 1998). In contrast, the notion of a non-replicative state of persisters during latency is strongly challenged by two interesting studies published recently. Using an unstable plasmid as a reporter, Sherman and colleagues found that *M. tuberculosis* bacilli actively replicate during the chronic phase of infection in a mouse model – a phase when neither the host develops any symptoms of disease nor the number of live bacteria changes (Gill *et al.*, 2009). Recently, Fortune and colleagues determined that mutations in *M. tuberculosis* populations accumulate at the same rate in latent and active infections of non-human primates, and both were similar to a logarithmically growing *in vitro* culture, implying active DNA replication and thus cell division of the pathogen in latent infection (Ford *et al.*).

The replicative state of the bacilli in asymptomatic infection of animal models reflects a dynamic host-pathogen interface. This interestingly is fully consistent with an emerging picture of a spectrum of disease status- in terms of bacterial load, inflammation and lesion morphologies – as against the dogmatic view of a bimodal existence of infection in either latent or active form (DB *et al.*, 2009, Rhoades *et al.*, 2005, Barry *et al.*, 2009). Interestingly, comparative studies of latent and active TB not only fail to establish a clear immunological distinction but also reveal highly heterogeneous lesion morphologies reflecting localized and highly diverse host pathogen interactions within an infected organ irrespective of the clinical symptoms (Barry et al., 2009). It is thus reasonably evident that in an asymptomatic infection *M. tuberculosis* could persist in diverse physiological states – from non-replicative to fully replicative states – each with distinct host-pathogen interactions. Furthermore, persistence of actively growing bacilli in asymptomatic infection could conceivably occur through delicately balanced host-pathogen interaction, which keeps the inflammation below the symptomatic threshold, but has the greatest chance of tipping the balance to cause the active disease.

Mechanisms of persistence of *M. tuberculosis* during chemotherapy, like latency, also remains unclear, but data from clinical trials indicate a strong positive correlation between bacterial burden and duration of chemotherapy [reviewed in (Connolly *et al.*, 2007)]. Consistent with these data, the Center for Disease Control of the United States recommends an extension of chemotherapy from six to nine months in case of patients with cavitary TB (CDC, 2003). Besides the total burden, the most intriguing aspect of long-term chemotherapy in TB is that the clearance of the pathogen follows a biphasic pattern as clearly demonstrated by Mitchison and colleagues (Jindani et al., 2003) (Fig. 1). While > 95% of the population could be cleared in the first few days of the beginning of treatment, the remaining fraction required a prolonged exposure (Jindani et al., 2003).

In summary, the persistence of *M. tuberculosis* in a chronic infection and chemotherapy are likely to be facilitated by multiple mechanisms including the adaptive changes in the bacilli in response to dynamic microenvironments during colonization and active growth. These

changes could either be in surface structure or physiology that lead to decreased antibiotic permeability, as well as controlled host-pathogen interaction and inflammation. Therefore, addressing questions such as where and how *M. tuberculosis* colonizes during chronic infection and gaining insight into the growth phase-dependent adaptive changes are critical for a comprehensive understanding of its persistence.

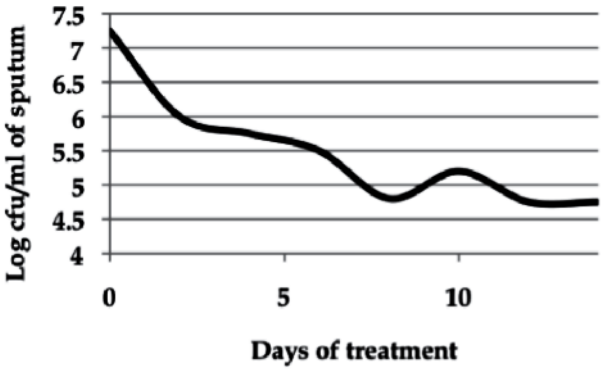

Fig. 1. Representation of the data published by Jindani et al. (4), showing the pattern of *M. tuberculosis* clearance in patients treated with isoniazid and rifampicin.

4. Chronic infections, bacterial persistence and biofilms

The hallmark of a successful pathogen is to colonize the host for a long period of time against the challenges of host immune system, and persist against the antibiotics pressure. Although numerous bacterial and fungal pathogens including *M. tuberculosis* easily qualify for this category, the question as to how they establish such infections remains unanswered for most species.

However, a large and ever-growing body of evidence provide a compelling argument that the persistence of most, if not all, microbial species in general is achieved through their ability to grow in self-organized, surface associated, sessile communities called biofilms (Costerton et al., 1999, Fux et al., 2005, Hall-Stoodley et al., 2004, Kolter & Greenberg, 2006, Marrie *et al*., 1982, McNeill & Hamilton, 2003, Donlan & Costerton, 2002). Moreover, several long-term colonizers in humans like *P. aeruginosa*, *S. aureus*, *S. epidermis*, *C. albicans*, *H. influenzae* and *E. coli* grow as extracellular or intracellular biofilms inside the cell, on the tissues, or on medical implantation devices (Blankenship & Mitchell, 2006, Anderson *et al*., 2004, Davies, 2002, Fey & Olson, Foreman & Wormald, Post, 2001). Furthermore, evidence of direct association between chronic persistence and biofilm formation is found in *S. epidermis* through mutation in a single gene that disrupted both phenotypes (Vuong *et al*., 2004).

The mechanisms of biofilm formation are primarily investigated in genetically tractable species like *B. subtilis*, *Vibrio spp*. and *Pseudomonas* spp. (Kolter & Losick, 1998, O'Toole *et al*., 1999, Hall-Stoodley & Stoodley, 2002). Despite the distinction in their specific genetic requirements and structural constituents, biofilms of each species are formed through common developmental mechanisms that involve surface attachment, cell-to-cell communication, and synthesis of extracellular matrix (ECM), which encapsulates the resident cells (Kolter & Losick, 1998, Hall-Stoodley & Stoodley, 2002, Hogan & Kolter, 2002,

Chu *et al.*, 2006, Blankenship & Mitchell, 2006, Branda et al., 2005, Danese *et al.*, 2000, Higgins *et al.*, 2007). The constituent microbes in biofilms must reside in, and therefore adapt to, highly complex, heterogeneous and dynamic microenvironments that conceivably could foster phenotypic diversity in the population, a scenario unlikely to be encountered by single-cell planktonic counterparts (Kolter & Losick, 1998). Overall, the encapsulated growth along with phenotypic diversity in the population can be argued as the primary contributors to the extraordinary persistence of biofilms against environmental challenges including antibiotics (Mah & O'Toole, 2001).

The changes in intercellular interactions, cellular physiology and structural compositions associated with development of pathogenic biofilms can also have a profound effect on the outcome of both acute and chronic infections. Accumulation of a set of two quorum sensing signals, CAI-1 and AI-2, in high density cultures of *Vibrio cholerae* negatively co-regulate genes for ECM synthesis as well as virulence (Higgins et al., 2007). This suggests that formation of biofilms and creation of suitable microenvironments in the host through virulence factors are intricately related steps that constitute the colonization phase of an acute infection of *V. cholerae,* and their concomitant down-regulation at high density could possibly be an exit strategy of the pathogen. However, in a chronic infection of *S. aureus* in a mouse model Shirtliff and colleagues found that early and late stages of biofilms elicit distinct host responses (Prabhakara *et al.*, 2011). While early stage biofilms triggered a Th1-mediated acute inflammatory response- possibly to create conducive tissue microenvironment for colonization – the old biofilms induced Th2-mediated humoral response that was ineffective on the pathogen – perhaps an immune evasive mechanism that facilitates the chronic survival (Prabhakara et al., 2011).

Taken together, biofilms represent a natural but highly complex life-style of most microbial species, promote persistence of constituent cells in robust structures, and provide unique microenvironments that facilitate extensive phenotypic diversity.

5. Could *M. tuberculosis* infections persist as biofilms?

While the long-term persistence of *M. tuberculosis* against the host immune system and antibiotics has striking similarity with the chronic infections of biofilm forming pathogens, it remains unclear if the tubercle bacilli form biofilms in the host. It is, however, noteworthy that *in vitro* cultures of all mycobacterial species grow in complex structures that eventually develop as pellicles on the liquid-air interface, unless a detergent is added as dispersal agent in the medium. Interestingly, such growth pattern of mycobacteria have frequently been noted in the literature as aggregation of cells driven by their surface hydrophobicity, and largely been ignored ever since Dubos and colleagues reported a method to grow dispersed culture of tubercle bacilli without diminishing their virulence (Dubos *et al.*, 1946). However, the emerging concept of microbial persistence in biofilms have recently led several groups to investigate the detergent-free *in vitro* growth of mycobacterial species from the perspective of organized multicellular structures (Hall-Stoodley & Lappin-Scott, 1998, Carter *et al.*, 2003). In one of the first genetic studies of surface associated growth of mycobacteria, Kolter and colleagues observed that an *M. smemgatis* mutant deficient in biosynthesis of acetylated glycopeptidolipid was also unable to attach and grow on an abiotic surface, thus demonstrating a specific genetic requirement for surface-associate mycobacterial growth (Recht & Kolter, 2001). Ojha *et al.* subsequently reported that a

mutation in one of the non-essential chaperone, of *M. smegmatis* specifically retarded the maturation stages of pellicle formation, observed at 4- and 5-day of incubation, without affecting early attachment and growth during first three days of incubation (Ojha *et al.*, 2005). The mutant was also indistinguishable from its wild-type parent in planktonic growth. The maturation defect of the mutant was linked to defective synthesis in mycolic acids as a consequence of the loss of a KasA (enzyme involved in mycolic acid biosynthesis) interaction, which is induced in this phase of wild-type culture (Ojha et al., 2005). The regulated synthesis of mycolic acids was surprising because it is highly abundant in the cell wall, although it is consistent with the subsequent observation of induced synthesis of extracellular free mycolic acids during the maturation stage of the pellicles (Ojha *et al.*, 2010). The free mycolic acids (FM) are released through regulated hydrolysis of mycolyl esters of Trehalose, Trehalose 6',6' dimycolate (TDM), and by cutinase-like serine esterase (Ojha et al., 2010), although other mycolyl esters could also contribute to the FM pool through similar mechanism. One possible candidate could be mycolyl diacyl glycerol (MDAG), a mycolyl ester of glycerol, which is also found in low abundance in impaired biofilms of an *lsr2* mutant of *M. smegmatis* (Chen *et al.*, 2006). The accumulation of FM is likely facilitated through a three-step mechanism: 1) mediated upregulation in *de novo* synthesis of the nascent mycolic acids, 2) processing of nascent mycolic acids into a subset of mycolyl esters through housekeeping mechanisms, and 3) hydrolysis of these mycolyl esters through substrate-specific esterases. The elevated levels of extracellular free mycolic acids during the maturation of *M. smegmatis* pellicles is also consistent with the obvious waxy appearance of the structures, and thus could likely constitute the structure component of the ECM. The significance of *M. smegmatis* growth in pellicles is further highlighted by distinct phenotypes of the bacilli in these structures that are absent in planktonic cell suspension. For example, increased intercellular transfer of genetic materials, and extraordinary tolerance to anti-TB drugs (Ojha et al., 2010, Nguyen *et al.*, 2010). Most importantly, the fundamental properties of mycobacterial pellicles are conserved in *M. tuberculosis* when grown in detergent-free media under defined condition (Ojha et al., 2008). The pathogenic species not only has specific genetic requirement for forming the pellicles (Fig. 2A and B), but also produces large abundance of free mycolic acids in the structure, which expectedly harbor large numbers of drug tolerant bacilli (Ojha et al., 2008) (Fig. 3). Taken together, these *in vitro* studies strongly support the possibility that surface-associated multicellular structures of mycobacteria have all the characteristics of biofilms, developing through distinct growth phases, having specific genetic requirements, and conferring high tolerance to antibiotics. Despite a recent surge in understanding the multicellular structures of mycobacteria, multicellular structures of *M. tuberculosis* in the host have been difficult to define. This in part can be attributed to the conventional image of *M. tuberculosis* as an intracellular pathogen living in phagosome, which is too restrictive for exuberant growth of the pathogen in multicellular structure. However, while *M. tuberculosis* might be restricted to the phagosome in early stages of infection, at later t imes, especially when lesions contain liquefied caseum and when patients are highly infective, it is likely that many of the bacilli experience an extracellular environment. In a comprehensive histopathological study of TB lesions from 1,500 autopsies, Geroges Canetti documented several lesions including open cavities that had numerous extracellular bacilli growing in multicellular structures (Canetti, 1955). Interestingly, in this 7-year study Canetti microscopically analyzed lesions of various

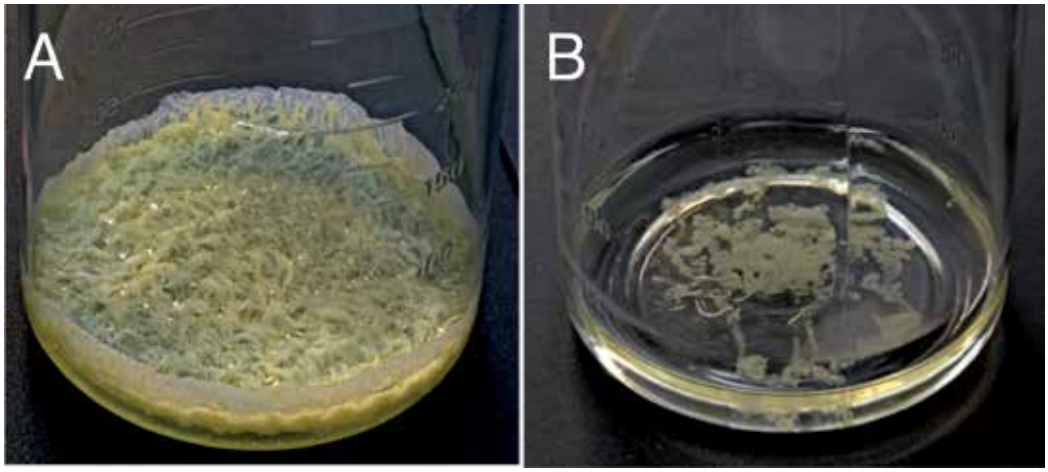

Fig. 2. A. Growth of *M. tuberculosis* biofilms on the liquid-air interface in detergent-free Sauton's medium. B. Insertion of a mariner transpososn (Himar) in Rv1013 abolishes the formation of biofilms, although the growth of the mutant in planktonic state remains unaltered (68).

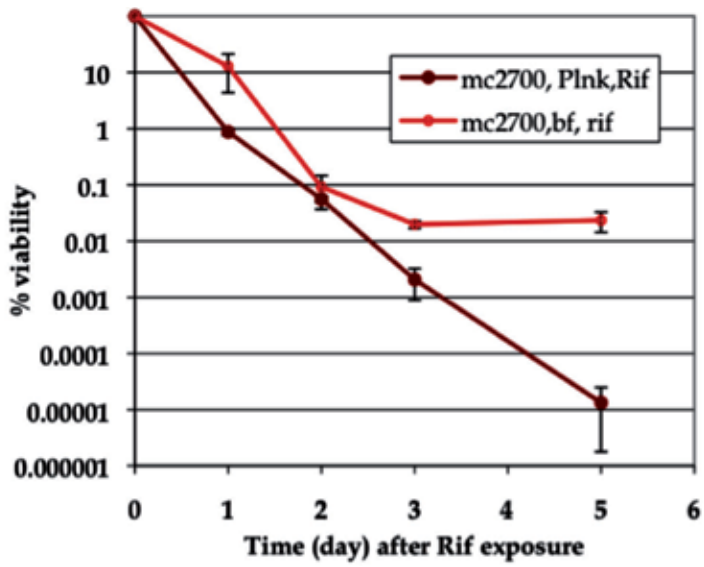

Fig. 3. Presence of rifampicin tolerant persisters is significantly higher in biofilm populations of *M. tuberculosis* than in their planktonic counterparts, as reported by Ojha et al. (68).

morphotypes in detail, with equal focus on both the tissue structures as well as bacterial growth (Canetti, 1955). The goal of the undertaking was to bridge the partition between immunopathology and bacteriology of tuberculosis that a student of the disease was always confronted with. This partition ironically continues to be a relevant issue in questions as to how and where bacilli persist in the both latent and active TB. In a recent attempt to locate the bacilli persisting after antibiotic treatment, Orme and colleagues observed the persisters as microcolonies in the acellular rim of granulomas (Lenaerts *et al*., 2007). Although it was

not clear in their study whether these bacilli were alive or dead, these are reminiscent of the extracellular multicellular structures of bacilli reported in Canetti's study.

6. Exploring *M. tuberculosis* biofilms *in vivo*

The *in vitro* studies on mycobacterial persistence in biofilms provide a compelling argument that the extracellular multicellular structures of *M. tuberculosis* in liquefied lesions could be primary foci of persister cells. A basic approach in such a study will involve three critical components: a) imaging *M. tuberculosis* bacilli in intact lesions, b) identifying molecular signatures like free mycolic acids of biofilms in multicellular structures of bacilli, and c) genetically correlating persistence with multicellular structures. Imaging bacilli in intact lesions could be a potentially challenging and expensive approach but is important because conventional processing of tissues with harsh organic chemicals used in typical histopathological protocols likely distort the bacillary architecture. Moreover, the modified cell wall of mycobacteria in biofilms could also render them undetectable by acid-fast staining. Confocal Laser Scanning Microscopy (CLSM) of fluorescently marked *M. tuberculosis* in lesions resected from an animal model that closely mimic human infections, like non-human primates, represents an attractive approach to imaging *M. tuberculosis in vivo* biofilms. The imaging studies could then be followed with detection and analysis of extracellular molecules including lipids and proteins that are associated with multicellular structures. Although the abundance of free mycolic acids in biofilms *in vitro* makes this an excellent candidate, the search should remain open, in case different surface molecules are used for cohesion of the structures *in vivo*. Finally, a systematic *in vivo* analysis of genetically defined mutants that fail to form *in vitro* biofilms could be a powerful strategy for gaining mechanistic insights and identifying drug targets that can dismantle the biofilm structure. It is noteworthy that transposon insertion in *pks16* (Rv1013) and *helY* can impair the development of *M. tuberculosis*, although it is unclear whether the effects of genes products are directly on structural formation or indirectly on adaption of resident bacteria within the structure (Ojha et al., 2008). Mutants in the former category could be especially useful for *in vivo* studies avoiding indirect effects of gene products resulting from changes in the morphologies of the structures.

7. Conclusions

Although a short and effective treatment of *M. tuberculosis* infection remains a big challenge to mankind, a solution is unlikely to appear without mechanistic insights into the persistent nature of the pathogen. At the origin of such studies lies a growth model that would reflect the spontaneous behavior of the pathogen. Use of detergents in the process of growing dispersed *in vitro* cultures has arguably misrepresented the physical existence of *M. tuberculosis* in its natural context. In the absence of detergent, the pathogen forms drug tolerant multicellular biofilms, and the complex structures of biofilms undoubtedly hold a treasure of information about the mechanisms that shape their behavior. It is time we focused on these observations to develop new strategies to combat Man's deadliest microbial enemy – *M. tuberculosis*.

8. References

(1953) REPORT of panel discussion on survival and revival of tubercle bacilli in healed tuberculous lesions. *Am Rev Tuberc* 68: 477-495.

Aaron, L., D. Saadoun, I. Calatroni, O. Launay, N. Memain, V. Vincent, G. Marchal, B. Dupont, O. Bouchaud, D. Valeyre & O. Lortholary, (2004) Tuberculosis in HIV-infected patients: a comprehensive review. *Clin Microbiol Infect* 10: 388-398.

Anderson, G. G., K. W. Dodson, T. M. Hooton & S. J. Hultgren, (2004) Intracellular bacterial communities of uropathogenic Escherichia coli in urinary tract pathogenesis. *Trends Microbiol* 12: 424-430.

Barry, C. E., 3rd, H. I. Boshoff, V. Dartois, T. Dick, S. Ehrt, J. Flynn, D. Schnappinger, R. J. Wilkinson & D. Young, (2009) The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 7: 845-855.

Blankenship, J. R. & A. P. Mitchell, (2006) How to build a biofilm: a fungal perspective. *Curr Opin Microbiol* 9: 588-594.

BMC, (1972) Controlled clinical trial of short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. *Lancet* 1: 1079-1085.

Branda, S. S., S. Vik, L. Friedman & R. Kolter, (2005) Biofilms: the matrix revisited. *Trends Microbiol* 13: 20-26.

Canetti, G., (1955) *Tubercle Bacillus in the pulmonary lesion of man: histobacteriology and its bearing on the therapy of pulmonary tuberculosis*. Springer Publishing Company, New York.

Carter, G., M. Wu, D. C. Drummond & L. E. Bermudez, (2003) Characterization of biofilm formation by clinical isolates of Mycobacterium avium. *J Med Microbiol* 52: 747-752.

CDC, (2003) Treatment of tuberculosis. In: Morbidity and Mortality Weekly Report. Center for Disease Control and Prevention, pp.

Chen, J. M., G. J. German, D. C. Alexander, H. Ren, T. Tan & J. Liu, (2006) Roles of Lsr2 in colony morphology and biofilm formation of Mycobacterium smegmatis. *J Bacteriol* 188: 633-641.

Chu, F., D. B. Kearns, S. S. Branda, R. Kolter & R. Losick, (2006) Targets of the master regulator of biofilm formation in Bacillus subtilis. *Mol Microbiol* 59: 1216-1228.

Connolly, L. E., P. H. Edelstein & L. Ramakrishnan, (2007) Why is long-term therapy required to cure tuberculosis? *PLoS Med* 4: e120.

Corper, H. & M. Cohn, (1933) The viability and VIrulence of old cultures of tubercle bacilli: studies on twelve-year broth cultures maintained at incubator temperature. *Am Rev Tuberc* 28: 856-874.

Costerton, J. W., P. S. Stewart & E. P. Greenberg, (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-1322.

Danese, P. N., L. A. Pratt, S. L. Dove & R. Kolter, (2000) The outer membrane protein, antigen 43, mediates cell-to-cell interactions within Escherichia coli biofilms. *Mol Microbiol* 37: 424-432.

Davies, J. C., (2002) Pseudomonas aeruginosa in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev* 3: 128-134.

DB, Y., G. Hannah & W. RJ, (2009) Eliminating Latent Tuberculosis. *Trends Microbiol* 17: 183-188.

Donlan, R. M. & J. W. Costerton, (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15: 167-193.

Dubos, R., B. Davis, G. Middlebrook & C. Pierce, (1946) The effect of water of water soluble lipids on the growth and biological properties of tubercle bacilli. *Am Rev Tuberc* 54: 204-212.

Dye, C., K. Lonnroth, E. Jaramillo, B. G. Williams & M. Raviglione, (2009) Trends in tuberculosis incidence and their determinants in 134 countries. *Bull World Health Organ* 87: 683-691.

Feldman, W. H. & A. H. Baggenstoss, (1939) The occurrence of virulent tubercle bacilli in presumably non-tuberculous lung tissue. *Am J Pathol* 15: 501-515.

Fey, P. D. & M. E. Olson, Current concepts in biofilm formation of Staphylococcus epidermidis. *Future Microbiol* 5: 917-933.

Ford, C. B., P. L. Lin, M. R. Chase, R. R. Shah, O. Iartchouk, J. Galagan, N. Mohaideen, T. R. Ioerger, J. C. Sacchettini, M. Lipsitch, J. L. Flynn & S. M. Fortune, Use of whole genome sequencing to estimate the mutation rate of Mycobacterium tuberculosis during latent infection. *Nat Genet* 43: 482-486.

Foreman, A. & P. J. Wormald, Different biofilms, different disease? A clinical outcomes study. *Laryngoscope* 120: 1701-1706.

Fox, W., G. A. Ellard & D. A. Mitchison, (1999) Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. *Int J Tuberc Lung Dis* 3: S231-279.

Fux, C. A., J. W. Costerton, P. S. Stewart & P. Stoodley, (2005) Survival strategies of infectious biofilms. *Trends Microbiol* 13: 34-40.

Gill, W. P., N. S. Harik, M. R. Whiddon, R. P. Liao, J. E. Mittler & D. R. Sherman, (2009) A replication clock for Mycobacterium tuberculosis. *Nat Med* 15: 211-214.

Gomez, J. E. & J. D. McKinney, (2004) M. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis (Edinb)* 84: 29-44.

Haapanen, J. H., I. Kass, G. Gensini & G. Middlebrook, (1959) Studies on the gaseous content of tuberculous cavities. *Am Rev Respir Dis* 80: 1-5.

Hall-Stoodley, L., J. W. Costerton & P. Stoodley, (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2: 95-108.

Hall-Stoodley, L. & H. Lappin-Scott, (1998) Biofilm formation by the rapidly growing mycobacterial species Mycobacterium fortuitum. *FEMS Microbiol Lett* 168: 77-84.

Hall-Stoodley, L. & P. Stoodley, (2002) Developmental regulation of microbial biofilms. *Curr Opin Biotechnol* 13: 228-233.

Harrington, M., (2010) From HIV to tuberculosis and back again: a tale of activism in 2 pandemics. *Clin Infect Dis* 50 Suppl 3: S260-266.

Higgins, D. A., M. E. Pomianek, C. M. Kraml, R. K. Taylor, M. F. Semmelhack & B. L. Bassler, (2007) The major Vibrio cholerae autoinducer and its role in virulence factor production. *Nature* 450: 883-886.

Hobby, G. L. & T. F. Lenert, (1957) The in vitro action of antituberculous agents against multiplying and non-multiplying microbial cells. *Am Rev Tuberc* 76: 1031-1048.

Hogan, D. A. & R. Kolter, (2002) Pseudomonas-Candida interactions: an ecological role for virulence factors. *Science* 296: 2229-2232.

Jindani, A., C. J. Dore & D. A. Mitchison, (2003) Bactericidal and sterilizing activities of antituberculosis drugs during the first 14 days. *Am J Respir Crit Care Med* 167: 1348-1354.

Kolter, R. & E. P. Greenberg, (2006) Microbial sciences: the superficial life of microbes. *Nature* 441: 300-302.

Kolter, R. & R. Losick, (1998) One for all and all for one. *Science* 280: 226-227.

Lenaerts, A. J., D. Hoff, S. Aly, S. Ehlers, K. Andries, L. Cantarero, I. M. Orme & R. J. Basaraba, (2007) Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. *Antimicrob Agents Chemother* 51: 3338-3345.

Loring, W. E. & H. M. Vandiviere, (1956) The treated pulmonary lesion and its tubercle bacillus. I. Pathology and pathogenesis. *Am J Med Sci* 232: 20-29.

Loring, W. W., I. Melvin, H. M. Vandiviere & H. S. Willis, (1955) The death and resurrection of the tubercle bacillus. *Trans Am Clin Climatol Assoc* 67: 132-138.

Mah, T. F. & G. A. O'Toole, (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34-39.

Malhotra, V., D. Sharma, V. D. Ramanathan, H. Shakila, D. K. Saini, S. Chakravorty, T. K. Das, Q. Li, R. F. Silver, P. R. Narayanan & J. S. Tyagi, (2004) Disruption of response regulator gene, devR, leads to attenuation in virulence of Mycobacterium tuberculosis. *FEMS Microbiol Lett* 231: 237-245.

Marrie, T. J., J. Nelligan & J. W. Costerton, (1982) A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. *Circulation* 66: 1339-1341.

McCune, R. M., F. M. Feldmann, H. P. Lambert & W. McDermott, (1966) Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med* 123: 445-468.

McKinney, J. D., K. Honer zu Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan, D. Swenson, J. C. Sacchettini, W. R. Jacobs, Jr. & D. G. Russell, (2000) Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 406: 735-738.

McNeill, K. & I. R. Hamilton, (2003) Acid tolerance response of biofilm cells of Streptococcus mutans. *FEMS Microbiol Lett* 221: 25-30.

Nguyen, K. T., K. Piastro, T. A. Gray & K. M. Derbyshire, (2010) Mycobacterial biofilms facilitate horizontal DNA transfer between strains of Mycobacterium smegmatis. *J Bacteriol* 192: 5134-5142.

O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver & R. Kolter, (1999) Genetic approaches to study of biofilms. *Methods Enzymol* 310: 91-109.

Ojha, A., M. Anand, A. Bhatt, L. Kremer, W. R. Jacobs, Jr. & G. F. Hatfull, (2005) GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell* 123: 861-873.

Ojha, A. K., A. D. Baughn, D. Sambandan, T. Hsu, X. Trivelli, Y. Guerardel, A. Alahari, L. Kremer, W. R. Jacobs, Jr. & G. F. Hatfull, (2008) Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol Microbiol* 69: 164-174.

Ojha, A. K., X. Trivelli, Y. Guerardel, L. Kremer & G. F. Hatfull, (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *J Biol Chem* 285: 17380-17389.

Opie, E. & J. Aronson, (1927) Tubercle bacilli in latent tuberculous lesions and in lung tissues without tuberculous lesions. *Arch Pathol* 4: 1-21.

Parish, T., D. A. Smith, S. Kendall, N. Casali, G. J. Bancroft & N. G. Stoker, (2003) Deletion of two-component regulatory systems increases the virulence of Mycobacterium tuberculosis. *Infect Immun* 71: 1134-1140.

Park, H. D., K. M. Guinn, M. I. Harrell, R. Liao, M. I. Voskuil, M. Tompa, G. K. Schoolnik & D. R. Sherman, (2003) Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. *Mol Microbiol* 48: 833-843.

Parrish, N. M., J. D. Dick & W. R. Bishai, (1998) Mechanisms of latency in Mycobacterium tuberculosis. *Trends Microbiol* 6: 107-112.

Post, J. C., (2001) Direct evidence of bacterial biofilms in otitis media. *Laryngoscope* 111: 2083-2094.

Prabhakara, R., J. M. Harro, J. G. Leid, M. Harris & M. E. Shirtliff, (2011) Murine immune response to a chronic Staphylococcus aureus biofilm infection. *Infect Immun* 79: 1789-1796.

Recht, J. & R. Kolter, (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in Mycobacterium smegmatis. *J Bacteriol* 183: 5718-5724.

Rhoades, E. R., R. E. Geisel, B. A. Butcher, S. McDonough & D. G. Russell, (2005) Cell wall lipids from Mycobacterium bovis BCG are inflammatory when inoculated within a gel matrix: characterization of a new model of the granulomatous response to mycobacterial components. *Tuberculosis (Edinb)* 85: 159-176.

Rustad, T. R., M. I. Harrell, R. Liao & D. R. Sherman, (2008) The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS One* 3: e1502.

Saini, D. K., V. Malhotra, D. Dey, N. Pant, T. K. Das & J. S. Tyagi, (2004) DevR-DevS is a bona fide two-component system of Mycobacterium tuberculosis that is hypoxia-responsive in the absence of the DNA-binding domain of DevR. *Microbiology* 150: 865-875.

Saltini, C., (2006) Chemotherapy and diagnosis of tuberculosis. *Respir Med* 100: 2085-2097.

Vandiviere, H. M., W. E. Loring, I. Melvin & S. Willis, (1956) The treated pulmonary lesion and its tubercle bacillus. II. The death and resurrection. *Am J Med Sci* 232: 30-37; passim.

Via, L. E., P. L. Lin, S. M. Ray, J. Carrillo, S. S. Allen, S. Y. Eum, K. Taylor, E. Klein, U. Manjunatha, J. Gonzales, E. G. Lee, S. K. Park, J. A. Raleigh, S. N. Cho, D. N. McMurray, J. L. Flynn & C. E. Barry, 3rd, (2008) Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect Immun* 76: 2333-2340.

Vuong, C., S. Kocianova, J. M. Voyich, Y. Yao, E. R. Fischer, F. R. DeLeo & M. Otto, (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* 279: 54881-54886.

Wayne, L. G. & L. G. Hayes, (1996) An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence. *Infect Immun* 64: 2062-2069.

Wayne, L. G. & K. Y. Lin, (1982) Glyoxylate metabolism and adaptation of Mycobacterium tuberculosis to survival under anaerobic conditions. *Infect Immun* 37: 1042-1049.

11

Cell Wall Deficiency in Mycobacteria: Latency and Persistence

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1. Introduction

It is believed that persistence of small populations of *Mycobacterium tuberculosis* in hosts underlies latent tuberculosis. Very little is known about the morphological and physiological nature of tubercle bacilli in latent TB. It is under discussion whether and how tubercle bacilli adapt to latent state and remain alive in face of damaging stressful conditions such as antibiotics and host immune factors. In this respect, cell wall deficiency (existence without rigid walls) in mycobacteria and its occurrence *in vivo* suggests one of the possible pathways by which tubercle bacilli can survive, replicate and persist within the body for a long period, harboring latent tuberculosis with a risk for disease reactivation, in case of reversion to classical TB bacilli upon changes in host immune status. Essentially, cell wall deficiency, or the ability of bacteria to exist as populations of self-replicating forms with defective or entirely missing cell walls (L-forms), is considered an adaptive strategy of bacteria to survive and reproduce under unfavorable circumstances.

This chapter elaborates on some special aspects of the L-form phenomenon and its importance for discovering new fundamental aspects of TB bacillary morphology and physiology, as well as understanding the mechanisms of latent tuberculosis.

2. History

L-forms were first observed by Emmy Klieneberger-Nobel, in 1945, whose typical "fried eggs"-shaped colonies, duplicating Mycoplasma, were isolated from cultures of *Streptobacillus moniliformis*. The wall-less variants of L-forms she named after the institution she worked in – England's Lister Institute.

Fig. 1. Emmy Klieneberger-Nobel – the founder of bacterial L-forms.

The period between 1882 and 1940, after Robert Koch discovered the cause of tuberculosis, was marked by series of papers reporting about the appearance of L-form elements in cultures of mycobacteria, such as filterable forms, branching filaments, syncytial growth, large spheres and "variegated mycelia", all of which characterize mycobacterial growth. Mattman summarized the known data about the ability of *M. tuberculosis* to convert to cell wall deficient forms and suggested a "L-cycle" for mycobacteria (Mattman et al., 1960; Mattman, 1970, 2001).

Fig. 2. Lida Mattman

Despite the long history in tuberculosis research, the nature of cell wall deficiency and its association with persistence in life of mycobacteria still remain obscure. Unfortunately, over the last several decades, investigations on these unusual forms of tubercle bacilli have been ignored and neglected. Information about forming of mycobacterial L-forms *in vitro* (in the laboratory), as well *in vivo* (within the body) is based mainly on studies concerning their morphological appearance. Two periods in L-form research of mycobacteria should be distinguished: before introduction of chemotherapy against tuberculosis, and after. Observations made in the beginning of 20th century on mycobacterial pleomorphism and L-form elements provide evidence for existence of L-forms without contact with antimicrobial drugs (Calmette & Valti, 1926; Much, 1931). In the following decades, examinations regarding modification of morphology and L-form transformation by antimicrobials became the starting point of additional information on mycobacterial properties (Dorozhkova & Volk, 1972; Dorozhkova & Volk, 1973; Kochemasova et al., 1968; Mattman et al. 1960; Wang & Chen, 2001).

3. Basic characteristics of cell wall deficient L-forms

3.1 L-conversion, morphology and ultrastructure

Bacterial L-form conversion, i.e. existence without rigid walls, is universal but difficultly recognized phenomenon in nature (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981). The term „cell wall deficiency" implies alterations in the constitution of bacterial cell wall, resulting from deletion and faulty synthesis of wall components (Mattman, 2001). It is considered that imbalance of cells' ability to degrade and synthesize its classical thick wall results in cell wall deficiency. Since the peptidoglycan is the stress-bearing structure of bacteria, its loss, respectively the loss of rigidity, is a distinctive characteristic of cell wall deficient forms (L-forms). In fact, morphological variability is an indicative and common feature of all L-forms, regardless of what bacterial species they originated from. Although these forms have been observed in patients' specimens for many decades, most are ignored and generally regarded as diagnostically insignificant staining artifacts or debris

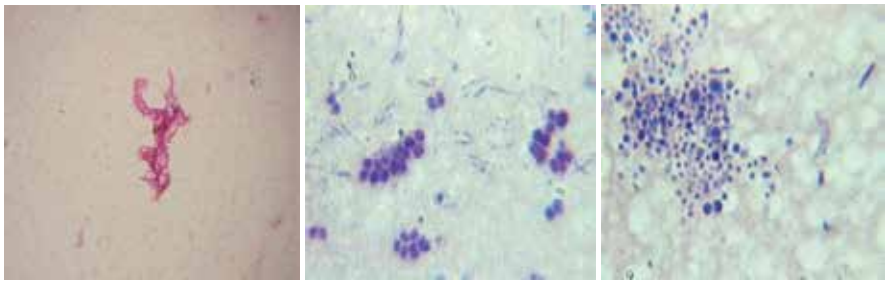
(Domingue, 2010). It is assumed that these pleomorphic forms represent various stages in the life cycle of stressed bacteria.

M. tuberculosis is known to exhibit extreme pleomorphism in certain circumstances. Various morphological forms of mycobacteria were observed by many authors and were described as "mycococcus form" (Csillag, 1964), large "amoeba-like cells" (Imaeda, 1975), giant non-cellular structures or so called "budding yeast-like structures" (Koch, 2003), "elementary bodies and filament structures" (Merkal et al, 1973) "endospores" (Ghosh et al.,2009; Traag et al, 2010) and "ovoid cells"(Shleeva et al., 2011). Mycobacteria are unique among procaryotes with their cell wall structure, containing tightly packed mycolic acids that provide TB bacilli with efficient protection and remarkable capacity to resist to various exogenous stress conditions. The high concentration of lipids in cell wall of mycobacteria is associated with general insusceptibility to chemical/toxic agents and most antibiotics. The mycolic acids and glycolipids in cell wall of mycobacteria also impedes the entry of nutrient substrates, causing the organisms to grow slowly (Draper, 1998). However, mycobacterial cell wall appears to be a dynamic structure that can be remodeled, as the microorganism is either growing, or persisting in different environments (Kremer & Besra, 2005). Under unfavorable conditions, where mycobacteria are exposed to different damaging factors particularly in face of host defense mechanisms, they may produce cell wall deficient forms (L-forms) (Markova et al.2008a; Markova et al. 2008b). A variety of papers reported about production of mycobacterial L-forms experimentally *in vitro,* using different inducing factors. Wide range of substances (cell wall inhibitors) as antibiotics, lytic enzymes and some amino acids affecting cell wall and especially biosynthesis of peptidoglycan have been used as L-inducing factors (Beran et al., 2006; Hammes et al., 1973; Hines and Styer, 2003; Naser et al., 1993; Udou et al., 1983). Indeed, it is important to understand how mycobacteria regulate the cell wall composition in response to changing environment. In some wall deficient cells pieces of cell wall are synthesized and dutifully pulled through the pores of cell membrane but somehow lack structural detail that would permit them to link together. Mitchel & Moyle have added another interesting aspect to consider, which may explain why a cell is unable to resynthesize its cell wall, once losing it. They postulate that perhaps the building blocks are sufficiently soluble to diffuse spontaneously into the culture medium than remain together against the wall where their union is facilitated (Mitchel & Moyle, 1956).

The ability of strains from *M. tuberculosis* complex to produce L-phase variants after nutrient starvation stress was demonstrated in our experiments (n. d.). Morphological transformations of tubercle bacilli from acid fast to polymorphic non-acid-fast and coccoid forms of varying size were observed (Fig. 3). In contrast to classical tubercle bacilli, which typically appear as straight or slightly curved red stained rods in Ziehl-Neelsen stained smears, mycobacterial L-forms showed marked polymorphism and variability in staining reaction. L-form variants of mycobacteria lost acid fastness completely and resembled the morphology of various other bacteria (Fig. 3 b, c).

It is known that acid fastness is dependent on the integrity of the tubercle bacilli. Sometimes, persistent *M. tuberculosis* bacteria bearing cell wall alterations may remain undetected by the classic Ziehl-Neelsen staining (Seiler et al, 2003). Appearance of polymorphic non-acid fast forms and coccoids in cultures of mycobacteria has been observed by other authors

(Chandrasekhar & Ratnam , 1992; Csillag, 1964; Juhasz, 1962; Miller, 1932; Xalabarder, 1958).



a b c

Fig. 3. Ziehl-Neelsen stained smears: (a) control TB bacilli; (b, c) non-acid fast polymorphic cells of *M. tuberculosis* L-forms (n.d.)

Morphological forms of different sizes and shapes (short coccobacilli and long rods, oval or round coccoid cells, large spherical bodies and giant filaments) in mycobacterial L-form cultures obtained after starvation stress, were observed by us with scanning electron microscopy (Fig.4, n. d.). Very small granular elements placed on membrane filters with pore size diameter of 0.22µm, evidencing their ability to pass through bacterial filters i.e. filterable L-form cells, were detected (Fig. 4 f). The filterable forms are considered as minimal reproductive cells, which can be formed from large L-bodies in all possible ways. It is believed that such filterable bodies contain a bacterial genome and minimal metabolic

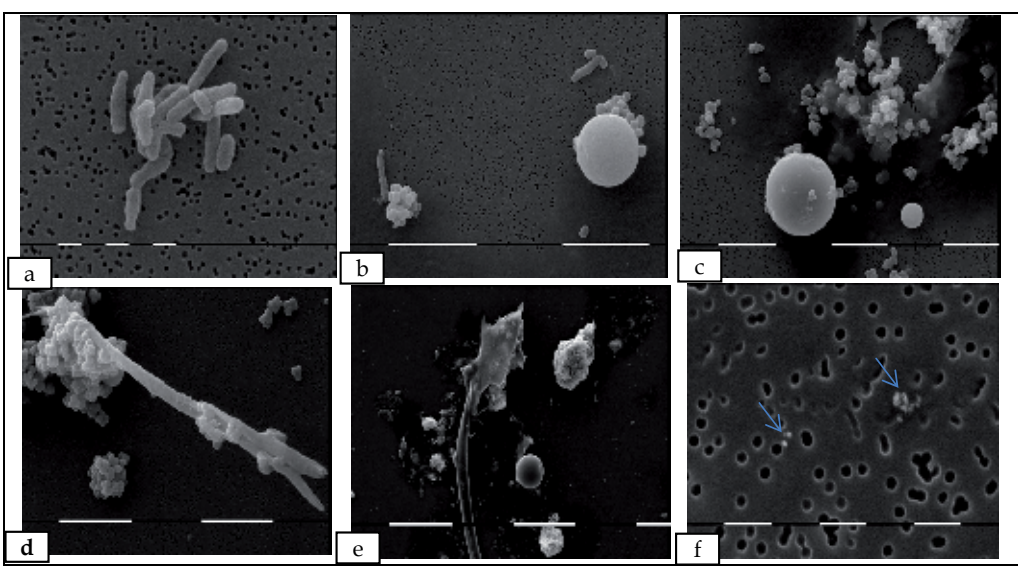


Fig. 4. Scanning electron microscopy of classical tubercle bacilli (a) and mycobacterial L-forms obtained after stress treatments *in vitro* of *M.tuberculosis* (b), *M.bovis* (c, d) and *M.bovis* BCG (e, f), (n.d.).

capability sufficient to initiate reproduction (Domingue, 2010; Klieneberger-Nobel, 1951; Prozorovski et al., 1981).

Findings from transmission electron microscopy yielded additional valuable information about the ultrastructure morphology of mycobacterial L-forms. Examinations of *M. tuberculosis* L-forms obtained *in vitro* after starvation stress (n.d.) or during experimental infection in rats (Markova et al., 2008a) revealed typical fine structure of L-form population. L-phase growth consisted of cells of variable shape and size, completely devoid of bacterial cell wall and bound only by a single unit membrane (Fig. 5). Large and elementary bodies of different electron microscopy density, as well as very small granules and vesicular forms were observed (Fig. 5 b, c, d). Some vesicular forms either appeared empty or contained electron-dense granules (Fig. 5 c, f). Of considerable interest was the observation of large bodies of so called "mother" cells, filled with numerous small spherical L-elements (Fig. 5 f). Such "mother" cells are often internally vesiculated and may produce also small, empty bodies, or membrane bound vesicles. Fragmentation of the cytoplasmic mass in numerous granular forms was the mode of L-form reproduction that was noted. Cytoplasmic condensation at the periphery of the large bodies ending in formation of protrusions and buds was often seen. Budding, another mode of L-form replication, was observed as well. It should be noted that nucleoid and ribosomal areas within L-bodies were of variable electron densities and intracellular location .The nucleoids were variable, being sometimes compact and sometimes scattered throughout the cytoplasm. Enucleated L-bodies were also seen. Ribosomes were either packed together or diffusely scattered, usually at the periphery of the cells. Electron-dense L-bodies of different size and giant filamentous forms were found in clinical isolates of *M. tuberculosis* (Fig.5 g, h; Michailova *et al*, 2005).

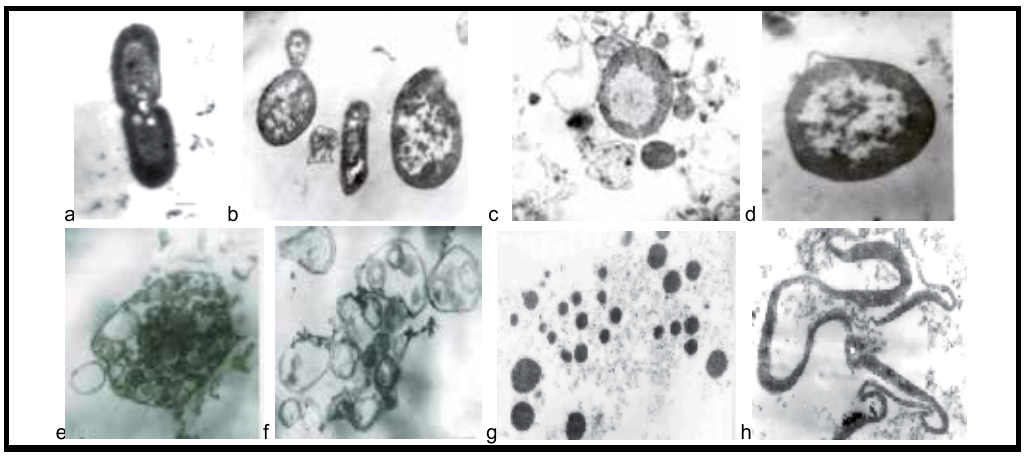


Fig. 5. Transmission electron microscopy of classical tubercle bacilli (**a**), and L-forms of *M. tuberculosis* obtained *in vitro* after starvation stress (**b ,c ,d**; n. d.), isolated from rats, experimentally infected with *M. tuberculosis* (**e, f**; Markova et al.,2008a) and observed in clinical strains, isolated from patients (**g, h;** Michailova et al., 2005).

3.2 Modes of reproduction and morphogenesis of L-cycle

The normal existence of bacteria appears to be a dynamic state of morphological and physiological changes, and the reproducibility in response to established conditions for

growth is considered as a "life style". Under certain circumstances, bacteria can enter unbalanced growth and undergo complex life cycles, involving different morphological transformations, known as the bacterial L-cycle. Conversion to L-forms is assumed to be a general property of bacteria and as adaptive reaction to unfavourable environmental factors, which interfere with the normal reproduction, as well as permit the growth of cell wall deficient variants (Dienes & Weinberger, 1951). Loss of rigidity due to the lack of murein layer in L-forms, results in uncoordinated propagation and appearance of highly pleomorphic forms. In contrast to classical bacteria, L-forms can reproduce by great variety of unusual modes, such as irregular binary fission, budding, protrusion-extrusion of elementary bodies and granules from large bodies, multiple division with intracellular fragmentation of cytoplasm or combination of all types (Prozorovski et al., 1981). Variations in the development of morphological units of different sizes and shapes, typical for L-forms, appear in accordance with the changing environmental conditions (Markova et al, 2010). The newly reorganized L-form population continues to exist and replicate by unusual modes, displaying various cells and elements such as elementary and large spherical bodies, granular and filterable forms, vesicular and empty bodies, giant filaments and others. Those giant filaments and large bodies may be serving a two-fold purpose, playing a role in L-form reproduction, as well as protecting them from unfavourable environment.

Our observation of giant L-bodies ("mother" cell) within mycobacterial L-form population releasing, through protrusion or budding, numerous previously generated granules, is also perhaps noteworthy of mentioning. Such granular elements, often released from the terminal sides of filaments (Fig 4 d, e; n. d.), were found to develop into bigger coccoid or large L-bodies, although transformation of granules into rod shaped forms was also noticed. The segmentation of L-bodies and breaking up into small elements, which germinate again, as well as the processes of regeneration initiated by the fusion of certain elements (Klieneberger-Nobel, 1951), challenge the conventional vision about bacterial replication. Although the modes of L-form replication were less effective, it should be noted that, at a point of their development and adaptation, L-forms started multiplying with remarkable rapidity, by releasing numerous small granules from collapsing giant L-structures. The small forms grew into large bodies which subsequently either increased in diameter, or disintegrated into even smaller L-form bodies. The observed by us different arrangements of *M. tuberculosis* L-forms coccoid cells of varied size (singly, in pairs or in irregular clusters) suggest either capability of L-forms to divide in different planes by binary fission or the possibility that they arose *en masse* from huge L-form bodies. In our opinion, L-life style is best understood by taking into consideration the unusual modes of replication, exhibited by L-forms. L-forms behave like an entire population, within which the role of individual organisms and organelles is difficult to determine (Markova et al., 2010). Of all structures in the L-cycle, syncytium, designed as "symplasm" and consisting of numerous nuclei embedded in a cytoplasm within one L-body (Mattman, 2001), is the most incredible. As noted by Mattman, fifty mycobacteria can be made within one sac (L-syncytium). Syncytia were observed to be formed from coalescing aggregates of bacteria, when the cell walls disintegrate and the cytoplasm starts to coalesce. The granules emerging from the symplasm grow into young cells, which reproduce further by fission or by other modes. According to Norris, syncytium-like structures may create a favorable environment for development of a complex prebiotic ecology, in which rearranged hyperstructures give rise to even more complex life forms (Norris, 2011).

It is assumed that cell wall deficient bacterial forms survive storage and unfavorable conditions much longer than classical bacteria (Mattman, 2001). Domingue suggests the role of small electron-dense bodies (filterable granules) as notoriously resistant forms of pathogenic bacteria (Domingue, 1997). Xalabander noted that L-forms of mycobacteria were remarkably different from L-forms of other species in their resistance to physical and chemical agents. Similar to prions, mycobacterial L-forms escape destruction by body's immune system, and are seemingly imperishable. Xalabander also noted that these L-forms contain both RNA and DNA proteins, but do not stain well by ordinary mycobacteria dyes (Xalabander, 1958; 1963). On other hand, it is supposed that the smallest and most resistant to environmental stresses filterable L-granules, containing DNA may exert nuclear functions (Klieneberger-Nobel, 1951). Moreover, chromosomal DNA, especially within L-symplasm, should be regarded as a substantial mass of the nucleoid body, which can dynamically interact with other components (Allan et al, 2009). This problematic question is still under discussion and yet, no matter how small and at first glance, enucleated, some of these L-forms will revert back to virulent mycobacteria.

Shleeva et al. (2010) believe that dormancy in mycobacteria is related to the formation of different cell forms with various characteristics (less differentiated cyst-like forms, weakly differentiated resting cells and highly differentiated spore-like forms) within a population. According to the same authors, passing into a dormant state is associated with drastically decreased metabolic activity of cells, enhanced resistance to harmful factors, and absence of cell division. The resting cells retain their viability but lose capacity for germination and growth, becoming "nonculturable". It is a generally accepted postulate that TB bacilli are in a true dormant state, undergoing no replication. Dormant cells switch on the mechanisms of division arrest and may persist, due to survival of a small number of bacteria (Kaprelyants et al., 1993; Postgate & Hunter, 1962; Shleeva et al., 2010). Recent data, however, cast doubt on the assumption of such 'inactive' latent state, as there is constant metabolic activity within the TB bacilli (Zumla et al., 2011). Evidence about the role of molecular chaperones and intercellular signalling molecules in control of metabolic activity and composition of the cell wall has been provided by Henderson et al. (2010).

From the view point of the L-cycle theory, a transition of mycobacteria from acid-fast to non-acid fast state, along with appearance of polymorphic cell wall deficient cells, occurs in response to stress. L-forms develop through several stages and result in formation of polymorphic or coccoid fast growing cells. The initial phase of L-conversion probably corresponds to an "invisible" stage, where bacteria cease forming colonies on solid media and growing in liquid media. We suppose that formation and persistence of giant L-forms structures (filaments, syncytia and "mother" cell) sheltering and embodying many individuals inside a common envelope, represents a unique mechanism of survival and may resemble "invisible" or cryptic state of L-form development. However, at some point of L-form development, these giant spherical or filamentous forms start to disintegrate and are no longer visible, giving place to an abundance of granular and coccoid forms, which sometimes become the prevailing elements within L-population. Coccoid forms of mycobacteria, called "mycococcus", were obtained *in vitro* by Csillag in 1964. Mycococcii were grown from *M. tuberculosis* and were similar to the morphology of staphylococci (Csillag, 1964). Genetic analysis of mycobacterial coccoids however, performed by us through amplification of 16SrRNA gene fragment, 16S-23S rRN gene Internal Transcribed

Spacer sequences and IS *6110* PCR, verified them as *M. tuberculosis* (n. d.). DNA sequencing analysis is currently in progress (n. d.).We consider that the invisible L-conversion phase is followed by a state of active reproduction of non- acid fast and non-recognizable as mycobacteria L-forms usually with coccoid morphology. Taken together, these data may argue that the curious morphology and growth characteristics of mycobacterial L-forms, their extremely different habit of existence define them as specific type of unrecognizable and hidden persisters. As seen in Fig.6, L-form conversion cycle of mycobacteria is schematically outlined with emphasis on ability of different L-structures to form colonies. In this sense, L-form persistence phenomenon substantially differs from the current understanding for latency as persistence of few ''non-replicating''or ''dormant'' bacteria.

3.3 Growth characteristics and colonial morphology

In contrast to classical tubercle bacilli, we found that L-form variants, obtained after nutrient starvation stress of *M. tuberculosis in vitro,* grew and developed colonies phenomenally faster, mimicking rapidly replicating bacteria.

The morphology of growths underwent progressive changes, which resulted in formation of typical L-form colonies with "fried egg" appearance (Fig.7 b, c).

As pointed out by other authors, dark centers of "fried egg" colonies usually consist of dense granular elements, which are deeply embedded in the medium but at the periphery of the colony large pleomorphic bodies are frequently found (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981). The shape of L-form colonies resulted from the variety of individual structural units and the way that they divided (Mattman, 2001). It should be pointed out that fully developed L-type colonies appeared between 36 and 48 hours after plating on Middlebrook semisolid agar, in contrast to control *M. tuberculosis* microcolonies.

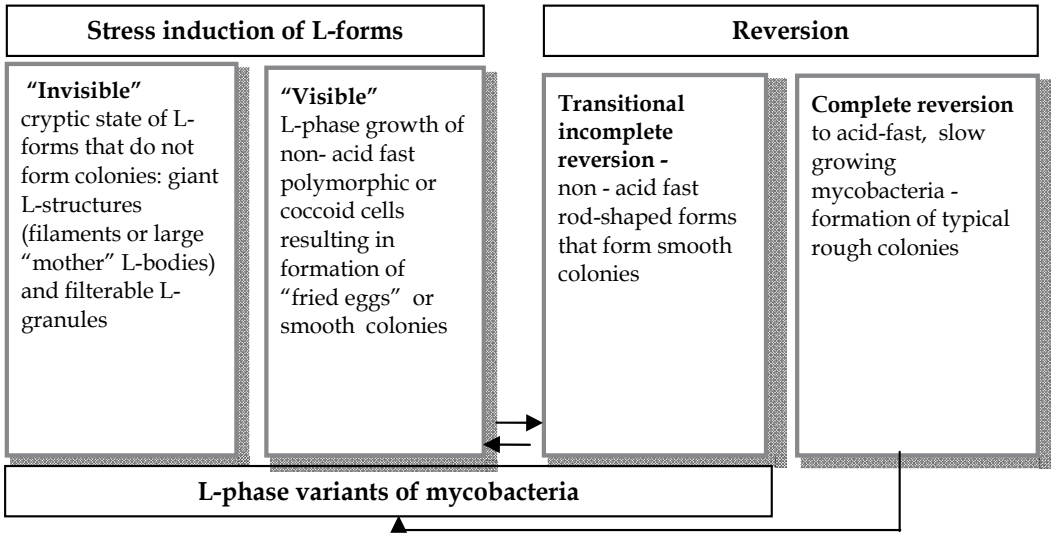
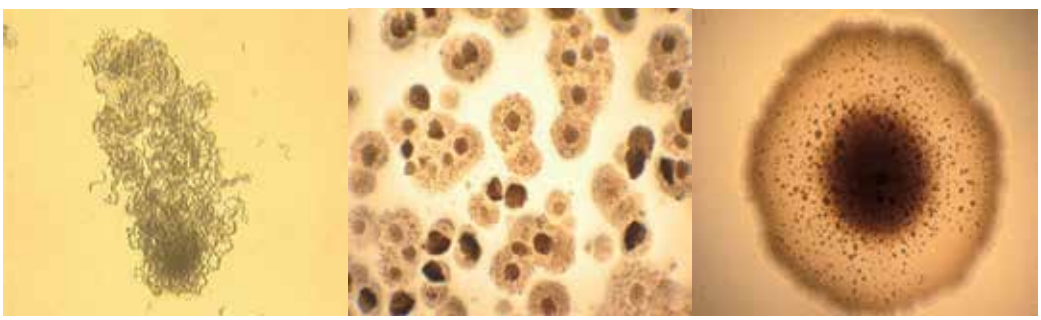


Fig. 6. Morphological phases during L-form conversion and reversion of mycobacteria.

We suggest that the lack of cell walls and easier permeation of nutrients is the reason for the unique ability of mycobacterial L- forms to grow faster in comparison to classical tubercle bacilli. Pla Y Armengol (1931) found that a large inoculum of tubercle bacilli grows rapidly on all routine media, appearing as large L-body spheres and also vegetated mycelia. In our study, L-form variants were adapted without difficulties to grow on conventional nutrient agar. Light and electron microscopy also provided interesting results about the appearance of non-acid fast coccoid cell morphology of stressed *M. tuberculosis*, that support observations of other authors. The appearance of non-acid fast coccoids in cultures of mycobacteria has been reported by others in the beginning of the last century but the phenomenon was not clearly explained and proven at that time (Csillag, 1964; Juhasz, 1962; Xalabander, 1958;). More surprising was the fact that mycobacterial coccoid L-forms not only mimicked the morphology of staphylococci or other coccus- shaped bacteria, but also exhibited extremely rapid growth and colonial development in contrast to classical TB bacilli (n. d.). Coccoid cells were initially mistaken by us as contaminants, but the specific DNA testing (amplification of 16SrRNA gene fragment, 16S-23S rRNA gene Internal Transcribed Spacer sequences, IS*6110* PCR and DNA sequencing analysis) identified them as *M. tuberculosis* (n. d.). We suppose that non-acid fast coccoid L-form variants of mycobacteria resulted probably from the more regular mode of multiplication, synchronization and stabilization of L-form cells under specific condition of cultivation. Thus, it can be presumed why such coccoid forms of *M. tuberculosis* remain often unrecognized or are mistaken for contaminants.



a. b. c.

Fig. 7. Light microscopy of (a) control *M. tuberculosis* rough microcolony and (b, c) typical "fried eggs" shaped colonies of *M.tuberculosis* L-forms obtained after nutrient starvation stress (n. d.).

Standard plating techniques are often inadequate for accurate enumeration of microbial dormant forms, because some of them may be in a "nonculturable" state (Shleeva et al., 2010). When it comes to L-forms, they are considered to be both "difficult-to-cultivate" and "difficult-to-identify". Because of their altered morphology and fully changed bacterial life cycle, L-forms are difficult to be identified in clinical materials. The isolation of arising *in vivo* L-forms is generally possible only with special procedures ensuring their enrichment and resuscitation to actively growing state i.e. having an ability to form colonies (Michailova et al., 2000a; Zhang et al., 2001; Zhang, 2004). The use of specially supplemented liquid and semisolid media, as well special techniques, like so called "blind" passages, are absolutely necessary for isolation of L-forms from specimens (Michailova et al., 2005; Markova et al., 2008a).

3.4 Yin-Yang hypothesis for co-existence of classical and L- forms within natural mycobacterial population

The Yin-Yang hypothesis is based on the idea that classical and cell wall deficient forms co-exist within natural mycobacterial populations. The Chinese concept of the complementary alternating forces of Yin and Yang provides opportunities to better understand the natural phenomenon of heterogeneity and correspondence between both subpopulations in mycobacteria. The Yin-Yang point of view, suggesting the hypothesis for coexistence of classical and cell wall deficient forms, is illustrated in Fig.6.

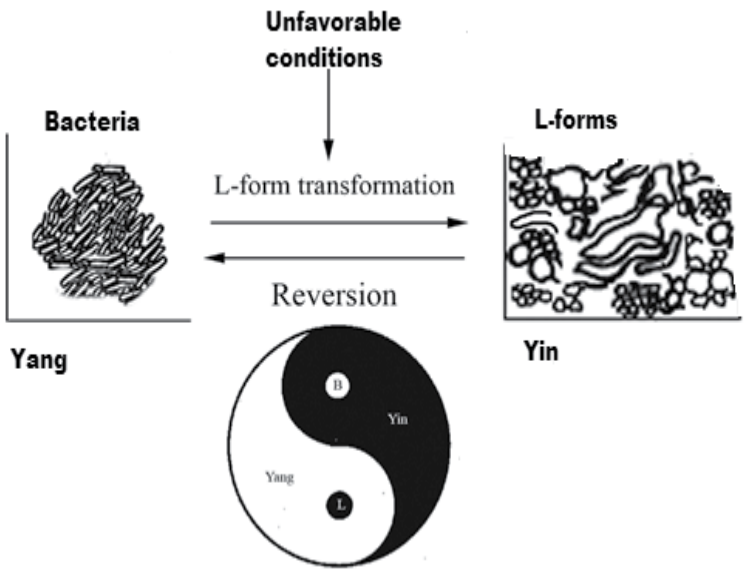


Fig. 8. Alternating "Yin-Yang"–life phases of classical walled bacteria and cell wall deficient L-forms: "Yang" - monomorphic population of classical rod shaped bacteria under optimal conditions ensuring yield and nutrients for growth and cell division; "Yin"- polymorphic population of L-forms. Polymorphism ensures survival advantages and arsenal of various notoriously resistant to environmental assaults L-form structures under unfavorable conditions.

The morphological diversity within bacterial populations is often related to heterogeneous environments observed under natural conditions. Population-based morphological variability and cell wall deficiency of *M. tuberculosis* might be considered as a natural phenomenon ensuring the adaptive strategy of this pathogen for environmental change (Markova, 2009; Mattman, 2001). By making use of our Yin-Yang concept, by utilizing available scientific data on this subject and our own findings, we try to figure out how classical walled and cell wall deficient subpopulations interact under different conditions. We found that both classical walled and cell wall deficient L-forms coexist within clinical strains of *M. tuberculosis* freshly isolated from sputum of patients has been demonstrated in our study (Michailova et al., 2005). This finding supports the concept that natural mycobacterial population usually consists of prevalent classical forms and small numbers of L- forms. There is data reporting about coexistence of classical walled and cell wall deficient

L-forms within natural populations of other bacteria as well as about relations of concurrence and interference between them under different conditions (Boris *et al*., 1969; Fodor and Roger, 1966). Extreme morphological plasticity of bacteria has been found to provide survival advantages (Justice *et al*., 2008; Young, 2007). It is assumed that L-forms occur along with resistance to factors that trigger their appearance (Prozorovski, 1981).

We found that under stress *in vitro* and *in vivo* (Markova et al, 2008a) the balance within mycobacteria was shifted in favor of cell wall deficient forms and the population continued to exist, replicating predominantly as L-forms. Our *in vitro* experiments aimed to induce L-conversion of *M. tuberculosis,* by means of nutrient starvation stress (n. d.). Once the process in favor of L-form development was induced and shifted, further selective separation of L-form variants was made, based on the unique ability of mycobacterial L- forms to grow faster in comparison to classical tubercle bacilli due to the lack of cell walls and easier permeation of nutrients. Selection of mycobacterial L-forms was achieved technically through transfers of stressed mycobacterial cultures at weekly intervals on semisolid Middlebrook agar. Due to their growth advantage, mycobacterial L-form variants became the prevailing subpopulation, overgrowing classical TB bacilli during the performed five passages, which resulted in isolation of L-form cultures. As has been demonstrated in our previous study, similar L-form transformation of *E. coli* was found to appear under conditions of starvation and, more surprisingly, after lethal heat stress (Markova et al., 2010). However, it has been found that cell wall deficient forms of *E. coli* developed slower than classical walled forms. In contrast with interactions between both subpopulations during L-form transformation of *E. coli*, we recognized the opposite relations between classical and L-forms in mycobacteria, which were strongly influenced by the special biochemical structure and physiology of TB bacilli.

4. Formation and persistence of mycobacterial L-forms in vivo

Animal models for the study of tuberculosis include guinea pigs, mice, rabbits and nonhuman primates. Despite the difficulty in modeling human latency in experimental animals, the understanding of both host and microbial factors that contribute to the establishment and maintenance of a persistent *M. tuberculosis* infection has progressed and the information gathered is pertinent to human latent tuberculosis (Flynn & Chan, 2001). Formation of *M. tuberculosis* L-forms *in vivo* were demonstrated by means of biological experiments on guinea pigs (Li, 1990; Markova et al., 2008b ; Ratnam &Chandrasekhar, 1976; Snitinskaia et al., 1990;), mice (Belianin et al., 1997) and rats (Markova et al., 2008a).

In our study, we established a rat model of experimental tuberculosis that produces mycobacterial cell-wall deficient forms *in vivo* (Markova et al, 2008a). Although rats are not a common animal model for TB research, we attempted, on basis of our previous experience with other bacterial L- form experimental infections (Markova et al, 1997; Michailova et al., 2000), to use the capability of these animals to exhibit high innate resistance to infections, thus ensuring inhibition of classical bacterial forms and inducing the occurrence of cell-wall deficient forms. After intraperitoneal and intranasal infection with *M. tuberculosis*, samples from lung, spleen, liver, kidney, mesenterial and inguinal lymph nodes and broncho-alveolar and peritoneal lavage liquid were taken and plated simultaneously on Löwenstein-Jensen medium or inoculated into specially supplemented for L-forms Dubos broth at weekly intervals over five weeks. Mycobacterial L-form cultures were isolated throughout

the whole period of the experiment, including the last two weeks, when typical mycobacterial colonies consisting of classical bacilli were not isolated on Löwenstein-Jensen medium. If we had used only the classical isolation procedure with Löwenstein-Jensen media alone, we would have been led to falsely believe that mycobacteria were completely eliminated. However, mycobacteria continued to persist as L-forms at the late stage of infection. We believe that the established by us rat model of experimental tuberculosis can mimick latent infection.

Mycobacteria can convert to cell wall deficient forms (L-forms) inside macrophages. After intraperitoneal administration of BCG, samples of peritoneal lavage fluid from guinea pigs were obtained at day 1, 14 and 45. In order to study whether and how *M. bovis* BCG can transform in L-forms and persist *in vivo*, series of events during interaction of live BCG bacilli with peritoneal macrophages in guinea pigs were evaluated and observed by transmission electron microscopy (Markova et al, 2008b). At the late intervals of infection, an interesting phenomenon of L-form formation inside macrophages was observed. The percent of the formed L-forms at day 14 was about 15% and at day 45, we did not find any BCG bacilli with normal morphology - all observed bacteria were in L-form state. Examination of BCG bacilli inside macrophages revealed morphological peculiarities typical of cell wall deficient bacterial L-forms, as well as different modes of L-form multiplication. As shown in Fig. 9 (d, e, f), pleomorphic and relatively large BCG L-form bodies were found inside vacuoles which were found to persist for a long time inside macrophages due to the ineffectual phagocytosis, digestion and clearance**.** Fusion of small phagosomes containing L-forms and formation of larger ones was seen as well . Additional point of interest was the observation that many mitochondria (M) with enlarged size and endoplasmic reticulum dilation (ER) were clustered closely and around L-forms . The observed process of organelle translocation appeared to be related to the intracellular life of L- forms - survival and multiplication. Microbial digestion, respectively a process of complete phagocytosis of L-forms, was not observed. Some intra-phagosomally located L-forms inside macrophages were surrounded by multi-membranes (Fig. 9 f) and so packed within membranes they were released to the extracellular space. The observed cycle of L-form attachment and engulfment by new phagocytes at the late stage of infection suggests that L-forms probably exploit apoptotic-like pathway as means of returning to the extracellular environment and for subsequent rounds of new entry and uptake by macrophages. Obviously, such apoptosis-like pathway may protect L-forms from humoral and cellular host defense factors during their trafficking from intracellular to extracellular compartment and vice versa. It is generally assumed that apoptosis has developed as a host defence mechanisms against infection, but it is not completely clear what advantages apoptosis can provide to bacteria (Keane et al., 2000; Riendeau & Kornfeld, 2003; Rosenberger & Finlay, 2003). A number of authors have presented evidence that cell-wall defective variants can be formed within macrophages (Mattman, 2001; Michailova et al., 2000a; Thacore & Willett, 1966). Thacore and Willett (1966) have reported about formation of spheroplasts of *M. tuberculosis* within tissue culture cells.

Since *M. bovis* BCG is an attenuated live strain, little is known about how long it can survive in the vaccinated individuals . Reports about detection and isolation of BCG bacilli from patients with AIDS many years after vaccination (Armbruster et al., 1990; Reynes et al., 1989; Smith et al., 1992) give rise to questions about the mechanisms by which BCG bacilli persist

in vivo for a long time. As far as cell wall deficiency facilitates the bacterial survival under unfavorable conditions, L-forms of different bacterial species have been shown to survive and persist for an extended period inside macrophages due to the ineffectual phagocytosis, digestion and clearance (Markova et al., 1997; Michailova et al., 1993; Michailova et al., 2000b; Michailova et al., 2007). The finding that of all the bacteria, L-forms predominate and are crucial to the survival of mycobacteria *in vivo* (Mattman, 2001; Michailova et al., 2005) needs to be taken into account when developing and putting in use new viable mycobacterial vaccines, especially considering that L-forms of *M. bovis* BCG bacilli have been found in the blood of persons vaccinated against TB with BCG vaccine (Xalabarder, 1958). This provides us with insight of the importance of L-form conversion phenomenon for the behavior, persistence and safety of live BCG vaccines.

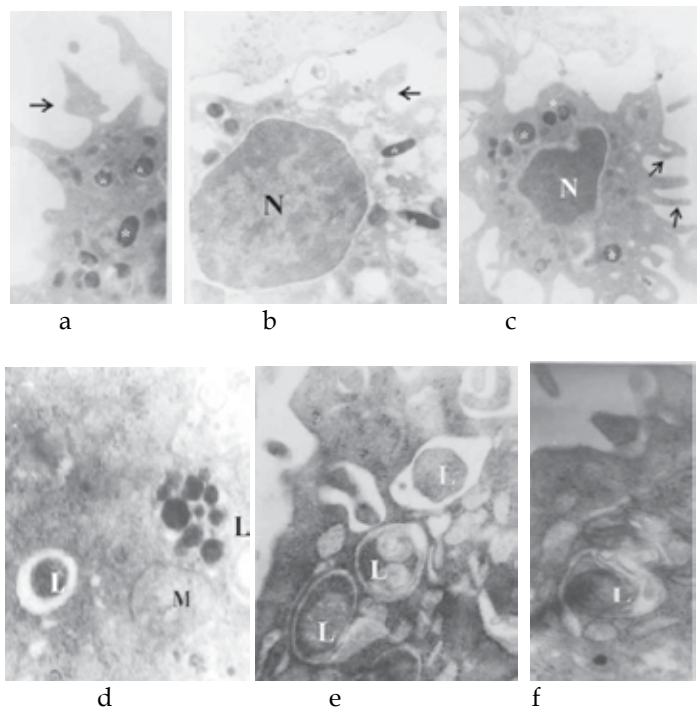


Fig. 9. Formation of BCG L-form cells (L) within the vacuoles in guinea pigs peritoneal macrophages**: a, b , c** -at day 1 after BCG installation, the interactions of BCG bacilli (*) with peritoneal cells demonstrated initial phases of phagocytosis including attraction, adhesion and attachment of bacteria to the phagocytes and processes of bacterial enclosing and engulfment; **d, e, f**- at day 45 after BCG installation, formation of BCG L-form cells (L) within the vacuoles near to mitochondria (M); **d**, **e** - L-form multiplication inside macrophages; **f**- BCG L-form large bodies , surrounded by multi membranes; Bar = 0.5 μm (Markova et al., 2008)

5. Reversion of mycobacterial L-forms to classical TB bacilli

Reversion of L-forms to normal parental bacteria is an important property, which is inducible by changing the condition of cultivation *in vitro* or occurs spontaneously *in vivo*

under favorable for the pathogen circumstances. Mattman defined the essential factors for reversion, the most popular of which are omission of the inducing agent, changes in nutrition, concentrating populations, inoculation in to experimental animals and others (Mattman, 2001). Of special interest is the reversion stimulated by products from microbes. Rathham & Chandrasekhar reported about reversion of filterable variants of tubercle bacillus from sputum by culturing with Freund's adjuvant (Rathham & Chandrasekhar, 1976). Although atypical forms are genetically programmed to develop a cell wall, it is not yet clear how compromised cell wall deficient bacteria mobilize the energy necessary for reversion to bacterial walled phase. It is interesting to note that the reversion of mycobacterial L-forms to normal TB bacilli appeared to be more difficult and slower, when compared to other bacteria.

There is a widespread assumption, which perceives the dormant state of *M. tuberculosis* as a *reversible* state or as ability of mycobacteria to reverse into active state and to reactivate the disease (Shleeva et al., 2010). Recently, it has been found that bacteria possess a specific system for autoregulation of growth and development, which participates in control of cell differentiation at the level of regulation of the functional activity of subcellular components and of the cell as a whole (Shleeva et al., 2010). Resuscitation-promoting factors have also been identified and their role in latency and reactivation of tuberculosis have been investigated (Biketov et al., 2007; Zhang et al., 2001;). Five genes encoding Rpf-like proteins have been found in *M. tuberculosis* genome, which may act in reactivation of "nonculturable " forms of *M. tuberculosis* (Kana et al., 2008; Mukamolova et al., 2002; Tufariello et al., 2004). Shleeva et al. (2003) found that cell-free culture liquid of an exponential-phase *Mycobacterium tuberculosis* culture or the bacterial growth factor Rpf exerted a resuscitating effect, substantially increasing the growth capacity of the nonculturable cells in liquid medium. During resuscitation of nonculturable cells, a transition from ovoid to rodlike cell shape occurred.

6. Clinical significance and role of mycobacterial L-forms

Arguments for and against significance of L-forms as infecting and persisting agents, respectively their role in human and animal diseases, are limited because of difficulties in their isolation, cultivation and identification. However, a lot of papers, reviews (Allan et al., 2009; Beran et al., 2006; Domingue and Woody, 1997; Domingue, 2010; Gumpert & Taubeneck, 1983; Onwuamaegbu et al., 2005; Zhang, 2004) and several monographs (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981), support the concept that L-forms can be induced *in vivo*, can persist there for a significant span of time and can be the cause for latent, chronic and relapsing/recurrent infections, as well as for diseases of unknown infectious-allergic or autoimmune origin.

However, of all the bacteria, L-forms predominate and are crucial to the survival of *M. tuberculosis in vivo.* Therefore they are thought of as carriers of a tubercular constitution (Mattman, 2001). The understanding of cell wall deficiency in *M. tuberculosis* may occur as consequence of a long-lasting interaction with the host and as a strategy to ensure its survival and persistence *in vivo* is still limited, as mycobacteria are quite difficult to detect, especially when in their viral-like, cryptic state. Although the mechanisms of spontaneously occurring *in vivo* cell wall deficient forms are difficult to explain, many authors have considered that mycobacteria, undergoing L-form transformation, are of clinical significance

for the incidence of relapses and are a prognostic unfavorable indicator (Berezovski & Salobai, 1988; Dorozhkova. et al., 1989, Dorozhkova et al., 1990; Khomenko et al., 1980). Observation of atypical, non-acid fast and cell wall deficient forms of *M. tuberculosis* in patient specimens suggests their occurrence *in vivo*. Kochemasova succeeded in isolating *M. tuberculosis* L-forms from cerebrospinal fluid, from resected sections of different organs of tuberculosis patients, as well as from urine of patients with renal tuberculosis during long lasting chemotherapy (Berezovski & Golanov, 1981; Kochemasova et al., 1970; Kochemasova, 1975). L-variants of *M. tuberculosis* were observed during antibacterial therapy of tuberculosis meningitis by Kudriavtsev et al. (1974). Of special interests were the reports by different authors about isolation of *Mycobacterium tuberculosis* L-forms from sputum and caverns of patients with pulmonary tuberculosis (Takahashi, 1979a, 1979b; Tsybulkina, 1979;). Zhu et al. (2000) found cell wall deficient forms of *M. tuberculosis in* biological material,particularly sputum and blood from patients with pulmonary tuberculosis. The first report of L-forms from *Mycobacterium scrofulaceum* infection, occurring in an 11 –year- old boy, was made by Korsak (1975). L-colonies consisting of non- acid fast coccoids and large spheres grew from autopsy materials (dermal lesions, brain, spleen, kidney, lung and intesties), sometimes making syncitya and reverting to acid fast bacilli.

Regardless of the huge progress in TB research and the development of new molecular technologies, pathogenesis of latent tuberculosis is still not well understood. The dynamic hypothesis of Cardona (2009) suggests that latent tuberculosis infection is caused by the constant endogenous reinfection of latent bacilli. Considering this hypothesis, constant "escape" of bacilli from granulomas before fibrosis is the primary source of bacteria, reactivation would never occur after a specific time period, unless the host suffered an immunosuppressive episode (Cardona & Ruiz-Manzano, 2004). Of special interest is the finding that foamy macrophages are able to maintain a stressful environment that keeps the bacilli in non-replicating state, but on the other hand, allow them to escape from granulomas, making them more resistant to future stressful conditions (Cardona et al., 2000; Cardona et al.; 2003; Cardona, 2009).

Currently, asymptomatic latent tuberculosis is defined not by identification of bacteria, but by host immune response tests. Although individuals with latent tuberculosis harbor viable bacteria, it is difficult to identify them (Young et al., 2009; Manabe & Bishai, 2000). Among the unresolved mysteries of latent tuberculosis is the nature and anatomical situation of persisting tubercle bacilli (Grange 1992). The common observation that acid-fast bacilli are frequently absent in smears is an indication that pathology may result from *in vivo* propagation of cell wall deficient mycobacteria (Domingue, 1982; Judge& Mattman, 1982). Thus, if diagnosis by finding these forms (cell wall free, non acid-fast persisting bacilli) becomes practice, it may have valuable application in diagnosis of latent tuberculosis.

There are many tuberculous syndromes in which the aetiology is occult or imitative of other diseases (Domingue, 1982; Judge & Mattman, 1982). Traditional concept of the mycobacterial aetiology of sarcoidosis and especially the assumption that cell wall deficient forms rather than bacillary are involved has been supported by several reports. Varying acid fast spindle-shaped or yeast-like structures, termed *pleomorphic chromogens*, and cell wall deficient forms of *M. tuberculosis* complex were detected in lymph node tissue from subjects with sarcoidosis (Alavi and Moscovic, 1996; Moscovic,1978). Cantwell also suggested that acid-fast organisms, found in skin lymph nodes and lung tissue from patients with

sarcoidosis, were mycobacterial cell wall deficient forms (Cantwell, 1982a,b). Judge & Mattman grew mycobacterial cell wall deficient forms (predominantly coccoid forms, larger L forms and short acid-fast rods) from blood of patients with sarcoidosis (Judge & Mattman, 1982). Polymerase chain reaction (PCR) was used to detect mycobacterial DNA in clinical samples from patients with sarcoidosis and in half the sarcoidosis patients was found *M tuberculosis* DNA (Saboor et al, 1992). A report, describing the molecular characterization of *M. tuberculosis* complex isolates from patients with sarcoidosis and tuberculosis, showed that half of the isolates from sarcoidosis patients did not resemble the spoligotypes of the isolates from patients with tuberculosis (Gazouli et al., 2005). Cell wall-defective mycobacteria were isolated also from skin lesions and cerebrospinal fluid of patients with sarcoidosis and identified to be *M. a. paratuberculosis* or other *M. avium-intracellulare* complex members (El-Zaatari et al., 1996). A relationship between cell wall deficient forms of *M. a. paratuberculosis* and Crohn's disease has been found by some authors, although this aetiological agent has not yet been conclusively proven (Hermon-Taylor and Bull, 2002; Hulten et al., 2001 a, b, c; Sechi et al. ,2001; Schwartz et al., 2000).

Future research in the field of cell wall deficiency in mycobacteria promises an increased accent on its association with latent and persistent bacterial state, which should be supported with modern molecular biological evidences. In order to better understand the nature of L-conversion phenomenon, it will be important to correlate *in vitro* with *in vivo* (experimental animals and patients) findings.

Since many researchers do not believe in existence of L-conversion phenomenon in mycobacteria, molecular genetic studies are relatively scarce (Hulten et al., 2000a,b; Lu et al, 2009; Melnikoava &, Mokrousova, 2006; Vishnevskaia et al. , 2001; Wang et al., 2007; Wall et al., 1993). On the other hand, L-forms are "difficult-to-identify" by most of the standard DNA-based tests, probably due to their unusual life style and irregular division. The relative scarcity and rather inaccessibility of the genetic material in L-forms make them generally difficult for genetic studies. De Wit & Mitchison (1993) indicated that mycococci derived from mycobacteria did not exist. The authors examined stored cultures of the mycococcus form of *M. bovis* BCG and *M. phlei* which were prepared by Csillag in 1972 and 1969 and found that restriction fragment patterns of the DNA of the variant forms and the parent mycobacteria were not similar. Traag et al (2009) also found no evidence that mycobacteria produced free-living "spores" (i.e cocci). However, the verification of L-forms isolated from experimental animals as genuine *M. tuberculosis* but not as contaminating bacteria became possible in our study, with species - specific spoligotyping test (spacer oligonucleotide typing technique) and after some modification of the initial steps in preparing the L-form cultures (Markova et al., 2008a). Spoligotyping results provided interesting insight into the occurrence of certain polymorphisms, *i.e.* insertion or deletion of spacer signals in some of the L-form isolates. In our laboratory, we have also gained much experience in experiments to obtain stable mycobacterial L-forms *in vitro* and have already developed a reproducible protocol, which allows obtaining sufficient biomass of L-cultures to get enough DNA. Under screening is a spectrum of the most examined genes for detection, identification and characterization of *Mycobacterium tuberculosis* complex in stable mycobacterial L-form cultures. The next necessary step after gene screening would be the sequencing analysis, in order to understand what kind of genetic events happen during L-transformation and which mechanisms lead to cell wall deficiency.

7. Conclusion

In conclusion, tubercle bacilli may use L-form conversion as unique adaptive strategy to survive and reproduce under unfavorable conditions in hosts. Possibility for persistence and reversion of L-forms to classical TB bacilli *in vivo* elaborates on some specific aspects of L-conversion phenomenon and link them to the mechanisms at play in latent tuberculosis. Morphologically modified and non-acid fast L-forms of mycobacteria are difficult to identify and often remain unrecognized, or are mistaken for contaminants. "L-form persistence phenomenon" of actively growing and propagating by unusual modes cell wall deficient cells differs definitively from the current understanding for latency as persistence of a few''non-replicating'' or ''dormant'' bacteria. Mycobacterial L-forms give rise to many unsolved questions concerning their biology and behavior *in vivo*, as well as about the genetic regulatory mechanisms leading to their appearance. Cell wall deficiency in mycobacteria remain an interesting topic that needs to be re-examined in the context of modern molecular biology.

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9. References

Alavi, H. & Moscovic, E. (1996). Immunolocalization of cell-wall-deficient forms of *Mycobacterium Tuberculosis* complex in sarcoidosis and in sinus histiocytosis of lymph nodes draining carcinoma. *Histology and Histopathology*, Vol.11, No3, (July 1996), pp.683–694, ISSN: 1699-5848

Allan, E.; Hoishen, C. & Gumpert, J. (2009). Bacterial L-forms. *Advances in Applied Microbiology*, Vol.68, (May 2009), pp. 1- 39, ISSN: 0065-2164

Armbruster, C.; Junker, W.; Vetter, N.& Jaksch, G. (1990). Disseminated bacilli Calmette-Guerin infection in an AIDS patient 30 years after BCG vaccination. *The Journal of Infectious Diseases,* Vol. 162, No5 (November 1990), pp.1216, ISSN: 0022-1899

Belianin, I.; Nikolaeva, G. & Martynova, L. (1997). Action of dissolved ozone on mycobacterium tuberculosis and alveolar macrophages in experimental tuberculosis. *Problemy Tuberkuleza*, No1, pp.56-59, ISSN: 0032-9533

Beran, V.; Havelkova, M.; Kaustova, J.; Dvorska, L. & Pavlik I. (2006). Cell wall deficient forms of mycobacteria: a review. *Veterinarni Medicina*, Vol. 51, No 7, pp.365–389, ISSN: 03758427

Berezovski, B. & Golanov, V. (1981). *Mycobacterium tuberculosis* L forms in patients with abacillary lung caverns. *Problemy Tuberkuleza*, No6, (June 1981), pp.22-25, ISSN: 0032-953

Berezovskii, B. & Salobai, R. (1988). The role of L variants of Mycobacteria in the development and clinical course of recurrences of pulmonary tuberculosis. *Problemy Tuberkuleza*, No4, (July 1988), pp.32-35, ISSN: 0032-9533

Biketov, S.: Potapov, V.; Ganina, E.; Downing, K., Kana, B. & Kaprelyants, A. (2007). The role of resuscitation promoting factors in pathogenesis and reactivation of Mycobacterium tuberculosis during intra- peritoneal infection in mice. *BMC Infectious Diseases*, Vol.7 (December 2007), pp.146, ISSN:1471-2334

Boris, M.; Teubner, D. & Shinefield, H. (1969). Bacterial interference with L-forms. *Journal of Bacteriology*, Vol.100, No2, (November 1969), pp.791-795, ISSN: 0021-9193

Calmette, A.& Valti, J. (1926). Virulent filterable elements of the tubercle bacillus, *Annals of Medicine*, Vol.19, (March 1926), pp.553, ISSN:16512219

Cantwell, A. (1982a).Variably acid-fast bacteria in a rare case of coexistent malignant lymphoma and cutaneous sarcoid-like granulomas. *International Journal of Dermatology*, Vol.21, No9, (November 1982), pp. 99–106, ISSN: 00119059

Cantwell, A.(1982b). Histologic observations of variably acid-fast pleomorphic bacteria in systemic sarcoidosis: a report of 3 cases. *Growth,* Vol.46, No2, pp.113–125, ISSN: 0017-4793

Cardona, P. & Ruiz-Manzano, J. (2004). On the nature of Mycobacterium tuberculosis-latent bacilli. European Respiratory Journal, Vol.24, No6, (December 2004), pp.1044-51, ISSN: 0903-1936

Cardona, P.; Gordillo, S.; Díaz, J.; Tapia, G.; Amat, I.; Pallarés, A.; Vilaplana, C.; Ariza, A. & Ausina, V. (2003). Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*. *Infection and Immunity*, Vol.71, No pp.5845–5854, ISSN: 0019-9567

Cardona, P. (2009). A dynamic reinfection hypothesis of latent tuberculosis infection. *Infection*, Vol. 37, No2, (April 2009), pp.80-86, ISSN: 0300-8126

Cardona, P.; Llatjós, R.; Gordillo, S.; Díaz, J.; Ojanguren, I.; Ariza, A.& Ausina, V. (2000). Evolution of granulomas in mice infected aerogenically with *Mycobacterium tuberculosis*. *Scandinavian Journal of Immunology*, Vol.52, No2 (August 2000), pp.156–163, ISSN: 0300-9475

Chandrasekhar, S., & Ratnam, S. (1992). Studies on cell-wall deficient non-acid fast variants of *Mycobacterium tuberculosis. Tubercle and Lung Diseases* , Vol. 73, No5, (October 1992), pp.273-279, ISSN: 0962-8479

Csillag, A. (1964). The Mycococcus Form of Mycobacteria. *Journal of General Microbiology*, Vol. 34, No 2, pp. 341-352, ISSN: 0022-1287

de Wit, D. & Mitchison, D. (1993). DNA analysis demonstrates that mycococcus forms are not mycobacteria. *Tubercle and Lung Diseses,* 1993; Vol.74, No2, (April 1993), pp.96–99, ISSN:0962-8479

Dienes, L. & Weinberger, H. (1951). The L- forms of Bacteria. *Bacteriology Reviews,* Vol. 15, No4 (December 1951), pp.245-288, ISSN: 1082-0132

Domingue, G. (1982). *Cell-wall Deficient Bacteria: Basic Principles and Clinical Significance*. Reading MA: Addison Wesley Publishing Co, ISBN: 0-201-10162-9, Reading MA

Domingue, G. (2010). Demystifying pleomorphic forms in persistence and expression of disease. *Discovery Medicine*, Vol.10, No52, (September 2010), pp.234-246, ISSN: 1539-6509

Domingue, G.& Woody, H. (1997). Bacterial persistence and expression of disease. *Clinical Microbiology Reviews* Vol. 10, No2, (April 1997), pp. 320-344, ISSN 1098-6618

Dorozhkova, I. & Volk A. (1972). Dihydrostreptomycin as a factor inducing L-forms in *M. tuberculosis*. *Antibiotiki*, Vol.17, No10, (October 1972), pp.915–922, ISSN:0003-5637

Dorozhkova, I & Volk, A. (1973). Induction of L-forms of *Mycobacterium tuberculosis* under the action of ethambutol, ethionamide and their combinations with some antibiotic and tuberculostatic drugs *Antibiotiki*, Vol. 18, No2 (February 1973), pp.144–148. ISSN: 0003-5637

Dorozhkova, I.; Karachunskii, M. ; Abdullaeva, E.; Gamzaeva, N. & Kochetkova, E. (1989).Isolation of mycobacteria L forms as a prognostic criterion of recurrence and aggravation of tuberculosis in patients with extended residual tuberculous lesions of the lungs. *Problemy Tuberkuleza*, No3, pp. 14-18, ISSN: 0032-9533 Dorozhkova, I.;

Krudu, V. & Popesku, T. (1990). Clinical value of detection of L forms of Mycobacterium tuberculosis in patients with residual tuberculous changes in the lungs. *Problemy Tuberkuleza,* No12, pp.5-8, ISSN: 0032-953

Draper, P.(1998). Draper, P.(1998). The outer parts of mycobacterial envelope as permeability barriers. *Frontiers in Bioscience* Vol. 15, (December 1998), No3, pp. 1253-1261 ISSN: 1093-9946

El-Zaatari, F.; Naser, S.; Markesich, D; Kalter, D.; Engstand, L. & Graham, D.(1996). Identification of *Mycobacterium avium* complex in sarcoidosis. *Journal of Clinical Microbiology*, Vol.34, No9. (September 1996), pp.2240–2245, ISSN: 0095-1137

Flynn, J. & Chan, J. (2001). Tuberculosis: latancy and reactivation. *Infection and Immunity*, Vol. 69, No7, (July 2001), pp.4195-4201, ISSN: 0019-9567

Fodor, M. & Roger, H. (1966). Antagonism between vegetative cells and L-forms of *Bacillus lichniformis* 6346. *Nature*, Vol. 211, No5049, (August 1966), pp. 658-659. ISSN: 0028-0836

Gazouli, M.; Mantzaris, G.; Archimandritis, A.; Nasioulas, G. & Anagnou, N. (2005). Single nucleotide polymorphisms of OCTN1, OCTN2, and DLG5 genes in Greek patients with Crohn's disease. *World Journal of Gastroenterology,* Vol.11, No47, (December 2005), pp.7525-7530, ISSN: 1007:9327

Ghosh, J.; Larsson, P.; Singh, B.; Pettersson, BMF, Islam NM, Sarkar SN, Dasgupta S, Kirsebom LA (2009) Sporulation in mycobacteria. *Proc Natl Acad Sci USA*, Vol.106, No26, (June 2009), pp.10781–10786, ISSN: 1091-6490

Grange, J.(1992).The mystery of the mycobacterial 'persistor'. *Tubercle and Lung Diseases*, Vol. 73, No5, (October 1992), pp. 249-51 ISSN: 0962-8479

Gumpert, J. & Taubeneck, U. (1983).Characteristic properties and biological significance of stable protoplast type L-forms. *Experientia Supplimentum,* Vol.46, pp.227-41, ISSN: 0071-335X

Hammes, W.; Schleifer, K. & Kandler, O. (1973). Mode of action of glycine on the biosynthesis of peptidoglycan. *Journal of Bacteriology,* Vol. 116, No2, (November 1973), pp. 1029–1053, ISSN: 0021-9193

Henderson, B.; Lund, P. & Coates, A. (2010). Multiple moonlighting functions of mycobacterial molecular chaperones.*Tuberculosis* (Edinb), Vol.90, No2, (March 2010), pp.119–124, ISSN: 1472-9792

Hines, M. & Styer, E. (2003). Preliminary characterization of chemically generated *Mycobacterium avium* subsp. *paratuberculosis* cell wall deficient forms (Spheroplasts). *Veterinary Microbiology*, Vol.95, No4, (September 2003), pp.247–258, ISSN: 0378-1135

Hermon-Taylor, J. & Bull T. (2002). Crohn's disease caused by *Mycobacterium avium* subspecies *paratuberculosis*: A public health tragedy whose resolution is long overdue. *Journal of Medical Microbiology*,Vol 51, No1 (January, 2002), pp.3–6, ISSN: 0022-2615

Hulten, K.; Karttunen, T; El-Zimaity, H.; Naser, S.; Almashhrawi, A; Graham, D.; El-Zaatari, F (2000a): *In situ* hybridization method for studies of cell wall deficient *M. paratuberculosis* in tissue samples. *Veterinary Microbiology*, Vol.77, No3-4, (December 2000), pp. 513–518, ISSN: 0378-1135

Hulten, K.; Karttunen, T.; El-Zimaity, H.; Naser, S.; Collins, M.; Graham, D. & El-Zaatari, F. (2000b). Identification of cell wall deficient forms of *M. avium* subsp. *paratuberculosis* in paraffin embedded tissues from animals with Johne's disease by *in situ* hybridization. *Journal of Microbiological Methods*, Vol.42, No2, (October 2000), pp.185–195, ISSN: 1872-8359

Hulten, K.; El-Zimaity, H.; Karttunen, T.; Almashhrawi, A.; Schwartz, M.; Graham, D.; El-Zaatari, F. (2001). Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's diseased tissues by *in situ* hybridization. American Journal of Gastroenterology, Vol96, No5 (May 2001), pp.1529–1535, ISSN: 0002-9270

Imaeda, T. (1975). Ultrastructure of L-phase variants isolated from a culture of *Mycobacterium phlei*. *The Journal of Medical Microbiology*.Vol.8, No 3, pp. 389–395, ISSN: 0022-2615

Judge, M. & Mattman, L.(1982). Cell wall-deficient mycobacteria in tuberculosis, sarcoidosis and leprosy. In. *Cell wall deficient bacteria*. Domingue GJ, ed., pp. 257-29, Addison – Wesley , ISBN: 0-201-10162-9,MA, USA

Juhasz, S. (1962). Aberrant forms of *Mycobacterium phlei* produced by streptomycin and their multiplication on streptomycin-free media. *Journal of General Microbiology*, Vol. 28, (April 1962), pp.9-13, ISSN:0022-1287

Justice, S.; Hunstad, D.; Cegelski, L. & Hultgren, S. (2008). Morphological plasticity as bacterial survival strategy. *Nature Reviews Microbiology*, Vol.6, No2, (February 2008), pp.162-168, ISSN: 1740-1526 Kana, B.; Gordhan, B.; Downing, K.; Sung, N.; Vostroknutova, G.; Machowski, E.; Tsenova, L.; Young, M.; Kaprelyants, A.; Kaplan, G. & Mizrahi, V. (2008). The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*, *Molecular Microbiology*, Vol.67, No3, (February 2008), pp. 672–684, ISSN: 0950-382X

Kaprelyants, A.; Gottschal, J.& Kell, D. (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiology Reviews*, Vol. 10, No3-4, (April 1993) pp. 271–285, ISSN: 1574-6976

Keane, J.; Remold, H. & Kornfeld, H. (2000). Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *Journal of Immunology*, Vol. 164, No4, (February 2000), pp.2016-2020, ISSN: 0022-1767

Khomenko, A.; Karachunskii, M.; Dorozhkova, I.; Chukanov, V., Balta, I. (1980). L-transformation of the mycobacterial population in the process of treating patients with newly detected destructive pulmonary tuberculosis. *Problemy Tuberkuleza*, No2, (February 1980), pp.18–23, ISSN: 0032-9533

Klieneberger-Nobel, E. (1951). Filterable forms of bacteria. *Bacteriology Review*, Vol. 15, No2, (June 1951), pp. 77-103, ISSN: 1082-0132

Koch, A. (2003).Cell wall-deficient (CWD) bacterial pathogens: Could amylotrophic lateral sclerosis (ALS) be due to one? *Critical Review in Microbiology*, Vol.29, No3, pp. 215–221, ISSN: 1549-7828

Kochemasova, Z.; Dykhno, M.; Prozorovski, S.; Kassirskaia, N,. & Bakanova, D. (1968). L-transformation of *M. tuberculosis* strains isolated from patients treated with antibiotics. *Problemy Tuberkuleza*, Vol.46, No8, pp.64-67, ISSN: 0032-9533.

Kochemasova, Z.; Kudriavtsev, A.; Dykhno, M. &, Kassirskaia, D. (1970). Isolation of L-forms of mycobacteria from pathological material of tuberculosis patients. *Problemy Tuberkuleza,* Vol.48, No12, pp. 63-65, ISSN: 0032-953

Kochemasova, Z.; Dykhno, M.; Kassirskaia, N. & Bakalova, D. (1975). L-forms in the urine of patients with renal tuberculosis. *Problemy Tuberkuleza,* No4, pp.68-70, ISSN: 0032-953

Korsak, T. (1975). Occurrence of L-forms in a case of generalized mycobacteriosis due to *Mycobacterium scrofulaceum*. *Acta Tuberculosea et Pneumologica Belgica*, Vol.66, No6, (November 1975), pp.445–469,

Kremer, L. & Besra, G. (2005).A waxy tale, by *Mycobacterium tuberculosis* pp. 287-305. *In* Tuberculosis and the tubercle bacillus. ASM Press, ISBN: 0001-7078, Washington

DC; Kudriavtsev, A.; Kochemasova, Z.; Dykhno, M.; Kassirskaia, N. & Bakanova D. (1974). Characteristics of the course of meningeal tuberculosis with the presence of L-forms of *Mycobacterium tuberculosis* in the cerebrospinal fluid. *Problemy Tuberkuleza,* No2, pp.35-39, ISSN: 0032-953

Li, G. (1990). Induction of L-forms of *M. tuberculosis in vivo* in guinea pigs. *Zhonghua Jie He He Hu Xi Za Zhi*). Vol. 13, No6, (December 1990), pp.351-354, ISSN:0254-6450

Lu, J.; Ye, S.; Li, C.; Sai, W.& Li, W. (2009). Sequence analysis on drug-resistant gene of rpoB in Mycobacterium tuberculosis L-forms among pneumoconiosis patients complicated with tuberculosis. *Zhonghua Liu Xing Bing Xue Za Zhi*, Vol.30, No5, (May 2009), pp.486-488, ISSN:0254-6450

Manabe, Y. & Bishai, W. (2000). Latent Mycobacterium tuberculosis-persistence, patience, and winning by waiting. *Nature Medicine*, Vol.6, No12, (December 2000), pp.1327-1329, ISSN : 1078-8956 ISSN: 10010939

Markova, N. (2009). Hidden face of tuberculosis. *Bioscience Hypotheses*, Vol.2, No6, (July 2009), pp. 441-442, ISSN: 1756-2392

Markova, N.; Michailov, L.; Vesselinova, A.; Kussovski, V.; Radoucheva, T.; Nikolova S. & Paskaleva, I. (1997). Cell wall–deficient forms (L–forms) of *Listeria monocytogenes* in experimentally infected rats. *Zentralblatt für Bakteriologie,* Vol.286, Vol.1, (June 1997), pp.46-55, ISSN: 1438-4221

Markova, N.; Michailova, L.; Jourdanova, M.; Kussovski, V.; Valcheva, V.; Mokrousov, I. & Radoucheva, T. (2008a). Exhibition of persistent and drug-tolerant L-form habit of *Mycobacterium tuberculosis* during infection in rats. *Central European Journal of Biology*, Vol. 3, No 4, (December 2008), pp. 407-416, ISSN: 1895-104X Markova, N.; Michailova, L.; Kussovski , V. & Jourdanova, M. (2008b). Formation of persisting cell wall deficient forms of *Mycobacterium bovis* BCG during interaction with peritoneal macrophages in guinea pigs. *Electronic Journal of Biology*, Vol. 4, No1, pp.1-10, ISSN: 1860-3122

Markova, N.; Slavchev, G.; Michailova, L. & Jourdanova, M. (2010). Survival of *Escherichia coli* under lethal heat stress by L-form conversion. *International Journal of Biological Sciences*, Vol. 6, No4, (June 2010), pp.303-315, ISSN: 1449-2288

Mattman, L.; Tunstall, L.; Mathews, W. & Gordon D. (1960). L-variation in mycobacteria. *American Review of Respiratory Diseases,* Vol. 82, No2, (August 1960), pp. 202-211, ISSN: 0003-0805

Mattman, L. (1970). Cell wall-deficient forms of mycobacteria. *Annals of the New York Academy of Sciences* Vol.174, No 2, (October 1970), pp. 852–861, ISSN: 0077-8923

Mattman, LH. (2001). *Cell wall Deficient Forms. Stealth Pathogens*, 3rd ed., CRC Press Inc, ISBN: 0-8493-8767 Boca Raton, FL, USA

Melnikoava, N. &, Mokrousova, I. (2006). Study of rifampicin resistance in L-forms of Mycobacterium tuberculosis, by analyzing rpoB gene mutations. *Problemy Tuberkuleza,* No11, pp. 22-24, ISSN: 1728-2993

Merkal, R.; Rhoades, K.; Gallagher, J. & Ritchie, A. (1973). Scanning electron microscopy of mycobacteria. *American Review of Respiratory Diseases,* Vol. 108, No2, (August 1973), pp.381–387, ISSN: 0003-0805

Michailova, L. ; Stoitsova, S. ;Markova, N. ;Kussovski, V. ;Jourdanova, M. & Dimova, I. (2000a). Interaction of alveolar macrophages with *Staphylococcus aureus* and induction of microbial L-forms during infection in rats. *International Journal of Medical Microbiology*, Vol. 290, No 3, (July 2000), pp.259-267, ISSN: 1438-4221

Michailova, L.; Kussovski, V.; Radoucheva, T.; Jordanova, M.; Berger, W.; Rinder, H. & Markova, N. (2005). Morphological variability and cell wall deficiency in

Mycobacterium tuberculosis 'heteroresistant' strains. *International Journal of Tuberculosis and Lung Diseases*, Vol. 9, No8, (August 2005), pp.907-914, ISSN: 1027-3719

Mihailova, L.; Markova, N.; Radoucheva, T.; Veljanov, D. & Radoevska, S. (1993). Cell interaction of *Listeria monocytogenes* L-forms and peritoneal exudative cells in rats. *Canadian Journal Microbiology,* Vol. 39, No11, (November 1993), pp.1014-1021, ISSN: 0008-4166

Michailova, L.; Markova, N.; Radoucheva, T.; Stoitsova, S.; Kussovski, V. & Jordanova, M. (2000b). Atypical behaviour and survival of *Steptococcus pyogenes* L-forms during intraperitoneal infection in rats. *FEMS Immunology and Medical Microbiology* , Vol. 28, No1, (May 2000), pp.55-65, ISSN: 1574-695X Michailova, L.; Kussovski, V.; Radoucheva, T.; Jordanova, M. & Markova, N. (2007). Persistence of *Staphylococcus aureus* L-forms during experimental lung infection in rats. *FEMS Microbiology Letters,* Vol. 268, No1, (March 2007), pp. 88-97, ISSN: 0378-1097

Miller, F. (1932). The induced development of non-acid fast forms of bacillus tuberculosis and other mycobacteria. *Journal of Experimental Medicine*, Vol.56, No3 (August 1932, pp 411–424, ISSN::0022-1007

Mitchel, P. & Moyle, J. (1956). Autolytic protoplast release in Bacterium coli. *Nature,* Vol.3, No178, (November 1956), pp.993, ISSN: 0028-0836

Moscovic, E. (1978). Sarcoidosis and mycobacterial L-forms: a critical reappraisal of pleomorphic chromogenic bodies (Hamazaki corpuscles) in lymph nodes. *Pathology Annual,* Vol.13, No2, pp.69–164, ISSN: 0079-0184

Much, H. (1931). Die Variation des Tuberkelbacillus in Form und Wirkung. *Beitrage zur Klinik der Tuberkulose,* Vol.77, No 1, (March, 1931), pp.60-71, ISSN: 0366-0966

Mukamolova, G.; Turapov, O.; Young, D.; Kaprelyants, A.; Kell, D. & Young, M. (2002). A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Molecular Microbiology*, Vol. 46, No3, (November 2002), pp.623-635, ISSN: 0950-382X

Naser, S.; McCarthy, C.; Smith, G.& Tupponce, A. (1993). Low temperature protocol for efficient transformation of *Mycobacterium smegmatis* spheroplasts. *Current Microbiology*, Vol. 27, No3, (September 1993), pp. 153– 156, ISSN: 1432-0991

Norris, V. (2011). Speculations on the initiation of chromosome replication in Escherichia coli: the dualism hypothesis. *Medical Hypothesis*, Vol.76, No5, (May 2011), pp.706-716, ISSN: 03069877

Onwuamaegbu, M.; Belcher, R. & Soare, C. (2005). Cell wall-deficient bacteria as a cause of infections: a review of the clinical significance. *Journal of International Medical Research,* Vol. 33, No1 (January 2005), pp. 1- 20, ISSN: 0300-0605

Pla Y Armengol, R. (1931). Die verschiedenen Formen des Tuberkuloseerregers. *Beiträge zur Klinik der Tuberkulose*, Vol.77, pp.47-55

Postgate, J. & Hunter, J. (1962). The survival of starved bacteria. *Journal of General Microbiology*, Vol. 29, (October 1962) pp.233–267, ISSN: 0022-1287

Prozorovski, S.; Kaz, L. & Kagan, G. (1981). *L-formsof bacteria (mechanisms of formation, structure, role in pathology).* Medicine Publishing, ISBN: 50500-347, Moscow

Ratnam, S. &Chandrasekhar, S. (1976).The effect of gravitational forces on the viability of spheroplasts of mycobacteria. *Canadian Journal of Microbiology*, Vol. 22, No 9, (September 1976), pp.1397-1399, ISSN: 0008-4166

Reynes, J.; Perez, C.; Lamaury, I.; Janbon, F. & Bertrand, A. (1989). Bacille Calmette-Guérin adenitis 30 years after immunization in a patient with AIDS. *The Journal of Infectious Diseases*, Vol. 160, No4, (October 1989), pp. 727, ISSN: 0022-1899

Riendeau, C. & Kornfeld, H. (2003). THP-1 Cell Apoptosis in Response to Mycobacterial Infection. *Infection and Immunity*, Vol.71, No1, (January 2003), pp. 254-259, ISSN: 0019-9567

Rosenberger, C. & Finlay, B. (2003). Phagocyte sabotage: Disruption of macrophage signalling by bacterial pathogens. *Nature Reviews Mol Cell Biology,* Vol. 4, No5, (May 2003), pp.385-396, ISSN: 1471-0072 Saboor, S.; Johnson, N. & McFadden, J. (1992). Detection of mycobacterial DNA in sarcoidosis and tuberculosis with polymerase chain reaction. *Lancet*, Vol 339, No8800, (April 1992), pp.1012-1015, ISSN: 1474-547X

Schwartz, D.; Shafran, I.; Romero, C.; Piromalli, C.; Biggerstaff, J.; Naser, N.; Chamberlin, W. & Naser, S.(2000). Use of short-term culture for identification of *Mycobacterium avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients. *Clinical Microbiology and Infection*,Vol 6, No6 (June 2000), pp.303–307, ISSN: 1198-743X

Sechi, L.; Mura, M.; Tanda, F.; Lissia, A.; Solinas, A.; Fadda, G.& Zanetti, S. (2001): Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by *in situ* hybridization. *Journal of Clinical Microbiology*, Vol. 39, No12, (December 2001), pp.4514–4517, ISSN: 0095-1137

Seiler, P.; Ulrichs, T.; Bandermann, S.; Pradl, L.; Jorg, S.; Jörg, S.; Krenn, V.; Morawietz, L.; Kaufmann, S.& Aichele, P. (2003). Cell-wall alterations as an attribute of *Mycobacterium tuberculosis* in latent infection. *Journal of Infectious Diseases*, Vol. 188, (November 2003), No9, pp.1326-1331, ISSN: 0022-1899

Shleeva, M.; Kudykina, Y.; Vostroknutova, G.; Suzina, N.; Mulyukin, A.; Arseny, S. & Kaprelyants, A. (2011). Dormant ovoid cells of *Mycobacterium tuberculosis* are formed in response to gradual external acidification. *Tuberculosis*, Vol. 91, No2, (March, 2011), pp.146-154, ISSN: 1472-9792

Shleeva, M.; Salina, E. & Kaprelyants, A. (2010). Dormant of Mycobacteria. *Mikrobiologiya*, Vol. 79, No. 1, pp.3–15, ISSN: 0026-2617

Shleeva, M.; Mukamolova, G.; Telkov, M.; Berezinskaya, T.; Syroeshkin, A.; Biketov, S. & Kaprelyants, A. (2003). Formation of nonculturable cells of *Mycobacterium tuberculosis* and their resuscitation, *Microbiology,* Vol. 72, No 1, (January 2003), pp.76–83, ISSN: 0026-2617

Smith, E.; Thybo, S.& Bennedsen, J. (1992). Infection with *Mycobacterium bovis* in a patient with AIDS: a late complication of BCG vaccination. , *Scandinavian Journal of Infectious Diseases,* Vol. 24, No1, (January 1992), pp. 109-110, ISSN: 0036-5548

Snitinskaia, O.; Sibirnaia, R. & Beliakova, O. (1990). The significance of L-form mycobacteria in the development of pulmonary tuberculosis. *Vrachebnoe Delo,* No 3 (March 1990), pp.38-40, ISSN: 0049-6804

Takahashi, S. (1979a). L-phase growth of mycobacteria. 1. Cell walldeficient form of mycobacteria. *Kekkaku,* Vol.54, No2, (February 1979), pp. 63-70, ISSN: 0022-9776 .

Takahashi, S. (1979b). L-phase growth of mycobacteria. 2. Consideration on the survival of tubercle bacillus in caseous . *Kekkaku,* Vol.54, No4, (April 1979), pp. 231-235, ISSN: 0022-9776.

Thacore, H. & Willett, H. (1966). The formation of spheroplasts of Mycobacterium tuberculosis in tissue cultures cells. *American Review of Respiratory Diseases*, Vol. 93, No5, (May 1966), pp.786-796, ISSN: 0003-0805

Traag, B.; Driksb, A.; Stragierc, P.; Bitterd, W.; Broussarde, G.; Hatfulle, G.; Chuf, F.; Adamsf, K.; Ramakrishnanf, L. & Losicka, R. (2010). Do mycobacteria produce

endospores? *Proc Natl Acad Sci USA*, Vol. 107, No 2, (January 2010), pp. 878–881, ISSN 1091-6490

Tsybulkina, I. (1979). Isolation of Mycobacterium tuberculosis and its L-forms from caverns and sputum of patients with pulmonary tuberculosis. *Problemy Tuberkuleza*, No11, (November 1979), pp.64-68, ISSN: 0032-953

Tufariello, J.; Jacobs, W. & Chan, J. (2004). Individual *Mycobacterium tuberculosis* resuscitation-promoting factor Homologues are dispensable for growth *in vitro* and *in vivo*. *Infection and Immunity,* Vol. 72, No1, (January 2004), pp. 515–526, ISSN: 0019-9567

Udou T.; Ogawa M.& Mizuguchi, Y. (1982). Spheroplast formation of *Mycobacterium smegmatis* and morphological aspects of their reversion to the bacillary form. *Journal of Bacteriology*, Vol. 151, No2 (August 1982), pp. 1035–1039, ISSN: 0021-9193

Vishnevskaia, E.; Bobchenok, A. & Melnikova, N. (2001). Identification of L-forms of Mycobacterium tuberculosis complex by polymerase chain reaction (PCR). *Problemy Tuberkuleza*, No4, pp.38-40, ISSN*:* 0032-953 Wall, S.; Kunze, Z.; Saboor, S.; Soufleri, I.; Seechurn, P.; Chiodini, R. & McFadden J. (1993). Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. *Journal of Clinical Microbiology*, Vol.31, No5, (May 1993),pp.1241–1245, ISSN: 0095-1137

Wang, He; Luo, Zhen-hua; Xu, Yan; Liang, Jing-Ping.(2007). Study on genes for drug resistance of cell-wall deficient M.tuberculosis. *Chinese Journal of Antibiotics*.No10, pp. 636-664, ISSN 1001-8689

Wang, H. & Chen, Z. (2001). Observations of properties of the L-form of M. tuberculosis induced by the antituberculosis drugs. *The Chinese Journal of Tuberculosis and Respiratory Diseases* (Zhongua Jie He He Hu Xi Za Zhi), Vol. 24, No 1 (January 2001), pp. 52-55, ISSN: 10010939

Xalabarder, C. (1958). Electron microscopy of tubercle bacilli. *Excerpta Medica, Sect XV Chest Diseases*, Vol. 11, No1 (October 1958), pp. 467-473, ISSN: 0014-4320

Xalabarder, C.(1963). The nature of so-called atypical mycobacteria. *Neumol Cir Torax*, Vol.24, (July 1963), pp. 259-274, ISSN: 0028-3746 Young, D.; Gideon, H. & Wilkinson, R. (2009). Eliminating latent tuberculosis. *Trends in Microbiology*, Vol. 17 No 5, (May 2009), pp.183-188, ISSN 0966-842X

Young, K. (2007). Bacterial morphology: why have different shapes? *Current Opinion in Microbiology,* Vol. 10 No 6 (December 2007), pp.596-600, ISSN: 1369-5274

Zhang, Y.; Yang, Y.; Woods, A.; Cotter, R. & Sun, Z. (2001). Resuscitation of Dormant *Mycobacterium tuberculosis* by Phospholipids of Specific Peptides. *Biochemical and Biophysical Research Communications,* Vol. 284, No2, (June 2001), pp.542–547, ISSN 0006-291X

Zhang, Y. (2004). Persistent and dormant tubercle bacilli and latent tuberculosis. *Frontiers in Bioscience*, Vol. 9, No1, (May 2004), pp.1136-1156, ISSN: 1093-9946

Zhu, M.; Xie, P. & Zhang, Y. (2000). Detecting mycobacteria and their L-forms in peripheral blood from pulmonary tuberculosis patients by cultivation with hemolyzed-centrifugated blood in liquid medium. *The Chinese Journal of Tuberculosis and Respiratory Diseases* (Zhonghua Jie He He Hu Xi Za Zhi), Vol.23, No9, (September 2000), pp.556-558, ISSN: 10010939

Zumla, A.; Atun, R.; Maeurer, M.; Mwaba, P.; Ma, Z.; O'Grady, J.; Bates, M.; Dheda, K.; Hoelscher, M. & Grange, J. (2011). Viewpoint: Scientific dogmas, paradoxes and mysteries of latent M. tuberculosis infection. *Tropical Medicine and International Health*, Vol.16, No1, (Januaray 2011), pp.79-83, ISSN: 13602276

12

Mycobacterium tuberculosis: Dormancy, Persistence and Survival in the Light of Protein Synthesis

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1. Introduction

The chapter introduces and discusses the metabolic robustness and survival strategies of mycobacteria with focus on glyoxylate shunt and protein synthesis. Primarily, it is an attempt to understand the significance of glyoxylate pathway, which provides an adaptive advantage in metabolically starved situation. The other issue addressed here are the key players behind resistance mechanism in all thriving forms of the bacteria. The protein synthesis process, its challenges and advantages that can effectively be harnessed have been discussed in details. It also gives a comprehensive account of the strength, weaknesses opportunities and threats in targeting mycobacterium both in active and dormant state. Last but not the least we also highlight further perspectives to control the pathogenesis by the bacteria.

Definitions:

Dormancy: the reversible state of bacterial metabolic shutdown (Kell et al., 1998; Mukamolova et al., 1998; Barer & Harwood, 1999).

Persistence: the phenomenon whereby otherwise drug susceptible microorganisms have the capacity to survive indefinitely within mammalian tissues despite continued exposure to the appropriate drug or drugs (Mc et al., 1957).

Latency: *in vivo* situation where bacteria and the host have established a balanced state without causing apparent symptoms in the host, as in latent infection (Orme, 2001a)

1.1 A prelude to mycobacterium: The culprit in stealth

The enigma caused by the mycobacterium has been a challenge to the scientific community by virtue of their adaptive skills and evasion mechanism to combat immunologically educated host. It is a gram positive, acid fast pathogenic bacterium with unique cell wall. It

can survive even within the hostile environment of alveolar macrophages. Statistically speaking as per the WHO fact sheet 2010/2011, Tuberculosis is reported to kill 1.7 million people in 2009 globally. It accounts for about 4700 death per day (Organization, 2010). Further complications in eradicating these 'notorious culprits of mass destruction' is the emergence of drug resistant forms as MDR-TB and XDR-TB i.e. multi drug resistance and extensive drug resistance respectively. Their smart stealth behaviour to get away from host defence together with metabolic fine-tuning in hostile environment makes it world's most successful pathogen in action.

1.2 *Modus operandi* of hide out

It has been quite puzzling to understand the metabolic fluctuations in the changing pathophysiological microenvironment of mycobacteria. Our understanding pertaining to the process for acquisition of essential nutrients for thriving in these environments by intracellular bacterium is still in the stage of infancy. Detailed analyses have revealed a transformative process where environmental hostility brings about a lifestyle change following a reductionist agenda to minimize nutritional needs leading to dormant and/or persistent cells, collectively described as latent tuberculosis (Gomez & McKinney, 2004; Lewis, 2007). A very recent transcriptiome based analysis brought an interesting scenario to light that in *Mycobacterium tuberculosis*, low numbers of drug-tolerant persisters are present from the lag and early exponential phases, which increase sharply at late exponential and stationary phases roughly accounting for 1% of the total population. This further established a new understanding that dormancy is not an all or none phenomenon, and it is collectively governed by both deterministic and stochastic mechanisms (Keren et al., 2011).There are several models to study the phenomenon but one recent model based on multiple stress dormancy, that generates a lipid loaded drug tolerant dormant pathogen looks quite promising (Deb et al., 2009). A recent study using same model by Daniel *et.al* mimicking the microenvironment inside the human granuloma by incubating mycobacterium infected macrophages under hypoxic phase revealed that under these conditions macrophages produce lipid droplets containing Triacyl glycerol (TAG) which is smartly utilized by these bacteria too and exhibit dormancy like phenotype (Daniel et al., 2011). Pandey *et.al* from their work also demonstrated that Mycobacterium can effectively degrade cholesterol derived from host and use it for their carbon and energy source thus maintaining chronic infections in murine models and establishing persistence (Pandey & Sassetti, 2008).

1.3 Scheme of the process

Latent tuberculosis is characterized by a plethora of converging events; on one hand the immunological modulators govern the process in dynamic fashion, while on the other hand metabolic plasticity is at its best. The core strategy is to undergo a downshift in the needs in order to survive with minimal metabolic activity (Wayne & Hayes, 1996). Looking from a metabolomics perspective a variety of genes undergo fluctuations in their expression profile falling mainly under three major groups; respiratory enzymes, stress related proteins and proteins involved in fatty acid metabolism (Honer zu Bentrup & Russell, 2001). The shift to anaerobiosis leads to a metabolic shuffle triggering alternative pathway of glyoxylate shunt to meet the challenges ahead.

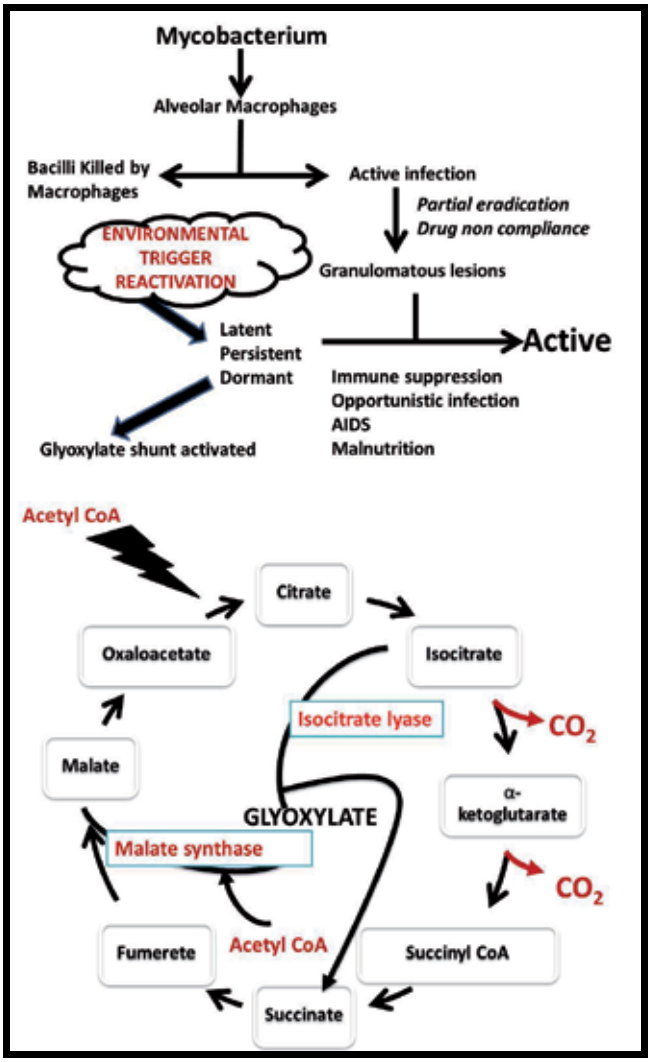


Fig. 1. Mycobacterium survival: the lipid lunch through glyoxylate shunt.

2. Glyoxylate pathway: The saviour of the anarchist

Tuberculosis is characterized by periods in which the disease may be non-obvious or even clinically inapparent but even in these regimens mycobacteria persist with the potential to reactivate the disease opportunistically. Persistence may be defined as a stage where the metabolic downshift to anaerobiosis brings about a nutritionally suspended condition. In the granulomas the bacterium does not replicate and becomes inert yet surviving in occult forms to get activated in immune-compromised situations. In persistent phase the *ala carte* is changed from glucose to delicious lipids, glycolysis is decreased and the glyoxylate shunt is upregulated allowing anaplerotic maintenance of the tricarboxylic acid (TCA) cycle (McKinney et al., 2000). The glyoxylate shunt converts isocitrate to succinate and glyoxylate, catalyzed by the enzyme isocitrate lyase (ICL), followed by the addition of Acetyl-CoA to

glyoxylate to form malate by malate synthase (MS) (Sharma et al., 2000). The glyoxylate shunt allows the bacteria to avoid the carbon dioxide generating steps of the Krebs cycle, enabling them to shunt carbons from fats to carbohydrate synthesis (Wayne, 1994). The drugs used today in combination therapy for treating tuberculosis were discovered 40 years ago and none of them has been effective against these robust persisters (Reddy et al., 2009). The global mandate today is focused to reduce the treatment time line which is six to nine months at present. Targeting pathways that get triggered during persistence phase can yield potential leads. As humans do not have functional glyoxylate pathway the enzymes of the pathway are promising drug target (Kumar & Bhakuni, 2008). The relative abundance of genes pertaining to fatty acid degradation (more than 150) in mycobacterial genome highlights the importance of survival on lipids derived from host *in vivo* (Cole, 1999).

2.1 The arsenal of survival

Enzymes of the glyoxylate shunt operate by diverting carbon from beta-oxidation of fatty acids into the glyoxylate pathway to sustain a nutrient starved intracellular infection they have been implicated for their roles in both virulence and persistence in candida (Lorenz & Fink, 2001) and salmonella (Allen et al., 2000) too. One protein present at significantly higher level in the phagocytized population was identified by microsequencing as isocitrate lyase, a key enzyme of the glyoxylate cycle (Manabe et al., 1999). Furthermore, cDNA selection technique to identify genes upregulated upon phagocytosis revealed Isocitrate lyase as one of the 11 genes identified and was the only metabolic gene in the set (Graham & Clark-Curtiss, 1999).Expression of ICL is upregulated under certain challenged growth conditions (Honer Zu Bentrup et al., 1999) and during infection of macrophages by Mycobacterium spp. It has also been demonstrated that ICL is important for survival of *M. tuberculosis* in the lungs of mice during the persistent phase of infection (2–16 weeks), but is not essential during the acute phase (0–2 weeks) of infection (McKinney et al., 2000). Recent report suggests that suppressing the apoptosis of host macrophage may be one of the important mechanisms for their increased intracellular survival (LI Jun-ming, 2008). Common to all ICL is a signature sequence 'KKCGH'. It has the nucleophilic cysteine residue that sits on a flexible loop which undergoes large conformational change after binding of substrate resulting in complete closing of the active site from the bulk solvent (Smith et al., 2004). Interestingly it is quite important to note that although the genome of *M. tuberculosis* encodes orthologues of two of the three enzymes of the methylcitrate cycle, methylcitrate synthase and methylcitrate dehydratase, it does not appear to contain a distinct 2-methyl isocitrate lyase (MCL). ICL from *M. tuberculosis* can clearly function as a MCL thus metabolizing both Acetyl and Propionyl CoA generated by β-oxidation of even and odd chain fatty acids facilitating the lipid lunch (Gould et al., 2006).

The other enzyme downstream in the pathway is Malate Synthase G (MtbMS) that drives the reaction ahead. A single malate synthase gene called *glcB* (Rv1837c) has been identified in *M. tuberculosis* encoding MtbMS (Smith et al., 2004). MtbMS is a very important housekeeping enzyme involved in persistence of the bacteria. Its intracellular/extracellular localization acting as adhesion and virulence factor together with persistence is quite enigmatic. A recent study further showed the existence of active dimeric form of the enzyme that may add to multiplicity of function that the enzyme exhibits (Kumar & Bhakuni, 2010). It was further revealed in a recent study that the enzyme in mycobacterium differ substantially as compared to *Escherichia coli* by having differences in molecular assembly

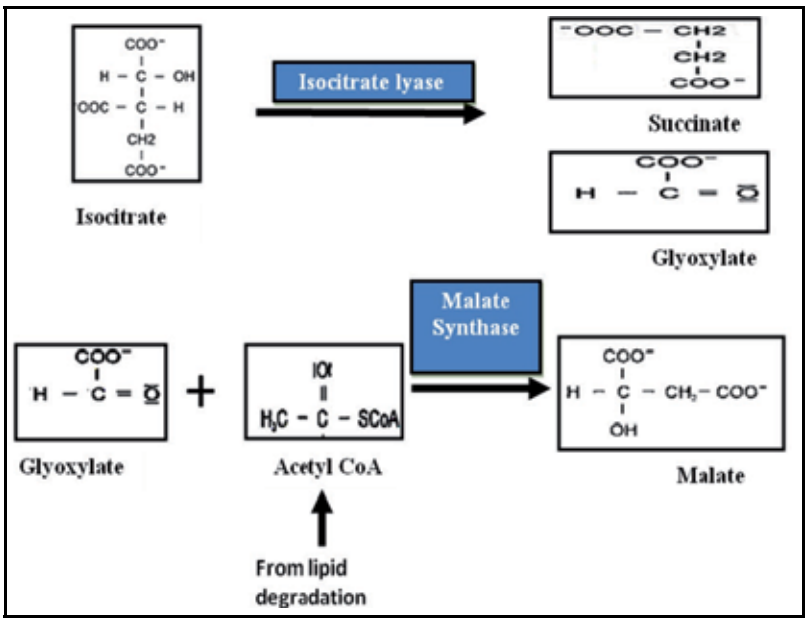
governed by subtle ionic interactions (Kumar & Bhakuni, 2011). Antibodies to MS have been discovered in 90% of patients during incipient subclinical tuberculosis (Singh et al., 2003; Singh et al., 2005). Bishai (Gyanu Lamichhane, 2003) reported the random insertion of transposons into the MtbMS gene (*glcB*, annotated Rv1837c) resulted in a non-viability under normal growth conditions; in contrast, Sassetti (Sassetti & Rubin, 2003) determined that MtbMS is not required for tuberculosis infection in mice using a similar transposon insertion method. However, attempts to knockout Mtb *glcB* have been unsuccessful (J. D. McKinney, personal communication). This consolidates the role of MS in metabolic up keeping of the bacterium when hostile environment prevails.

2.2 The rescue saga

In enzymology, an isocitrate lyase (EC 4.1.3.1) is an enzyme that catalyzes the reversible aldol cleavage of threo-DS(+)-isocitric acid to succinic and glyoxylic acids (Rua et al., 1989)

$$\text{Isocitrate} \rightleftharpoons \text{Succinate} + \text{Glyoxylate}$$

Hence, this enzyme has one substrate, isocitrate, and two products, succinate and glyoxylate. This enzyme belongs to the family of lyases, specifically the oxo-acid-lyases, which cleave carbon-carbon bonds. The systematic name of this enzyme class is isocitrate glyoxylate-lyase (succinate-forming). Isocitrate lyase is the first enzyme unique to the metabolic pathway known as the glyoxylate cycle which is required for the assimilation of fatty acids and acetate (Kelly et al., 2002). Recent reports have alluded to an additional role for this enzyme in *M. tuberculosis* metabolism, specifically for growth on propionate. A product of beta-oxidation of odd-chain fatty acids is propionyl-CoA. Clearance of propionyl-CoA and the



Acetyl-CoA + H_2O + Glyoxylate $\rightleftharpoons$ (S)-Malate + CoA

Fig. 2. Reaction scheme of glyoxylate shunt.

by-products of its metabolism via the methylcitrate cycle are vital due to their potentially toxic effects but no homolog of this enzyme has been found in the mycobacterium genome. This unique phenomenon points out on the dual role of isocitrate lyase in the glyoxylate and methylcitrate cycles in *M. tuberculosis* (Gould et al., 2006). ICL-deficient bacteria could not grow on propionate, suggesting that ICL might function as ICLs in the glyoxylate cycle and as MCLs in the methylcitrate cycle (Munoz-Elias et al., 2006).

The enzyme malate synthase (EC 4.1.3.2) catalyses the condensation reaction between the carbonyl group of glyoxylate and the methyl group of acetyl-CoA to form a thio-ester which, after hydrolysis, generates L-malate and CoA (Dixon et al., 1960).

Catalysis by ICL and MS ensures the bypass of two oxidative steps of the tricarboxylic acid cycle, permitting net incorporation of carbon during growth of most microorganisms on acetate or fatty acids as the primary carbon source. Thus, the glyoxylate bypass conserves carbon and ensures an adequate supply of tricarboxylic acid cycle intermediates for biosynthetic purposes when cells convert lipids to carbohydrates (Sharma et al., 2000).

2.3 Magnesium the magic molecule in metabolic recession

Magnesium serves as key ingredient in the recipe of lipid lunch as both the pivotal enzymes are activated in the presence of magnesium as a cofactor. There have been two schools of thoughts as far as role of magnesium on these enzymes are concerned the one thinks that magnesium is activating substrate by binding to it (magnesium-substrate complex) while other view rests on the idea that magnesium binds to the active site inducing conformational change and having catalytic role for efficient catalysis (2007).

The plant and bacterial ICL have an absolute requirement of Mg^{2+} ions for functional activity. Mg^{2+} ions were postulated to be necessary for catalysis on the active site of ICL however, a higher concentration of these ions has been found to have inhibitory effect on the enzyme (Beeckmans et al., 1997). Later on, Giachetti et al. (Giachetti & Vanni, 1991) performed detailed kinetic studies with *Pinus pinea* ICL and concluded that the Mg^{2+}-isocitrate complex and not isocitrate is the true substrate of enzyme. This conclusion has been supported by studies on ICL from several other sources. In the absence of divalent cations, only negligible activity was measured for the purified ICL, whereas addition of Mg^{2+} or Mn^{2+} supported enzyme activity. Mn^{2+} was able to replace Mg^{2+}, yielding 39% of the activity obtained with Mg^{2+}. Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} were not able to support significant ICL activity (Honer Zu Bentrup et al., 1999). A variety of metal ion combinations were studied for their ability to inhibit ICL activity. It is known for the isocitrate lyases of *Corynebacterium glutamicum* and *Acinetobacter calcoaceticus* that Mn^{2+} can partially substitute for Mg^{2+} (Hoyt et al., 1988; Hoyt et al., 1991).In the absence of divalent cations only negligible activity was measured for the purified MS. Mg^{2+} at 5 mM was found to be the most effective cation. Mn^{2+} was able to replace Mg^{2+}, yielding 40% of the activity obtained with Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} were not able to support significant MS activity (Smith et al., 2003). Zn^{2+} and Cd^{2+} were found to bring about structural alterations thereby inhibiting the function of enzyme in case of MtbIcl (Kumar & Bhakuni, 2008).

3. The shunt and the hunt

The structural intricacies have been deciphered for both the key enzymes successfully and all the information is at our disposal (Sharma et al., 2000; Smith et al., 2003; Anstrom & Remington, 2006). In the era of structure based drug design where high throughput screening, molecular modelling, *in silico* docking strategies have accelerated drug development timeline promising rescue of the hijacked host from the persistent mycobacteria. The TB Alliance has been strategically focusing to combat these molecular targets. ICL is a tetrameric protein with four subunits of 428 amino acids each. The high-resolution structure of ICL from *M. tuberculosis* has been solved to 2.0 Å resolution (Sharma et al., 2000). The enzyme structure in complex with inhibitors, 3-nitropropionate with glyoxylate and 3-bromopyruvate has also been resolved. 3-bromopyruvate inhibits ICL-activity by forming a covalent adduct with the nucleophilic Cys191 (Sharma et al., 2000). The inhibitor bound ICL structures, on one hand, provide crucial information regarding the active site microenvironment, and on the other hand, produce valuable information on the type of interactions prevalent at those localized site adding momentum strength to the drug discovery process. Several ICL inhibitors are being tested, which mainly include 3-nitropropionate (McFadden & Purohit, 1977), 3-bromopyruvate (Ko & McFadden, 1990), 3-phosphoglycerate (Ko et al., 1989), mycenon (Hautzel et al., 1990) and itaconate (McFadden & Purohit, 1977). However, *in vivo* application of these inhibitors is yet a dream because of their potent toxicity and low activity. Sesterterpene sulphate, which has recently been shown to effectively inhibit ICL in *Candida albicans* (Lee et al., 2008) is also promising.

The second enzyme of the glyoxylate shunt is encoded by a single gene identified in TB called *glcB* and encoding a 741 amino acid 80 kDa protein malate synthase (Smith et al., 2003). The enzyme catalyzes the Mg^{2+}-dependent condensation of glyoxylate and acetyl-coenzyme A and hydrolysis of the intermediate to yield malate and coenzyme A (Anstrom & Remington, 2006). The structure of MS from *M. tuberculosis* in complex with the substrate glyoxylate has been solved to 2.1 Å resolution structural analysis indicated that malate synthase is a much more druggable target by virtue of its deeper and more hydrophobic binding domain (Smith et al., 2003). Screening against this target will have a better chance of identifying tractable inhibitors as lead molecules (www.tballiance.org). Further refinement in understanding the mechanistic implications were brought forth by revised position of bound malate which is consistent with a reaction mechanism that does not require reorientation of the electrophilic substrate during the catalytic cycle(Anstrom & Remington, 2006) . These insights have been crucial in the inhibitor ergonomics. High throughput screening has been completed with a 1.4 million compound library and hits have been identified. The endeavour ahead is to confirm the potential hits and efficiently evaluate these, thus paving the pathway for identification of analogues and series for future optimization. It's also important to mention that high throughput screening initiatives for identifying inhibitors has not yielded very promising outcomes reason being the druggability of these potential targets. The challenge here is to design inhibitors that are equipped with permeability parameters that can reach the action targets.

Evolutionarily enzymes of glyoxylate shunt are highly conserved and have unique signature active site sequences which offer leverage to rational drug design approach thereby coming up with a broad spectrum more pharmacologically attractive target relevant to the treatment

of not only tuberculosis, but also candidiasis and melioidosis and many other pathogens (Kumar, 2009). The rationing mechanism for supply of the precursor amino acid and nucleic acids required for growth can be halted and paused by inhibitors of metabolic pathways and thus making it tough for the bug to proliferate and persist. The search for elixir of life continues with the effort to evolve a drug that has bactericidal activity both on the active and persistent form and a robust delivery mechanism that has ability to penetrate diverse metabolic niche to eradicate these killer bugs.

3.1 Sporulation speculations and beyond

The best form of dormancy has been studied in gram positive bacteria forming spores. As far as mycobacterium is concerned the existence of spore is debatable. A recent study by a group led jointly by Leif Kirsebom and Santanu Dasgupta in Uppsala University put forward the evidence for existence of spores in mycobacterium (Ghosh et al., 2009). The study revealed existence of endospores in very late stationary phase cultures of *Mycobacterium marinum*, a common model for acute mycobacterial infections. Utilizing transmission electron microscopy the distinct outer coat and cortex of the spores could be identified. Furthermore, heat tolerance, malachite green staining, and the presence of dipicolinic acid was also shown. They were able to isolate rRNA from these sporulating cells and identified it as *M. marinum* rRNA. Extensive bioinformatics analysis identified possible homologues of spore forming genes in mycobacterial genome. Thus they suggested that mycobacteria can form spores under stress and presented the hypothesis that sporulation might be one of the adaptations causing dormancy. When genes encoding GFP or mCherry were introduced on plasmids or integrated in to mycobacterium genome fluorescent spore were seen confirming their mycobacterial origin.

However, a rebuttal to this work by several US labs challenging the concept of endospore formation in mycobacteria was published (Traag et al., 2010) which was responded in to a subsequent rebuttal (Singh et al., 2010). The controversies continue with more supporting evidences yet to be deciphered about a phenomenon not so common to many other species.

4. Understanding the pathophysiology in light of protein synthesis

Mycobacterium can maintain itself in the fluctuating environment inside the host and the diversity of its survival mechanism makes it one of the most successful pathogens in the world. The ability of the bacteria to enter a stealth mode and exhibit occult form in combination with its starvation strategies suggests that there exists a complex series of events that is modulated by differential gene expression and protein synthesis. When mycobacterium switches its lifestyle from luxury to mere survival the DNA replication goes to a standby mode (Wayne, 1994) and the protein synthesis process undergoes drastic changes (Hu et al., 1998). Though the knowledge in this research area is limited, understanding the bacterium in the light of protein synthesis and protein turnover opens new avenues to understand this 'culprit in disguise' better.

4.1 Intricacies of macromolecular orchestration

The protein synthesis process is a hotspot for action of antibiotic drugs, which interact directly with mycobacterial ribosome and sabotage 'protein synthesis', the most vital

process of the cells. The mechanism of translation and the function of the ribosome in mycobacteria can thus be visited collectively in the light of action of the drugs which include aminoglycosides, macrolides, tetracycline and peptide antibiotics etc. (Inderlied, 1991). At the same time the study of the resistance mechanism in mycobacteria against these drugs, often involving certain mutations on rRNA or r-proteins, would shed light on the process.

In a study where several independent streptomycin-resistant mutants of *M. tuberculosis* H37Rv were isolated, it was shown that while in the wild-type streptomycin inhibited the incorporation of ^{14}C-amino acids into proteins, very little or no inhibition was observed in either high-level or low-level streptomycin-resistant strains (Shaila et al., 1973). This result on one hand points towards the activity of the drug against mycobacteria and on the other hand brings forward the potential problems arising from the development of resistance phenotypes. The Bottger-group has made very important contribution in the field in identifying potential drug candidates against mycobacteria (Hobbie et al., 2005; Hobbie et al., 2006a; Hobbie et al., 2006b; Hobbie et al., 2007). In addition, they have also identified potential hotspots for mutations leading to resistance against these drugs. Thus, these studies all together call for a continuous quest for identification of new and potential drug candidates for treatment of mycobacterial infections.

Protein synthesis involves a coordinated action of the ribosome, mRNA, tRNAs and translation factors; thus it needs an intelligent setup and precise orchestration between different components. The process, also known as translation, is divided into four distinct steps; namely initiation, elongation, termination and ribosome-recycling. The process initiates by association of large ribosomal subunit with an mRNA programmed small subunit bound with initiator tRNA and initiation factors. Once a 70S initiation complex is successfully formed, the initiation factors dissociate and the process proceeds to elongation. In the first step of elongation the elongation factor–Tu (EF-Tu) brings tRNA loaded with respective amino acids to the decoding center (DC) of the ribosome. This site includes highly conserved regions of 16S rRNA arranged in a highly ordered structure. Correct codon anticodon pairing between the tRNA and mRNA through initial selection and tRNA accommodation leads the process towards the most significant step of protein synthesis, i.e. peptide bond formation. A primarily rRNA (23S rRNA) constituted region of the large subunit, called the Peptidyl-transferase site, catalyses this step. After formation of the peptide bond the tRNA carrying the peptide chain moves one codon along the mRNA, a process called 'tRNA translocation', conducted by elongation factor-G (EF-G). Then the elongation steps repeat and the cycle continues until a stop codon on mRNA reaches the decoding center. The stop codons signal release factors to bind on the mRNA and release the peptide from the tRNA. The process ends by splitting of the ribosomal subunits, which recycle for the next round of protein synthesis. The detailed mechanism of these steps although studied mainly with components from gram-negative bacteria *E. coli*, can be extrapolated to gram-positive mycobacteria due to high degree of sequence conservation in the translation components in all eubacteria. It should be mentioned that although the protein synthesis machinery is highly conserved in general, the fine differences that exist in this system between the pathogenic bacteria and the host organisms provide ample opportunity for targeting the bacterial translation system in a rather specific manner.

Although in recent years, we have acquired a lot of knowledge on the mechanism of bacterial protein synthesis from high resolution crystal structure and fine-tuned biochemical

experiments, very little information specific to mycobacteria have been available. Generally it is believed that bacterial growth rate is highly dependent on the rate of protein synthesis and varies according to the number of translationally active ribosomes in the bacterial cells (Dennis & Bremer, 1974). Thus, mycobacterium, a remarkably slow growing pathogenic bacterium, provides a very interesting system for studying the rate of protein synthesis in different stages of its life.

In 2008, a strong step was taken to study the basic components of mycobacterial protein synthesis. It is known that although very similar, mycobacterial ribosomes differ from the well characterized ribosomes from *E. coli* to some extent. Firstly, the mutations on rRNA lead to somewhat different phenotype in these two bacteria. Secondly, the ribosomal stalk, a visible protuberance on the large subunit of the ribosome composed of the multicopy protein L12, differs in its composition and structural conformation between the two. In *E. coli,* the ribosomal stalk is composed of two dimers of L12 protein in contrast to three dimers in *M. smegmatis.* Also in *E. coli,* the stalk changes its conformation from compact to extended form depending on its state of interaction with the translation factors. In comparison, the stalk in mycobacteria seems to be extended most of the time, the functional significance of such behaviour is not known. It should also be mentioned that mycobacterial species often harbour only one or two rRNA operons in contrast to seven rRNA operons in *E. coli.* Thus mycobacteria provide a very good system to test the effect of mutations in the significant positions of rRNA towards the viability of the bacteria (Hobbie et al., 2007; Long et al., 2009). Also, homogeneous populations of the ribosomes carrying mutations in the rRNA could be isolated and characterized relatively easily from the mutated mycobacterial strains.

The study, based on translation components from *M. smegmatis* enriched the knowledge about the translation machinery in mycobacteria. A combined effort by the research groups of Erik C. Böttger and Marina Rodnina led the study where the genes for various translation factors were mapped, cloned, and expressed in heterologous (*E. coli*) expression system. Further, these factors were used together with mycobacterial ribosomes in a complete *in vitro* translation system complemented with some components from *E. coli*. In fast kinetics assay, the rate of initiation and elongation was measured and compared with those obtained from purely *E. coli* origin. Under these conditions, the rates of fundamental reactions of initiation and elongation of protein synthesis were found remarkably similar in the two systems. Thus, these results suggested that under *in* vitro conditions the basic mechanisms of protein synthesis are highly conserved in these two widely separated species of bacteria (Bruell et al., 2008). At the same time, this study provides an alternative system to identify and test the action of the antibiotics. However, it fails to provide any insight about the rate of protein synthesis in slow growing phases of mycobacteria and calls for detailed investigation of the same under controlled *in vivo* conditions using methods such as radio-labelled amino acid incorporation or beta-galactosidase synthesis assay.

4.2 Combat targets and troubleshooting

There are basically three main hotspots that can confer resistance to inhibitors against protein synthesis. The first is by mutations in the rRNA, the second being by mutations in the ribosomal proteins, and finally by post-transcriptional modification, especially methylation of the ribosomal RNA. Concerted efforts have revealed in the recent past that antibiotics might interfere with chemical probe binding to specific nucleotides in the rRNA

(Moazed & Noller, 1987). These studies suggested that antibiotics could act by interacting with highly conserved hotspots of the bacterial rRNA and thereby interfering with their natural functions. There are also evidences that antibiotics inhibit enzymes essential for creating modifications on rRNA vital for its function. Identification of the sites for resistant mutation often sheds light on the antibiotic action. Resistance to streptomycin which acts by causing misreading in the genetic code followed by inhibition of translation initiation and dubious proofreading (Moazed & Noller, 1987) is attributed to mutations in the aminoglycoside modifying enzyme (Benveniste & Davies, 1973) as well as in *rpsL* gene coding for r-protein S12 (Funatsu & Wittmann, 1972; Allen & Noller, 1989). Specific to mycobacteria, it has been shown that mutations in the *rpsL* gene that replaces Lys 43 or Lys 88 by arginine are associated with streptomycin resistance (Honore & Cole, 1994). There have been also evidences of mutations in 16S rRNA clustered in two regions in the 16SrRNA leading to resistance to streptomycin (Douglass & Steyn, 1993).

Macrolides are bacteriostatic in nature and inhibit the petidyl transferase function of the 50S ribosomal subunit by blocking the peptidyl exit-tunnel. The clinically acquired resistance against macrolides is described as the MLS phenotype (resistant to Macrolide, Lincomycin and Stretogramin B) which is thought to be collectively mediated by methylases coded by *erm* genes (Leclercq & Courvalin, 1991). These methylases brings about changes in the conserved loop of domain V of 23S rRNA (A2058 equivalent of *E.coli*) which is implicated to have definitive role in peptidyl transfer. Recent studies have shown paromomycin, an aminoglycoside, to be effective both *in vitro* and *in vivo* against MDR-TB (Kanyok et al., 1994). A study in *E.coli* has mapped the changes in rRNA that confer resistance by transfecting mutated rRNA operons in case of paromomycin(De Stasio et al., 1989). It would be interesting to see if similar mutations in mycobacteria would also confer resistance for this antibiotic.

In case of tetracycline studies it was revealed that the drug does not inhibit the growth of *M. tuberculosis* but inhibits protein synthesis *in vitro* suggesting that intact cells are impermeable to the drug (Bottger, 1994). Ribosomal mutations that confer tetracycline resistance are difficult to map because the mutations occur frequently in the system which pumps the drugs out.

Protein synthesis: The portfolio for inhibition and innovating novel combat strategies

The contemporary treatment of tuberculosis includes aminoglycosides (streptomycin, amikacin, kanamycin, and capreomycin) and oxazolidinones (linezolid).

- **Initiation:** Streptomycin / Rifampicin
- **Elongation and translocation:** Fusidic acid
- **Amino acyl tRNA inhibitors:** Tetracyclines
- **Proofreading inhibitors:** Aminoglycosides
- **Peptidyl transfer inhibitor:** Chloroamphenicol, Macrolides
- **EFG inhibitors:** Fusidic acid
- Inhibitors binding to ribosome

50S: Chloroamphenicol, Oxazolidinones, Macrolides
30S: Aminoglycosides and Tetracyclines

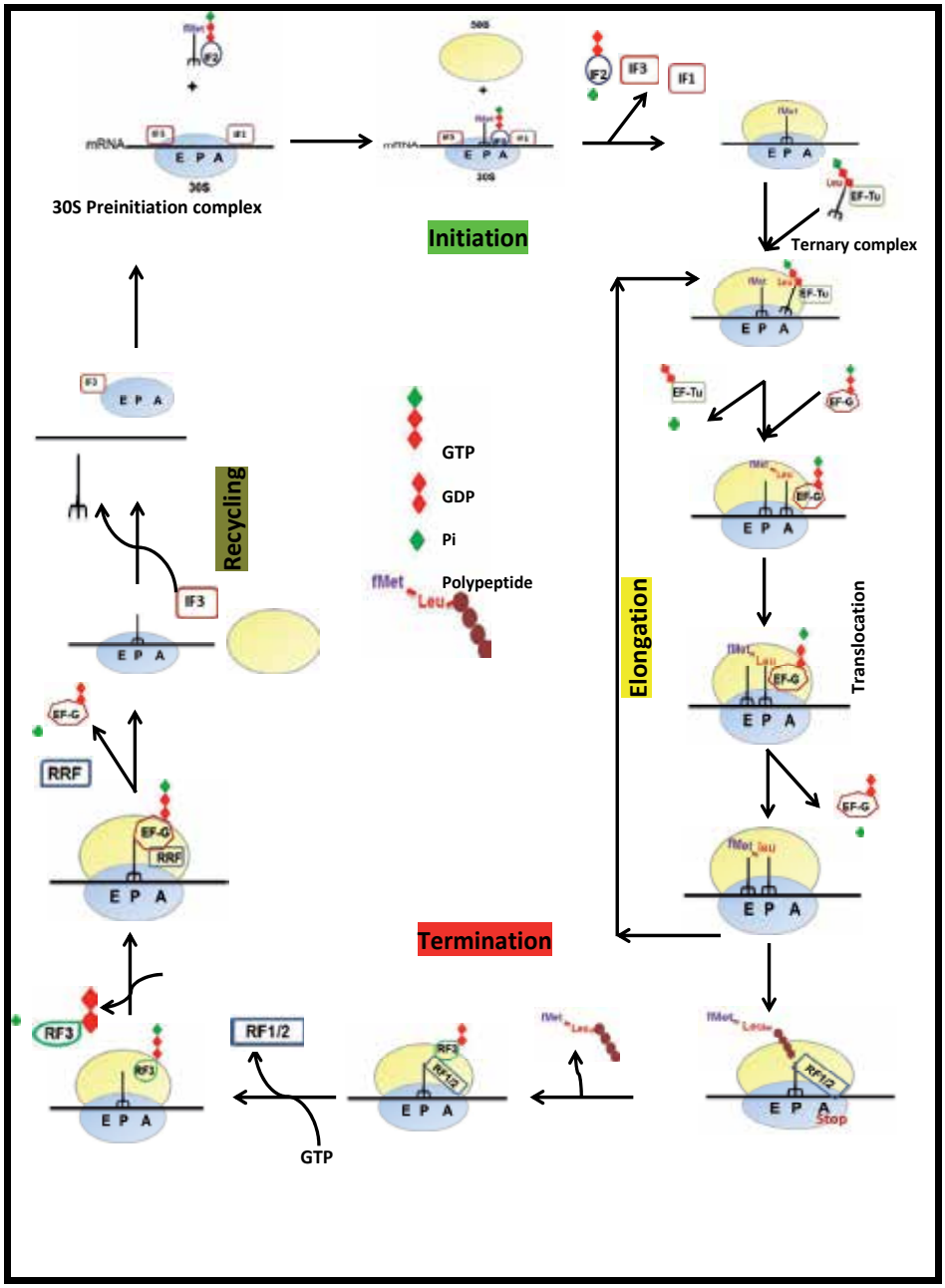


Fig. 3. Translation scheme showing various steps that can be targets of therapeutic intervention. (Adopted from Mandava CS, Ribosomal Stalk Protein L12 Structure Function and Application, thesis submitted to Uppsala University, Sweden 2011)

Nevertheless two important resistant determinants have been found in *E. coli TetO* and *TetM* genes. TetM acts by protecting the ribosome from antibiotic attack and the resistance to tetracycline encoded by these genes are located on plasmids or transposable elements

(Roberts, 1994). A detailed account of the process in slow growing mycobacteria is still not available, which would yield valuable insight about the antibiotic in treatment of mycobacteria.

4.3 The Vendetta in light of protein turnover

The complex milieu, in which the mycobacterium survives, necessitates dynamic adjustments and fine-tuning in protein synthesis and turn-over. During the transfer from active to dormant stage a change in the nature of the proteins expressed is bound to occur. It remains an open question whether protein synthesis continues in a slow rate in the dormant stage or stops completely following the change of the growth rate in mycobacteria. It is very important to understand how protein synthesis machinery work in the dormant or persistent stage since under favourable conditions this dormant form can again get triggered and reactivated leading to a full blown infection in the immune compromised host; thus posing a big threat. One recent report suggested that the protein synthesis is almost shut down in dormant mycobacterium and the conditions can be reversed by providing stimuli like oxygen or heat shock to anaerobic stationary cultures (Hu et al., 1998). It also illustrates the fact that the mycobacteria are quite responsive to stimuli and can trigger protein synthesis quite rapidly even in the dormant state. Hu *et al*. showed a 98% decrease in protein synthesis using ^{35}S-met pulse labeling experiments with microaerophilic cultures of mycobacterium tuberculosis. However, it is still not understood fully whether there is complete shutdown of protein synthesis or a minimal protein synthesis continues in this state to facilitate survival. Another recent report by Murphy *et.al*, suggests that during dormancy *M. tuberculosis* performs limited protein synthesis and the pathogen spends its available resources in maintaining cell wall, membrane potential, genome integrity as well as resisting host defence systems (Murphy & Brown, 2007). The transcriptiome analysis further revealed five times more downregulated genes in persisters than upregulated ones (1,408 versus 282) under dormant condition suggesting that the growth and energy metabolism pathways are significantly downregulated (Keren et al., 2011). Even the synthesis of the ribosomal proteins tends to get down regulated in the state of dormancy as revealed in several different models (Wayne & Hayes, 1996; Betts et al., 2002; Keren et al., 2011). Although these studies are insufficient to resolve whether proteins synthesis comes to a complete halt or not in the dormant stage, these also open up a new question regarding the availability of the vital proteins in this stage. In other words, these studies indicate towards a longer turn-over of the proteins in this stage.

Continuous translation is not the only crucial process that defines the abundance of a protein or enzyme in the cell; instead it is often regulated by the parameters controlling its propensity to degradation and stabilization. Thus, regulation of protein amounts in a non-favourable and fluctuating environment can be more dependent on turnover of proteins rather than the synthesis (Neher et al., 2003). Our knowledge regarding protein turnover in dormant phases in mycobacteria is still in infancy. However research in the last decade has thrown some light on it by attempting to examine the protein turnover at global level. Two seminal papers (Rao & Li, 2009; Rao et al., 2009) have helped in understanding the molecular basis of persistence in mycobacterium. Rao et.al showed that the protein turnover was much more effective to maintain relative protein abundance in the dormant phases of mycobacteria. These studies also help explain how the bacteria manage to exist as a submissive pathogen in the host for a prolonged period. The molecular detail behind the

prolonged protein-turnover mechanism in mycobacteria is not available yet. We speculate that under the dormant and stress condition, the protein degradation systems also slow down significantly as the protein synthesis system, thereby maintaining a good-enough concentration of the vital proteins in the cell and ensure survival of the bacteria.

Using the two-dimensional electrophoresis-based proteomics approach, a protein has been identified in *Escherichia coli* (Almiron et al., 1992), which expresses preferentially under starvation conditions. This protein is homologous to a family of proteins called Dps (DNA binding Protein from Starved cells) that are known to protect DNA under various kinds of environmental stresses. Although Dps kind of proteins have, so far, not been yet reported in mycobacteria, it is highly possible that similar system exists. Using comparative modelling it was possible to demonstrate that Dps from *Mycobacterium smegmatis* could form a dodecamer structure similar to the Dps from *Escherichia coli* (Gupta et al., 2002). The intriguing properties related to protein stability, DNA binding property and protection was further revisited to consolidate the structure function attribute of this unique protein (Ceci et al., 2005). These studies put emphasis on the need of global proteome analysis not only for actively growing cells but also for cells under dormant conditions. The proteomics can also be useful to identify the proteins related to starvation response of the pathogen.

Applying a systems biology approach the contemporary knowledge reveals approximately 4000 genes in mycobacterium genome and proteomic studies on the persistence reveals only very few which are differentially expressed during dormancy. A big proportion of these correspond to stress response proteins and metabolic enzymes (Cho et al., 2006). Unfortunately, there seems to be no exact correlation between the transcriptiome and the proteome data, which would otherwise be very useful (Mattow et al., 2006). The research group led by KVS Rao at ICGEB, New Delhi came up with an innovative strategy to comprehend the host key players that are involved in the onset and persistence of mycobacterium. They employed exhaustive genome-wide small interfering RNA (Si RNA) and identified 275 key molecules that are intricately associated in a network. This has led to a better understanding of host intracellular adaptation as result of bacterial diversification and host-pathogen interactions (Kumar et al., 2010). A bird's eye view from this aspect is bound to yield innovative insights in the pathophysiology of host pathogen interactions.

5. Future perspective and therapeutic interventions

There has been a great interest in looking for better alternatives and treatment modules throughout the globe to address the issue of this deadly pathogen. The focus has further intensified because of its proven role in immunodeficiency syndromes such as AIDS. The Global TB Alliance estimates that the disease will be a financial burden with expenditure ranging from $ 1-3 trillion in coming decades for poor countries (www.tballiance.org). Developing countries are at the hotspots where 94% of TB cases and 98% of death takes place. The present treatment employs the strategies being used since decades with longer treatment regimen. The standard treatment regimen of six to nine month was based on clinical practices dating almost half a century back. The puzzle is that the exact molecular mechanism still needs to be deciphered with evidence that Isoniazid one of the key cocktail medicine only acts on actively growing form (Fox et al., 1999) and not against the anaerobic occult form (Thadepalli et al., 1979) makes situation draconian. The first real rescue for tuberculosis came in the form of Streptomycin in 1943 that was found to bind to 16S rRNA

and block the initiation of translation (Waksman & Schatz, 1943). As early as in 1948 reports of streptomycin resistance hit the scene where streptomycin seems no more effective at all (1948). It was in 1950's that the field had many new drugs hitting the market and the situation looked quite under control. The most recent drug to hit the space is still 35 years old. Modern therapy relies on a combination of potent bactericidal agents, such as isoniazid, rifampicin and pyrazinamide, in a treatment with six month duration. Isoniazid and Rifampicin are the two most effective drugs in the treatment bouquet that is administered throughout the treatment (Zhang, 2005). These are complemented with Ethambutol and Pyrazinamide in the early phase of treatment. Since there has been no new drugs developed in the last four decades and with excessive use of antibiotics there has emerged a new family of multidrug and extremely drug resistance strains leading to what we call MDR and XDR-TB. MDR refers to a resistance against the frontline drugs i.e. Isoniazid and Rifampicin whereas XDR is a complex form which is coupled to MDR and resistant against second line of potent antibiotics like Amikacin, Kanamycin and Capreomycin etc. (Organization, 2010). The perennial problem of non-compliance led the WHO to come up with universal treatment adherence programs, through a process currently known as the directly observed treatment short-course (DOTS).

The complex milieu of *Mycobacterium tuberculosis* and its intelligent survival instinct by virtue of adaptive diversification in tuberculosis disease have been a major obstacle that hinders the development of shorter treatment regimens to eradicate the disease. Currently the TB Alliance, a global initiative in newer TB drug development is trying to come up with affordable, shorter, safer and effective alternatives to target tuberculosis. At present they have 20 promising projects in the pipeline which includes three crucial drug candidates and a novel treatment regimen under phase three clinical trials (REMOX TB) that would considerably shorten the treatment span by at least two months without compromising efficacy. Another blue- eyed boy is a potential compound TMC207 that promises to be active on both drug sensitive and drug resistance form is under phase two trials. A novel cocktail NC001 is also under clinical trial which would shorten treatment regimen and alternatively provide affordable treatment without compromise (Alliance, 2010).

On the vaccination front BCG still occupies the central position being one of the most widely used vaccine against TB to be administered globally without any serious side effects. Its low cost of production ability to confer lifelong protection without booster dose in single immunization, availability of oral alternatives makes it popular in infant immunization programs. BCG has been effective to prevent meningeal TB in children, but it does not confer immunity to pulmonary TB in adults (Orme, 2001b). An interesting investigation in India during the 1960's by WHO in order to establish the efficacy of the BCG vaccine on two separate groups comprising 375,000 people in the province of Madras where one group was vaccinated against TB and the other group was not finally concluded that: "The efficacy of the TB vaccine is 0%" (www.whale.to/a/tb_q.html). Since then in last decade most of the European countries has removed it from their immunization programme. The result is further substantiated by the argument that contracting TB doesn't provide any immunity against a second infection; and if a natural infection doesn't provide protection then a vaccination certainly won't provide protection either. As of now no promising vaccine has come in to the scene so far for tuberculosis in spite of various attempts in coming up with a DNA vaccine (Young et al., 1988; Lowrie et al., 1999; Orme, 2001b). The vaccine development has again come to picture after lacunae of several decades where the

development pipeline now includes seven vaccine candidates that are being tested in humans. Two non- replicating viral vector vaccines have very recently entered the first phase efficacy trial in infants (the first such trial in 80 years) and in human immunodeficiency virus–infected adults (Beresford & Sadoff, 2010). Yet we have a long way to go for an effective vaccine which would take care of all the thriving forms of this enigmatic bacterium.

6. The odyssey ahead

The geography of tuberculosis has achieved a global dimension with transmigration across the boundaries as a result TB pandemic has taken the world in its clutches thus making it a true global concern. The TB Alliance, a global initiative has embarked an integrated innovative approach to combat and sabotage one of the oldest, deadly, and most resilient enemies of the mankind. At the level of individual it impacts them in the most productive phase of their life thereby sucking their income and decreasing the productivity and thus inflicting a great loss in human capital. On average one person infects fifteen others before finally getting successfully treated; the death statistics is alarming with one death every twenty second. The right to health and hope is one of the fundamental rights that is robbed by this enemy of the mankind. Bill Gates propounded at the World Health Assembly in 2005: "Today, we have tuberculosis drugs you have to take for 9 months. Why can't we find one that works in 3 days?" this is still a dream.

The research arena has also received momentum with the deciphering of the Mycobacterial genome in 1998 coupled with advanced molecular biology tools, structural genomics, target based drug design, high throughput screening, *in silico* experiments, whole cell screening and advanced imaging technologies to study real time changes and system biology platforms coming under one umbrella. The idea now is to leverage on the existing portfolio having more than two dozens of potent molecules and drug regimen in pipeline at various stages of clinical trials. The TB development has galvanized further by the coming together of WHO, TB Alliance and DNDi (Drug for Neglected Disease initiative) sharing a common podium. TDR for research on diseases of poverty has been working under the joint conglomeration of WHO, UNICEF, WORLD BANK and UNDP to address this concern globally. Nelson Mandela commented that "we cannot win the battle against AIDS, if we do not also fight TB; TB is too often a death sentence for people with AIDS". The WHO targets to treat 80% of the MDR-TB patients by 2015 with an estimated cost of $15 billion. With initiatives at full bloom the scenario looks promising and hopeful in global attempts to address tuberculosis.

The conquest of the Mycobacterium to the mankind needs to be the priority of the synergistic efforts by the scientific community. In nutshell the enemy of humanity needs to be taken in to clutches by innovative approaches from drug development that includes quest for effective molecular scaffolds and their derivatives both old and new as well as reengineering delivery strategies for the drug to penetrate the recalcitrant stubborn microbes. On one hand there are challenges to finding safer, cheap, less toxic, shorter regimen and compatible drugs quickly while on the other the socio economic feasibilities that can deliver these magic bullets to the neediest ones have to be ensured. Revamping the health care system with Government, business houses and NGO's is the need of the hour to combat these perpetrators of human misery from the globe. Wave of optimism exists as we

attempt to streamline the drug discovery process together with policy issues, social engineering and outreach initiatives.

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8. References

(1948). STREPTOMYCIN treatment of pulmonary tuberculosis. *Br Med J*, Vol. 2, No. 4582, pp. 769-782

(2007). *Biological Inorganic Chemistry* University Science Books,U.S,

Allen, J. H., Utley, M., van Den Bosch, H., Nuijten, P., Witvliet, M., McCormick, B. A., Krogfelt, K. A., Licht, T. R., Brown, D., Mauel, M., Leatham, M. P., Laux, D. C. and Cohen, P. S. (2000). A functional cra gene is required for Salmonella enterica serovar typhimurium virulence in BALB/c mice. *Infect Immun*, Vol. 68, No. 6, pp. 3772-3775

Allen, P. N. and Noller, H. F. (1989). Mutations in ribosomal proteins S4 and S12 influence the higher order structure of 16 S ribosomal RNA. *J Mol Biol*, Vol. 208, No. 3, pp. 457-468

Alliance, T. (2010). ANNUAL REPORT. No. pp.

Almiron, M., Link, A. J., Furlong, D. and Kolter, R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli. *Genes Dev*, Vol. 6, No. 12B, pp. 2646-2654

Anstrom, D. M. and Remington, S. J. (2006). The product complex of M. tuberculosis malate synthase revisited. *Protein Sci*, Vol. 15, No. 8, pp. 2002-2007

Barer, M. R. and Harwood, C. R. (1999). Bacterial viability and culturability. *Adv Microb Physiol*, Vol. 41, No. pp. 93-137

Beeckmans, S., Khan, A. S. and Van Driessche, E. (1997). Role of Mg2+ in the structure and activity of maize (Zea mays L.) isocitrate lyase: indications for hysteretic behaviour. *Biochem J*, Vol. 327 (Pt 1), No. pp. 171-176

Benveniste, R. and Davies, J. (1973). Mechanisms of antibiotic resistance in bacteria. *Annu Rev Biochem*, Vol. 42, No. pp. 471-506

Beresford, B. and Sadoff, J. C. Update on research and development pipeline: tuberculosis vaccines. *Clin Infect Dis*, Vol. 50 Suppl 3, No. pp. S178-183

Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. and Duncan, K. (2002). Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol*, Vol. 43, No. 3, pp. 717-731

Bottger, E. C. (1994). Resistance to drugs targeting protein synthesis in mycobacteria. *Trends Microbiol*, Vol. 2, No. 10, pp. 416-421

Bruell, C. M., Eichholz, C., Kubarenko, A., Post, V., Katunin, V. I., Hobbie, S. N., Rodnina, M. V. and Bottger, E. C. (2008). Conservation of bacterial protein synthesis machinery: initiation and elongation in Mycobacterium smegmatis. *Biochemistry*, Vol. 47, No. 34, pp. 8828-8839

Ceci, P., Ilari, A., Falvo, E., Giangiacomo, L. and Chiancone, E. (2005). Reassessment of protein stability, DNA binding, and protection of Mycobacterium smegmatis Dps. *J Biol Chem*, Vol. 280, No. 41, pp. 34776-34785

Cho, S. H., Goodlett, D. and Franzblau, S. (2006). ICAT-based comparative proteomic analysis of non-replicating persistent Mycobacterium tuberculosis. *Tuberculosis (Edinb)*, Vol. 86, No. 6, pp. 445-460

Cole, S. T. (1999). Learning from the genome sequence of Mycobacterium tuberculosis H37Rv. *FEBS Lett*, Vol. 452, No. 1-2, pp. 7-10

Daniel, J., Maamar, H., Deb, C., Sirakova, T. D. and Kolattukudy, P. E. (2011). Mycobacterium tuberculosis Uses Host Triacylglycerol to Accumulate Lipid Droplets and Acquires a Dormancy-Like Phenotype in Lipid-Loaded Macrophages. *PLoS Pathog*, Vol. 7, No. 6, pp. e1002093

De Stasio, E. A., Moazed, D., Noller, H. F. and Dahlberg, A. E. (1989). Mutations in 16S ribosomal RNA disrupt antibiotic--RNA interactions. *EMBO J*, Vol. 8, No. 4, pp. 1213-1216

Deb, C., Lee, C. M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., Pawar, S., Rogers, L. and Kolattukudy, P. E. (2009). A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One*, Vol. 4, No. 6, pp. e6077

Dennis, P. P. and Bremer, H. (1974). Differential rate of ribosomal protein synthesis in Escherichia coli B-r. *J Mol Biol*, Vol. 85, No. 3, pp. 407-422

Dixon, G. H., Kornberg, H. L. and Lund, P. (1960). Purification and properties of malate synthetase. *Biochim Biophys Acta*, Vol. 41, No. pp. 217-233

Douglass, J. and Steyn, L. M. (1993). A ribosomal gene mutation in streptomycin-resistant Mycobacterium tuberculosis isolates. *J Infect Dis*, Vol. 167, No. 6, pp. 1505-1506

Fox, W., Ellard, G. A. and Mitchison, D. A. (1999). Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. *Int J Tuberc Lung Dis*, Vol. 3, No. 10 Suppl 2, pp. S231-279

Funatsu, G. and Wittmann, H. G. (1972). Ribosomal proteins. 33. Location of amino-acid replacements in protein S12 isolated from Escherichia coli mutants resistant to streptomycin. *J Mol Biol*, Vol. 68, No. 3, pp. 547-550

Ghosh, J., Larsson, P., Singh, B., Pettersson, B. M., Islam, N. M., Sarkar, S. N., Dasgupta, S. and Kirsebom, L. A. (2009). Sporulation in mycobacteria. *Proc Natl Acad Sci U S A*, Vol. 106, No. 26, pp. 10781-10786

Giachetti, E. and Vanni, P. (1991). Effect of Mg2+ and Mn2+ on isocitrate lyase, a non-essentially metal-ion-activated enzyme. A graphical approach for the discrimination of the model for activation. *Biochem J*, Vol. 276 (Pt 1), No. pp. 223-230)

Gomez, J. E. and McKinney, J. D. (2004). M. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis (Edinb)*, Vol. 84, No. 1-2, pp. 29-44

Gould, T. A., van de Langemheen, H., Munoz-Elias, E. J., McKinney, J. D. and Sacchettini, J. C. (2006). Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in Mycobacterium tuberculosis. *Mol Microbiol*, Vol. 61, No. 4, pp. 940-947

Graham, J. E. and Clark-Curtiss, J. E. (1999). Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective

capture of transcribed sequences (SCOTS). *Proc Natl Acad Sci U S A*, Vol. 96, No. 20, pp. 11554-11559

Gupta, S., Pandit, S. B., Srinivasan, N. and Chatterji, D. (2002). Proteomics analysis of carbon-starved Mycobacterium smegmatis: induction of Dps-like protein. *Protein Eng*, Vol. 15, No. 6, pp. 503-512

Gyanu Lamichhane, M. Z., Natalie J. Blades, Deborah E. Geiman, Annette Dougherty, Jacques Grosset, Karl W. Broman, and William R. Bishai (2003). A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: Application to Mycobacterium tuberculosis. *PNAS*, Vol. 100 (12), No. pp.

Hautzel, R., Anke, H. and Sheldrick, W. S. (1990). Mycenon, a new metabolite from a Mycena species TA 87202 (basidiomycetes) as an inhibitor of isocitrate lyase. *J Antibiot (Tokyo)*, Vol. 43, No. 10, pp. 1240-1244

Hobbie, S. N., Bruell, C., Kalapala, S., Akshay, S., Schmidt, S., Pfister, P. and Bottger, E. C. (2006a). A genetic model to investigate drug-target interactions at the ribosomal decoding site. *Biochimie*, Vol. 88, No. 8, pp. 1033-1043

Hobbie, S. N., Kalapala, S. K., Akshay, S., Bruell, C., Schmidt, S., Dabow, S., Vasella, A., Sander, P. and Bottger, E. C. (2007). Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucleic Acids Res*, Vol. 35, No. 18, pp. 6086-6093

Hobbie, S. N., Pfister, P., Bruell, C., Sander, P., Francois, B., Westhof, E. and Bottger, E. C. (2006b). Binding of neomycin-class aminoglycoside antibiotics to mutant ribosomes with alterations in the A site of 16S rRNA. *Antimicrob Agents Chemother*, Vol. 50, No. 4, pp. 1489-1496

Hobbie, S. N., Pfister, P., Brull, C., Westhof, E. and Bottger, E. C. (2005). Analysis of the contribution of individual substituents in 4,6-aminoglycoside-ribosome interaction. *Antimicrob Agents Chemother*, Vol. 49, No. 12, pp. 5112-5118

Honer Zu Bentrup, K., Miczak, A., Swenson, D. L. and Russell, D. G. (1999). Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. *J Bacteriol*, Vol. 181, No. 23, pp. 7161-7167

Honer zu Bentrup, K. and Russell, D. G. (2001). Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol*, Vol. 9, No. 12, pp. 597-605

Honore, N. and Cole, S. T. (1994). Streptomycin resistance in mycobacteria. *Antimicrob Agents Chemother*, Vol. 38, No. 2, pp. 238-242

Hoyt, J. C., Johnson, K. E. and Reeves, H. C. (1991). Purification and characterization of Acinetobacter calcoaceticus isocitrate lyase. *J Bacteriol*, Vol. 173, No. 21, pp. 6844-6848

Hoyt, J. C., Robertson, E. F., Berlyn, K. A. and Reeves, H. C. (1988). Escherichia coli isocitrate lyase: properties and comparisons. *Biochim Biophys Acta*, Vol. 966, No. 1, pp. 30-35

Hu, Y. M., Butcher, P. D., Sole, K., Mitchison, D. A. and Coates, A. R. (1998). Protein synthesis is shutdown in dormant Mycobacterium tuberculosis and is reversed by oxygen or heat shock. *FEMS Microbiol Lett*, Vol. 158, No. 1, pp. 139-145

Inderlied, C. B. (1991). *Antibiotics in Laboratory Medicine*, Williams & Wilkins,

Kanyok, T. P., Reddy, M. V., Chinnaswamy, J., Danziger, L. H. and Gangadharam, P. R. (1994). In vivo activity of paromomycin against susceptible and multidrug-resistant Mycobacterium tuberculosis and M. avium complex strains. *Antimicrob Agents Chemother*, Vol. 38, No. 2, pp. 170-173

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R. and Barer, M. R. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek*, Vol. 73, No. 2, pp. 169-187

Kelly, B. G., Wall, D. M., Boland, C. A. and Meijer, W. G. (2002). Isocitrate lyase of the facultative intracellular pathogen Rhodococcus equi. *Microbiology*, Vol. 148, No. Pt 3, pp. 793-798

Keren, I., Minami, S., Rubin, E. and Lewis, K. Characterization and Transcriptome Analysis of Mycobacterium tuberculosis Persisters. *MBio*, Vol. 2, No. 3, pp.

Keren, I., Minami, S., Rubin, E. and Lewis, K. (2011). Characterization and Transcriptome Analysis of Mycobacterium tuberculosis Persisters. *MBio*, Vol. 2, No. 3, pp.

Ko, Y. H. and McFadden, B. A. (1990). Alkylation of isocitrate lyase from Escherichia coli by 3-bromopyruvate. *Arch Biochem Biophys*, Vol. 278, No. 2, pp. 373-380

Ko, Y. H., Vanni, P. and McFadden, B. A. (1989). The interaction of 3-phosphoglycerate and other substrate analogs with the glyoxylate- and succinate-binding sites of isocitrate lyase. *Arch Biochem Biophys*, Vol. 274, No. 1, pp. 155-160

Kumar, D., Nath, L., Kamal, M. A., Varshney, A., Jain, A., Singh, S. and Rao, K. V. Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. *Cell*, Vol. 140, No. 5, pp. 731-743

Kumar, R. (2009). Glyoxylate Shunt : Combating Mycobacterium at Forefront. *International Journal of Integrative Biology*, Vol. 7, No. 2, pp. 69-72

Kumar, R. and Bhakuni, V. (2008). Mycobacterium tuberculosis isocitrate lyase (MtbIcl): role of divalent cations in modulation of functional and structural properties. *Proteins*, Vol. 72, No. 3, pp. 892-900

Kumar, R. and Bhakuni, V. (2010). A functionally active dimer of mycobacterium tuberculosis malate synthase G. *Eur Biophys J*, Vol. 39, No. 11, pp. 1557-1562

Kumar, R. and Bhakuni, V. (2011). Comparative analysis of malate synthase G from Mycobacterium tuberculosis and E. coli: Role of ionic interaction in modulation of structural and functional properties. *Int. J. Biol. Macromol.*, Vol. No. pp.

Leclercq, R. and Courvalin, P. (1991). Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. *Antimicrob Agents Chemother*, Vol. 35, No. 7, pp. 1267-1272

Lee, D., Shin, J., Yoon, K. M., Kim, T. I., Lee, S. H., Lee, H. S. and Oh, K. B. (2008). Inhibition of Candida albicans isocitrate lyase activity by sesterterpene sulfates from the tropical sponge Dysidea sp. *Bioorg Med Chem Lett*, Vol. 18, No. 20, pp. 5377-5380

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*, Vol. 5, No. 1, pp. 48-56

LI Jun-ming, L. N., ZHU Dao-yin, WAN La-gen, HE Yong-lin , YANG Chun (2008). Isocitrate lyase from Mycobacterium tuberculosis promotes survival of Mycobacterium smegmatis within macrophage by suppressing cell apoptosis. *Chinese Medical Journal*, Vol. 121, No. 12, pp.

Long, K. S., Poehlsgaard, J., Hansen, L. H., Hobbie, S. N., Bottger, E. C. and Vester, B. (2009). Single 23S rRNA mutations at the ribosomal peptidyl transferase centre confer resistance to valnemulin and other antibiotics in Mycobacterium smegmatis by perturbation of the drug binding pocket. *Mol Microbiol*, Vol. 71, No. 5, pp. 1218-1227

Lorenz, M. C. and Fink, G. R. (2001). The glyoxylate cycle is required for fungal virulence. *Nature*, Vol. 412, No. 6842, pp. 83-86

Lowrie, D. B., Tascon, R. E., Bonato, V. L., Lima, V. M., Faccioli, L. H., Stavropoulos, E., Colston, M. J., Hewinson, R. G., Moelling, K. and Silva, C. L. (1999). Therapy of tuberculosis in mice by DNA vaccination. *Nature*, Vol. 400, No. 6741, pp. 269-271

Manabe, Y. C., Saviola, B. J., Sun, L., Murphy, J. R. and Bishai, W. R. (1999). Attenuation of virulence in Mycobacterium tuberculosis expressing a constitutively active iron repressor. *Proc Natl Acad Sci U S A*, Vol. 96, No. 22, pp. 12844-12848

Mattow, J., Siejak, F., Hagens, K., Becher, D., Albrecht, D., Krah, A., Schmidt, F., Jungblut, P. R., Kaufmann, S. H. and Schaible, U. E. (2006). Proteins unique to intraphagosomally grown Mycobacterium tuberculosis. *Proteomics*, Vol. 6, No. 8, pp. 2485-2494

Mc, C. R., Lee, S. H., Deuschle, K. and Mc, D. W. (1957). Ineffectiveness of isoniazid in modifying the phenomenon of microbial persistence. *Am Rev Tuberc*, Vol. 76, No. 6, pp. 1106-1109

McFadden, B. A. and Purohit, S. (1977). Itaconate, an isocitrate lyase-directed inhibitor in Pseudomonas indigofera. *J Bacteriol*, Vol. 131, No. 1, pp. 136-144

McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr. and Russell, D. G. (2000). Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, Vol. 406, No. 6797, pp. 735-738

Moazed, D. and Noller, H. F. (1987). Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, Vol. 327, No. 6121, pp. 389-394

Mukamolova, G. V., Yanopolskaya, N. D., Kell, D. B. and Kaprelyants, A. S. (1998). On resuscitation from the dormant state of Micrococcus luteus. *Antonie Van Leeuwenhoek*, Vol. 73, No. 3, pp. 237-243

Munoz-Elias, E. J., Upton, A. M., Cherian, J. and McKinney, J. D. (2006). Role of the methylcitrate cycle in Mycobacterium tuberculosis metabolism, intracellular growth, and virulence. *Mol Microbiol*, Vol. 60, No. 5, pp. 1109-1122

Murphy, D. J. and Brown, J. R. (2007). Identification of gene targets against dormant phase Mycobacterium tuberculosis infections. *BMC Infect Dis*, Vol. 7, No. pp. 84

Neher, S. B., Sauer, R. T. and Baker, T. A. (2003). Distinct peptide signals in the UmuD and UmuD' subunits of UmuD/D' mediate tethering and substrate processing by the ClpXP protease. *Proc Natl Acad Sci U S A*, Vol. 100, No. 23, pp. 13219-13224

Organization, W. H. (2010). 2010/2011 TUBERCULOSIS GLOBAL FACTS. No. pp.

Orme, I. M. (2001a). The latent tubercle bacillus (I'll let you know if I ever meet one). *Int. J. Tuberc. Lung Dis.*, Vol. 5, No. pp. 589-593

Orme, I. M. (2001b). The search for new vaccines against tuberculosis. *J Leukoc Biol*, Vol. 70, No. 1, pp. 1-10

Pandey, A. K. and Sassetti, C. M. (2008). Mycobacterial persistence requires the utilization of host cholesterol. *Proc Natl Acad Sci U S A*, Vol. 105, No. 11, pp. 4376-4380

Rao, P. K. and Li, Q. (2009). Protein turnover in mycobacterial proteomics. *Molecules*, Vol. 14, No. 9, pp. 3237-3258

Rao, P. K., Singh, C. R., Jagannath, C. and Li, Q. (2009). A systems biology approach to study the phagosomal proteome modulated by mycobacterial infections. *Int J Clin Exp Med*, Vol. 2, No. 3, pp. 233-247

Reddy, T. B., Riley, R., Wymore, F., Montgomery, P., DeCaprio, D., Engels, R., Gellesch, M., Hubble, J., Jen, D., Jin, H., Koehrsen, M., Larson, L., Mao, M., Nitzberg, M., Sisk, P., Stolte, C., Weiner, B., White, J., Zachariah, Z. K., Sherlock, G., Galagan, J. E., Ball, C.

A. and Schoolnik, G. K. (2009). TB database: an integrated platform for tuberculosis research. *Nucleic Acids Res*, Vol. 37, No. Database issue, pp. D499-508

Roberts, M. C. (1994). Epidemiology of tetracycline-resistance determinants. *Trends Microbiol*, Vol. 2, No. 10, pp. 353-357

Rua, J., De Arriaga, D., Busto, F. and Soler, J. (1989). Effect of glucose on isocitrate lyase in Phycomyces blakesleeanus. *J Bacteriol*, Vol. 171, No. 11, pp. 6391-6393

Sassetti, C. M. and Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A*, Vol. 100, No. 22, pp. 12989-12994

Shaila, M. S., Gopinathan, K. P. and Ramakrishnan, T. (1973). Protein synthesis in Mycobacterium tuberculosis H37Rv and the effect of streptomycin in streptomycin-susceptible and -resistant strains. *Antimicrob Agents Chemother*, Vol. 4, No. 3, pp. 205-213

Sharma, V., Sharma, S., Hoener zu Bentrup, K., McKinney, J. D., Russell, D. G., Jacobs, W. R., Jr. and Sacchettini, J. C. (2000). Structure of isocitrate lyase, a persistence factor of Mycobacterium tuberculosis. *Nat Struct Biol*, Vol. 7, No. 8, pp. 663-668

Singh, B., Ghosh, J., Islam, N. M., Dasgupta, S. and Kirsebom, L. A. (2010). Growth, cell division and sporulation in mycobacteria. *Antonie Van Leeuwenhoek*, Vol. 98, No. 2, pp. 165-177

Singh, K. K., Dong, Y., Belisle, J. T., Harder, J., Arora, V. K. and Laal, S. (2005). Antigens of Mycobacterium tuberculosis recognized by antibodies during incipient, subclinical tuberculosis. *Clin Diagn Lab Immunol*, Vol. 12, No. 2, pp. 354-358

Singh, K. K., Dong, Y., Hinds, L., Keen, M. A., Belisle, J. T., Zolla-Pazner, S., Achkar, J. M., Nadas, A. J., Arora, V. K. and Laal, S. (2003). Combined use of serum and urinary antibody for diagnosis of tuberculosis. *J Infect Dis*, Vol. 188, No. 3, pp. 371-377

Smith, C. V., Huang, C. C., Miczak, A., Russell, D. G., Sacchettini, J. C. and Honer zu Bentrup, K. (2003). Biochemical and structural studies of malate synthase from Mycobacterium tuberculosis. *J Biol Chem*, Vol. 278, No. 3, pp. 1735-1743

Smith, C. V., Sharma, V. and Sacchettini, J. C. (2004). TB drug discovery: addressing issues of persistence and resistance. *Tuberculosis (Edinb)*, Vol. 84, No. 1-2, pp. 45-55

Thadepalli, H., Bach, V. T. and Webb, D. W. (1979). Antimicrobial activity of antituberculosis agents against anaerobic bacteria. *Chest*, Vol. 75, No. 5, pp. 569-570

Traag, B. A., Driks, A., Stragier, P., Bitter, W., Broussard, G., Hatfull, G., Chu, F., Adams, K. N., Ramakrishnan, L. and Losick, R. (2010). Do mycobacteria produce endospores? *Proc Natl Acad Sci U S A*, Vol. 107, No. 2, pp. 878-881

Waksman, S. A. and Schatz, A. (1943). Strain Specificity and Production of Antibiotic Substances. *Proc Natl Acad Sci U S A*, Vol. 29, No. 2, pp. 74-79

Wayne, L. G. (1994). Dormancy of Mycobacterium tuberculosis and latency of disease. *Eur J Clin Microbiol Infect Dis*, Vol. 13, No. 11, pp. 908-914

Wayne, L. G. and Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence. *Infect Immun*, Vol. 64, No. 6, pp. 2062-2069

Young, D., O'Neill, K., Jessell, T. and Wigler, M. (1988). Characterization of the rat mas oncogene and its high-level expression in the hippocampus and cerebral cortex of rat brain. *Proc Natl Acad Sci U S A*, Vol. 85, No. 14, pp. 5339-5342

Zhang, Y. (2005). The magic bullets and tuberculosis drug targets. *Annu Rev Pharmacol Toxicol*, Vol. 45, No. pp. 529-564

13

Lipid Surrounding of Mycobacteria: Lethal and Resuscitating Effects

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1. Introduction

Inside of the host macrophages *Mycobacterium tuberculosis* cells are supposed to face various hostile conditions as the result of immune response: action of reactive oxygen and nitrogen intermediates, hydrolases, increased acidity, and antimicrobial peptides activities [Russell, 2010]. However mycobacterial cells have developed certain mechanisms to resist these defenses. Transcriptome analysis of *M. tuberculosis* showed that even negligible changes of environmental factors cause considerable alterations in global gene expression profile [Boshoff, 2004; Cole, 1998]. It's important that such alterations were observed both in the presence of chemical agents (respiration inhibitors, antituberculosis drugs (ATD), ATP synthesis inhibitors), and during incubation of mycobacteria in modified conditions (medium pH, nature of nutrient, nutrient depletion and starvation, hypoxia, exposure to nitric oxide). As the result, altered properties of the whole cell enable mycobacteria to resist these effects. For instance, increase of the incubation temperature activated synthesis of heat shock proteins which led to higher thermal resistance of the cells; exposure of mycobacteria to the acid environment induces expression of *aprABC* locus responsible for restructuration of lipids of the mycobacterial cell wall and storage lipids that are required for intraphagosome survival [Abramovitch, 2011; Sung N, 2004]. Low concentrations of antibiotics in cultivation medium, that don't affect the cell growth, activate genes responsible for protein pump synthesis, which provides a removal of the antibiotics from the bacterial cell. This is one of the main mechanisms of the ATD resistance. Substitution of the nutrient, e.g. substitution of glycerol to FA (free FA or as part of phospholipids (PL)), activates genes responsible for synthesis of enzymes that switch metabolism to a different pathway of nutrient utilization. In this case mycobacterial cell involves two forms of isocitrate lyase, and utilization of the nutrient in the tricarboxylic acid cycle goes through the glyoxylate shunt [Munoz-Elias, 2005]. Upregulation of the genes encoding isocitrate lyase was shown for *M. tuberculosis* cells cultivated in anaerobic conditions [Lu, 2005], for cells isolated from human lung granulomas [Fenhalls, 2002] and from infected macrophages [Schnappinger, 2003]. All these data prove glyoxylate shunt to be an essential mechanism for survival of mycobacterial cells in phagosomes inside of the host macrophage, where they

use lipids as the main nutrient source. And besides that bacillus has designed the way to use its own lipids to control a state of the immune cell due to release of them into the macrophage internal space followed by exocytosis and transfer to nearby macrophages [Russell, 2009]. The other successful strategy for mycobacteria to survive inside of the host cells is believed to be a transition into nonreplicating dormant state so that they could resuscitate when appropriate conditions appear.

An effect of PL on growth of mycobacteria has been studied for a long time and the data obtained are insufficient and contradictory. On the one hand, phosphatidylcholine (PC) in the form of liposomes was demonstrated to serve as the nutrient source for a pathogenic strain of *M. tuberculosis* H37Rv, but not to effect a growth of nonpathogenic strain H37Ra [Kondo, 1976]. On the other hand, the same research group had found that lysophosphatidylcholine, formed as the result of hydrolysis of PL under action of bacterial phospholipases, suppresses mycobacterial growth [Kondo, 1985]. For *Mycobacterium smegmatis* (rapidly growing nonpathogenic species of the genus *Mycobacterium*, commonly used as a model for *M. tuberculosis*) an influence of PL has been poorly investigated, but it was shown that fatty acids inhibit its growth [Kanetsuna, 1985].

In the present chapter we summarize results obtained by the authors to discuss correlation between bacilli state in vitro (active cell division, inhibition of growth, dormant state, reactivation) and amount of lipid substances (secreted or added externally) in surrounding medium.

2. An effect of lipid substances (PL and FA) on growth of mycobacterial cells in actively replicating state

As it was mentioned above, mycobacterial cells are able to use FA as a nutrient source both in vivo, and in vitro by means of glyoxylate shunt [McKinney, 2000; Munoz-Elias, 2005]. It is well known that mycobacterial cells have lipases of various types [Deb, 2006; Mishra, 2008], as well as phospholipase A2 [Raynaud, 2002; Stonehouse, 2002], which was determined due to fully sequenced *M. tuberculosis* H37Rv genome and due to separation of these enzymes. Being equipped with such enzymes mycobacteria is capable of the hydrolysis of PL with the formation of FA. Therefore primarily we investigated the influence of PL of different classes on the growth rate of pathogenic and nonpathogenic mycobacteria.

2.1 An effect of lipids on growth and susceptibility to ATD of *M. smegmatis* mc^{2}155

At first we used rapidly growing nonpathogenic *M. smegmatis* mc²155 as the model of *M. tuberculosis*. We have cultivated the cells in medium with or without PL (PC, cardiolipin (CL)) to study their susceptibility to two ATD: isoniazide (INH) and rifabutin (RFB). Growth curve measured as optical density (wavelength 600 nm) of the cultivation medium (meat-peptone broth (MPB)) during growth of mycobacteria for 48 hours is represented in fig. 1. When PC and PC/CL (1:4) liposomes (large unilamellar vesicles) applied, a slight stimulation of growth compared with control was observed. Moreover stimulation with pure PC was stronger than with mixture of PC/CL.

Addition of INH (5 μg/ml) inhibit growth of *M. smegmatis* cells at the beginning, which is reflected in longer lag phase but after 30 hours mycobacteria grow almost with the same rate

as without INH. Cultivation of *M. smegmatis* cells with liposomal form of INH is similar at the beginning: there is no growth during first 30 hours and cell division during the later cultivation. However growth rate in the presence of liposomal form of INH was shown to be higher than in the presence of free INH. It's noteworthy that capacity of liposomes to reduce the effect of INH depends on the lipid composition of liposomes and was more pronounced for the mixture of PC/CL (1:4) compared to free PC (fig. 1). Analogous results were obtained for RFB (fig. 1). It's obvious, that RFB 1 μg/ml fully inhibited growth of *M. smegmatis*, and its liposomal form was less effective.

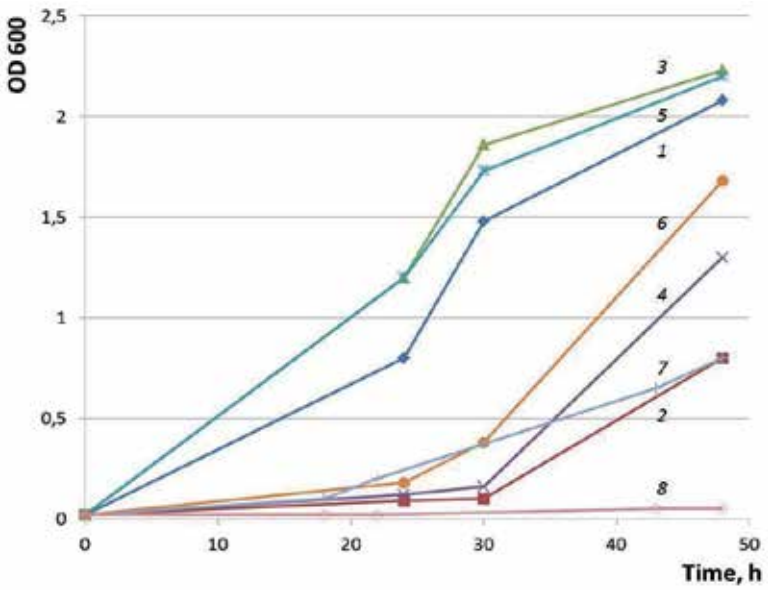

Fig. 1. A growth curve of *M. smegmatis*. Optical density at 600 nm (D600) of cultural medium (meet-peptone broth) with in control (0,99% NaCl) (1) and in the presence of 2) – INH, 5 μg/ml; 3) - PC, 200 μg/ml; 4) - INH, 5 μg/ml + PC, 200 μg/ml; 5) – PC/CL 1:4, 200 μg/ml; 6) – PC/CL 1:4, 200 μg/ml + INH, 5 μg/ml; 7) RFB (1 μg/ml) + PC (200 μg/ml); 8) RFB (1 μg/ml).

Thus, our data indicate that PL in culture medium decrease both ATD (INH and RFB) effect of *M. smegmatis* growth inhibition. INH and RFB have different target in mycobacterial cell: INH inhibit enzymes, responsible for elongation of fatty acid part in mycolic acids [Takayama, 1972], while RFB inhibit DNA-dependent RNA polymerase [Wehrli, 1971]. Our results allow us to conclude that there must be a general mechanism of ATD susceptibility reduction in the presence of PL for mycobacteria. This mechanism has to be realized before ATD reaches their targets.

To determinate the cause of the similar influence of PL on effect of different ATD, we investigated the growth rates of *M. smegmatis* in synthetic laboratory medium Sauton, and in modified analogues, in which glycerol was supplemented on other nutrient sources (acetate or PL), in the presence or absence of one of the involved ATD. We selected such concentrations of substrates that the growth rate of the samples differed slightly in the moment of ATD incorporation (24 h of cultivation). The data is represented in fig. 2 as the

ratio (%) between optical density of *M. smegmatis* in culture medium, containing different nutrient source in the presence of INH or RFB and control, i.e. optical density of culture medium in the absence of ATD.

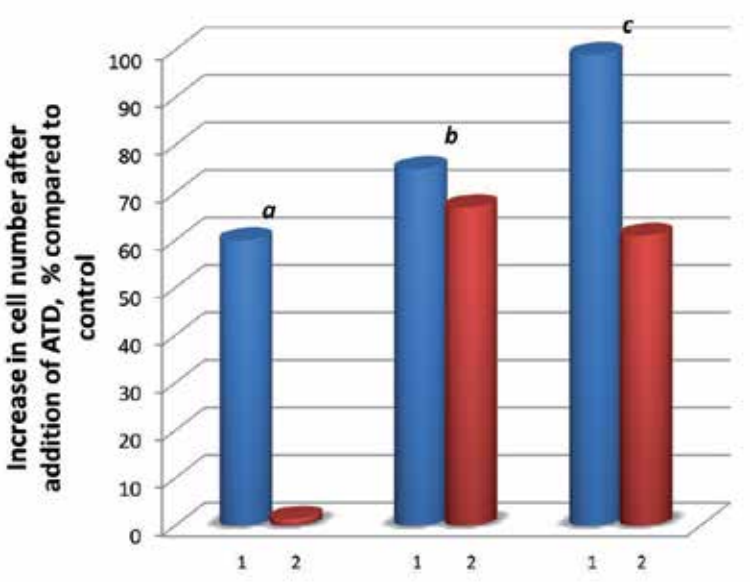

Fig. 2. Alteration of optical density of *M. smegmatis* cultivation medium (% of control numbers) in the presence of ATD. 1. INH (40 μg/ml), 2. RFB (7 μg/ml) with different nutrient source: a) glycerol (0,5 g/l) ; b) acetate (0,7 g/l); c) PC (1 g/l).
(Reproduced from [Shakina Y.V., 2007])

Fig.2 demonstrates, the during the growth in medium, that contains glycerol the main carbon source, *M. smegmatis* cells possess a strong resistance to INH, but are highly susceptible to RFB. In other words, when hydrocarbon substrates are utilized in full Krebs cycle, mycobacteria retain susceptibility to RFB. But when glyoxylate pathway is activated, i.e. during the growth of *M. smegmatis* in medium with acetate or PL as the main carbon source, mycobacteria become fully resistant to INH and susceptibility to RFB drops considerably.

It is possible, that mechanism of phenotypic resistance of *M. smegmatis* to two ATDs, that have different targets, may be attributed to expression of transport proteins, that are capable of binding the ATD that entered into the cell, and transport it through the cell wall out. Morris et al. [Morris, 2005] has shown that polyketide (tetracycline), macrolide (erythromycin) and aminoglycoside (streptomycine) antibiotics induce *whiB7* gene of *M. tuberculosis*. Similar effect was detected when mycobacteria were cultivated with FA [Morris, 2005]. *whiB7* affects *tap* (Rv 1258c), which in turn is responsible for synthesis of protein pumps that provide efflux of antibiotics out of cell.

2.2 An effect of lipids on growth and viability of *Mycobacterium tuberculosis* H37Rv

Then we have studied a growth and viability of pathogenic *Mycobacterium tuberculosis* H37Rv *in vitro* during cultivation in liquid Dubo's medium when PL and products of their hydrolysis (lyso-components) in various concentrations were added (table 1). PL were incorporated in the cultivation medium in the form of large unilamellar vesicles. The cells were grown in atomized system BACTEC, in which the growth is detected due to the alterations in oxygen uptake level by means of fluorescent indicator, that is quenched by high oxygen concentrations (fig. 3).

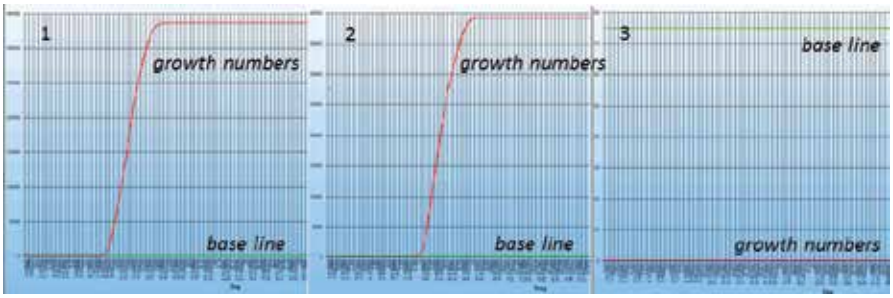

Fig. 3. Growth curves of *M. tuberculosis* H37Rv in the presence of PL liposomes obtained by means of BACTEC. 1-H37Rv; 2-H37Rv+CL (50 μg/ml); 3-H37Rv+CL (750 μg/ml). Reproduced from [Andreevskaya, 2010]

Viability of the cells was observed using colony counting on solid medium (Dubo's agar) after isolation of the passage that was exposed to the lipids in the liquid medium (fig. 4). It was found that addition of negatively-charged CL into cultivation medium had an effect on the growth rate and viability of *M. tuberculosis* H37Rv, while electro-neutral PC wasn't active. As it follows from the data shown in table 1 the effect of CL was dose-dependent: low concentration (50 μg/ml) caused the 1-2 day delay in growth of mycobacterial cells, and 250-330 μg/ml concentration fully inhibited growth. Applying Murohashi staining method we demonstrated that such concentration of CL cause lysis of *M. tuberculosis* H37Rv cells (fig. 5) [Sorokoumova, 2009].

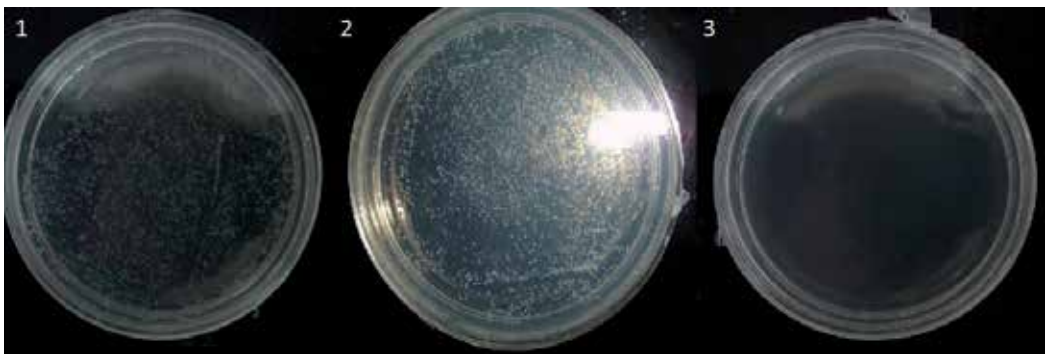

Fig. 4. A photography of macrocolonies of *M. tuberculosis* H37Rv on Dubo's agar by the 18th day of cultivation. 1-H37Rv; 2-H37Rv+CL (50 μg/ml); 3-H37Rv+CL (750 μg/ml). Reproduced from [Sorokoumova, 2009]

#	Lipid	Concentration of the lipid added		The start of growth, days	A presence of growth (+) An absence of growth (-)
		μg/ml	μM		
1	Control	-	-	7-8	+
2	CL	50	35	8-9	+
		125	90	8-9	+
		250		16-18	+ (considerably lower than control)
		300 – 700	200 and more	-	-
3	LisoCL		35	7-8	+
			200	7-8	+
4.	Bisliso-CL		35	7-8	+
			200	7-8	+
5	Linoleic acid		10	7-8	+
			90	7-8	+
			200	14-15	+
			400	20	+ (slight growth)
			600	-	-
2	Phosphatidic acid		50	8-9	+
			335	-	-
			502	-	-
3.	Phosphatidylglycerol		50	8-9	+
			335	-	-
			502	-	-
4	Lisophosphatidyl-glycerol		50	6-7	+
			335	2-7	+
			502-600	2-7	+
5.	Lisophosphatidyl-glycerol + linoleic acid		50	5-7	+
			335	1-4, 8-9	+
			502	1-4	-
			670	-	-
6	PC	500		-	+
7	LisoPC		90	7-8	+
			900	16	+
			9000	16	+
8	Linoleic acid and lisoPC (1:1, mole),		90	7-8	+
			900		

Table 1. An effect of different concentrations of PL and the products of their hydrolysis on the start and intensity of *M. tuberculosis* H37Rv growth in Dubo's medium registered with BACTEC MGIT960.

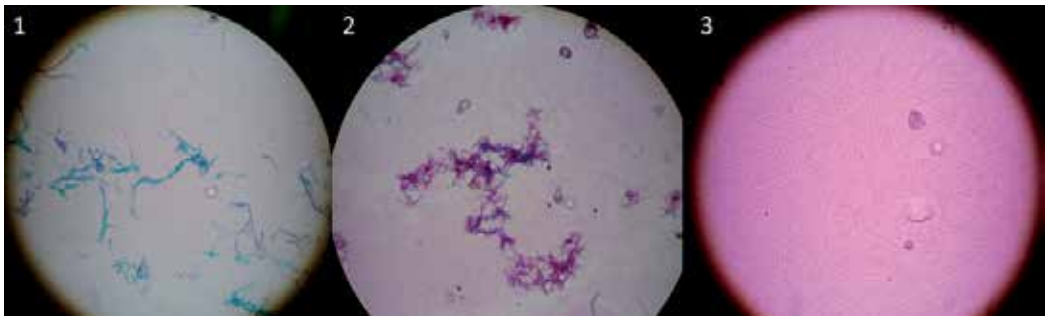

Fig. 5. Murohashi staining of *M. tuberculosis* cells (14th day of growth). 1-H37Rv; 2-H37Rv+CL (50 μg/ml); 3-H37Rv+CL (750 μg/ml). Reproduced from [Mikulovish, 2010]

These data correlate well with the results of mycobacterial DNA detection by real time PCR, according to which appearance CL in culture medium didn't lead to increase of DNA number [Sorokoumova, 2009] (fig. 6).

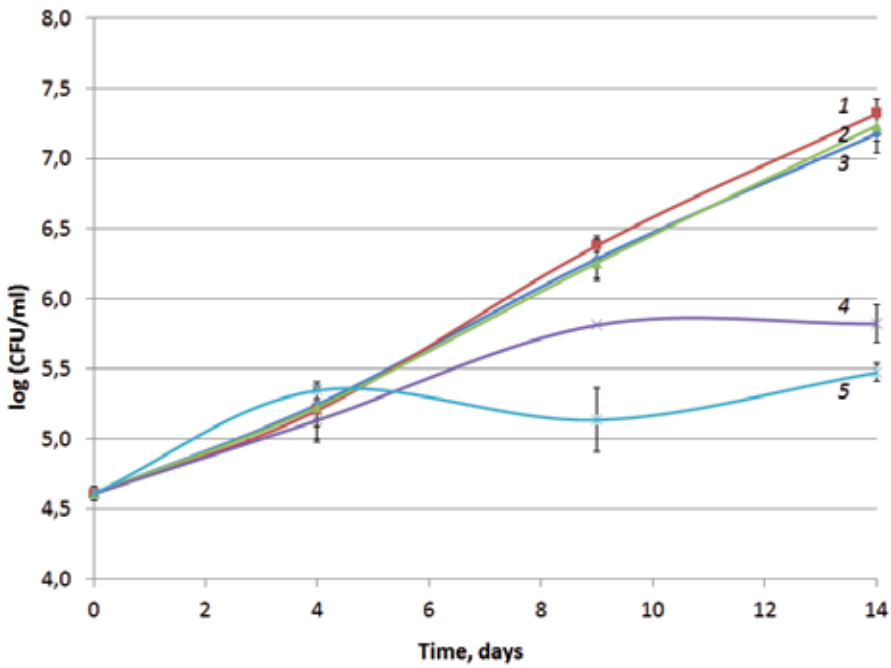

Fig. 6. Growth curves of *M. tuberculosis* H37Rv in Dubo's medium in the presence of CL liposomes. Growth was detected with real time PCR. 1-H37Rv; 2-H37Rv+CL (50 μg/ml); 3-H37Rv+CL (250 μg/ml) ; 4-H37Rv+CL (500 μg/ml); 5-H37Rv+CL (750 μg/ml). Reproduced from [Sorokoumova G.M., 2009]

CL is an instable compound that transforms in several substances in water phase. Both in Dubo's medium, containing high concentration of iron ions, and in Tris-buffer, which doesn't have an iron, the products of hydrolysis were mainly formed: lyso-, bislysocomponents and FA (fig. 7). Along with this components such products of

destruction as phosphatidic acid, phosphatidylglycerol, as well as the products of their hydrolysis (lysophosphatidic acid and lysophosphatidylglycerol appear in cultivation medium. Mentioned substances were isolated by preparative TLC from water phase, in which CL was cultivated without *M. tuberculosis* at 37^0 C for 4 or 6 days, and were identified by MS [Smirnova, 2011]. Summarized results of their effect on *M. tuberculosis* growth is represented in table 1.

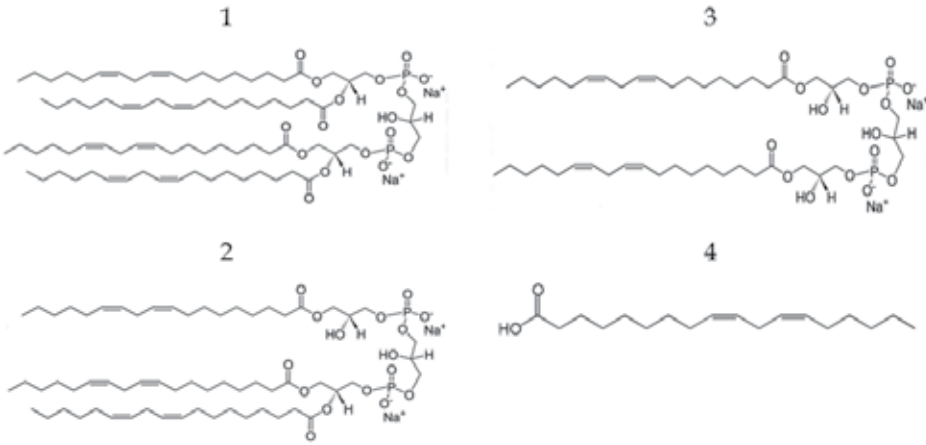

Fig. 7. Structural formulas of 1 – cardiolipin, 2 – lysocardiolipin, 3 – bislysocardiolipin, 4 – linoleic acid.

It's interesting, that lysophosphatidylglycerol, the product of phosphatidylglycerol hydrolysis, doesn't have any inhibitory activity in involved concentration interval, as well as a mixture of lysophosphatidylglycerol with linoleic acid with concentration lower than 335 μM, which is inhibitory for phosphatidylglycerol. Thus, conducted experiments have shown that only negatively-charged PL (CL, phosphatidic acid, phosphatidylglycerol), but not their lysocomponents, that have negative charge as well, are able to cause the delay in growth and suppress viability of the mycobacterial cells almost in the same concentration range (250 - 335 μM). Based on the literature one may speculate the mechanisms of negatively-charged PL activity, that inhibit the bacterial cell growth due to:

- their interaction with the transcription factors [Carman, 2007];
- an influence of CL on binding of DNA-binding A-protein, regulating interaction of ATP with mycobacterial DNA; this interaction initiate the replication of bacterial DNA [Yamamoto, 2002];
- an inhibitory effect of CL on activity of topoisomerase during binding with it in complex, which prevents cell division [Mizushima, 1992].

To conclude, the same PL seems to have various effects on the growth of different species mycobacteria. Perhaps, it may be explained by a different destiny of PL once they were introduced into 'mycobacteria space': weather they serve only as FA source, or may play a role in metabolism of microorganism by themselves. Therefore we further investigated the alterations in lipid surrounding of mycobacteria in different states: in actively replicating and nonculturable.

3. Role of lipid components in formation and reactivation of nonculturable mycobacterial cells

3.1 Mycobacterial lipids released into extracellular space during formation of dormant cells

In vitro model of *Mycobacterium smegmatis* dormant state obtained due to absence of potassium ions in growth medium, created by Kaprelyants, A.S. with colleagues., let us to show that during formation of nonculturable ATD-resistant cells they lose up to 90% of their mass, which reflects in relatively balanced decrease in both lipid and protein amounts [Nazarova, 2011] (fig. 8). Nevertheless at the end of this process nonculturable cells have larger percentage of proteins rather than lipids. Qualitative lipid composition of the cells doesn't noticeably change during transition into nonculturable state with the exception of the drop of the trehalose monomycolate level. Trehalose monomycolate is a precursor of the cord-factor trehalose dimycolate, which is inherent for mycobacterial cells in all of the states.

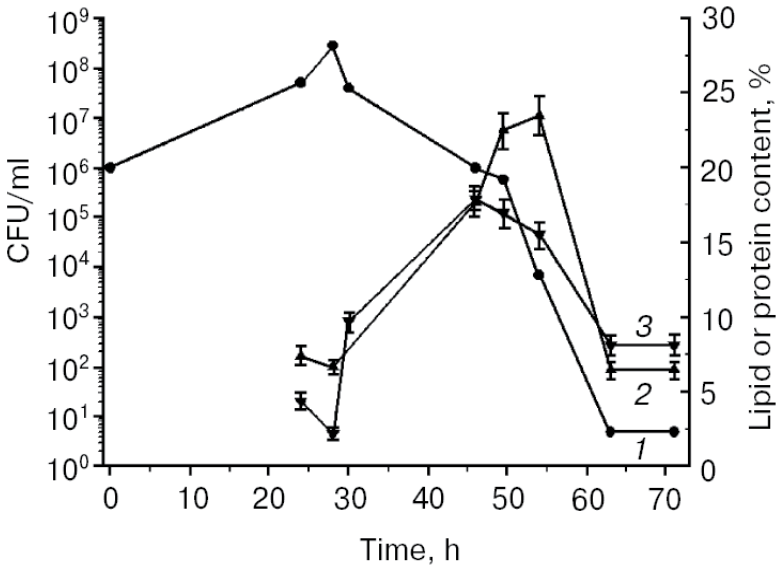

Fig. 8. *Mycobacterium smegmatis* growth in mH-deB medium (1), lipid (2), and protein (3) content in cells during transition into nonculturable state depending on time based on dry biomass. Reproduced from [Nazarova, 2011].

Cell mass loss during transition into dormant state certainly might lead to appearance of detached cell lipids in surrounding medium. To detect such compounds in growth medium, we conducted a chloroform extraction of culture liquid, separated from the cells through the 200 nm filter and lyophilized. Nonculturable forms of *M. smegmatis* were obtained as the result of cultivation in mH-deB medium. We used the cells, grown in nutrient and potassium ion rich Sauton's medium, for control. *M. smegmatis* cells upon metabolically active growth as well as upon formation of nonculturable cells release into culture liquid a considerable range of neutral lipids: FA, mycolic acids (MA), diacylglycerols (DAG), monoacylglycerols (MAG) (fig. 9). It is noteworthy that mycobacteria in none of the states don't release triacylglycerols (TAG), intracellular energy storage component. Qualitative

lipid composition of culture liquids both in the process of active growth and during transition into nonculturable state doesn't change. However only during development of nonculturable cells level of released fatty acids enlarges twice (from 33 μM to 59 μM). These data were obtained after isolation of free FA with preparative TLC and quantification of their amount with titration.

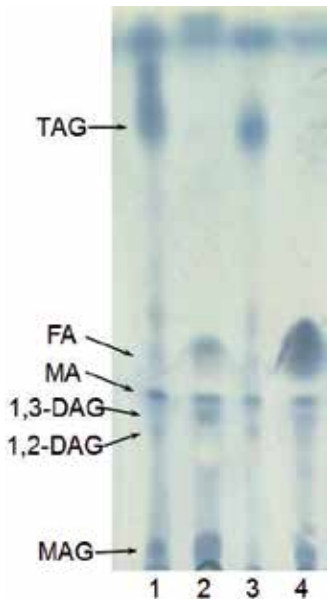

Fig. 9. TLC of lipids in system heptane–diethyl ether–acetic acid (55 : 45 : 1 v/v). Lipid extract of *M. smegmatis* cells cultivated for 24 h in Sauton's medium 1); 2) lipid extract of culture liquid of *M. smegmatis* cells grown for 24 h in Sauton's medium; 3) lipid extract of *M. smegmatis* cells cultivated for 24 h in mH-deB medium; 4) lipid extract of culture liquid after cultivation of *M. smegmatis* cells for 24 h in mH-deB medium. Development by phosphomolybdic acid solution with following plate heating at 100°C. Represented from [Nazarova, 2011].

Analysis of *M. smegmatis* culture liquid free-FA content by GLC-MS showed that oleic acid was a prevalent component. NMR-spectra of FA isolated from a culture liquid and of the oleic acid standard appeared to be identical.

To prove a biogenic nature if the found in culture medium FA we incorporated a radioactive carbon isotope in lipids of mycobacteria as the result of [^{14}C]aspartic acid metabolism. After addition of tagged compound to the *M. smegmatis* cells grown in mH-deB for 46.5 h, a part of the sample was taken for control and the rest was left to cultivate for other day at 37°C. Gained lipid extracts of culture medium were divided on separate substance with reverse-phase and thin-layer chromatography. Measurement of radioactivity of different zones of TLC-plate demonstrated that in zone, corresponding to oleic acid there is an accumulation of radioactivity (12 490 cpm) as compared to control sample (356 cpm). Incorporation of [^{14}C]aspartic acid followed by accumulation of radioactivity in extracellular FA confirms that at least partially FA detected in growth medium are actually secreted.

Level of free FA in growth medium in active state decreases (from 29 μM to 5 μM), which indicates an involvement of FA in metabolism and biosynthesis of required lipids for metabolically active mycobacterial cells.

We consider complex of potassium absence and accumulation of FA in growth medium as a cause of transition of mycobacteria into dormant state in the present model. Bovine serum albumin (BSA) is conventionally required for binding of FA excess in culture liquid in laboratory experiments. When applying BSA A1470 Sigma Cohn Analog™, mentioned FA accumulation and formation of nonculturable cells occur, whereas in case when applying Fatty Acid Free-BSA MP Biomedicals, LLC, secreted FA are entirely bound and dormant state is not achieved.

To determine the amount of FA, that were introduced initially into growth medium, we have measured the level of free FA, present in the used for experiments Tween-80, and content of the FA bound with BSA.

The mixture of low concentration Tween 80 and water was titrated with 0,014M NaOH using 0.01% thymol blue solution as an indicator. This method let us to estimate the quantity only of free FA, not exposing an ester group for hydrolysis. FA were shown to be brought into growth medium together with Tween 80 in amount of 15% of detergent mass (table 2).

We applied a technique, based on combination of methods described by Chen, R.F., and Dole, V.P., for determination of quantity of FA bound with BSA [Chen, 1967; Dole, 1956]. Briefly: 25 ml of extraction mixture (isopropyl alcohol : heptane : 1N H_2SO_4 (40:10:1)) were added to 5 ml of water solution of BSA (0,1 g/ml), shaken, let to stand for 30 minutes. Then 10 ml of heptane and 15 ml of 0,1N H_2SO_4 were added to the mixture and shaken again. The upper phase is transferred to another flask, that contains 25ml of 0,01N H_2SO_4, shaken again. The upper heptane fraction is separated and titrated as described above. It was shown that BSA A1470 Sigma Cohn Analog™ possesses only 15%-binding capability, while Fatty Acid Free-BSA MP Biomedicals, LLC - 87,5% (of the maximum possible) (table 2).

Thus, addition of BSA (Cohn An.), possessing only 15 % (moles) binding capability of the maximum possible, result in the formation of nonculturable cells. Remaining 15%is necessary for the binding of 57,3 μM of FA, incorporated together with Tween 80. Therefore we may conclude that 33-59 μM of free FA found in the medium can't be bound with BSA, and as the result have to be accumulated in culture liquid, which may lead to the formation of nonculturable cells in response to stressful conditions. As it was mentioned earlier, these FA have biogenic nature, and might be the product of Tween 80 hydrolysis.

In the case of more pure BSA (MP Biomedicals, LLC) it is obvious that despite the presence of free FA in Tween 80 and negligible traces in BSA, it is still capable of binding up to 205,2 μM of FA (i.e. 68,4% of maximum possible 300 μM). Therefore FA, that might be secreted during the lifetime of mycobacterial cells are removed from them with BSA, which enables more successful adaptation to stressful conditions, and dormant cells do not form.

Thus in conducted experiments we demonstrated secretion of lipid substances including FA into surrounding medium during growth upon stressful conditions which results in transition of bacilli into dormant state. Moreover extracellular FA are accumulated in this process.

Component of the medium		Concentration in culture liquid, μM
Tween 80		382
Admixed FA in Tween 80		57.3
Percentage of admixed FA in Tween 80		15% (57.3 · 100%/382)
Maximum binding capacity of BSA at pH 6,6-7 *		300
BSA (Cohn An.)		75
FA bound with BSA (Cohn An.)		255
Binding capacity of BSA (Cohn An.)		15% ((300-255) · 100%/300)
BSA (MP)		75
FA bound with BSA (MP)		37.5
Binding capacity of BSA (MP)		87.5% ((300-37.5) · 100%/300)
Free FA in growth medium	24 h.	33.2
	48 h.	41.4
	72 h.	59

* Maximum binding capability of BSA (FA mole / BSA mole) was shown to be ~ 4 [Spector, 1969].

Table 2. Amounts of FA introduced into growth medium along with Tween 80 and BSA with consideration of experimentally used concentrations. Amounts of free FA detected in growth medium.

It is well known that *M. smegmatis* and *M. tuberculosis* dormant cells can be reactivated with resuscitation promoting factor (Rpf) [Shleeva, 2004; Shleeva, 2011]. An influence of FA on resuscitation appears to be interesting in the context of the shown data on their role in the process of adaptation to stressful conditions.

3.2 An effect of lipid substances on reactivation of mycobacterial dormant cells

M. smegmatis dormant nonculturable cells have been studied to be reactivated by their incorporation into fresh medium containing potassium ions and various lipid substances (triacylglycerols, PL, FA) [Nazarova, 2011]. Oleic acid is observed to have the strongest resuscitation effect, and its active concentrations are 0.18-10.6 μM. Such low concentration range sufficient for reactivation of dormant cells indicates a signal function of this FA in resuscitation.

The capability of phospholipids to stimulate growth of M. tuberculosis cells in the late stationary phase was known previously [Zhang, 2001]. Since FA are components of PL, we supposed that both phospholipids and FA may be able to stimulate reactivation of dormant cells. To study the role of the lipid compounds in resuscitation process, nonculturable cells were transferred into fresh medium containing potassium ions and a certain amount of lipid component, which effect on cell reactivation was estimated by the method of final dilutions.

As it was found different types of lipids are objectively capable to "trigger" the process of resuscitation of nonculturable cells. Addition of oleic acid compared to different length acids, higher alcohols, and TAG in concentration of 1 μg/ml to 10^6 nonculturable cells was the most efficient for reactivation (Fig. 10). The phospholipid and oleic acid effects on

resuscitation were concentration dependent (Fig. 11). It is important that the oleic acid concentrations (0.05-3 μg/ml) were in significantly lower range compared to phospholipids (50-250 μg/ml) (Fig. 11). Such low concentration excludes the use of free FA in this process as a nutrient source, and that they are the substances responsible for reactivation, while phospholipids serve as their sources (due to activity of bacterial phospholipases). FA are known to function as signal substances in a number of important processes in bacterial cells. Thus, arachidonic acid serves as chemoattractant for *Dictyostelium discoideum* [Schaloske, 2007]. Another example of FA signal function is their secretion into extracellular space by the *Xanthomonas campestris*, plant pathogen, and their following absorption resulting in increased expression of genes associated with cell pathogenicity [Barber, 1997]. So, we have revealed a new, not previously described signal function of oleic acid as an agent stimulating reactivation of nonculturable mycobacterial forms.

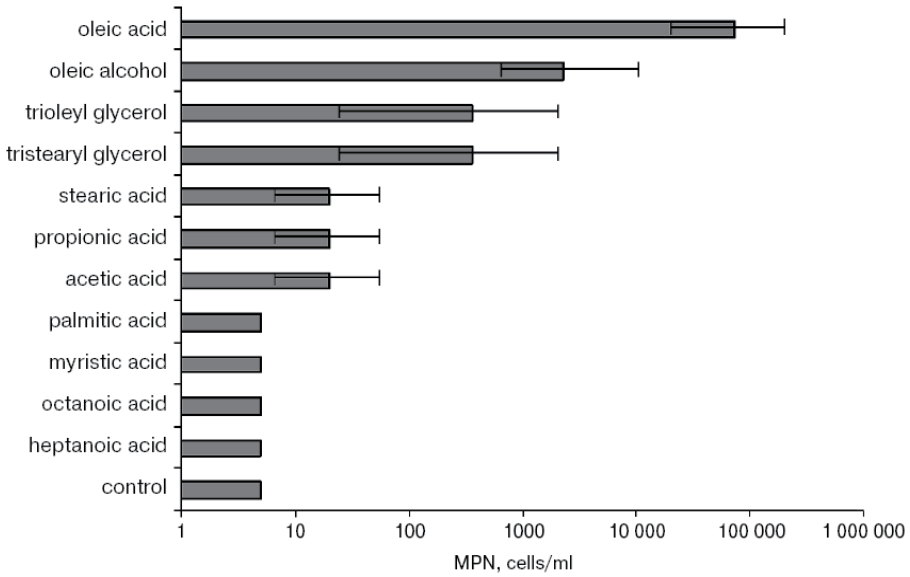

Fig. 10. Effect of FA and substances of lipid nature (in concentration of 1 μg/ml) on resuscitation of *M. smegmatis* nonculturable cells. Represented from [Nazarova, 2011].

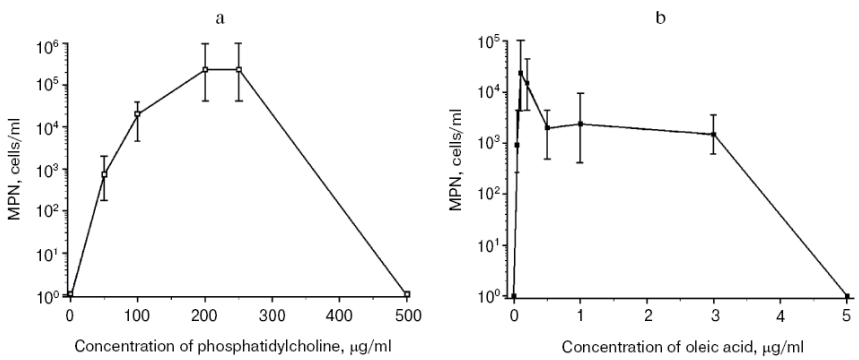

Fig. 11. Effect of phosphatidylcholine (a) and oleic acid (b) in different concentrations on resuscitation of *M. smegmatis* nonculturable cells. Represented from [Nazarova, E.V., 2011].

All of the presented results support an idea that amount of FA in surrounding medium play a determinative role in formation and reactivation of *M. smegmatis* nonculturable cells (fig. 12). Thus 0.18-10.6 μM of oleic acid possess a resuscitation effect. A little larger number of FA (33-59 μM) accumulates in culture liquid during transition into nonculturable state. And finally, as it was shown previously, oleic acid in amount of 92 μM inhibits growth of mycobacterial cells.

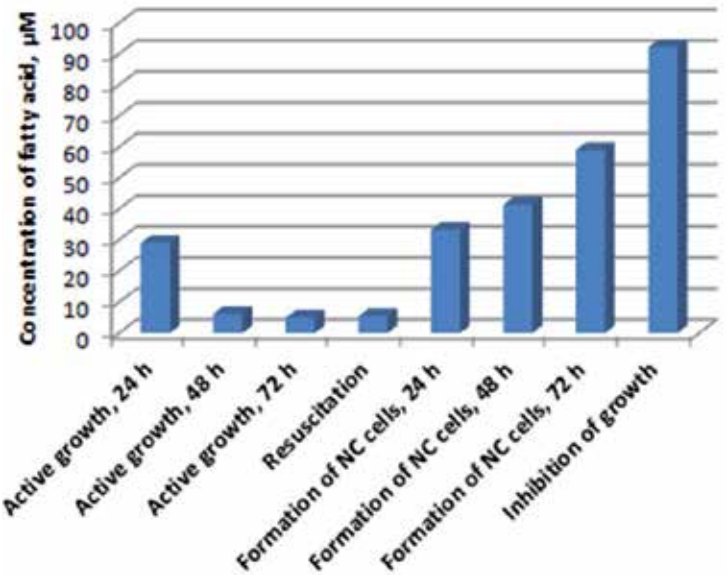

Fig. 12. Correlation between a state of *M. smegmatis* and a quantity of extracellular FA.

4. Conclusion

Pathogen *M. tuberculosis* by itself as well as particular bacterial lipids are reported to induce loading of macrophages with lipid bodies resulting in formation of foamy macrophages [Russell, 2009]. So it seems that mycobacterial lipids passed by macrophages to each other cause such differentiation of immune cells influencing on total tissue remodelling of the infection site. Inside of such altered foamy macrophages mycobacteria have an access and actually use host lipids from the lipid drops, which leads to their transition into a state similar to the nonreplicating state [Peyron, 2008]. Cultivation of mycobacteria in medium enriched with lipid substances showed that bacilli do form cells with changed morphology and resistant to anti-tuberculosis drugs in such conditions [Nazarova, 2010].

When as a consequence of total 'lipidation' of granuloma macrophages mycobacteria find themselves in the excess of surrounding lipids, this excess induces a dormant state in pathogens. If immune system of the host is active enough, granuloma either undergoes resolution, or remains balanced. But in case of active disease development caseum is accumulated in the centre of some granulomas; this leads to necrosis and collapse of granuloma, and to release of virulent bacilli into airways [Kaplan, 2003]. Since during this release mycobacteria appear to be finally not in stressful conditions and not surrounded or surrounded with a little amount of lipid substances, they might reactivate, as it can be

supported by our results where only 0.18-10.6 μM of oleic acid is required for resuscitation of dormant bacilli.

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6. References

Abramovitch, R.B., Rohde K.H., Hsu, F.F., & Russell, D.G. (2011). aprABC: a *Mycobacterium tuberculosis* complex-specific locus that modulate pH-driven adaptation to the macrophage phagosome. *Mol.Microbiol.,* Vol. 80, No. 3, (May 2011), pp. 678-694

Andreevskaya, S.N., Smirnova, T.G., Zhogina, Yu.A., Smirnova, D.I., Mikulovich, J.L, Kalashnikova, T.Yu., Sorokoumova, G.M., Selishcheva, A.A., Chernousova, L.N., & Shvets, V.I. (2010). Effect of exogenous cardiolipin on the growth and viability of *Mycobacterium tuberculosis* H37Rv in vitro. *Dokl. Biol. Sci*., Vol. 434, No. 5, (October 2010), pp. 371-374

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson,T.J.G., Slater, H., Dow, J.M., Williams, P., & Daniels, M.J. (1997). A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol*., Vol. 24, No. 3, (May 1997), pp. 555–566

Boshoff, H.I., Myers, T.G., Copp, B.R., McNeil, M.R., Wilson, M.A., & Barry III, C.E. (2004). The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action. *J. Biol. Chem.,* Vol. 279, No. 38, (September 2004), pp. 40174-40184

Carman, G.M., & Henry, S.A. (2007). Phosphatidic acid plays a central role in the transcriptional adaptational regulation of glycerophospholipid synthesis in *Sacchatomyces cerevisiae*. *J. Biol. Chem.,* Vol. 282, No. 52, (November 2007), pp. 37293-37297

Chen, R.F. (1967). Removal of Fatty Acids from Serum Albumin by Charcoal Treatment. *The Journal of Biological Chemistry*, Vol. 242, No. 2, (January 1967), pp. 173-181

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, Vol. 393, No. 6685, (June 1998), pp.537-544

Deb, C., Daniel, J., Sirakova, T.D., Abomoelak, B., Dubey, V.S., & Kolattukudy, P.E. (2006). A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. *J. Biol. Chem.*, Vol. 281, No. 7, (February 2006), pp. 3866-3075

Dole, V.P. (1956). A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest*., Vol. 35, No. 2, (February 1956), pp. 150-154

Fenhalls, G., Stevens, L., Moses, L., Bezuidenhout, J., Betts, J.C., Helden Pv.P., Lukey, P.T., & Duncan, K. (2002). In situ detection of *Mycobacterium tuberculosis* transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. *Infect Immun*., Vol. 70, No. 11, (November 2002), pp.6330-6338

Kanetsuna, F. (1985). Bactericidal effect of fatty acids on mycobacteria, with particular reference to the suggested mechanism of intracellular killing. *Microbiol Immunol*., Vol. 29, No. 2, pp.127-141

Kaplan, G., Steyn, L.M., Bekker, L.G., Post, F.A., & Wainwright, H.C. (2003). *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect. Immun*., Vol. 71, No. 12, (December 2003), pp. 7099–7108

Kondo, E., & Kanai, K. (1976). An attempt to cultivate mycobacteria in simple synthetic liquid medium containing lecithin-cholesterol liposomes. *Jpn. J. Med. Sci. Biol*, Vol. 29, No. 3, (June 1976), pp.109-121

Kondo, E., & Kanai, K. (1985). Mechanism of bactericidal activity of lysolecithin and its biological implication. *Jpn. J. Med. Sci. Biol*, Vol. 38, No. 4, (August 1985), pp.181-194

Lu, Y., Wu, Y.R., & Han, B. (2005). Anaerobic induction of isocitrate lyase and malate synthase in submerged rice seedlings indicates the important metabolic role of the glyoxylate cycle. *Acta Biochim. Biophys. Sin. (Shanghai)*, Vol. 37, No. 6, (June 2005), pp.406-414

McKinney, J.D., Honer zu Bentrup, K., Munoz-Elias, E.J., Miczak, A., Chen, B., Chan, W.T., Swenson, D., Sacchettini, J.C., Jacobs, Jr. W.R., & Russell, D.G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, Vol. 406, No. 6797, (August 2000), pp. 735–738

Mikulovich, J.L., Andreevskaya, S.N., Smirnova, T.G., Sorokoumova, G.M., Chernousova, L.N., Selishcheva, A.A., & Shvets, V.I. (2010). The influence of cardiolipin liposomes on growth and survival of *Mycobacterium tuberculosis* H37Rv in vitro, *Proceedings of The Moscow International Scientific and Practical Conference "Biotechnology: Ecology of Big Cities"*, Moscow, Russia, March 15-17, 2010, pp. 453-454

Mishra, K.C., de Chastellier, C., Narayana, Y., Bifani, P., Brown, A.K., Besra, G.S., Katoch, V.M., Joshi, B., Balji, K.N., & Kremer, L. (2008). Functional role of the PE domain immunogenicity of the *Mycobacterium tuberculosis* triacylglycerol hygrolase LipY. *Infect Immun*, Vol. 76, No. 1, (January 2008), pp. 127-140

Mizushima, T., Natori, S., & Sekimizu, K. (1992). Inhibition of *Escherichia coli* DNA topoisomerase I activity by phospholipids. *Biochem. J.,* Vol. 285, Part. 2, (July 1992), pp. 503-506

Morris, R. P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., Ehrt, S., Liu, Y., Heifets, L., Pieters, J., Schoolnik, G., & Thompson, C.J. (2005). Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.*, Vol. 102, No. 34, (August 2005), pp. 12200-12205

Munoz-Elias, E.J., & McKinney, J.D. (2005). *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nature Med.*, Vol. 11, No. 6, (June 2005), pp. 638-644

Nazarova, E.V., Zhogina, Y.A., Morozova, N.S., Shleeva, M.O., Sorokoumova, G.M., Selischeva, A.A., Kaprelyants, A.S., & Shvets, V.I. (2010) The Influence of carbon source on antituberculous drug resistance of mycobacteria, *Proceedings of The Moscow International Scientific and Practical Conference "Biotechnology: Ecology of Big Cities"*, Moscow Russia, March 15-17, 2010, pp. 454-455

Nazarova, E.V., Shleeva, M.O., Morozova, N.S., Kudykina, Yu.K., Vostroknutova, G.N., Ruzhitsky, A.O., Selishcheva, A.A., Sorokoumova, G.M., Shvets, V.I., & Kaprelyants A.S. (2011). Role of lipid components in formation and reactivation of *Mycobacterium smegmatis* "nonculturable" cells. *Biochemistry (Moscow)*, Vol. 76, No. 6, (June 2011), pp. 636-644

Peyron, P., Vaubourgeix, J., Poquet, Y., Levillain, F., Botanch, C., Bardou, F., Daffé, M., Emile, J.F., Marchou, B., Cardona, P.J., de Chastellier, C., & Altare, F. (2008). Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. *PLoS Pathog.*, Vol. 4, No. 11, (November 2008), pp. e1000204

Raynaud, C., Guilhot, C., Rauzier, J., Bordat, Y., Pelicic, V., Manganelli, R., Smith, I., Gicquel, B., & Jackson M. (2002). Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*. *Mol. Microbiol.*, Vol. 45, No. 1, (July 2002), pp. 203-217

Russell, D.G., Cardona, P.-J., Kim, M.-J., Allain, S., & Altare F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunology*, Vol. 10, No. 9, (September 2009), pp. 943 - 948

Russell, D.G., VanderVen, B.C., Lee, W., Abramovitch, R.B., Kim, M.J., Homolka, S., Niemann, S., & Rohde, K.H. (2010). *Mycobacterium tuberculosis* wears what it eats. *Cell Host Microbe*, Vol. 8, No. 1, (July 2010), pp. 68-76

Schaloske, R.H., Blaesius, D., Schlatterer, C., & Lusche, D.F. (2007). Arachidonic acid is a chemoattractant for *Dictyostelium discoideum* cells. *J. Biosci.*, Vol. 32, No. 7, (December 2007), pp. 1281–1289

Schnappinger, D., Ehrt, S., Voskuil, M.I., Liu, Y., Mangan, J.A., Monahan, I.M., Dolganov, G., Efron, B., Butcher, P.D., Nathan, C., & Schoolnik, G.K. (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J. Exp. Med.*, Vol. 198, No. 5, (September 2003), pp. 693-704

Shakina, Y.V., Troshkina, O.A., Petrova, E.E., Salina, E.G., Sorokoumova, G.M., Shvets, V.I., Kaprelyants, A.S., & Selishcheva, A.A. (2007). Susceptibility of *Mycobacterium smegmatis*, grown on the media, contained various carbon substrates, to antituberculosis drugs. *Zh. Mikrobiol. (Moscow)*, No. 6, pp. 7-11

Shleeva, M., Mukamolova, G.V., Young, M., Williams, H.D., & Kaprelyants, A.S. (2004). Formation of 'non-culturable' cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology*, Vol. 150, Pt. 6, (June 2004), pp. 1687–1697

Shleeva, M.O., Kudykina, Y.K., Vostroknutova, G.N., Suzina, N.E., Mulyukin, A.L., & Kaprelyants, A.S. (2011). Dormant ovoid cells of *Mycobacterium tuberculosis* are formed in response to gradual external acidification. *Tuberculosis (Edinb)*, Vol. 91, No. 2, (March 2011), pp. 146-154

Smirnova, T.G., Mikulovich, J.L., Andreevskaya, S.N., Sorokoumova, G.M., Chernousova, L.N., Selishcheva, A.A., & Shvets V.I. (2011). Cardiolipin lysoderivatives suppress

the survival both susceptible and resistant strains of *Mycobacterium tuberculosis*. *Russian Journal of Biopharmaceuticals*, Vol. 3, No. 2, pp. 19-27

Sorokoumova, G.M., Andreevskaya, S.N., Smirnova, T.G., Petrova, E.E., Zhogina, Yu.A., Kalashnikova, T.Yu., Chernousova, L.N., Selishcheva, A.A., & Shvets V.I. (2009). Effects of liposomes of different lipid composition on in vitro growth of *Mycobacterium tuberculosis* H37Rv. *Bull Exp Biol Med*, Vol. 148, No. 5, (November 2009), pp. 550-552

Spector, A.A., John, K., & Fletcher, J.E. (1969). Binding of long-chain fatty acids to bovine serum albumin. *Journal of Lipid Research*, Vol. 10, No. 1, (January 1969), pp. 56-67

Stonehouse, M.J., Cota-Gomez, A., Parker, S.K., Martin, W.E., Hankin, J.A., Murphy, R.C., Chen, W., Lim, K.B., Hackett, M., Vasil, A.I., & Vasil, M.L. (2002). A novel class of microbial phosphocholine -specific phospholipases C. *Mol Microbiol*, Vol. 46, No. 3, (November 2002), pp. 661-676

Sung, N., Takayama, K., & Collins, M.T. (2004). Possible association of GroES and antigen 85 proteins with heat resistance of *Mycobacterium paratuberculosis*. *Appl. Environmental Microbiol*, Vol. 70, No. 3, (March 2004), pp. 1688-1697

Takayama, K., Wang, L., & David, H.L. (1972). Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. *Antimicrob Agent Chemother*, Vol. 2, No. 1, (July 1972), pp. 29-35

Wehrli, W., & Staehelin, M. (1971). Actions of the rifamycins. *Bacteriological Reviews*, Vol. 35, No. 3, (September 1971), pp. 290-309

Yamamoto, K., Muniruzzaman, S., Rajagopalan, M., & Madiraju, M.V. (2002). Modulation of *Mycobacterium tuberculosis* DnaA protein-adenine-nucleotide interactions by acidic phospholipids. *Biochem J.*, Vol. 363, Pt. 2, (April 2002), pp.305-311

Zhang, Y., Yang, Y., Woods, A., Cotter, R.J., & Sun, Z. (2001). Resuscitation of dormant *Mycobacterium tuberculosis* by phospholipids or specific peptides. *Biochem. Biophys. Res. Commun*., Vol. 284, No. 2, (June 2001), 542–547

14

Heat Shock Proteins in *Mycobacterium tuberculosis*: Involvement in Survival and Virulence of the Pathogen

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1. Introduction

Tuberculosis (TB) is an infectious disease of global concern. Worldwide TB kills two million people each year. About 90% of those infected with *Mycobacterium tuberculosis* have asymptomatic, latent TB infection (sometimes called LTBI) (Smith, 2003; Wayne & Sohaskey, 2001). Years after initial infection, the bacilli may resume growth, the outcome of which is active TB. If left untreated, the death rate for active TB cases is more than 50%. Approximately 95% of new cases and 98% of deaths occur in developing nations, where human immunodeficiency virus (HIV) infections are common, this is generally because of the unavailability of proper treatment. The causative agent, *M. tuberculosis* has a cell wall which has a very low permeability for most antibiotics and chemotherapeutic agents. Another critical problem is the development of multi-drug resistant TB (MDR-TB) or extremely drug resistant TB (XDR-TB) (Chiang et al., 2010; Eismont, 2009; WHO report, 2010). Every year in the world, around 440,000 new MDR-tuberculosis cases are found due to bacilli that are resistant to the two main antitubercular drugs, isoniazid and rifampicin. The XDR-TB is a recently developed form. The mortality rate in the case of XDR-TB can go from 50 to 100%. *M. tuberculosis* mutants, resistant to any single drug are naturally present in any large bacterial population, irrespective of exposure to drugs. Despite the availability of effective chemotherapy and the moderately protective vaccine, new anti-TB agents are urgently needed to decrease the global incidence of TB (Cox et al., 2006; Ducati et al., 2006).

2. Mycobacterial infection and survival of pathogen inside the host

On infection, *M. tuberculosis* resides mainly in the host macrophage, inside an endocytic vacuole called the phagosome. The pathogenic mycobacteria inhibit phagosome-lysosome fusion (Hestvik et al., 2005; Pieters & Gatfield, 2002). Lack of maturation of phagosomes containing pathogenic *M. tuberculosis* within macrophages has been widely recognized as a crucial factor for the persistence of mycobacterial pathogen. Mycobacteria have been shown to remain within phagosomes for a long time after infection by EM analysis (Jordao et al., 2008). It is unclear whether blocking of phagosome–lysosome fusion is essential for *M.*

tuberculosis survival (Armstrong & Hart, 1975). After phagocytosis and replication of pathogenic bacteria within macrophages, the infected cells migrate into tissues where additional immune cells are recruited to form a granuloma which consists of T cells and *M. tuberculosis*-infected macrophages (Grosset, 2003). The granuloma subsequently develops central areas of necrosis called caseum. This mass of cells of immune system and the bacteria are all dead cells. The surviving bacilli exist in a latent state and can become reactivated to develop active disease (Grosset, 2003). The latent infection in the asymptomatic individuals serves as a large reservoir of the bacterium. The biology of the latent state of the bacterium is not completely understood, however it is accepted that the latent state bacilli are metabolically less active (Wayne & Sohaskey, 2001).

Inside the macrophages, *M. tuberculosis* encounters many stress conditions like nitric oxide generated by inducible nitric-oxide synthase, nutrient starvation or carbon limitated condition, and reactive oxygen species (ROS) by the phagosomal NADPH oxidase (Farhana, 2010; Ehrt, 2009; Butler, 2010; Beste, 2007; Axelrod, 2008). A large number of studies have been undertaken to understand the survival of *M. tuberculosis* under stress such as heat, reduced oxygen or hypoxia, nutrient starvation, reactive nitrogen intermediates (RNI), antimicrobial molecules and downshift in pH (Chan et al., 1992; Farhana et al., 2010; Firmani & Riley, 2002; Lowrie, 1983; Wayne & Sohaskey, 2001). It has been suggested that the bacteria enter the non-growth or stationary phase during such stress conditions (Wayne & Sohaskey, 2001). *M. tuberculosis* also survives the lethal effects of RNI and antimicrobial molecules produced by activated macrophages and other cell types (Chan et al., 1992). The intracellular pathogen has the ability to survive inside the host macrophage in spite of the microbicidal effector functions of the macrophages. The bacterium responds to the stress conditions by genome wide changes in gene expression including the induction of a transient expression of a well conserved set of genes encoding heat shock or heat stress proteins.

3. Heat shock proteins

Heat shock proteins (Hsps) are well conserved and universal in all organisms. The expression of Hsps is highly increased under stress conditions such as hypoxia, nutrient starvation and oxygen radical (Richter et al., 2010; Tyedmers et al., 2010). Heat shock and other types of stresses lead to protein aggregation and unfolding of proteins. However, the most deleterious effect is the collapse of intermediary filament, tubulin and actin networks which leads to complete loss of localization and breakdown of intracellular transport and fragmentation of ER (Toivola et al., 2010). Hsps have cytoprotective roles, and under the stress conditions they maintain the cellular organization and homeostasis. Hsps are expressed at significant level in all eukaryotic and prokaryotic cells under normal conditions at physiological temperature. There is a high level of conservation of Hsps indicating a fundamental role played by these proteins in cellular processes (Moseley, 1997; Richter et al., 2010). Hsps were initially discovered in *Drosophila melanogaster* larvae as chromosomal puffs when it was exposed to heat shock (Tissieres et al., 1974). Subsequently, several Hsps were discovered in the following years. Many heat shock proteins work as molecular chaperones that are essential for maintaining cellular functions by preventing misfolding and aggregation of nascent polypeptides and by facilitating protein folding of conformationally altered proteins (Lanneau et al., 2010; Tyedmers et al., 2010).

The predominant class of Hsps is of molecular chaperones (Ellis & Hemmingsen, 1989). The molecular chaperones are further grouped into five major families based upon their molecular masses. These families are Hsp100, Hsp90, Hsp70, Hsp60 and small heat shock protein (sHsps) (Richter et al., 2010). The classification is based on their related functions and sizes, using the conventional nomenclature adopted after the Cold Spring Harbor Meeting of 1996 (Hightower & Hendershot, 1997). The molecular chaperones not only facilitate the proper folding of proteins but many times direct improperly folded proteins for destruction. In recent years, multiple chaperone-assisted degradation pathways have emerged, in which chaperones associate with a protease present inside the cell to degrade a misfolded protein (Gottesman, 2003, Kettern et al., 2010). Several other small heat inducible molecular chaperones, like Hsp33 are also known (Jakob et al., 1999).

The Hsp100 family consists of a group of ATPases associated with cellular activities (AAA+) family of ATP-dependent chaperones that transfers aggregated protein into a proteolytic chamber of an associated protease. These energy-dependent proteases, also known as caseinolytic proteases, Clp or Ti, are involved in a number of cellular activities, such as the degradation of proteins misfolded as a result of various types of stresses, the regulation of short-lived proteins and the housekeeping removal of dysfunctional proteins, which include denatured and aggregated polypeptides (Gottesman et al., 1997a, Gottesman et al., 1997b). The members of Hsp100 family include ClpA, ClpB, ClpC, ClpE, ClpX, ClpY and others (Kirstein et al., 2009). Hsp100 proteins have either one or two copies of a conserved ATPase, AAA+ core domain. Hsp100 family is further divided into two subclasses. The class I family members that include ClpA-E and L, contain two ATPase domains. The class II family members contain one ATPase domain, and include ClpX and ClpY (Lindquist & Craig, 1988; Schirmer et al., 1996). The Clp proteins form hexameric structure with one nucleotide binding site in each monomer of Class II and two nucleotide binding sites in Class I (Schirmer et al., 1996). These ATP-dependent chaperones associate with a protease, ClpP or ClpQ forming an oligomeric enzyme which assembles into ring-like or barrel like structure, containing a cavity within the centre of the macromolecular structure (Gottesman, 2003). The central cavity is also known as the proteolytic chamber, where unfolded protein substrates are translocated and subsequently degraded by the proteolytic site (Gottesman, 2003). Degradation of structured protein substrates requires the presence of ATP (Baker & Sauer, 2006). Unlike the other class I Clp proteins, ClpB does not associate with any protease to direct substrates for degradation (Lee et al., 2004).

Hsp90 is present mostly in cytosol of bacteria and eukaryotes, and is upregulated under stress (Welch & Feramisco., 1982). This chaperone is different in a way that it is not very promiscuous in substrate binding as it does not bind unfolded proteins rather it binds to native like proteins (Jakob et al., 1995). Under stress conditions two of the Hsp90 family proteins, namely yeast Sti1 and the propyl isomerise, Cpr6 are upregulated (Pearl & Prodromou., 2006).

Hsp70 family consists of highly conserved chaperones. All Hsp70 proteins bind ATP and under physiological conditions prevent the aggregation of proteins, and also refold aggregated proteins (Kiang & Tsokos, 1998). The activity of Hsp70 is regulated by co-factors. Much of the functional diversity of Hsp70s is driven by a diverse class of cofactors named J proteins or Hsp40 (Kampinga & Craig, 2010). The major members of the Hsp70 family include HSC 70 (heat shock cognate 70), mitochondrial GRP 75 and GRP 78 (Shi & Thomas,

1992). Hsp70 proteins in the endoplasmic reticulum are involved in two distinct chaperone functions in the normal cell. In the first, the Hsp70 family chaperone transfers the newly synthesized, unfolded protein to Hsp60 family of chaperonins, leading to eventual folding of the proteins. In the second case, Hsp70 chaperones carry proteins to different cellular compartments for the proper folding of the proteins (Kiang & Tsokos, 1998, Shi & Thomas, 1992).

Chaperonins are ring shaped proteins involved in promoting the ATP dependent folding of proteins under normal as well as under stress conditions. GroE machinery is the most prominent chaperonin in bacteria (Horwich et al., 2006). It consists of 14 GroEL subunits arranged in a cylinder of two heptameric rings, which is further attached to a heptameric ring of GroES (Horwich et al., 2006). GroE can bind to several different types of non-native proteins. The non-native protein is encapsulated in the GroE cylinder. GroEL internalizes the protein for the length of ATP hydrolysis cycle, during which the protein can refold to its native state (Viitanen et al., 1992). The closely related proteins in the mitochondria are called as Hsp60 and Hsp10.

Small Hsps (sHsps) are the most poorly conserved group among Hsps. Their most common trait is an α-crystallin domain. The most prominent member is the eye lens protein α-crystallin or Acr (Horwitz, 2003). sHsps are ATP-independent chaperones that form a large oligomeric structure often composed of 24 subunits. sHsps interact with partially folded targeted proteins to prevent their aggregation under stress conditions (Haslbeck et al., 2005). sHsp are also shown to be important in protecting the cell against the numerous injuries like heat stress, oxidative stress and apoptosis inducing factors (Arrigo, 1998).

4. Hsps and virulence in pathogenic microorganisms

Hsps play a central role in managing the damaged or aggregated proteins inside the cells. They have been linked to the virulence of several pathogenic microbes. *Candida albicans* expresses a bonafide heat shock response that is regulated by the evolutionarily conserved, essential heat shock transcription factor Hsf1. Hsf1 is thought to play a fundamental role in thermal homeostasis, adjusting the levels of essential chaperones to changes in growth temperature (Brown et al., 2010). In *Plasmodium falciparum* heat shock protein 70 is thought to play an essential role in parasite survival and virulence inside the host; Hsp70 is also being tried as a target for designing potential anti-malarial drugs (Cockburn et al., 2010). *Histoplasma capsulatum* is the causative agent of histoplasmosis in humans. A 62 kDa Hsp (Hsp60) of *H. capsulatum* is an immunodominant antigen which has been shown to play an important role in the adaptation of the fungus to temperature stress (Guimaraes et al., 2010). *Staphylococcus aureus* and *Staphylococcus epidermidis* can cause serious chronic infections in humans. An important factor involved in the pathogenesis of *S. aureus* is its ability to be internalized by phagocytes thereby evading the host immune system. Heat shock cognate protein, Hsc70 was identified as playing an important role in the internalization mechanism of *S. aureus* (Hirschhausen et al., 2010). ClpB gene from *Enterococcus faecalis* is linked to thermotolerance and virulence of the bacteria (de Oliveira et al., 2010). The Clp proteases appear to be critical for cell development in *Caulobacter crescentu*, and stress induction in *Bacillus subtilis* (Gerth et al., 2004). ClpC has been linked to the tight regulation of virulence genes in *Listeria monocytogenes*; it has been shown to be required for adhesion and invasion of the pathogen (Nair et al., 2000). ClpC has also been shown to be important for the

virulence and survival of *L. monocytogenes* in macrophages (Rouquette et al., 1998). In *Salmonella typhimurium* the Clp protease, ClpP is involved in maintaining the level of Sigma factors inside the bacterium; disruption of ClpP leads to decreased virulence in mice (Webb et al., 1999). ClpP mutation significantly attenuated the virulence of *Streptococcus pneumoniae* in murine intraperitoneal infection model (Kwon et al., 2003). Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar typhimurium results in loss of virulence in mice; these mutants were more sensitive to the intracellular environment of the macrophage (Gahan & Hill, 1999).

5. Hsps in *M. tuberculosis*

The ability of *M. tuberculosis* to survive under oxidative stress *in vivo* is an important aspect of its pathogenesis. Heat shock proteins are essential molecular chaperones for maintaining cellular functions during normal as well as stress conditions. The heat shock proteins also play a role in antigen presentation, and activation of lymphocytes and macrophages (Tsuchiya et al., 2009). The virulence of mycobacterium is dependent upon multiple genes that are expressed for the successful survival of the pathogen inside the macrophage. Expression of many heat shock proteins have been shown to increase under stress conditions in *M. tuberculosis* (Monahan et al., 2001; Sherman et al., 2001; Stewart et al., 2002; Voskuil et al., 2004). Proteome analysis of *M. tuberculosis* showed increased expression of Hsps such as 16 kDa α-crystallin (HspX), GroEL-1 and GroEL-2 inside macrophages. Hypoxia and starvation induce stationary phase in *M. tuberculosis*, under these conditions there is increased expression of hspX and acr2 (Sherman et al., 2001; Voskuil et al., 2004). Exposure of *M. tuberculosis* to heat shock induced the expression of hsp70 regulon, groEL, groES and acr protein (Stewart et al., 2002). The deletion of HspR, a repressor of Hsp70 proteins in *M. tuberculosis* has important impact on virulence. A HspR deletion mutant over-expressed Hsp70 proteins, and was fully virulent in the initial stages of infection; however the ability of the bacteria to establish a chronic infection was impaired as compared to the wild type (Stewart et al., 2001). The expression of Hsp65 and Hsp71 of *M. bovis* was increased under heat shock (Patel et al., 1991).

The synthesis of Hsps is increased after infection, some of which are immunodominant antigens in *M. tuberculosis* and *M. leprae* (Young et al., 1988). Hsp70 is an immunodominant antigen in *M. tuberculosis, M. leprae, Leishmania donovani, Plasmodium faciparum and Trypanosoma cruzi* (Kaufmann, 1994; Kiang & Tsokos, 1998). The heat shock protein, DnaK and many other proteins show increased expression during survival in carbon-starved stationary phase in *Mycobacterium smegmatis* (Blokpoel et al., 2005). In addition to a significant role in immune response, Hsps may also play a direct role in the virulence of *M. tuberculosis.* The over-expression of Hsps in *M. tuberculosis* leads to a better survival at higher temperature as compared to the wild type because of the protective effect of higher levels of Hsp (Stewart et al., 2001). Heat shock protein 22.5 (Hsp22.5) is a member of heat shock regulon which was shown to be activated under stress conditions, including survival in macrophages and during the late phase of chronic tuberculosis in murine lungs (Abomoelak et al., 2010). Deletion of Hsp22.5 resulted in the modulation of transcription of important genes like dormancy regulon, ATP synthesis, respiration, protein synthesis, and lipid metabolism (Abomoelak et al., 2010). Heat shock in *M. tuberculosis* has been shown to induce the expression of Acr2, a novel member of the α-crystallin family of molecular

chaperones (Wilkinson et al., 2005). The expression of acr2 increased within 1 h after infection of monocytes or macrophages. A deletion mutant (Δacr2) was unimpaired in log phase growth and persisted in IFN-γ-activated human macrophages (Wilkinson et al., 2005). GroES, also known as cpn.10 is found as a major constituent in the culture filtrate of *M. tuberculosis*, suggesting that it is exposed to the intraphagosomal milieu; it may be playing an important role in the survival of bacteria inside the phagosome (Sonnenberg & Belisle, 1997). Wayne and Sohaskey (2001) suggested that the decreased effectiveness of rifampin in the non-replicative state could be because of the stabilizing effect of chaperonin. Thus, a combination therapy of rifampin and a chaperonin inhibitor has the potential to shorten the therapeutic regimen. CD43, a large sialylated glycoprotein found on the surface of haematopoietic cells is involved in efficient macrophage binding and immunological responsiveness to *M. tuberculosis. M. tuberculosis* employs Cpn60.2 (Hsp65, GroEL), and to a lesser extent DnaK (Hsp70) as an adhesin that binds CD43 on the macrophage surface (Hickey et al., 2010). The crystal structure of the chaperonin 60 of *M. tuberculosis*, also called Hsp65 or chaperonin 60.2 has been solved (Qamra & Mande, 2004). Another *M. tuberculosis* small heat shock protein 16.3 (Hsp16.3) accumulates as the dominant protein in the latent stationary phase of tuberculosis infection and its expression is increased in response to stress (Valdez et al., 2002). It contains the core 'α-crystallin' domain found in all sHsps and protects against protein aggregation *in vitro* (Valdez et al., 2002). Protein phosphorylation is frequently used by organisms to adjust to environmental variations. Hsp16.3 and Hsp70 are immunodominant proteins synthesized during the *M. tuberculosis* infection. It was shown that these Hsps possess autophosphorylation activity (Preneta et al., 2004). *M. tuberculosis* genome has revealed the presence of heat shock proteins ClpP1, ClpP2, ClpC1, ClpX and ClpC2. The ClpC1 of *M. tuberculosis* has been shown to have an inherent ATPase activity, and to prevent protein aggregation as a chaperone in the absence of any adaptor protein (Kar et al., 2008). *M. tuberculosis* ClpC1 has also been shown to interact with ClpP2 (Singh et al., 2006). *M. tuberculosis* ClpC1 has been shown to interact with ResA, an anti sigma factor which is degraded by ClpC1P2 protease *in vitro* (Barik et al., 2010). Knockdown of ClpC1 in *M. smegmatis* and *M. tuberculosis* showed inhibition of RseA degradation indicating a regulatory role of Clp proteins in *M. tuberculosis* (Barik et al., 2010). ClpX is predicted to be essential for *in vivo* survival and pathogenicity and is conserved in *M. tuberculosis, M. leprae, M. bovis* and *M. avium paratuberculosis* (Ribeiro-Guimaraes & Pessolani, 2007). ClpX of *M. tuberculosis* was not able to substitute ClpC1 in ClpC1P2 protease complex (Barik et al., 2010). Knockdown of ClpX in *M. smegmatis* did not prevent the degradation of RseA indicating that ClpP2 does not associate with ClpX for its proteolytic activity (Barik et al., 2010). Leprosy and tuberculosis patients with active disease had shown the presence of antibodies recognizing ClpC in dot ELISA (Misra et al., 1996). The expression of ClpX was found to be upregulated in *M. tuberculosis* upon macrophage infection (Dziedzic et al., 2010). FtsZ is a protein known to assemble at the midcell division site in the form of a Z-ring. It is crucial for initiation of the cell division process in eubacteria. ClpX has been shown to interact with FtsZ in *M. tuberculosis* (Dziedzic et al., 2010). The crystal structure of *M. tuberculosis* caseinolytic protease, ClpP1 showed a disordered conformation of the residues in the catalytic triad, which makes the protein inactive (Ingvarsson et al., 2007). ClpP of *M. tuberculosis* has been studied as a target for drug designing (Tiwari et al., 2010). *M. avium*, the causative agent of paratuberculosis (Johne's disease) and an economic problem for beef,

dairy and sheep industries showed increased expression of ClpB gene during infection (Hughes et al., 2007). ClpB, of *M. bovis* BCG showed reactivity with sera of TB patients suggesting it to be an antigen target of the human immune response to mycobacteria (Bona et al., 1997). There are specific transcription factors that are involved in the regulation as well as transcription of Hsps during different conditions. ClgR, a clp gene regulator of *M. tuberculosis* activates the transcription of at least ten genes, including four that encode protease systems ClpP1/C, ClpP2/C, PtrB and HtrA-like protease Rv1043c, and three that encode chaperones Acr2, ClpB and the chaperonin Rv3269 (Estorninho et al., 2010). This transcriptional activation and regulatory function of ClgR is very important in the replication of bacteria inside the macrophages. It has been shown that ClgR deficient *M. tuberculosis* is not able to resist the pH inside macrophage post infection (Estorninho et al., 2010). The mechanism by which mycobacteria return to a replicating state after a non-replicating state, when exposed to low oxygen tension conditions is not clearly understood. ClgR is also implicated in the resumption of replicating state after hypoxia in *M. tuberculosis* (Sherrid et al., 2010).

6. Hsps as antigens in *M. tuberculosis*

Hsps may be released extracellularly upon necrotic cell death or independent of cell death. The mechanism of the release of Hsps is not clear (Tsan & Gao, 2004). The human as well bacterial Hsps stimulate immune response. The bacterial Hsps might modulate immunity by rapidly and directly increasing cytokine production in macrophages. T cells reacting to Hsp65 appear to play an important role in the control of *M. leprae* infection (de la Barrera et al., 1995). Hsp65 directly activates monocytes during mycobacterial infection. It leads to the production of TNF (tumor necrosis factor), IL-6 and IL-8. These cytokines are important in developing antigen specific T-cell mediated host immunity (Friedland et al., 1993). The murine intraepithelial lymphocytes (IEL), when exposed to soluble extract from *M. tuberculosis* showed elevated expression of IL-3, interferon-γ and IL-6 (Mendez-Samperio et al., 1995). Peripheral blood mononuclear cells (PBMC) from TB patients showed proliferative response to the Hsp65 (Mendez-Samperio et al., 1995). The *in vitro* immune responses to *M. tuberculosis* Hsp65 were checked in TB patients, and their PBMC showed high IFN-γ levels (Antas et al., 2005). When guinea pigs were vaccinated or infected with *M. bovis* (BCG) and virulent *M. tuberculosis*, cellular and humoral immune responses to mycobacterial stress proteins Hsp65 and Hsp70 were detected (Bartow & McMurray, 1997).

The C-terminal portion of heat shock protein Hsp70 was shown to be responsible for stimulating Th1-polarizing cytokines in human monocytes to produce IL-12, TNF-α, NO, and C-C chemokines (Wang et al., 2002). Hsp70 induces the expression of IL-10 and inhibits T-cell proliferation *in vitro*. Hsp70 appears to have immunosuppressive properties rather than inflammatory potential (Motta et al., 2007).

Hsp71 and Hsp65 are the major active components of the soluble extract of *M. tuberculosis*. Murine IELs were induced to divide and to secrete cytokines by Hsp71 and Hsp65 (Beagley et al., 1993). *M. tuberculosis* Hsp70, *M. leprae* Hsp65, and *M. bovis* BCG Hsp65 increased the levels of cytokines IL-1α, IL-1β, IL-6, TNFα, and GMCSF in macrophages (Retzlaff et al., 1994). *M. tuberculosis* contains multiple genes encoding Cpn 60 proteins, and these chaperonins have been involved in directly activating human monocytes and vascular

endothelial cells. Among them the Cpn 60.2 protein activates human PBMCs by a CD14-independent mechanism, whereas Cpn 60.1 is partially CD14 dependent and Cpn 60.1 is a more potent cytokine stimulator than Cpn 60.2 (Lewthwaite et al., 2001). Cpn 60.1 is said to play more important role in *M. tuberculosis* virulence than Cpn 60.2 (Lewthwaite et al., 2001).

M. tuberculosis can manipulate and inhibit the host response to ensure survival within macrophage. The anti-inflammatory cytokine IL-10 is shown to inhibit phagosome maturation in macrophages infected with *M. tuberculosis* (O'Leary et al., 2010). HspX/Acr is among the dormancy regulon whose expression is increased in hypoxia and on nitric oxide exposure essential for the survival of bacteria during persistence *in vivo*. Acr is also an immunodominant antigen during infection (Roupie et al., 2007). The T cells primed with Hsp65 of *M. tuberculosis*/*M.bovis* showed a response to the epitpoes shared by human Hsp65 and mycobacterial Hsp65, demonstrating that if activated these T cells can develop autoimmunity (Munk et al., 1989). The immune system, once primed for one Hsp might recognize other Hsps as well. Sarcoidosis (SA) is a multisystem granulomatous autoimmune disorder. The clinical and histopathological pictures of SA and TB are similar. *M. tuberculosis* heat shock proteins have been considered as proteins involved in the genesis of SA (Chen & Moller, 2008; Rajaiah & Moudgil, 2009). *M. tuberculosis* Hsps are also proposed to have a role in apoptosis which might be important in the pathogenesis of SA and TB granuloma formation (Dubaniewicz et al., 2006a; Dubaniewicz et al., 2006b). There is high cross reactivity between human and mycobacterial Hsp65. This could be the reason for the development of SA, however another hypothesis is that the BCG vaccination can develop autoimmunty in a pre-disposed host (Dubaniewicz, 2010). T-cells from SA patients produced a CD4+ response to multiple mycobacterial antigens including Hsps. These T cells were present at the site of active SA inside the human body (Oswald-Richter et al., 2010). The sera of the patients of rheumatoid arthritis (RA) showed increased levels of IgG and IgA antibody to the mycobacterial Hsp65 (Tsoulfa et al., 1989). The synovial membrane from rats and humans with arthritis appeared positive for mycobacterial Hsp65 showing the possible role of mycobacterial antigens in autoimmune diseases like arthritis (de Graeff-Meeder et al., 1990; Karopoulos et al., 1995). A survey of antigen-specific antibody isotypes from rheumatoid patients showed that anti-mycobacterial Hsp65 antibodies clearly do not appear to be disease specific markers for RA; however this does not exclude the possibility of mycobacterial Hsp65 in the pathogenesis of RA (Lai et al., 1995; Minota, 1997). The mycobacterial 71kDa Hsp antigen in lower concentration inhibits arthritis and at higher concentrations completely protects rats from arthritis (Kingston et al., 1996). Mycobacterial Hsp65 has also shown cross-reactive epitopes of epidermal cytokeratins which is a protein from epidermal keratinocytes of the normal human skin (Rambukkana et al., 1992). *Mycobacterium paratuberculosis* Hsp65 has been implicated as a possible cause of Crohn's disease, an inflammatory bowel disease (el-Zaatari et al., 1995).

As seen in several studies, Hsp65 is involved in the development of autoimmune response because of its highly conserved sequence. Another important finding about Hsp65 is that the effector cells activated with Hsp65 strongly inhibited colony formation from live BCG-infected autologous macrophages (Ab et al., 1990). In the case of *M. lepare* infection, the T-cells from leprosy patients are exposed to a large variety of different antigens including *M. lepare* Hsp70, *M. tuberculosis* Hsp70 and Hsp65 (Janson et al., 1991). When patients with

multiple sclerosis and tuberculosis were assessed, it was seen that Hsp70 is an antigen in TB as well as an autoantigen in multiple sclerosis (Salvetti et al., 1996). Immunizations with recombinant Hsp65, and Hsp65 rich *M. tuberculosis* in C57BL/6J mice induced atherogenesis indicating the involvement of Hsp65 in atherogenesis (George et al., 1999).

7. Hsps in vaccine development against TB

Hsps are among the proteins that are expressed at high level during the TB infection and are highly conserved. They could mediate the T cell sensitization required for the production of antibodies and can be used in the development of vaccine(s) against TB (Lussow et al., 1991). Inside the host, T cells are involved in activating macrophages and controlling the mycobacterial infection. Both the macrophage and mycobacterium synthesize heat shock proteins in order to facilitate their survival, and these Hsps possess potent immunogenicity (Munk & Kaufmann, 1991). The Hsp70 of *M. tuberculosis* has been shown to have anti-inflammatory properties and immunosuppressive role in a graft rejection system (Borges et al 2010). Hsp70 is recognized by human $CD4^+$ T-cells and it leads to the secretion of TNF, IL-6 and IL-1β (Asea et al., 2000). Mycobacterial Hsp70 can be used in subunit vaccine design since it contains a variety of T-cell epitopes (Oftung et al., 1994). Studies have been done to map the epitopes of Hsp70, so as to eliminate the autoimmune response in humans (Adams et al., 1993). A synthetic peptide, non-covalently bound to *M. tuberculosis* Hsp70 generated a very strong specific proliferative T-cell response in the spleen of mice (Roman & Moreno, 1996).

Hsp16 also induces T-cells to proliferate and secret cytokines, and therefore can be used as a potential subunit vaccine candidate (Agrewala & Wilkinson, 1999). A DNA vaccine combination expressing mycobacterial Hsp65 and IL-12 provided high degree of protection against TB (Okada et al., 2007). The vaccine was delivered by the hemagglutinating virus of Japan (HVJ)-envelope and liposome. This vaccine provided remarkable protection in mice and monkeys compared to the BCG vaccine, demonstrating the potential of Hsps to be used in vaccine development (Okada, 2006; Okada & Kita; Okada et al., 2007; Okada et al., 2009). A prime-boost strategy was investigated in cattle, using a combination of three DNA vaccines coding for Hsp65, Hsp70, and another mycobacterial protein Apa for priming, followed by a boost with BCG prior to experimental challenge with virulent *M. bovis* (Skinner et al., 2003).

Hsp65 as an antigen can confer protection equal to that from live BCG vaccine (Silva, 1999). The mycobacterial Hsp65 and Hsp70 acted as carrier molecules in mice previously primed with *M. tuberculosis* and showed high and long-lasting titers of IgG (Barrios et al., 1992; Perraut et al., 1993). The mycobacterial Hsp65 conjugated to peptides or oligosaccharides in the absence of adjuvants, induced antibodies which cross-reacted well with Hsp homologues from other prokaryotes, but weakly with the human Hsp homologue (Barrios et al., 1994). The PBMCs and T-cell lines from *M. leprae* and *M. bovis* BCG vaccinated subjects showed proliferation in response to Hsp18 and Hsp65 of *M. leprae*, Hsp65 of *M. bovis* BCG, and the Hsp70 of *M. tuberculosis* (Mustafa et al., 1993). The response of T cells to these Hsps makes them eligible for their application in the next generation of subunit vaccines (Mustafa et al., 1993).

8. Hsps in TB diagnosis

In high-TB incidence countries, TB control relies on diagnosis which is mainly based on clinical symptoms or laboratory diagnosis using sputum smear microscopy. TB smear microscopy is highly insensitive for HIV-co-infected individuals and for children due to the reduced pulmonary bacillary loads in these patients. TB diagnosis by smear microscopy is usually further confirmed by culture. However, this requires extended incubation times and is significantly more expensive than smears, requiring specialized equipment and highly trained personnel (Parsons et al., 2004; Storla et al., 2008). Thus, there is a basic need for the development of fast and inexpensive ways of TB diagnosis.

Using recombinant DNA techniques, synthetic peptides, antigen-specific antibodies and T cells, several major antigens of *M. tuberculosis* have been identified which include hsp60, hsp70, Ag85, ESAT-6 and CFP10 (Mustafa, 2001). In addition, Hsp65, Hsp71, 14-kDa Hsp and GroE proteins can play an important role in the diagnosis of TB. The identification of these markers can contribute to the clinical diagnosis of TB and may also provide additional insight into the pathogenesis of TB (Kashyap et al., 2010). sHsp18 has been shown to be a major immunodominant antigen of *M. leprae* (Lini et al., 2008). Hsp65 has been shown to be an attractive marker for TB (Bothamley et al., 1992; Haldar et al., 2010; Lee et al., 1994; Rambukkana et al., 1991). The 65 kDa heat shock protein is detected even in the cerebrospinal fluid of tuberculous meningitis patients, indicating its potential use as a diagnostic marker for tuberculous meningitis (Mudaliar et al., 2006). In case of TB ascites, the ascitic fluid has shown the presence of Hsp65, Hsp71 and Hsp14 as very useful diagnostic markers (Kashyap et al., 2010). A multiplex PCR against Hsp65 gene coding for 65 kDa antigen for early detection has been tested. The technique was able to distinguish between strains of the *M. tuberculosis* complex and non-tuberculous mycobacteria (Bhattacharya et al., 2003).

The response to recombinant 10-kDa heat shock protein of *M. leprae* was evaluated by indirect ELISA in sera from leprosy patients, household contacts, tuberculosis patients and healthy controls. However, this test seems to have a low sensitivity and specificity for leprosy detection and tuberculosis patients sera cross-reacted with *M. leprae* antigen as well (Rojas et al., 1997).

9. Conclusion

Treatment for *M. tuberculosis* has to be lengthy, since populations of this bacillus differ in metabolic activity. In addition, the treatment has to consist of a variety of drugs, since spontaneous chromosome mutations can give rise to drug resistance. As heat shock proteins are involved in crucial housekeeping activity, their inactivation may be lethal for the cells. Despite the impressive progress in the understanding of structure and function of Hsps, the biological significance of these proteins in the survival of bacteria inside macrophage is still not completely clear. These proteins appear to be playing an important role in protecting the bacteria from the environment. Further understanding of Hsps is required for the development of new anti-tuberculosis drugs and vaccines.

10. References

Ab BK, Kiessling R, Van Embden JD, Thole JE, Kumararatne DS, et al. (1990). Induction of antigen-specific CD4+ HLA-DR-restricted cytotoxic T lymphocytes as well as

nonspecific nonrestricted killer cells by the recombinant mycobacterial 65-kDa heat-shock protein. *Eur J Immunol* 20: 369-77

Abomoelak B, Marcus SA, Ward SK, Karakousis PC, Steinberg H, Talaat AM. (2010). Characterization of a novel heat shock protein (Hsp22.5) involved in the athogenesis of *Mycobacterium tuberculosis*. *J Bacteriol* 193: 3497-505

Adams E, Britton WJ, Morgan A, Goodsall AL, Basten A. (1993). Identification of human T cell epitopes in the Mycobacterium leprae heat shock protein 70-kD antigen. *Clin Exp Immunol* 94: 500-6

Agrewala JN, Wilkinson RJ. (1999). Influence of HLA-DR on the phenotype of CD4+ T lymphocytes specific for an epitope of the 16-kDa alpha-crystallin antigen of *Mycobacterium tuberculosis*. *Eur J Immunol* 29: 1753-61

Antas PR, Cardoso FL, Pereira KC, Franken KL, Cunha KS, et al. (2005). T cell immune responses to mycobacterial antigens in Brazilian tuberculosis patients and controls. *Trans R Soc Trop Med Hyg* 99: 699-707

Armstrong JA, Hart PD. (1975). Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med* 142: 1-16

Arrigo AP. (1998). Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. *Biol Chem* 379: 19-26

Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, et al. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6: 435-42

Axelrod S, Oschkinat H, Enders J, Schlegel B, Brinkmann V, et al. (2008). Delay of phagosome maturation by a mycobacterial lipid is reversed by nitric oxide. *Cell Microbiol* 10: 1530-45

Baker TA, Sauer RT. (2006). ATP-dependent proteases of bacteria: recognition logic and operating principles. *Trends Biochem Sci* 31: 647-53

Barik S, Sureka K, Mukherjee P, Basu J, Kundu M. (2010). RseA, the SigE specific anti-sigma factor of *Mycobacterium tuberculosis*, is inactivated by phosphorylation-dependent ClpC1P2 proteolysis. *Mol Microbiol* 75: 592-606

Barrios C, Lussow AR, Van Embden J, Van der Zee R, Rappuoli R, et al. (1992). Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guerin priming. *Eur J Immunol* 22: 1365-72

Barrios C, Tougne C, Polla BS, Lambert PH, Del Giudice G. (1994). Specificity of antibodies induced after immunization of mice with the mycobacterial heat shock protein of 65 kD. *Clin Exp Immunol* 98: 224-8

Bartow RA, McMurray DN. (1997). Cellular and humoral immune responses to mycobacterial stress proteins in experimental pulmonary tuberculosis. *Tuber Lung Dis* 78: 185-93

Beagley KW, Fujihashi K, Black CA, Lagoo AS, Yamamoto M, et al. (1993). The *Mycobacterium tuberculosis* 71-kDa heat-shock protein induces proliferation and

cytokine secretion by murine gut intraepithelial lymphocytes. *Eur J Immunol* 23: 2049-52

Beste DJ, Laing E, Bonde B, Avignone-Rossa C, Bushell ME, McFadden JJ. (2007). Transcriptomic analysis identifies growth rate modulation as a component of the adaptation of mycobacteria to survival inside the macrophage. *J Bacteriol* 189: 3969-76

Bhattacharya B, Karak K, Ghosal AG, Roy A, Das S, et al. (2003). Development of a new sensitive and efficient multiplex polymerase chain reaction (PCR) for identification and differentiation of different mycobacterial species. *Trop Med Int Health* 8: 150-7

Blokpoel MC, Smeulders MJ, Hubbard JA, Keer J, Williams HD. (2005). Global analysis of proteins synthesized by *Mycobacterium smegmatis* provides direct evidence for physiological heterogeneity in stationary-phase cultures. *J Bacteriol* 187: 6691-700

Bona M, Nayak R, Wu M, Mincek M, Ellner JJ. (1997). Immunological screening of a genomic M. bovis BCG library expressed in *M. smegmatis* and identification of the *M. bovis* BCG analog of ClpB. *Tuber Lung Dis* 78: 133-41

Borges TJ, Porto BN, Teixeira CA, Rodrigues M, Machado FD, et al. (2010). Prolonged survival of allografts induced by mycobacterial Hsp70 is dependent on CD4+CD25+ regulatory T cells. *PLoS One* 5: e14264

Bothamley GH, Rudd R, Festenstein F, Ivanyi J. (1992). Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. *Thorax* 47: 270-5

Brown AJ, Leach MD, Nicholls S. (2010). The relevance of heat shock regulation in fungal pathogens of humans. *Virulence* 1: 330-2

Butler RE, Cihlarova V, Stewart GR. (2010) Effective generation of reactive oxygen species in the mycobacterial phagosome requires K+ efflux from the bacterium. *Cell Microbiol* 12: 1186-93

Chan J, Xing Y, Magliozzo RS, Bloom BR. (1992). Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 175: 1111-22

Chen ES, Moller DR. (2008). Etiology of sarcoidosis. *Clin Chest Med* 29: 365-77

Chiang CY, Centis R, Migliori GB. (2010). Drug-resistant tuberculosis: past, present, future. *Respirology* 15: 413-32

Cockburn IL, Pesce ER, Pryzborski JM, Davies-Coleman MT, Clark PG, et al. (2010). Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1. *Biol Chem* 392: 431-8

Cox H, Kebede Y, Allamuratova S, Ismailov G, Davletmuratova Z, et al. (2006). Tuberculosis recurrence and mortality after successful treatment: impact of drug resistance. *PLoS Med* 3: e384

de Graeff-Meeder ER, Voorhorst M, van Eden W, Schuurman HJ, Huber J, et al. (1990). Antibodies to the mycobacterial 65-kd heat-shock protein are reactive with synovial tissue of adjuvant arthritic rats and patients with rheumatoid arthritis and osteoarthritis. *Am J Pathol* 137: 1013-7

de la Barrera S, Fink S, Finiasz M, Minnucci F, Valdez R, et al. (1995). Lack of cytotoxic activity against *Mycobacterium leprae* 65-kD heat shock protein (hsp) in multibacillary leprosy patients. *Clin Exp Immunol* 99: 90-7

de Oliveira NE, Abranches J, Gaca AO, Laport MS, Damaso CR, et al. (2010). clpB, a class III heat-shock gene regulated by CtsR, is involved in thermotolerance and virulence of *Enterococcus faecalis*. *Microbiology* 157: 656-65

Dubaniewicz A. (2010). *Mycobacterium tuberculosis* heat shock proteins and autoimmunity in sarcoidosis. *Autoimmun Rev* 9: 419-24

Dubaniewicz A, Kampfer S, Singh M. (2006a). Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis (Edinb)* 86: 60-7

Dubaniewicz A, Trzonkowski P, Dubaniewicz-Wybieralska M, Singh M, Mysliwski A. (2006b). Comparative analysis of mycobacterial heat shock proteins-induced apoptosis of peripheral blood mononuclear cells in sarcoidosis and tuberculosis. *J Clin Immunol* 26: 243-50

Ducati RG, Ruffino-Netto A, Basso LA, Santos DS. (2006). The resumption of consumption -- a review on tuberculosis. *Mem Inst Oswaldo Cruz* 101: 697-714

Dziedzic R, Kiran M, Plocinski P, Ziolkiewicz M, Brzostek A, et al. (2010). *Mycobacterium tuberculosis* ClpX interacts with FtsZ and interferes with FtsZ assembly. *PLoS One* 5: e11058

Ehrt S, Schnappinger D. (2009). Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol* 11: 1170-8

Eismont NV. (2009). Deaths of patients with tuberculosis in combination with HIV infection from various causes, other than tuberculosis. *Probl Tuberk Bolezn Legk*: 54-9

Ellis RJ, Hemmingsen SM. (1989). Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem Sci* 14: 339-42

el-Zaatari FA, Naser SA, Engstrand L, Burch PE, Hachem CY, et al. (1995). Nucleotide sequence analysis and seroreactivities of the 65K heat shock protein from *Mycobacterium paratuberculosis*. *Clin Diagn Lab Immunol* 2: 657-64

Estorninho M, Smith H, Thole J, Harders-Westerveen J, Kierzek A, et al. (2010). ClgR regulation of chaperone and protease systems is essential for *Mycobacterium tuberculosis* parasitism of the macrophage. *Microbiology* 156: 3445-55

Farhana A, Guidry L, Srivastava A, Singh A, Hondalus MK, Steyn AJ. (2010). Reductive stress in microbes: implications for understanding *Mycobacterium tuberculosis* disease and persistence. *Adv Microb Physiol* 57: 43-117

Firmani MA, Riley LW. (2002). Reactive nitrogen intermediates have a bacteriostatic effect on *Mycobacterium tuberculosis in vitro*. *J Clin Microbiol* 40: 3162-6

Friedland JS, Shattock R, Remick DG, Griffin GE. (1993). Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clin Exp Immunol* 91: 58-62

Gahan CG, Hill C. (1999). The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. *Int J Food Microbiol* 50: 93-100

George J, Shoenfeld Y, Afek A, Gilburd B, Keren P, et al. (1999). Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein-65. *Arterioscler Thromb Vasc Biol* 19: 505-10

Gerth U, Kirstein J, Mostertz J, Waldminghaus T, Miethke M, et al. (2004). Fine-tuning in regulation of Clp protein content in *Bacillus subtilis*. *J Bacteriol* 186: 179-91

Gottesman S. (2003). Proteolysis in bacterial regulatory circuits. *Annu Rev Cell Dev Biol* 19: 565-87

Gottesman S, Maurizi MR, Wickner S. (1997a). Regulatory subunits of energy-dependent proteases. *Cell* 91: 435-8

Gottesman S, Wickner S, Maurizi MR. (1997b). Protein quality control: triage by chaperones and proteases. *Genes Dev* 11: 815-23

Grosset J. (2003). *Mycobacterium tuberculosis* in the extracellular compartment: an underestimated adversary. *Antimicrob Agents Chemother* 47: 833-6

Guimaraes AJ, Nakayasu ES, Sobreira TJ, Cordero RJ, Nimrichter L, et al. 2010. Histoplasma capsulatum heat-shock 60 orchestrates the adaptation of the fungus to temperature stress. *PLoS One* 6: e14660

Haldar S, Bose M, Chakrabarti P, Daginawala HF, Harinath BC, et al. (2010). Improved laboratory diagnosis of tuberculosis - The Indian experience. *Tuberculosis (Edinb)*

Haslbeck M, Franzmann T, Weinfurtner D, Buchner J. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol* 12: 842-6

Hestvik AL, Hmama Z, Av-Gay Y. (2005). Mycobacterial manipulation of the host cell. *FEMS Microbiol Rev* 29: 1041-50

Hickey TB, Ziltener HJ, Speert DP, Stokes RW. (2010). *Mycobacterium tuberculosis* employs Cpn60.2 as an adhesin that binds CD43 on the macrophage surface. *Cell Microbiol* 12: 1634-47

Hightower LE, Hendershot LM. (1997). Molecular chaperones and the heat shock response at Cold Spring Harbor. *Cell Stress Chaperones* 2: 1-11

Hirschhausen N, Schlesier T, Schmidt MA, Gotz F, Peters G, Heilmann C. (2010). A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cell Microbiol* 12: 1746-64

Horwich AL, Farr GW, Fenton WA. (2006). GroEL-GroES-mediated protein folding. *Chem Rev* 106: 1917-30

Horwitz J. (2003). Alpha-crystallin. *Exp Eye Res* 76: 145-53

Hughes V, Smith S, Garcia-Sanchez A, Sales J, Stevenson K. (2007). Proteomic comparison of *Mycobacterium avium* subspecies paratuberculosis grown *in vitro* and isolated from clinical cases of ovine paratuberculosis. *Microbiology* 153: 196-205

Ingvarsson H, Mate MJ, Hogbom M, Portnoi D, Benaroudj N, et al. (2007). Insights into the inter-ring plasticity of caseinolytic proteases from the X-ray structure of *Mycobacterium tuberculosis* ClpP1. *Acta Crystallogr D Biol Crystallogr* 63: 249-59

Jakob U, Lilie H, Meyer I, Buchner J. (1995). Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock *in vivo*. *J Biol Chem* 270: 7288-94

Jakob U, Muse W, Eser M, Bardwell JC. (1999). Chaperone activity with a redox switch. *Cell* 96: 341-52

Janson AA, Klatser PR, van der Zee R, Cornelisse YE, de Vries RR, et al. (1991). A systematic molecular analysis of the T cell-stimulating antigens from *Mycobacterium leprae* with

T cell clones of leprosy patients. Identification of a novel *M. leprae* HSP 70 fragment by *M. leprae*-specific T cells. *J Immunol* 147: 3530-7

Jordao L, Bleck CK, Mayorga L, Griffiths G, Anes E. (2008). On the killing of mycobacteria by macrophages. *Cell Microbiol* 10: 529-48

Kampinga HH, Craig EA. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat Rev Mol Cell Biol* 11: 579-92

Kar NP, Sikriwal D, Rath P, Choudhary RK, Batra JK. (2008). *Mycobacterium tuberculosis* ClpC1: characterization and role of the N-terminal domain in its function. *FEBS J* 275: 6149-58

Karopoulos C, Rowley MJ, Handley CJ, Strugnell RA. (1995). Antibody reactivity to mycobacterial 65 kDa heat shock protein: relevance to autoimmunity. *J Autoimmun* 8: 235-48

Kashyap RS, Saha SM, Nagdev KJ, Kelkar SS, Purohit HJ, et al. (2010) Diagnostic markers for tuberculosis ascites: a preliminary study. *Biomark Insights* 5: 87-94

Kaufmann SH. (1994). Heat shock proteins and autoimmunity: a critical appraisal. *Int Arch Allergy Immunol* 103: 317-22

Kettern N, Dreiseidler M, Tawo R, Hohfeld J. (2010). Chaperone-assisted degradation: multiple paths to destruction. *Biol Chem* 391: 481-9

Kiang JG, Tsokos GC. (1998). Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 80: 183-201

Kingston AE, Hicks CA, Colston MJ, Billingham ME. (1996). A 71-kD heat shock protein (hsp) from *Mycobacterium tuberculosis* has modulatory effects on experimental rat arthritis. *Clin Exp Immunol* 103: 77-82

Kirstein J, Moliere N, Dougan DA, Turgay K. (2009). Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat Rev Microbiol* 7: 589-99

Kwon HY, Kim SW, Choi MH, Ogunniyi AD, Paton JC, et al. (2003). Effect of heat shock and mutations in ClpL and ClpP on virulence gene expression in *Streptococcus pneumoniae*. *Infect Immun* 71: 3757-65

Lai NS, Lan JL, Yu CL, Lin RH. (1995). Antibody to *Mycobacterium tuberculosis* 65 kDa heat shock protein in patients with rheumatoid arthritis--a survey of antigen-specific antibody isotypes and subclasses in an endemic area of previous tuberculosis infection. *Ann Rheum Dis* 54: 225-8

Lanneau D, Wettstein G, Bonniaud P, Garrido C. (2010). Heat shock proteins: cell protection through protein triage. *ScientificWorldJournal* 10: 1543-52

Lee BW, Tan JA, Wong SC, Tan CB, Yap HK, et al. (1994). DNA amplification by the polymerase chain reaction for the rapid diagnosis of tuberculous meningitis. Comparison of protocols involving three mycobacterial DNA sequences, IS6110, 65 kDa antigen, and MPB64. *J Neurol Sci* 123: 173-9

Lee S, Sowa ME, Choi JM, Tsai FT. (2004). The ClpB/Hsp104 molecular chaperone-a protein disaggregating machine. *J Struct Biol* 146: 99-105

Lewthwaite JC, Coates AR, Tormay P, Singh M, Mascagni P, et al. (2001). *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infect Immun* 69: 7349-55

Lindquist S, Craig EA. (1988). The heat-shock proteins. *Annu Rev Genet* 22: 631-77

Lini N, Rehna EA, Shiburaj S, Maheshwari JJ, Shankernarayan NP, Dharmalingam K. (2008). Functional characterization of a small heat shock protein from *Mycobacterium leprae*. *BMC Microbiol* 8: 208

Lowrie DB. (1983). How macrophages kill tubercle bacilli. *J Med Microbiol* 16: 1-12

Lussow AR, Barrios C, van Embden J, Van der Zee R, Verdini AS, et al. (1991). Mycobacterial heat-shock proteins as carrier molecules. *Eur J Immunol* 21: 2297-302

Mendez-Samperio P, Gonzalez-Garcia L, Pineda-Fragoso PR, Ramos-Sanchez E. (1995). Specificity of T cells in human resistance to *Mycobacterium tuberculosis* infection. *Cell Immunol* 162: 194-201

Minota S. (1997). Autoimmune diseases and stress proteins. *Rinsho Byori* 45: 19-26

Misra N, Habib S, Ranjan A, Hasnain SE, Nath I. (1996). Expression and functional characterisation of the clpC gene of *Mycobacterium leprae*: ClpC protein elicits human antibody response. *Gene* 172: 99-104

Monahan IM, Betts J, Banerjee DK, Butcher PD. (2001). Differential expression of mycobacterial proteins following phagocytosis by macrophages. *Microbiology* 147: 459-71

Moseley PL. (1997). Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol* 83: 1413-7

Motta A, Schmitz C, Rodrigues L, Ribeiro F, Teixeira C, et al. (2007). *Mycobacterium tuberculosis* heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation *in vitro*. *Immunology* 121: 462-72

Mudaliar AV, Kashyap RS, Purohit HJ, Taori GM, Daginawala HF. (2006). Detection of 65 kD heat shock protein in cerebrospinal fluid of tuberculous meningitis patients. *BMC Neurol* 6: 34

Munk ME, Kaufmann SH. (1991). The immune response to *Mycobacterium tuberculosis*. *Behring Inst Mitt*: 27-35

Munk ME, Schoel B, Modrow S, Karr RW, Young RA, Kaufmann SH. (1989). T lymphocytes from healthy individuals with specificity to self-epitopes shared by the mycobacterial and human 65-kilodalton heat shock protein. *J Immunol* 143: 2844-9

Mustafa AS. (2001). Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis. *Curr Pharm Biotechnol* 2: 157-73

Mustafa AS, Lundin KE, Oftung F. (1993). Human T cells recognize mycobacterial heat shock proteins in the context of multiple HLA-DR molecules: studies with healthy subjects vaccinated with *Mycobacterium bovis* BCG and *Mycobacterium leprae*. *Infect Immun* 61: 5294-301

Nair S, Milohanic E, Berche P. (2000). ClpC ATPase is required for cell adhesion and invasion of *Listeria monocytogenes*. *Infect Immun* 68: 7061-8

Oftung F, Geluk A, Lundin KE, Meloen RH, Thole JE, et al. (1994). Mapping of multiple HLA class II-restricted T-cell epitopes of the mycobacterial 70-kilodalton heat shock protein. *Infect Immun* 62: 5411-8

Okada M. (2006). [Novel vaccines against *M. tuberculosis*]. *Kekkaku* 81: 745-51

Okada M, Kita Y. (2010). Anti-tuberculosis immunity by cytotoxic T cells * granulysin and the development of novel vaccines (HSP-65 DNA+IL-12 DNA). *Kekkaku* 85: 531-8

Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, et al. (2007). Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB. *Vaccine* 25: 2990-3

Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, et al. (2009). Novel prophylactic and therapeutic vaccine against tuberculosis. *Vaccine* 27: 3267-70

O'Leary S, O'Sullivan MP, Keane J. (2010). IL-10 Blocks Phagosome Maturation in *Mycobacterium tuberculosis*-infected human macrophages. *Am J Respir Cell Mol Biol* 45: 172-80

Oswald-Richter KA, Beachboard DC, Zhan X, Gaskill CF, Abraham S, et al. (2010). Multiple mycobacterial antigens are targets of the adaptive immune response in pulmonary sarcoidosis. *Respir Res* 11: 161

Parsons LM, Somoskovi A, Urbanczik R, Salfinger M. (2004). Laboratory diagnostic aspects of drug resistant tuberculosis. *Front Biosci* 9: 2086-105

Patel BK, Banerjee DK, Butcher PD. (1991). Characterization of the heat shock response in *Mycobacterium bovis* BCG. *J Bacteriol* 173: 7982-7

Pearl LH, Prodromou C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu Rev Biochem* 75: 271-94

Perraut R, Lussow AR, Gavoille S, Garraud O, Matile H, et al. (1993). Successful primate immunization with peptides conjugated to purified protein derivative or mycobacterial heat shock proteins in the absence of adjuvants. *Clin Exp Immunol* 93: 382-6

Pieters J, Gatfield J. (2002). Hijacking the host: survival of pathogenic mycobacteria inside macrophages. *Trends Microbiol* 10: 142-6

Preneta R, Papavinasasundaram KG, Cozzone AJ, Duclos B. (2004). Autophosphorylation of the 16 kDa and 70 kDa antigens (Hsp 16.3 and Hsp 70) of *Mycobacterium tuberculosis*. *Microbiology* 150: 2135-41

Qamra R, Mande SC. (2004). Crystal structure of the 65-kilodalton heat shock protein, chaperonin 60.2, of *Mycobacterium tuberculosis*. *J Bacteriol* 186: 8105-13

Rajaiah R, Moudgil KD. (2009). Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev* 8: 388-93

Rambukkana A, Das PK, Krieg S, Young S, Le Poole IC, Bos JD. (1992). Mycobacterial 65,000 MW heat-shock protein shares a carboxy-terminal epitope with human epidermal cytokeratin 1/2. *Immunology* 77: 267-76

Rambukkana A, Yong S, Das PK. (1991). Identification of a novel B-cell epitope of restricted specificity on the hsp 65-kDa protein of *Mycobacterium tuberculosis*. *FEMS Microbiol Immunol* 3: 39-45

Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW. (1994). Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. *Infect Immun* 62: 5689-93

Ribeiro-Guimaraes ML, Pessolani MC. (2007). Comparative genomics of mycobacterial proteases. *Microb Pathog* 43: 173-8

Richter K, Haslbeck M, Buchner J. (2010). The heat shock response: life on the verge of death. *Mol Cell* 40: 253-66

Rojas RE, Demichelis SO, Gimenez MF, Molinari ML, Segal-Eiras A. (1997). Cross-reactivity of anti-10kD heat shock protein antibodies in leprosy and tuberculosis patients. *Medicina (B Aires)* 57: 581-6

Roman E, Moreno C. (1996). Synthetic peptides non-covalently bound to bacterial hsp 70 elicit peptide-specific T-cell responses *in vivo*. *Immunology* 88: 487-92

Roupie V, Romano M, Zhang L, Korf H, Lin MY, et al. (2007). Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. *Infect Immun* 75: 941-9

Rouquette C, de Chastellier C, Nair S, Berche P. (1998). The ClpC ATPase of Listeria monocytogenes is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. *Mol Microbiol* 27: 1235-45

Salvetti M, Ristori G, Buttinelli C, Fiori P, Falcone M, et al. (1996). The immune response to mycobacterial 70-kDa heat shock proteins frequently involves autoreactive T cells and is quantitatively disregulated in multiple sclerosis. *J Neuroimmunol* 65: 143-53

Schirmer EC, Glover JR, Singer MA, Lindquist S. (1996). HSP100/Clp proteins: a common mechanism explains diverse functions. *Trends Biochem Sci* 21: 289-96

Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK. (2001). Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. *Proc Natl Acad Sci U S A* 98: 7534-9

Sherrid AM, Rustad TR, Cangelosi GA, Sherman DR. (2010). Characterization of a Clp protease gene regulator and the reaeration response in *Mycobacterium tuberculosis*. *PLoS One* 5: e11622

Shi Y, Thomas JO. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol Cell Biol* 12: 2186-92

Silva CL. (1999). The potential use of heat-shock proteins to vaccinate against mycobacterial infections. *Microbes Infect* 1: 429-35

Singh A, Mai D, Kumar A, Steyn AJ. (2006). Dissecting virulence pathways of *Mycobacterium tuberculosis* through protein-protein association. *Proc Natl Acad Sci U S A* 103: 11346-51

Skinner MA, Buddle BM, Wedlock DN, Keen D, de Lisle GW, et al. (2003). A DNA prime-*Mycobacterium bovis* BCG boost vaccination strategy for cattle induces protection against bovine tuberculosis. *Infect Immun* 71: 4901-7

Smith I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* 16: 463-96

Sonnenberg MG, Belisle JT. (1997). Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. *Infect Immun* 65: 4515-24

Stewart GR, Snewin VA, Walzl G, Hussell T, Tormay P, et al. (2001). Overexpression of heat-shock proteins reduces survival of *Mycobacterium tuberculosis* in the chronic phase of infection. *Nat Med* 7: 732-7

Stewart GR, Wernisch L, Stabler R, Mangan JA, Hinds J, et al. (2002). Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology* 148: 3129-38

Storla DG, Yimer S, Bjune GA. (2008). A systematic review of delay in the diagnosis and treatment of tuberculosis. *BMC Public Health* 8: 15

Tissieres A, Mitchell HK, Tracy UM. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosome puffs. *J Mol Biol* 84: 389-98

Tiwari A, Gupta S, Srivastava S, Srivastava R, Rawat AK. (2010). A ClpP protein model as tuberculosis target for screening marine compounds. *Bioinformation* 4: 405-8

Toivola DM, Strnad P, Habtezion A, Omary MB. (2010). Intermediate filaments take the heat as stress proteins. *Trends Cell Biol* 20: 79-91

Tsan MF, Gao B. (2004). Cytokine function of heat shock proteins. *Am J Physiol Cell Physiol* 286: C739-44

Tsoulfa G, Rook GA, Bahr GM, Sattar MA, Behbehani K, et al. (1989). Elevated IgG antibody levels to the mycobacterial 65-kDa heat shock protein are characteristic of patients with rheumatoid arthritis. *Scand J Immunol* 30: 519-27

Tsuchiya K, Kawamura I, Mitsuyama M. (2009). Survival strategy of intracellular bacteria in macrophages. *Tanpakushitsu Kakusan Koso* 54: 996-1001

Tyedmers J, Mogk A, Bukau B. (2010). Cellular strategies for controlling protein aggregation. *Nat Rev Mol Cell Biol* 11: 777-88

Valdez MM, Clark JI, Wu GJ, Muchowski PJ. (2002). Functional similarities between the small heat shock proteins *Mycobacterium tuberculosis* HSP 16.3 and human alphaB-crystallin. *Eur J Biochem* 269: 1806-13

Viitanen PV, Gatenby AA, Lorimer GH. (1992). Purified chaperonin 60 (groEL) interacts with the nonnative states of a multitude of *Escherichia coli* proteins. *Protein Sci* 1: 363-9

Voskuil MI, Visconti KC, Schoolnik GK. (2004). *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb)* 84: 218-27

Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, et al. (2002). Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J Immunol* 169: 2422-9

Wayne LG, Sohaskey CD. (2001). Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* 55: 139-63

Webb C, Moreno M, Wilmes-Riesenberg M, Curtiss R, 3rd, Foster JW. (1999). Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium*. *Mol Microbiol* 34: 112-23

Welch WJ, Feramisco JR. 1982. Purification of the major mammalian heat shock proteins. *J Biol Chem* 257: 14949-59

WHO global tuberculosis control report (2010). Summary. *Cent Eur J Public Health* 18: 237

Wilkinson KA, Stewart GR, Newton SM, Vordermeier HM, Wain JR, et al. (2005). Infection biology of a novel alpha-crystallin of *Mycobacterium tuberculosis*: Acr2. *J Immunol* 174: 4237-43

Young D, Lathigra R, Hendrix R, Sweetser D, Young RA. (1988). Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A* 85: 4267-70

15

Mammalian Heme Peroxidases and *Mycobacterium tuberculosis*

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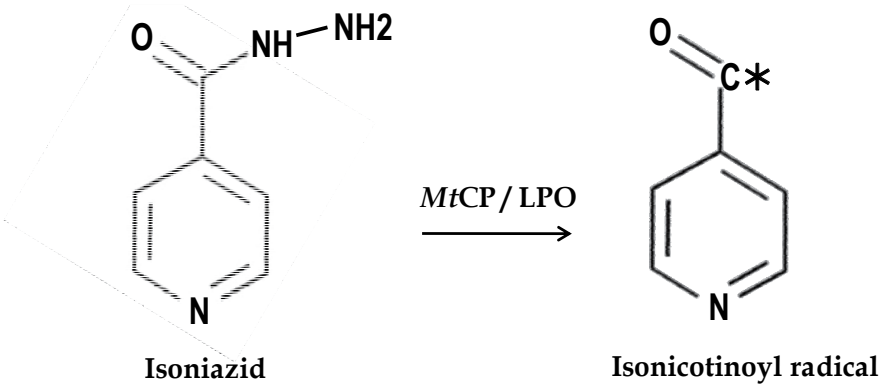
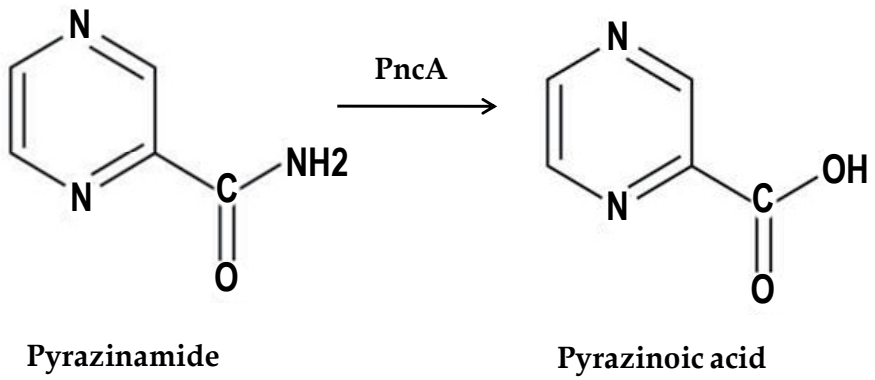
1. Introduction

Tuberculosis (TB) is a lethal infectious disease which is caused by *Mycobacterium tuberculosis*. The alarming rate at which the incidence of bacterial resistance to known antibiotics has been rising is a serious cause of concern. At present, the two well known anti-tuberculosis drugs, isonicotinic acid hydrazide (INH, isoniazid) and pyrazinamide (PZA, pyrazin-2-carboxamide) which are important components of the current course of the first-line TB chemotherapy suffer from increasing bacterial resistance. The other drugs of the combination therapy include rifampicin and ethambutol. It may be noted that both INH and PZA are prodrugs and require specific enzymes to convert them into drugs. INH is activated by a bacterial heme enzyme catalase peroxidase (*Mt*CP) into a free radical form (Scheme I) (Zhang et al., 1992). The structure of unliganded *Mt*CP is known (Bertrand et al., 2004) and detailed information is available about the substrate-binding site and the residues that might be involved in the binding and conversion of INH into a beneficial product. However, a precise mode of binding and the mechanism of action are not yet clearly understood because the structure of INH bound *Mt*CP is not yet determined. On the other hand, PZA is metabolized into its active form pyrazinoic acid (POA) by amidase activity of the *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase (PncA) (Scheme II) (Konno et al., 1967). Although the crystal structure of pyrazinamidase in complex with POA is known but the structure of the complex with the original compound PZA is not yet determined. Therefore, the mode of binding of PZA with PncA has not so far been revealed. As shown by the crystal structure of the complex of LPO with INH, the binding of INH to lactoperoxidase (LPO) occurs through the distal heme cavity where INH interacts with a conserved water molecule W1 which is hydrogen bonded to ferric iron (Singh et al., 2010). Similarly, as revealed by the structure determination of the complex formed between LPO and PZA, PZA has been located in the substrate-binding site and interacts with substrate recognition residues of LPO (PDB ID: 3R4X) indicating a possible role of LPO in the conversion of PZA into an active form. Although the crystal structure of the PZA bound PncA is not known but a piece of information is available on the possible mode of ligand binding based on the molecular modeling data (Petrella et al., 2011). Therefore, it is of great interest that both prodrugs, INH and PZA bind to LPO specifically at the substrate-binding site on the distal heme side as the substrates bind to LPO (Singh et al., 2009) so that these

compounds are converted into useful antimicrobial products. Since LPO is able to bind and oxidize both of these compounds, the role of LPO in the treatment of TB appears to be quite plausible. It may be mentioned here that the peroxidase activity of *Mt*CP was shown to be associated with the activation of isoniazid (Zhang et al., 1992). It may also be mentioned here that the role of LPO has already been demonstrated in the bacterial clearance of airways by inhaling INH because LPO and H_2O_2 are present in the mucus of airways (Sawatdee et al., 2006). Thus, understanding the mode of binding of INH and PZA to LPO as well as the mechanisms of action of LPO with respect to these compounds will provide important insights on the possible mode of bindings of INH and PZA to bacterial enzymes *Mt*CP and PncA respectively.



Scheme 1.



Scheme 2.

2. Lactoperoxidase

Lactoperoxidase (EC.1.11. 1.7) (LPO) belongs to the family of mammalian heme peroxidases which also includes myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO). LPO is present in exocrine secretions such as milk, saliva and tears. Although it is produced at different sites in human body by various glands such as mammary, salivary and lachrymal with varying amino acid sequences but these were found to be chemically and immunologically similar (Kussendrager & van Hooijdonk, 2000). The

primary function of LPO is to catalyze the bielectronic oxidation of pseudohalide (SCN^- ion) or halides (I^-, Br^- and Cl^-) to pseudohypohalide ($OSCN^-$ ion) or hypohalides (OI^-, OBr^- and OCl^- ions) **(**Oram & Reiter, 1996; Hoogendoorn et al., 1977) at the expense of hydrogen peroxide (H_2O_2) in order to generate reactive products with a wide range of antimicrobial activities (Reiter & Harnulv, 1984; Reiter & Perraudin, 1991; Wolfson & Sumner, 1993). LPO also catalyzes the bielectronic oxidation (by two 1-electron steps) of a number of physiologically relevant aromatic organic compounds (Ciaccio et al., 2004; Zhang & Dunford 1993; Monzani et al., 1997; Metodiewa et al., 1989; Metodiewa et al., 1989; Ferrari et al., 1993; Doerge & Decker, 1994; Sipe, 1994; Cavalieri et al., 1997; Ghibaudi et al., 2000; Ramakrishna et al., 1993).

The biological significance of lactoperoxidase is related to its involvement in the natural host defense system against invading micro-organisms (Reiter & Harnulv, 1984; Reiter & Perraudin, 1991; Wolfson & Sumner, 1993). Apart from that, it was also reported to be involved in the antiviral activity (Mikola et al., 1995; Pourtois et al., 1990; Shin et al., 2005), degradation of various carcinogens and protection of animal cells against peroxidative effects (Tenovuo et al, 1985). It may be noted that the reaction products generated by the catalytic action of lactoperoxidase are harmless to mammalian cells (Reiter & Harnulv, 1984).

Lactoperoxidase is a heme-containing single chain protein with 595 amino acid residues. Its molecular mass is approximately 68 kDa. LPO is a basic protein with an isoelectric point of 8.2. The carbohydrate content of this protein molecule is about 10% for the four glycosylation sites (Carlstrom, 1969). LPO contains a covalently linked prosthetic group in the catalytic centre which is a derivative of protoporphyrin IX (Thanabal & La Mar, 1989). The iron content of LPO is 0.07% (Paul & Ohlsson, 1985) corresponding to one iron atom per LPO molecule which is a part of the heme prosthetic group. The overall molecular structure of LPO is stabilized by a calcium ion which is strongly bound to LPO molecule through seven-fold coordination.

2.1 Mechanism of action

LPO catalyzes a set of reactions where the resting ferric enzyme (Fe^{3+}) is oxidized rapidly by hydrogen peroxide to form compound I (Kussendrager & van Hooijdonk, 2000), an oxyferryl porphyryl radical species where an oxygen is coupled by a double bond to the iron (Dolphin et al., 1973) which subsequently oxidizes two aromatic substrate molecules as follows:

$$\text{LPO} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + S \rightarrow \text{Compound II} + S^* \quad (2)$$

$$\text{Compound II} + S \rightarrow \text{LPO} + S^* + H_2O \quad (3)$$

Where S is an aromatic substrate and S* is an 1-electron oxidized form of substrate.

3. Structure of lactoperoxidase

Lactoperoxidase folds into an oval-shaped structure which is largely α-helical with 20 α-helices and two small anti-parallel β-strands (Figure 1) (Singh et al., 2008). The central core of the protein consists of five long α-helices, H2, H5, H6, H8 and H12. The N-terminal

segment of LPO does not form any repetitive structure till amino acid residue number 75 from where α-helix H1 starts which is a short helix. It is connected to H2 through a long chain. The α-helix H2 is connected to two unique α-helices H2a and H2b which are absent in MPO (Zeng & Fenna, 1992). These helices are followed by two short α-helices, H3 and H4. The α-helices H5 and H6 are connected through a V-shaped loop which is flanked by two short extended chains. A core α-helix H8 forms a triangle with helices H5 and H6. Heme group is sandwiched between helices H2 and H8. This is connected to the region consisting of helices H12, H13, H14, H15, H16 and parts of helices H17, H18 and H19. This region represents the crown of the back face of the core region. Three helices H2, H5 and H6 also form a triangle below which lies the heme group. The other two core α-helices, H8 and H12 which run parallel to each other and form the lower wall on which the heme moiety rests. The heme moiety is located nearly at the centre of the protein. The δ-heme side is accessible through a channel from the surface of the protein. The structure is stabilized by a calcium ion which forms a seven fold co-ordination. In the structure of LPO, Ser-198 is phosphorylated and seems to facilitate the entry of calcium ion into the core of protein molecule.

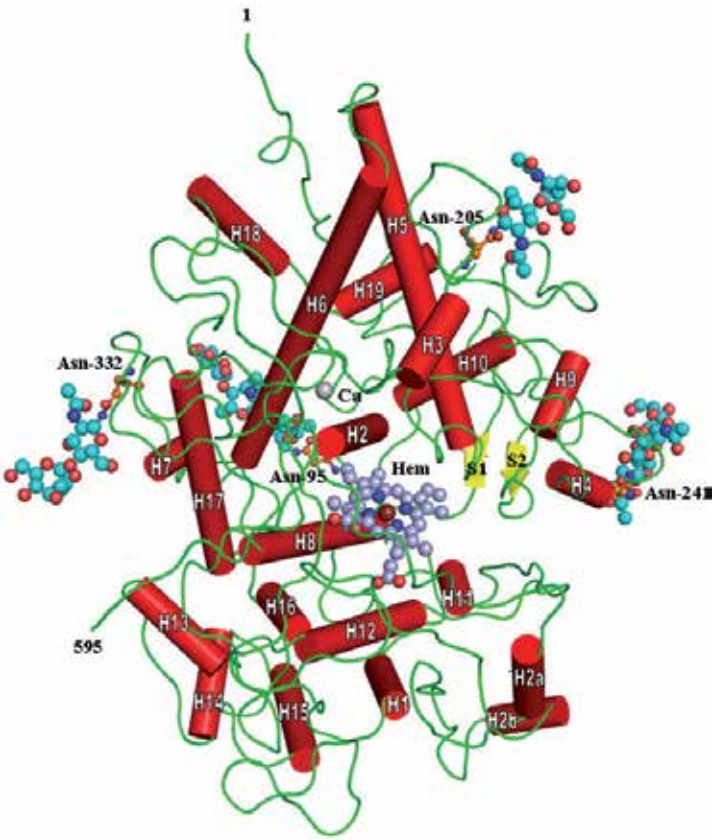


Fig. 1. Molecular structure of lactoperoxidase. Cylinders indicate α-helices which are labelled. β-strands are indicated as arrows. Heme group is shown in the centre as a ball and stick model. The positions of four glycan moieties are also indicated.

3.1 Heme moiety

The heme moiety in LPO is a derivative of protoporphyrin IX (Thanabal & La Mar, 1989) in which the methyl groups on pyrrole rings A and C are modified to allow formation of ester linkages with carboxylic groups of Glu258 and Asp108 respectively (Figure 2).

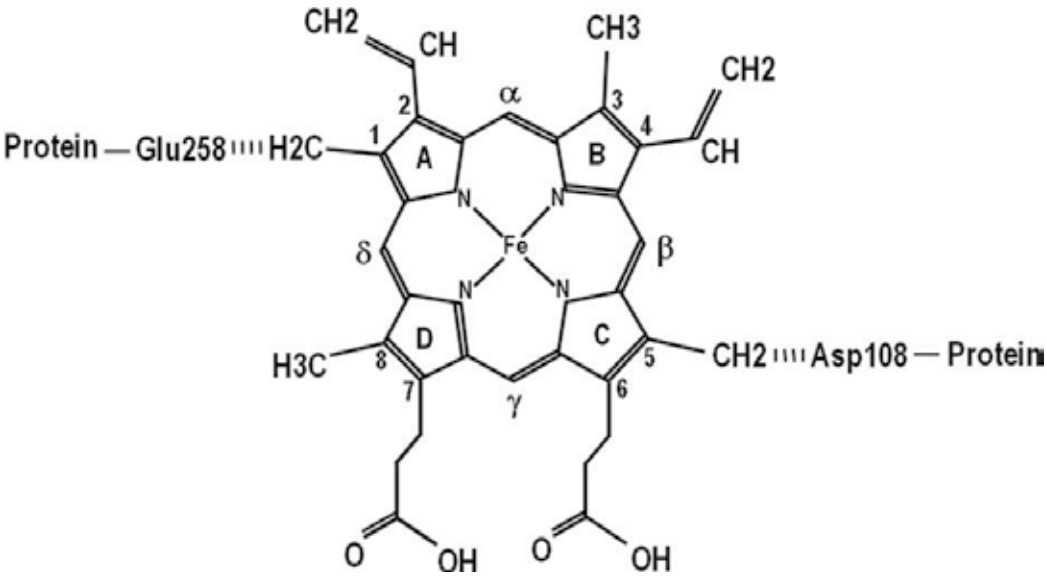


Fig. 2. The structure of the heme moiety showing a standard nomenclature. The two covalent linkages to protein are indicated.

The ferric iron atom is coordinated to four heme nitrogen atoms in a slightly distorted planar arrangement. The fifth coordination is provided by proximal His351 while on the sixth side a conserved water molecule W1 is located at a hydrogen bonded distance from the heme iron. The heme moiety is deeply buried inside the protein molecule while the heme cavity is surrounded by a number of α-helices from three sides. The two β-strands, S1 and S2 are situated on the upper side of the opening to the heme cavity (Figure 1). Overall, the plane of heme protoporphyrin IX moiety is slightly distorted from planarity. The pyrrole rings A, C and D are essentially planar while pyrrole ring B is slightly distorted from planarity. The iron position is shifted slightly towards the proximal side. The carboxyl group of the pyrrole ring D propionate interacts with the guanidinium groups of Arg348 and Arg440. In contrast, the ring C propionate interacts with Asp112 $O^{\delta 2}$, Ala112 N and a water molecule.

3.2 Substrate specificity

The substrate binding site is formed on the distal heme side. In the native structure of LPO, the substrate-binding space is occupied by six water molecules W1, W2′, W3′, W4′¸ W5′ and W6′ (Figure 3). In the resting state, W1 is linked to ferric iron at a hydrogen bonded distance.

When H_2O_2 is supplied, it expels W1 and forms the sixth coordination. On this side, His109 works as proton donor-acceptor residue as it is linked to a chain of water molecules that facilitate proton relay (Figure 4). When ligands bind to LPO in the substrate binding site on the distal heme side, it plays an essential role in the enzymatic action. The substrate-binding site is surrounded by heme moiety on one side while residues, His109, Phe113, Phe254 and Arg255 occupy the opposite side. The front end of the site consists of Gln105 while Glu258 supports it from below. The other wall is made up of residues, Phe381, Phe422, Gln423 and Pro424. The substrate-binding site is connected by a long channel formed by hydrophobic aromatic residues including Pro234, Pro236, Phe380, Phe381 and Phe254 on one side while Leu421, Phe422, Gln423 and Pro424 on the opposite (Figure 5). The length of substrate diffusion channel in LPO is approximately 22Å while its diameter is about 10Å. The substrate-binding site is connected to the surface of the protein through this diffusion channel.

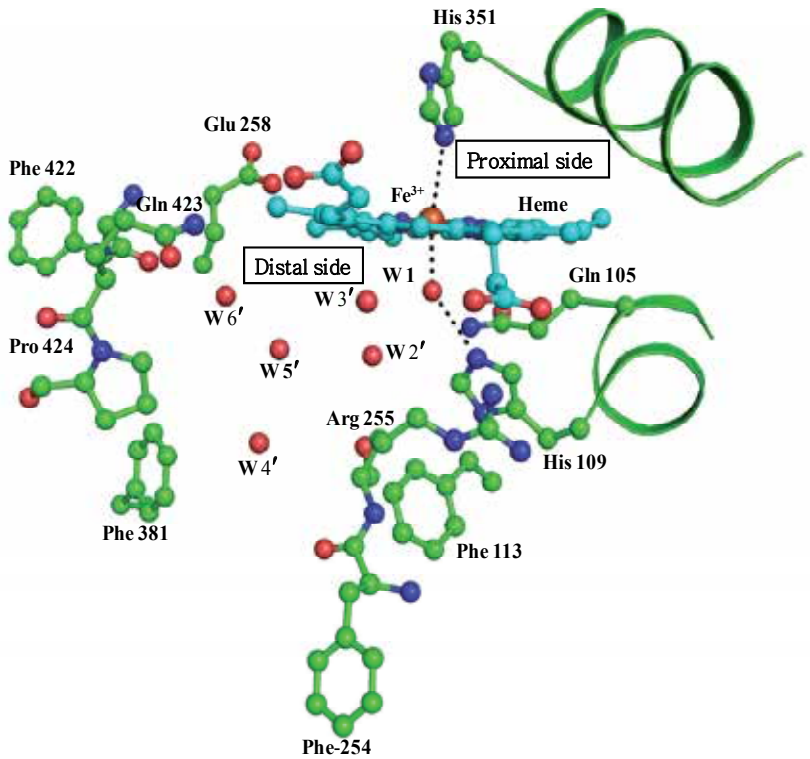


Fig. 3. The structure of the substrate-binding site on the distal heme side in LPO and the positions of six conserved water molecules, W1, W2′, W3′, W4′¸ W5′ and W6′ as observed in the unliganded structure. The proximal and distal sites have been indicated.

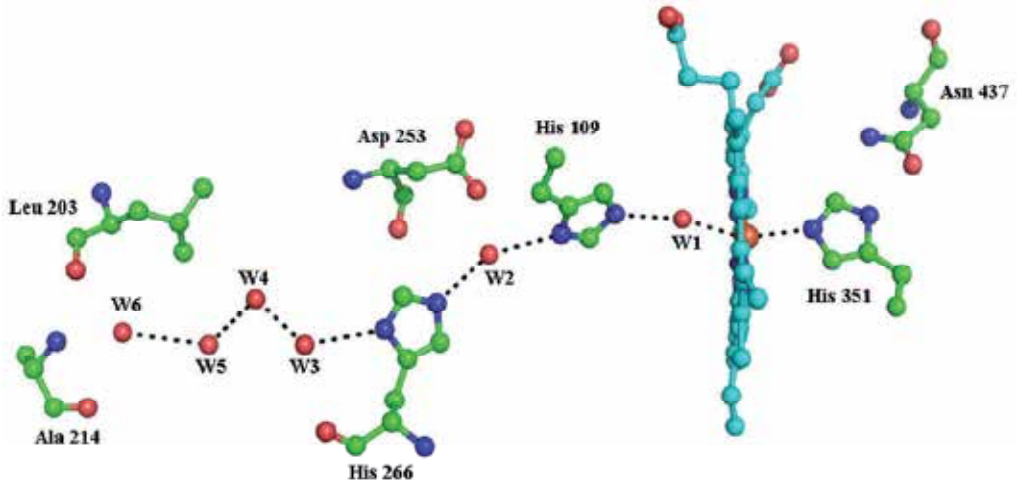


Fig. 4. Hydrogen bonded chain involving His351, Fe^{3+}, W1, His109, W2, His266, W3, W4, W5 and W6 where W1 is hydrogen bonded to Fe^{3+} and W6 is located near the protein surface.

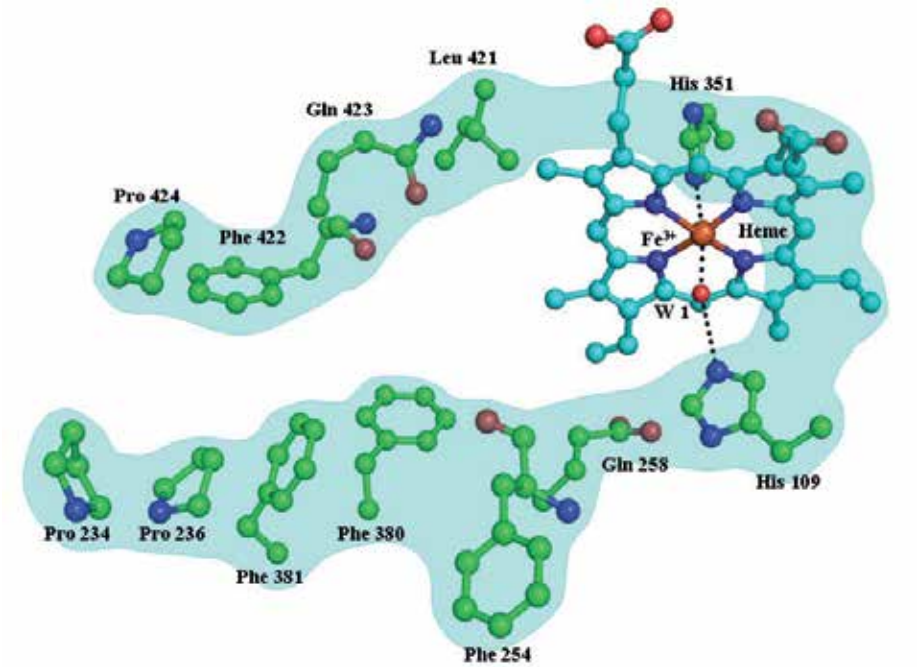


Fig. 5. A schematic representation of the diffusion channel and substrate-binding site in LPO drawn using PDB-ID: 3GC1.

3.3 Specificity for aromatic compounds

The substrate diffusion channel in LPO is long and surrounded by aromatic residues from all sides. As a result it allows the diffusion of small aromatic compounds from surface to the substrate binding site on the distal heme side. The aromatic compounds such as acetyl

salicylic acid (ASA) and salicylhydroxamic acid (SHA) have been shown to bind to LPO at the substrate-binding site (Figure 6) (Singh et al., 2009). It has been observed that the aromatic compounds which bind to LPO but do not expel the conserved water molecule, W1 from its original position as has been observed in the case of acetyl salicylic acid act as substrates (Figure 6A) while those that expel the conserved water molecule, W1 and coordinate directly with the heme iron act as inhibitors as found in the case of salicylhydroxamic acid (Figure 6B). Therefore, the mode of binding of substrates and inhibitors differ in terms of their positioning in the substrate-binding site.

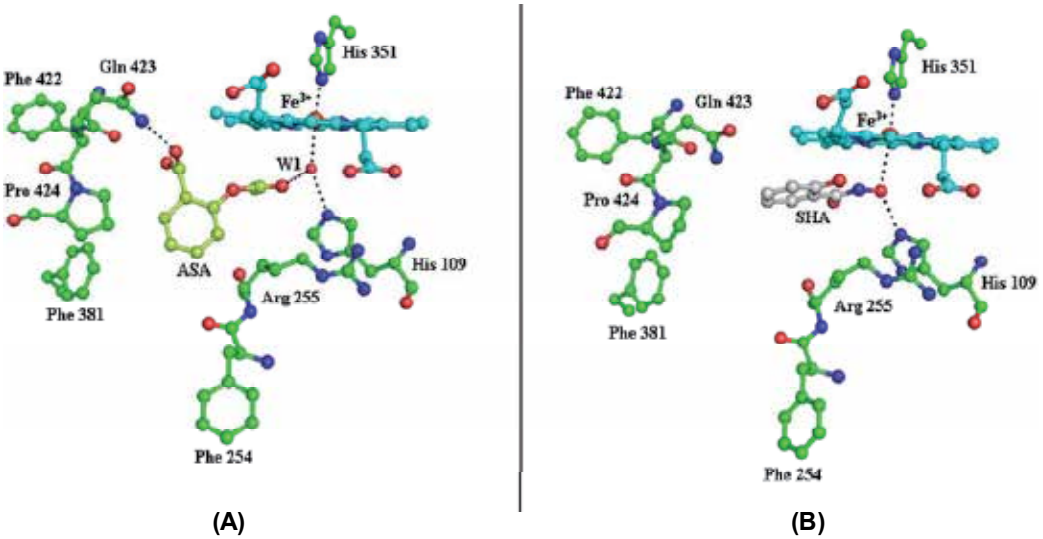


Fig. 6. (A) Acetyl salicylic acid (ASA) bound to LPO as a substrate at the substrate-binding site (PDB-ID: 3GCL). The hydrogen bonded interactions are indicated by dotted lines. (B) Salicylhydroxamic acid (SHA) bound to LPO as an inhibitor in the substrate-binding site (PDB-ID: 3GCJ). The hydrogen bonded interactions are indicated by dotted lines.

3.4 Structure of the LPO complex with Isoniazid

Since LPO has been shown to bind to small aromatic compounds, the binding properties of LPO with INH were examined using surface plasmon resonance technique. The protein molecule was immobilized on the chip while INH was used as a solution in a mobile phase. These measurements gave a value of 1.1×10^{-6} M for the dissociation constant (Kd) for the binding of LPO with INH. In order to determine the interactions between LPO and INH, the crystal structure of the INH-bound LPO was determined (Singh et al., 2010). The structure of the LPO-INH complex showed that INH upon binding to LPO in the substrate-binding site on the distal heme side expelled four water molecules W2′, W4′, W5′ and W6′ from the substrate-binding site on the distal heme side. Two out of six conserved water molecules, W1 and W3′ remained unperturbed. INH binds to LPO with an appropriate orientation as its pyridine ring nitrogen atom is at a hydrogen bonded distance from the conserved water molecule W1 (Figure 7). Therefore, when H_2O_2 is introduced into the solution containing LPO and INH, it expels the water molecule W1 and the reaction gets initiated. The product isonicotinyl radical is formed as described by

equations 1 to 3. This product is similar to that produced by *Mycobacterium tuberculosis* catalase peroxidase (*Mt*CP) (Bertrand et al., 2004).

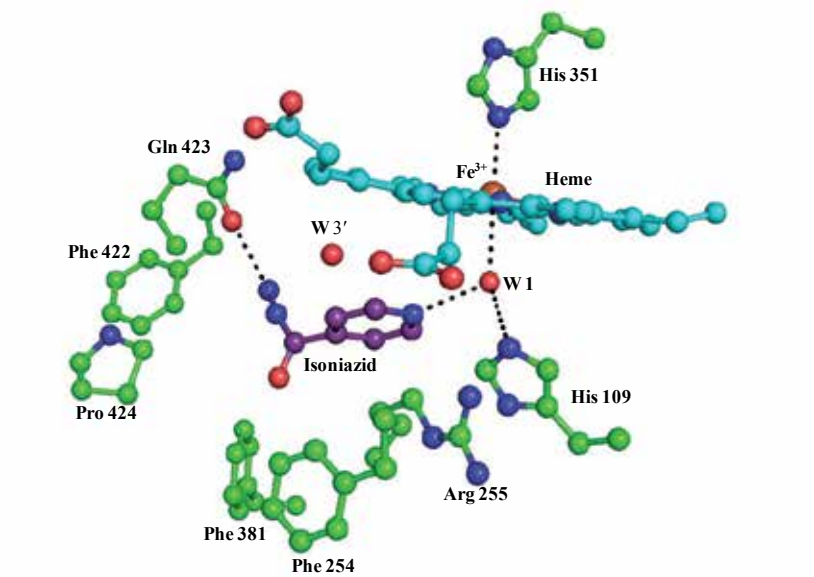


Fig. 7. Showing binding of INH to LPO as a substrate. The dotted lines indicate hydrogen bonds.

3.5 Complex of LPO with Pyrazinamide

As revealed by binding studies using surface Plasmon resonance technique, pyrazinamide (PZA) has also been found to bind to LPO with a slightly lower affinity than that of INH (dissociation constant, Kd = 1.2×10^{-5} M). The structure determination of PZA-bound LPO revealed that it occupies a position in the centre of the substrate-binding site on the distal heme side. Upon binding to pyrazinamide, three water molecules, W4′, W5′ and W6′ were expelled from the substrate-binding site. It retained three water molecules, W1, W2′ and W3′. The nearest nitrogen atom of PZA is about 5.5Å away from the oxygen atom of conserved water molecule W1. PZA and conserved water molecule W1 are separated from each other by another water molecule W2′. The carboxamide nitrogen atom of PZA forms a hydrogen bond with W2′ which in turn is hydrogen bonded at W1 (Figure 8). It reflects a slightly weaker affinity of PZA towards the position of W1. However, when H_2O_2 is introduced, it is expected to move closer to H_2O_2 and the product may be formed. The position occupied by PZA in the substrate-binding site appears to be suitable for the catalytic action by LPO. However, the nature of product is not characterized clearly.

3.6 Structure of catalase peroxidase

Mycobacterium tuberculosis catalase peroxidase (*Mt*CP) is a dimeric bi-functional heme-dependent enzyme of molecular mass of 160 kDa. Its primary function is of catalase activity. However, its role as a peroxidase is well established and its peroxidative activity is comparable with those of other mono-functional heme peroxidases (Metcalfe et al., 2008;

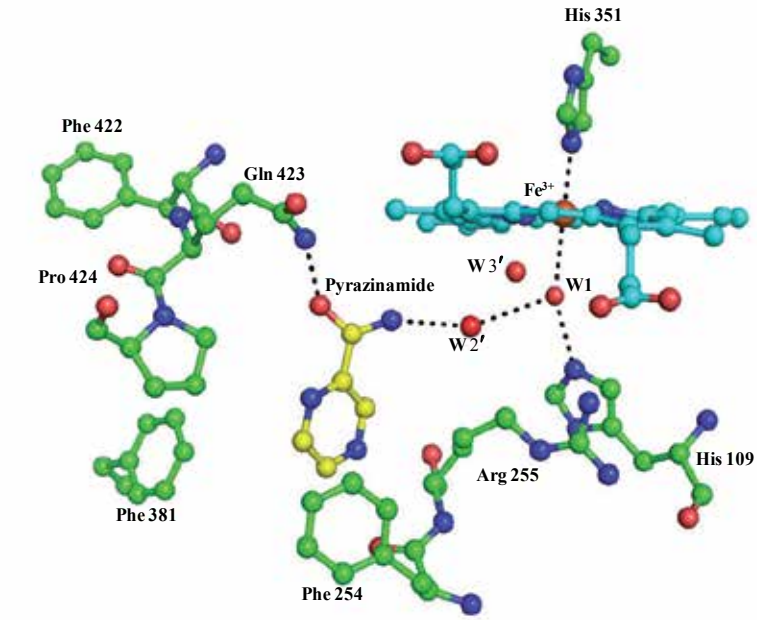


Fig. 8. Binding of PZA to LPO as a substrate in the substrate-binding site (PDB-ID: XYZ). The dotted lines indicate hydrogen bonded interactions.

Pierattelli et al., 2004). Sofar, crystal structure of only unliganded-*Mt*CP has been determined (Bertrand et al., 2004). However, modeling and computational studies have indicated the binding of INH at the δ-meso heme edge which superseded the existing proposition of it being in a surface loop structure of the enzyme (Mo et al., 2004). Yet another important observation pertains to the role of residue Ser315 in the binding of INH. It was suggested that Ser315 might interact with INH but it would not be involved in the catalytic action. It was further indicated that His108 on the distal heme side was involved in enzyme catalytic action (Figure 9). A comparison of the binding of INH with LPO where Gln423 forms a hydrogen bond with amino nitrogen atom of the hydrazide moiety. However, it is not responsible for the catalytic action as it is important for promoting an appropriate orientation of the substrate. On the other hand, it has been shown that His109 plays the role of proton donor/acceptor as it forms two hydrogen bonds, one with pyridine ring nitrogen atom of INH and another with conserved water molecule W1. These modes of binding of INH show a striking similarity in the substrate-binding sites on the distal heme side of the two enzymes, *Mt*CP and LPO indicating similar mechanisms of actions.

3.7 Complex of pyrazinamidase with PZA

Pyrazinamidase (PncA) activates PZA into POA. The crystal structure of *Mycobacterium tuberculosis* pyrazinamidase has been determined (Petrella et al., 2011) which shows that PncA folds into an α/β single domain protein. It has an iron binding site involving residues Asp49, His51, His57 and His71 and consists of a catalytic triad with residues Cys138, Asp8 and Lys96. The substrate binding cavity in pyrazinamidase is a part of the cleft which is shown schematically in Figure 10. The amino acid residues of the active site are located on

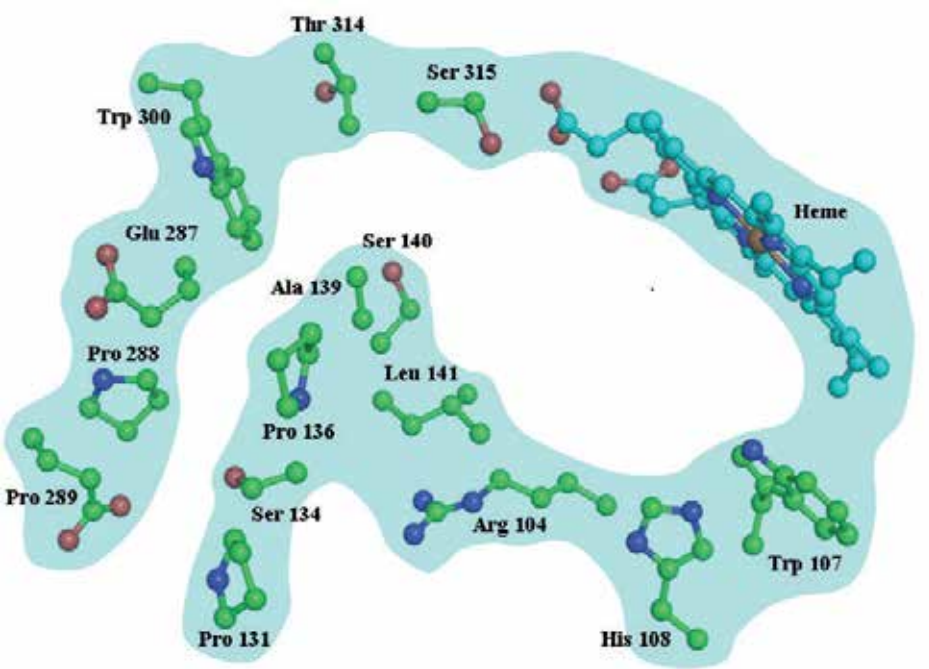


Fig. 9. Schematic representation of the diffusion channel and substrate-binding site in *Mt*CP (PDB-ID: 1SJ2). Ser315 as Gln423 in LPO is involved in the interaction with INH while His108 as His109 in LPO is involved in the catalytic mechanism.

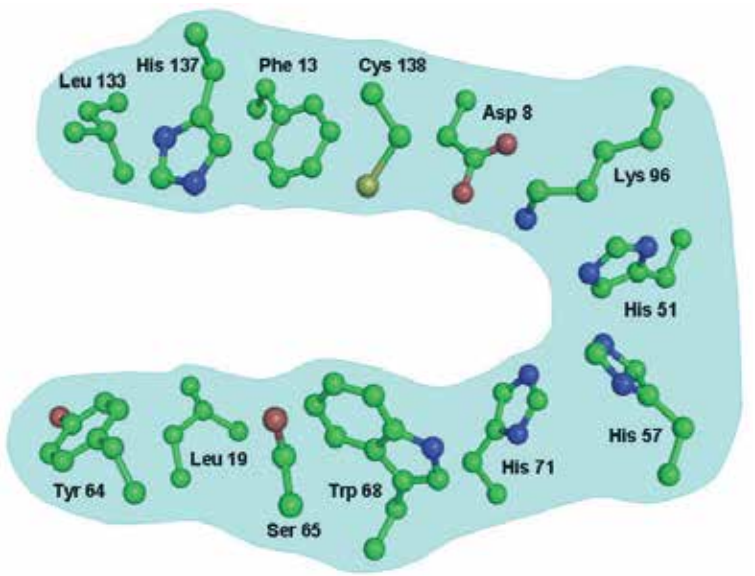


Fig. 10. Schematic representation for the binding site in PncA (PDB-ID: 3PL1) showing significant similarities to those of LPO and *Mt*CP.

one of the cleft. Although crystal structure of PZA-bound PncA is not known but it is suggested that the binding of PZA involves hydrogen bonded interaction between pyridyl nitrogen atom of PZA and the conserved water molecule W1 which is coordinated to Fe^{2+} ion. Another possible interaction is provided by carbonyl oxygen atom of PZA with peptide Ala134 – Cys138. Overall, the role played by Fe^{2+} ion and His57 is critical in stabilizing the structure of substrate-binding site. Finally the conversion of PZA to POA and its accumulation results in lowering the intracellular pH to suboptimal level and thus causing the inactivation of some of the critically important proteins such as fatty acid synthase resulting in the killing of bacteria (Zimhony et al., 2000).

4. Discussion

Both prodrugs INH and PZA bind to LPO in the substrate-binding site on the distal heme side. In the unliganded structure of LPO, the space of the substrate-binding site is filled with six water molecules, W1, W2′, W3′, W4′¸ W5′ and W6′. Upon binding to INH, four water molecules W2′, W4′¸ W5′ and W6′ are expelled from the site. In the structure of the complex of LPO with PZA, three water molecules W4′, W5′ and W6′ are displaced. The conserved water molecule W1 occupies a position on distal heme side in the centre between the positions of heme-iron and $N^{\delta 2}$ atom of His109. His109 is linked to a chain of six other conserved water molecules, W1, W2, W3, W4¸ W5 and W6 together with His266 and Asp253 residues interlinked between them. The pyridine ring nitrogen atom is hydrogen bonded to W1 which in turn is hydrogen bonded to $N^{\delta 2}$ of His109 and heme iron. The position and orientation of INH are fixed in the substrate-binding site because of a hydrogen bonded interaction between amino nitrogen atom of INH and $N^{\varepsilon 2}$ of Gln423. The position of PZA also fixed in the substrate-binding site on the distal heme side where amino nitrogen atom of PZA forms a hydrogen bond with water molecule W2′ which in turn is hydrogen bonded to the conserved water molecule W1. As in the complex of LPO with INH, W1 is hydrogen bonded to heme iron and His109 $N^{\delta 2}$. On the other side carbonyl oxygen atom of PZA is hydrogen bonded to Gln423 $N^{\varepsilon 2}$. However, unlike INH where pyridine nitrogen atom is hydrogen bonded to W1, in this case carboxamide nitrogen atom is hydrogen bonded to W1 via another water molecule W2′. The main difference between the two complexes is that the INH molecule interacts directly with heme water molecule W1 while PZA binds to heme water molecule W1 via another water molecule W2′.

As far as the ligand binding sites are concerned all the three enzymes, LPO, *Mt*CP and PncA show considerable similarities with strong preferences for the binding of small aromatic compounds. There is a clear evidence that INH is converted in a similar manner into active form by both LPO and *Mt*CP indicating a direct role of LPO in the treatment of TB. Similarly, PZA also makes a good substrate for LPO which can be converted into active form as an antibacterial agent. However, the final active forms produced by LPO and PncA may not be same and the mechanism of action may be different. Nevertheless, LPO seems to have a role in the treatment of tuberculosis through its interactions with INH and PZA and it should be exploited.

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6. References

Bertrand, T., Eady, N. A., Jones, J. N., Jesmin, Nagy, J. M., Jamart-Gregoire, B., Raven, E. L., & Brown, K. A. (2004). Crystal structure of Mycobacterium tuberculosis catalase-peroxidase. *Journal of Biological Chemistry*, Vol.279, No.37, (September 2004), pp. 38991-38999, ISSN 0021-9258

Carlstrom, A. (1969). Lactoperoxidase: Identification of multiple molecular forms and their interrelationships. *Acta Chemica Scandinavica*, Vol.23, No.1, pp. 171-184, ISSN 0001-5393

Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K., Ramanathan, R., Cerny, R. L. & Rogan, E. G. (1997). Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proceedings of the National Academy of Sciences, USA*, Vol.94, No.20, (September 1997), pp. 10937-10942, ISSN 0027-8424

Ciaccio, C., De Sanctis, G., Marini, S., Sinibaldi, F., Santucci, R., Arcovito, A., Bellelli, A., Ghibaudi, E., Ferrari, R. P., & Coletta, M. (2004). Proton linkage for CO binding and redox properties of bovine lactoperoxidase. *Biophysical Journal*, Vol.86, No.1 Pt 1, (January 2004), pp. 448-454, ISSN 0006-3495

Doerge, D. R. & Decker, C. J. (1994). Inhibition of peroxidase-catalyzed reactions by arylamines: mechanism for the anti-thyroid action of sulfamethazine. *Chemical Research in Toxicology*, Vol.7, No.2, (March-April 1994), pp. 164-169, ISSN 0893-228X

Dolphin, D., Muljiani, Z., Rousseau, K., Borg, D. C., Fajer, J., & Felton, R. H. (1973). The chemistry of porphyrin pi-cations. *Annals of the New York Academy of* Sciences, Vol.206, (October 1973), pp. 177-200, ISSN 0077-8923

Ferrari, R. P., Laurenti, E., Casella, L., & Poli, S. (1993). Oxidation of catechols and catecholamines by HRP and LPO: ESR spin stabilization approach combined with optical methods. *Spectrochimica Acta*, Vol.49A, pp. 1261–1267, ISSN 1386-1425

Ghibaudi, E. M., Laurenti, E., Beltramo, P. & Ferrari, R. P. (2000). Can estrogenic radicals, generated by lactoperoxidase, be involved in the molecular mechanism of breast carcinogenesis? *Redox Report*, Vol.5, No.4, pp. 229-235, ISSN 1351-0002

Hoogendoorn, H., Piessens, J. P., Scholtes, W., & Stoddard, L. A. (1977) Hypothiocyanite ion; the inhibitor formed by the system lactoperoxidase-thiocyanate-hydrogen peroxide. I. Identification of the inhibiting compound. *Caries Research*, Vol.11, No.2, pp. 77-84, ISSN 0008-6568

Konno, K., Feldmann, F. M., & McDermott, W. (1967). Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *American Review of Respiratory Diseases*, Vol.95, (March 1967), pp 461-469, ISSN 0003-0805

Kussendrager, K. D, & van Hooijdonk, A. C. (2000). Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *British Journal of Nutrition*, Vol.84, Suppl 1, (November 2000), pp. S19-25, ISSN 0007-1145

Metcalfe, C., Macdonald, I. K., Murphy, E. J., Brown, K. A., Raven, E. L., & Moody, P. C. (2008).The tuberculosis prodrug isoniazid bound to activating peroxidases. *Journal of Biological Chemistry*, Vol.283, No.10 (March 2008), pp. 6193-6200, ISSN 0021-9258

Metodiewa, D., Reszka, K. & Dunford, H. B. (1989). Evidence for a peroxidatic oxidation of norepinephrine, a catecholamine, by lactoperoxidase. *Biochemical and Biophysical Research Communications*, Vol.160, No.3, (May 1989), pp.1183-1188, ISSN 0006-291X

Metodiewa, D., Reszka, K. & Dunford, H. B. (1989). Oxidation of the substituted catechols dihydroxyphenylalanine methyl ester and trihydroxyphenylalanine by lactoperoxidase and its compounds. *Archives of Biochemistry and Biophysics*, Vol.274, No.2, (November 1989), pp. 601-608, ISSN 0003-9861

Mikola, H., Waris, M., & Tenovuo, J. (1995). Inhibition of herpes simplex virus type 1, respiratory syncytial virus and echovirus type 11 by peroxidase-generated hypothiocyanite. *Antiviral Research*, Vol.26, No.2, (March 1995), pp. 161-171, ISSN 0166-3542

Mo, L., Zhang, W., Wang, J., Weng, X. H., Chen, S., Shao, L. Y., Pang, M. Y. & Chen, Z. W. (2004). Three-dimensional model and molecular mechanism of Mycobacterium tuberculosis catalase-peroxidase (KatG) and isoniazid-resistant KatG mutants. *Microbial Drug Resistance*, Vol.10, No.4, 269-279, ISSN 1076-6294

Monzani, E., Gatti, A. L., Profumo, A., Casella, L. & Gullotti, M. (1997). Oxidation of phenolic compounds by lactoperoxidase. Evidence for the presence of a low-potential compound II during catalytic turnover. *Biochemistry*, (February 1997), Vol.36, No.7, pp. 1918-1926, ISSN 0006-2960

Oram, J. D., & Reiter, B. (1966). The inhibition of streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide. The oxidation of thiocyanate and the nature of the inhibitory compound. *Biochemical Journal*, (August 1966), Vol.100, No.2, pp. 382-388, ISSN 0264-6021

Paul, K. G., & Ohlsson, P. I. (1985). The chemical structure of lactoperoxidase, In: *The Lactoperoxidase System, Chemistry and Biological Significance*, Pruitt, K. M., Tenovuo, J. O. (ed.), pp. 15-29, Marcel Dekker, New York, ISBN 0824772989

Petrella, S., Gelus-Zietal, N., Maudry, A., Laurans, C., Boudjelloul, R., & Sougakoff, W. (2011). Crystal structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural and acquired resistance to pyrazinamide. *PLoS One*, Vol.6, No.1, (January 2011), pp. e15785, ISSN 1932-6203

Pierattelli, R., Banci, L., Eady, N. A., Bodiguel, J., Jones, J. N., Moody, P. C., Raven, E. L., Jamart-Gregoire, B., & Brown, K. A. (2004). Enzyme-catalyzed mechanism of isoniazid activation in class I and class III peroxidases. *Journal of Biological Chemistry*, Vol.279, No.37, (September 2004), pp. 39000-39009. ISSN 0021-9258

Pourtois, M., Binet, C., Van Tieghem, N., Courtois, P., Vandenabbeele, A., Thiry, L. (1990). Inhibition of HIV infectivity by lactoperoxidase-produced hypothiocyanite. *Journal de biologie buccale*, Vol.18, No.4, (December 1990), pp. 251-253. ISSN 0301-3952

RamaKrishna, N. V., Li, K. M., Rogan, E. G., Cavalieri, E. L., George, M., Cerny, R. L. & Gross, M. L. (1993). Adducts of 6-methylbenzo[a]pyrene and 6-fluorobenzo[a]pyrene formed by electrochemical oxidation in the presence of

deoxyribonucleosides. *Chemical Research in Toxicology*, Vol.6, No.6, (November-December 1993), pp. 837-845, ISSN 0893-228X

Reiter, B., & Harrnulv, G. (1984). Lactoperoxidase antibacterial system: natural occurrence, biological functions and practical applications. *Journal of Food Protection*, Vol.47, (Month 1984), pp. 724-732, ISSN 0362-028X

Reiter, B., & Perraudin, J. P. (1991). Lactoperoxidase: biological functions. In: *Peroxidases in Chemistry and Biology*. pp. 143-180, Boca Raton: CRC Press, ISBN 0849369630

Sawatdee, S., Worakul, N., & Srichana, T. (2006) Preparation of isoniazid as dry powder formulations for inhalation by physical mixing and spray drying. *Malaysian Journal of Pharmaceutical Sciences*, Vol.4, No.1, pp. 43-63, ISSN 2180-429X

Shin, K., Wakabayashi, H., Yamauchi, K., Teraguchi, S., Tamura, Y., Kurokawa, M. & Shiraki, K. (2005). Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice. *Journal of Medical Microbiology*, Vol.54, No.8, (August 2005), pp. 717-723, ISSN 0022-2615

Singh, A. K., Kumar, R. P., Pandey, N., Singh, N., Sinha, M., Bhushan, A., Kaur, P., Sharma, S., & Singh, T. P. (2010) Mode of binding of the tuberculosis prodrug isoniazid to heme peroxidases: binding studies and crystal structure of bovine lactoperoxidase with isoniazid at 2.7 Å resolution. *Journal of Biological Chemistry,* Vol.285, No.2, (January 2010), pp. 1569-1576, ISSN 0021-9258

Singh, A. K., Singh, N., Sharma, S., Singh, S. B., Kaur, P., Bhushan, A., Srinivasan, A. & Singh, T. P. (2008). Crystal structure of lactoperoxidase at 2.4 Å resolution. *Journal of Molecular Biology*, Vol.376, No.4, (February 2008), pp. 1060-1075, ISSN 0022-2836

Singh, A. K., Singh, N., Sinha, M., Bhushan, A., Kaur, P., Srinivasan, A., Sharma, S. & Singh, T. P. (2009). Binding modes of aromatic ligands to mammalian heme peroxidases with associated functional implications: crystal structures of lactoperoxidase complexes with acetylsalicylic acid, salicylhydroxamic acid, and benzylhydroxamic acid. *Journal of Biological Chemistry,* Vol.284, No.30, (July 2009), pp. 20311-20318, ISSN 0021-9258

Sipe, H. J., Jr., Jordan, S. J., Hanna, P. M. & Mason, R. P. (1994). The metabolism of 17 beta-estradiol by lactoperoxidase: a possible source of oxidative stress in breast cancer. *Carcinogenesis*, (November 1994), Vol.15, No.11, pp. 2637-2643, ISSN 0143-3334

Tenovuo, J., Makinen, K. K. & Sievers, G. (1985). Antibacterial effect of lactoperoxidase and myeloperoxidase against Bacillus cereus. *Antimicrobial Agents and Chemotherapy*, (January 1985) Vol.27, No.1, pp. 96-101, ISSN 0066-4804

Thanabal, V., & La Mar, G. N. (1989). A nuclear Overhauser effect investigation of the molecular and electronic structure of the heme crevice in lactoperoxidase. *Biochemistry*. Vol.28, No.17, (August 1989), pp. 7038-7044, ISSN 0006-2960

Wolfson, L. M., & Sumner, S. S. (1993). Antibacterial activity of the lactoperoxidase system: a review. *Journal of Food Protection*, Vol.56, pp. 887-892, ISSN 0362-028X

Zeng, J., & Fenna, R. E. (1992). X-ray crystal structure of canine myeloperoxidase at 3 Å resolution. *Journal of Molecular Biology*, Vol.226, No.1, (July 1992), pp. 185-207, ISSN 0022-2836

Zhang, H., and Dunford, H. B. (1993). Hammett pσ correlation for reactions of lactoperoxidase compound II with phenols. *Canadian Journal of Chemistry*, Vol.71, No.12, pp. 1990-1994, ISSN 0008-4042

Zhang, Y., Heym, B., Allen, B., Young, D., & Cole, S. (1992). The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature*, Vol.358, No.6387, (August 1992), pp. 591-593, ISSN 0028-0836

Zimhony, O., Cox, J. S., Welch, J. T., Vilcheze, C., & Jacobs, W. R. Jr. (2000). Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. *Nature Medicine*, Vol.6, No.9, (September 2000), pp. 1043-1047, ISSN 1061-4036

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Thiol-Dependent Peroxidases in *Mycobacterium tuberculosis* Antioxidant Defense

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1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is the causative agent of tuberculosis disease. According to the Global Tuberculosis Control 2010 report of the World Health Organization http://www.who.int/tb/publications/global_report/en/, approximately one-third of the world's population is latently infected with *M. tuberculosis* and about two million people die of this disease every year. The emergence of multi- and extensively- drug resistant strains to the currently available drugs makes the development of new therapeutic strategies a priority. However, the mechanisms underlying pathogenesis, virulence and persistence of infections caused by *M. tuberculosis* are not completely understood (Nathan, 2009; Lawn & Zumla, 2011).

M. tuberculosis is one of the most successful human pathogens. It has evolved diverse strategies to ensure growth and survival inside the hostile environment of macrophages, its primary host cells (Ehrt & Schnappinger, 2009; Meena & Rajni, 2010). The molecular mechanisms of *M. tuberculosis* pathogenesis are under active investigation, since they could provide the basis for a rationalized drug design. These include inhibition of phagosome maturation into phagolysosomes (Armstrong & Hart, 1971; MacMicking *et al*., 2003), inhibition of the acidification of *Mycobacterium*-harboring phagosomes (Sturgill-Koszycki *et al*., 1994), DNA repair and protein repair or degradation (Boshoff *et al*., 2003; Gandotra *et al*., 2007; Lee *et al*., 2009), as well as decomposition of cytotoxic reactive nitrogen and oxygen species formed upon phagocytosis (Nathan & Shiloh, 2000; Shiloh & Nathan, 2000; Bedard & Krause, 2007). These should be considered as complementary survival mechanisms. Herein, we will focus in the antioxidant systems of *M. tuberculosis*, and particularly, in thiol-dependent peroxidases.

2. Formation of reactive oxygen and nitrogen species by activated macrophages

Upon phagocytosis, NADPH oxidase (NADPHox) assembles into an enzymatically active complex that transfers electrons from NADPH to molecular oxygen producing superoxide

anion radical ($O_2^{\bullet-}$) inside the phagosomes (Babior, 1984; Groemping & Rittinger, 2005). The charged nature of this radical at physiological pH (hydroperoxyl radical ($HO_2^{\bullet}$) pK_a = 4.75, (Blelski & and Allen, 1977)) determines its low diffusion capability through membranes. In turn, INFγ-mediated induction of iNOS leads to the formation of nitric oxide ($^{\bullet}NO$), a small lipophilic moiety that can diffuse into the phagosome (Xie *et al.*, 1993; Martin *et al.*, 1994; MacMicking *et al.*, 1997). $O_2^{\bullet-}$ can spontaneously or enzymatically dismutate into hydrogen peroxide (H_2O_2)(De Groote *et al.*, 1997; Fridovich, 1997). Reactions of the latter species with reduced transition metal centers (particularly containing Fe^{2+} or Cu^{+}) yield the strong and non-selective oxidizing compound, hydroxyl radical ($^{\bullet}OH$) through Fenton reactions. Moreover, the diffusion-controlled reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$ forms peroxynitrite[1], an

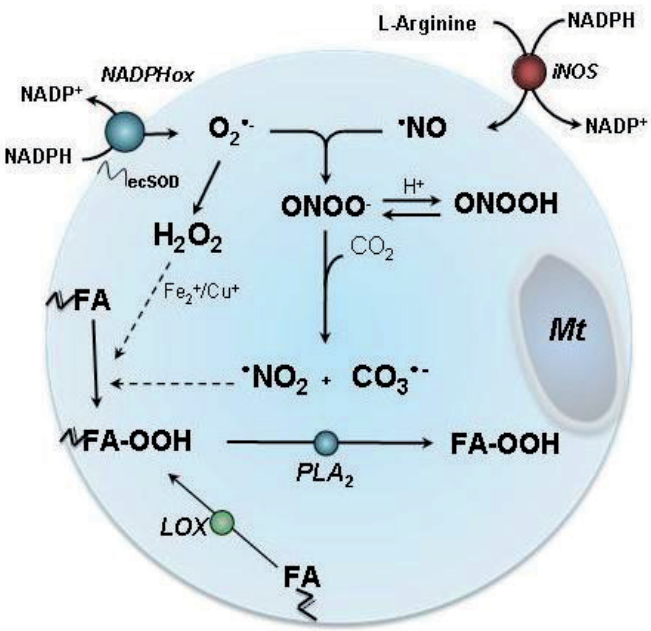

Fig. 1. **Peroxide sources in *M. tuberculosis* (*Mt*)-harboring phagosomes of activated macrophages.** Details on the pathways leading to the production of peroxides (H_2O_2, peroxynitrite and fatty acid hydroperoxides (FA-OOH), in bold) among other reactive nitrogen and oxygen species are given in the text. Dashed lines indicate reactions involving several steps and intermediates.

[1] IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH.

oxidizing and nitrating moiety (Ferrer-Sueta & Radi, 2009; Alvarez *et al.*, 2011). In the absence of direct targets, peroxynitrous acid (pK_a = 6.5-6.8, (Goldstein & Czapski, 1995; Pryor & Squadrito, 1995; Kissner *et al.*, 1997)) homolyses into nitrogen dioxide ($^{\bullet}NO_2$) and $^{\bullet}OH$ in 30% yields (k = 0.9 s^{-1} at pH 7.4 and 37 ^{o}C) (Goldstein & Czapski, 1995; Gerasimov & Lymar, 1999). However, the importance of this reaction *in vivo* is probably limited, since in cells, most peroxynitrite is expected to be involved in direct reactions. For instance, peroxynitrite can react with carbon dioxide (CO_2) present in mM concentrations in biological systems (k = 4.6 x 10^4 M^{-1} s^{-1} at pH 7.4 and 37 ^{o}C), leading to the formation of up to 35% carbonate ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ radicals, which are also oxidizing species (Lymar & Hurst, 1995; Denicola *et al.*, 1996; Bonini *et al.*, 1999; Augusto *et al.*, 2002). $^{\bullet}OH$ and $^{\bullet}NO_2$ can participate in lipid peroxidation reactions, resulting in fatty acid hydroperoxide formation (Barber & Thomas, 1978; Prutz *et al.*, 1985). These can also be synthesized by enzymatic mechanisms through lipoxygenase (LOX)-catalyzed reactions (Sevanian *et al.*, 1983). Fatty acid hydroperoxides can be released from membranes by the action of phospholipase A_2 (PLA_2)(Bonney *et al.*, 1985). Free arachidonic acid is toxic for *M. tuberculosis* acting in a synergistic way with reactive nitrogen species (Akaki *et al.*, 2000). Although the mechanism of synergism has not been resolved, the fact that free fatty acid- dependent toxicity to *Helicobacter pylori* increases in peroxidase-deficient strains indicates that fatty acid hydroperoxides could participate in cytotoxicity (Wang *et al.*, 2006). In summary, inside the phagosomes of activated macrophages, and among other reactive species, different peroxides can be formed, including H_2O_2, peroxynitrite and fatty acid hydroperoxides (Figure 1). All of these species have been reported to be cytotoxic against microorganisms including bacteria (Clifford & Repine, 1982; Denicola *et al.*, 1993; Hurst & Lymar, 1997; Evans *et al.*, 1998; Wang *et al.*, 2006). The enzymatic mechanisms that allow reactive nitrogen and oxygen species detoxification, in general, and peroxide reduction, in particular, thus enabling the bacterium to infect and persist inside the phagosome of activated macrophages, is a field of active investigation.

3. Singular aspects of the antioxidant defense systems of *M. tuberculosis*

The antioxidant defenses of *Mycobacterium tuberculosis* are unusual in many aspects. As most other Actinobacteria, it lacks glutathione, and contains millimolar concentration of 1-D-myo-inosityl-2-deoxy-2-(N-acetyl-L-cysteinyl)amino-D-glucopyranoside, or mycothiol (MSH), as main low molecular weight thiol (Newton & Fahey, 2002). MSH is maintained in the reduced form by mycothione reductase using NADPH as electron donor (Patel & Blanchard, 2001). It participates in drug detoxification pathways by forming adducts with alkylating agents and antibiotics that are subsequently cleaved by MSH S-conjugate amidase to generate a mercapturic acid (excreted outside the cell) and glucosamine inositol (used to regenerate MSH) (Newton *et al.*, 2000). MSH can function as a resource for metabolic precursors and for energy production (Bzymek *et al.*, 2007). Mycothiol-deficient *M. smegmatis* strains are more sensitive to $^{\bullet}NO$- and H_2O_2-mediated toxicity than wild type strains (Rawat *et al.*, 2002; Miller *et al.*, 2007). However, there is currently no evidence for MSH acting as a reducing substrate for any peroxidase. Mycobacteria, among other organisms, also synthesize ergothioneine, which is a thiourea derivative of histidine containing a sulfur atom in the imidazole ring. Its synthesis is increased in *M. smegmatis* mutants in MSH synthesis suggesting a compensation mechanism (Ta *et al.*, 2011), although the actual function of this unusual thiol remains to

be investigated (Seebeck, 2010). Related to enzymatic mechanisms of reactive oxygen species detoxification, *M. tuberculosis* expresses a Fe-dependent superoxide dismutase, SODA (Rv3846), which is released to the extracellular medium and is considered to be important for pathogenesis (Edwards *et al.*, 2001); it also express a Cu-dependent SODC (Rv0432) that is not essential for intracellular growth within macrophages and seems to play a minor role in pathogenicity (Dussurget *et al.*, 2001). *M. tuberculosis* contains different thioredoxin-related enzymes which are maintained at reduced state by thioredoxin reductase and NADPH (Jaeger *et al.*, 2004). In spite of the absence of glutathione, *M. tuberculosis* genome codifies for different glutaredoxin-like proteins whose functional role awaits further investigation (Cole *et al.*, 1998). The bacterium expresses a heme-dependent peroxidase (catalase peroxidase, KatG) and several thiol-dependent peroxidases of the peroxiredoxin (Prx) type (see below). Moreover, *M. tuberculosis* lacks a functional OxyR, that in *E. coli* controls the transcription of a regulon of ~ 20 antioxidant genes (Zahrt & Deretic, 2002). The regulation of oxidative stress responses in *M. tuberculosis* is at least partially dependent on the alternative sigma factor H/antisigma factor H, a zinc-thiolate redox sensor (Raman *et al.*, 2001).

4. Catalase peroxidase, the heme-dependent peroxidase of *M. tuberculosis*

M. tuberculosis constitutively expresses a catalase peroxidase (EC 1.11.1.6) (*Mt*KatG, Rv1908)(Diaz & Wayne, 1974). The enzyme has attracted considerable attention due to its role in the activation of the first line antituberculosis prodrug isonicotinic acid hydrazide (isoniazid, INH) and the fact that loss-of-function mutations are a major mechanism of resistance to INH (Zhang *et al.*, 1992). *In vitro* generated *Mt*KatG negative strains were non pathogenic. Virulent catalase-negative clinical isolates overexpressed the thiol-dependent peroxidase alkyl hydroperoxidase reductase C (AhpC), indicating the need of another peroxidase to assure protection of the pathogen against oxidizing species (Sherman *et al.*, 1996). More recently, a mechanism of INH resistance in *M. tuberculosis* through down-regulation of KatG was proposed based on the observation that mutations in the *furA*[2]*-katG* intergenic region conferred INH resistance (Ando *et al.*, 2011). The protein has been identified in the cytosol, membrane fraction and culture filtrates of *M. tuberculosis* (Gu *et al.*, 2003; Mawuenyega *et al.*, 2005; Malen *et al.*, 2007). It displays a broad peroxidase activity, as well as a high catalase activity (k_{cat}/K_M = 2 x 10^6 $M^{-1}s^{-1}$)(Johnsson *et al.*, 1997), catalyzing the dismutation of H_2O_2 into dioxygen and water. It also reduces peroxynitrite (k = 1.4 x 10^5 M^{-1} s^{-1} at pH 7.4 and 25 ^oC (Wengenack *et al.*, 1999)). The catalytic mechanism of H_2O_2 reduction by KatG involves the initial two-electron oxidation of the enzyme to compound I ((Fe IV=O)$^{\bullet}$). KatG contains a unique post-translational modification in the form of a three amino acid adduct (Met255-Tyr229-Trp107) with a specific role in the catalase reaction since mutation of any of the three residues virtually eliminates catalase but not peroxidase activity (Jakopitsch *et al.*, 2004; Ghiladi *et al.*, 2005). It has been proposed that catalase activity in KatG is associated with a radical formation in the Met-Tyr-Trp adduct, whereas during the peroxidase activity a tyrosyl radical is formed (Zhao *et al.*, 2010). In the case of peroxynitrite reduction, oxidation of resting state KatG to compound II (Fe IV=O) plus $^{\bullet}NO_2$ has been proposed (Wengenack *et al.*, 1999).

[2] FurA is a negative regulator of KatG expression in *Mycobacterium smegmatis* (Zahrt *et al.*, 2001)

In addition to *Mt*KatG, the genome of *M. tuberculosis* codifies for a putative lignin peroxidase (Rv1900c) and other putative non-heme non-thiol -dependent peroxidases whose functional characterization is lacking (Cole *et al.*, 1998)(http://www.webtb.org/).

5. Thiol-dependent peroxidases of *M. tuberculosis*

5.1 Thiol-dependent peroxidases

Peroxidases with catalytic activities dependent on critical cysteine residues are called thiol-dependent peroxidases. These enzymes catalyze the reduction of H_2O_2, organic hydroperoxides and/or peroxynitrous acid (ONOOH) to water, organic alcohols and nitrite, respectively, at the expense of a reducing substrate, usually thioredoxin (Trx) or a Trx-related protein, via a double-displacement or ping-pong kinetic mechanism (Flohe *et al.*, 2003; Wood *et al.*, 2003; Trujillo *et al.*, 2007).

$$\begin{matrix} ROOH \\ H_2O_2 + \\ ONOOH \end{matrix} \quad Trx(SH)_2 \xrightarrow[\text{peroxidase}]{\text{Thiol}} H_2O + \begin{matrix} ROH \\ H_2O \\ NO_2^- + H^+ \end{matrix} + TrxS_2 \qquad (1)$$

where ROOH is organic peroxide; ONOOH is peroxynitrous acid; NO_2^- is nitrite; ROH is organic alcohol; $Trx(SH)_2$ is reduced thioredoxin and $TrxS_2$ is oxidized thioredoxin.

The oxidizing part of the catalytic cycle involves a SN_2 reaction occurring through a nucleophilic attack of the deprotonated thiol at the so called peroxidatic cysteine residue (C_P) on one of the peroxide oxygens. In the transition state, the negative charge is distributed among the two oxygen and the sulfur atoms, and the reaction is completed by the break of the peroxide bond forming an alcoxide as leaving group, which may protonate depending on its basicity. Thus, the thiolate in C_P suffers a two-electron oxidation to sulfenic acid (E-SOH).

$$\begin{matrix} ROOH \\ H_2O_2 + \\ ONOOH \end{matrix} \quad C_P\text{-}S^- \longrightarrow \begin{matrix} ROH \\ H_2O \\ NO_2^- + H^+ \end{matrix} + C_P\text{-}SO^- \qquad (2)$$

The rest of the catalytic cycle differs depending on the kind of thiol-dependent peroxidase. In most cases, it consists on the formation of a disulfide bridge through the reaction between the sulfenic acid intermediate in C_P and another cysteine residue, which is called the resolving cysteine residue (C_R), which is then reduced by thioredoxin (Trx) (or another thiol-disulfide oxidoreductase protein) that is maintained at reduced state by thioredoxin reductase and NADPH (Poole, 2007). For all thiol-dependent peroxidases tested so far, the acidity constants of the peroxidatic thiols are quite high (pK_a ~ ‹5 - 6.3, (Bryk *et al.*, 2000; Ogusucu *et al.*, 2007; Trujillo *et al.*, 2007; Nelson *et al.*, 2008; Hugo *et al.*, 2009)). Thus, under physiological conditions they are expected to be mostly under thiolate form, the reactive species. However, the rate constants of reactions of C_P in thiol-dependent peroxidases with peroxide substrates are several orders of magnitude faster than the corresponding reactions of low molecular weight or most protein thiolates, indicating the existence of protein factors involved in specific peroxide reduction by these enzymes that are only starting to be unraveled (Trujillo *et al.*, 2007; Flohe *et al.*, 2010; Hall *et al.*, 2010; Ferrer-Sueta *et al.*, 2011).

Other intriguing aspect related to thiol-dependent peroxidase catalytic mechanism is the molecular mechanisms of the oxidizing substrate specificity: although in most cases thiol-dependent peroxidases can catalyze the reduction of a broad range of peroxides, preferential substrates vary, and do not reflect the expected trend that correlates thiolate reactivity with leaving group pK_a[3] (Trujillo *et al.*, 2007) that was reported for the reactivities of other thiolate with peroxides (Trindade *et al.*, 2006; Trujillo *et al.*, 2007).

Thiol-dependent peroxidases can be classified into two main groups[4] based on sequence homology: glutathione peroxidases (Gpxs) and peroxiredoxins (Prxs). Since there are not genes for enzymes of the GPx type in *M. tuberculosis* genome, but there are several members of the Prx family, we will focus in the latter group of enzymes through the rest of this chapter.

5.2 Peroxiredoxins (EC 1.11.1.15)

Prxs are a family of thioredoxin-scaffold enzymes with thiol-dependent peroxidase activity (Chae *et al.*, 1994). They are ubiquitous, present in all living kingdoms and in different cellular compartments. They are also abundant, with concentrations usually in the μM range (Hofmann *et al.*, 2002). Due to their peroxidase activity, these enzymes play a role in antioxidant defenses. Moreover, at the light of the signaling role ascribed to H_2O_2 and other peroxides, Prxs are also regarded as key players in redox signaling processes and regulation of transcription factors (Rhee *et al.*, 2005; Hall *et al.*, 2009; Brigelius-Flohe & Flohe, 2011; Rhee & Woo, 2011). Peroxiredoxins have been functionally classified into 1-Cys Prxs and 2-cysteine Prxs according to the number of cysteine residues that participate in catalysis (Poole, 2007). The first part of the catalytic cycle is common for all kinds of Prxs and consists on the reduction of the peroxide substrate with concomitant oxidation of the C_P to a sulfenic acid derivative. In the case of 1-Cys Prxs, this sulfenic acid is reduced by different reducing pathways that depend on the particular 1-Cys Prx and that for most of them are still unclear. In 2-Cys Prxs, the sulfenic acid in C_P reacts with another Cys residue also required for catalysis, C_R that can be either in the same or in a different protein subunit (atypical or typical 2-Cys Prxs, respectively), forming a disulfide bridge that is reduced by Trx or a Trx-related protein. More recently, a Prx classification base on sequence homology has been proposed in the peroxiredoxin classification index (PREX) database (http://csb.wfu.edu/prex/index.php)(Nelson *et al.*, 2011; Soito *et al.*, 2011). Subfamilies thus identified are denoted by the name of one or more canonical member, as indicated below:

- **Alkyl hydroperoxide reductase C (AhpC) - Peroxiredoxin 1 (Prx1)**. This subfamily is both the largest and the most widely distributed, with members found in archaea, bacteria, and all classes of eukaryotes These proteins are functionally classified as typical 2-Cys Prxs.

[3] pK_a value of the alkoxide formed upon peroxide reduction

[4] Other thiol-dependent peroxidases non-structurally related to Gpxs and Prxs exist. For example, in many bacteria, a thiol-dependent organic hydroperoxide reductase (Ohr) is involved in organic hydroperoxide detoxification. However, the ohr gene is absent in *M. tuberculosis* genome

- **Bacterioferritin comigratory protein (Bcp)- Peroxiredoxin Q (PrxQ) (for *Escherichia coli* Bcp and plant PrxQ, respectively).** Present mostly in bacteria, also in yeast and plants but not in mammals. They can function either as atypical 2-Cys Prxs or as 1-Cys Prxs.
- **Thiol peroxidase (Tpx) (for *E. coli* Tpx).** Tpx subfamily members are all bacterial and are almost exclusively classified as atypical 2-Cys Prxs.
- **Peroxiredoxin 5 (for *Homo sapiens* Prx5).** Members of this subfamily are found from bacteria to mammals with members present in plants, fungi, and yeast. They are functionally classified as either 1-Cys Prxs or atypical 2-Cys Prxs.
- **Peroxiredoxin 6 (for *H. sapiens* Prx6).** Members of this subfamily are found in bacteria, plants, yeast and mammals. In general, they function as 1-Cys Prxs.
- **Alkyl hydroperoxide reductase E (AhpE) (for *M. tuberculosis* AhpE).** Found in aerobic gram-positive bacteria of the order Actinomycetes and some archaea. AhpE from *Mycobacterium tuberculosis* has been functionally classified as a 1-Cys Prx, but information regarding the catalytic mechanisms of other members of this group is lacking.

Further information regarding this sequence-based classification of Prxs can be found in the PREX database and references therein.

Peroxiredoxins are now known to be, at least in some cases, very efficient peroxidases (Trujillo *et al.*, 2007; Parsonage *et al.*, 2008; Manta *et al.*, 2009). The local sequence motif at the active site, ProXXXThrXXCys, is very conserved among different Prx subfamilies, although Thr is replaced by Ser in few known Prx sequences and a peroxidatic selenocysteine (Sec) instead of Cys has been reported in a Prx from *Eubacterium acidamidophilum* (Sohling *et al.*, 2001; Hofmann *et al.*, 2002; Poole, 2007; Nelson *et al.*, 2011). Prxs also contain a highly conserved Arg. These conserved residues along with several backbone interactions determine a low pK_a value of C_P and contribute to the catalytic mechanism of Prxs, in which transition state stabilization has been proposed to be involved (Hall *et al.*, 2010), although the precise mechanism of catalysis is still to be unraveled. Prx concentrations in different cells and tissues are frequently regulated, and usually increase under conditions of oxidative stress. Moreover, their catalytic activities are also regulated by different mechanisms, including protein phosphorylation (Chang *et al.*, 2002; Woo *et al.*, 2010) and inactivation due to overoxidation of the C_P, which involves the two- electron oxidation of the sulfenic form of the enzyme to sulfinic acid (Yang *et al.*, 2002). Recent data from our group indicated that the mechanism of C_P overoxidation is similar to that of oxidation, with the deprotonated sulfenate (or its tautomeric sulfoxide form) and the protonated peroxide as the reacting species (Hugo *et al.*, 2009; Reyes *et al.*, 2011).

$$\begin{matrix} \text{ROOH} \\ \text{H}_2\text{O}_2 + \\ \text{ONOOH} \end{matrix} \quad \text{E-SO}^- \longrightarrow \begin{matrix} \text{ROH} \\ \text{H}_2\text{O} \\ \text{NO}_2^- + \text{H}^+ \end{matrix} \quad + \ \text{E-SO}_2^- \tag{3}$$

In 2-Cys Prxs, the susceptibility to overoxidation depends on the structural GGLG and YF motifs present mostly in eukaryotic 2-Cys Prxs (Yang *et al.*, 2002) but also in some prokaryotic organisms including cyanobacteria (Pascual *et al.*, 2010). These structural motifs make disulfide formation with C_R to occur at a slower rate and thus, 2-Cys Prxs that possess them are more prone to oxidative inactivation (Wood *et al.*, 2003). Cysteine sulfinic acid,

previously considered as an irreversible post-transductional modification, is now known to be reversed by enzymatic mechanisms in different 2-Cys Prxs (Chang *et al.*, 2004; Iglesias-Baena *et al.*, 2011) and has been suggested to be involved in signaling processes (Iglesias-Baena *et al.*, 2010). Moreover, overoxidized forms of some members of the Prx family gained function as molecular chaperones (Moon *et al.*, 2005; Lim *et al.*, 2008).

5.3 Peroxiredoxins from *M. tuberculosis*

The genome of *M. tuberculosis* codifies for different thiol-dependent peroxidases of the Prx type, namely AhpC, TPx, AhpE, and two putative Bcps proteins (Cole *et al.*, 1998), which have been detected in the cytosolic, membrane and culture medium fractions (Figure 2). We will describe below the main functional characteristics of *M. tuberculosis* Prxs as well as reported evidences of their participation in peroxide detoxification in cellular or animal models of tuberculosis disease.

5.3.1 Alkyl hydroperoxide reductase C (*Mt*AhpC, Rv2428)

AhpCs are thiol-dependent peroxidase member of the AhpC-Prx1 subfamily of Prxs. *Mt*AhpC is functionally classified as a typical 2-Cys Prx, although site directed mutagenesis experiments revealed that it has three instead of two Cys residues involved in catalysis: C_P (Cys 61), the putative C_R (Cys 174) and a third Cys (Cys 176) whose role in catalysis is not completely clear but could provide an alternative route of disulfide bond formation (Guimaraes *et al.*, 2005). Whereas Cys 61 plays a central role in catalysis, the ehzyme remains partially functional in the absence of Cys 174 and 176 and possible adopts a 1-Cys-like mechanism (Chauhan & Mande, 2002; Koshkin *et al.*, 2004). *Mt*AhpC has been detected both in the bacterial cytosol (Covert *et al.*, 2001) and as a membrane associated protein (Gu *et al.*, 2003). AhpC forms part of bacterial alkyl hydroperoxide reductase (Ahp) system (Storz *et al.*, 1987). In enterobacteria, this system commonly consists of two components, AhpC and a flavin-containing disulfide reductase (AhpF) that reduces AhpC at NADH expense, and both enzymes are jointly up-regulated under oxidative stress conditions targeting the oxyR regulon (Tartaglia *et al.*, 1989). However, AhpF is lacking in all mycobacteria. In this context, two reducing systems for *M. tuberculosis* AhpC (*Mt*AhpC) have been proposed. Firstly, alkyl hydroperoxide reductase D (AhpD), that contains a CXXC motif, can reduce *Mt*AhpC. The *ahpD* gene is found immediately downstream of *ahpC*, in the position occupied by *ahpF* in *S. typhimurium* genome, and both proteins are controlled by the same promoter (Hillas *et al.*, 2000). Oxidized AhpD is regenerated by dihydrolipoamide acyltransferase (DlaT); in turn, dihydrolipoamide dehydrogenase (Lpd) mediates the reduction of DlaT at NADH expense and completes the catalytic cycle (Bryk *et al.*, 2002). *dlaT* (Rv2215) encodes the E2 component of the piruvate deshydrogenase complex, and *lpdC* (Rv0462), the only functional Lpd in *M. tuberculosis* (Argyrou & Blanchard, 2001), most probably codifies the E3 components of the piruvate deshydrogenase complex (Tian *et al.*, 2005). Secondly, thioredoxin C (TrxC), but not thioredoxin B (TrxB) or A (TrxA), was also able to act as AhpC reducing substrates (Jaeger *et al.*, 2004), and the catalytic cycle is completed by thioredoxin reductase (*Mt*TR) and NADPH. Catalytic efficiency of TrxC-mediated AhpC reduction was ~ 100 fold lower than that measured using AhpD as reducing substrate (2.5×10^4 *versus* 2.7×10^6 $M^{-1}s^{-1}$, respectively)(Jaeger *et al.*, 2004). However, the preferential reducing substrate would be determined not only by catalytic efficiencies but also by the steady-state concentrations of

reducing substrates at reduced state. *Mt*TrxC is consistently seen as a major spot in bacterial proteomes while the spot corresponding to *Mt*AhpD is of much lower intensity (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 1999), indicating a lower concentration of *Mt*AhpD compared to *Mt*TrxC in these cells. Moreover, *Mt*TR is also an abundant protein in Mycobacteria (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 1999), and it is expected to keep TrxC at reduced state as long as NADPH is not limiting (Jaeger *et al.*, 2004). These data suggest that, despite the lower catalytic efficiency of *Mt*TrxC compared to *Mt*AhpD in *Mt*AhpC reduction, both enzymatic pathways could be contributing to *Mt*AhpC-mediated peroxide detoxification *in vivo*. Concerning the oxidizing substrate specificity, AhpC are broad-spectrum peroxidases that catalyze the reduction of H_2O_2, organic hydroperoxides and peroxynitrite. The catalytic efficiency of *t*-BuOOH reduction (an artificial hydroperoxide used as a mimic of natural organic hydroperoxides) by *Mt*AhpC was reported as ~ 10^4 M^{-1} s^{-1} (Jaeger *et al.*, 2004). The enzyme could also reduce another artificial organic hydroperoxide, cumene hydroxperoxide at similar rates. H_2O_2 and linoleic acid hydroperoxides, but not phosphatidylcholine hydroperoxide, were also reduced by *Mt*AhpC. This enzyme, together with other bacterial AhpC enzymes, where the first Prxs for which a peroxynitrite reductase activity was demonstrated (k = 1.3 x 10^6 M^{-1} s^{-1} at pH 6.85 and RT (Bryk *et al.*, 2000)). H_2O_2 was a preferential substrate of *Mt*AhpC, although precise activity measurements were difficult to estimate due to the basal activity of the TR/Trx system (Jaeger *et al.*, 2004). In the case of another AhpC protein (from *Salmonella typhimurium*) the catalytic efficiency for H_2O_2 reduction was reported as 3.7 x 10^7 M^{-1} s^{-1} (Parsonage *et al.*, 2008). Thus, oxidizing substrate selectivity of bacterial AhpC seems to follow the same trend as for other members of the AhpC-Prx1 subfamily, where reduction of peroxynitrite is somewhat slower than that of H_2O_2 at near-physiological pHs, and occur with similar pH independent rate constants[5] (Manta *et al.*, 2009). There is no data regarding the pK_a of C_P and redox potential of AhpC from *M. tuberculosis*. The pK_a values of C_P in *S. tiphymurium* AhpC was first determined as < 5 (Bryk *et al.*, 2000) and more recently reported as 5.8 (Nelson *et al.*, 2008), indicating that C_P would be mostly deprotonated at physiological pH. The midpoint reduction potential of the enzyme was reported as -178 ± 0.4 mV, somehow lower than that reported for mammalian Prx 3 ($E^{o\prime}$ = -290 mV) (Cox *et al.*, 2010) and plant 2-Cys Prxs and PrxQ ($E^{o\prime}$ = −288 to −325 mV)(Dietz *et al.*, 2006). Data regarding redox potential of *Mt*AhpD is lacking. In turn, *M. tuberculosis* Trx redox potentials have not been investigated so far, but redox potential of other bacterial Trxs has been reported to be low (-270 mV for *E. coli* Trx (Krause *et al.*, 1991). Since the standard midpoint reduction potential for H_2O_2 reduction to water and for ONOOH reduction to nitrite and water are 1.77 and 1.6 V, respectively (Latimer, 1938; Koppenol & Kissner, 1998), the thermodynamic driving forces would highly favor the flux of electrons from Trxs to these peroxides through AhpC. In addition to its peroxidase

[5] According to the mechanism of reaction in which the thiolate form of the C_P reacts with the protonated peroxide, and considering that all the reported pK_a values of Prxs including AhpC are < 6.3 (*68*), (*70*), (*71*), and that the pK_a for the first H_2O_2 deprotonation is far above physiological pH, the pH-independent rate constant for H_2O_2-mediated Prx oxidation is practically the same as the rate constant determined at physiological pH. However, the pK_a value of peroxynitrous acid is around 6.8 (reported values of ONOOH pK_a = 6.5-6.8 (*23*), (*24*), (*25*)) and therefore, only 50 % or 20 % of peroxynitrite would be as be protonated at pH 6.8 or 7.4, respectively. Thus, the pH-independent rate constant of Prx oxidation by peroxynitrite would be 2 or 5 times higher than the value determined at pH 6.8 or 7.4.

activity, some bacterial AhpCs have other functions: *Helicobacter pylori* AhpC can form high molecular weight aggregates with chaperone activity under oxidative stress conditions (Huang *et al.*, 2010). Moreover, AhpC from some Gram negative microorganisms show a deglutathionylating activity that depends on C_R rather than on C_P (Yamamoto *et al.*, 2008).

Size exclusion chromatography indicated that wild-type *Mt*AhpC performs as a heterogeneous mixture of oligomers under non-reducing conditions, whereas under reduced state the enzyme is a homogeneous oligomer formed by 10- or 12-subunits. The C176S mutant form of AhpC is dimeric under oxidized state, and forms oligomers of 10-12 subunits upon reduction. The crystallographic structure of C176S *Mt*AhpC trapped as an intermediate of its catalytic cycle (where condensation had already occurred but still the enzyme was under its oligomeric form) was consistent with the formation of a ring shaped oligomer of 12 subunits, a hexamer of dimers (Guimaraes *et al.*, 2005). The relationship between *Mt*AhpC oligomerisation and activity has not been addressed. In the case of *Salnonella typhimurium* AhpC, decameric under reduced state, the analysis of mutated forms of the enzyme at the decamer-building interface indicated that the oligomerization is quite important, but not essential to activity (Parsonage *et al.*, 2005).

The role of *Mt*AhpC in the detoxification of peroxides *in vivo* was first suggested by the fact that pathogenic, INH-resistant strains lacking KatG over-expressed *Mt*AhpC, which would represent a compensatory mechanism allowing the bacteria to get rid of cytotoxic peroxides (Sherman *et al.*, 1996). Overexpression of MtAhpC in those strains was associated to mutations in the gene promoter (Wilson & Collins, 1996). Thus, *Mt*AhpC was proposed as a potential drug target. However, data obtained using *M. tuberculosis* strains lacking *Mt*AhpC are not straightforward. AhpC expression in virulent strains of *M. tuberculosis* grown *in vitro* was repressed and increased under conditions of static growth, probably reflecting adaptation of the bacterium during its infection cycle (Springer *et al.*, 2001). AhpC expression was also induced by hypoxia (Sherman *et al.*, 2001). *S. typhimurim* lacking ahpC became hypersusceptible to reactive nitrogen species and *Mt*AhpC complemented the defect. The enzyme also protected human cells from toxicity caused by reactive nitrogen species (Chen *et al.*, 1998). Whereas inactivation of *Mt*AhpC caused no effect on bacterial growth during acute infection in mice and had no effect on *in vitro* sensitivity to H_2O_2, it caused an increase susceptibility to organic hydroperoxide and peroxynitrite-mediated toxicity (Springer *et al.*, 2001; Master *et al.*, 2002). Inactivation of *Mt*AhpC caused a decrease in the survival of *M. tuberculosis* in non-stimulated macrophages but not in macrophages stimulated with interferon-γ (Master *et al.*, 2002). Strains lacking DlaT showed retarded growth, were highly susceptible to killing by acidified nitrite *in vitro*, showed decreased intracellular survival during macrophage infection and were less virulent in a mouse model of tuberculosis (Shi & Ehrt, 2006). Overall, these data indicate the importance of both *Mt*AhpC and *Mt*DlaT, its reductant through *Mt*AhpD, for *M. tuberculosis* to overcome oxidative stress encountered inside its primary host cells and to establish a successful infection.

5.3.2 Thiol peroxidase (*Mt*TPx, Rv1932)

The second Prx from *M. tuberculosis* to be identified belonged to the TPx subfamily (Jaeger *et al.*, 2004), enzymes widely distributed among Gram-positive and Gram-negative bacteria. In the case of *E. coli* Tpx, the enzyme is localized in the periplasmic space. In *M. tuberculosis*,

TPx was firstly characterized as an extracellular antigen that induces a strong proliferative response in animals (Weldingh *et al.*, 1998) . *Mt*TPx was repeatedly found in culture filtrates; it has also been found associated to membranes and in cytosolic fractions (Rosenkrands *et al.*, 2000; Covert *et al.*, 2001; Malen *et al.*, 2007; Malen *et al.*, 2010).

TPxs are atypical 2-Cys Prxs. They typically contain three cysteine residues where Cys60 is C_P, C93 is C_R and Cys80[6] is catalytically irrelevant. However, site directed mutagenesis studies revealed that *Mt*TPx lacking Cys 93 remained active for a limited period of time before getting inactivated by C_P overoxidation to sulfinic acid, and therefore the role of Cys93 is likely the formation of an intramolecular disulfide with the sulfenic acid in C_P and to avoid C_P overoxidation under conditions of restricted availability of reducing substrates (Trujillo *et al.*, 2006). *Mt*TPx reacts very rapidly with peroxynitrite (k = 1.5 x 10^7 M^{-1} s^{-1} at pH 7.4 and 25 ^oC)[7]. Reduction of *t*-BuOOH was slower (k ~ 1 x 10^5 M^{-1} s^{-1} at pH 7.4 and 25 ^oC). Reduction of H_2O_2 was faster than that of *t*-BuOOH, although the exact number was difficult to estimate. The enzyme was hardly active towards linolenic acid hydroperoxide and could not reduce phosphatidylcholine hydroperoxide. Concerning the reductive part of the catalytic cycle, both *Mt*TrxB and *Mt*TrxC reduced *Mt*TPx with similar catalytic efficiencies (4.6 and 5.8 x 10^4 M^{-1} s^{-1}, respectively). Since according to proteomic data currently available *Mt*Trx C would be much more abundant than *Mt*TrxB, the former would play a major role as *Mt*TPx reducing substrate. Mycothiol plus mycothione reductase/NADPH were not able to reduce *Mt*TPx (Jaeger *et al.*, 2004).

The crystallography structure of *Mt*Tpx (Rho *et al.*, 2006) and on the inactive mutant C60S *Mt*Px (Stehr *et al.*, 2006), as for other bacterial TPxs, indicated that Cys60 in *Mt*TPx forms part of a typical catalytic triad with Thr57 and Arg130. The enzyme is dimeric both in the crystal structure and in solution (Rho *et al.*, 2006; Stehr *et al.*, 2006). In C60S *Mt*TPx, a cocrystallized acetate molecule interacted with Ser60, Arg130 and Thr57 (Stehr *et al.*, 2006). Similarly, the wild type enzyme also showed anions near the active site. Co-crystallization with anions is frequently observed in Prxs; it has been proposed the existence of an anion-binding site in the neighborhood of reactive thiols in proteins, that could participate in transition state stabilization and thus, in the acceleration of peroxides reduction in general (Hall *et al.*, 2010; Ferrer-Sueta *et al.*, 2011).

M. tuberculosis strains lacking functional *Mt*TPx had a lower peroxidase activity than their wild type counterparts, indicating that the enzyme importantly contributes to the total peroxidase activity in *M. tuberculosis*. Moreover, *Mt*TPx mutants were more sensitive to H_2O_2 and •NO-mediated toxicity, but the effect was recovered when they were complemented with the tpx gene. Strains lacking *Mt*TPx failed to grow and survive in macrophages, particularly after activation by interferon-γ. Growth was significantly restored in the macrophages from iNOS knockout mice. This is consistent with the ability of the enzyme to rapidly reduce peroxynitrite *in vitro*. Moreover, strains lacking *Mt*TPx

[6] Cysteine numbers correspond to the sequence in TPx from *M. tuberculosis*.

[7] The pK_a value of C_P in *Mt*TPx or other bacterial TPx has not been reported previously. Considering a pK_a value of <6.3, as for all other Prxs investigated so far, more than 90 % of C_P would be as thiolate and 20% of peroxynitrite as ONOOH at pH 7.4. Thus, the pH-independent rate constant of C_P oxidation by peroxynitrite would be 5 times higher than the value determined at pH 7.4, 7.5 x 10^7 $M^{-1}s^{-1}$. It would be even higher if the pK_a of C_P of *Mt*TPx was > 6.3.

failed to initiate an acute infection and to maintain a persistent infection, and were less virulent than wild type strains (Hu & Coates, 2009). In the *M. bovis* strain BCG, TPx is induced in response to exposure to diamide, an agent that causes thiol oxidation (Dosanjh *et al.*, 2005).

5.3.3 Alkyl hydroperoxide reductase E (*Mt*AhpE, Rv2238c)

The genome of *M. tuberculosis* also codifies for a one-cysteine Prx, alkyl hydroperoxide reductase E, which is highly conserved among many Mycobacteria (Cole *et al.*, 1998; Passardi *et al.*, 2007). *Mt*AhpE belongs to a novel family of Prxs, comprising bacterial and archaean AhpE and AhpE-like enzymes (Passardi *et al.*, 2007; Soito *et al.*, 2011). This protein has been identified in the membrane fraction of *M. tuberculosis* H37Rv using a proteomics approach (Gu *et al.*, 2003). The expression of *Mt*AhpE increases during the dormant phase of tuberculosis disease (Murphy & Brown, 2007). Although *Mt*AhpE shows greater sequence similarity with mammalian typical two-Cys Prxs than with one-Cys Prxs (Passardi *et al.*, 2007; Soito *et al.*, 2011), it has only one Cys residue and functions by a one-Cys mechanism. Accordingly, in the oxidized form of the enzyme C_P is as sulfenic acid, as revealed by crystallographic studies and by mass spectrometry analysis (Li *et al.*, 2005; Hugo *et al.*, 2009). We have reported the peroxidase activity of *Mt*AhpE, being the first member of the AhpE family to be functionally characterized (Hugo *et al.*, 2009). The physiological reducing substrate(s) for *Mt*AhpE (as well as AhpE-like Prxs) is/are still unknown, but its catalytic activity was demonstrated using the artificial substrates dithiotreitol (DTT) and thionitrobenzoic acid (TNB). Neither N-acetylcysteine nor glutathione could reduce oxidized *Mt*AhpE but led to mixed disulfides formation. Concerning oxidizing substrate specificity, *Mt*AhpE reduces peroxynitrite three orders of magnitude faster than H_2O_2 (1.9×10^7 *versus* 8.2×10^4 M^{-1} s^{-1} at pH 7.4 and 25 ^oC, respectively[8]). These rate constants were measured directly by taking advantage of the decrease in Trp-dependent fluorescence intensity that the enzyme exhibits upon oxidation. Moreover, the kinetics of peroxide-mediated inactivation by overoxidation of C_P to sulfinic acid was measured following the increase in the enzyme's intrinsic fluorescence intensity (k = 40 $M^{-1}s^{-1}$ for H_2O_2–mediated overoxidation)(Hugo *et al.*, 2009). This value was very similar to that previously calculated for mammalian Prx 1 oxidative inactivation by H_2O_2 (57 M^{-1} s^{-1}) (Wood *et al.*, 2003; Stone, 2004). The pK_a of the thiol (in reduced *Mt*AhpE) and of the sulfenic acid (in oxidized *Mt*AhpE) were reported to be 5.2 and 6.6, respectively. Thus, taking into account the intrabacterial pH of wild-type *M. tuberculosis* (6.8–7.5 (Vandal *et al.*, 2008)), >95 % of the reduced and >50 % of the oxidized form of C_P in *Mt*AhpE would be deprotonated, and therefore, at their reactive forms with peroxides (Hugo *et al.*, 2009). More recently, we have performed a comprehensive study on *Mt*AhpE oxidizing substrate specificity as well as on its oxidative inactivation (Reyes *et al.*, 2011). For most peroxides tested, oxidation as well as oxidative inactivation rates

[8] Considering a mechanism of reaction where thiolate and sulfenate as well as protonated peroxides are the reactive species, the reported pK_a values of the thiol and sulfenic acid in reduced and oxidized *Mt*AhpE (Hugo *et al.*, 2009) and the pK_a of the H_2O_2 and peroxynitrite above indicated, pH independent rates constants can be calculated as very similar (for H_2O_2) and ~ 5 fold higher (for peroxynitrite) that the corresponding values measured at pH 7.4.

correlated with leaving group pK_a, indicating that both reactions occur by similar mechanisms, i.e. reaction of the thiolate or sulfenate anion at C_P with the protonated peroxide. In contrast, the hydroperoxide at position 15 of arachidonic acid (15-HpETE) and linolenic acid-derived hydroperoxides reacted surprisingly fast, with rate constants of ~10^8 and ~10^5 M^{-1} s^{-1} for *Mt*AhpE oxidation and overoxidation, respectively. The molecular basis for the fast reactivity of *Mt*AhpE with fatty acid hydroperoxides is intriguing. The quaternary structure of *Mt*AhpE in solution is tightly regulated by the oxidation state of the C_P, the enzyme being a dimer under reduced state and slowly forming high molecular weight aggregates upon oxidation (Hugo *et al.*, 2009). Analysis of the reported crystallographic structure of the protein under reduced state (Li *et al.*, 2005) showed a hydrophobic grove present in the dimeric enzyme, and formed by residues from both subunits, which is proposed as an anchoring site for fatty acid hydroperoxide binding (Reyes *et al.*, 2011). These data set *Mt*AhpE (and probably other AhpE-like Prxs) as potential Prxs specialized for fatty acid hydroperoxide detoxification. However, the roles of *Mt*AhpE in reduction of these or other peroxides *in vivo*, as well as in macrophage infection or bacterial virulence, remain to be investigated.

Prx	reductant	pK_a of C_P	$k_{2\ H2O2}$ (M^{-1} s^{-1})	$k_{2\ ONOOH}$ (M^{-1} s^{-1})	$k_{2\ t\text{-}BuOOH}$ (M^{-1} s^{-1})	$k_{2\ LOOH}$ (M^{-1} s^{-1})
AhpC	AhpD, TrxC	[a]5.8 (C_P-SH)	[a]3.7 x 10^7	[b]1.3 x 10^6	1-2.3 x 10^4	[c]6.9 x 10^3
TPx	TrxB, TrxC	ND	ND	1.5 x 10^7	0.9-3.4 x10^5	0
AhpE	ND	5.2 (C_P-SH) 6.6 (C_P-SOH)	8.2 x 10^4	1.9 x 10^7	8 x10^3	[d]1.8 x 10^8 [e]2.7 x 10^8

[a]For *St*AhpC; [b]At pH 6.85 and RT; [c] Calculated from (Jaeger *et al.*, 2004; Parsonage *et al.*, 2008), for linoleic acid hydroperoxide; [d]For 15-HpETE; [e]For α-linolenic acid hydroperoxide; ND is non determined. In the case of H_2O_2 reduction by *Mt*AhpC and *Mt*TPx, reactions were faster than with *t*-BuOOH, but precise rate constants were difficult to estimate (Jaeger *et al.*, 2004).

Table 1. Functional data on Prxs from *M. tuberculosis*: acidity constants, reducing substrates and kinetics of peroxide reduction.

5.3.4 Bacterioferritin comigratory proteins (Bcp, Rv2521; BcpB, Rv1608c)

The genome of *M. tuberculosis* also codifies for two putative Prxs of the Bcp type (Cole & Barrell, 1998). Evidence for the first Bcp (Rv2125) expression at a protein level exists, both in the membrane fraction (Gu *et al.*, 2003) and in the cytosol of H37Rv strains (Mawuenyega *et al.*, 2005). The protein has been shown to be target of modification by the small protein Pup, a post-translational modification that targets proteins for degradation by the *M. tuberculosis* proteosome (Pearce *et al.*, 2006; Festa *et al.*, 2010). To note, pupylation and proteosome function are essential for the virulence of this bacterium, for reasons still unknown (Darwin *et al.*, 2003; Gandotra *et al.*, 2007). In the case of BcpB (Rv1608c), it was identified associated to the membrane fraction of *M. tuberculosis* H37Rv (Gu *et al.*, 2003). The genes for both putative Bcps are considered as non-essential according to mutagenesis analysis in H37Rv strain (Sassetti *et al.*, 2003). Structural and functional data regarding both putative Bcps from *M. tuberculosis* and their role in infection processes await further investigation.

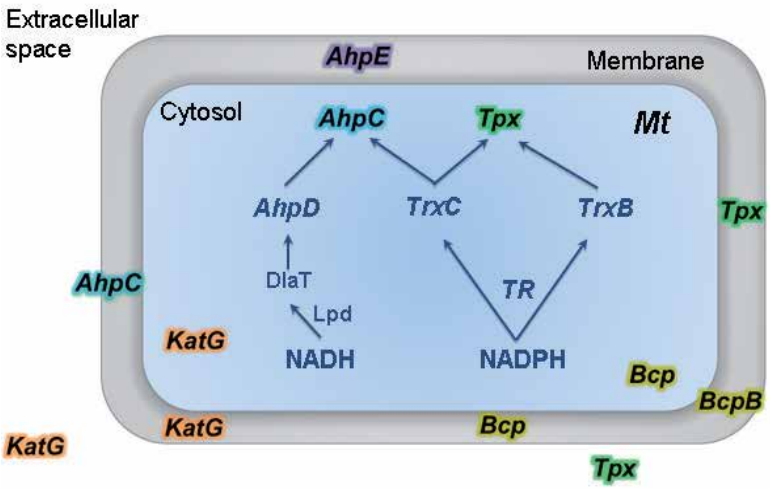

Fig. 2. **Cellular localization and reducing substrates of peroxidases from *M. tuberculosis*.** The five Prxs and the heme peroxidase KatG have distinct, although overlapping cellular distributions. *Mt*KatG (orange) has been found in the cytosol, membrane and extracellular space. *Mt*AhpC (blue) is a cytosolic enzyme also that was also found associated to the bacterial membrane. *Mt*TPx (green) was detected in culture media repeatedly. It has also been found in membrane fractions and in the cytosol. *Mt*AhpE (violet), and the putative BcpB and Bcp (yellow) were detected in cell membrane fractions, and the latter also in the cytosol. Reducing systems for *Mt*AhpC and *Mt*Tpx (in grey) are shown without considering their cellular localization. *Mt*AhpE and *Mt*Bcps reducing substrates are still unknown.

6. Conclusions

M. tuberculosis is an extremely successful pathogen, despite of being exposed to cytotoxic peroxides formed inside the phagosome of activated macrophages, its primary host cells. The bacterium expresses a heme-dependent peroxidase, KatG, and various thiol-dependent peroxidases of the Prx type. From the data reviewed herein, it becomes clear that Prxs from *M. tuberculosis* differ in cellular location, and have diverse oxidizing and reducing substrate specificities, that may explain in part the presence of different subfamilies of Prxs in the bacterium. Available data indicate that at least two of them (*Mt*AhpC and *Mt*TPx) play a role in pathogenesis. The third one, *Mt*AhpE, has an outstanding reactivity with fatty-acid derived hydroperoxides, but since natural reducing substrate(s) has not been identified so far, its peroxidase catalytic activity *in vivo* remains to be confirmed. Similarly, further investigation is required to characterize the two putative Bcp proteins from *M. tuberculosis.*

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8. References

Akaki, T., Tomioka, H., Shimizu, T., Dekio, S. & Sato, K. (2000). Comparative roles of free fatty acids with reactive nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages against Mycobacterium tuberculosis. *Clin Exp Immunol* 121(2): 302-310.

Alvarez, M. N., Peluffo, G., Piacenza, L. & Radi, R. (2011). Intraphagosomal peroxynitrite as a macrophage-derived cytotoxin against internalized Trypanosoma cruzi: consequences for oxidative killing and role of microbial peroxiredoxins in infectivity. *J Biol Chem* 286(8): 6627-6640.

Ando, H., Kitao, T., Miyoshi-Akiyama, T., Kato, S., Mori, T. & Kirikae, T. (2011). Downregulation of katG expression is associated with isoniazid resistance in Mycobacterium tuberculosis. *Mol Microbiol* 79(6): 1615-1628.

Argyrou, A. & Blanchard, J. S. (2001). Mycobacterium tuberculosis lipoamide dehydrogenase is encoded by Rv0462 and not by the lpdA or lpdB genes. *Biochemistry* 40(38): 11353-11363.

Armstrong, J. A. & Hart, P. D. (1971). Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J Exp Med* 134(3 Pt 1): 713-740.

Augusto, O., Bonini, M. G., Amanso, A. M., Linares, E., Santos, C. C. & De Menezes, S. L. (2002). Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic Biol Med* 32(9): 841-859.

Babior, B. M. (1984). Oxidants from phagocytes: agents of defense and destruction. *Blood* 64(5): 959-966.

Barber, D. J. W. & Thomas, J. K. (1978). Reaction of radicals with lecithin bilayers. *Radiat Res* 74: 51-65.

Bedard, K. & Krause, K. H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87(1): 245-313.

Blelski, B. H. J. & and Allen, A. O. (1977). Mechanism of the Disproportionation of Superoxide Radicals. *J Phyl Chem* 81: 1048-1050.

Bonini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M. & Augusto, O. (1999). Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J Biol Chem* 274(16): 10802-10806.

Bonney, R., J.;, Opas, E. E. & Humes, J. L. (1985). Lipoxygenase pathways of macrophages. *Fed Proc* 44: 2933-2936.

Boshoff, H. I., Reed, M. B., Barry, C. E., 3rd & Mizrahi, V. (2003). DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. *Cell* 113(2): 183-193.

Brigelius-Flohe, R. & Flohe, L. (2011). Basic Principles and Emerging Concepts in the Redox Control of Transcription Factors. *Antioxid Redox Signal*.

Bryk, R., Griffin, P. & Nathan, C. (2000). Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407(6801): 211-215.

Bryk, R., Lima, C. D., Erdjument-Bromage, H., Tempst, P. & Nathan, C. (2002). Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. *Science* 295(5557): 1073-1077.

Bzymek, K. P., Newton, G. L., Ta, P. & Fahey, R. C. (2007). Mycothiol import by Mycobacterium smegmatis and function as a resource for metabolic precursors and energy production. *J Bacteriol* 189(19): 6796-6805.

Clifford, D. P. & Repine, J. E. (1982). Hydrogen peroxide mediated killing of bacteria. *Mol Cell Biochem* 49(3): 143-149.

Cole, S. T. & Barrell, B. G. (1998). Analysis of the genome of Mycobacterium tuberculosis H37Rv. *Novartis Found Symp* 217: 160-172; discussion 172-167.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S. & Barrell, B. G. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 393(6685): 537-544.

Covert, B. A., Spencer, J. S., Orme, I. M. & Belisle, J. T. (2001). The application of proteomics in defining the T cell antigens of Mycobacterium tuberculosis. *Proteomics* 1(4): 574-586.

Cox, A. G., Peskin, A. V., Paton, L. N., Winterbourn, C. C. & Hampton, M. B. (2010). Correction to Redox Potential and Peroxide Reactivity of Human Peroxiredoxin 3. *Biochemistry* 49 (44):9677–9677.

Chae, H. Z., Chung, S. J. & Rhee, S. G. (1994). Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269(44): 27670-27678.

Chang, T. S., Jeong, W., Choi, S. Y., Yu, S., Kang, S. W. & Rhee, S. G. (2002). Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. *J Biol Chem* 277(28): 25370-25376.

Chang, T. S., Jeong, W., Woo, H. A., Lee, S. M., Park, S. & Rhee, S. G. (2004). Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. *J Biol Chem* 279(49): 50994-51001.

Chauhan, R. & Mande, S. C. (2002). Site-directed mutagenesis reveals a novel catalytic mechanism of Mycobacterium tuberculosis alkylhydroperoxidase C. *Biochem J* 367(Pt 1): 255-261.

Chen, L., Xie, Q. W. & Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol Cell* 1(6): 795-805.

Darwin, K. H., Ehrt, S., Gutierrez-Ramos, J. C., Weich, N. & Nathan, C. F. (2003). The proteasome of Mycobacterium tuberculosis is required for resistance to nitric oxide. *Science* 302(5652): 1963-1966.

De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y. & Fang, F. C. (1997). Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci U S A* 94(25): 13997-14001.

Denicola, A., Freeman, B. A., Trujillo, M. & Radi, R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys* 333(1): 49-58.

Denicola, A., Rubbo, H., Rodriguez, D. & Radi, R. (1993). Peroxynitrite-mediated cytotoxicity to Trypanosoma cruzi. *Arch Biochem Biophys* 304(1): 279-286.

Diaz, G. A. & Wayne, L. G. (1974). Isolation and characterization of catalase produced by Mycobacterium tuberculosis. *Am Rev Respir Dis* 110(3): 312-319.

Dietz, K. J., Jacob, S., Oelze, M. L., Laxa, M., Tognetti, V., de Miranda, S. M., Baier, M. & Finkemeier, I. (2006). The function of peroxiredoxins in plant organelle redox metabolism. *J Exp Bot* 57(8): 1697-1709.

Dosanjh, N. S., Rawat, M., Chung, J. H. & Av-Gay, Y. (2005). Thiol specific oxidative stress response in Mycobacteria. *FEMS Microbiol Lett* 249(1): 87-94.

Dussurget, O., Stewart, G., Neyrolles, O., Pescher, P., Young, D. & Marchal, G. (2001). Role of Mycobacterium tuberculosis copper-zinc superoxide dismutase. *Infect Immun* 69(1): 529-533.

Edwards, K. M., Cynamon, M. H., Voladri, R. K., Hager, C. C., DeStefano, M. S., Tham, K. T., Lakey, D. L., Bochan, M. R. & Kernodle, D. S. (2001). Iron-cofactored superoxide dismutase inhibits host responses to Mycobacterium tuberculosis. *Am J Respir Crit Care Med* 164(12): 2213-2219.

Ehrt, S. & Schnappinger, D. (2009). Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol* 11(8): 1170-1178.

Evans, M. V., Turton, H. E., Grant, C. M. & Dawes, I. W. (1998). Toxicity of linoleic acid hydroperoxide to Saccharomyces cerevisiae: involvement of a respiration-related process for maximal sensitivity and adaptive response. *J Bacteriol* 180(3): 483-490.

Ferrer-Sueta, G., Manta, B., Botti, H., Radi, R., Trujillo, M. & Denicola, A. (2011). Factors affecting protein thiol reactivity and specificity in peroxide reduction. *Chem Res Toxicol* 24(4): 434-450.

Ferrer-Sueta, G. & Radi, R. (2009). Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol* 4(3): 161-177.

Festa, R. A., McAllister, F., Pearce, M. J., Mintseris, J., Burns, K. E., Gygi, S. P. & Darwin, K. H. (2010). Prokaryotic ubiquitin-like protein (Pup) proteome of Mycobacterium tuberculosis [corrected]. *PLoS One* 5(1): e8589.

Flohe, L., Jaeger, T., Pilawa, S. & Sztajer, H. (2003). Thiol-dependent peroxidases care little about homology-based assignments of function. *Redox Rep* 8(5): 256-264.

Flohe, L., Toppo, S., Cozza, G. & Ursini, F. (2010). A Comparison of Thiol Peroxidase Mechanisms. *Antioxid Redox Signal*. 15(3):763-80.

Fridovich, I. (1997). Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem* 272(30): 18515-18517.

Gandotra, S., Schnappinger, D., Monteleone, M., Hillen, W. & Ehrt, S. (2007). In vivo gene silencing identifies the Mycobacterium tuberculosis proteasome as essential for the bacteria to persist in mice. *Nat Med* 13(12): 1515-1520.

Gerasimov, O. V. & Lymar, S. V. (1999). The yield of hydroxyl radical from the decomposition of peroxynitrous acid. *Inorg Chem* 38 (19):4317-4321.

Ghiladi, R. A., Medzihradszky, K. F. & Ortiz de Montellano, P. R. (2005). Role of the Met-Tyr-Trp cross-link in Mycobacterium tuberculosis catalase-peroxidase (KatG) as revealed by KatG(M255I). *Biochemistry* 44(46): 15093-15105.

Goldstein, S. & Czapski, G. (1995). Direct and indirect oxidations by peroxynitrite. *Inorg. Chem.* 34: 4041-4048.

Groemping, Y. & Rittinger, K. (2005). Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J* 386(Pt 3): 401-416.

Gu, S., Chen, J., Dobos, K. M., Bradbury, E. M., Belisle, J. T. & Chen, X. (2003). Comprehensive proteomic profiling of the membrane constituents of a Mycobacterium tuberculosis strain. *Mol Cell Proteomics* 2(12): 1284-1296.

Guimaraes, B. G., Souchon, H., Honore, N., Saint-Joanis, B., Brosch, R., Shepard, W., Cole, S. T. & Alzari, P. M. (2005). Structure and mechanism of the alkyl hydroperoxidase AhpC, a key element of the Mycobacterium tuberculosis defense system against oxidative stress. *J Biol Chem* 280(27): 25735-25742.

Hall, A., Karplus, P. A. & Poole, L. B. (2009). Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. *FEBS J* 276(9): 2469-2477.

Hall, A., Parsonage, D., Poole, L. B. & Karplus, P. A. (2010). Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. *J Mol Biol* 402(1): 194-209.

Hillas, P. J., del Alba, F. S., Oyarzabal, J., Wilks, A. & Ortiz De Montellano, P. R. (2000). The AhpC and AhpD antioxidant defense system of Mycobacterium tuberculosis. *J Biol Chem* 275(25): 18801-18809.

Hofmann, B., Hecht, H. J. & Flohe, L. (2002). Peroxiredoxins. *Biol Chem* 383(3-4): 347-364.

Hu, Y. & Coates, A. R. (2009). Acute and persistent Mycobacterium tuberculosis infections depend on the thiol peroxidase TpX. *PLoS One* 4(4): e5150.

Huang, C. H., Chuang, M. H., Wu, Y. H., Chuang, W. C., Jhuang, P. J. & Chiou, S. H. (2010). Characterization of site-specific mutants of alkylhydroperoxide reductase with dual functionality from Helicobacter pylori. *J Biochem* 147(5): 661-669.

Hugo, M., Turell, L., Manta, B., Botti, H., Monteiro, G., Netto, L. E., Alvarez, B., Radi, R. & Trujillo, M. (2009). Thiol and sulfenic acid oxidation of AhpE, the one-cysteine peroxiredoxin from Mycobacterium tuberculosis: kinetics, acidity constants, and conformational dynamics. *Biochemistry* 48(40): 9416-9426.

Hurst, J. K. & Lymar, S. V. (1997). Toxicity of peroxynitrite and related reactive nitrogen species toward Escherichia coli. *Chem Res Toxicol* 10(7): 802-810.

Iglesias-Baena, I., Barranco-Medina, S., Lazaro-Payo, A., Lopez-Jaramillo, F. J., Sevilla, F. & Lazaro, J. J. (2010). Characterization of plant sulfiredoxin and role of sulphinic form of 2-Cys peroxiredoxin. *J Exp Bot* 61(5): 1509-1521.

Iglesias-Baena, I., Barranco-Medina, S., Sevilla, F. & Lazaro, J. J. (2011). The dual-targeted plant sulfiredoxin retroreduces the sulfinic form of atypical mitochondrial peroxiredoxin. *Plant Physiol* 155(2): 944-955.

Jaeger, T., Budde, H., Flohe, L., Menge, U., Singh, M., Trujillo, M. & Radi, R. (2004). Multiple thioredoxin-mediated routes to detoxify hydroperoxides in Mycobacterium tuberculosis. *Arch Biochem Biophys* 423(1): 182-191.

Jakopitsch, C., Ivancich, A., Schmuckenschlager, F., Wanasinghe, A., Poltl, G., Furtmuller, P. G., Ruker, F. & Obinger, C. (2004). Influence of the unusual covalent adduct on the kinetics and formation of radical intermediates in synechocystis catalase peroxidase: a stopped-flow and EPR characterization of the MET275, TYR249, and ARG439 variants. *J Biol Chem* 279(44): 46082-46095.

Johnsson, K., Froland, W. A. & Schultz, P. G. (1997). Overexpression, purification, and characterization of the catalase-peroxidase KatG from Mycobacterium tuberculosis. *J Biol Chem* 272(5): 2834-2840.

Jungblut, P. R., Schaible, U. E., Mollenkopf, H. J., Zimny-Arndt, U., Raupach, B., Mattow, J., Halada, P., Lamer, S., Hagens, K. & Kaufmann, S. H. (1999). Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. *Mol Microbiol* 33(6): 1103-1117.

Kissner, R., Nauser, T., Bugnon, P., Lye, P. G. & Koppenol, W. H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem Res Toxicol* 10(11): 1285-1292.

Koppenol, W. H. & Kissner, R. (1998). *Chem Res Toxicol* 11: 87-90.

Koshkin, A., Knudsen, G. M. & Ortiz De Montellano, P. R. (2004). Intermolecular interactions in the AhpC/AhpD antioxidant defense system of Mycobacterium tuberculosis. *Arch Biochem Biophys* 427(1): 41-47.

Krause, G., Lundstrom, J., Barea, J. L., Pueyo de la Cuesta, C. & Holmgren, A. (1991). Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in Escherichia coli thioredoxin. *J Biol Chem* 266(15): 9494-9500.

Latimer, W. M., *Oxidation Potentials*, second ed. (1952). Prentice-Hall, New York.

Lawn, S. D. & Zumla, A. I. (2011). Tuberculosis. *Lancet* 378(9785):57-72.

Lee, W. L., Gold, B., Darby, C., Brot, N., Jiang, X., de Carvalho, L. P., Wellner, D., St John, G., Jacobs, W. R., Jr. & Nathan, C. (2009). Mycobacterium tuberculosis expresses methionine sulphoxide reductases A and B that protect from killing by nitrite and hypochlorite. *Mol Microbiol* 71(3): 583-593.

Li, S., Peterson, N. A., Kim, M. Y., Kim, C. Y., Hung, L. W., Yu, M., Lekin, T., Segelke, B. W., Lott, J. S. & Baker, E. N. (2005). Crystal Structure of AhpE from Mycobacterium tuberculosis, a 1-Cys peroxiredoxin. *J Mol Biol* 346(4): 1035-1046.

Lim, J. C., Choi, H. I., Park, Y. S., Nam, H. W., Woo, H. A., Kwon, K. S., Kim, Y. S., Rhee, S. G., Kim, K. & Chae, H. Z. (2008). Irreversible oxidation of the active-site cysteine of peroxiredoxin to cysteine sulfonic acid for enhanced molecular chaperone activity. *J Biol Chem* 283(43): 28873-28880.

Lymar, S. V. & Hurst, J. K. (1995). Rapid reaction between peroxynitrite anion and carbon dioxide: implication for biological activity. *J Am Chem Soc* 117: 8867-8868.

MacMicking, J., Xie, Q. W. & Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* 15: 323-350.

MacMicking, J. D., Taylor, G. A. & McKinney, J. D. (2003). Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* 302(5645): 654-659.

Malen, H., Berven, F. S., Fladmark, K. E. & Wiker, H. G. (2007). Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. *Proteomics* 7(10): 1702-1718.

Malen, H., Pathak, S., Softeland, T., de Souza, G. A. & Wiker, H. G. (2010). Definition of novel cell envelope associated proteins in Triton X-114 extracts of Mycobacterium tuberculosis H37Rv. *BMC Microbiol* 10: 132.

Manta, B., Hugo, M., Ortiz, C., Ferrer-Sueta, G., Trujillo, M. & Denicola, A. (2009). The peroxidase and peroxynitrite reductase activity of human erythrocyte peroxiredoxin 2. *Arch Biochem Biophys* 484(2): 146-154.

Martin, E., Nathan, C. & Xie, Q. W. (1994). Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J Exp Med* 180(3): 977-984.

Master, S. S., Springer, B., Sander, P., Boettger, E. C., Deretic, V. & Timmins, G. S. (2002). Oxidative stress response genes in Mycobacterium tuberculosis: role of ahpC in resistance to peroxynitrite and stage-specific survival in macrophages. *Microbiology* 148(Pt 10): 3139-3144.

Mawuenyega, K. G., Forst, C. V., Dobos, K. M., Belisle, J. T., Chen, J., Bradbury, E. M., Bradbury, A. R. & Chen, X. (2005). Mycobacterium tuberculosis functional network analysis by global subcellular protein profiling. *Mol Biol Cell* 16(1): 396-404.

Meena, L. S. & Rajni (2010). Survival mechanisms of pathogenic Mycobacterium tuberculosis H37Rv. *FEBS J* 277(11): 2416-2427.

Miller, C. C., Rawat, M., Johnson, T. & Av-Gay, Y. (2007). Innate protection of Mycobacterium smegmatis against the antimicrobial activity of nitric oxide is provided by mycothiol. *Antimicrob Agents Chemother* 51(9): 3364-3366.

Mollenkopf, H. J., Jungblut, P. R., Raupach, B., Mattow, J., Lamer, S., Zimny-Arndt, U., Schaible, U. E. & Kaufmann, S. H. (1999). A dynamic two-dimensional polyacrylamide gel electrophoresis database: the mycobacterial proteome via Internet. *Electrophoresis* 20(11): 2172-2180.

Moon, J. C., Hah, Y. S., Kim, W. Y., Jung, B. G., Jang, H. H., Lee, J. R., Kim, S. Y., Lee, Y. M., Jeon, M. G., Kim, C. W., Cho, M. J. & Lee, S. Y. (2005). Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H2O2-induced cell death. *J Biol Chem* 280(31): 28775-28784.

Murphy, D. J. & Brown, J. R. (2007). Identification of gene targets against dormant phase Mycobacterium tuberculosis infections. *BMC Infect Dis* 7: 84.

Nathan, C. (2009). Taming tuberculosis: a challenge for science and society. *Cell Host Microbe* 5(3): 220-224.

Nathan, C. & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* 97(16): 8841-8848.

Nelson, K. J., Knutson, S. T., Soito, L., Klomsiri, C., Poole, L. B. & Fetrow, J. S. (2011). Analysis of the peroxiredoxin family: using active-site structure and sequence information for global classification and residue analysis. *Proteins* 79(3): 947-964.

Nelson, K. J., Parsonage, D., Hall, A., Karplus, P. A. & Poole, L. B. (2008). Cysteine pK(a) values for the bacterial peroxiredoxin AhpC. *Biochemistry* 47(48): 12860-12868.

Newton, G. L., Av-Gay, Y. & Fahey, R. C. (2000). A novel mycothiol-dependent detoxification pathway in mycobacteria involving mycothiol S-conjugate amidase. *Biochemistry* 39(35): 10739-10746.

Newton, G. L. & Fahey, R. C. (2002). Mycothiol biochemistry. *Arch Microbiol* 178(6): 388-394.

Ogusucu, R., Rettori, D., Munhoz, D. C., Netto, L. E. & Augusto, O. (2007). Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxynitrite: rate constants by competitive kinetics. *Free Radic Biol Med* 42(3): 326-334.

Parsonage, D., Karplus, P. A. & Poole, L. B. (2008). Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc Natl Acad Sci U S A* 105(24): 8209-8214.

Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus, P. A. & Poole, L. B. (2005). Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry* 44(31): 10583-10592.

Pascual, M. B., Mata-Cabana, A., Florencio, F. J., Lindahl, M. & Cejudo, F. J. (2010). Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins sensitive to oxidative stress. *J Biol Chem* 285(45): 34485-34492.

Passardi, F., Theiler, G., Zamocky, M., Cosio, C., Rouhier, N., Teixera, F., Margis-Pinheiro, M., Ioannidis, V., Penel, C., Falquet, L. & Dunand, C. (2007). PeroxiBase: the peroxidase database. *Phytochemistry* 68(12): 1605-1611.

Patel, M. P. & Blanchard, J. S. (2001). Mycobacterium tuberculosis mycothione reductase: pH dependence of the kinetic parameters and kinetic isotope effects. *Biochemistry* 40(17): 5119-5126.

Pearce, M. J., Arora, P., Festa, R. A., Butler-Wu, S. M., Gokhale, R. S. & Darwin, K. H. (2006). Identification of substrates of the Mycobacterium tuberculosis proteasome. *EMBO J* 25(22): 5423-5432.

Poole, L. B. (2007). The catalytic mechanism of peroxiredoxins. *Subcell Biochem* 44: 61-81.

Prutz, W. A., Monig, H., Butler, J. & Land, E. J. (1985). Reactions of nitrogen dioxide in aqueous model systems: oxidation of tyrosine units in peptides and proteins. *Arch Biochem Biophys* 243(1): 125-134.

Pryor, W. A. & Squadrito, G. L. (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* 268(5 Pt 1): L699-722.

Raman, S., Song, T., Puyang, X., Bardarov, S., Jacobs, W. R., Jr. & Husson, R. N. (2001). The alternative sigma factor SigH regulates major components of oxidative and heat stress responses in Mycobacterium tuberculosis. *J Bacteriol* 183(20): 6119-6125.

Rawat, M., Newton, G. L., Ko, M., Martinez, G. J., Fahey, R. C. & Av-Gay, Y. (2002). Mycothiol-deficient Mycobacterium smegmatis mutants are hypersensitive to alkylating agents, free radicals, and antibiotics. *Antimicrob Agents Chemother* 46(11): 3348-3355.

Reyes, A. M., Hugo, M., Trostchansky, A., Capece, L., Radi, R. & Trujillo, M. (2011). Oxidizing substrate specificity of Mycobacterium tuberculosis alkyl hydroperoxide reductase E: kinetics and mechanisms of oxidation and overoxidation. *Free Radic Biol Med* 51(2):464-473.

Rhee, S. G., Chae, H. Z. & Kim, K. (2005). Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 38(12): 1543-1552.

Rhee, S. G. & Woo, H. A. (2011). Multiple Functions of Peroxiredoxins: Peroxidases, Sensors and Regulators of the Intracellular Messenger H(2)O(2), and Protein Chaperones. *Antioxid Redox Signal* 15(3):781-794.

Rho, B. S., Hung, L. W., Holton, J. M., Vigil, D., Kim, S. I., Park, M. S., Terwilliger, T. C. & Pedelacq, J. D. (2006). Functional and structural characterization of a thiol peroxidase from Mycobacterium tuberculosis. *J Mol Biol* 361(5): 850-863.

Rosenkrands, I., King, A., Weldingh, K., Moniatte, M., Moertz, E. & Andersen, P. (2000). Towards the proteome of Mycobacterium tuberculosis. *Electrophoresis* 21(17): 3740-3756.

Sassetti, C. M., Boyd, D. H. & Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* 48(1): 77-84.

Seebeck, F. P. (2010). In vitro reconstitution of Mycobacterial ergothioneine biosynthesis. *J Am Chem Soc* 132(19): 6632-6633.

Sevanian, A., Muakkassah-Kelly, S. F. & Montestruque, S. (1983). The influence of phospholipase A2 and glutathione peroxidase on the elimination of membrane lipid peroxides. *Arch Biochem Biophys* 223(2): 441-452.

Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E., 3rd & Stover, C. K. (1996). Compensatory ahpC gene expression in isoniazid-resistant Mycobacterium tuberculosis. *Science* 272(5268): 1641-1643.

Sherman, D. R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M. I. & Schoolnik, G. K. (2001). Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. *Proc Natl Acad Sci U S A* 98(13): 7534-7539.

Shi, S. & Ehrt, S. (2006). Dihydrolipoamide acyltransferase is critical for Mycobacterium tuberculosis pathogenesis. *Infect Immun* 74(1): 56-63.

Shiloh, M. U. & Nathan, C. F. (2000). Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr Opin Microbiol* 3(1): 35-42.

Sohling, B., Parther, T., Rucknagel, K. P., Wagner, M. A. & Andreesen, J. R. (2001). A selenocysteine-containing peroxiredoxin from the strictly anaerobic organism Eubacterium acidaminophilum. *Biol Chem* 382(6): 979-986.

Soito, L., Williamson, C., Knutson, S. T., Fetrow, J. S., Poole, L. B. & Nelson, K. J. (2011). PREX: PeroxiRedoxin classification indEX, a database of subfamily assignments across the diverse peroxiredoxin family. *Nucleic Acids Res* 39(Database issue): D332-337.

Springer, B., Master, S., Sander, P., Zahrt, T., McFalone, M., Song, J., Papavinasasundaram, K. G., Colston, M. J., Boettger, E. & Deretic, V. (2001). Silencing of oxidative stress response in Mycobacterium tuberculosis: expression patterns of ahpC in virulent and avirulent strains and effect of ahpC inactivation. *Infect Immun* 69(10): 5967-5973.

Stehr, M., Hecht, H. J., Jager, T., Flohe, L. & Singh, M. (2006). Structure of the inactive variant C60S of Mycobacterium tuberculosis thiol peroxidase. *Acta Crystallogr D Biol Crystallogr* 62(Pt 5): 563-567.

Stone, J. R. (2004). An assessment of proposed mechanisms for sensing hydrogen peroxide in mammalian systems. *Arch Biochem Biophys* 422(2): 119-124.

Storz, G., Christman, M. F., Sies, H. & Ames, B. N. (1987). Spontaneous mutagenesis and oxidative damage to DNA in Salmonella typhimurium. *Proc Natl Acad Sci U S A* 84(24): 8917-8921.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. & Russell, D. G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* 263(5147): 678-681.

Ta, P., Buchmeier, N., Newton, G. L., Rawat, M. & Fahey, R. C. (2011). Organic hydroperoxide resistance protein and ergothioneine compensate for loss of mycothiol in Mycobacterium smegmatis mutants. *J Bacteriol* 193(8): 1981-1990.

Tartaglia, L. A., Storz, G. & Ames, B. N. (1989). Identification and molecular analysis of oxyR-regulated promoters important for the bacterial adaptation to oxidative stress. *J Mol Biol* 210(4): 709-719.

Tian, J., Bryk, R., Shi, S., Erdjument-Bromage, H., Tempst, P. & Nathan, C. (2005). Mycobacterium tuberculosis appears to lack alpha-ketoglutarate dehydrogenase and encodes pyruvate dehydrogenase in widely separated genes. *Mol Microbiol* 57(3): 859-868.

Trindade, D. F., Cerchiaro, G. & Augusto, O. (2006). A role for peroxymonocarbonate in the stimulation of biothiol peroxidation by the bicarbonate/carbon dioxide pair. *Chem Res Toxicol* 19(11): 1475-1482.

Trujillo, M., Clippe, A., Manta, B., Ferrer-Sueta, G., Smeets, A., Declercq, J. P., Knoops, B. & Radi, R. (2007). Pre-steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase upon oxidation. *Arch Biochem Biophys* 467(1): 95-106.

Trujillo, M., Ferrer-Sueta, G., Thomson, L., Flohe, L. & Radi, R. (2007). Kinetics of peroxiredoxins and their role in the decomposition of peroxynitrite. *Subcell Biochem* 44: 83-113.

Trujillo, M., Mauri, P., Benazzi, L., Comini, M., De Palma, A., Flohe, L., Radi, R., Stehr, M., Singh, M., Ursini, F. & Jaeger, T. (2006). The mycobacterial thioredoxin peroxidase can act as a one-cysteine peroxiredoxin. *J Biol Chem* 281(29): 20555-20566.

Vandal, O. H., Pierini, L. M., Schnappinger, D., Nathan, C. F. & Ehrt, S. (2008). A membrane protein preserves intrabacterial pH in intraphagosomal Mycobacterium tuberculosis. *Nat Med* 14(8): 849-854.

Wang, G., Hong, Y., Johnson, M. K. & Maier, R. J. (2006). Lipid peroxidation as a source of oxidative damage in Helicobacter pylori: protective roles of peroxiredoxins. *Biochim Biophys Acta* 1760(11): 1596-1603.

Weldingh, K., Rosenkrands, I., Jacobsen, S., Rasmussen, P. B., Elhay, M. J. & Andersen, P. (1998). Two-dimensional electrophoresis for analysis of Mycobacterium tuberculosis culture filtrate and purification and characterization of six novel proteins. *Infect Immun* 66(8): 3492-3500.

Wengenack, N. L., Jensen, M. P., Rusnak, F. & Stern, M. K. (1999). Mycobacterium tuberculosis KatG is a peroxynitritase. *Biochem Biophys Res Commun* 256(3): 485-487.

Wilson, T. M. & Collins, D. M. (1996). ahpC, a gene involved in isoniazid resistance of the Mycobacterium tuberculosis complex. *Mol Microbiol* 19(5): 1025-1034.

Woo, H. A., Yim, S. H., Shin, D. H., Kang, D., Yu, D. Y. & Rhee, S. G. (2010). Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. *Cell* 140(4): 517-528.

Wood, Z. A., Poole, L. B. & Karplus, P. A. (2003). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300(5619): 650-653.

Wood, Z. A., Schroder, E., Robin Harris, J. & Poole, L. B. (2003). Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28(1): 32-40.

Xie, Q. W., Whisnant, R. & Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med* 177(6): 1779-1784.

Yamamoto, Y., Ritz, D., Planson, A. G., Jonsson, T. J., Faulkner, M. J., Boyd, D., Beckwith, J. & Poole, L. B. (2008). Mutant AhpC peroxiredoxins suppress thiol-disulfide redox deficiencies and acquire deglutathionylating activity. *Mol Cell* 29(1): 36-45.

Yang, K. S., Kang, S. W., Woo, H. A., Hwang, S. C., Chae, H. Z., Kim, K. & Rhee, S. G. (2002). Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation

of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* 277(41): 38029-38036.

Zahrt, T. C. & Deretic, V. (2002). Reactive nitrogen and oxygen intermediates and bacterial defenses: unusual adaptations in Mycobacterium tuberculosis. *Antioxid Redox Signal* 4(1): 141-159.

Zahrt, T. C., Song, J., Siple, J. & Deretic, V. (2001). Mycobacterial FurA is a negative regulator of catalase-peroxidase gene katG. *Mol Microbiol* 39(5): 1174-1185.

Zhang, Y., Heym, B., Allen, B., Young, D. & Cole, S. (1992). The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. *Nature* 358(6387): 591-593.

Zhao, X., Suarez, J., Khajo, A., Yu, S., Metlitsky, L. & Magliozzo, R. S. (2010). A radical on the Met-Tyr-Trp modification required for catalase activity in catalase-peroxidase is established by isotopic labeling and site-directed mutagenesis. *J Am Chem Soc* 132(24): 8268-8269.

17

Characteristic Conformational Behaviors of Representative Mycolic Acids in the Interfacial Monolayer

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1. Introduction

Mycobacterial mycolic acid (MA) are long chain 2-alkyl branched, 3-hydroxy fatty acid with two intra-chain groups in the so-called meromycolate chain. On the basis of the nature of the functional groups in the meromycolate chains, MAs are categorized into three major groups: α-MA with no oxygen-containing intra-chain groups, methoxy-MA (MeO-MA) in which the distal group has a methoxy gorup and Keto-MA in which the distal group has a carbonyl group (Fig. 1) (Watanabe et al., 2001; 2002). MAs are characteristic components of mycobacterial cell envelopes, where a major proportion are covalently bonded to the underlying cell wall arabinogalactan (Goren & Brennan, 1979; McNeil et al., 1991; Minnikin, 1982).

In the structural models of the mycobacterial cell envelope proposed previously (Minnikin, 1982; Rastogi, 1991), MAs covalently linked to penta-arabinosyl residues of cell wall arabinogalactan are arranged perpendicular to the cell wall, forming a highly structured monolayer. Recent computer simulation work supported such arrangement of MAs as proposed in the model (Hong & Hopfinger, 2004). This outer leaflet of mycobacterial cell envelope is considered to provide the cells with a special permeability barrier responsible for various physiological and pathogenic features of mycobacterial cells (Daffé et al., 1999). There are various other lipids in the mycobacterial cell envelope and they may also take part in the permeability function of the cell envelope as suggested (Minnikin, 1982; Puech et al., 2001; Rastogi, 1991). Recently, a *Mycobacterium tuberculosis* (*M. tb*) mutant whose MA comprises only α-MA (Dubnau et al., 2000), a recombinant mutant having over-produced MeO-MA with no Keto-MA (Yuan et al., 1998) and a mutant having 40 % less cell wall mycolate (Daffé et al., 1999) have been described. These results show that *M. tb* can be viable with highly modified mycolic acid composition and that its pathogenicity may be related to the types of MAs. Those papers also suggest that MAs on the cell envelope have determining effect on the permeability barrier function of the cell wall outer hydrophobic layer barrier and different MAs may contribute to the cell wall permeability barrier functions in different ways.

In very early studies (Staellberg-Stenhagen & Stenhagen, 1945), the multi-component nature of mycolic acids was not yet known, but it was shown that the total MA formed a stable monolayer on the water surface. It was concluded that MA had extended linear structures, a feature later confirmed by structural analysis (Minnikin, 1982; Minnikin et al., 2002; Rastogi,

1991). Both in the monolayer on the water surface and in the proposed cell envelope lipid structure models, MA is considered to take the same structural arrangement, with the hydrophilic 3-hydroxy and 2-carboxyl groups touching the hydrophilic surface and with the aliphatic chains stretching out in parallel, and normal to the hydrophilic surface. Therefore, detailed studies on the artificial MA layers on water surface should help elucidation of the roles and the nature of actual mycolate layers in the mycobacterial cell envelope.

Recently, limited Langmuir monolayer studies have been performed on a selection of MA (Hasegawa et al., 2000; 2002; Hasegawa & Leblanc, 2003; Hasegawa et al., 2003). Those studies reported that, in a compressed monolayer, α-mycolic acid from *Mycobacterium avium*, apparently took a conformation with three parallel chains, and on further compression, an extended structure, but that the corresponding *M. tb* mycolate appeared to take an extended conformation. As regards the MeO and Keto MAs from *M. tb*, they were reported to take triple chain folded conformations (Hasegawa & Leblanc, 2003; Hasegawa et al., 2003). Regrettably, their monolayer experiments were limited at a single temperature of 25 °C whereas temperature is one of the important factors that influence biological activities of the living cells.

In this chapter, the temperature effect on the Langmuir monolayer packing of all three α-, Keto-, and MeO-MAs from representative slow growing mycobacteria are analyzed to elucidate the conformational behavior of MAs in the monolayer and to understand their roles in the mycobacterial cell envelope.

2. Air/water interface as a biological model membrane

All the biological cells are covered with membranes, through which selective materials are allowed to diffuse in and/or out. It is needless to say that almost all biological activities are intermediated by membranes.

In a real biological membrane system, there always exists specific intermolecular interaction among the membrane-forming components, which is essential for biological activity however sometimes hinders simple understanding of the mechanism. Air/water interface across which dielectric constant changes drastically is a similar self-assembly field to biological interface without particular intermolecular interaction and therefore an ideal model for a biological membrane to study how a certain membrane component behaves there.

Interfacial tension or surface tension, when the interface is between air and water, γ is an essential thermodynamic property for studying interfacial phenomena. A monolayer formed at the air/water interface by a water-insoluble film-forming material is called "Langmuir monolayer." They have been widely studied by using a so-called trough, a shallow container of wide base area equipped with surface tensiometer and a movable barrier for changing the surface area. The performance of amphiphilic molecules such as MAs, lipids, or other biologically significant substance, at interfaces depends not only on the nature of the interface but also strongly on the environmental conditions such as temperature, pressure, pH and so on. Therefore, we studied the temperature effect on the surface tension and the molecular area in the interfacial monolayer of the representative MAs.

3. Structural features of representative mycolic acid samples

The structures of MAs have been characterized (Watanabe et al., 2001; 2002) and MAs are grouped into three major groups, i.e., α-MAs in which X and Y in Fig. 1 are two cyclopropyls or one cyclopropyl and one double bond, MeO-MAs in which X is a methoxy group with a methyl group at the adjacent distal carbon, and Keto-MAs in which X is a keto group with a methyl group at the distal adjacent carbon. In *Mycobacterium avium-intracellulare* complex (MAC), further oxidized wax-ester MA is also known. Further, natural mixtures have both *cis*- and *trans*-cyclopropane rings, the latter having an adjacent methyl group (Fig. 1).

The stereochemistry of the proximal carbon and that of the distal carbon of the *cis*-cyclopropyl group have been determined to be *R* and *S*, respectively, according to the knowedge that the *cis*-cyclopropyl group is derived from the same biosynthetic intermediate of the known stereochemistry (Al Dulayymi et al., 2005). The absolute configurations of the hydroxy-bearing carbon and the carboxyl-bearing carbon in -CH_2-CH(COOH)-CH(OH)-CH_2- are both *R* as reported (Asselineau & Asselineau, 1966; Tocanne & Asselineau, 1968) and as demonstrated by us by easy preparation of its stable chair form acetonide by reduction of MA methyl ester and subsequent acetonide formation (yield 74%).

The non-oxygenated MA samples assayed were so-called type-1 α-MAs (α1-MAs, X and Y both *cis*-cyclopropyls) from *M. tb* (strain Aoyama B), *M. kansasii* (strain 304) and MAC (strain KK41-24) and so-called type-3 α-MAs (α3-MAs), from BCG (strain Tokyo 172) (X *cis*-double bond, Y *cis*-cyclopropyl in Fig. 1) and from MAC (X *cis*-cyclopropyl, Y *cis*-double bond). Their intrachain groups are either *cis*-cyclopropyl or *cis*-double bond but the methylene chain segment lengths vary greatly.

The oxygenated MA samples were type-1 MeO-MA and Keto-MAs from *M. tb* (strain Aoyama B) and *Mycobacterium bovis* BCG (strain Tokyo 172). The structural characteristics and compositions of the MAs studied are summarized in Fig. 1 and Table 1.

4. Experimental details

4.0.0.1 Preparation of the mycolic acid samples

MA samples used in our study were prepared by hydrolysis of purified relevant α-MA, MeO-MA and Keto-MA methyl esters. The procedures for separation and purification of the methyl esters including argentation thin-layer chromatography (TLC) to remove minor components with double bonds and the analytical details are described elsewhere (Watanabe et al., 2001; 2002). Hydrolysis was performed by heating a sealed tube containing MA methyl ester (70 mg), powdered KOH (200 mg) and 2-propanol (2 ml) in an oil bath kept at 80-85 °C for 2 hours with stirring. The hydrolysate was acidified with 2 N H_2SO_4 and treated with hexane, and the mycolic acid obtained was purified by TLC with hexane/AcOEt (4:1, v/v) to remove the byproduct epimer.

4.0.0.2 Other reagents

Distilled reagent grade chloroform (Wako chemicals) was used as the spreading medium. Water was distilled once and deionized by Milli-Q Plus (resistance 18.2 MΩ cm).

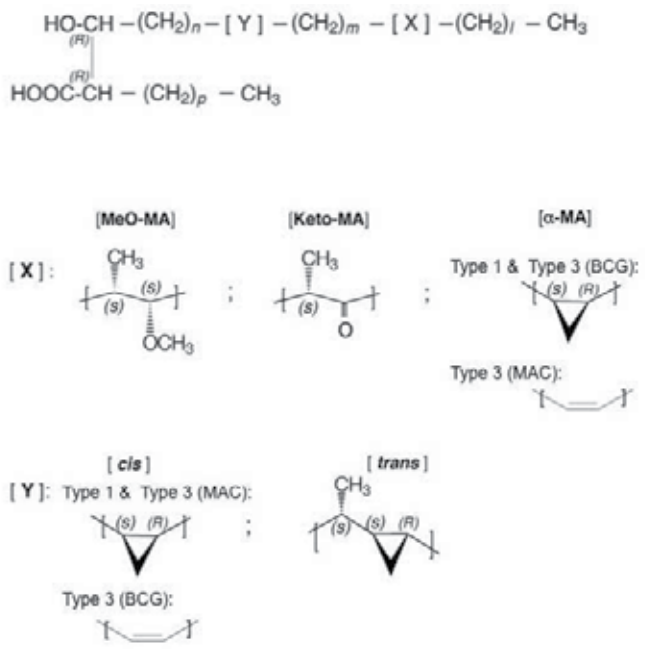


Fig. 1. General structure of MA.

Sample	Origin	Distal group [X]	*p-n-m-l*[a]	*cis* / *trans* ratio of [X][b]
Type 1 α	*M. tb*	*cis*-cyclopropyl	23-11-14-19 23-13-14-19	1/0
	M. kansasii	*cis*-cyclopropyl	21-15-14-19 [21-15-16-17 or 21-17-14-17]	1/0.03
	MAC	*cis*-cyclopropyl	21-17-14-17	1/0.14
Type 3 α	BCG	*cis*-double bond	23-13-14-19	1/0
	MAC	*cis*-cyclopropyl	21-17-14-17	1/0.14
Type 1 MeO	*M. tb*	*cis*-cyclopropyl	23-17-16-17	1/0.4
		trans-cyclopropyl	23-18-16-17	
	BCG	*cis*-cyclopropy	23-17-16-17	1/0.03
		trans-cyclopropyl	23-18-16-17	
Type 1 Keto	*M. tb*	*cis*-cyclopropyl	23-15-18-17	1/0.4
		trans-cyclopropyl	23-16-18-17	
	BCG	*cis*-cyclopropyl	23-15-18-17 23-17-18-17	1/0.33
		trans-cyclopropyl	23-16-18-17	

a. Values of major components.
b. Ratios implied by ^{1}H-NMR spectra.

Table 1. Structural features of the MA samples.

4.0.0.3 Surface pressure vs. mean molecular surface area (π vs. A) isotherms measurement

The Langmuir monolayers were prepared by spreading a chloroform solution of MA (1 ml, ca. 6×10^{-5} M) on the water surface. Surface pressure (π) vs. mean molecular area (A) isotherms of the Langmuir monolayer of MA spread on water were measured by a Lauda film balance (FW1). Here, π is defined as $\pi = \gamma^0 - \gamma$, where γ^0 is the surface tension of water with no monolayer. The area of the water surface was about 562 cm^2 in this trough. The compression rate of the monolayer was 14 $Å^2$ $molecule^{-1}$ min^{-1}. π vs. A isotherms were measured at various temperatures in the range of $10 \sim 46$ °C. The subphase temperature was controlled within the accuracy of ± 0.2 °C. The room temperature was thermostatted at 23 ± 1 °C. Each measurement was repeated 3∼5 times.

4.0.0.4 Ellipsometry:

Ellipsometry (Tompkins & McGahan, 1999) was performed on a Nanofilm EP^3 (NFT Co., Göttingen, Germany) with a home-built trough installed on the stage. The trough was thermostatted at the temperatures as specified in Table 2 (error within $\pm$ 0.2 °C). The monolayer was prepared and compressed with a Teflon-coated barrier to the target π values. The refractive index was taken to be 1.48 in evaluation of monolayer thickness.

5. Conformational behavior of MAs in the monolayer

The behavior of MAs in the interfacial monolayer is most effectively shown by phase diagram of the monolayer. The phase diagrams are constructed by plotting the surface pressures at the bends on the π vs. A isotherms which correspond to the phase transitions π^{tr} and to film collapsing π^{cp} against the temperature (Villeneuve et al., 2005; 2007; 2010). As they will be shown later, at low temperature (T) and low surface pressure (π: defined as the decrement of surface tension due to monolayer formation), all these MAs form a condensed four-chain (so called W-shape) structure in which the 2-alkyl side chain and the three methylene-chain segments are parallel to each other. The two functional groups appear to allow the meromycolate chain to fold up into a compact parallel arrangement. As T and π are increased, α- and MeO-MAs tend to take stretchedout structures in which the distal functional group in the meromycolate chain leaves the near-hydroxy group location. The conformation of Keto-MA is little affected by the changes in T and π and the four-chain form is retained.

5.1 α-MA monolayers

The phase diagrams (π vs. T diamgram), where surface pressure of phase transition π^{tr} and that of film collapse π^{cp} are plotted against T and the diagram where mean molecular areas at π^{tr} and π^{cp} are plotted against T are shown in Figs. 2-6. Those diagrams, some of which are quite simple and others more complex, demonstrate that π^{tr}, π^{cp}, A^{tr} and A^{cp} all changed depending on the temperature. As shown, all those diagrams for the present α-MAs from different origins gave analogous features: In Figs 2a, 3a, 4a, 5a and 6a, each of the π^{cp} vs. T diagram gave a cusp in the range of $32 \sim 39$ °C. The T at the cusp were different in different samples, and at that point, A^{cp} vs. T curve was discontinuous, as shown in Figs. 2b, 3b and 4b, though it is not quite obvious in Figs. 5b and 6b. One characteristic feature noted in those diagrams is that the π^{cp} values of *M. tb* complex α-MAs, such as α1-MA from *M. tb* and α3-MA from BCG were much higher, and accordingly the A^{cp} values much smaller than the

corresponding values of α1-MAs from *M. kansasii* and MAC. At the temperature just below the cusp in the range of 32 ∼ 39 °C, the π^{cp} values for *M. tb* α1-MA, BCG α3-MA, *M. kansasii* α1-MA and MAC α1-MA were 60, 58, 42 and 34 mN m^{-1}, respectively, and the A^{cp} values were 36, 36, 43 and 41 Å^2 molecule^{-1}, respectively.

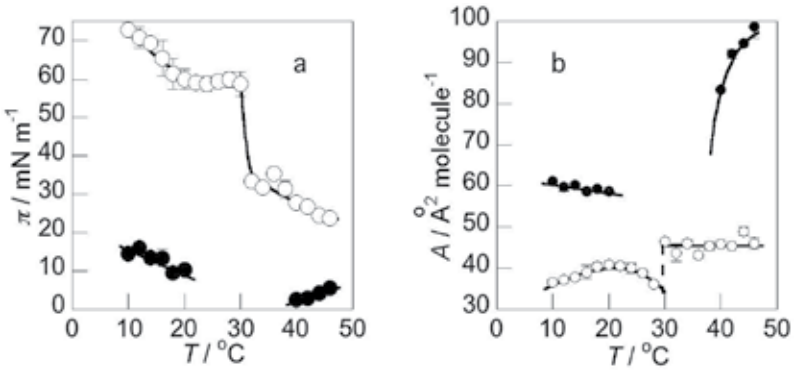


Fig. 2. (a) Phase diagram and (b) A^{cp}, A^{tr} vs. T curves of *M. tb* α1-MA. (—◯—) film collapse; (—●—) phase transition.

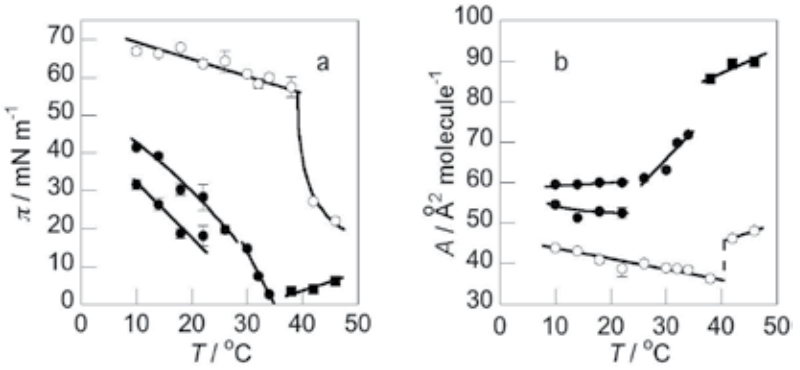


Fig. 3. (a) Phase diagram and (b) A^{cp}, A^{tr} vs. T curves of BCG α3-MA. (—◯—) film collapse; (filled symbols) phase transition.

All the phase diagrams of the α-MAs in the present study, having varied hydrocarbon segment lengths and different X and Y combinations (Fig. 1), showed the same features. As the surface pressure was increased by compression, the molecules apparently changed from the 4-chain W-shapes to extended ones, having a mean molecular area corresponding to that of two hydrocarbon chains. As described in the literature (Villeneuve et al., 2010), when no restriction is applied, the W-shape conformation obtained by Monte Carlo (MC) calculation retains its form during a series of 2.5 ps molecular dynamics (MD) runs, suggesting that the sparsely scattered α-MA molecules on the water surface are in the W-shape before compression is started. When the film is compressed, in a very low surface pressure and low temperature region of the phase diagram the first phase-transition point appears on the π vs. A isotherms, where the original condensed phase changes to another condensed phase. In the A^{cp}, A^{tr} vs. T diagrams, when the temperature is between 10 °C and 20 °C, the A^{tr} values remain more or less at around 60 Å^2 molecule^{-1}, which shows that at that stage the molecules are in W-shape. At this first phase-transition point, the molecules of W-shaped conformation is

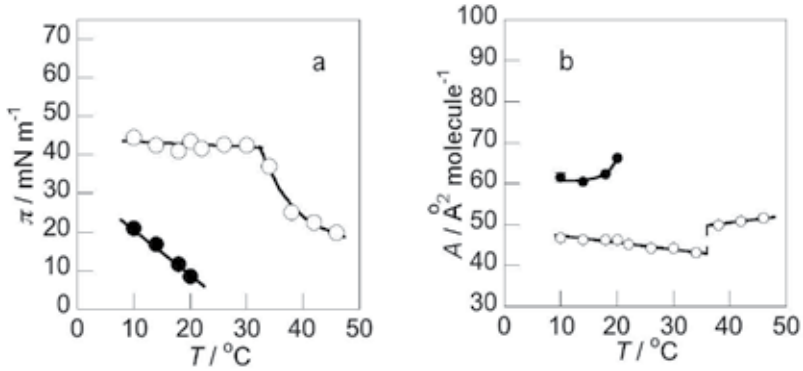


Fig. 4. (a) Phase diagram and (b) A^{cp}, A^{tr} vs. T curves of *M. kansasii* α1-MA. (—◯—) film collapse; (—●—) phase transition.

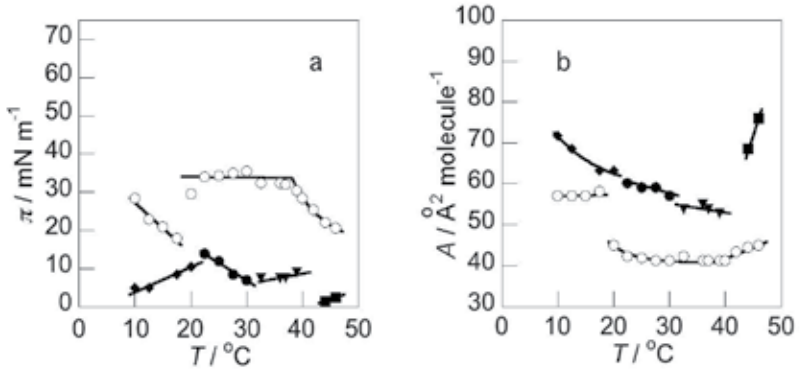


Fig. 5. (a) Phase diagram and (b) A^{cp}, A^{tr} vs. T curves of MAC α1-MA. (—◯—) film collapse; (filled symbols) phase transition.

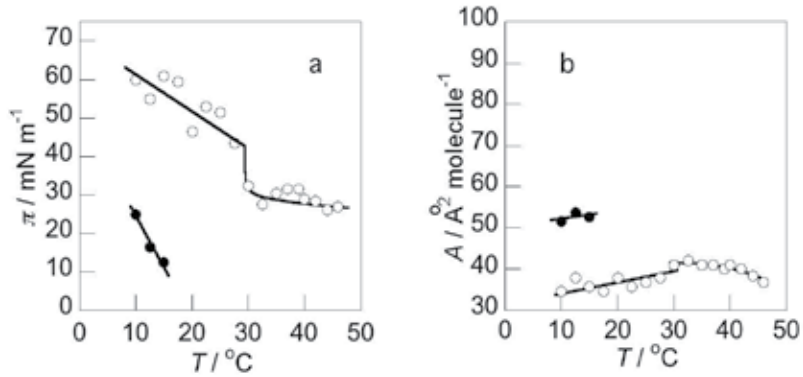


Fig. 6. (a) Phase diagram and (b) A^{cp}, A^{tr} vs. T curves of MAC α3-MA. (—◯—) film collapse; (—●—) phase transition.

considered to start unfolding, thereby inducing conversion of the *trans* form to the *gauche* form, which then start extending. The geometry change should be facile, because DFT calculations demonstrated a very small energy barrier between the two geometries and the

sharp NMR signals of the relevant atoms implied that the conversion between the two should take place easily. Though an exothermic transition is noted in the phase diagrams, the π^{tr} values tend to decrease generally in the π vs. T diagrams as the temperature increases, which means that the transition taking place at the surface pressure is endothermic. This also implies that the conversion from *trans* to *gauche* geometry takes place during this phase transition. The decrease in the π^{tr} values, however, is not so marked as in the case of MeO-MA (Villeneuve et al., 2007) as will be shown below, probably because the change in the conformation of α-MAs does not involve breaking of hydrogen bonding.

Elasticity and fluidity of the biological membranes are important factors in relation to their functions. The elasticity modulus

$$E = -A(\partial\pi/\partial A)_{T,p} \tag{1}$$

or E values of α-MAs are in the range of 80 to 200 mN m^{-1}, which roughly correspond to the values of liquid condensed film of common fatty acids, such as stearic acid (Villeneuve et al., 2005). When π is 10 mN m^{-1} and T below 20 °C, where the molecules are considered to be taking the W-shape according to the phase diagrams, the E values of those MAs are equivalent to those at higher temperatures where the molecules are in extended conformations. Thus, α-MA forms a monolayer which is fluid in whatever conformation the α-MAs might be taking and in which the α-MAs is ready to change the molecular area in response to outside pressure and temperatures.

5.2 Keto-MA monolayers

The phase diagrams of Langmuir monolayer and the A^{cp}, A^{tr} vs. T diagrams for Keto-MAs are quite different from those for α-MAs as shown in Fig. 7. The phase diagrams for Keto-MAs are much simpler than those for the α-MAs and the values of A^{cp} are much larger for the Keto-MAs than for the α-MAs. Keto-MA forms a condensed monolayer more rigid than a liquid condensed film but less stiff than a solid condensed film over a wide range of temperature and surface pressure. A^{cp} of Keto-MA is shown to be about 80 Å^2 molecule^{-1}. Moreover, the monolayer is in a condensed state as indicated by the elastic modulus, e.g., about $E = 1000$ mN m^{-1} for *M. tb* and about $E = 300$ mN m^{-1} for BCG. Accordingly, it seems reasonable to assume that in the Keto-MA molecules the meromycolate chain bends at the cyclopropane and at the carbonyl group to form a 4-chain structure whose four hydrocarbons are packed tightly in parallel, with the carbonyl group touching the water surface and hydrated.

5.3 MeO-MA monolayers

The phase diagrams of monolayer and the mean molecular area vs. T diagrams for MeO-MAs from *M. tb* and BCG are shown in Fig. 8. In each of the phase diagram, a characteristic phase transition is shown, of which surface pressure greatly decreased with an increase in temperature. This phase transition is reversible as has been confirmed by repeated compression-expansion measurement of the π vs. A isotherm. A^{tr} takes values from 70 to 90 Å^2 molecule^{-1} for *M. tb* and from 70 to 110 Å^2 molecule^{-1} for BCG*. Even with such large mean molecular areas, the monolayers are in condensed states. For example the elastic moduli below π^{tr} of the two MA samples are $E = 100 \sim 350$ mN m^{-1}. On the other hand, A^{cp} takes values less than 60 Å^2 molecule^{-1} down to 38 Å^2 molecule^{-1} for both MeO-MA samples*.

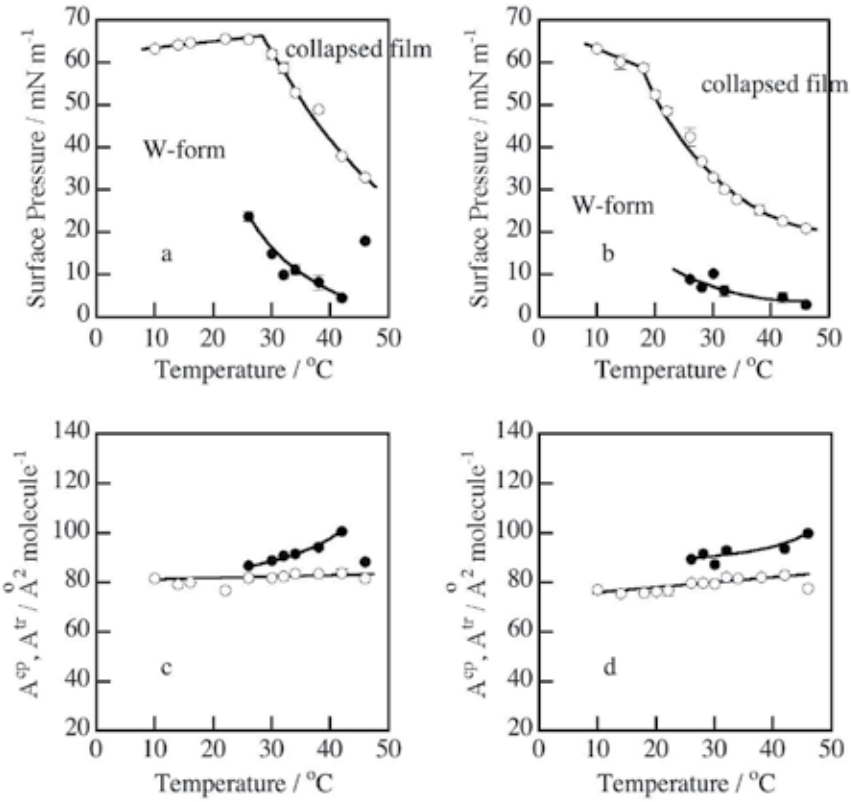


Fig. 7. Phase diagrams and A^{cp}, A^{tr} vs. T curves of Keto-MAs. (a) Phase diagrams for *M. tb* Keto-MA; (b) BCG Keto-MA; (c)A^{cp}, A^{tr} vs. T diagrams for *M. tb* Keto-MA; (d) BCG Keto-MA; (—◯—) film collapse; (—●—) phase transition.

These results strongly suggest that MeO-MAs take W-shaped conformations under low T and low π condition.

Enthalpy changes associated with these phase transitions are tentatively (because the systems consist of known and unknown species) evaluated by applying the Clausius-Clapeyron equation derived for insoluble monolayer to the π^{tr} vs. T and A^{tr}, A^{cp} vs. T curves (Motomura, 1967).

$$\left(\frac{\partial \pi^{tr}}{\partial T}\right)_p = \frac{1}{T}\frac{\Delta_l^s h}{\Delta_l^s A}, \tag{2}$$

where

$$\Delta_l^s h = h_{fs} - h_{fl} \tag{3}$$

$$\Delta_l^s A = A_{fs} - A_{fl} \tag{4}$$

Here, h_{fl} and h_{fs} are the partial molar enthalpies, and A_{fl} and A_{fs} are the areas per mole at equilibrium, in different states. Here, the more condensed state is denoted by 's'. The values of A^{tr} and A^{cp} are employed as A_{fl} and A_{fs} for evaluation of $\Delta_l^s A$. The results are shown in Fig. 9 together with the values of enthalpy change associated with the phase transition

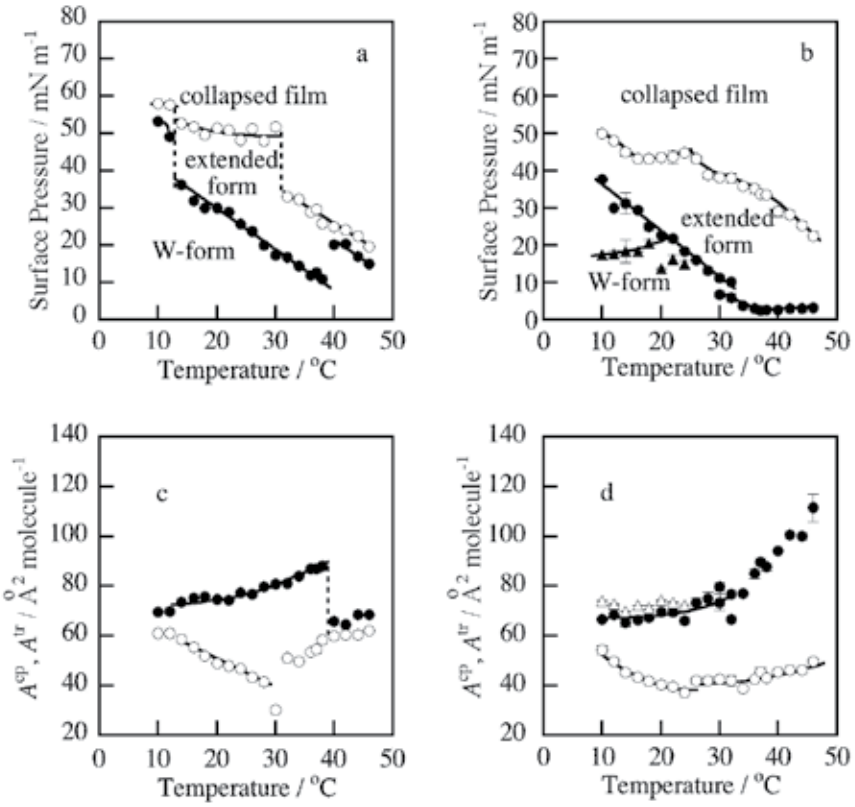


Fig. 8. Phase diagrams and A^{cp}, A^{tr} vs. T curves of MeO-MAs. (a) Phase diagrams for *M. tb* MeO-MA; (b) BCG MeO-MA; (c)A^{cp}, A^{tr} vs. T diagrams for *M. tb* MeO-MA; (d) BCG MeO-MA; (open circle) film collapse; (filled symbols) phase transition.

observed in the low temperature and low surface pressure region of the phase diagrams for *M. tb* α1-MA (Fig. 2a) and BCG α3-MA (Fig. 3a).

In the entire temperature range, the $\Delta_l^s h$ values for the MeO-MAs are larger than those for the α-MAs. This is probably because the methoxy group is hydrated in the interfacial layer and when the MeO-MA transforms to an extended conformation, hydrogen bondings must be broken.

One possible reason that may explain the difference in the behavior of the MAs is the difference in the hydrophilic nature of the functional group [X]. The adhesion energy of hydrophilic groups to water surface such as ether, ketone and alcohol groups is said to be fairly independent of other parts of the molecule and at 20 °C, those of diisopropyl ketone and of diamyl ether are reported to be 74 and 68 mJ m^{-2}, respectively (Timmons & Zisman, 1968). The difference may not be much, but may play some role in the conformational behaviors of MeO- and Keto-MAs in the water surface monolayers. α-MAs having no hydrophilic group at the [X]-position may be more free to take a stretched-out structure, even at a lower surface pressure.

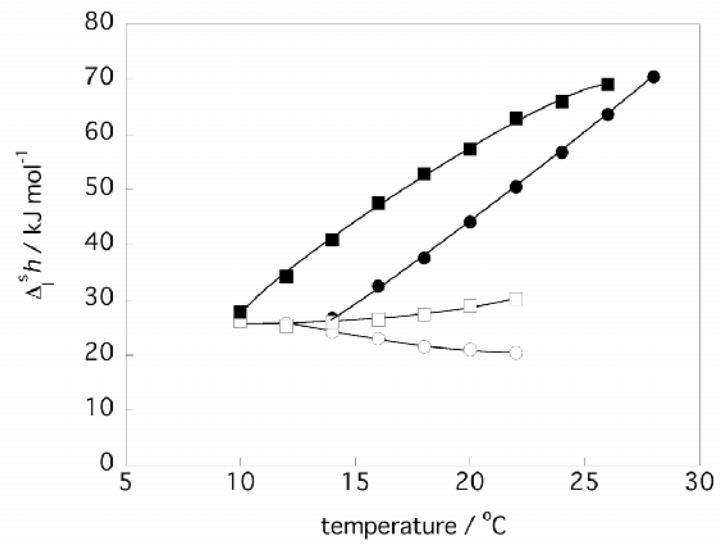


Fig. 9. Enthalpy change associated with phase transition. *M. tb* α 1-MA (—◯—); *M. tb* MeO-MA (—●—); BCG α 3-MA (—□—); BCG MeO-MA (—■—).

* The A^{tr} and A^{cp} must be corrected to the values mentioned here from the ones reported in the literature (Villeneuve et al., 2007).

5.4 Other data supporting conformational behavior of Keto- and MeO-MAs deduced from the monolayer study

5.4.0.5 Ellipsometry

Conformational transition from a 4-chain structure to an extended one suggested from the π vs. A study is supported by the *in-situ* ellipsometry. Results of the ellipsometric measurement are summarized in Table 2. Thus the thickness of the monolayer of MeO-MA at $T = 32$ °C and $\pi = 30$ mN m^{-1} is larger by a factor of $1.7 \sim 2$ than that of MeO-MA at $T = 18$ °C and $\pi = 18$ mN m^{-1} and those of Keto-MA at $T = 18$ °C and $\pi = 30$ mN m^{-1} and $T = 32$ °C and $\pi = 20$ mN m^{-1}. The thickness of MeO-MA monolayer changes from 2.91 nm for *M. tb* and 2.78 nm for BCG at $T = 18$ °C and $\pi = 18$ mN m^{-1}, which is almost the same value with the thickness of Keto-MA, drastically to 4.96 nm (*M. tb*) and 5.62 nm (BCG) at $T = 32$ °C and $\pi = 30$ mN m^{-1}. As for Keto-MA whose A^{cp} values imply that its carbonyl group is hydrated at the water surface to give a four-chain molecular conformation, the monolayer thickness is unchanged irrespective of the surface pressure or temperature (Table 2).

Origin	Keto-MA			MeO-MA		
	T / °C	π / mN m^{-1}	thickness / nm	T / °C	π /mN m^{-1}	thickness / nm
M. tb	18	30	2.90 ± 0.07	18	18	2.91 ± 0.05
				32	30	4.96 ± 0.88
BCG	18	30	2.90 ± 0.05	18	18	2.78 ± 0.04
	32	20	2.80 ± 0.08	32	30	5.62 ± 0.18

Table 2. Thickness of Langmuir monolayer estimated by ellipsometry.

5.4.0.6 Computer simulation

The MAs subjected to MC calculations and MD simulations were *cis*-cyclopropyl MeO-MA with *n-m-l* of 17-16-17, *trans*-cyclopropyl MeO-MA with *n-m-l* of 18-16-17, *cis*-cyclopropyl Keto-MA with *n-m-l* of 15-18-17 and 17-18-17 and *trans*-cyclopropyl Keto-MA with *n-m-l* of 16-18-17 (Table 1). The structural models of MeO- and Keto-MA produced by MC calculations were all of 4-chain structure as illustrated in Figs. 10a and b. The fact suggests that this type of arrangement of carbon chain segments is appropriate for energetically stable conformations. After the molecular dynamics (MD) simulation, Keto-MA having 4-chain structure normally retained the original 4-chain form (Fig. 10e) and seldom gave extended structures. On the other hand, MeO-MA, whose starting structure is as in Figs. 10a and b, often gave extended structures (Fig. 10c), though some models retained 4-chain structures as seen in Fig. d (Villeneuve et al., 2007).

Intra-molecular hydrogen bonding involving either the oxo or methoxy group and the 3-hydroxy carboxylic acid group may contribute to some extent to the retaining of the 4-chain structure. However, one of the major causes for the difference observed in the results of MD simulation of the two types of MAs seems to be in the difference in the lengths of the methylene chain segments or in the *n-m-l* values. As described previously (Watanabe et al., 2002), the major component of the MeO-MA is *cis*-cyclopropyl-containing MeO-MA acid with the *n-m-l* value of 17-16-17, and the minor component with a *trans*-cyclopropane with the *n-m-l* value of 18-16-17. In those MeO-MAs, n is larger than m. In the starting models for MD of MeO-MA, having the energetically stabilized 4-chain structure produced by MC (Figs. 10a and b), a bulky group consisting of a methoxy group and the adjacent methyl group is at a position to obstruct the compact arrangement of the 4 chains, as demonstrated by the molecular minimized energy levels: for the models in the literature (Villeneuve et al., 2007), the minimized energy levels of *cis*- and *trans*-MeO-MAs are $-13 \sim -19$ kcal mol^{-1} and $-7 \sim -12$ kcal mol^{-1}, respectively, whereas those of *cis*- and *trans*-Keto-MAs are $-24 \sim -32$ kcal mol^{-1} and $-28 \sim -38$ kcal mol^{-1}. The vibrations of the bulky group locating at or above the location of the 3-hydroxy carboxylate group during MD simulation may disturb the neat arrangement of the neighbouring chains to induce faster and more efficient deviation from the original 4-chain structure. In Keto-MA, the *n-m-l* value for the major *cis*-cyclopropane containing component is 15-18-17 or 17-18-17 and that for the major *trans*-cyclopropane containing component is 16-18-17, n being smaller than m. Thus, the oxo and the adjacent methyl groups are normally at the end or stretching out of the square pillar-like 4-chain structure. It allows a more compact solid arrangement of the methylene chains in the molecule and the more quiet vibration of the alpha-methyl oxo group may tend to be less disturbing for the 4-chain arrangement during the MD.

The fact that the oxo group is normally situating at the end or out of the 4-chain pillar structure may contribute to the more stable 4-chain structure of Keto-MA in Langmuir monolayers; it assures that the oxo group touches and bonds to the water surface firmly. On the other hand, in MeO-MA, the methoxy group may often be above the level of the location of the carboxyl group, which touches the water surface. Therefore, although the hydrophilicity of the methoxy group approaches that of an oxo group, the methoxy group may not be able to interact decisively with the water surface.

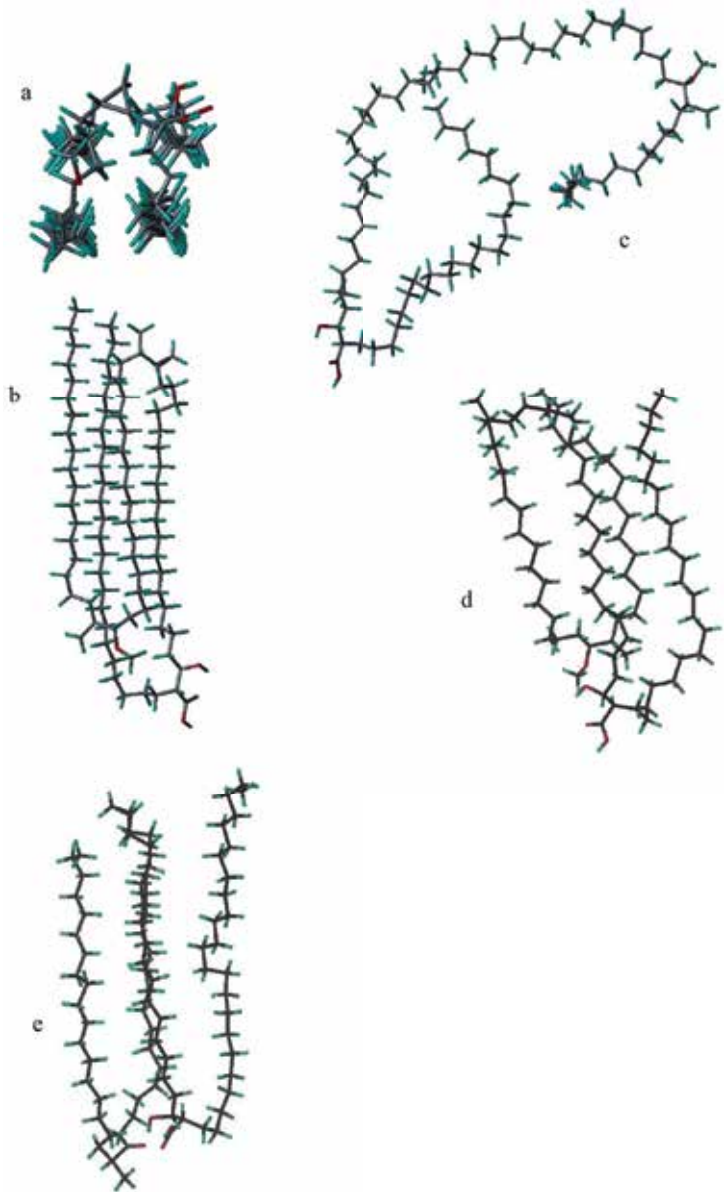


Fig. 10. Structures of MAs in MD study. (a) and (b) Top and side views of MeO-MA molecule obtained by MC followed by minimization; (c) MeO-MA taking a stretched-out structure after 20 ps in MD; (d) MeO-MA retaining a 4-cain structure after 20 ps in MD; (e) structure of Keto-MA obtained after 20 ps in MD.

6. A tentative interpretation of the model membrane in terms of biological activity

6.1 Generality

In relating the present results to the biological role of mycolic acids, the most valuable finding is the special behavior of the Keto-MA. It has exceptional rigidity in monolayers, over a

wide temperature range (Fig. 7), apparently assuming a W-shaped conformation with four hydrocarbon chains packing together in parallel. To our knowledge, this is the first time that fatty acid packing of this type has been detected in nature. The initial studies (Minnikin & Polgar, 1967, 1; 2) on the location of functional groups in cyclopropyl MA showed that these fatty acids are assembled according to a generalized template, with groups spaced at regular intervals separated by relatively uniform lengths of hydrocarbon chains (Table 1); these results have been thoroughly substantiated in recent studies (Watanabe et al., 2001; 2002). The current results offer the first justification for this exquisite regular architecture of mycobacterial MAs. The true reason for the presence of oxygenated functions in MAs is also revealed as being necessary for conformational stabilization probably through hydrophilic interactions. In this model monolayer study, the hydrophilic interaction is most likely to be with the aqueous sub-phase. In the cell envelope of mycobacteria, however, the interaction of the keto group could either be with the covalently attached arabinogalactan or, more intriguingly, with the mycolic acid 3-hydroxy group. This latter interaction could take place in an inter- or intramolecular fashion.

Biological activities of living cells rely upon, not only the relevant chemical reactions but also on relating physicochemical or physical processes. In this study, we have shown that MAs of different chemical structures form Langmuir monolayers having distinctive physicochemical features and each MA exhibits multiple phase transitions depending upon the temperature and the surface pressure. Dubnau (Dubnau et al., 2000) et al. reported that in a *M. tb* strain whose cell wall-linked mycolate consists solely of α-MA, the permeation rate was very low, and Yuan et al. (Yuan et al., 1998) reported that in a recombinant mycobacterial strain whose Keto-MA is completely replaced by MeO-MA showed poor growth in macrophages and a decreased rate of permeation for hydrophilic substances. Those studies imply that different mycolates in the cell envelope contribute differently to the permeability function of the mycolate layer of the cell envelope. It seems possible that each component mycolate takes different conformation in the cell envelope mycolate layer as suggested by the present study, and that the different forms of different mycolates have different effects on the cell function, though we should be careful in applying the monolayer results to the natural cell wall mycolate layer functions.

6.2 Roles of α-MAs

Recent papers revealed better defined features of the outer membrane lipid bilayer of mycobacterial cells by the cyro-electron tomography (Hoffmann et. al., 2008; Niederweis et al., 2010; Zuber et al., 2008). On the basis of the thickness of the layer, in those papers, the cell-bound MAs constituting the basis of the inner leaflet of the outer membrane lipid bilayer are suggested to be in the W-shape. However, if MAs in the lipid bilayer are to take basically the W-shape, it does not necessarily mean that all the MAs are to stay in the W-shape. In the biological lipid bilayers, at biological temperatures, it is well known that the component molecules are able to move quite easily to shift their locations in the layer or to change the conformation. Our present monolayer studies and computer simulation results showed that the α-MAs are ready to change the conformation from the W-shape to any of the various extended shapes as required or favored by its environment. In the outer membrane lipid layer, α-MAs, a large molecular weight component, may change the conformation to various extended shapes and probably by dynamically waving and bending the variously extended

long hydrocarbon chains, may take an initiative active part in making the lipid layer more mobile and biologically compatible.

The observations of α-MAs, especially of the flexible conformational behavior of the molecules and of various characteristics closely and directly related to the physical nature of the lipid layer may imply importance of the presence of α-MAs always in about 50 % of the whole cell-bound MAs in mycobacterial cells.

6.3 Roles of oxygenated-MAs

The possible special influence of MAs with a *trans*-cyclopropane ring on membrane function or pathogenicity of the cells has been highlighted (Glickman et al., 2000; 2001). When the features of the Langmuir monolayers of MeO- and Keto-MAs from BCG in the present study were compared with those of the corresponding MAs from *M. tb* in our study (Villeneuve et al., 2005), some differences were noted. The collapse pressures of Keto-MA and the surface pressures of MeO-MA at the phase transition from the 4-chain conformation to the extended one were lower in those of Keto- and MeO-MAs from BCG, respectively, than in those from *M. tb*, at all the temperatures assayed. The structures of the molecular components of the MAs from the two mycobacteria are essentially identical and the difference is only in the ratios. The ratios between the *cis*-cyclopropane and *trans*-cyclopropane contents are 1/0.03 and 1/0.22, respectively, in MeO-MAs from BCG and *M. tb* and is 1/0.33 and 1/3.5, respectively, in Keto-MAs from BCG and *M. tb*. The differences noted in the respective phase diagrams or isotherms may be attributed to subtle differences in the properties of the *cis*-cyclopropane rings and the *trans*-cyclopropane rings with an adjacent methyl branch. Apparently an increased ratio of *trans*-isomers seems to stabilize the four-chain conformation of the oxygenated MAs. The MD studies did not demonstrate any clear difference between the conformational behaviors of *cis*-cyclopropane-containing and *trans*-cyclopropane-containing MAs. This confirms the previous conclusion (Villeneuve et al., 2005) that *trans*-cyclopropane units, with an adjacent methyl branch, are able to allow folding of MAs in a similar manner to that allowed by *cis*-cyclopropane rings. Further studies on individual molecular species of oxygenated mycolates should be of great value to clear this problem.

Many factors are involved in the process of the onset of infectious diseases. In the case of tuberculosis, the primary and characteristic factor relating to the onset of the disease should be the intrinsic capacity of the *M. tb* cells to resist and reject the attacks by the defense mechanisms of human host cells. If the mycolate layer of the cell envelope is to play a determining role in the permeability barrier function, as suggested (Minnikin, 1982; Puech et al., 2001; Rastogi, 1991), then the layer is to take an active part in regulating the in and out passages of essential factors vital for the living bacteria and thus to control the viability of the bacterial cells. The component MAs, therefore, may be considered to be responsible for the viability of the *M. tb* cells in human cells and detailed analysis of the physicochemical features of the component MAs may help to clear part of the problems relating to the human tuberculosis.

MAs from pathogenic *M. tb* and from non-pathogenic BCG are the same in the chemical structures of each component and slightly different in the ratios between the *trans*-cyclopropane-containing and *cis*-cyclopropane-containing components. One marked difference between the MAs from the two is in the ratios between the non-oxygenated MA (alpha-MA) and the oxygenated MAs. The ratio in the former is roughly 1:1, whereas that

in BCG reaches 1:3.5, in which the oxygenated MA is often mostly Keto-MA (Watanabe et al., 2001). The actual surface pressure in the cell envelope mycolate layer is unknown, but whatever the environmental conditions may be, as demonstrated in the present study, Keto-MAs form compact, relatively solid domains with a minimum thickness in the mycolate monolayer. Such Keto-MA units may provide a relatively impermeable stable foundation in the outer leaflet of the cell envelope. A larger number of, or a larger proportion of this type of less permeable compact domains in BCG cell envelope may provide the cells with the features that distinguish BCG cells from *M. tb* cells. The presence of such solid domains may provide BCG cells with slower and lower multiplication rate and fairly good or moderate resistance to the killing system of the host cells so that the cells can survive quietly for a long time, which is an essential and necessary requirement for a good live vaccine.

The extended structures of MeO-MA, produced by MD simulation, are not of two long straight methylene chains. As exemplified in Fig. 1c, the long chains curve and bend, implying that they are ready to change their conformation in response to the environmental conditions. Probably MeO-MA and also alpha-MA are to be considered to provide less condensed organelles suitable for facilitating selective permeability and interaction with complex cell surface free lipids.

7. References

Al Dulayymi, J. R., Baird, M. S., Roberts, E. (2005), The synthesis of a single enantiomer of a major α-mycolic acid of *M. tuberculosis*, *Tetrahedron* Vol. 6, pp. 11939-11951.

Asselineau, C. & Asselineau, J. (1966), Stéréochimique de l'acide corynomycolique, *Bull. Soc. Chim. France* pp. 1992-1999.

Jackson, M., Raynaud, C., Lanèelle, M.-A., Guilhot, C., Laurent-Winter, C., Ensergueix, D., Gicquel, B., Daffé, M. (1999), Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *mycobacterium tuberculosis* cell envelope, *Mol. Microbiol.* Vol. 31, pp. 1573-1587.

Dubnau, E., Chan, J., Raynaud, C., Mohan, V. P., Lanèelle, M.-A., Yu, K., Quémard, A., Smith, I., Daffée, M., (2000), Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice, *Mol. Microbiol.* Vol. 36, pp. 630-637.

Glickman, M. S., Cox, J. S., Jacobs Jr., W. R., (2000), A novel mycolic acid cyclopropane synthetase is required for cording, persistence and virulence of *Mycobacterium tuberculosis*, *Mol. Cell* Vol. 5, pp. 717-727.

Glickman, M. S., Cahill, S. M., Jacobs Jr., W. R. (2001), The *Mycobacterium tuberculosis cmaA2* gene encodes a mycolic acid *trans*-cyclopropane synthetase, *J. Biol. Chem.* Vol. 276, pp. 2228-2233.

Goren, M. B. & Brennan, P. J. (1979), Mycobacterial lipids: Chemistry and biologic activities, In: *Tuberculosis*, Youmans, G.P. (Ed.), page numbers (first-last), Saunders, ISBN, Philadelphia.

Hasegawa, T., Nishijo, J., Watanabe, M., Funayama, K., Imae, T. (2000), Conformational characterization of a-mycolic acid in a monolayer film by the Langmuir-Blodgett technique and atomic force microscopy, *Langmuir* Vol. 16, pp. 7325-7330.

Hasegawa, T., Nishijo, J., Watanabe, M., Umemura, J., Ma, Y., Sui, G., Huo, Q., Leblanc, R. M. (2002), Characteristics of long-chain fatty acid monolayers studied by infrared external-reflection spectroscopy, *Langmuir* Vol. 18, pp. 4758-4764.

Hasegawa, T. & Leblanc, R. M. (2003), Aggregation properties of mycolic acid molecules in monolayer films: a comparative study of compounds from various acid-fast bacterial species *Biochim. Biophys. Acta* Vol. 1617, pp. 89-95.

Hasegawa, T., Amino, S. Kitamura, S., Matsumoto, R., Katada, S., Nishijo, J. (2003), Study of the molecular conformation of α- and keto-mycolic acid monolayers by the Langmuir-Blodgett technique and Fourier transform infrared reflection-adsorption spectroscopy, *Langmuir* Vol. 19, pp. 105-109.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., Engelhardt, H. (2008), Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure, *PNAS* Vol. 105, pp. 3963-3967.

Hong, X. & Hopfinger, A. J. (2004), Construction, molecular modeling, and simulation of *mycobacterium tuberculosis* cell walls, *Biomacromolecules* Vol. 5, pp. 1052-1065.

Motomura, K. (1967), Thermodynamics and phase transitions in monolayers, *J.C.I.S.* Vol. 23, pp. 313-318.

M. McNeil, M. Daffé, P. J. Brennan (1991), Location of the mycolyl ester substituents in the cell walls of mycobacteria, *J. Biol. Chem.* Vol. 266, pp. 13217-13223.

Minnikin, D. E. & Polgar, N. (1967), The mycolic acids from human and avian tubercle bacilli, *Chem. Comm.* pp. 916-918.

Minnikin, D. E. & Polgar, N. (1967), The mycolic acids from human and avian tubercle bacilli, *Chem. Comm.* pp. 1172-1174.

D. E. Minnikin (1982). Lipids: Complex Lipids, Their Chemistry, Biosynthesis and Roles, In: *The Biology of the Mycobacteria Vol. 1*, Ratledge, C. & Stanford, J. L. (Ed.), pp. 95-184, Academic Press, New York.

Minnikin, D. E., Kremer, L., Dover, L. G., Besra, G. S. (2002), The methyl-branched fortifications of Mycobacterium tuberculosis, *Chem. Biol.* Vol. 9, pp. 545-553.

Niederweis, M., Danielchanka, O., Huff, J., Hoffmann, C., Engelhardt, H. (2010), Mycobacterial outer membranes: in search of proteins, *Trends in Microbiology* Vol. 18, pp. 109-116.

Puech, V., Chami, M., Lemassu, A., Lanèelle, M.-A., Schiffler, B., Gounon, P., Bayan, N., Benz, R., Daffé, M. (2001), Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane, *Microbiology* Vol. 147, pp. 1356-1382.

Rastogi, N. (1991), Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug-resistance, *Res. Microbiol.* Vol. 142, pp. 464-476.

Staellberg-Stenhagen, S. & Stenhagen, E. (1945) A monolayer and X-ray studies of mycolic acid from the human tubercle bacillus, *J. B. C.* Vol. 150, pp. 255-262.

Timmons, C. O. & Zisman, W. A. (1968) The relation of initial spreading pressure of polar compounds on water to interfacial tension, work of adhesion, and solubility, *J.C.I.S.* Vol. 28, pp. 106-117.

Tocanne, J. F. & Asselineau, C. (1968), Étude stéréochimique des acides aliphatiques α-ramifiés β-hydroxylés. Configuration de l'acide corynomycolique, *Bull. Soc. Chim. Fr.* pp. 4519-4525.

Tompkins, H. G. & McGahan, W. A. (1999). *Spectroscopic Ellipsometry and Reflectometry: A User's Guide*, Wiley-Interscience, New York.

Villeneuve, M. Kawai, M., Kanashima, H., Watanabe, M., Minnikin, D. E., Nakahara, H. (2005), Temperature dependence of the Langmuir monolayer packing of mycolic acids from *Mycobacterium tuberculosis*, *Biochim. Biophys. Acta* Vol. 1715, pp. 71-80.

Villeneuve, M. Kawai, M., Watanabe, M., Aoyagi, Y., Hitotsuyanagi, Y., Takeya, K., Gouda, H., Hirono, S., Minnikin, D. E., Nakahara, H. (2007), Conformational behavior of oxygenated mycobacterial mycolic acids from *Mycobacterium bovis* BCG, *Biochim. Biophys. Acta* Vol. 1768, pp. 1717-1726.

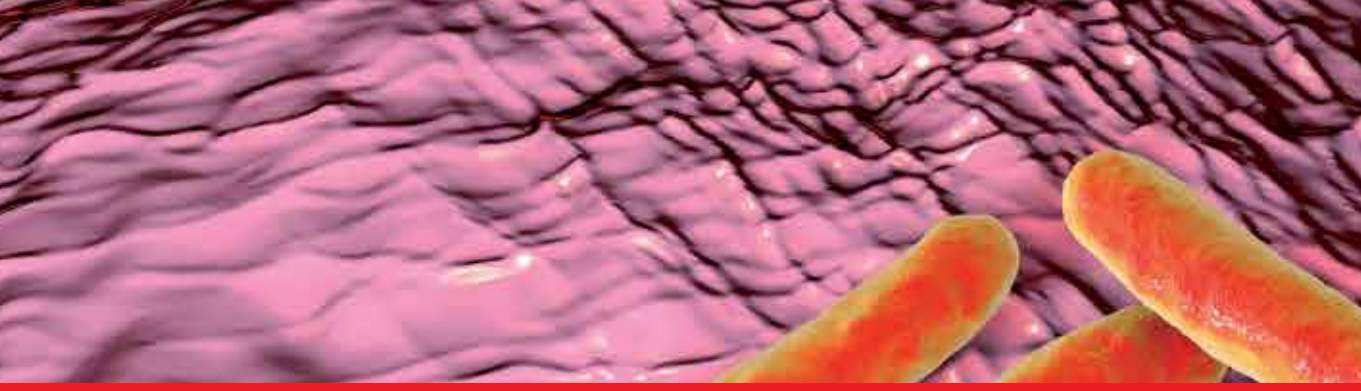
Villeneuve, M. Kawai, M., Watanabe, M., Aoyagi, Y., Hitotsuyanagi, Y., Takeya, K., Gouda, H., Hirono, S., Minnikin, D. E., Nakahara, H. (2010), Differential conformational behavior of α-mycolic acids in Langmuir monolayers and computer simulations, *Chemistry and Physics of Lipids* Vol. 163, pp. 569-579.

Watanabe, M., Aoyagi, Y. Ridell, M. Minnikin, D. E. (2001), Separation and characterization of individual mycolic acids in representative mycobacteria, *Microbiology* Vol. 147, pp. 1825-1837.

Watanabe, M., Aoyagi, Y. , Mitome, H. ,Fujita, T., Naoki, H., Ridell, M., Minnikin, D. E. (2002), Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids, *Microbiology* Vol. 148, pp. 1881-1902.

Yuan, Y., Zhu, Y. Q., Crane, D. D., Barry III, C. E. (1998), The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *mycobacterium tuberculosis*, *Mol. Microbiol.* Vol. 29, pp. 1449-1458.

Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G., Daffé, M. (2008), Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state, *Journal of Bacteriology* Vol. 190, 5672-5680.



Edited by Pere-Joan Cardona

Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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