IntechOpen

Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods

Edited by Sahra Kırmusaoğlu

Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods

Edited by Sahra Kırmusaoğlu

Published in London, United Kingdom







IntechOpen






Supporting open minds since 2005

Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods
http://dx.doi.org/10.5772/intechopen.78751
Edited by Sahra Kırmusaoğlu

Contributors
Muhammad Usman Qamar, Muhammad Hidayat Rasool, Shah Jahan, Muhammad Shafique, Bilal Aslam, Laura Castrillon Rivera, Alejandro Palma Ramos, Jorge Ismael Castañeda-Sánchez, Maria Elisa Drago-Serrano, Eng Hwa Wong, Mansab Ali Saleemi, Navindra Kumari Palanisamy, Christian Agyare, Cynthia Amaning Danquah, Yaw Duah Boakye, Francis Adu, Newman Osafo, Angel Manteca, Paula Yagüe, Sahra Kırmusaoğlu

First published in London, United Kingdom, 2019 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods
Edited by Sahra Kırmusaoğlu
p. cm.
Print ISBN 978-1-78985-789-4
Online ISBN 978-1-78985-790-0
eBook (PDF) ISBN 978-1-83962-103-1

Meet the editor

Dr. Kırmusaoğlu, PhD, is an Assistant Professor of microbiology at the Department of Molecular Biology and Genetics, T.C. Haliç University, where she had previously held an administrative position as vice head of the same department. She specialized in microbiology at Abant Izzet Baysal University (Biology Department), Turkey. Her previous experience includes the position of laboratory manager at microbiology laboratories in several research and private hospitals. Throughout her career, she collaborated with academicians/researchers from AIBU, METU and Istanbul University Cerrahpaşa Faculty of Medicine, and participated in various research projects. She also worked as a lecturer in the Medical Laboratory Techniques Program held at the Department of Medical Services and Techniques, T.C. Istanbul Kavram Vocational Training School, before transitioning to T.C. Haliç University as an Assist.Prof. Dr. Kırmusaoğlu's research interests include pathogenic bacteria, bacterial biofilms, antibiofilm and antimicrobial agents, antibacterial resistance, synergistic activities of antimicrobials in combination with new agents such as chemical and natural bioactive compounds against pathogenic bacteria such as antibiotic-resistant and biofilm-producing microorganisms, regulation of antibiotic resistance, and virulence genes. She is also interested in research on antibiofilm medical devices for preventing biofilm infections associated with indwelling devices that could lead to systemic infections. She has published several international research articles, book chapters, and congress proceedings. More specifically, Dr. Kırmusaoğlu participated as an author in several books, e.g. *Microbial Biofilms: Importance and Applications and The Rise of Virulence and Antibiotic Resistance in Staphylococcus aureus* with her chapters "Staphylococcal biofilms: pathogenicity, mechanism and regulation of biofilm formation by quorum sensing system and antibiotic resistance mechanisms of biofilm embedded microorganisms" and "The mechanism of methicillin resistance and the influence of methicillin resistance on biofilm phenotype of Staphylococcus aureus." The chapter entitled "The methods for detection of biofilm and screening antibiofilm activity of agents" was written by Dr. Kırmusaoğlu. She is also the editor of *Disinfection* and *Bacterial Pathogenesis and Antibacterial Control*. In addition to these, she wrote a book *Genel Biyoloji Laboratuvar Kılavuzu (General Biology Laboratory Manual)* published by Hipokrat Publisher. She has been also contributing to an ongoing book project titled *Staphylococcus and Streptococcus* as an editor, and has contributed to a chapter translation of the book *Sherris Medical Microbiology by Ryan et al*. as one of the translation authors of *Sherris Tıbbi Mikrobiyoloji*, which is a Turkish translated book edited by Prof. Dr. Dürdal Us and Prof. Dr. Ahmet Başustaoğlu.

Contents

Preface

To prevent bacterial adherence caused by virulence factors such as biofilms, invasion, and infection, newly developed antimicrobials in combination with certain agents or bioactive compounds present in natural products, such as pharmacological nutrients and plant extracts as well as certain novel technologies, are being used and vastly researched nowadays.

Several factors such as natural selection, mutations in genes, the presence of efflux pumps, impermeability of the cell wall, structural changes in enzymes and receptors, biofilm formation, and quorum sensing cause microorganisms to develop resistance against antimicrobials. Multidrug-resistant pathogens that are the main causes of morbidity and mortality in the worldwide have the ability to synthesize a number of enzymes such as extended spectrum-β-lactamases, induced β-lactamases, carbapenamases, metallo-β-lactamases, New Delhi metallo-β-lactamases, and many others. Due to the wide range of antimicrobial resistance multidrug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Pseudomonas aureginosa* have emerged throughout the worldwide, and mechanisms of resistance must be further investigated to treat untreatable infections caused by resistant pathogens. To overcome antimicrobial resistance, new strategies must be developed, and minimum doses of antimicrobials in combination with other agents such as synergistic compounds and nutrient extracts can be used.

Determining virulence factors such as biofilms and the level of antimicrobial activities of antimicrobial agents alone and in combination with appropriate doses against microorganisms is very important for the diagnosis, inhibition, and prevention of microbial infection, especially for recurrent ones.

This book contains seven chapters from valued experts in Turkey, Mexico, Spain, Netherlands, Pakistan, Malaysia, and Kumasi-Ghana. The goal of this book is to provide information on all the topics mentioned. It can be useful for researchers who are interested in the study of microbial responses against antimicrobials, and the development of new strategies for the treatment of infections caused by MDR and biofilm producer microorganisms.

I would like to thank all the authors who contributed to this book with their chapters. Especially, I would like to express my special thanks to Prof. Dr. Bekir Kocazeybek for his valued support. Finally, I would also like to thank Ms. Maja Bozicevic who

assisted me in this project as an Author Service Manager and IntechOpen Publisher for their concern and encouragement in publishing this book.

Assist. Prof. Sahra Kırmusaoğlu
Haliç University, Faculty of Arts and Sciences,
Department of Molecular Biology and Genetics,
Beyoğlu-Istanbul, Turkey

Section 1

Antimicrobials and Antimicrobial Resistance

Chapter 1

Introductory Chapter: The Action Mechanisms of Antibiotics and Antibiotic Resistance

Sahra Kırmusaoğlu, Nesrin Gareayaghi and Bekir S. Kocazeybek

1. Antibiotics

Pathogenic microorganisms can infect tissues of human by destroying cellular functions. Microorganisms themselves or their toxins can damage host cells. Microbial infections are treated with antimicrobials by either inhibiting the microbial growth or killing the microorganism. Antibiotics are widely being used not only in the treatment of acute and chronic infections, but also in the prophylactic treatment [1]. Targets of antimicrobials are cell membrane, cell wall, protein synthesis, nucleic acid synthesis, and biological metabolic compound synthesis (**Figure 1**) [2]. Over usage of antibiotics, mutations in the genes, carrying resistance genes in chromosomes and plasmids, gaining resistance genes carried by transposons, insertion sequences (IS) and conjugation from the same or other species of microorganisms cause bacteria develop resistance to antimicrobials [3].

2. The action mechanisms of antibiotics and antibiotic resistance

2.1 Injury to cell membrane

Plasma membrane of microorganism that has selective permeability contributes active transport to gain energy as ATP. Cytoplasmic content and gradient such as micro and macromolecules and ions are controlled by active transportation via integral transporter proteins. When selective membrane permeability is disrupted by antimicrobials, ions are lost and cellular ion gradient is distorted, so, the organism undergoes cellular damage and death [4].

Plasma membranes of bacteria are constructed by fatty acids that can be synthesized in cell or taken from environment as building blocks. Targets of antimicrobials are metabolic steps of fatty acid synthesis and membrane phospholipids. Polymyxin B, that is a bactericidal antibiotic, has been used as one of very few drugs in the treatment of Gram-negative bacteria such as *Pseudomonas* [2]. Polymyxin B that has detergent-like peptides having lipophilic and hydrophilic groups disrupts phosphatidylethanolamine of membrane. Valinomycin, that is an ionophore, disrupts cellular membrane potential that contributes oxidative phosphorylation by forming pores in cellular membrane. Daphtomycin that is widely used in bloodstream, wound, and soft skin infections caused by β-lactam especially vancomycin-resistant *Staphylococcus aureus* disrupts membrane potential by depolarization, that means potassium ions are released from cytoplasm to extracellular matrix [4].

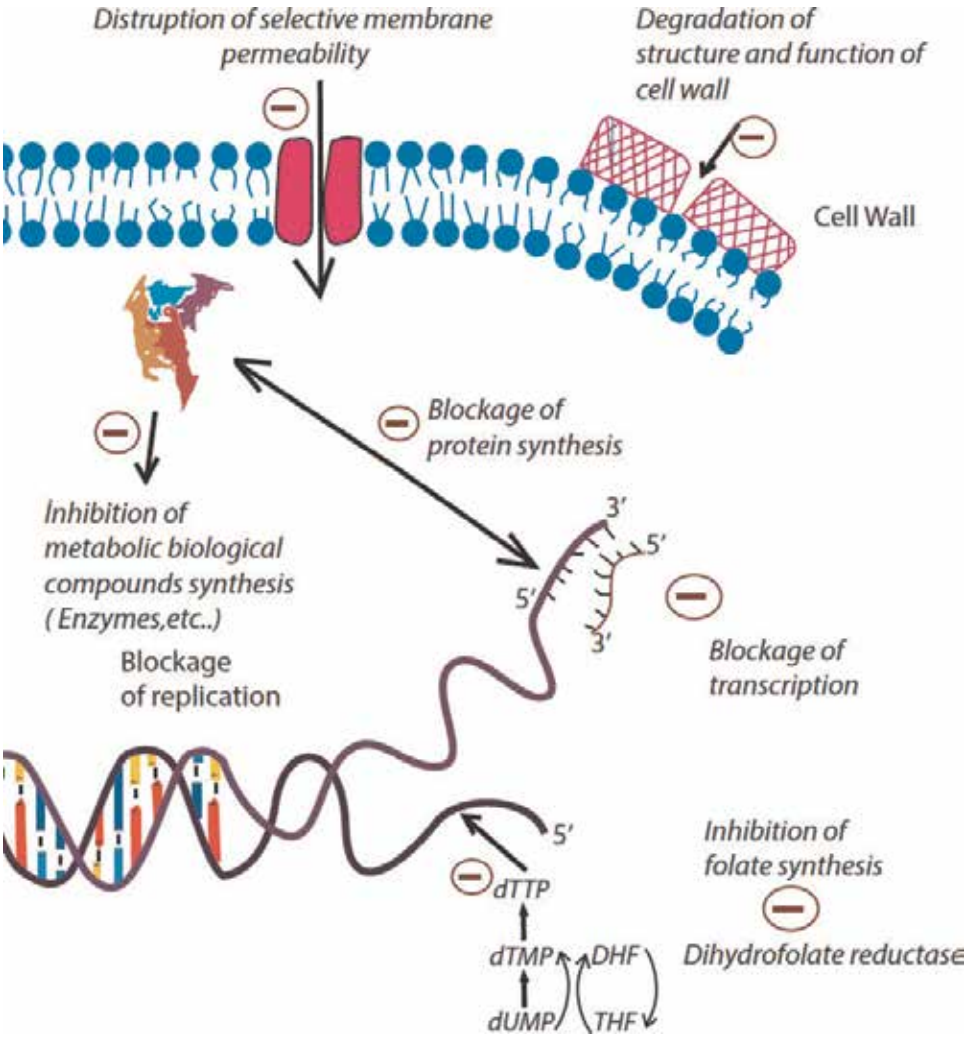

Figure 1.
Targets of antimicrobials.

Daptomycin, amphotericin B, colistin, imidazoles, and triazoles also act as inhibitors of cell membrane [2, 4].

2.2 The effect of antibiotics against cell wall

2.2.1 Cell wall synthesis

Cell walls of microorganisms are constructed by peptidoglycan. Glycan polysaccharide strands are linked by crosslink that bind polypeptides bound to N-acetyl muromic acid (NAM) of each polysaccharide strands.

After bactoprenol, which is a membrane-bound acceptor, transfers UDP-NAM-pentapeptide and UDP-NAG from cytoplasm to outer site of cell membrane, transglycosylation, and transpeptidation reactions are catalyzed by penicillin-binding proteins (PBPs) bound to cell membrane as a DD-peptidases to construct peptidoglycan [3].

2.2.2 Degradation of structure and function of cell wall

Certain antibiotics such as β-lactam antibiotics react with PBPs having high affinity to β-lactams by binding to PBPs as a substrate. These drugs are structural analogs of acyl-D-alanyl-D-alanine that binds to active site of PBP as a substrate of PBP during transpeptidation reaction. Transpeptidation reaction is blocked by these antibiotics inactivating transpeptidase domain of PBPs. Microorganisms are killed by these cell wall inhibitors that inhibit peptidoglycan biosynthesis [3].

2.2.3 β-lactamases and the mechanisms of β-lactam resistance

β-lactamase, that is an enzyme synthesized by many species of Gram-positive and Gram-negative bacteria, inactivates β-lactams degrading amide bond of β-lactam ring of β-lactam antibiotics [4] (bnm MRSA and MSSA). β-lactamases can be mediated by either plasmids or chromosomes, whereas penicillinases of *Staphylococcus aureus* are plasmid mediated, many Gram-negative bacteria are chromosomally mediated. β-lactamases that are plasmid mediated have tendency to be transferred between distinct species of bacteria. Chromosomally mediated β-lactamases can be either produced constructively as in the species of *Bacteroides* and *Acinetobacter* or induced as in the species of *Enterobacter*, *Citrobacter*, and *Pseudomonas*. Extended-spectrum β-lactamases (ESBLs) that are one class of β-lactamases having distinct ability to hydrolyze β-lactam rings of cefotaxime, ceftazidime, and aztreonam can be seen in a few species of Gram-negative bacteria such as *Klebsiella pneumoniae* and *Escherichia coli* [4].

β-lactamase is synthesized by *bla*Z gene and regulated by *bla*I and *bla*RI located in plasmid or transposon. When β-lactam is not used, by binding of BlaI synthesized by *bla*I gene to promoter-operator region of *bla*I-*bla*RI operon, β-lactamase is not transcriped by *bla*Z gene. But in the treatment with β-lactam antibiotics, active site of BlaRI which is a signal transducer integral protein of β-lactam is blocked by β-lactam. After this blockage of active site, intracellular zinc metalloprotease domain of BlaRI releases BlaI bound to *bla*I-*bla*RI operator. By upregulation of *bla*Z gene, β-lactamase enzyme is synthesized, so the microorganism develops resistance to β-lactam used [3].

2.3 Inhibition of metabolic biological compounds synthesis

Biological metabolic reactions are catabolized by enzymes that are activated by substrates. Synthesis of metabolic biological compounds can be inhibited by drugs as a competitive inhibition manner. Drugs that are structural analogs of substrates act as substrates for the enzymes used in metabolic reactions.

Para-aminobenzoic acid (PABA) is a substrate for folic acid synthesis that is a coenzyme in the reactions of purines, pyrimidine, and amino acids synthesis [2].

Sulfanilamide and 3,4,5-trimethoxybenzylpyrimidine are the examples of drugs inhibiting synthesis of metabolic biological compounds. Sulfonamides (Sulfa drugs) have been used in many infections such as urinary tract infections. Sulfonamide is widely used in combination with other compounds. Silver sulfadiazine, one of the combined drugs, is used in burn infections [2].

Trimethoprim sulfamethoxazole (TMP-SMZ) is another combined drug used widely because of its synergistic activity. Trimethoprim and sulfamethoxazole block distinct steps of DNA and RNA precursor synthesis, and protein. Sulfamethoxazole that is sulfonamides showing structurally analogy with PABA blocks the reaction

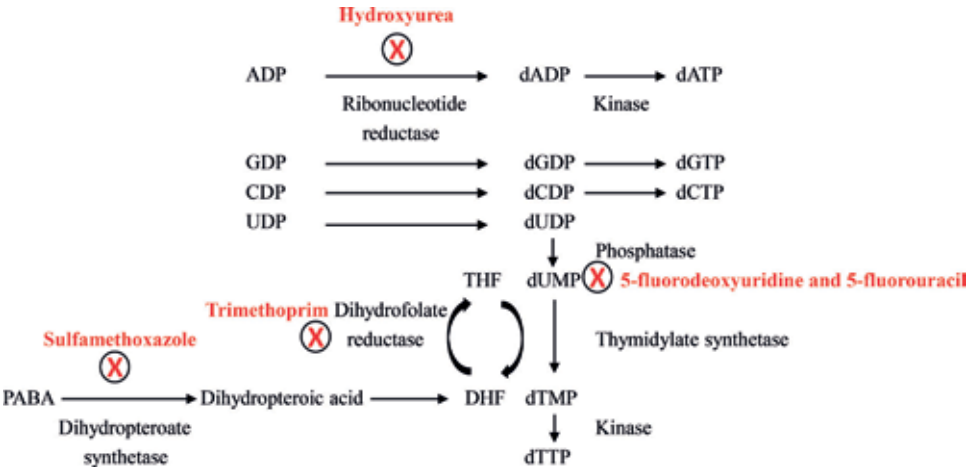

Figure 2.
The synthesis of deoxynucleoside triphosphate precursors (dNTP: dATP, dGTP, dCTP, and dTTP), and the inhibition of tetrahydrofolate (THF) and dNTP synthesis by antibiotics.

synthesizing dihydrofolic acid (DHF) from PABA, whereas trimethoprim that is sulfonamides showing structurally analogy with DHF blocks the reaction synthesizing tetrahydrofolic acid (THF) from DHF (**Figure 2**) [2].

2.4 Inhibition of nucleic acid synthesis

Antibiotics can inhibit replication, transcription, and folate synthesis of microorganisms.

2.4.1 Blockage of replication

2.4.1.1 Replication of DNA

Deoxyribonucleotide precursors are synthesized for the polymerization of deoxynucleotides. By kinase enzyme, deoxynucleoside triphosphates (dNTP: dGTP, dCTP, and dATP) are synthesized from deoxynucleoside diphosphates (dNDP: dGDP, dCDP and dADP) that are synthesized from ribonucleosides by ribonucleotide reductase. But deoxythymidine triphosphate (dTTP) is synthesized by different pathway. Deoxyuracil diphosphate (dUDP) that is synthesized from uracil diphosphate (UDP) by ribonucleotide reductase is converted to deoxyuracil monophosphate (dUMP). Thymidylate synthetase catalyzes a reaction that converts dUMP methylated by tetrahydrofolate (THF) to dTMP. In this step, tetrahydrofolate (THF) is synthesized from dihydrofolate (DHF) by dihydrofolate reductase. Finally, dTTP is synthesized from dTMP by kinase (**Figure 2**).

DNA gyrase opens DNA strands for the polymerization of deoxynucleotides by DNA polymerase according to the each circular template strand of chromosome [5].

2.4.1.2 Inhibition of replication and the mechanisms of resistance

Kinolons, such as nalidixic acid and ciprofloxacin that is used in the treatment of infections caused by *Pseudomonas* spp., prevent the formation of replication fork by inhibiting DNA gyrase, as a result of binding to *gyr*A subunit. Novobiocin and coumermycin prevent the formation of replication fork by inhibiting DNA gyrase, as a result of binding to *gyr*B subunit. When *gyr*A and *gyr*B genes of bacteria are

mutated, bacteria develop resistant against these antibiotics [5]. Norfloxacin, gatifloxacin, gemifloxacin, and moxifloxacin are the other groups of floroquinolones having broader spectrum of activity. Floroquinolones are nontoxic. Ciprofloxacin, norfloxacin, gatifloxacin, and gemifloxacin are used to treat urinary tract infection and pneumonia [2].

Synthesis of deoxynucleotide precursors that are used in the replication of DNA can be blocked by trimethoprim, hydroxyurea, 5-fluorodeoxyuridine, and 5-fluorouracil.

Trimethoprim is an inhibitor of folate synthesis. Trimethoprim, that is a structural analog of DHF, prevents the synthesis of THF by inhibiting dihydrofolate reductase. So, dTMP that is a precursor of deoxynucleotide polymer is not synthesized. There are many mechanisms of trimethoprim resistance. If *thyA* gene of microorganism undergoes to mutation that inactivates thymidylate sentetaz dTMP and DHF are not synthesized, as a result of inability in the transfer of methyl group from THF to dUMP. Due to dihydrofolate reductase is not synthesized in the microorganism of which *thyA* gene is mutated, this microorganism is not inhibited by trimethoprim. If the microorganism changes the binding site of the dihydrofolate reductase, the trimethoprim cannot bind to dihydrofolate reductase. So, bacteria develop resistance to trimethoprim. Another mechanism of trimethoprim resistance is carrying gene causing resistance to trimethoprim [5].

Hydroxyurea inhibits ribonucleotide reductase that catalyzes deoxynucleoside diphosphate from ribonucleoside diphosphate (**Figure 2**). If microorganism of gene coding ribonucleotide reductase is mutated, microorganism develops resistance to hydroxyurea [5].

Precursor synthesis can be blocked by 5-fluorodeoxyuridine and 5-fluorouracil with competitive inhibition. Monophosphate forms of 5-fluorodeoxyuridine and 5-fluorouracil are structural analogs of dUMP that is the substrate of thymidylate synthase. They inhibit the synthesis of dTMP. If the microorganism of gene coding thymidylate synthase is mutated, the microorganism develops resistance to 5-fluorodeoxyuridine and 5-fluorouracil [5].

Dideoxynucleotides that are used as drugs are similar with deoxynucleotide precursors, except that the hydroxyl group is absent in their 3′ carbon. Dideoxynucleotides that mimic deoxynucleotide precursors corpore into DNA and then, stop replication, due to it cannot be bound by the next deoxynucleotide.

Mitomycin C blocks replication by binding guanine bases that are located in both template strands of DNA [5].

2.4.2 Blockage of transcription

2.4.2.1 Transcription

Genetic information is transcripted from DNA to RNA by RNA polymerase that catalyzes a reaction, binds ribonucleotides with phosphodiester bond. RNA polymerase is constructed by 2α, 1β, 1β′, 1ω, and 1σ subunit. Transcription that is initiated by σ subunit that binds to promoter elongates until it is terminated by termination protein P (Rho) that is a RNA-DNA helicase releasing transcript from template DNA by breaking hydrogen bonds produced between template DNA and transcript [5].

2.4.2.2 Inhibition of transcription

Rifampin, that is a derivative of rifamycine family of antibiotics, blocks initiation of transcription by binding to β subunit of RNA polymerase. Rifampin is

used in the treatment of infections caused by *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and bacteria for which treatment is hard is not toxic to humans, due to it does not inhibit eukaryotic RNA polymerase. Mutated gene that codes RNA polymerase containing a distinct β subunit structure causes the microorganism to resist against ripampin. Streptolydigin also blocks initiation of transcription by binding with the β subunit of RNA polymerase. Bicyclomycin, the target of which is termination protein P, prevents termination of transcription. Bleomycin produces nicks on DNA. Bleomycin is not suitable in the usage for humans, because they are not specific to bacteria and has high toxic effect against humans and animals; whereas, it is used for the experiments of transcription. Azaserin blocks transcription by inhibition of ribonucleoside triphosphate synthesis [5].

2.5 Inhibition of protein synthesis

2.5.1 Translation

Protein is translated from mRNA by tRNA in ribosome. Translation is initiated by the binding of formylmethionine tRNA-aminoacyl-tRNA (fMet-$tRNA_f^{Met}$), translation initiation region (TIR) of mRNA and initiation factor 2 (IF-2) to P site of 30S subunit of ribosome and the formation of 70S complex as a result of the release of IF-2. Translation continues with the binding of a new aminoacyl tRNA to A site, transferring the polypeptide from tRNA bond to P site to tRNA bond to A site by peptidyl transferase, and translocating of polypeptidyl tRNA from A to P site by elongation factor-G (EF-G), until translation is terminated by termination protein P (Rho) [5].

2.5.2 Inhibitors mimicking tRNA

Puromycin that mimics aminoacyl tRNA enters into ribosome and is added to polypeptide grown, but it is not translocated from A site to P site of ribosome. Polypeptide containing puromycin at the carboxyl terminal is released from ribosome and translation is terminated. Puromycin is toxic to humans and animals, as it inhibits translation of eukaryotes [5].

2.5.3 Inhibitors binding to 23S rRNA

2.5.3.1 Chloramphenicol

Chloramphenicol, that is a bacteriostatic agent and the inhibitor of 23S rRNA, inhibits transcription by preventing peptidyl transferase reaction, as a result of preventing the binding of aminoacyl tRNA to A site of ribosome. Due to its ability enter into blood-brain barrier, chloramphenicol is used in the treatment of many central nervous system infections such as bacterial meningitis. If the gene of ribosomal protein is mutated or bacteria has enzyme inactivating chloramphenicol, coded by *cat* gene of Tn9, bacteria resists to chloramphenicol. The product of *cat* gene that acetylates chloramphenicol inactivates chloramphenicol [5].

2.5.3.2 Erythromycin

Erythromycin, that belongs to macrolide class of antibiotics, inhibits translation by binding to 23S rRNA. As a result of the blockage of E site, that is the

exit site for peptidyl-tRNA by erythromycin, premature polypeptide is released in translocation step. Macrolites, such as erythromycin, clarithromycin, azithromycin, and roxithromycin, are used in the treatment of Gram-positive and Gram-negative bacteria such as *Legionella*, *Mycoplasm*, and *Rickettsia*, due to usefulness of antibiotics. Mutational changes in 23S rRNA and efflux pumps, and conformational changes of 23S RNA that is caused by methylation of adenine localized in 23S rRNA by Erm methylase cause bacteria to resist against erythromycin [5].

2.5.3.3 Thiostrepton

Thiostrepton and other thiopeptide antibiotics block translation by binding to 23S RNA in the peptidyl transferase reaction and preventing the binding of EF-G that is a translocase translocating polypeptidyl tRNA from A to P site. Thiostrepton is used against Gram-positive bacteria. But the usage of thiostrepton is limited to veterinary and agriculture [5].

2.5.4 Inhibitors binding to A site of aminoacyl tRNA

2.5.4.1 Tetracyclin

Tetracyclin causes futile cycle to release aminoacyl tRNA from A site of ribosome by binding of release factors mimicking aminoacyl tRNA to A site. Tetracyclin is a broad spectrum antibiotic used to treat infections caused by Gram-positive and Gram-negative bacteria. *tet*M gene transferred from conjugative transposon Tn916 codes not only an enzyme that causes resistance by methylating certain bases of 16S rRNA, but also membrane proteins that pump tetracyclin to out of the cell, consequently resistance is developed. Ribosome protector proteins coded by *tet*O and *tet*Q gene bind to A site of ribosome by mimicking EF-G, consequently tetracyclin is released from A site [5].

2.5.5 Inhibitors of translocation

2.5.5.1 Aminoglycosides

Aminoglycosides that contain kanamycin, neomycin, gentamycin, streptomycin, amikacin, and tobramycin effect translocation by binding to A site. Aminoglycosides that have broad spectrum of activity cause false reading of mRNA and translation errors. Mutants that are resistant to aminoglycosides are seen rare. Aminoglycoside resistance is caused by the genes that inactivate aminoglycosides by phosphorylation, acetylation, and adenylation of them. *neo* gene that phosphorylates kanamycin and neomycin cause resistance of Gram-negative bacteria against kanamycin and neomycin [5].

2.5.5.2 Fusidic acid

Fusidic acid inhibits turnover of EF-G by preventing the release of EF-G from ribosome. Mutations that are occurred in *fus*A gene of *E. coli* cause resistance against fusidic acid. Certain acetyltransferases causing resistance against chloramphenicole can inhibit fusidic acid by binding to fusidic acid [5].

Author details

Sahra Kırmusaoğlu[1*], Nesrin Gareayaghi[2] and Bekir S. Kocazeybek[3]

1 Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, Haliç University, Istanbul, Turkey

2 Istanbul Sisli Hamidiye Etfal Training and Research Hospital, Blood Center, University of Health Sciences, Istanbul, Turkey

3 Department of Medical Microbiology, Cerrahpasa Faculty of Medicine, Istanbul University Cerrahpasa, Istanbul, Turkey

*Address all correspondence to: kirmusaoglu_sahra@hotmail.com

IntechOpen

References

[1] Sköld O. Antibiotics and Antibiotic Resistance. Hoboken, New Jersey: Wiley & Sons, Inc; 2011

[2] Tortora GJ, Funke BR, Case CL. Microbiology: An Introduction, Global Edition. 12th ed. London: Pearson Education, Inc; 2015

[3] Kırmusaoğlu S. MRSA and MSSA: The mechanism of methicillin resistance and the influence of meticillin resistance on biofilm phenotype of *Staphylococcus aureus*. In: Enany SME, Alexander LEC, editors. The Rise of Virulence and Antibiotic Resistance in *Staphylococcus aureus*. Croatia: InTech; 2016. pp. 25-41

[4] Brooks GF, Carroll KC, Butel JS, Morse SA, Mietzner TA. Jawetz, Melnick, & Adelberg's Medical Microbiology. 26th ed. New York; Chicago: McGraw Hill Education; 2013

[5] Snyder L, Champness W. Molecular Genetics of Bacteria. Washington: American Society of Microbiology Press; 2007

Chapter 2

Antimicrobial Resistance

Muhammad Usman Qamar, Muhammad Hidayat Rasool, Shah Jahan, Muhammad Shafique and Bilal Aslam

Abstract

Klebsiella pneumoniae (*K. pneumoniae*) pose an emerging threat to public health sector worldwide. They are one of the potent nosocomial pathogens and cause variety of infections including pneumonia, septicaemia, wound infections, urinary tract infections and catheter-associated infections. From the last two decades, these pathogens are becoming more powerful due to the acquisition of resistomes on different types of plasmids and transposons. There are four main mechanisms of antibacterial resistance such as efflux pump, target alteration, membrane permeability and notably enzymes hydrolysis. *K. pneumoniae* produce different types of enzymes but most importantly extended spectrum-β-lactamase (ESBL), carbapenemase and metallo-β-lactamase (MBL). *K. pneumoniae* carbapenemases (KPCs) and New Delhi metallo-β-lactamase (NDM) producing isolates displayed resistance not only against the β-lactam drugs (penicillins, cephalosporins and carbapenems) but also to other classes of antibiotics (aminoglycosides and quinolones). Therapeutic options available to treat serious infections caused by these extensively drug-resistant pathogens are limited to colistin, tigecycline and fosfomycin. Hence, combination therapy has also been recommended to treat such bacteria with clinical side effects, therefore, new treatment regime must be required. Moreover, we are relying on conventional diagnostic tools, however, novel techniques must be required for robust identification of multi-drug-resistant bacteria.

Keywords: *Klebsiella pneumoniae*, antimicrobial resistant mechanisms, mortality and morbidity, economic burden

1. Introduction

Klebsiella pneumoniae is one of the notorious pathogens with increase number of severe infections and shortage of effective treatment regimes. They are extremely resilient pathogens because their success seems to follow the model of "the best defence for a pathogen is a good defence" rather than "the best defence for a pathogen is a good offense". *K. pneumoniae* is a Gram negative, non-motile, capsulated and an imperative member of *Enterobacteriaceae* family. They were firstly identified in late nineteenth century and given the name Friedlander's bacterium [1]. They are hermaphrodite and cause both community acquired infections (soil, surface water) and significantly hospital acquire infections (medical devices, catheters). Importantly, they can reside on different body mucosal surfaces of oropharynx and gastrointestinal tract and cause variety of infections including pneumonia, bacteraemia, septicaemia, gastritis, wound infections, urinary tract infections, and soft tissue surgical infections [2]. From the last 2 decades, *K. pneumoniae* strains

acquired extra genetic traits and become more virulent and antibiotic resistant [3]. They develop resistance against a wide range of antibiotics including carbapenems. Therefore, they produce (or synthesise) extended spectrum-β-lactamases (ESBL), carbapenemases, metallo-β-lactamases (MBLs), New Delhi metallo-β-lactamases (NDM) and have many other resistant mechanisms such as efflux pump and membranous target alteration.

2. Antimicrobial resistance

Antimicrobial resistance (AMR) defined when a bacterial strain shows resistance against antibiotics that normally inhibit or stop their growth and allow them to withstand against drugs. Last 2 decades, there are various AMR pathogens have been emerged around the globe such an ESBL producing pathogens, multidrug resistant and extensively drug resistant *Mycobacterium tuberculosis* (MDR, XDR-MTB), MBL producing bacteria, methicillin resistance *Staphylococcus aureus* (MRSA) and the advent of NDM producing bacteria. These isolates are almost impossible to treat with the existent treatment regime [4]. AMR emerged even since the discovery of first antibiotic "penicillin" in 1940 due the natural process of bacteria. Certainly, the genes that confer resistance upon some strains of bacteria precede antibiotics by millions of years [5]. Currently, AMR is becoming a big health issue around the world due to the irrational, overused and misuse of antibiotics which can lead to extra financial burden, prolong hospital stay and even fatal consequences. Unfortunately, currently we deficiency of the novel effective antimicrobial compounds, peptides and pipelines antibiotics to combat with emerging superbugs which result us facing growing enemy with largely depleted armoury [6].

3. Mortality, morbidity and economic burden due to antimicrobial resistant bacteria

AMR pathogens are one of the main cause of morbidity and mortality in both developed and developing countries. According to a published data, around 700,000 people die due to infections caused by AMR pathogens every year worldwide and most of the data is still underreported because of poor reporting and surveillance studies [7]. On the other hand, as per The Centre of Disease and Control (CDC), above 2 million people get infected due to AMR bacteria with a mortality rate of 23,000 each year [8]. Similarly, an Indian study revealed that around 60,000 neonates die due to AMR infections per year [9]. Likewise, a study conducted in Pakistan in 2009 was also documented that 40% neonates die due to MDR and XDR pathogens (*Escherichia coli*, *K. pneumoniae, Enterobacter cloacae*) isolated from blood samples [10]. Another study from Pakistan testified that 37 out of 78 new-borns died due to infections caused by MDR-*Acinetobacter spp.,* [11]. Qamar et al., conducted a study in Islamabad, Pakistan also reported 4/9 children died due to NDM positive isolates mainly of *E. coli* and *K. pneumoniae* [12]. Recently, Khan et al., from Karachi, Pakistan also reported that 22 with <1 month of age and 10 were elderly patients died due to bacteraemia and septicaemia which was mainly caused by NDM positive *K. pneumoniae* [13]. Highest mortality rate is mainly reported in developing countries as compare to developed countries largely in immunocompromised persons. Major risk factors in public sectors hospitals of developing countries are, poor hygienic practices of health care workers, irrational use of antibiotics, poor sanitation and hygienic facilities, nosocomial pathogens, hospital and wards are overcrowded with patients and attended and lack of health care workers, floors

and rooms are spilled with sputum and operation theatres are in deliberated condition and are poorly equipped, contaminated drinking water, more than one patients on single bed, presence of insects and flies and most importantly low health care budget by the Government [14].

Every year, millions of dollars are spent to tackle the burden of AMR bacterial infections around the globe. European Union invested around €1.5 billion annually to tackle the infections caused by AMR pathogens which are responsible for around 23,000 mortalities only in Europe [15]. However, United States of America also expenditures above $20 billion to treat the 20 million patients who get infected with AMR infection each year [16].

4. Mechanisms of antimicrobial resistance

Since 1990s, bacteria have been becoming gradually more lethal, due to acquisition of resistomes and develop resistance to different sets of antibiotics. Now a day, many bacterial pathogens notably *K. pneumoniae* are intrinsically resistant to various antibiotics or they acquire or encoded antimicrobial resistant genes mainly on plasmids as well on chromosomes [17]. There are different mechanisms such as conjugation, transformation and transduction in which susceptible strains can acquire resistance genes with transposons that eventually support different resistance genes to incorporate with host chromosome or plasmids. Presently, there are four fundamental mechanisms of bacterial drug resistance (1) target alteration (2) modification in membrane permeability (3) efflux pump and (4) antibiotic degradation by enzymes as shows in **Figure 1** [18]. In *K. pneumoniae* both the efflux pump and enzymatic degradation of different antibiotics are more efficient as compare to other two mechanisms.

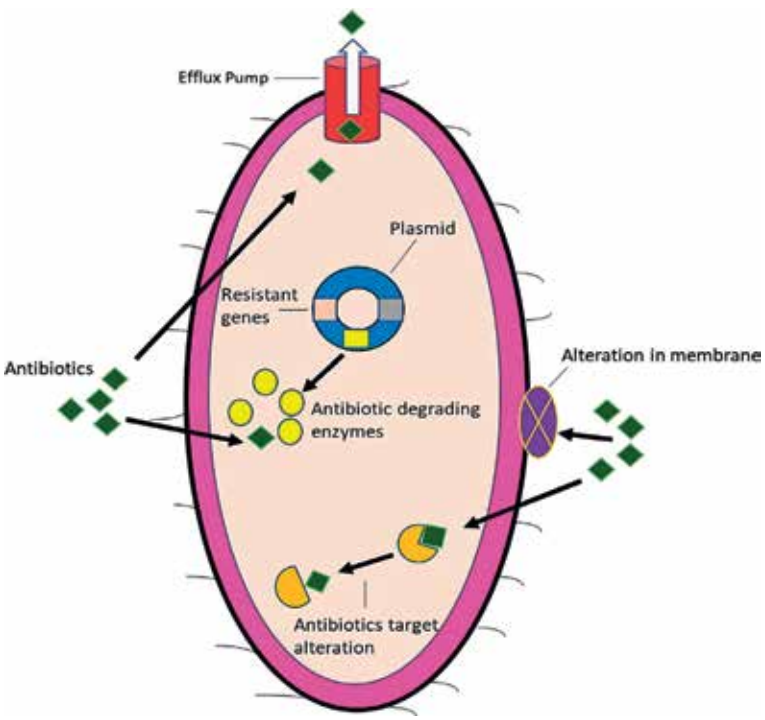

Figure 1.
Mechanism of antimicrobial resistance.

4.1 Target alteration of the antibiotics

In general, many antibiotics bind to different bacterial target sites with high affinity. So, most of the bacteria including *K. pneumoniae* alter the target sites and structure to block or inhibit the efficient binding sites [19]. In *K. pneumoniae*, antibiotic resistance occurs due to target modification in lipopolysaccharides (LPS), which is interceded by the activation of PmrA/PmrB two component regulatory system (TCRS). Moreover, another TCRS, PhoP/PhoQ contributes to produce polymyxin resistance by indirectly activating the PmrA/PmrB TCRS via PmrD linker protein. Similarly, inactivation of the PhoQ/PhoP negative regulator encoded by *mgrB* gene has also been identified to play a prominent role in polymyxin resistance in *K. pneumoniae* [20]. There are various pathogens that cause different infections and develop resistance even against single point mutation of resistant genes that encode an antibiotic target. These resistant strains then further produce numerous copies of bacteria [21].

4.2 Modification in membrane permeability

Outer membrane of the bacteria is first line barrier to protect the microorganisms against unfavourable environment including chemical and biological materials. In comparison, majority of the Gram-negative rods (GNR) are intrinsically less permeable to various antibiotics because of outer membrane barrier than Gram positive bacteria [22]. In the cell wall of *Enterobacteriaceae* family including *K. pneumoniae* contains various outer membrane proteins (Omps) such as OmpA, OmpF and OmpC and the hydrophilic antibiotics can cross through these Omps by diffusion. These proteins are involved in adhesion to other cell surfaces, maintenance of cell structure and regulation of transport of bactericidal agents and nutrition. OmpF expression is regulated at the posttranscriptional level via the *mic*F RNA, and many different environmental stresses impact the OmpC/OmpF ratio via changes in *mic*F levels. The expression of *mic*F is increased along with that of OmpF at high OmpR-P concentrations. The expression of *mic*F is also induced by toxic agents, such as paraquat and weak acids, by the SoxS, MarA, and Rob transcription factors, which bind to the same sequence element in the *mic*F promoter and they down regulate the synthesis of OmpF at transcriptional level (**Figure 2**) [23]. Therefore, the bacteria showed resistance by limiting the antibiotics entry in to the cell by the down regulation or replacement of porins with more selective channels. However, recent data showed that the loss of porins expression play a significant role to produce resistance to new drugs (cephalosporins and carbapenems), to which resistance is manly mediated by the enzyme degradation [24]. OmpA is one of the major protein of *K. pneumoniae* that prevent the activation of airway epithelial and loss of this protein make the bacteria susceptible. There are two important Omps in *K. pneumoniae*; OmpK35 and OmpK36 that allow the hydrophilic molecules such as nutrients and carbapenems to diffuse into bacteria. Moreover, *K. pneumoniae* also contain two alternative proteins; KpnO and OmpK26. Any mutation in OmpK36, OmpK26 and KpnO leads to increased resistance to cephalosporin and carbapenems [25].

4.3 Increased efflux pumps

Efflux pumps are the active transport mechanisms that expel out antibiotics from bacterial cell and play a vital role to develop intrinsic resistance against broad range of antibiotics in GNR [18]. Currently, there are several MDR efflux pumps are present in bacteria including FuaABC in *Stenotrophomonas*

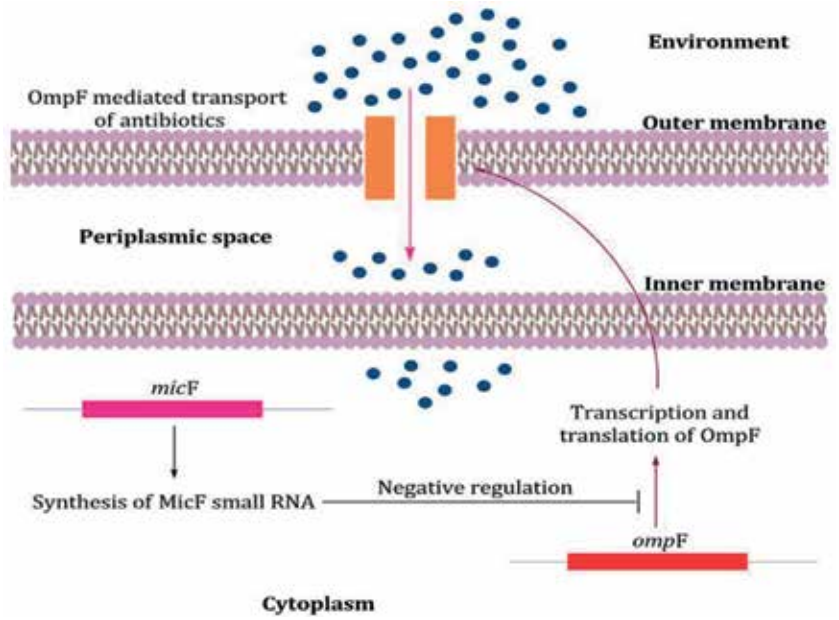

Figure 2.
Down regulation mechanism of outer membrane proteins (OMPs) in gram negative bacteria.

maltophilia, MedeA in *Streptococcus mutans* and AcrAB, KexD and more recently OqxAB efflux pumps in *K. pneumoniae* [26, 27]. In *K. pneumoniae*, most efficient antimicrobial resistant efflux pump is AcrAB, which is not only expel out the β-lactam, quinolones and chloramphenicol but also the host derived antimicrobial agents such as human antimicrobial peptides and human bronchoalveolar lavage fluid [25]. Recently, newly emerging OqxAB efflux pump has been recognised in different parts of the world that conferring resistance to quinolones and chloramphenicol [27].

4.4 Enzymatic hydrolysis of the antibiotics

In *K. pneumoniae*, the most potent and significant antimicrobial resistant mechanism is enzymatic hydrolysis of wide range of antibiotics including β-lactam, quinolones, aminoglycosides and macrolides [18]. β-lactamases are the enzymes that hydrolyze the effect of β-lactam drugs by attacking on their β-lactam ring. Currently, a total of 4037 β-lactam enzymes have been identified and mong these 1327 are class A, class B (n = 581), class C (n = 1359) and 660 belong to class D [28]. *K. pneumoniae* encode for many β-lactamases such as ESBLs, carbapenemases and MBLs. ESBLs and carbapenemases contain important subclasses of β-lactamase enzymes such as imipenemase (IMP), Verona integron encoded metallo-β-lactamase (VIM), *K. pneumoniae* carbapenemases (KPC), oxacillinase (OXA) and recently NDM [29].

4.4.1 Classification of β-lactamases

These enzymes are broadly classified into two main scheme one is Ambler Molecular classification scheme which is based on amino acid sequence and is widely acceptable and secondly, Bush Jacoby classification scheme which is based on biochemical properties [30]. Ambler class is further divided in to four major sub-classes A, B, C, and D based on conserved and distinguished amino acid

motifs. Among these, class A, C, and D are the serine carbapenemases that hydrolyzed their substrate through active site by forming acyl groups and require serine as a cofactor for their activity. However, class B enzymes are the MBLs that utilised at least one zinc ion at their active site to hydrolyze the β-lactam antibiotics. MBLs are further classified into three super families B1, B2 and B3 [31]. However, Bush-Jacoby-Medeiros classification scheme is divided into three major groups; class C (Group 1 cephalosporinases), class A and D (Group 2 Serine β-lactamases) and class B (Group 3 MBLs). These are further divided in to several different subgroups as shows in **Table 1** [30].

4.4.2 Extended spectrum-β-lactamase producing K. pneumoniae

ESBL are the enzymes that hydrolyze the extended spectrum drugs mainly cephalosporins. ESBL producing *Enterobacteriaceae* mainly the *K. pneumoniae* and *E. coli* is becoming one of the serious threat to the public health sectors. According to World Health Organisation (WHO), around 50% of ESBL producing *K. pneumoniae* becomes the endemic in many parts of the world and it causes 30% resistance rate in the community [32]. In 1965, first β-lactamase enzyme Temoneria (TEM-1) and sulphydryl variable (SHV-1) has been identified. In 1983, first case SHV-2 and TEM-3 has been documented in Germany and France respectively. Similarly, in 1989, USA also reported ESBL producing *K. pneumoniae* and *E. coli*. Since then, incidence of these enzymes markedly sporadic from one region to another [33]. ESBL producing *K. pneumoniae* displayed resistance to clinically effective extended spectrum cephalosporins and monobactam and left limited treatment options. Moreover, ESBL producing *K. pneumoniae* has become major ESBL carrying bacteria associated with nosocomial infections and outbreak during 1990 to 2000. Later, these pathogens acquired variants of ESBL such as CTX-M, TEM and SHV. There are various types of ESBLs have been identified throughout the world. Up till now, TEM (n = 227) are the most predominant type of ESBLs followed by CTX-M (n = 207), SHV (n = 203), VEB (n = 19) and PER (n = 9) [28]. Majority of these ESBLs have been reported in *Enterobacteriaceae* notably *K. pneumoniae* and *E. coli*. ESBLs producing *K. pneumoniae* have also become an important hospital acquired pathogen. In China, around 50% of the hospital acquired infections are caused by CTX-M-14 and CTX-M-15 ESBL producing *K. pneumoniae* [34]. Recently, these ESBL producing *K. pneumoniae* were also reported from community such as water, soil and veterinary settings [35]. In Asia, SHV is the predominant genotype of ESBL and early SHV-5 and SHV-12 enzymes were documented in Korea and Japan. However, in Europe and south east Asia (India and Pakistan), CTX-M-2, CTXM-3, CTX-M-14 and CTX-M-15 producing *Enterobacteriaceae* at its rise. However, in Japan, SHV-12 and CTX-M-3 and CTXM-14 have been predominantly reported in *K. pneumoniae* and *E. coli*. In Indo-Pakistan region, most of the clinical isolates of *K. pneumoniae* encoded for TEM, CTX-M and SHV. Two highly populated countries in the world (China and India) represents the largest reservoir of CTX-M in the world [36].

4.4.3 AmpC producing K. pneumoniae

AmpC are the β-lactam enzymes that belong to the ambler class C of β-lactam. These enzymes hydrolyze cephalosporins antibiotics and produce resistance against penicillin, second and third generation cephalosporins and cephamycin. Moreover, these enzymes can also convey resistance to combination of these antibiotics along with β-lactam inhibitors. The versatility of the

Molecular class (subclass)	Bush Jacoby groups	Main substrate	Defining characteristic(s)	Representative	Bacteria
A	2a	Penicillins	Increased hydrolysis of benzylpenicillin	PC1	
A	2b	Penicillins and early Cephalosporins	Hydrolysis of the penicillins and cephalosporins	TEM and SHV	*Enterobacteriaceae* (*K. pneumoniae, E. coli, E. cloacae*)
A	2be	Extended spectrum-β-lactam drugs, monobactam	Hydrolysis of extended spectrum-β-lactam drugs	TEM, SHV and CTX-M	*Enterobacteriaceae* (*K. pneumoniae, E. coli, E. cloacae*)
A	2br	Penicillins	Resistant to β-lactam inhibitor	TEM and SHV	*Enterobacteriaceae* (*K. pneumoniae, E. coli, E. cloacae*)
A	2c	Carbenicillin	Hydrolysis of carbenicillin	CARB	*Enterobacteriaceae* (*K. pneumoniae, E. coli, E. cloacae*)
A	2ce	Carbenicillin and Cefepime	Hydrolysis of carbenicillin and cefepime	RTG	
B (B1)	3a	Carbapenems	Carbapenems but not aztreonam	IMP, VIM and NDM	*Enterobacteriaceae* (*K. pneumoniae, E. coli*), non-fermenters (*P. aeruginosa, A. baumannii*)
B (B2)	3b	Carbapenems	Prefer to hydrolyze carbapenems	CphA and Sfh	
C	1	Cephalosporins	More hydrolyze the cephalosporins	AmpC,	
C	1e	Cephalosporins	Highly hydrolyzed the ceftazidime	GC1, CMY-37	
D	2d	Oxacillin	Hydrolysis of oxacillin	OXA-1, OXA-10	*A. baumannii*
D	2de	Extended spectrum-β-lactam drugs	Hydrolysis of extended spectrum-β-lactam drugs	OXA-11, OXA-50	*A. baumannii*
D	2df	Carbapenems	Hydrolysis of oxacillin and carbapenems	OXA-23 and OXA-48	*A. baumannii*

Table 1.
Classification of the β-lactamases.

K. pneumoniae is to encode various β-lactam resistant genes on their transferable plasmids that able them to spread AmpC like resistomes in to other species [30]. Plasmid mediated AmpC gene was firstly identify during 1980s in

K. pneumoniae. The most common genes of AmpC families that have been reported in *K. pneumoniae* are DHA, FOX, CMY and MOX types in different parts of the world. Although they are plasmid mediated however, first case of chromosomal mediated AmpC *bla*CMY-2 containing *K. pneumoniae* was reported in 2009. AmpC producing *K. pneumoniae* along with porin loss and increase efflux pump display increase resistance against β-lactam drugs which can lead to the carbapenem resistance in future [37].

4.4.4 Class A carbapenemases producing K. pneumoniae

Since the sporadic spread of ESBL producing *K. pneumoniae* in different regions, carbapenems which is considering last resort to treat infections caused by such pathogens have widely used. The extensive usage of carbapenems have led the emergence of plasmid mediated carbapenemase genes such as IMP, VIM, KPC and NDM. Carbapenemases are the potent enzymes that hydrolyzed the carbapenem drugs and left no treatment option available [32]. There are various chromosomal mediated (SME, SFC and NMC-A) and plasmid mediated carbapenemases (KPC, GES, IMI) have been identified. It has determined that a carbapenemase has variants extending from KPC-1 to KPC-33 till now [28]. KPC gene is mainly located on different types of transmissible plasmids such as IncF, IncX, IncA/C, IncR and IncI2 which lead to the horizontal transfer of antimicrobial resistomes. These plasmids also acquired resistant genes of other antibiotics such as aminoglycosides, tetracycline and quinolones. Most of the KPC genes are associated with *Tn4401* transposons which jumped from one plasmid to other [38]. the endemic of KPC producing *K. pneumoniae* identified in different parts of the world including USA, UK, Japan, China, Greece, India, Spain, Germany and Italy. Moreover, out breaks of the KPC also documented in different countries like USA, France (travelling history from the endemic area), Spain, Netherlands, UK, Finland and Sweden [39].

4.4.5 Class B carbapenemase producing K. pneumoniae

Class B are the MBLs that require Zn^{+} as a cofactor for their activity. There are many types of MBLs have been identified such as IMP, VIM, and NDM in *Enterobacteriaceae*. These enzymes cause resistance to various antibiotics including carbapenems and left no treatment option [40]. NDM is one the potent enzyme of MBL that newly emerged in clinically isolates of *K. pneumoniae* and *E. coli* from a Swedish patient who had been hospitalised in New Delhi, India in 2008. NDM producing bacteria displayed resistance not only against β-lactam drugs but also to other 14 classes of antibiotics. NDM producing bacteria notable *K. pneumoniae* and *E. coli* are major cause of morbidity and mortality in developing countries. Qamar et al. reported 4/9 children died due to NDM producing bacteria [12]. Similarly, another study from Karachi, Pakistan also documented 57% mortality in neonates due to NDM producing bacteria mainly *K. pneumoniae* [13]. Up till now there are 19 (NDM-1 to NDM-19) variants have been identified in different parts of the world [41]. Among these NDM-1, NDM-5 and NDM-7 are most prevalent variants with a hyper variable region. NDM are mainly plasmid mediated as compared to chromosome and located on different size and number of plasmids such as IncF, IncX, IncA/C. NDM producing *K. pneumoniae* are belong to various sequence types such as ST11, ST147, ST258, ST340, ST15 and ST16. More than 50% of the NDM producing

K. pneumoniae have been identified from ST11 and ST147. NDM genes are mainly associated with IS*Aba125* transposons in *Enterobacteriaceae* family which hypothesised originated from the Tn*125* of *A. baumannii* [42]. Since the discovery of the NDM in Asia it is widely spread in different parts of the world including USA, UK, Middle East, Europe, Africa and Australia. Main reservoir for NDM are the Asian countries like India, Pakistan and China and the Balkan States [39].

4.4.6 Class D carbapenemases producing K. pneumoniae

These carbapenemase enzymes swiftly hydrolyzed the isoxazolylpenicillins drugs such as oxacillin, cloxacillins and dicloxacillin than benzylpenicillin. There are more than 574 variants have been identified around the globe but few of them have the carbapenemase activity. Therefore, class D are reclassified in to 12 main groups; Oxacillinase (OXA)-23, OXA-24, OXA-48, OXA-51, OXA-58, OXA-143, OXA134, OXA-211, OXA-213, OXA-214, OXA-229, OXA-235 [43]. Among these most potent variants is OXA-48 which was firstly identified in *K. pneumoniae* in Turkey in 2003. Later, this gene spread in different parts of the world such as Libya, Egypt, India, Spain Switzerland, France, Germany, Argentine, Israel, Saudi Arabia, Japan. The highest percentage of OXA-48 has been reported in France, Spain and Saudi Arabia (78%) followed by 88% in Lebanon and 56% in Pennsylvania [39]. Co-existence of OXA-48 like *K. pneumoniae* has been reported from different countries like Switzerland (OXA-48/NDM-1), Turkey (OXA-48/NDM-1) and India (OXA-181/VIM-5). OX-48 like genes are mainly present on transferable and conjugative group of plasmids (IncL/M and IncL) [39, 44].

5. Tacking of antibiotic resistance

AMR is becoming a menace to global health. According to United Nation (UN), in 2050, if we do not tackle the AMR now, more than 10 million people will die each year and the global income cost would be $100 trillion. So, in 2050, one person will die after every 3 seconds [7]. Firstly, it is need of the hour to give a public awareness through paper and electronic media to stop the irrational use of antibiotics. Secondly, people should improve their hygiene practices which will reduce the 60% antibiotics burden to reduce the diarrhoea. Thirdly, more than 70% world's antibiotics are being utilised in agriculture and live stocks, so overuse of antibiotics must be avoided in these settings. Fourthly, Global surveillance of AMR should be conducted and maintained throughout the world. Fifthly, there are many countries which do not have the rapid and effective diagnostics facilities of microbiology which allow the physician to prescribe the broad-spectrum empirical therapy. Sixthly, there is an urgent need to develop an alternative method to treat the AMR such as vaccines, phage therapy, probiotics, antibodies and lysins. Seventhly, there are shortages of microbiologists, infectious diseases specialists, infections control specialists, vegetarians, pharmacists and epidemiologists. Eighthly, A global funding must be required to support and encourage the less commercially attractive. Ninthly, the world gives better incentive to pharmaceutical companies to develop new and existing antibiotics. Finally, world leading organisations such as WHO, G20, UN must work together with coordination.

Author details

Muhammad Usman Qamar[1*], Muhammad Hidayat Rasool[1], Shah Jahan[2], Muhammad Shafique[1] and Bilal Aslam[1]

1 Department of Microbiology, Faculty of Life Sciences, Government College University, Faisalabad, Pakistan

2 Department of Immunology, University of Health Sciences, Lahore, Pakistan

*Address all correspondence to: musmanqamar@gcuf.edu.pk

IntechOpen

References

[1] Merino S, Camprubi S, Alberti S, Benedi VJ, Tomas JM. Mechanisms of *Klebsiella pneumoniae* resistance to complement-mediated killing. Infection and Immunity. 1992;**60**:2529-2535

[2] Rock C, Thom KA, Masnick M, Johnson JK, Harris AD, Morgan DJ. Frequency of *Klebsiella pneumoniae* carbapenemase (KPC)-producing and non-KPC-producing *Klebsiella Species* contamination of healthcare workers and the environment. Infection Control. Hospital Epidemiology. 2016;**35**:426-429

[3] Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: Going on the offense with a strong defense. Microbiology and Molecular Biology Reviews: MMBR. 2016;**80**:629-661

[4] Ventola CL. The antibiotic resistance crisis: Part 1: Causes and threats. P & T: A Peer-Reviewed Journal for Formulary Management. 2015;**40**:277-283

[5] Read AF, Woods RJ. Antibiotic resistance management. Evolution, Medicine, and Public Health. 2014;**2014**:147

[6] Viswanathan R, Singh AK, Basu S, Chatterjee S, Sardar S, Isaacs D. Multi-drug resistant gram negative bacilli causing early neonatal sepsis in India. Archives of Disease in Childhood Fetal and Neonatal Edition. 2012;**97**:182-187

[7] de Kraker MEA, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050? PLOS Medicine. 2016;**13**:e1002184

[8] Lim C, Takahashi E, Hongsuwan M, Wuthiekanun V, Thamlikitkul V, Hinjoy S, et al. Epidemiology and burden of multidrug-resistant bacterial infection in a developing country. Life. 2016;**5**:e18082

[9] Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, et al. Antibiotic resistance-the need for global solutions. The Lancet Infectious Diseases. 2013;**13**:1057-1098

[10] Hannan A, Qamar MU, Usman M, Waheed KAI, Rauf K. Multidrug resistant microorganisms causing neonatal septicaemia: In a tertiary care hospital Lahore, Pakistan. African Journal of Microbiology Research. 2013;**7**:1896-1902

[11] Saleem AF, Ahmed I, Mir F, Ali SR, Zaidi AK. Pan-resistant Acinetobacter infection in neonates in Karachi, Pakistan. Journal of Infection in Developing Countries. 2009;**4**:30-37

[12] Qamar MU, Nahid F, Walsh TR, Kamran R, Zahra R. Prevalence and clinical burden of NDM-1 positive infections in paediatric and neonatal patients in Pakistan. The Paediatric Infectious Disease Journal. 2015;**34**:452-454

[13] Khan E, Irfan S, Sultan BA, Nasir A, Hasan R. Dissemination and spread of New Delhi Metallo-beta-lactamase-1 superbugs in hospital settings. The Journal of the Pakistan Medical Association. 2016;**66**:999-1004

[14] Qamar MU, Hannan A, Arshad MU, Arshad M. Metallo-β-lactamase producing *Enterobacter cloacae*: An emerging threat in neonates. African Journal of Microbiology Research. 2014;**8**:525-528

[15] Davies SC, Fowler T, Watson J, Livermore DM, Walker D. Annual report of the chief medical officer: Infection and the rise of antimicrobial resistance. Lancet. 2013;**381**:1606-1609

[16] Hampton T. Report reveals scope of us antibiotic resistance threat. Journal

of the American Medical Association. 2013;**310**:1661-1663

[17] Tenover FC. Mechanisms of antimicrobial resistance in bacteria. American Journal of Infection Control. 2006;**34**:3-10; 64-73

[18] Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology. 2015;**13**:42-51

[19] Billal DS, Feng J, Leprohon P, Légaré D, Ouellette M. Whole genome analysis of linezolid resistance in *Streptococcus pneumoniae* reveals resistance and compensatory mutations. BMC Genomics. 2011;**12**:512

[20] Haeili M, Javani A, Moradi J, Jafari Z, Feizabadi MM, Babaei E. MgrB alterations mediate colistin resistance in *Klebsiella pneumoniae* isolates from Iran. Frontiers in Microbiology. 2017;**8**:1-8

[21] Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, et al. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLOS Pathogens. 2010;**6**:e1000944

[22] Kojima S, Nikaido H. Permeation rates of penicillins indicate that *Escherichia coli* porins function principally as nonspecific channels. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**:2629-2634

[23] Tran QT, Williams S, Farid R, Erdemli G, Pearlstein R. The translocation kinetics of antibiotics through porin OmpC: Insights from structure-based solvation mapping using WaterMap. Proteins. 2013;**81**:291-299

[24] Djahmi N, Dunyach-Remy C, Pantel A, Dekhil M, Sotto A, Lavigne J-P. Epidemiology of carbapenemase-producing *Enterobacteriaceae* and *Acinetobacter baumannii* in Mediterranean countries. BioMed Research International. 2014;**2014**:11

[25] Li B, Zhao Y, Liu C, Chen Z, Zhou D. Molecular pathogenesis of *Klebsiella pneumoniae*. Future Microbiology. 2014;**9**:1071-1081

[26] Hu R-M, Liao S-T, Huang C-C, Huang Y-W, Yang T-C. An inducible Fusaric acid tripartite efflux pump contributes to the fusaric acid resistance in *Stenotrophomonas maltophilia*. PLoS One. 2012;**7**:e51053

[27] Bialek-Davenet S, Lavigne J-P, Guyot K, Mayer N, Tournebize R, Brisse S, et al. Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in *Klebsiella pneumoniae*. Journal of Antimicrobial Chemotherapy. 2015;**70**:81-88

[28] Naas T, Oueslati S, Bonnin RA, Dabos ML, Zavala A, Dortet L, et al. Beta-lactamase database (BLDB)—Structure and function. Journal of Enzyme Inhibition and Medicinal Chemistry. 2017;**32**:917-919

[29] Livermore DM. Defining an extended-spectrum beta-lactamase. Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases. 2008;**14**(Suppl 1):3-10

[30] Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrobial agents and chemotherapy. 2010;**54**:969-976

[31] Öztürk H, Ozkirimli E, Özgür A. Classification of beta-lactamases and penicillin binding proteins using ligand-centric network models. PLoS One. 2015;**10**:e0117874

[32] Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: A major worldwide source and shuttle for antibiotic resistance. FEMS Microbiology Reviews. 2017;**41**:252-275

[33] Gupta A, Ampofo K, Rubenstein D, Saiman L. Extended spectrum beta lactamase-producing *Klebsiella pneumoniae* infections: A review of the literature. Journal of Perinatology: Official Journal of the California Perinatal Association. 2003;**23**:439-443

[34] Zhang J, Zhou K, Zheng B, Zhao L, Shen P, Ji J, et al. High prevalence of ESBL-producing *Klebsiella pneumoniae* causing community-onset infections in China. Frontiers in Microbiology. 2016;**7**:1830

[35] Toubiana J, Timsit S, Ferroni A, Grasseau M, Nassif X, Lortholary O, et al. Community-onset extended-spectrum β-lactamase-producing *Enterobacteriaceae* invasive infections in children in a University Hospital in France. Medicine. 2016;**95**:e3163

[36] Hawkey PM. Prevalence and clonality of extended-spectrum-beta-lactamases in Asia. Clinical Microbiology and Infection: The official Publication of the European Society of Clinical Microbiology and Infectious Diseases. 2008;**14**:159-165

[37] Zamorano L, Miro E, Juan C, Gomez L, Bou G, Gonzalez-Lopez JJ, et al. Mobile genetic elements related to the diffusion of plasmid-mediated AmpC beta-lactamases or carbapenemases from *Enterobacteriaceae*: Findings from a multicentre study in Spain. Antimicrobial Agents and Chemotherapy. 2015;**59**:5260-5266

[38] Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. Genetic structures at the origin of acquisition of the beta-lactamase blaKPC gene. Antimicrobial Agents and Chemotherapy. 2008;**52**:1257-1263

[39] Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: Epidemiology, genetic context, treatment options, and detection methods. Frontiers in Microbiology. 2016;**7**:895

[40] Qamar MU, Saleem S, Toleman MA, Saqalein M, Waseem M, Nisar MA, et al. In vitro and in vivo activity of Manuka honey against NDM-1-producing *Klebsiella pneumoniae* ST11. Future Microbiology. 2018;**13**:13-26

[41] Qamar MU, Walsh TR, Toleman MA, Saleem S, Jahan S. First identification of clinical isolate of a novel "NDM-4" producing *Escherichia coli* ST405 from urine sample in Pakistan. Brazilian Journal of Microbiology [Publication of the Brazilian Society for Microbiology]. 2018;**49**:949-950

[42] Toleman MA, Spencer J, Jones L, Walsh TR. blaNDM-1 is a chimera likely constructed in *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy. 2012;**56**:2773-2776

[43] Jeong SH, Lee KM, Lee J, Bae IK, Kim JS, Kim HS, et al. Clonal and horizontal spread of the blaOXA-232 gene among *Enterobacteriaceae* in a Korean hospital. Diagnostic Microbiology and Infectious Disease. 2015;**82**:70-72

[44] Kilic A, Baysallar M. The first *Klebsiella pneumoniae* isolate co-producing OXA-48 and NDM-1 in Turkey. Annals of Laboratory Medicine. 2015;**35**:382-383

Section 2

Control Strategies of Microbial Biofilm

Chapter 3

Alternative Approaches to Combat Medicinally Important Biofilm-Forming Pathogens

Mansab Ali Saleemi, Navindra Kumari Palanisamy and Eng Hwa Wong

Abstract

Bacteria have developed the capability to produce structured communities (or cluster of cells) via adherence to surface to form biofilms that facilitate or prolong their survival under extreme environmental condition. Bacterial biomass adheres to inanimate and biotic surfaces in the hospital setting as well as in the environment. In the healthcare system, the biofilm formation on medical devices allows bacteria to sustain as a reservoir and becomes more resistant to antimicrobial agents. However, biofilm formation facilitates pathogens to sabotage the host defenses that are linked to long-term retention within the host cell. Therefore, in this review, we provide some steps leading to the formation of biofilm within the host and on inanimate surfaces, also emphasizing various medically significant pathogens and debate current developments on novel approaches that aimed to prevent biofilm formations and its dispersion to patients.

Keywords: biofilm, antibiotic resistant, distribution, control, therapeutic strategy

1. Introduction

Biofilm formation is structured accumulation of fastidious microorganisms attached on inanimate objects or compact surfaces that extensively have been examined in the past decades because they particularly cause infections and more often responsible for chronic infections [1–3]. They are predominantly problematic due to their antimicrobial resistant properties and their ability to evade host defense mechanisms, which substantially hinders disease treatment in the hospital [1–4]. Bacterial biofilms are ubiquitous in nature and harbor phenotypic adaptations in the environment with respect to broader perspective [1]. The nature of single cell organisms enables them to adhere to each other and form a "complex structure," which assists to survive under adverse environmental condition. The biofilm formation occurs from planktonic bacteria due to environmental changes and involves in conjugation gene transfer "multiple regulatory network" from one bacterium to another in response to environmental stress [5–9]. This type of cell-to-cell adhesion and gene transformation changes the expression of surface molecules, virulence factors, and nutrient utilization that enables their survival under unfavorable environmental condition [8, 10–17].

Bacteria are cocooned within the biofilm and form extracellular matrix, which represents 90% of the biomass [18]. The matrix as a stabilizing scaffold for the three-dimensional structure is composed of extracellular polymeric substance (EPS) along with extracellular DNS and carbohydrate binding protein [19–21]. Nutrients are trapped by the resident bacteria in the matrix and water is retained efficiently via H-bond interaction with hydrophilic polysaccharides [18, 22]. The composition of extracellular polymeric substance (EPS) is modified in response to alterations in nutrient availability [23, 24] by certain enzyme secretion of bacteria, thus tailoring biofilm formation to the more specific environment [23, 25]. Therefore, the skeletal components of the extracellular matrix are highly hydrated and provide high tensile strength that enables bacteria to exchange their DNA by conjugation and promote cell-to-cell interaction while defending the biomass from predation, radiation, desiccation, oxidizing molecules, and other dangerous agents [18, 26–28].

The multifaceted nature of biofilms that allow the bacteria to form a community, i.e., division of labor and express their virulence factors in response to local oxygen and nutrient availability, makes them resistant against different antimicrobial agents [29, 30]. Some studies have shown that there are presence of nondividing metabolically inactive recalcitrant bacteria within the biomass [29, 31], which play very crucial role to cause tolerance against broad-spectrum antimicrobial drugs. The matrix protein inside the host cell protects bacterial biofilm against innate immune defenses, i.e., phagocytosis and opsonization [32]. The spread of other virulence factors inside the host cell and drug resistance marker is due to the cell-to-cell interaction [15]. Thus, biofilm-forming pathogens retained and adhere to the infected surface and cause recalcitrant and chronic infection, i.e., upper respiratory tract infection (particularly, *Pseudomonas aeruginosa*) [33, 34], dental decay (mixed culture of *Streptococcus mutans,* and other pathogens) [35], ventilated-induced and other device-associated infections (*Escherichia coli*, *Klebsiella* spp., *Enterococcus faecalis*, *Staphylococcus aureus,* etc.) [36, 37], urinary tract infections [*Proteus* spp., uropathogenic *E. coli* (UPEC)] [38]. In particular, immunocompromised patients are the most common target to all these biofilm-forming pathogens, causing a devastating impact on patients, and in many cases, leading to death. Here, we analyze the formation of intracellular and extracellular biofilm which is the underlying factor for various medically important microorganisms. Given the recalcitrance and prevalence of infections caused by biofilm-forming pathogens, we discuss knowledge about the most current progresses in the advancement of novel strategies of biofilm.

2. Extracellular formation of biofilm

2.1 Bacterial attachment on surfaces and what does make it adhere to object surface?

Bacterial biofilm growth, subsequent maturation, and aggregation consist of irreversible and reversible stages, which involve various conserved and species-specific aspects. At the first stage, the bacteria are introduced on the surface; a process of at least a part of stochastic that is driven by gravitational forces and Brownian motion, and usually influenced by nearby hydrodynamic forces [39, 40]. Microorganisms encounter with repelling or attractive forces—within the niche that alter depending on ionic strength, pH, nutrient levels, and temperature. Bacterial cell wall composition, along with medium properties, affects direction and velocity toward or away by the contact surface of pathogens [39]. Motile bacteria utilize flagella in order to overcome repulsive and hydrodynamic forces, by

having a competitive advantage. The main function of flagella is to provide motility and initial cell attachment to the surface for various pathogens, including *Listeria monocytogenes*, *E. coli*, *Vibrio cholerae*, and *P. aeruginosa* [41–45]. In some species of bacteria, chemotaxis plays very important role in direct attachment to nutrient composition, for instance, mutations arise in CheR1 methyltransferase, which have been observed to vary the response of amino acid of *P. aeruginosa* and impair maturation of bacterial biofilm and attachment [46]. Some earlier studies have been shown that chemotaxis in *E. coli* is dispensable [5]; moreover, current observations revealed that the disruption occurs in the chemotaxis methyl accepting protein II and informs biofilm defects particularly in uropathogenic *E. coli* cells [47]. With respect to intercepting surface, bacterial attachment is facilitated by additional secreted molecules such as adhesin protein and extracellular adhesive appendages.

Initially, the attachment is reversible and dynamic during which pathogens can separate and rejoin planktonic biomass if agitated through repulsive forces [48], hydrodynamic forces—detach bacteria off from the surface. Some bacteria attained irreversible attachment in order to maintain a firm grip on the cell surface. Serotypes of other *E. coli* and uropathogenic *E. coli* depend intensely on the type 1 pili [5, 40, 49–51]. Uropathogenic *E. coli* harbors several pili systems (means CUP system), which mediate adhering to a specific niche [38]. Attachment on the bacterial surface is facilitated by the adhesion protein (FimH), which identifies mannosylated moieties [50–52]. The adhesive protein (FimH) plays a critical role in the pathogenesis of uropathogenic *E. coli* because it facilitates adherence and causes invasion to epithelial cells of bladder in human, adheres to the human uroplakin and is also critical in preclinical murine cystitis model, which causes human disease [51, 53, 54]. FimH is much more consistent to play a critical role in the virulence of human disease under positive selection [52–56].

Furthermore, antigen 43, curli fibers, and type 1 pili have been observed to facilitate attachment and cell-to-cell interaction on inanimate surfaces [57]. Curli fiber also mediates attachment to the extracellular matrix components in eukaryotes such as plasminogen, fibronectin, and laminin [58]. *Pseudomonas aeruginosa,* for instance, uses various additional organelles, which assist in adherence to the surface, irreversibly. Contrary to UPEC and *P. aeruginosa*, Gram-positive bacteria (*Enterococci*) are lactose producing, nonmotile, and recently identified to contain nonadhesive (pili) that mediate attachment to the extracellular matrix components in eukaryotes. Examples of these include Ace (*E. faecalis*) and SagA (*E. faecium*), which attach to the collagen protein [59] and surface protein (Esp). This has been observed to stimulate abiotic formation of biofilm on the contact surface specifically in *E. faecalis* [60]. Current studies showed the existent of biofilm-associated pili (Ebp) and also confirmed their contribution toward urinary tract infections, endocarditis, and biofilm formation and attachment [61].

2.2 Maturation of biofilm

Cell-to-cell interaction triggers specific intrinsic responses that cause changes in the gene expression, upregulating factors favorable to sessility especially for those involved in extracellular matrix protein formation [40]. However, relatively very little information is obtained about the matrix constituents with respect to *E. coli* pathogen. Initially, cellulose was recognized as essential components in *E. coli* pellicle biofilms and later on expressed with curli fibers in gastrointestinal *E. coli* strains [62]. Curli fiber plays a critical role in pellicles, for instance, curli fiber (amyloid) that leads to the pellicle biofilm formation. It also acts as a curlicide to prevent pellicle formation, and some of them have deficient to form pellicles (known as curli mutants) [63]. Further studies revealed that colonic acid and polyglucosamine

(PGA) take part in biofilm architecture [64], while the PGA being predominant among the clinical strains, particularly in UPEC isolates. Thus, more detailed investigations are required for further characterization of extracellular matrix protein in *E. coli*. The composition of extracellular matrix protein has been extensively analyzed in *P. aeruginosa* and varies depending on external environmental conditions [65]. The primary components of EPS are Psl and Pel [25]. Psl enhances the attachment of *P. aeruginosa* to epithelial cells [66] and mucin, while the expression of Pel increased in small colony variants (SCV) isolated from the cystic fibrosis patients associated with *Pseudomonas* persistence in the airways of lung [67]. Moreover, intercellular interactions and biofilm stabilizations in *P. aeruginosa* are critical in response to environmental DNA (eDNA) [68].

Mature *P. aeruginosa* biofilm formations are more resistant to treatment with DNase as compared to young biofilms, demonstrating that eDNA remains stable because the components of EPS are not abundant during the initial stage of biofilm when the bacterial cells come to attach each other. In contrast, the concentration of eDNA increases during biofilm maturation stage due to the occurrence of bacterial cell lysis in response to quorum sensing mechanism of *Pseudomonas* quinolone signal (Pqs) [69]. In *Pseudomonas*, type IV pili play an essential role in the migrating pathogens to form aggregation in the area of high eDNA binding attraction [70]. The amount of eDNA to form biofilm structure has already been observed in *E. faecalis*. Some reports identified that biofilm formation in this organism is influenced by the affected autolysis of cells and intracellular release of DNA [71, 72]. Initial study reported that the mutant reduced the biofilm formation by 30% due to the lack of autolysin gene, Atn [59]. In another study investigated, it showed that specific stage of bacterial biofilm formation required temporal regulation by Atn for the release of DNA [73].

2.3 Matrix escape mechanisms

Bacterial mature biofilm provides a suitable living environment to the resident microorganisms for making compact surface adherence community, so as to share products and actively exchange their genetic materials by conjugation. Moreover, as biofilms mature, dispersal becomes a choice. In addition to passive dispersal caused by shear stress, the pathogen develops different ways to recognize environmental changes, which make it to stay within the biofilm. Bacterial biofilm dispersal occurs as a result of various clues such as oxygen fluctuations, modifications in nutrient availability, and increases in toxic products [74]. Biofilm dispersal is induced by the increase of extracellular iron in uropathogenic *E. coli* [75], while in *Pseudomonas* spp., it is due to the increased quantities of various nitrogen and carbon source [76]. The amounts of small molecules such as alterations in environment and changes in gene expression are monitored by various sensory systems [77]. Among various other signals, for instance, universal cyclic-di-GMP has been used in *P. aeruginosa* and *E. coli* causing implication in a shift between motility and sessility. Typically, an increase in the level of cyclic-di-GMP is favorable to sessility, while a reduction in cyclic-di-GMP induces upregulation of motility [78].

Recently, some results reported the factors responsible for such changes such as downregulation of extracellular polymeric substance, reduction of cyclic-di-GMP in bacterial biofilm communities, and upregulation of swarming and swimming motility [25]. Certain type of enzymes (such as alginate lyase) also participates in pathogen detachment from surface especially in *P. aeruginosa* [79], whereas in *E. coli,* the enzyme (CsrA) is responsible to repress the synthesis of PGA [80]. Along with that downregulation of EPS, certain molecules of surfactant are produced causing a reduction in cell-to-cell interaction. Moreover, studies identified that

flagellated populations within the biofilms of *P. aeruginosa* migrate to other void surface in order to make colonies [65]. Initially, these colonies loosely attach to compact surface, but after maturation process, they make a hard shell in the surrounding and use the infected surface as a source of nutrient. Sometimes, live cells use dead cells as a source of carbon. When bacteria become dead, then live cells accumulate on it, bind to each other by sharing their genetic materials and form a compact layer that is usually very hard to break. Dead cells are also responsible for creating cavity within the bacterial biomass. The bacteria within the biofilm can be scattered by applying dispersal mechanism.

Due to dispersing nature of bacteria, they may have the ability to restart the biofilm formation process after encountering a favorable environmental condition [81]. This is another sophisticated mechanism of dispersal revealed by using *B. subtilis,* which could be prevalent among the bacterial species. Researchers reported that the pathogen (*B. subtilis*) lost its cellular integrity within 5–8 days and also found that disassembly of biofilm is associated with a mixture of different amino acids ($_{D}$-tyrosine, $_{D}$-methionine, etc.) that are formed during bacterial stationary growth phase [82]. These $_{D}$-types of amino acids interfere with bacterial attachment to cell surface and perturbation to fiber dissociation, without influencing matrix component expression or bacterial growth [83]. In *B. subtilis*, the performance of biofilm is disrupted by the addition of $_{D}$-type amino acid mixture [83]. Further studies showed that another factor such as norspermidine, which is produced by *B. subtilis,* works together with $_{D}$-type amino acid leading to biofilm disassembly [84]. So, this type of association—norspermidine/$_{D}$-type amino acid—is essential for the eradication of bacterial biofilm and makes them vulnerable to antimicrobial agents used in the hospital.

3. Bacterial intracellular biofilms

Gathering evidence have showed that numerous bacterial pathogenic species formerly considered as extracellular can retain within the host cell by adapting intracellular bacterial lifestyle that includes the bacterial communities having biofilm-like properties. First, a murine model of infection was used to assess the bacterial communities for UPEC [85]. Type 1 pili in uropathogenic *E. coli* bind to the receptor on superficial bladder cells [86], triggering to induce bacterial internalization. Toll-like receptor-4 (TLR-4)-dependent process used to expel out from inside the UPEC [87], but certain bacteria elude exocytic procedure and leave out from the cytoplasm of host cell, where they duplicate into intracellular bacterial communities (IBCs) [85]. Several developmental stages lead to the process of IBCs that indicate distinct morphological features [85]. After passing first 6 h ensuing bladder inoculation, UPEC rapidly divides (replication time 30–35 min) causing small clusters associated with loosely attached rods (during early IBCs), having a coccoid shape and an average bacterial length of about 0.7 mm. The bacterial exponential growth rate dramatically drops between 6 and 8 h, exceeding replication time to 60 min. This is the second stage where bacteria accumulate and are tightly packed within the biofilm and organized a compact sphere-shaped structure (mature-stage IBCs) (**Figure 1**).

The amount of IBCs is found between 3 and 700 in an infected patient's bladder—IBCs are composed of 10^4–10^5 bacterial cells [88]. There are numerous fibers surrounded on IBC bacteria that originate from the surface of pathogen and enclose pathogens in individualized sections. One of the main components present on the surface of IBCs called polysaccharide (sialic acid) that provides protection from the attack of immune system and environmental stress. The heterogeneous nature

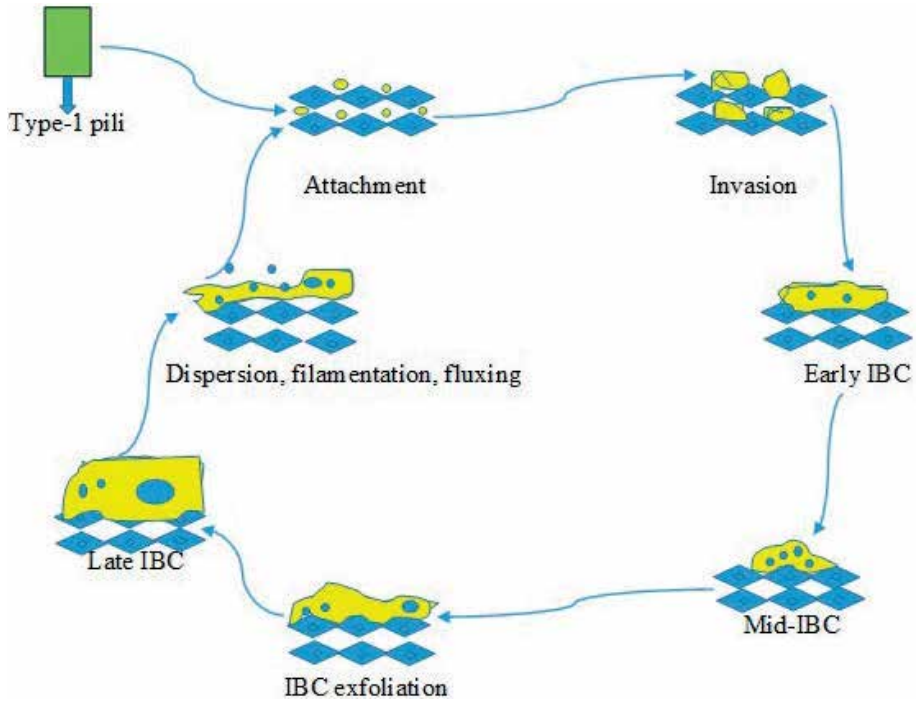

Figure 1.
Schematic diagram of the development of IBC cascade in uropathogenic E. coli (UPEC), taken by scanning electron microscope (SEM) images indicating different structural changes from attachment to dispersion and fluxing.

of IBCs, such as extracellular bacterial biofilm, composed of different subpopulation having distinct gene expression systems [89]. As IBCs expand, they induce the bacterial biofilm to cause interruption against cell membrane of host, producing a pod-like structure on the infected cell surface. Ultimately, UPEC detaches as filament or single rod at the IBC boundary and the infected cells are flux out into the lumen of bladder where can invade epithelial cells and restart the process through binding [85]. The inhibitor (SuIA) of cell division has been observed to be crucial for dispersal and filamentation of UPEC from the bacterial biofilm. The patients suffered from urinary tract infections (UTIs) are more likely observed with the UPEC filaments in their urine, but not in comparison with healthy controls [90].

The formation of IBC is prevented by intense molecular blockages and during acute infection—development of chronic cystitis—the IBC numbers are higher, representing the significance of intracellular pathways in the pathogenesis of UTIs [88]. The cycle of IBC is dependent on FimH, causing interruption in the expression of type-1 pili after invasion to host cell, and disrupts normal development of IBC due to attenuation of UPEC [54]. The two-component system (QseBC) is a key factor influencing curli expression, formation of IBC and type-1 pili. Some studies indicated that the intracellular pathway of UPEC is necessary for the TCA cycle completion [47]. The techniques such as qPCR and DNA microarray analyses interpreting the UPEC expression patterns within IBC pathogen exposed that acquisition of iron in bacteria is upregulated, representing the significance of system biomass formation [91]. While in clinical isolates of UPEC, the iron acquisition patterns are prevalent [92]. Moreover, the pathogen *Klebsiella pneumoniae* is more commonly seen in community- and hospital-acquired infection. About up to 5% forms intracellular communities and is more predominant in hospitalized diabetic patients [93]. Likewise to UPEC, the *Klebsiella pneumoniae* invasion is mediated by

type-1 pili and formation of IBC, although the differences occur in the expression kinetic of pili and filaments [90]. The ability to occupy an intracellular niche and persist within the host cell through transitioning from single microbial cell to the multicellular community is not confined to uropathogens. Researchers showed that by using different animal models and cell line of acute lung infection, the cluster formation occurs inside the lung airways due to *P. aeruginosa*, morphology similar to *Klebsiella* and UPEC (IBCs) [94]. The biofilm formation ability could be evolutionary adaptation of pathogens that enable the bacteria to persist within the host cell. All these findings represent the formation of IBC, a process that enables the bacteria to rapidly expand inside the host cell and take part in bacterial persistence.

4. Postantibiotic period: treatment strategy for biofilm

Broad-spectrum antibiotics are the drug of choice for the treatment of bacterial infections. Conventional antibiotics act as either killing the bacterial cell (bactericidal) or inhibiting the cell division (bacteriostatic). Numerous evidence shows that the use of antibiotics extensively causes damage to the host microbiota, producing a condition where invading bacteria can prevail and enhance the selective pressure against drug resistance [95]. Furthermore, surgery proceeded by administering antibiotics is highly successful in order to minimize the infection prophylactically. In certain cases, the perfect treatment of choice for foreign material associated with biofilm infections is the removal of infectious device. In some cases like pacemakers, cardiac implants and implantable prostheses, device removal is difficult [37]. Biofilm formation nature of bacteria that make them recalcitrant against different antimicrobial drugs is a result of prolonged treatment. There is a need for the irradiation or complete removal of these kinds of pathogens. Antibiotic resistance is not only due to increased resistance markers transmitted within the bacterial biofilm community, but also due to high metal ion concentration, low pH, and the presence of persistent cells that are metabolically inactive and inactivate the antibiotics

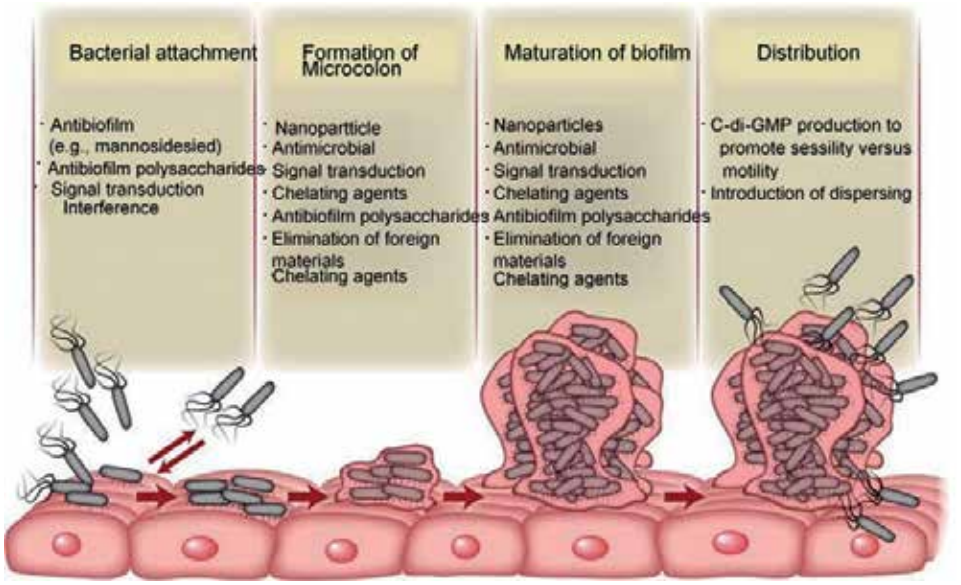

Figure 2.
Schematic diagram about the different stages in the development of biofilm and indicating the strategies to preventing and damaging the bacterial biofilm production at particular stages.

[31]. All these characteristics make bacterial biofilm more tolerant/resistant to antimicrobial drugs up to 1000-fold more when compared to planktonic bacterial cells [96]. Therefore, an alternative strategy must be investigated to combat the antibiotic resistant strains and make them vulnerable to antimicrobial drugs. Here below, we have mentioned some of the recent developments in strategies that are considered to prevent formation of biofilm by bactericidal mechanism or targeting distinct developmental stages of biofilm (**Figure 2**).

5. Bacterial killing strategies

5.1 Elimination of foreign material (indwelling devices) and abscess

There are studies that have reported that the presence of any foreign body (indwelling medical devices such as implants or prostheses or catheters) in low inoculums of *Staphylococcus aureus* (10^2 CFU/ml) in animal tissues was sufficient to form abscesses in the patients (95%) despite significant existence of leukocytes. In fact, this could be associated with the existence of any foreign material considerably intracellular bactericidal effects of body immune cells (leukocytes) and down-regulated the mechanism of phagocytosis [97]. The polymorphonuclear leukocytes cannot perform well in the presence of any foreign body because it provides a surface ideal for the bacterial attachment. Therefore, the existence of any foreign material considerably increases the chances of bacterial biofilm formation. This leads to the pathogen becoming more persistent and resistant against conventional antibiotics. Thus, potential therapeutic strategy is required for the elimination of such type of bacterial biofilm formations. Certain precautionary measures could be employed, for instance, to replace the infected devices used for medical purposes in the patients with a new one. Otherwise, it would be hard to overcome the problem regardless of applying various effective antimicrobial drugs in response to fastidious pathogens. Changing dialysis catheter if it is infected by the pathogens is another measure that could be taken. When pathogen forms biomass on the catheter, it could be the source of bacterial colonization leading to bacteremia which may be caused by a deadly bacterial strain. For the cure of catheter-associated infections caused by bacterial biofilm formation, it is important to change the catheter infected with pathogens along with administration of antibiotic intravenously during a short time in order to eradicate the pathogen before it invades into the bloodstream. However, in some cases, it is hard to change the catheter temporarily; therefore, antimicrobial drugs and other alternative therapy may be the best option for the minimal release of pathogens from the infected site.

5.2 Phage therapy

An alternative approach to antibiotic treatment is phage therapy [98]. Phages are present in a wide range in the environment. It can be isolated easily and ubiquitous in nature. Their host ranges from specific to narrow, they are able to self-replicate, and therefore, a small dosage may be sufficient to disturb the host microorganisms. Furthermore, high mutation rate of phage facilitates adaptation as conforming bacterial host aggregate mutations to fix in a specific environment. Phage therapy has various advantages during lytic cycle phage that does not enter prophage cycle and rarely transfers or contains a virulence gene, thus causing destruction of bacterial cell rapidly. Many phages are associated with EPS degrading protein [99] or spread during stationary growth phase; these features allow to persist inside the bacterial biofilm [100].

5.3 Antimicrobial peptides

This is another alternative approach used for the improvement of new type of antimicrobial drug, usually produced by innate immune response mechanism [101]. Contrary to that, their mechanism of action and antimicrobial spectrum activity must be defined more accurately before applying as a therapeutic strategy. Cathelicidin, for instance, possesses most essential type of antibacterial peptides. The biofilm formation of multidrug-resistant *Pseudomonas* strains, isolated from cystic fibrosis (CF) patients, is reduced considerably by BMAP-28, BMAP-27, and BMAP-29 [102]. According to a recent study by Pompilio et al. [102], antimicrobial activity of tobramycin against multidrug-resistant strains is less than cathelicidin peptides. This study indicates that the multidrug-resistant strains are vulnerable to cathelicidins due to antibiofilm agents. Another important group that can be used to assess the inhibitory effects is called lytic peptides. These peptides assist in attachment of lipopolysaccharides (LPS) to the cell membrane of pathogen and cause cell membrane disruption. The study on *Staphylococcus aureus* indicated that *in vitro* formation of biofilm is prohibited by the lytic peptide (PTP-7) and easily penetrates the bacterial biofilm causing death of the bacteria at a rate of 99.9%. This peptide has the capacity to bear extreme acidic environment and inhibit the biofilm formation of *Staphylococcus aureus* [103].

5.4 Silver nanoparticles

Many researchers have done research on the antimicrobial property of silver nanoparticles. Fey [37] found that the silver nanoparticles are the best alternative strategy to combat the bacterial biofilms. For example, antimicrobial agents (silver nanoparticles) have been incorporated with medical devices and have showed to inhibit the device-associated bacterial biofilms. Silver was frequently used as an antimicrobial agent for different pathogens over a 100 years; for instance, during World War 1, it was extensively used to sterilize the wound infections [104]. The antimicrobial activity of silver nanoparticles depends on the positively charged ions of metal and electrostatic interactions between negatively charged cell membrane of bacteria [105]. The thiol group in silver is the main cause of death in bacteria that play an important role in the inactivation of enzyme [106]. This is the reason why silver nanoparticles are increasingly used in response to various bacterial infections. The antimicrobial agents contain different properties such as high aspect ratios, nonimmunogenic, biocompatible, nonbiodegradable, ultralight weight, and easy cell membrane penetration. Due to such remarkable properties, we can apply silver nanoparticles in various applications such as infection therapy, gene therapy, and as antioxidants. The size of silver nanoparticles is typically smaller than 100 nm. The mechanism of action of silver nanoparticle is to interrupt the cell membrane of bacteria, generate the reactive oxygen species (ROS), interrupt the metabolic pathway, prevent the replication of DNA, disrupt the bacterial electron transport chain (ETC) [106], and release the toxic ions outside the bacterial cells that lead to the death of bacteria. There are large numbers of studies conducted regarding toxicity mechanism of silver nanoparticles in rabbits. There is a study that showed that silver nanoparticles inhibited bacterial biofilm formation against *Staphylococcus aureus,* without accumulating inside the host tissue [106, 107].

5.5 Polysaccharides

Bacterial cell-to-cell interaction mediated by the exopolysaccharides is a serious threat to the formation of biofilm and stabilization. Mutants incapable to export or

synthesize such exopolysaccharides are usually deficient in the formation of biofilm and adherence and hence are extremely sensitive to killing through host immune defenses and antimicrobial drugs [108]. Recent studies showed that certain bacterial exopolysaccharides destabilize or prevent biofilm formation by some pathogenic species. For instance, the existence of *Pseudomonas aeruginosa* prevented biofilm formation of *S. epidermidis* in in vitro experiments [109]. Polysaccharides along with nonbactericidal antibiofilm characteristics have been separated from acellular biofilm (or biomass) extracts of various species [108]. The antibiofilm properties of *Pseudomonas aeruginosa* have the ability to act as signaling molecules that effect the expression of genes in susceptible pathogens, change the physical features of isolated bacterial cells, and prevent the protein-carbohydrate interactions. Most polysaccharides with antibiofilm properties allow a broad-spectrum inhibition of biofilm, while some are proficient of scattering preformed biofilms. So far, there are evidence suggests that polysaccharide with antibiofilm features acts as a surfactant molecule that alters the physical properties of abiotic surfaces and bacterial cells. Some results also show that polysaccharides might modulate the expression of genes of the recipient pathogenic bacteria by acting as signaling molecules [110]. Another potential mode of action of polysaccharide is to prevent competitively the multivalent protein-carbohydrate interactions [66]. As a result, polysaccharides with antibiofilm properties might block tip adhesins of pili and fimbriae, or block sugar or lectin-binding proteins that are present on the outer surface of pathogens. In pathogen *P. aeruginosa*, for instance, lectin-dependent adhesion to human cell is proficiently repressed by galactomannans [111]. This kind of polysaccharides that inhibit the biofilm could be a prominent strategy appropriate for the prevention of bacterial infections. Some scientist showed that antibiofilm polysaccharides can be used as an adjuvant because of enhancing antibiotic drug functions [108].

5.6 Interference with signal transference

Many studies have been carried out on biofilm inhibition caused by interruption of the pathogen signaling cascades. This is possible provided that the two-component systems in bacteria establish a dominant means of translating and intercepting the environmental changes. Signal transduction inhibition system plays a critical role in response to antimicrobial therapy because of this type of signaling cascade interruption. Not only does it kill the pathogen, but it also interferes with the gene expression. Two-component system (QseBC) is the best alternative candidate for targeting the drugs, particularly in Gram-negative biofilm-forming pathogens [112]. QseC/QseB establishes a significant association between the bacterial environmental signaling and the host stress response. The pathogen (*E. coli*) responds to autoinducer-3 in the intestine that is formed by the human stress hormones (such as epinephrine and norepinephrine) and gut flora. The cascade of signaling transduction comprises chemotaxis by activation of QseC and by using the serine receptor Tsr. In the quest for novel antimicrobial drugs and therapeutic targets, two-component system (QseBC) can play an important role to inhibit biofilm formation by blocking the binding of epinephrine or norepinephrine to QseC, as a result to reduce QseB/QseC signaling and decrease virulence and motility [113]. Studies have also suggested that the removal of QseC in EHEC and UPEC causes an excessive activation of response regulator QseB, owing to particular QseC phosphatase activity required for deactivation of QseB. The optimal strategy behind targeting the phosphate activity is to interfere with common gene expression in QseC containing pathogens [47]. Some other studies focused on the FsrATC/FsrA inhibitors in *E. faecalis*. The expression of gelE-sprE and FsrBDC

control by the FsrC/FsrA leads to increase in the production of serine protease and gelatinase, both are crucial for the proper eDNA production [71].

5.7 Antimatrix agents

Apart from that, extracellular matrix with disrupting components is also very important to target the bacterial aggregates. Various observations exploited the inhibiting enzymes potentially involved in the modification or synthesis of cell wall-secreted or associated with EPS components. In these studies, use of engineered or naturally occurring enzyme and use of phage therapy as an enzyme delivery vehicle or to interrupt with matrix integrity by taking benefits from metal chelators have been recommended.

5.8 Chelating agents

Metal cations such as iron, magnesium, and calcium have been associated with stabilizing the matrix integrity [114]. Chelating agents indicated to cause interruption in the bacterial cell membrane stability besides disrupting the bacterial biomass structure [39]. *In vitro* study showed that biofilm formation was inhibited in various *Staphylococcus* species by sodium citrate [115]. Furthermore, eradication of bacterial biofilms in *in vitro* experiments is also facilitated by tetrasodium EDTA, while disodium EDTA only reduced the bacterial biofilm formations in *P. aeruginosa* and *Staphylococcus* species [116]. Current reports suggested that the solution of minocycline-EDTA was used to inhibit indwelling catheter-associated infections especially in children. There were no adverse side effects observed in patients treated with the solution of minocycline-EDTA but only a limited number (21%) of untreated group (control) developed infections [117]. Moreover, in hemodialysis patients, catheter-associated bloodstream infections were observed after applying minocycline-EDTA [118].

5.9 Enzyme

The main mechanism of active dispersal of bacterial biofilm is through the formation of extracellular enzymes (proteins) that act on several structural components (such as exopolysaccharides, surface proteins, and extracellular DNA) of the extracellular polymeric substances. These enzymes play an important role in the cell separation from the bacterial biofilm colonies and facilitate their planktonic discharge into the environment [119]. Through purifying and isolating these enzymes, therapist can apparently add them to preformed bacterial biofilms exogenously at raised concentrations, in order to make biofilm-associated bacteria more susceptible to antimicrobials/antibiotics and to achieve interventional dispersal of biofilms. For this purpose, several classes of enzymes (specifically proteases, glycoside hydrolases, and deoxyribonucleases) have been explored for the eradication of bacterial biofilms [119]. The enzymes dispersin-B and DNase-I have gained greater attention as possible antibiofilm agents, especially in response to Gram-positive bacteria. The DNase effect depends on its capability to interrupt the eDNA that is established within the bacterial biomass structure [73]. The treatment of DNase prevents biofilm formation in *Enterococcus* and *Staphylococcus* and dispersed bacterial biofilm [73]. For the treatment of patients with cystic fibrosis (CF), a recombinant enzyme (pulmozyme) is used in some cases [37]. However, treatment with dispersin-B represented to be more effective in response to *S. aureus* and *S. epidermidis* [77]. *In vitro* studies indicated that engineered dispersin-B used

bacteriophage machinery in order to replicate during the stationary phase of cell growth, hence causing disruption of complete *E. coli* biofilms [120].

6. Conclusion

Currently, the removal of bacterial biofilm is the most challenging task for the clinicians and microbiologists. Antibiotics are not the best choice for the treatment of infections caused by bacteria forming biofilm. Biofilm formation allows the pathogen to adhere to the host surface under extreme condition and is resistant against a wide range of antibiotics. The choice of drug depends on the characteristics of the biofilm such as composition, age, solidity, and type of pathogens. These are the major components influencing the microbial susceptibility. As the bacterial biofilm matures, it enhances the accumulation of exopolymeric substance (EPS), attaches with the oxygen and nutrient gradients that effect bacterial growth rates and metabolism of cells, becomes impermeable, and reduces the activity of antimicrobial agents. This leads to resistance to most antibiotic regime. Therefore, novel potential therapeutic strategies should be considered to curb bacterial biofilm formation at specific stage without harming the pathogen. Antiadhesion and antimatrix agents are exciting strategies that may be used pending further investigation.

List of abbreviations

EPS	extracellular polymeric substance
DNS	deoxyribonuclease
CBP	carbohydrate-binding protein
DNA	deoxyribonucleic acid
CUP	chaperone-usher pathway
UPEC	uropathogenic *E. coli*
PGA	polyglucosamine
SCV	small colony variants
eDNA	environmental deoxyribonucleic acid
PQS	*Pseudomonas* quinolone signal
c-di-GMP	cyclic di-GMP
AL	alginate lyase
BS	*Bacillus subtilis*
TLR-4	toll-like receptor-4
IBC	intracellular bacterial communities
UTIs	urinary tract infections
SEM	scanning electron microscope
TCA	tricarboxylic acid
qPCR	quantitative polymerase chain reaction
HAIs	hospital-acquired infections
CFU	colony forming unit
MDR	multidrug resistant
LPS	lipopolysaccharides
ETC	electron transport chain
ROS	reactive oxygen species
EHEC	enterohemorrhagic *E. coli*
EDTA	ethylene-diamine-tetra-acetic acid
CF	cystic fibrosis

DOI: http://dx.doi.org/10.5772/intechopen.80341

Author details

Mansab Ali Saleemi[1], Navindra Kumari Palanisamy[2] and Eng Hwa Wong[3]*

1 School of Biosciences, Taylor's University Lakeside Campus, Subang Jaya, Selangor Malaysia

2 Department of Medical Microbiology and Parasitology, Faculty of Medicine, Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia

3 School of Medicine, Taylor's University Lakeside Campus, Subang Jaya, Selangor, Malaysia

*Address all correspondence to: enghwa.wong@taylors.edu.my

IntechOpen

References

[1] Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews. Microbiology. 2004;**2**:95-108

[2] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science. 1999;**284**:1318-1322

[3] O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annual Review of Microbiology. 2000;**54**:49-79

[4] Kolter R. Biofilms in lab and nature: A molecular geneticist's voyage to microbial ecology. International Microbiology. 2010;**13**:1-7

[5] Pratt LA, Kolter R. Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type I pili. Molecular Microbiology. 1998;**30**:285-293

[6] Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, et al. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the csgD gene. Journal of Bacteriology. 2001;**183**:7213-7223

[7] Parsek MR, Singh PK. Bacterial biofilms: An emerging link to disease pathogenesis. Annual Review of Microbiology. 2003;**57**:677-701

[8] Lenz AP, Williamson KS, Pitts B, Stewart PS, Franklin MJ. Localized gene expression in *Pseudomonas aeruginosa* biofilms. Applied and Environmental Microbiology. 2008;**74**:4463-4471

[9] Monds RD, O'Toole GA. The developmental model of microbial biofilms: Ten years of a paradigm up for review. Trends in Microbiology. 2009;**17**:73-87

[10] Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature. 2001;**413**:860-864

[11] Stanley NR, Britton RA, Grossman AD, Lazazzera BA. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. Journal of Bacteriology. 2003;**185**:1951-1957

[12] Schembri MA, Kjaergaard K, Klemm P. Global gene expression in *Escherichia coli* biofilms. Molecular Microbiology. 2003;**48**:253-267

[13] Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Hoiby N. Dynamics and spatial distribution of beta lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy. 2004;**48**:1168-1174

[14] Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, et al. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Molecular Microbiology. 2004;**51**:659-674

[15] Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, et al. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cellular Microbiology. 2004;**6**:269-275

[16] Zhang L, Mah TF. Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. Journal of Bacteriology. 2008;**190**:4447-4452

[17] Klebensberger J, Birkenmaier A, Geffers R, Kjelleberg S, Philipp B. SiaA and SiaD are essential for

inducing auto aggregation as a specific response to detergent stress in *Pseudomonas aeruginosa*. Environmental Microbiology. 2009;**11**:3073-3086

[18] Flemming HC, Wingender J. The biofilm matrix. Nature Reviews. Microbiology. 2010;**8**:623-633

[19] Tielker D, Hacker S, Loris R, Strathmann M, Wingender J, Wilhelm S, et al. *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiology. 2005;**151**:1313-1323

[20] Branda SS, Chu F, Kearns DB, Losick R, Kolter R. A major protein component of the *Bacillus subtilis* biofilm matrix. Molecular Microbiology. 2006;**59**:1229-1238

[21] Diggle SP, Stacey RE, Dodd C, Camara M, Williams P, Winzer K. The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*. Environmental Microbiology. 2006;**8**:1095-1104

[22] Conrad A, Suutari MK, Keinanen MM, Cadoret A, Faure P, Mansuy-Huault L, et al. Fatty acids of lipid fractions in extracellular polymeric substances of activated sludge flocs. Lipids. 2003;**38**:1093-1105

[23] Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. Journal of Bacteriology. 2004;**186**:7312-7326

[24] Gjermansen M, Ragas P, Sternberg C, Molin S, Tolker Nielsen T. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. Environmental Microbiology. 2005;**7**:894-906

[25] Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathogens. 2009;**5**:e1000354

[26] Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrobial Agents and Chemotherapy. 2003;**47**:317-323

[27] Jefferson KK, Goldmann DA, Pier GB. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrobial Agents and Chemotherapy. 2005;**49**:2467-2473

[28] Mai-Prochnow A, Lucas-Elio P, Egan S, Thomas T, Webb JS, Sanchez-Amat A, et al. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several gram-negative bacteria. Journal of Bacteriology. 2008;**190**:5493-5501

[29] Lewis K. Persister cells and the riddle of biofilm survival. Biochemistry (Mosc). 2005;**70**:267-274

[30] Domka J, Lee J, Bansal T, Wood TK. Temporal gene expression in *Escherichia coli* K-12 biofilms. Environmental Microbiology. 2007;**9**:332-346

[31] Lewis K. Multidrug tolerance of biofilms and persister cells. Current Topics in Microbiology and Immunology. 2008;**322**:107-131

[32] Cerca N, Jefferson KK, Oliveira R, Pier GB, Azeredo J. Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. Infection and Immunity. 2006;**74**:4849-4855

[33] Koch C, Hoiby N. Pathogenesis of cystic fibrosis. Lancet. 1993;**341**:1065-1069

[34] Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiological Reviews. 1996;**60**:539-574

[35] Kuramitsu HK, Wang BY. The whole is greater than the sum of its parts: Dental plaque bacterial interactions can affect the virulence properties of cariogenic *Streptococcus mutans*. American Journal of Dentistry. 2011;**24**:153-154

[36] Venditti M, Biavasco F, Varaldo PE, Macchiarelli A, De Biase L, Marino B, et al. Catheter-related endocarditis due to glycopeptide-resistant *Enterococcus faecalis* in a transplanted heart. Clinical Infectious Diseases. 1993;**17**:524-525

[37] Fey PD. Modality of bacterial growth presents unique targets: How do we treat biofilm-mediated infections? Current Opinion in Microbiology. 2010;**13**:610-615

[38] Foxman B. The epidemiology of urinary tract infection. Nature Reviews. Urology. 2010;**7**:653-660

[39] Donlan RM. Biofilms: Microbial life on surfaces. Emerging Infectious Diseases. 2002;**8**:881-890

[40] Beloin C, Roux A, Ghigo JM. *Escherichia coli* biofilms. Current Topics in Microbiology and Immunology. 2008;**322**:249-289

[41] Toutain CM, Caizza NC, Zegans ME, O'Toole GA. Roles for flagellar stators in Biofilm formation by *Pseudomonas aeruginosa*. Research in Microbiology. 2007;**158**:471-477

[42] Lemon KP, Higgins DE, Kolter R. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. Journal of Bacteriology. 2007;**189**:4418-4424

[43] Klausen M, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Molecular Microbiology. 2003a;**50**:61-68

[44] Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, et al. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular Microbiology. 2003b;**48**:1511-1524

[45] Watnick PI, Kolter R. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Molecular Microbiology. 1999;**34**:586-595

[46] Schmidt J, Musken M, Becker T, Magnowska Z, Bertinetti D, Moller S, et al. The *Pseudomonas aeruginosa* chemotaxis methyltransferase CheR1 impacts on bacterial surface sampling. PLoS One. 2011;**6**:e18184

[47] Hadjifrangiskou M, Kostakioti M, Chen SL, Henderson JP, Greene SE, Hultgren SJ. A central metabolic circuit controlled by QseC in pathogenic *Escherichia coli*. Molecular Microbiology. 2011;**80**:1516-1529

[48] Dunne WM Jr. Bacterial adhesion: Seen any good biofilms lately? Clinical Microbiology Reviews. 2002;**15**:155-166

[49] Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, et al. Induction and evasion of host defenses by type-1-piliated uropathogenic *Escherichia coli*. Science. 1998;**282**:1494-1497

[50] Hung CS, Bouckaert J, Hung D, Pinkner J, Widberg C, DeFusco A, et al. Structural basis of tropism of *Escherichia coli* to the bladder during urinary tract infection. Molecular Microbiology. 2002;**44**:903-915

[51] Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ. Type 1 pilus-Mediated bacterial invasion of bladder epithelial cells. The EMBO Journal. 2000;**19**:2803-2812

[52] Nilsson LM, Yakovenko O, Tchesnokova V, Thomas WE, Schembri MA, Vogel V, et al. The cysteine bond in the *Escherichia coli* FimH adhesin is critical for adhesion under flow conditions. Molecular Microbiology. 2007;**65**:1158-1169

[53] Garofalo CK, Hooton TM, Martin SM, Stamm WE, Palermo JJ, Gordon JI, et al. *Escherichia coli* from urine of female patients with urinary tract infections is competent for intracellular bacterial community formation. Infection and Immunity. 2007;**75**:52-60

[54] Wright KJ, Seed PC, Hultgren SJ. Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. Cellular Microbiology. 2007;**9**:2230-2241

[55] Chen SL, Hung CS, Xu J, Reigstad CS, Magrini V, Sabo A, et al. Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli:* A comparative genomics approach. Proceedings of the National Academy of Sciences. 2006;**103**:5977-5982

[56] Chen SL, Hung CS, Pinkner JS, Walker JN, Cusumano CK, Li Z, et al. Positive selection identifies an in vivo role for FimH during urinary tract infection in addition to mannose binding. Proceedings of the National Academy of Sciences. 2009;**106**:22439-22444

[57] Hasman H, Chakraborty T, Klemm P. Antigen-43 mediated auto aggregation of *Escherichia coli* is blocked by fimbriation. Journal of Bacteriology. 1999;**181**:4834-4841

[58] Uhlich GA, Cooke PH, Solomon EB. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. Applied and Environmental Microbiology. 2006;**72**:2564-2572

[59] Mohamed JA, Teng F, Nallapareddy SR, Murray BE. Pleiotrophic effects of 2 *Enterococcus faecalis* sagA-like genes, salA and salB, which encode proteins that are antigenic during human infection, on biofilm formation and binding to collagen type I and fibronectin. The Journal of Infectious Diseases. 2006;**193**:231-240

[60] Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, et al. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Applied and Environmental Microbiology. 2001;**67**:4538-4545

[61] Kemp KD, Singh KV, Nallapareddy SR, Murray BE. Relative contributions of *Enterococcus faecalis* OG1RF sortase-encoding genes, srtA and bps (srtC), to biofilm formation and a murine model of urinary tract infection. Infection and Immunity. 2007;**75**:5399-5404

[62] Kai-Larsen Y, Luthje P, Chromek M, Peters V, Wang X, Holm A, et al. Uropathogenic *Escherichia coli* modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37. PLoS Pathogens. 2010;**6**:e1001010

[63] Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, et al. Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nature Chemical Biology. 2009;**5**:913-919

[64] Wang X, Preston JF 3rd, Romeo T. The pgaABCD locus of *Escherichia coli* promotes the synthesis of a

polysaccharide adhesin required for biofilm formation. Journal of Bacteriology. 2004;**186**:2724-2734

[65] Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. FEMS Immunology and Medical Microbiology. 2010;**59**:253-268

[66] Wittschier N, Lengsfeld C, Vorthems S, Stratmann U, Ernst JF, Verspohl EJ, et al. Large molecules as anti-adhesive compounds against pathogens. The Journal of Pharmacy and Pharmacology. 2007;**59**:777-786. [PubMed]

[67] Starkey M, Hickman JH, Ma L, Zhang N, DeLong S, Hinz A, et al. *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. Journal of Bacteriology. 2009;**191**:3492-3503

[68] Yang L, Barken KB, Skindersoe ME, Christensen AB, Givskov M, Tolker-Nielsen T. Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. Microbiology. 2007;**153**:1318-1328

[69] Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Molecular Microbiology. 2006;**59**:1114-1128

[70] Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, et al. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environmental Microbiology. 2008;**10**:2331-2343

[71] Thomas VC, Thurlow LR, Boyle D, Hancock LE. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. Journal of Bacteriology. 2008;**190**:5690-5698

[72] Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Molecular Microbiology. 2009;**72**:1022-1036

[73] Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of autolysin and Sortase A during *Enterococcus faecalis* DNA-dependent biofilm development. Infection and Immunity. 2009;**77**:3626-3638

[74] Hong SH, Lee J, Wood TK. Engineering global regulator Hha of *Escherichia coli* to control biofilm dispersal. Microbial Biotechnology. 2010;**3**:717-728

[75] Rowe MC, Withers HL, Swift S. Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperse upon the supply of iron. FEMS Microbiology Letters. 2010;**307**:102-109

[76] Karatan E, Watnick P. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiology and Molecular Biology Reviews. 2009;**73**:310-347

[77] Kaplan JB. Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. Journal of Dental Research. 2010;**89**:205-218

[78] Wood TK, Hong SH, Ma Q. Engineering biofilm formation and dispersal. Trends in Biotechnology. 2010;**29**:87-94

[79] Boyd A, Chakrabarty AM. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Applied

and Environmental Microbiology. 1994;**60**:2355-2359

[80] Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T. CsrA post-transcriptionally represses pgaABCD, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. Molecular Microbiology. 2005;**56**:1648-1663

[81] Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, et al. Cell death in *Pseudomonas aeruginosa* biofilm development. Journal of Bacteriology. 2003;**185**:4585-4592

[82] Lam H, Oh DC, Cava F, Takacs CN, Clardy J, de Pedro MA, et al. D-amino acids govern stationary phase cell wall remodeling in bacteria. Science. 2009;**325**:1552-1555

[83] Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. Science. 2010;**328**:627-629

[84] Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, et al. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell. 2012;**149**:684-692

[85] Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, et al. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proceedings of the National Academy of Sciences. 2004;**101**:1333-1338

[86] Eto DS, Jones TA, Sundsbak JL, Mulvey MA. Integrin mediated host cell invasion by type 1-piliated uropathogenic *Escherichia coli*. PLoS Pathogens. 2007;**3**:e100

[87] Bishop BL, Duncan MJ, Song J, Li G, Zaas D, Abraham SN. Cyclic AMP-regulated exocytosis of *Escherichia coli* from infected bladder epithelial cells. Nature Medicine. 2007;**13**:625-630

[88] Schwartz DJ, Chen SL, Hultgren SJ, Seed PC. Population dynamics and niche distribution of uropathogenic *Escherichia coli* during acute and chronic urinary tract infection. Infection and Immunity. 2011;**79**:4250-4259

[89] Anderson GG, Martin SM, Hultgren SJ. Host subversion by formation of intracellular bacterial communities in the urinary tract. Microbes and Infection. 2004;**6**:1094-1101

[90] Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Medicine. 2007;**4**:e329

[91] Reigstad CS, Hultgren SJ, Gordon JI. Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. The Journal of Biological Chemistry. 2007;**282**:21259-21267

[92] Henderson JP, Crowley JR, Pinkner JS, Walker JN, Tsukayama P, Stamm WE, et al. Quantitative metabolomics reveals an epigenetic blueprint for iron acquisition in uropathogenic *Escherichia coli*. PLoS Pathogens. 2009;**5**:e1000305

[93] Hansen DS, Gottschau A, Kolmos HJ. Epidemiology of *Klebsiella* bacteremia: A case control study using *Escherichia coli* bacteremia as control. The Journal of Hospital Infection. 1998;**38**:119-132

[94] Garcia-Medina R, Dunne WM, Singh PK, Brody SL. *Pseudomonas aeruginosa* acquires biofilm-like properties within airway epithelial cells. Infection and Immunity. 2005;**73**:8298-8305

[95] Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al.

Vancomycin-resistant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. The Journal of Clinical Investigation. 2010;**120**:4332-4341

[96] Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents. 2010;**35**:322-332

[97] Zimmerli W, Lew PD, Waldvogel FA. Pathogenesis of foreign body infection. Evidence for a local granulocyte defect. The Journal of Clinical Investigation. 1984;**73**(4):1191-1200

[98] Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends in Microbiology. 2009;**17**:66-72

[99] Sutherland IW, Hughes KA, Skillman LC, Tait K. The interaction of phage and biofilms. FEMS Microbiology Letters. 2004;**232**:1-6

[100] Burrowes B, Harper DR, Anderson J, McConville M, Enright MC. Bacteriophage therapy: Potential uses in the control of antibiotic-resistant pathogens. Expert Review of Anti-Infective Therapy. 2011;**9**:775-785

[101] Yang D, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: More than just microbicidal. Trends in Immunology. 2002;**23**:291-296

[102] Pompilio A, Scocchi M, Pomponio S, Guida F, Di Primio A, Fiscarelli E, et al. Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients. Peptides. 2011;**32**:1807-1814

[103] Kharidia R, Liang JF. The activity of a small lytic peptide PTP-7 on Staphylococcus aureus biofilms. Journal of Microbiology. 2011;**49**:663-668

[104] Chen X, Schluesener HJ. Nanosilver: A nanoproduct in medical application. Toxicology Letters. 2008;**176**:1-12

[105] Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomedicine. 2007;**3**:95-101

[106] Feng QL, Wu J, Chen GQ , Cui FZ, Kim TN, Kim JO. A mechanistic study of the anti-bacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. Journal of Biomedical Materials Research. 2000;**52**:662-668

[107] Secinti KD, Ozalp H, Attar A, Sargon MF. Nanoparticle silver ion coatings inhibit biofilm formation on titanium implants. Journal of Clinical Neuroscience. 2011;**18**:391-395

[108] Rendueles O, Kaplan JB, Ghigo JM. Antibiofilm polysaccharides. Environmental Microbiology. 2012;**15**(2):334-346. DOI: 10.1111/j.1462 2920.2012.02810.x.

[109] Pihl M, Davies JR, Chavez de Paz LE, Svensater G. Differential effects of Pseudomonas aeruginosa on biofilm formation by different strains of Staphylococcus epidermidis. FEMS Immunology and Medical Microbiology. 2010;**59**:439-446

[110] Kim Y, Oh S, Kim SH. Released exopolysaccharide (r-EPS) produced from probiotic bacteria reduce biofilm formation of enterohemorrhagic Escherichia coli O157:H7. Biochemical and Biophysical Research Communications. 2009;**379**:324-329. [PubMed]

[111] Zinger-Yosovich KD, Gilboa-Garber N. Blocking of *Pseudomonas aeruginosa* and *Ralstonia solanacearum* lectins by plant and microbial branched polysaccharides used as food additives. Journal of Agricultural and Food Chemistry. 2009;**57**:6908-6913. [PubMed]

[112] Wang X, Wang Q , Yang M, Xiao J, Liu Q , Wu H, et al. QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen Edwardsiella tarda. Fish & Shellfish Immunology. 2011;**30**:944-953

[113] Prüß BM. Involvement of Two-Component Signaling on Bacterial Motility and Biofilm Development. Journal of Bacteriology. 2017;**199**(18):e00259-e00217

[114] Raad II, Fang X, Keutgen XM, Jiang Y, Sherertz R, Hachem R. The role of chelators in preventing biofilm formation and catheter-related bloodstream infections. Current Opinion in Infectious Diseases. 2008;**21**:385-392

[115] Shanks RM, Sargent JL, Martinez RM, Graber ML, O'Toole GA. Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. Nephrology, Dialysis, Transplantation. 2006;**21**:2247-2255

[116] Bookstaver PB, Williamson JC, Tucker BK, Raad II, Sherertz RJ. Activity of novel antibiotic lock solutions in a model against isolates of catheter-related bloodstream infections. The Annals of Pharmacotherapy. 2009;**43**:210-219

[117] Chatzinikolaou I, Zipf TF, Hanna H, Umphrey J, Roberts WM, Sherertz R, et al. Minocycline-ethylene-diaminetetraacetate lock solution for the prevention of implantable port infections in children with cancer. Clinical Infectious Diseases. 2003;**36**:116-119

[118] Bleyer AJ, Mason L, Russell G, Raad II, Sherertz RJ. A randomized, controlled trial of a new vascular catheter flush solution (minocycline-EDTA) in temporary hemodialysis access. Infection Control and Hospital Epidemiology. 2005;**26**:520-524

[119] Fleming D, Rumbaugh KP. Approaches to dispersing medical biofilm. Microorganisms. 2017;**5**:15. DOI: 10.3390/microorganisms5020015

[120] Lu TK, Collins JJ. Dispersing biofilms with engineered enzymatic bacteriophage. Proceedings of the National Academy of Sciences. 2007;**104**:11197-11202

Chapter 4

Origin and Control Strategies of Biofilms in the Cultural Heritage

Laura E. Castrillón Rivera, Alejandro Palma Ramos, Jorge I. Castañeda Sánchez and María Elisa Drago Serrano

Abstract

Biodeterioration is defined as the undesirable change in the properties of materials caused by the activity of biological agents. This process is complex and involves alterations in the physicochemical and mechanical properties by the action of organisms and depends on the microorganisms involved, type of substrate, and environmental conditions. The biodeterioration of cultural heritage is the physical or chemical damage caused by microorganisms on objects, monuments, or buildings that belong to the cultural heritage. Among the main materials that can be affected are: stone, metal, ceramic, polymers, and other materials. Among the main undesirable effects to these materials are: discoloration, dissolution, rupture, and efflorescence among others. Biofilms represent the usual form of growth of bacteria and consist of communities of microorganisms that grow attached to an inert surface or a living tissue, surrounded by an extracellular matrix that they themselves synthesize. The importance of biodeterioration by biofilms is mainly related to changes in pH values, ionic concentrations, oxide-reduction reactions in the biofilm thickness, and in the interface with the substrate and enzymatic degradation. This chapter presents evidence of the participation of biofilms and associated mechanisms in biodeterioration as well as the main prevention and control strategies.

Keywords: biofilms, cultural heritage, biodeterioration, art biodeterioration

1. Cultural heritage

The concept of cultural heritage refers to the cultural inheritance that corresponds to a given community and as such is protected and communicated to both the present and the future generations. This concept is subjective and dynamic, and it does not depend on the objects or goods, but on the values that society in general attributes to them at each moment of history and that determine which goods are those that must be protected and preserved for posterity. Therefore, the identification, protection, conservation, and dissemination of the world's cultural heritage are one of the most recognized tasks of the United Nations Organization for Education, Science and Culture [1–3].

Cultural heritage is made up of tangible or intangible assets that history has left to a country and those citizens in the present grant it a special and relevant historical, scientific, symbolic or esthetic importance. This inheritance left by our ancestors and received today is the clear testimony of their existence and vision of the world [4].

The tangible heritage is also called material heritage, and there are movable and immovable property such as the objects of the artistic collections such as the collection of religious, ethnographic, technological, historical, artistic, and archeological and artisan objects. The real tangible heritage is made up of archeological sites, engineering works, places, buildings, and architectural ensembles.

The intangible cultural heritage includes the wealth of knowledge, also living expressions inherited from our ancestors and transmitted to our descendants, such as language, oral traditions, customs, performing arts, ways of life, rituals, festive events, knowledge, and practices related to nature and the universe, as well as knowledge and techniques linked to traditional crafts [5].

Cultural heritage is a nonrenewable resource with regard to its past, and that is why it manifests itself tangibly as an untouchable and irreplaceable resource of a people. This heritage is always linked to the human collective, since it is men and women who produce it, and therefore it is what gives identity, origin, and continuity to our people. Hence, it is the responsibility of all its conservation and restoration for which it is essential to know what are its main threats in order to prevent, delay its deterioration and, if necessary, restore this heritage.

The alteration of cultural assets is the characteristic of the continuous cycle of disintegration and reconstruction, and it is a natural condition since all matter follows a process of alteration, degradation, or decomposition which means that original physical, chemical, and optical qualities are lost and enter a process of instability promoted by factors or agents of deterioration that are of two types:

Intrinsic: it depends on the nature of the material, manufacturing technique, and procedures that were used to perform the work.

Extrinsic: It depends on the sources external to the object such as environmental factors (light, relative humidity, temperature, and air pollutants), anthropogenic factors (handling, use, consultation, vandalism, tourism, etc.), biological factors (microorganisms, plants, rodents, and insects), and catastrophic factors (floods, fires, etc.) [6–8].

Among the main mechanisms of deterioration, three processes are known:

Physical or mechanical processes where the behavior of the material is modified, where several mechanical forces participate (compression, traction, etc.). These change the behavior of a material without modifying its chemical composition.

Chemical processes: are those that compromise a chemical reaction that transforms the matter.

Biological processes: where living organisms, such as microorganisms, insects, rodents, plants, etc., can chemically attack the material or its mechanical resistance. This process is also known as biodeterioration, which has been defined as "undesirable changes in the properties of materials caused by the vital activity of organisms" [9].

2. Biofilms as biodeteriogens

It is expected that works of art last for a long period of time; however, these suffer deterioration, and previously it was believed that chemical and physical processes were the dominant factors in the degradation of materials. Since 1967 and in latter decades, dogma has changed, and today it is assumed that microorganisms only by their very presence can cause damage by esthetic destruction of the materials since they inhabit them and penetrate causing their loss due to their acid corrosion, enzymatic degradation, and mechanical attack [10]. These microorganisms can grow in nature in large, silty colonies known as biofilms where relationships and dependencies are established [11–13].

Biofilms are microbial monospecies or multispecies (consortium) communities that have demonstrated the most successful colonization among microorganisms are ubiquitous in nature and responsible for many diseases. They are considered growing communities of microorganisms embedded in a self-produced exopolysaccharides matrix and are attached to an inert surface or living tissue [14–16]. The microorganisms in biofilms have properties that are not shared by free organisms, and the requirements for the formation of biofilms are simple: surface, moisture, nutrients, and microorganisms. This complex microbial organization that can consist mainly of bacteria and fungi, offers several advantages for its survival, such as resistance to environmental stress through the formation of stable microcolonies, facilitates the exchange of genetic material, and there is accumulation of nutrients and water in its matrix that offers protection against toxic substances (biocides and antibiotics) and against desiccation as well as immune defenses in the case of the formation of biofilms in higher organisms [17].

The importance of biofilms in the biodeterioration of cultural heritage has been reported for several decades and is related to: (a) modifications in pH values and ionic concentrations, (b) reduction oxide conditions in the interface of biofilms and substrate, (c) covering the surface and masking its properties, (d) increasing the leaching of additives and monomers outside the polymer matrix by microbial degradation, (e) releasing enzymes that lead to embrittlement and loss of mechanical stability, (f) accumulating water that penetrates the matrix causing swelling and increased conductivity, and (g) excretion of lipophilic pigments among others [18, 19].

3. Biodeterioration of stone

The mineralogical nature of the stone, its surface properties, and environmental conditions act synergistically for its bioreceptivity (ability to be colonized by microorganisms), and its intensity will depend on the concentration of pollutants, microclimatic conditions, and anthropogenic eutrophication of the atmosphere [20].

The climatic conditions in which the monuments or architectural structures are exposed can be the wind that wears the rock eroding it, the solar radiation causing discoloration, the temperature, as well as the rain, snow and humidity that induce the process of physical and chemical wear. These factors affect the stability of the matrix of the stone or act through chemical corrosion forming minerals by oxidation and hydration reactions as well as by the dissolution of carbonates and solubilization of some elements of minerals with silicates [21, 22].

The microbial communities after the interaction with biotic and abiotic factors are developed using the stone as a substrate and are partially responsible for the chemical and physical deterioration of the same and alter the esthetic appearance and physical integrity of the material through different mechanisms (**Figure 1**). The effects of microbial activities on historic buildings may be: discoloration, water retention, growth stimulation of heterotrophic organisms and higher organisms, material breakage, disintegration of the material, formation of patinas, degradation (corrosion), wear and dissolution of the structure, alkaline dissolution, and alteration of stratified silicates [23].

The microorganisms that colonize the stone monuments can be distinguished according to their location in the stone. The so-called epilithic that are located on the surface of the rock and those that live inside the rock within fractures and cracks and pores in granites are known as endolithic [12]. The main microorganisms that play a potential role in biodeterioration are autotrophic and heterotrophic bacteria, fungi, algae, and lichens. Phototrophic microorganisms such as microalgae, cyanobacteria, and lichens are considered the pioneer colonizers of the surface of stone monuments.

Figure 1.
Biodeterioration on stone (photo: Laura Castillón).

Cyanobacteria and algae such as chlorophytes, chrysophytes, and diatoms are a morphologically diverse and widely distributed group endowed with remarkable adaptability to variable environmental conditions and effective protection mechanisms against various abiotic stresses that enable them to colonize almost all classes of extreme lithic habitats [24]. These microorganisms form pigmented scabs (patinas) and incrustations that affect the substrate esthetically and cause physical and chemical deterioration of the rock. The epilithic cyanobacteria play an important role in the dissolution of the limestone carbonate, being able to cause the detachment of parts of it, due to a decrease in the coherence of the crystals around the colonies [25, 26].

The external stones are an appropriate niche for the growth and development of pioneering microorganisms that include photoautotrophs, lithophiles, and chemolithotrophs. The colonization begins with cyanobacteria and algae, probably followed by lichens that synthesize extracellular organic matter, in addition, dead cells release their constituents that form sources of nutrients for the growth of heterotrophic microorganisms which are considered secondary colonizers [27]. The phototrophic metabolism of cyanobacteria and algae facilitates their growth in oligotrophic environments such as stone forming biofilms on rocky surfaces, and it is the characteristics of the substrate that determine the speed of their growth and therefore the intensity of biodeterioration [28]. Lichens are highly resistant to extreme temperatures and desiccation that allow their easy growth on the surface of the stone. The microbial populations present in the rocky substrate are the result of successive colonization by different heterotrophic microorganisms.

It is well known that stone surfaces are exposed to high levels of solar radiation, high temperature, and to prolonged periods of desiccation alternating with rainy and damp periods. Many cyanobacteria are known to tolerate environmental extremes like UV light and their resistance to desiccation and tolerance for high level of light intensities and UV radiation provide them a distinct advantage for their survival on exposed surfaces, and they synthesize UV sunscreen pigments including scytonemin, mycosporine like aminoacids and biopterin glucosides. There are several reports on the effect of UV radiation on nitrogenase activity as it relates to the role of cyanobacteria in the nitrogen economy of ecosystem [29].

Pigmentation as a mechanism of deterioration depends on the nature of the substrate, the presence of trace elements such as iron, zinc, etc., the acidity or basicity of the medium, and even environmental conditions. The microorganisms produce two types of pigments (a) endopigments: they are located inside the cell and only leave after the lysis of the same as photosynthetic pigments, such as chlorophyll and

phycobilins and (b) exopigments emitted outside the cell as fungal pigments (black, violet, blue, green, and purple). The black pigment known as melanin protects fungi against environmental threats and cellular lysis. Moreover, mycosporines and carotenoids (ß-carotene, s-carotene, phytoene, torulene, and torularhodin) may protect fungi against excessive UV radiation, act as antioxidants, osmoprotectants, and provide desiccation tolerance [30, 31].

The wear of materials is accelerated by the presence of biofilms containing active and latent microorganisms and their metabolic products, such as corrosive organic and inorganic acids as well as polymeric materials. Polymers, usually polysaccharides, act as gums that trap dust and other particulate materials increasing the disfiguring effects of the biofilm [32].

Beyond the type of microorganisms, the formation of the biofilm is a biodeterioration factor. The exopolysaccharide matrix plays a crucial role in this phenomenon since it produces mechanical stress on the stone through the pores of the mineral structure because it modifies the circulation of water within the material, its sensitivity to temperature variations, and the cycles of swelling and contraction dependent on the concentration of water within the matrix [27, 33].

Biofilms are also associated with the degradation of buildings and mural paintings by a phenomenon known as salt efflorescence, involves secondary minerals produced through the reaction of anions from excreted acids with cations from the stone which is available in the wall by the biological process or simply due to comigration with the infiltrated water. The solubilization of the calcareous material is detected by the presence of hygroscopic salts including carbonates, chlorides, nitrates, sulfates, etc., can be found on the surfaces of decayed monuments caused by chemical reactions (chemical agents in the air) or by enzymatic reactions of certain microorganisms. The most frequently isolated genus was *Bacillus*, followed *Staphylococcus*, *Kocuria*, *Micrococcus*, *Paenibacillus,* and *Arthrobacter* (bacteria of the sulfur and nitrogen cycle) [34].

The precipitation of salts is due to changes in temperature or humidity, and the salts can precipitate on the exposed surfaces and cause a destructive effect. Some salts when hydrated occupy a large space causing additional pressure that eventually leads to the loss of material and destruction due to cracking and detachment of walls or calcareous structures. In studies conducted by electron microscopy of salt efflorescence zones on walls, biofilms have been reported by members of *Firmicutes, Actinobacteria,* and *Ascomycota* [13, 35, 36].

From the nutritional point of view, organisms show a wide range of metabolic modalities where they use different sources of carbon (organic or inorganic compounds) as well as light as an energy source, and they are classified as photoautotrophic, chemoautotrophs, chemoheterotrophs, chemoorganotrophs, and photosynthetic.

The main mechanisms of biodeterioration associated with the different types of stone-colonizing organisms according to their nutrition are presented in **Table 1** [20].

Microbial colonization on bare stone surface is thought to be initiated by pioneering microorganisms which includes photoautotrophs, lithophiles, and chemolithotrophs. These organisms may secrete carbohydrates and growth factors which help in the formation of biofilm (a three-dimensional structure regulating temperature and humidity) and support the growth of successive microbial communities that is predominated by heterotrophic bacteria and fungi [20].

Limestones are carbonate rocks composed of calcite, and their main uses are in construction, chemical products, smelting, agrochemicals, and glass. This material is highly porous and hydrophilic in nature, and it is highly susceptible to water (such as acid rain) and environmental contaminants. Water often penetrates the pores of the stones causing damage by corrosive ions such as chlorine and acids. Biofilms,

Mechanisms of Biodeterioration	
Organism	**Mechanism**
Cyanobacteria	Formation of biofilms, coloured patinas and crusts, bio-waste as a result of calcium uptake, calcium salt precipitation and mineral formation
Lichens	Extraction of nutrients from the surface of the stone, formation of oxalate, production of carbonic acid associated with biodeterioration, physical intrusions in small pores.
Algae	Biofilm formation, color alteration, black scabs
Mosses and liverworts	Discoloration, green-gray patches, extraction of minerals from the stone
Sulfur-oxidizing and nitrifying bacteria	Formation of black crusts, secreted acids Salt efflorescence
Heterotrophic bacteria	Scab formation, patina, exfoliation, color alteration Bio-waste by secreted acids
Actinomycetes	White powder - grayish, patinas, white salt efflorescence Biofilm formation
Fungus	Fungal diagenesis, color alteration, oxalate formation, bio-waste by secreted acids, chelating properties by secreted acids, physical intrusion of hyphal penetration, biofilm formation and destabilization of the texture of the stone
Sulfur reducing bacteria	Conversion of sulphate into sulfite that acts as a nutrient for sulfur-oxidizing bacteria, salt efflorescence
Superior plants	Intrusion of roots within the cracks and pores, collapse and detach the structure of the stone.

Table 1.
Mechanisms of biodeterioration.

industrial and persistent pollutants, particulate matter, ash and often smog are deposited on the stone, and as a result, its deterioration is accelerated [23, 37].

The wear of the rocks and monuments can also be a consequence of the removal and solubilization of cations present in the minerals of the stone in particular iron and manganese of the mineral network by the negatively charged exopolysaccharide (EPS) of the biofilms or by some microbial proteins called siderophores by organic transport complexes and metallic organic chelates. Under low iron stress, siderophores chelate iron and supply to bacterial and fungi cells by outer membrane receptors, and the role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cells [38, 39].

The ability to grow by the dissimilatory oxidation of inorganic electron donors (ferrous iron, hydrogen, sulfur, and reduced inorganic sulfur anions) is widespread among acidophilic prokaryotes. Both oxygen and ferric iron can act as electron acceptors from many species of chemolithotrophic acidophiles, enabling them to exploit anoxic as well as aerobic environments [40].

In aerobic conditions, electron donors may include ferrous ions or sulfur compounds which are oxidized into ferric iron and sulfuric acid, respectively, yielding high energy. However, during anaerobic conditions, ferric ions can replace oxygen as the electron acceptor with multiple substrates donating an electron. This pathway yields less energy than aerobic conditions, but energy can still be produced for

growth. *A. ferrooxidans* is a chemolithoautotrophic bacterium which can use many different electron donors to support growth *Leptospirillum* spp. have been shown to use only ferrous iron as electron donor and are therefore (as a result of thermodynamic constraints) obligate aerobes.

The subsequent redox process is favored by the release of oxygen by photosynthetic bacteria, cyanobacteria as *Acidithiobacillus ferrooxidans, Bacillus* spp., *Leptospirillum* ssp., and chemoorganotrophic fungi such *Aureobasidium* spp. [24, 41, 42].

Mechanical damage to stone structures, monuments, and architecture is another type of biodeterioration mechanism which is due to the physical intrusion and penetration of bacteria, fungal hyphae in the gaps, pores, and fractures that destabilize the texture of the stone, causing mechanical deterioration or by the contraction, and expansion of the stem under fluctuations in humidity conditions. Also the mosses through the rhizoids can penetrate the rock causing holes (pitting) and the vascular plants through their mechanical deterioration through the growth of roots or chemically by the acidity and diverse exudates, alteration of the microclimatic parameters, increase of the risk of fires, and physical and visual obstruction [26, 43].

4. Biodeterioration of paintings

The chemistry of the manufacture of paintings and their function has now been transformed from art to science. The knowledge of the pictorial components allows to associate the type of microorganisms that can potentially colonize the paintings, and the different techniques used determines the final composition of the work.

The pictorial technique has multiple modalities: tempera, encaustic, fresco, mural, and oil among others. The first three techniques were used in the ancient Greco-Roman and Egyptian world. (a) Tempering consists basically of the mixture of earths or pigments with binder constituted by glue and water or by egg yolk and oils in any type of support, (b) encaustic or wax painting requires a previous preparation of the wall by means of wax, on which colors are applied to the tempera cast in wax using a hot palette, and (c) the fresco that consists essentially of painting on a surface of wet plaster, on the same wall, prepared by the artist himself [44]. This latter is the modality of frequent use and is performed on wet lime plaster that serves as a support for the various pigments dissolved in water which are chemically integrated into the wall, and so its durability is very high, and (d) oil is the best known pictorial technique and used on cloth or board. It consists of a mixture of colored pigments with oil, usually flax or walnut. Another technique related to painting on canvas is acrylic, which consists of a combination of acrylate molecules in emulsion with water [45].

The deterioration of a painting can have different origins such as: (a) alterations due to natural aging of the work that makes it more fragile, (b) defects inherent to the work such as low quality materials or bad techniques at the moment of being painted, and (c) influence of external conditions such as thermo-hygrometric conditions or other factors. The manifestations of the damage can be physical as lack of adhesion of the binder, or damage caused by the movements of the other, mechanical damage, etc. Chemical damage is manifested as a processes of gradual degradation and depolymerization or crosslinking of the materials of the work, damage caused by light, oxidation or biological attack [46–49].

The biodeteriorable character of the canvas is conditioned by the characteristics of the fabrics that are its support formed by cellulose fibers which is a polysaccharide whose constituent unit is D-glucose linked by glycosidic bonds β (1–4) forming

linear chains, which in turn are links in parallel fibers called microfibrils. For cellulose, degradation involved different enzymes whose combined action allows obtaining glucose molecules that can be assimilated by microorganisms as a carbon source. The degree of polymerization and its orientation, the length of these chains, their crystallinity, and their orientation are detected by microorganisms and could be susceptible to biodeterioration. The susceptibility to biological attack depends on the percentage content of cellulose, lignin, and other organic components. The purest cellulose can hardly be attacked. *Alternaria, Aspergillus, Fusarium*, *Memnoniella*, *Myrothecium, Neurospora, Penicillium, Scopulariopsis, Stachybotrys, Stemphylium,* and *Chaetomium* are the main fungi associated to this process and as cellulolytic bacteria: *Cellvibrio, Sporocytophaga, Myxococcoides, Cellufalcicula,* and also *Clostridium* sp. as anaerobic bacteria has been reported [50]. This cellulolytic process is favored in conditions of relative humidity of high air or condensation water where the fiber of the fabric loses consistency and elasticity becoming brittle and falls apart.

The filmogenic substances act as a binder and as a vehicle for the pigments; they are mainly of organic origin; they are applied in liquid form, and with drying, they solidify forming a hard and flexible layer, with the passage of time and under certain adverse conditions, this layer loses its property of cohesion of pigments and causes dusty surfaces or the separation of the layers.

The most important component of the paintings on canvas is the pigments; they are either natural or synthetic origin, and have three main functions: they provide color, opacity, and brilliance, and protect the surface in which they are applied and protect the binder from its destruction by UV radiation.

The adhesives are a fundamental element for the final result of the work whose function is to facilitate a uniform distribution of color and prevent the paint layer from being absorbed by the fabric, and these adhesives have been changing in the course of history and have been classified depending on their origin in animal (gelatin, albumin, casein, and wax) and vegetal (starch, resins, gums, and gluten).

Gelatin is obtained from collagen which is an existing protein in the skin and cartilage, albumin (protein of egg or blood plasma), casein (protein of milk), and wax (secreted by bees composed of a mixtures of esters, hydrocarbons, and fatty acids). The starch is a polysaccharide of vegetable origin, which is formed predominantly of amylose and amylopectin. Vegetal resins are a mixture of organic compounds principally terpenes and derivatives. Gums consisting of mixtures of water soluble polysaccharides produced by exudation, usually from the stem of tree and gluten, refer to the proteins in cereal grains found in the endosperm plant embryos during germination (Coppen, 1995).

The organic composition of all these adhesives favors the growth of microorganisms due to their high nutritional content, and therefore they are easily attacked by them [30].

The use of varnishes is required to provide protection against environmental attacks. For them, natural or synthetic varnishes (resins) are used, which must be applied in such a way that they form a resistant, colorless, and transparent film. According to its chemical composition, the name of the natural resins (soft) depends on the number of isoprene units that contain such terpene molecules as monoterpenes, sesquiterpenes, diterpenes, and triterpenes. The deterioration of natural resins causes chemical changes such as polymerizations (crosslinking of polymer chains, hydrolysis of polymer chains, and oxidation of the main chain or side groups which causes the resin to become more insoluble, losing its resistance and changes in its coloring [51]. Synthetic (acrylic) resins have good adhesive properties and are currently widely used in preservation treatments, and the monomers of these resins are generated by the esterification of an acrylic acid with several alcohols. Its general formula is: $CH_2CR1COOR2$ and due to its hydrophobic nature are more resistant to microbial attack because they are not used as a source of nutrients [52, 53].

Among the forms of deterioration of pictorial heritage on canvas related to biodeterioration agents are [54]:

Alterations of the canvas: hydrolysis, colorations, loss of strength, loss of support, cracks, scales, and deformations.

Alterations of the binder/adhesive: enzymatic degradation, colorations, disintegration, and pulverulence.

Alterations of the varnish: yellowing, tiling, whitening, and peeling.

The biological origin of deterioration in paintings has been widely reported in wall and easel paintings since the 1980s in different parts of the world [49, 54], and in general, the main fungal species associated with the biodeterioration of painted walls are *Penicillium* sps.*, A. niger, Rhizopus oryzae, Mucor, Trichophyton, Alternaria alternate,* and *Epidermophyton floccosum* [55, 56] and as biofilms *Acremonium*, *Cladosporium, Aspergillus, and Fusarium* [57]. In the case of canvases of oil paints, the bacterial strains of the phylum firmicutes such as *Bacillus* sp.*, Micrococcus luteus,* and *Paenisporosarcina* sp. and nonculturable bacteria of the phylum *Proteobacteria such as Stenotrophomonas* sp. [58] as well as *Halobacillus* sp.*, Halobacillus naozhouensis*, and *Nesterenkonia* sp. in wall paintings responsible for pigmentation by pink biofilms in Romanian monasteries [59].

The mere presence of microorganisms (colonization) in any type of surface does not determine their participation in the biodeterioration process, to be able to specify it, experimental strategies have been developed in the laboratory where traditional supports such as linen cloth prepared with layers enriched in gums of animal origin and linseed oil that are inoculated with suspensions of fungi and bacteria, later identifying the species that grow and deteriorate these materials, thus checking the postulates of Koch [60]. Another report associated with this proposal was made in the murals of the medieval church with the bacterium Arthrobacter responsible for the black spots as a result of the reaction of lead oxide of pigments and hydrogen sulfide produced by other bacteria responsible for spotting [61]. These models will allow to establish, under controlled conditions, which species colonize a given substrate and how the flora will change the substrate and how the substrate is modified by microbial colonization and how these modifications lead to the establishment of different microbial communities (microbial succession) [62].

There are several reports of the participation of biofilms as responsible for biodeterioration in the pictorial cultural heritage as in the case of the works of the Nerja and Treasure in Málaga [63], the church of St. Martins in Greene-Kreiensen, Germany [64], and the Mogao caves in Dunhuang, in Gansu Province of the People's Republic of China [65] among others. As an example of these works, mucilages have been analyzed in fragments detached from frescoes of the Santissima Annunziata Church in Siena Italy in damaged areas, and the presence of biofilms has been demonstrated where their growth is favored by external factors such as humidity, poor ventilation, and light which may be associated with biodeterioration, because their hydration retains particles and atmospheric pollutants that accelerate chemical corrosion by oxidation, reduction, and transformation of metal ions with the changes in the pigments in addition to the coexistence of saline efflorescence making the bioreceptives fresh and causing detachment of the paint layers [66].

Another threat has been reported to the cultural heritage of biodeterioration in frescoes caused by the presence of tourism such as the case of The Lascaux Cave, which is the most emblematic example of the damage that micro-organisms cause to mural paintings due to the amount of organic matter, respiration, and the sweat of visitors and workers that increased the concentration of CO2 favoring the growth of the fungus *Fusarium solani* and *Ochroconis lascauxensis* (black spots) and the alga *Bracteacoccus minor* forming a green patina (green disease) causing irreversible damage so it closed its access permanent visitors [62, 67].

At present, we must consider the use of commercial paints (canned) which, due to their composition, represent a carbon source for a large number of microorganisms and which can be a source of contamination and colonization for the surfaces in which they are applied. *Pseudomons, Flavobacterium, Escherichia, Bacillus, Enterobacter, Proteus, Micrococcus, Serratia, Aeromonas, or Stenotrophomonas maltophilia*, among others, have been reported in water-based paints, this contamination can occur during their production with the use of contaminated water or in the team [68–70].

5. Biodeterioration of textiles

Textiles are considered representative of cultural identity because they carry a significant value that transcends that of their materials and the work required for their manufacture. The desire of all cultures to express and communicate their social, esthetic and cultural values in their textile manifestations, and materials such as clothing or basketry (with ceremonial or ritual destination), are a unique cultural heritage, and the assignment of cultural value to a material object is the basis of conservation [71].

Textiles, such as clothing, fashion accessories, archeological objects, baskets, quilts, tapestries, embroideries, flags, funerary, and religious garb are often treasured for their artistic, technical, cultural and sentimental value, and for this reason, they are currently stored in collections in museums [72].

The textile heritage is very extensive, despite the loss to which it has been subjected throughout history, mainly due to the characteristics of its delicate materials and the interventions that have suffered and suffer from this type of pieces. The gradual deterioration of this material is very sensitive and can only be slowed down, the daily use of these fabrics, inadequate handling and bad storage conditions have caused the loss of unrepeatable textiles because the same materials and techniques are not available as well as the techniques that were used in their preparation that are already part of our past [73]. The state of preservation of textiles depends on the type of textile fiber, composition of the dye, age of the textile as well as its history of use and storage conditions.

It is called textile fiber to the set of filaments or strands susceptible to be used to form yarns (and of these fabrics). In the manufacture of the yarn for textiles, two types of fiber can be used: natural or synthetic. The natural fibers can be of animal or vegetable origin. In the first, they are generally of the protein type, such as wool from sheep's hair, goat, camelid, rabbit or another type of natural fiber such as silk from the silkworm. Among the natural fibers of plants include cotton, linen hemp, and jute among others. Synthetic fibers include polyester, polyamide polyurethane, polypropylene, polyacrylonitrile, and polyvinyl [74, 75].

In addition to the passage of time and the environmental characteristics of conservation as a possible source of deterioration, the development and presence of various types of organisms (microorganisms, rodents or insects) which are a threat to textiles and damage will depend on the type of fabric, its origin as well as storage conditions. The presence and permanence will depend on the availability of nutrients as well as light, humidity, and temperature conditions. The degradation of the materials that cause the damage by microorganisms is due to the processes of assimilation by fungi and bacteria that use these materials as a source of nutrients or to the degradation processes due to the effect of microbial metabolism.

The main manifestations of this process are the evident changes of the surface of these materials, discoloration, decrease in their resistance, changes in pH, and unpleasant odor. These damages can cause the total destruction of the material by the reduction of the degree of polymerization, decrease in its tensile strength, and elasticity. In general, natural fibers are more susceptible to microbial attack than synthetic fibers.

The main molecules responsible for the attack on textile fibers are organic acids, extracellular enzymes released or by exopigments of bacteria such as *Achromobacer* sp., *Bacillus* sp., *Brevibacterium* sp., *Corynebacterium* sp., *Pseudomonas* sp., *Rhodococcus* sp. *and Streptomyces* sp. or exopigments of fungi of the group *Aspergillus* sp., *Penicillium* sp., *Cryptococcus* sp., *Rhodotorula* sp., and others [76].

The biodeterioration mechanisms in textile objects will depend mainly on the type of fiber:

Cellulose fibers: The degradation of (1-4)-β-D-glucan or cellulose results from the activity of cellulolytic enzymes produced by several bacteria and especially fungi that hydrolyze cellulose to glucose by the enzymes: 1,4-β-D-glucan, cellobiohydrolase, endo-(1-4)-β-D-glucan glucanohydrolase, and glucohydrolase of β-D-glucosidases. These enzymes decrease the degree of polymerization of the long-chain cellulose molecules, resulting in a decrease in the strength of the fiber.

The presence of other components in fibers such as hemicellulose, pectins, other carbohydrates or substances added to fabrics (plasticizers) and even contaminants provide additional nutrients to microorganisms.

Among the genera of fungi associated with biodeterioration of cellulose are *Chaetomium, Myrothecium, Memnoniella, Stachybotrys, Verticillium, Alernaria, Trichoderma, Penicillium, Aspergillus, Aureobasidium, Cladosporium, Fusarium, Mucor, Paecilomyces, Rhizopus,* and *Trichothecium*. In the case of bacterial damage and with less significance, agents of degradation of cellulose are: *Arthrhobacter, Bacillus, Cellulomonas, Cellvibrio, Clostirdium, Cytophaga, Microbiospora, Nocardia, Pseudomonas, Sporocytophaga,* and *Streptomyces* [77].

Wool fibers: Keratin is the constituent protein of these fibers that form a polymer when disulfide bridges cross over this polymer. The mechanism of biodeterioration is by keratinolysis, sulfitolysis, proteolysis by peptidases, and deamination (metabolic processes with release of ammonia). The rate of degradation depends on the chemical composition, molecular structure, and degree of polymerization of the substrate and to a lesser degree on the structure of keratin [78].

Among the main biodeterioration agents are bacteria: *Arthrobacter, Bacillus (B. mesentericus, B. subtilis, B. cereus* and *B. mycoides), Cellulomonas, Cellvibrio, Clostridium, Cytophaga, Microbiospora, Nocardia, Pseudomonas, Sporocytophaga* and *Streptomyces* [79]. Degradation by fungi has been reported by the genera *Microsporum, Trichophyton, Fusarium, Rhizopus, Chaetomium, Aspergillus, Penicillium, Alternaria, Acremonium, Cephalothecium, Chrysosporium, Dematium, Oospora, Scopulariopsis, Stachybotrys, Trichoderma*, and *Ulocladium* [77, 80].

Silk fibers: They are produced by silkworms and are fibers of the fibroin protein that are joined to one another by rubber-like proteins, known as sericin that serves as protection from damage by light. This natural fiber is the most resistant to biodeterioration, and its decomposition depends on the proteolytic action on sericin and fibroin that are used as a carbon source by bacteria *Bacillus, Aeromonas, Arthobacter, Chyseomonas, Pseudomonas, Streptomyces, Serratia* and *Variovorax* and how biodeteriogenic fungal genera are: *Aspergillus, Chaetomium, Cladosporium, Penicillum* and *Rhizopus* [80].

There are very few works to which biofilms are directly associated as being responsible for biodeterioration in textile materials, and it may be the result that experimental designs have not been developed with these types of materials.

6. Biodeterioration of paper and parchments

The documentary production goes back in antiquity in different cultures that left numerous examples in different supports like tablets of mud, rolls of papyrus, parchment, sheet of amate and in more recent times the books. The invention

of paper gave man a faithful support where the written memory will inhabit his journey through history, and thanks to its consistency and durability, the texts of our ancestors are still today, a faithful witness of his time [81].

The main components of paper are fiber or fibrous material (hemp, cotton, linen, bagasse, rice straw a,nd wood) and functional additives (sizing, optical brighteners, and consolidating agents such as gelatin, cellulose acetate and carboxymethylcellulose). In this chapter, cellulose fiber is the major component with a lower proportion of lignin, hemicellulose, and other macromolecules, its quality depends on the source of the raw material used, and the procedure applied to obtain the fiber. Its mechanical resistance depends on its degree of polymerization and its interfiber links.

The inks are an important component of the documents and consist of a liquid that is fixed to the support endowed with an intense, durable, odorless and variable pH, is composed of a pigment, a diluent and a binder. Among the oldest ones are ferrous ink, whose components are iron sulfate, gallotanic acid and a binder, usually gum arabic. Over time the components of plant and animal origin have been replaced by synthetic compounds [82].

The books are composed of a support (parchment or paper), supported elements (inks), binding elements (seams and adhesives), protective structures (covers), and each of them with particular chemical characteristics that can be elements of degradation [83].

The microorganisms that commonly appear in the documentary supports are bacteria and fungi (yeasts and filamentous fungi), which transport moisture and attract pests by modifying the nutritive environment of the substrate. Both colonize a susceptible medium when in a poorly ventilated place, with adequate pH and low illumination, where temperature higher than 25°C and ambient humidity greater than 65% with accumulation of dust and/or soot in the different types of surfaces.

Filamentous fungi are the most biodeteriogenic microorganisms because they have structures called hyphae that are vegetative and reproductive [14, 84]. Vegetative hyphae are intertwined in paper fibers and through enzymatic processes that degrade cellulose, absorb nutrients, produce acids, and affect the coloration of the support resulting in fragility of the paper and often its complete destruction (**Figure 2**). On the other hand, the reproductive structures (spores) are a potential threat because they can remain in a latent state, they can be airborne, and they accumulate in layers of dust as long as the environmental conditions for their germination are reached, such as the formation of condensation points and local microclimates due to poor ventilation and heterogeneous temperature on the surface of the material.

Among the fungi identified are the Ascomycetes as well as mitosporic xerophilic fungi (which grow with a small amount of water) such as those of the genus *Aspergillus, Paecilomyces, Chrysosporium, Penicillium and Cladosporium* [85] and among the cellulolytic bacteria *Cellvibrio* and *Cellfacicula* as well as *Cytophaga* (myxobacteria).

In the case of the parchments that are composed of collagen (protein), its degradation depends on the oxidative chemical deterioration of amino acid chains and hydrolytic breakdown of the peptide structure and the production of pigments and organic acids that modify this material. The bacteria that have been described in this process are those of the genera *Bacillus, Staphylococcus, Pseudomonas, Virgibacillus* and *Micromonospora* as well as some alkalophilic bacteria such as *Actinobacteria* and among the proteolytic fungi reported are the Ascomycetes: *Chaetomium and Gymnoascus* and the genus *Acremonium*, *Aspergillus, Aureobasidium, Epicoccum, Trichoderma* and *Verticillium* [13, 78].

Figure 2.
Paper biodeterioration. (Photo: Laura Castrillón).

7. Prevention, conservation, restoration, and control

To preserve the tangible cultural heritage, there are two ways of action: the prevention of deterioration (conservation) and the repair of damage (restoration). With the preventive conservation anticipates the damage generated by extrinsic causes, alien to the nature of the pieces to conserve, but that in more or less long term could degrade their cultural value.

Therefore, prevention methods and strategies are usually not directly applicable to the object to be treated, but are directed to the environment to control microclimatic conditions in order to eradicate harmful agents or elements that can temporarily or permanently influence degradation [82].

The prevention methods inhibit or slow down the biological growth modifying the factors that can condition or inhibit their presence (humidity, temperature, light, and ventilation), if these factors cannot be controlled as in the case of monuments or archeological zones can be modified eliminating dust, dirt and deposits of residues of plant or animal nature.

In contrast and in general terms, conservation can be defined as the set of operations that aim to prolong the life of the material, thanks to the anticipation of damage or the correction of deterioration.

In the field of the conservation of cultural goods, the purpose is to maintain the physical and cultural properties of what has reached the category of cultural property, with the purpose that its value does not diminish and lasts beyond a limited time segment. Preventive conservation, as a methodology aims to control the deterioration of works of art before they occur to reduce the need to intervene. The deterioration must be minimized and the optimum conditions of exhibition, transportation, handling, cleaning, and storage must be maintained [86]. Among the main measures applied for preventive conservation that have been incorporated in the facilities are: air conditioning free of biodeteriogens agents, environmental fumigations, humidity, and temperature control.

The restoration aims to recover the physical and functional integrity of the work, thanks to the correction of the alterations that it has suffered. Consequently, the curative methods are of direct application because they try to amend all the damages they have experienced through their own history, whenever these suppose

mutilation or reduction of their documentary values. The restoration is more than an art is technical, thanks to the set of interdisciplinary scientific methods that give the work the authentic guarantee of the rigor of applied sciences to the field of conservation [87].

Restorers use the intervention techniques of biodeteriorated materials to eliminate the degradation products induced by microorganisms and if it is possible to delay their recurrence. The intervention treatment must be evaluated taking into account the identity of the biodeteriogens, degree and type of damage, safety of the treatment towards the materials of the object, risk for the worker, and possible environmental impacts. However, the growth of unwanted organisms will inevitably occur if the environmental conditions that favor their development persist.

Between the main methods of control of biodeteriogenic agents that grow as biofilms are:

Mechanical methods: they consist in the physical removal of fungi, bacteria, algae or any organism by shaving, abrasion, brushing, etc. Immediate but not lasting results are obtained, complete elimination is not achieved, and the results improve with the use of chemical agents.

Physical methods: modifying the temperature or pressure changes that are not suitable for the growth of organisms. Its biocidal effect depends on the denaturation or breaking of molecules of the organisms treated by breaking chemical bonds. Among these methods are electromagnetic radiation (microwave, ultraviolet rays, and gamma rays), anoxic treatments, and extreme temperatures. Its mechanism of action depends on its direct action with the genetic material or alteration of its structure and metabolic function.

Its main disadvantages are its high cost and the possible damage to the materials treated by its chemical alteration such as the pigmentation and hydrolysis of proteins and cellulose.

Chemical methods: these are the most commonly used intervention techniques through the use of biocides (disinfectants, bactericides or fungicides). Generally they are used in liquid or gas form, their mechanism of action is variable and they attack by disintegrating the bacterial or fungal membrane or by inhibiting their cellular processes, causing their death when they are used in the appropriate doses. Many products have been evaluated, however, due to their high risk and limited knowledge of the compatibility with the materials to which they apply their use has been limited. The selection of the biocide depends on the type of material, type of microorganism, and availability of the biocide. An additional problem is its long-term ineffectiveness [88].

A biocide can be a synthetic chemical, natural, of biological origin that is intended to destroy, counteract, neutralize, impede action or exercise control over any organism considered harmful to man. According to their action, they are divided into microbicides (bactericidal and fungicidal), growth inhibitors, and for the case of other organisms such as insects, rodents or birds, and there are also very toxic and lethal products such as pesticides, insecticides and/or repellents, acaricides, nematicides, avicides, rodenticides, etc.

A good biocide must have a broad spectrum of activity, be effective at low concentrations, be active over a wide range of pH, soluble in water, possess high persistence (effective over time), have low human and environmental toxicity, and have a low cost [89].

Among the main biocides used for the chemical treatment of tangible cultural goods are:

Thymol, orthophenylphenol (OFF), formaldehyde, pentachlorophenol, ethylene oxide, ethanol, etc., antibiotics and enzymes have also been used. For the best selection of the biocide, preliminary tests must be done to guarantee the innocuousness

of the operation for the safety of the operator and absence of risk for the object not only immediately but in the long term. For this reason, the use of very strong and long retention solvents (glycols, formamide, turpentine, and butylamine) in the porous bodies has been ruled out.

The forms of application of the biocides are: sublimation, pulverization or fumigation, according to their possible solid, liquid or gaseous state. The solids in solution or dispersion have a longer time of action although less exterminating capacity. The sublimable solids have little penetrability and, unless they are applied in high concentrations, they become repellent air fresheners with little lethal efficacy.

Solvents that are unstable to light and have a tendency to yellow or polymerize are also eliminated (ethylenic compounds such as dipentene, turpentine, acetylacetone, N-methylpyrrolidone). These rules are applicable to all porous objects, paintings, polychrome sculptures, mural paintings, etc. In the area of textiles, the most suitable solvent is water. But there are fibers very altered or with very bad coloration that are too sensitive. Then we must resort to organic solvents but avoiding chlorinated solvents, taking into account an eventual acidity and the release of hydrochloric acid. Water-based solvents must be used with extreme prudence, since many materials can suffer deterioration [90, 91].

Because the control of pests that use chemicals that are generally expensive and have side effects in people and can deteriorate the material, the choice of a biocide is increasingly difficult, therefore alternatives substances with biocidal properties have been sought for many years such as the use of natural plant products for which more and more reports justify their use. Currently, there is already a database of the accumulated experience of a Spanish group of the use of natural extracts for disinfection and disinfestation of cultural goods [92], as well as the use of essential oils from medicinal plants such as *Mentha piperita, Thymus vulgaris, Origanum compactum, Salvia officinalis, Artemisa absinthium and Lanandula angustifolia*, among others [93–95].

The development of nanotechnology is currently an emerging field in the conservation of cultural heritage, consequently the FP7 NANOFORART project has arisen (nano-materials for the conservation and preservation of movable and immovable artworks) and as an example of its applications, there are reports of the use of zinc oxide nanoparticles to control fungal biofilms or nanosilver coated cotton fabrics application for antimicrobial textile finishing [96–99].

Biological methods: another option for the control and restoration of works of art has been the use of microorganisms in the processes of biocleaning and biomineralización that are presented below:

Biocleaning: The accumulation of organic material on surfaces either by deposition of atmospheric particles, traces of colonization of microorganisms and organic substances allow the growth of bacteria and fungi. This accumulation can cause damages to the art work in response to the growth of microbial and are therefore considered biodeteriorating agents, however, in recent years, bacteria have been used for the conservation and restoration processes for the elimination of these organic materials. This procedure is known as biocleaning.

One of the advantages of the use of microorganisms over physicochemical treatments (which are very drastic) is that they use substrate-specific enzymes that do not degrade complex substances and adapt easily to environmental conditions. The microorganisms selected should be nonpathogenic and nonsporulating so that it is not a risk for workers after application and not be able to produce forms of resistance (spores).

Examples of these treatments have been documented for the removal in stone of black scale (hydrated calcium sulfate and carbon residues) caused by sulfur dioxide, hydrocarbons, and particulate matter (soot) emitted by the exhaust pipes

of vehicles, which were removed with the use of *Desulfovibrio desulfuricans* bacteria [100]. Good results have also been obtained for the elimination of nitrates in marble under anaerobic conditions with the use of *Pseudomonas denitrificans, Pseudomonas stuzeri, Pseudomonas pseudoalcaligenes* or *Paracoccus denitrificans* [101]. Biological cleaning of mural paints has also been explored to eliminate the remains of organic matter from old restorations or insoluble saline efflorescence with the use of *Pseudomonas stutzeri* [102].

Biomineralization: A modern and ecological alternative applicable to the restoration of historical monuments is the process known as biomineralization, specifically carbonatogenesis that can help in the restoration of cracks of statues or walls, since there are bacteria capable of mineralizing and filling these grooves when feeding them with means of culture that contain calcium salts in solution producing microcrystals of calcium carbonate that allow the restoration of damaged areas [103–105, 107, 108].

The carbonatogenesis or calcite production can occur either autotrophically or heterotrophically by the *Bacillus, Pseudomonas, Proteus, Myxococcus* and *Pantoea agglomerans* bacterial genera that allow obtaining a layer of a few millimeters thick by carbonatogenesis. Among the micro-environmental factors related to this process are: the concentration of dissolved inorganic carbon, the pH, the concentration of calcium ions and the presence of nucleation sites / or development of crystals for nucleation [23, 106].

8. Conclusion

In addition to the passage of time and environmental characteristics of the detriment of artistic and cultural heritage, this may also be the consequence of the microbial appearance favored by the enrichment of organic matter on the surfaces that colonize. The formation of biofilms is a strategy used by microorganisms to adapt to conditions that may be adverse for their growth. These biofilms are present in almost any type of surface, and the historical heritage is no exception. For the reason, it is important to know the characteristics that favor their formation, elimination and control to avoid being a threat to these objects.

As these biofilms represent a threat to this cultural heritage, the main challenges are to establish prevention measures for their appearance and to understand that if an intervention treatment is necessary, the resistance to treatment with biocides increases with this form of organization, which requires the use of high doses that compromise the physicochemical characteristics of the treated material.

The knowledge of the chemical composition of works of art made in different substrates such as stone, paintings, textile fibers, and paper composition allows us to understand what type of microbial colonization can be favored, and consequently be able to propose microbial growth as the responsible of biodeterioration. If these objects are valuable as a cultural heritage, it is the responsibility of all their conservation and restoration, for this reason the advanced techniques of identification and control of biofilms in cultural assets, currently, they are applied as strategies in the areas of preservation, restoration and control that will delay their natural deterioration and allow future generations to know this legacy.

Author details

Laura E. Castrillón Rivera*, Alejandro Palma Ramos, Jorge I. Castañeda Sánchez and María Elisa Drago Serrano
Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana, Mexico

*Address all correspondence to: lcrivera@correo.xoc.uam.mx

References

[1] García Cuetos P. El patrimonio cultural: Conceptos bàsicos. España: Ed. Prensas Universitarias de Zaragoza; 2011. ISBN: 978-84-15274-56-8

[2] Patrimonio Cultural: Instituto Andaluz del Patrimonio Histórico. Consejería de Cultura. Available from: www.iaph.es/web/canales/patrimonio-cultural [Accessed: Apr 5, 2018]

[3] UNESCO. Patrimonio Indicadores UNESCO de cultura para el Desarrollo. Available from: Vaillant CM. Materiales constituyentes de las colecciones de archivos y bibliotecas. In: Biodetrioro del patrimonio histórico documental: Alternativas para su erradicación y control. Río de Janeiro: Ed: Mast/FCRB; 2013

[4] Rivera CM. Patrimonio Cultural Tangible e Intangible. Available from: https://manuelrivera23.wordpress.com/blog/revista-ajayu/patrimonio-cultural-tangible-e-intangible/ [Accessed: May 7, 2018]

[5] ICOMOS, International Cultural Tourism Charter. Principles and guidelines for managing tourism at places of cultural and heritage significance. In: ICOMOS International Cultural Tourism Committee 2002. ISBN: 956-244-166-0

[6] Martianrena X. Conservación y Restauración. Cuadernos de Sección. Artes Plásticas y Documentales. 1992;**10**:177-224

[7] Moreno OM, Rogerio-Candelera MA, López NJT, Hernández JV. Estudio y conservación del patrimonio cultural. Coria Gráfica, SL: Actas. Ed.; 2015. ISBN: 978-84-608-2452-7

[8] Simmons EJ, Muñoz-Saba Y. Cuidado, manejjo y conservación de las colecciones biológicas. In: Conservación Internacional. Serie Manuales de campo 1. Bogotá Colombia. 2005. ISBN: 958-33-6969-1

[9] Hueck HJ. The biodeterioration of material as a part of hylobiology. Materials and Organisms. 1965;**1**:5-34

[10] Marijnissen RH. Degradation-conservation. Restauraction de 'Oeuvre D'Art. Ed Arcade; 1967. pp. 123-125

[11] Gacto M. Los microorganismos y el arte. Anales de Biologia. 2011;**33**:107-115

[12] Páramo AL, Narváez Z, De la Cruz E. Aislamiento e identificación de microorganismos en biopelículas provenientes del Castillo de Chapultepec, Ciudad de México. Nexo Revista Científica. 2011;**24**(2):83-91

[13] Sterflinger K, Piñar G. Microbial deterioration of cultural heritage and works of art-tilting at windmills? Applied Microbiology and Biotechnology. 2013;**97**:9637-9646

[14] Castrillón RLE, Palma RA, Padilla DMC. Biopelículas fúngicas. Dermatologia Revista Mexicana. 2013;**57**:350-361

[15] Castrillón RLE, Palma RA. Biofilms: A survival and resistance mechanism of microorganisms. In: Pana M, editor. Antibiotic Resistant Bacteria—A Continuous Challenge on the New Millennium. Rijeka . Ch 7: Intech Open Science; 2012. pp. 159-178

[16] Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ. Bacterial biofilms in nature and disease. Annual Review of Microbiology. 1987;**987**:435-464

[17] Castrillón RLE, Palma RA, Padilla DMC. Importancia de las biopelículas en la práctica médica. Dermatologia Revista Mexicana. 2010;**54**:14-24

[18] Morton LHG, Surman SB. Biofilms in biodetrioration—A review. International Biodeteriorarion & Biodegradation. 1994;**34**(3-4):203-221

[19] Videla HA. Manual of Biocorrosion. Boca Raton, PL, USA: Lewis Publishers/ CRC Press; 1996. p. 288

[20] Dakal TCH, Cameotra SS. Microbially induced deterioration of architectural heritages: Routes and mechanisms involved. Environmental Sciences Europe. 2012;**24**:36

[21] Tatis CRD, Barbosa LAL. Enfoque químico del deterioro y biodeterioro de rocas calcáreas conformantes de monumentos patrimoniales de importancia histórica y cultural. Luna Azul. 2013;**36**:247-284

[22] Warscheid T, Braams J. Biodeteriorarion of Stone: A review. International Biodeterioration & Biodegradation. 2000;**46**:343-368

[23] Cameotra SS, Dakal TC. Carbonatogenesis: Microbial contribution to the conservation of monuments and stone artwork. Conservation Science in Cultural Heritage. 2012;**12**:79-108

[24] Pandey VD. Rock-dwelling cyanobacteria: Survival strategies and biodeterioration of monuments. International Journal of Current Microbiology and Applied Sciences. 2013;**2**(2):519-524

[25] Macedo MF, Miller AZ, Dionisio A, Saiz-Jiménez C. Biodiversity of cyanobacteria an green algae on monuments in the mediterranean basin: An overview. Microbiology. 2009;**155**:3476-3490

[26] Videla AH, Guiamet SP, Gómez de Saravia SG. Biodeteriroro de materiales estructurales de sitios arqueológicos de la civilización maya. Revista Museo de la Plata. 2003;**44**:1-11

[27] Martino DP. What about biofilms on the surface of stone monuments? The Open Conference Proceedings Journal. 2016;(Suppl 1:M2):14-28

[28] Gómez de Saravia SG, Fontana JM, Guiamet SP. Estudio de biofilms fototróficos mediante microscopía óptica y microscopía electrónica de barrido. Revista Argentina de Microbiología. 2010;**42**:315

[29] Essays UK. Monuments and Caves: Effect of Cyanobacteria. 2013. Available from: https://www.ukessays.com/essays/biology/role-cyanobacteria-monuments-caves-3562.php?vref=1 [Accessed: Jun 8, 2018]

[30] López-Miras MM. Identificación y caracterización de comunidades microbianas presentas en pinturas sobre lienzo. Estudio de su capacidad como agentes de biodeterioro Tesis Doctoral Universidad de Granada. 2012. ISBN: 978-84-694-6935-4

[31] Salvatori O, Municchia CA. The role of fungi and lichens in the biodeterioration of stone monuments. The Open Conference Proceedings Journal. 2016;7(Suppl 1:M4):39-54

[32] Gaylarde CC, Morton GH. Deteriogenic biofilms on building and their control: A review. Biofouling. 1999;**14**(1):59-64

[33] Suihko ML, Alakomi HL, Gorbushina A, Fortune I, Marquardt J, Saarela M. Characterizaation of aerobic bacterial and fungal microbiota on surfact of historic Sctottish monuments. Systematic and Applied Microbiology. 2007;**30**:494-508

[34] Laiz L, Recio D, Hermosin B, Saiz-Jimenez C. Microbial communities in salt efflorescences. In: Ciferri O, Tiano P, Mastromei G, editors. Of Microbes and Art. Boston, MA: Springer; 2000

[35] Lepinay C, Mihajlovskia A, Seyer D, Touron S, Bousta F, Di Martino P. Biofilm communities survey at the areas of salt crystallization on the walls of a decorated shelter listed at UNESCO World cultural Heritage. International

Biodeterioration & Biodegradation. 2017;**122**:116-127

[36] Ortega-Morales BO, Nakamura S, Montejano-Zurita G, Camacho-Chab JC, Quintana P, De la Rosa-García SC. Implications of colonizing biofilms and microclimate on west stucco at North Acropolis, Tikal, Guatemala. Heritage Science. 2013;**1**:32

[37] Gómez-Cornelio S, Ortega-Morales O, Morón-Ríos A, Reyes-Estebanez M, de la Rosa-García S. Cambios en la composición de la comunidad fúngica de biopelículas sobre roca calcárea a través de una cronosecuencia en Campeche México. Acta Botánica Mexicana. 2016;**117**:59-77

[38] Neilands JB. Siderophores: Structure and function of microbial iron transport compounds. The Journal of Biological Chemistry. 1995;**270**(45):26723-26726

[39] Sajeed AS, Vidhale NN. Bacterial siderophore and their application: A review. International Journal of Current Microbiology and Applied Sciences. 2013;**2**(12):303-312

[40] Smith LS, Johnson BD. Growth of *Leptospirillum ferriphilum* in sulfur medium in co-culture with *Acidithiobacillus caldus*. Extremophiles. 2018;**22**:327-333

[41] Ortega-Morales O, Guezennec J, Hernandez-Duque G, Gaylarde CC, Gaylarde PM. Phototrophic biofilms on ancient Mayan buildings in Y ucatan, Mexico. Current Microbiology. 2000;**40**:81-85

[42] Schalk JI, Hannauer M, Braud A. New roles for bacterial siderophores in metal transport and tolerance. Environmental Microbiology. 2011;**13**(11):2844-2854

[43] Gutiérrez RJN, Nugkui TNM. Biodeterioro por plantas vasculares en el museo cementerio presbítero Matías Maestro, Lima-Perú. Revista Rebiol. 2016;**36**(2):40-53

[44] Sánchez FLP, Cardona ML. In: La pintura mural: Una pasión: análisis del mural fresco. San Diego, CA, USA. 2013. ISBN 13:9789588743479

[45] Triado JR, Subirana R. Las claves de la pintura. Barcelona: Ed. Planeta; 2002. 119 p

[46] Flemming CH. Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. Polymer Degradation and Stability. 1998;**59**(1-3):309-315

[47] Garófano MI. Materiales orgánicos naturales presents en pinturas y policromías. Naturaleza, usos y composición química. Revista del Instituto Andaluz del Patrimonio Histórico. 2011;**80**:56-71

[48] Ravikumar HR, Rai SS, Karigar CS. Biodegradation of paints: A current status. Indian Journal of Science and Technology. 2012;**5**(11):1977-1987

[49] Villarquide Jevenois A. La pintura sobre tela II. Alteraciones, materiales y tratamientos de restauración. Ed. Nerea SA; 2005. ISBN:84-89569-50-9

[50] Caneva G, Nugari MP, Salvadori O. La Biología de la restauración. España: Ed: Nerea SA; 2002. ISBN: 8489569487

[51] Doménech-Carbó MT. Novel analytical methods for characterising binding media and protective coatings in artworks. Analytica Chimica Acta. 2008;**621**:109-139

[52] Nugari MP, Priori GF. Resistance of acrylic polymers to microorganisms-first part. In: Vth International Congress on Deterioration and Conservation of Stone; 25-27 September; Lausanne. 1985. pp. 685-693

[53] Pankhurst ES, Davies MJ, Blake HM. The ability of polymers or materials containing polymers to provide a source of carbon for selected microorganisms. In: Harry Walters A, Hueck-van der Plas EH, editors. Biodeterioration of Material. Vol. 2. Elsevier Science & Technology. pp. 76-90

[54] Poyatos JF. Procesos de biodeterioro en pinturas sobre lienzo del museo de Bellas Artes de Granada: Examen visual y gráfico. Tesis Doctoral Univesidad de Granada. 2007. ISBN: 978-84-338-4436-1

[55] Elumalai P, Elumalai EK, David E. Fungi associated with deteriorations of painted wall surfaces: Isolation and identification. European Journal of Academic Essays. 2014;**1**(3):48-50

[56] Ogu TC, Okaa AI, Ozokpo AC, Onochie CC. Microbial deterioration of paints. African Journal of Education, Science and Technology. 2016;**3**(1):190-194

[57] Gorbushina AA, Hayrman J, Dornieden T, González-Delvalle M, Krumbein EW, Laiz L, Peersen K, Saiz-Jiménez C, Swings J. Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene-Kreiensen, Germany). International Biodeterioration & Biodegradation. 2004;**53**:13-24

[58] López-Miras MM, Martin-Sánchez I, Yebra-Rodríguez A, Romero-Noguera J, Bolívar-Galiano F, Ettenaurer J, Sterflinger K, Piñar G. Contribution of microbial communities detected on an oil painting on canvas to its biodeterioration. PLoS One. 2013;**8**(11):e80198

[59] Gomoiu I, Mohanu D, Radvan R, Dumbravician M, Neagu SE, Cojoc LR, Eanche MI, Chelmus A, Mohanu I. Environment impact on biopigmentation of mural paintings. Acta Physica Polonica. 2017;**131**:48-51

[60] Seves A, Romano H, Scilione G, Maifreni T, Sora S. A laboratory investigation of the microbial degradation of cultural heritage (ICMC). In: Cifferri O, Tiano P, Mastromei G, editors. Of Microbes and Art. Boston, MA: Springer; 2000. pp. 121-133. ISBN 978-1-4613-6904-2

[61] Petushkova JP, Lyalikova NN. Microbiological degradation of lead-containing pigments in mural paintings. Studies in Conservation. 1986;**31**:65-69

[62] Ciferri O. Microbial degradation of paintings. Applied and Environmental Microbiology. 1999;**65**(3):879-885

[63] Del Rosal Y, Lobo JV, Hernández MM, Roldán MM, Sáiz-Jiménez C. Biofilms en cuevas turísticas: la Cueva de Nerja y la Cueva del Tesoro. In: Andreo B, Durán JJ, editors. El karst y el hombre: las cuevas como Patrimonio Mundial. Nerja (Málaga): Asociación de Cuevas Turíisticas Españolas; 2016. pp. 103-114. ISBN: 978-84-617-4702-3

[64] Anna A. Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene-Kraiensen, Germany). International Biodeterioration & Biodegradation. 2004;**53**:13-24

[65] Ma Y, Zhang H, Du Y, Tian T, Xiang T, Liu X, Wu X, An F, Wang W, Ji-Dong G, Feng H. The community distribution of bacteria and fungi on ancient wall paintings of the Magao Grottoes. Scientific Reports. 2015;**5**:7752

[66] Milanesi C, Baldi F, Borin S, Ciampolini F, Cresti M. Biodeterioration of fresco by biofilm forming bacteria. International Biodtererioration & Biodegradation. 2006;**57**:168-173

[67] Martín-Sánchez PM, Jurado V, Porca E, Bastian F, Lacanette D, Alabouvette C, Saiz-Jimènez C. Airborne microorganisms in Lascaux Cave (France). International Journal of Speleology. 2014;**4**(3):295-303

[68] Fazenda JMR. Tintas e vernizes: ciência e tecnologia. São Paulo: Ed. Abrafati; 1995. ISBN: 9788521203742

[69] Obidi OF, Aboaba OO, Makanjuola MS, SCU N. Microbial evaluation and deterioration of paints and paint-products. Journal of Environmental Biology. 2008;**30**(5):835-840

[70] Rochoel RF, Giese EC, DRF H, Pelayoi JS, Melo BA. Micobiological contamination of water-based paints from an industry in the state of Paraná, Brazil. Semina: Ciencias Exatas e da Terram Londrina. 2008;**29**:85-92

[71] Ruiz de Haro MI. Orígenes, evolución y contextos de la tecnología textile: La producción del tejido en la prehistory y la protohistoria. Arqueología y Territorio. 2012;**9**:133-145

[72] Ramos ENJ, Valverde GM, Aliaga AMT, Gimènez TA. Deteriroro causado por microorganismos en textil arquelógico y lienzos. Revista Boliviana de Quimica. 2012;**29**(2):170-176

[73] Olmedo PM. Conservación del patrimonio textil. "Guía de Buenas Prácticas" DL MA 995-2017. España. 2017

[74] Giraldo MJF. Manual Técnico Textil. 4th ed. Medellín, Colombia; 2015 Available from: http://www.microdenier.com.co/manualtecnico.pdf

[75] Szostak-Kotova J. Biodeterioration of textiles. International Biodeterioration & Biodegradation. 2004;**53**:165-170

[76] Joshi VK, Attri D, Bala A. Microbial pigments. Indian Journal of Biotechnology. 2003;**2**:362-369

[77] Pekhanov E, Neverov A, Kubika S, Zaikov G. Biodegradation and biodeterioration of some natural polymers. Chemistry & Chemical Technology. 2012;**6**(3):263-280

[78] Kunert J. Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporum gypseum:* A comparison. Journal of Basic Microbiology. 1989;**29**:597-604

[79] Ararwal PN, Puvathingal JM. Microbiological deterioration of woolen materials. Textile Research Journal. 1969;**38**:38-42

[80] Gutarowska B, Pietrzak K, Machnowski W, Milczarek MJ. Historical textiles—A review of microbial deterioration analysis and disinfection methods. Textile Research Journal. 2017;**87**(2):2388-2406

[81] Asenjo MJL, Hidalgo MMC. El papel: 2000 años de historia. 2010. Available from: http://www.interempresas.net/Graficas/Articulos/37870-El-papel-2000-anos-de-historia.html [Accessed: May 17, 2018]

[82] Vaillant Callol M. In: Biodeterioro del Patrimonio Histórico Documental: Alterantivas para su erradicación y Control. Río de Janeiro: Ed. MAST/FCRB; 2013

[83] Forniés MZ, Garcìa QR. Factores de degradación intrínseco en los libros: la naturaleza del material bibliográfico. 2014. Available from: http://www.bid.ub.edu/es/32/fornies2.htm

[84] Méndez TLJ., López MR., Hernández HF. Actualidades en Micología Médica. México: Ed. Sefirot SA de CV; 2012. ISBN: 978-60-7772-833-7

[85] Pinzari F, Montanari M. Mould growth on library materials stored in compactus-type shelving units

(Chapter 11 pp. 193-206). In: Abdul-Whahab Al-Sulaiman SA, editor. Sick Building Síndrome in Public Bulidings and Workplaces. London, New York: Springer, Heidelberg Dordrecth; 2011

[86] Reyes Valderrama L. Biodeterioro del patrimonio cultural: el sorprendente apetito de algunos organismos por el arte y la historia. Hipotesis. 2013;**15**:70-75

[87] Viñas V, Viñas R. Las técnicas tradicionales de restauración: Un estudio del RAMP. Programa general de Información y UISIST. UNESCO. PGI-88/WS/17. 1988

[88] Sequeira S, Cabrita EJ, Macedo MF. Antifungals on paper conservation: An overview. International Biodeterioration & Biodegradation. 2012;**74**:67-86

[89] Borrego-Alonso S. Los biocidas vegetales en el control del biodeterioro del patrimonio documental. Perspectivas e impacto. Revista CENIC Ciencias Biológicas. 2015;**46**(3):259-269

[90] López LP. Óxido de etileno, utilización como agente esterilizante y riesgos para la salud del personal sanitario. Revista CES Salud Pública. 2014;**5**(2):154-162

[91] Masschelein-Kleiner L. Los Solventes. Publicaciones Centro Nacional de Conservación y Restauración DIBAH; 2004

[92] Morales R, Blanco P, Lalana P, Pardo de Santayana M, Valentín N. La ciencia y el arte IV. Ciencias experimentales y conservación del patrimonio. España: Ed: Ministerio de Educación Cultura y Deporte, Subdirección General de Documentación y Publicaciones; 2013

[93] Matusiak K, Machnowski W, Wryosek H, Polak J, Rajkowska K, Smigielski K, Kunicka-Styczynska A. Application of *Cinnamomun zeylanicum* essential oil in vapour phase for heritage textiles disinfection. International Biodeterioration & Biodegradation. 2017;**xxx**:1-9

[94] Valdés-Pérez O, Borrego-Aloso S, Vivar-González I, Anaya-Villapanda M, Molina-Veloso A. Actividad antifúngica del aceite esencial de clavo de olor en el control del biodeterioro fúngico de documentos. Revista CENIC Ciencias Biológicas. 2016;**47**(2):78-85

[95] Walentowka J, Folsowicz-Flaczyk J. Thyme essential oil for antimicrobial protection of natural textiles. International Biodeterioration & Biodegradation. 2013;**84**:407-411

[96] Baglioni P, Gorgi R, Chelazzi D. Nano-materials for the conservation and preservation of movable an inmovable artworks. In: Progress in Cultural Heritage—EUROMED 2012. 2012. pp. 313-318 Available from: https://www.researchgate.net/publication/270365605

[97] Balakumaran MD, Ramachandran R, Jagadeeswari S, Kalaichelvan PT. *In vitro* biological and characterization of nanosilver coated cotton fabric—An application for antimicrobial textile finishing. International Biodeterioration & Biodegradation. 2016;**107**:48-55

[98] Di Salvo S. Nanotechnology for cultural heritage. Science, Technology and Society: An International Journal. 2014;**2**(2):28-32

[99] Gambino M, Ali Ahmed MAA, Villa F. Zinc oxide nanoparticles hinder fungal biofilm development in an acient Egyptian tomb. International Biodeterioration & Biodegradation. 2017;**122**:92-99

[100] Capitelli F, Zanardini E, Randalli G, Mello E, Deffonchio D, Sorlini C. Improved methodology for bioremoval of black crust on historical stone artwork by use of sulphate reducing bacteria. Applied

and Environmental Microbiology. 2006;**72**:3733-3737

[101] Cappitelli F, Ranalli G, Zanardini E, Mello E, Sorlini C. Biotreatment of salts in stone at Matera Cathedral. In: Conference on Heritage, Microbiology and Science. Portsmouth, UK: University of Portsmouth; 29 June–2 July 2005. p. 51

[102] Bosh RP, Regidor RJL, Soriano SP, Deménech CMT, Montes ER. Ensayos de biolimpieza con bacterias en pinturas murales. Vol. 4-5. Arché: Publicación del Instituto de Restauración del Patarimonio de la UPV; 2010. pp. 117-124

[103] Le Métayer-Levrel G, Castanier S, Orial G, Joubiére JF, Perthuisot JP. Application of bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic patrimony. Sedimentary Geology. 1999;**126**:25-34

[104] Rinaldi A. Saving a fragil legacy. EMBO Reports. 2006;**11**(11):5-1079

[105] Zammit G, Sánchez-Moral S, Albertano P. Bacterially mediated mineralisation processes lead to biodeterirarion of artwork in Maltese catacombs. Science of the Total Environment. 2011;**409**:2773-2782

[106] Páramo ALA, Narváez ZJA, Ortega MBO. La bioprecipitación de carbonato de calcio por la biota nativa como método de restauración. Nexo Revista Científica. 2015;**28**(1):25-40

[107] Calvo A. Conservación y restauración de pintura sobre lienzo. In: De la A a la Z, editor. Conservación y Restauración: Materiales Técnicas y Procedimientos. Barcelona: Ed. Serbal; 1997. pp. 152-155. ISBN: 9788476281949

[108] Porqué es importante la conservación del patrimonio cultural? Available from: https://sabiendomsdenuestropatrimonio cultural.wordpress.com [Accessed: May 7, 2018]

Chapter 5

Antimicrobial Agents: Antibacterial Agents, Anti-biofilm Agents, Antibacterial Natural Compounds, and Antibacterial Chemicals

Yaw Duah Boakye, Newman Osafo, Cynthia Amaning Danquah, Francis Adu and Christian Agyare

Abstract

The surge in antimicrobial resistance coupled with the decline in the antimicrobial drug pipeline calls for the discovery and development of new agents to tackle antibiotic resistance and prevent a return to a post-antibiotic era. Several factors account for resistance of microbes; some are natural and others are acquired. Natural selection, presence of efflux pumps, impermeable cell wall, biofilm formation and quorum sensing are some of the factors. Though it is difficult to outwit the pathogens, the discovery and development of compounds with pleiotropic modes or mechanisms of action different from the conventional drugs currently being used can help us tackle antimicrobial resistance. Natural products have been known to be a rich source of bioactive compounds with diverse structures and functional group chirality. Various reports indicate medicinal plants with antibacterial, anti-biofilm, efflux pump inhibition, wound healing effects or properties and others used for upper respiratory and urinary tract infections. There is an urgent need to research into natural products particularly plants for antimicrobial agents including antibacterial agents, anti-biofilm agents, antibacterial natural compounds and antibacterial chemicals. This chapter throws more light on such antimicrobials.

Keywords: antimicrobials, biofilm inhibitors, natural products, antibacterials

1. Antibacterial agents

1.1 Introduction

The last decade has seen in a dramatic fashion, an accelerated microbiological evolution and resistance to antimicrobial agents. There is therefore the need to optimize appropriate stewardship of infection control in the light of an apparent

stagnation in the development of novel antimicrobial agents. This chapter therefore considers current anti-infective agents of the various classes that are clinically used in treating infections.

1.2 Beta lactam derivatives

The beta lactam derivatives stand as the oldest class of antibiotics used. The beta lactam ring has proven to be the major weapon in the fight against bacterial infections. Several novel molecules modeled after Alexander Fleming's penicillin and its derivatives have been developed. These drug moieties share the common characteristic of the beta lactam ring being an integral part of the structural make up and its effectiveness. These include the cephalosporins, monobactams, cephamycins, and the carbapenems (imipenem and meropenem). Beta lactams are indicated for a varied number of bacterial infections ranging from respiratory and urinary tract infections, ear and eye infections and gonorrhea to more life-threatening conditions like meningitis, septicemia and pneumonia. It is also widely adopted for prophylactic use in bacterial endocarditis, surgical site infections and in immuno-compromised situations [1].

Beta lactam antibiotics are bactericidal in their action. They inhibit the building of bacterial cell wall by interfering with the synthesis of peptidoglycan. Penicillin binding proteins which are bacterial enzymes which are essentially for bacterial cell wall synthesis are usually the targets of beta lactams [1]. Beta lactam antibiotics are generally available for parenteral administration with some also showing good absorption from the gastrointestinal tract. In patients with intact renal function, most beta lactams have a serum half-life of 1–2 h. Ceftazidime and temocillin break off this usual norm with a half-life of 4–6 h and an even higher half-life of 8–10 h for ceftriaxone. Penicillins and cephalosporins are eliminated primarily through glomerular filtration with varying levels of active transport across the renal tubules as well as the hepatobiliary system [2].

Owing to the general abuse of antibiotics, the beta lactams have suffered the challenge of the development of resistance in target pathogenic organisms. The production of beta lactamases has been a major determinant in the resistance observed especially in Gram-negative pathogens. Alterations in the beta-lactam targets, the penicillin binding proteins, are also important in Gram-positive pathogens. Efflux mechanisms and/or exclusion of these agents also contribute more often in conjunction with the other two mechanisms [3]. New agents of the beta-lactam group that have been approved following development have come from the cephalosporin class.

1.3 Cephalosporins

Cephalosporins are usually classified based on spectrum, generation, chemical structure, clinical pharmacology and resistance to beta-lactamases. The first cephalosporins were assigned first-generation cephalosporins; later, more expanded spectrum cephalosporins were designated as second-generation cephalosporins. Each more current generation has altogether more prominent activity against Gram-negative bacteria than the preceding generation and much of the time with diminished action against Gram-positive bacteria. Fourth-generation cephalosporins, however, have true broad-spectrum activity. The recent addition to the block, the fifth generation cephalosporins has become very crucial due to its activity against multidrug-resistant *Staphylococcus aureus* (MRSA) [4].

The drug of choice in this latest generation is ceftaroline. It is the only beta-lactam with MRSA activity. Ceftaroline, which is available as a pro-drug, ceftaroline prosamil is again unique for its expanded and extensive Gram-positive activity beyond all presently available cephalosporins. Ceftaroline active against the

Gram-positive organisms (*Streptococcus pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative species (*Haemophilus influenzae* and *Moraxella catarrhalis*), including resistant phenotypes. Data for its approval proved its effectiveness in the treatment of acute bacterial skin and skin structure infections and community acquired bacterial pneumonia [5]. Ceftaroline demonstrates dose-proportional pharmacokinetics similar to other renally excreted cephalosporins after intravenous administration. It is half-life after dose is 2.53 h with protein binding of approximately 20%. Ceftaroline also showed in clinical trials to have positive attributes with regards to antibacterial stewardship by having a low potential for development of resistance as well as favorable tolerability and safety profile [5].

Ceftolozane is a novel beta-lactam cephalosporin combined with beta-lactamase inhibitor, tazobactam for the management of complicated urinary tract and intra-abdominal infections. The peculiar chemistry and dosing accounts for its extensive coverage of Gram-negative organisms including multidrug-resistant *Pseudomonas aeruginosa* as well as extended-spectrum beta-lactamase producing organisms and some anaerobes. Its efficacy can be compared to levofloxacin in patients with complicated urinary tract infections, including pyelonephritis, and comparable to that of meropenem against complicated intra-abdominal infections. Ceftolozane-tazobactam has shown to be indispensable due to the lack of susceptibility to the usual mechanisms of resistance mostly by Gram-negative organisms such as the production of beta-lactamases, efflux pumps, alterations in penicillin binding proteins as well as porin loss [6].

1.4 Glycopeptides

Glycopeptide antibiotics are complex and rigid molecules that repress a late stage in bacterial cell wall peptidoglycan synthesis. The selective toxicity of glycopeptides is attributable to the fact that its 3D structure harbors a cleft into which peptides of a specific configuration found only in bacterial cell walls can fit. Glycopeptide has assumed a special role in the face of the general threat of antimicrobial activity resistance since its unique mechanism involving the attachment of a bulky inhibitor to a substrate with the goal that the active sites enzymes are unable to align themselves correctly, therefore renders resistance to glycopeptides more difficult to achieve than other antimicrobial agents [7].

A good number of glycopeptides have gone through development in recent years and have been approved for clinical use. Oritavancin, a lipoglycopeptide obtained from the naturally occurring chloroeremomycin of the eremomycin class of glycopeptides is very similar to vancomycin but possess two 4-epi-vancosamine monosaccharides, one supplanting vancosamine and the other connected to ring-6 via an amino acid residue. Based on the features of its pharmacophore and its stereochemistry, it has enhanced antimicrobial against Gram-positive organisms including those possessing both VanA- and VanB-mediated vancomycin-resistance [8]. Oritavancin is transcendently cleared by means of the reticuloendothelial system, accumulating most notably in macrophages of the liver (Kupffer cells), kidney, spleen and lungs, as well as in the intestinal mucosa, thymus, and lymph nodes. Subsequent release and elimination from these tissues does not occur readily and thus only trace amounts are recouped from urine and feces. Dosage adjustments are not required in hepatic and renal insufficiency [9].

Telavancin also derivative of vancomycin and a lipoglycopeptide has been shown to have a dual mechanism of action by causing an inhibition of the peptidoglycan synthesis and through membrane depolarization. Telavancin is reliably active against *Staphylococcus aureus*, including MRSA, vancomycin-intermediate-resistant *Staphylococcus aureus*, linezolid-resistant *Staphylococcus aureus*, and daptomycin-susceptible strains and therefore effective for the treatment of complicated skin and skin-structure infections. It has additionally proven effective in the treatment of

Gram-positive bacterial infections especially pneumonia. Its non-inferiority is compared with vancomycin, in the treatment of complicated skin and skin-structure infections and pneumonia. Telavancin is excreted by the kidneys, and thus, dosage adjustments are required in cases of renal failure. Telavancin is related with higher rates of renal events, changed taste, nausea and vomiting but however lesser rates of pruritus and infusion related events relative to vancomycin [10].

Dalbavancin has proven to be a valuable addition to the armamentarium of antimicrobial agents as it is the first once a week antibiotic with activity against a broad range of Gram-positive pathogens. Dalbavancin's uniqueness is its novel pharmacokinetic profile with a half-life of 170–210 h, which makes the once-weekly dosing optimal. Forty percent is eliminated via the renal route. Most of the drug is excreted as intact drug. Concentration was unchanged in patients with mild renal impairment. No adjustments are needed in hepatic insufficiency, as concentrations of the drug do not increase with severe hepatic impairment. It is still unknown if the drug penetrates the cerebrospinal fluid, or whether the drug is removed during hemodialysis. However, the high protein binding of dalbavancin would suggest both of these scenarios to be unlikely [11].

1.5 Oxazolidinones

Oxazolidinones are synthetic antimicrobial agent which inhibit bacterial protein synthesis. Linezolid, the first oxazolidinone to be approved for clinical use, has bacteriostatic activity against many important resistant pathogens including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and penicillin-resistant *Streptococcus pneumoniae* as seen from *in vitro* studies [12]. Tedizolid which is available as a prodrug (tedizolid phosphate) is the first in the class to be dosed once daily as it has a half-life of 12 h. It also has a profound oral bioavailability of about 90% and no dosage adjustment is required between intravenous and oral administration, nor is dosage adjustment needed based on hepatic or renal impairment. Its activity covers Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus.* It is indicated for the management of acute bacterial skin and skin structures infections. Tedizolid appears to depart from linezolid in the incidence of gastrointestinal and hematologic side effects and the apparent lack of drug interaction with selective serotonin reuptake inhibitors [13].

1.6 Fidaxomicin (macrocyclic antibiotics)

Fidaxomicin is the first in the new class of narrow spectrum macrocyclic antibiotics. It is derived from the organism *Dactylosporangium aurantiacum* as a fermentation product. Fidaxomicin's action leads to an inhibition transcription process by interfering with RNA polymerase. Fidaxomicin acts at a distinct site and step of RNA synthesis to that of the rifamycins and streptolydigin, and thus no overlapping antibiotic resistance has been identified. Its narrow spectrum of activity is against most Clostridial spp., including *Clostridium difficile*, and exhibits moderate activity against other Gram-positive organisms, such as staphylococci and enterococci. Systemic absorption is very limited with serum concentrations remaining generally low [14].

1.7 Aminoglycosides

Aminoglycosides are exceptionally potent, broad-spectrum antimicrobial agents with numerous alluring properties for the treatment of hazardous diseases. The antibacterial potency of the aminoglycosides is attributable to one or several

aminated sugars linked by glycosidic bonds to a dibasic cyclitol in their chemical structures. Aminoglycosides act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes. Widespread resistance against these agents including semi-synthetic additions such as amikacin and netilmicin has prompted the need for development of alternative agents. The three main resistance mechanisms identified include a decreased cell permeability; alterations at the ribosomal binding sites; and production of aminoglycoside modifying enzymes [12]. Plazomicin is a next-generation semisynthetic aminoglycoside derived from sisomicin, a naturally occurring aminoglycoside antibiotic. Plazomicin is active against Gram-negative and selected Gram-positive bacteria and it is active against enterobacteriaceae, multidrug-resistant enterobacteriaceae (MDR-EC), aminoglycoside-resistant enterobacteriaceae (AR-EC), carbapenem-resistant Enterobacteriaceae (CR-EC), colistin-resistant enterobacteriaceae (CRE), tigecycline-resistant enterobacteriaceae (TR-EC). It has no nephrotoxic and ototoxic effects that characterize agents in this class. Its superior pharmacokinetic profile also supports a more convenient once daily IV dosing [15].

1.8 Pleuromutilins

Pleuromutilins are antimicrobial agents that selectively restrain bacterial translation and derivatives of the naturally occurring tricyclic diterpenoid pleuromutilin. The interest in pleuromutilins has resurged following the resistance in more prominent classes like the beta-lactams. Pleuromutilins inhibit bacterial protein synthesis by binding to the central part of domain V of the 50S ribosomal subunit at the peptidyl transferase center ultimately affecting peptide bond formation [16]. Retapamulin used as a topical agent for the treatment of impertigo has bacteriostatic effect against *Staphylococcus aureus* and *Streptococcus pyogenes*. Retapamulin is well tolerated with the most commonly reported adverse effect being pruritus at the application site. Although comparative efficacy has not been established with mupirocin, it is an effective alternative with dosing advantage of twice a day application.

1.9 Tetracyclines

The tetracyclines are another old class of natural product antibiotics. Until the development of doxycycline, they were used topically. However subsequent systemic application allowed for its use in the treatment in respiratory tract infections. Tetracyclines also inhibit protein synthesis by inhibiting acyl-tRNA transfer on the bacterial 30S ribosome. Tetracyclines have a fused linear tetracyclic structure and form chelation complexes with divalent cations such as calcium and thus its use has been limited to adults albeit with side effects. They usually serve as alternative to patients who are intolerant of macrolides or macrolide-resistant pathogens [12].

Tigecycline is the foremost drug in the new glycycline subclass of antibiotics. In spite of the fact that it is structurally identifies with minocycline, modifications to the molecule has brought about an expanded spectrum of activity and decreased susceptibility to the development of resistance when compared with other tetracycline antibiotics. Tigecycline has a broad spectrum of activity, including activity against drug-resistant Gram-positive organisms [17].

1.10 Macrolides

Macrolides which have erythromycin as its first member inhibit protein synthesis by binding to the 23S RNA of the bacterial 50S ribosomal subunit at the exit of the peptide synthesis tunnel. Macrolides are most widely adopted for respiratory

tract infections as they have targeted activity against respiratory pathogens. Macrolide antibiotics achieve high tissue and intracellular concentrations, which helps to address bacteria that are intracellular. In addition, they have strong anti-inflammatory properties [12]. Telithromycin belongs to a class of drugs described as the ketolides which varies slightly from the existing class of the macrolides. The characteristic feature of the ketolides as opposed to other macrolides is the removal of the neutral sugar, L-cladinose from the three position of the macrolide ring and the subsequent oxidation of the 3-hydroxyl to a 3-keto functional group. Telithromycin is indicated for the treatment of upper respiratory tract infections such as community acquired pneumonia and sinusitis. Introduction of telithromycin was opportune due to the rise of microbial resistance in the existing macrolides as it appears to be effective against macrolide-resistant bacteria such as macrolide-resistant *Streptococcus pneumoniae* [18].

1.11 Quinolones

The quinolones although not obtained from microbial source, are derived indirectly from natural products, i.e., as a by-product of chloroquine synthesis, which is in itself an analogue of the plant alkaloid quinine. It has established itself as useful in the treatment of urinary tract infections. The introduction of fluorine group to the core structure, give rise to the fluoroquinolones like ciprofloxacin and moxifloxacin yielded products of an improved spectrum and better pharmacokinetic profiles. However, resistance to the class of antibiotics whose mechanism of action is to inhibit bacterial DNA gyrase and topoisomerase IV and even newer fluoroquinolones has prompted the need for the development of new and effective agents [12]. Delafloxacin is a new anionic fluoroquinolone used for the treatment of acute bacterial skin and skin structure infections caused by Gram-positive and Gram-negative organisms including MRSA and *Pseudomonas aeruginosa*. As with all fluoroquinolones, resistance is mediated through mutations in the target enzymes and drug efflux. However, delafloxacin has greater stability against target enzyme mutations in Gram-positive bacteria relative to other fluoroquinolones. Its availability in infusion and oral formulations, stability and enhanced antibacterial potency in acidic environments and overall tolerability gives a potentially better antimicrobial agent in the treatment of other infections [19].

2. Anti-biofilm agents

2.1 Biofilms

Microorganisms have a strong tendency to become associated with surfaces [20]. Bacteria, thus, live in communities, adhering to surfaces of implanted medical devices or damaged tissues. On these surfaces they encase themselves in a hydrated matrix of polysaccharide and protein forming a slimy layer known as biofilms [21]. Biofilm is a microbial culture which is identified as cells permanently bound to an interface or to other cells and are firmly attached to matrix consisting of polymers produced as a result of phenotypic alteration due to growth rate or transcription of genes. Bacteria tend to form biofilms in environments with rapid flow of matter. Planktonic bacteria can adhere to surfaces and initiate biofilm formation in the presence of shear forces that are higher than those of heart valves and exceed Reynolds numbers of 5000. The Reynolds number has no dimension and describes the turbulent flow of a liquid. If it is high, turbulent flow exists but if it is low then laminar flow conditions prevail. It is speculated that turbulent flow enhances bacterial adhesion to surfaces and biofilm formation by impinging the planktonic cells on the surface [22].

Biofilms do not form only between same species of microorganism but there can be inter-species adhesions. Interspecies binding outside the oral cavity have been described as well, most notably between pathogenic and commensal microorganisms of the urinary tract. Co-aggregation between aquatic bacteria has been reported, with *Micrococcus luteus* being mentioned as a bridging organism in the development of aquatic biofilms owing to its ability to co-aggregate with many aquatic heterotrophs [20, 23].

2.2 Bacterial biofilm formation

Genes are responsible for noted biochemistry of living things and biofilm production is no exception. Changes in microbial colony formation and organization may be due to mutations in one or more of certain genes. Mutations in a gene called *wspF*, which is part of a putative chemosensory signal-transduction operon result in cell aggregation and altered colony morphology. The phenotypic characteristics of *WspF* depend on the presence of *WspR*, which is a member of a family of signal transduction proteins known as response regulators. *WspR* contains a glycine-glycine-aspartic acid-glutamic acid-phenylalanine (GGDEF) domain known to catalyze formation of a cytoplasmic signaling molecule cyclic diguanylate (c-di-GMP). Mass sequencing of genomes in bacteria detected the highly abundant protein domains GGDEF and Glutamic acid-Alanine-Leucine (EAl) [24]. These two protein domains are involved in the turnover of c-diGMP *in vivo*.

The GGDEF domain stimulates c-diGMP production whereas EAL stimulates its degradation. Increased cellular levels of c-diGMP has been observed to correspond to increased biofilm formation in a *wspF* mutant while increased levels of EAL catalyze degradation of c-diGMP and reversed the phenotypes of a *WspF* mutant and inhibited biofilm initiation by wild-type cells, indicating that the presence of c-diGMP is necessary for biofilm formation. The *psl* and *pel* operons, which are involved in exopolysaccharide production and biofilm formation, were expressed at high levels in a *WspF* mutant [25].

2.3 Stages of biofilm formation

The process of biofilm formation is complex, but generally identified as consisting of five stages.

2.3.1 The conditioning film

The conditioning layer is the foundation on which a biofilm grows, and can be composed of many particles, organic or inorganic. Via gravitational pull or direction of flow of the bulk fluid, particles rest and become integral component of conditional layer. Hence, the conditioning layer which is basically made up of organic matter provides anchorage and nutrients for bacterial growth [26].

2.3.2 Reversible adhesion

When plankton bacteria come into close proximity with the conditioning layer, it attaches using bacteria appendages such as flagella, fimbriae, and pili. A fraction of the cells reaching the surface reversibly adheres. However, the ability of the plankton to adhere is dependent on factors such as available energy, bacteria orientation, temperature and pressure in the immediate environment. If repulsive forces are greater than the attractive forces, the bacteria will detach from the surface [26].

2.3.3 Irreversible adhesion

A number of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed. It has been reported that the physical appendages of bacteria overcome the physical repulsive forces. Henceforth, the attachments do come in close proximity with the bulk lattice of the conditioning layer culminating in oxidation and hydration which strengthen the bacteria–surface bond. There is a data supporting the assertion that microbial adhesion intensely relies on the hydrophobic–hydrophilic nature of interacting surfaces [26].

2.3.4 Cell growth

With the adhered cells undergoing binary division, the resulting clonally expanded cells spread from the point of adhesion leading to clusters formation. Ideally, such growth occurring in the biofilm yields a mushroom-like arrangement. Nutrient supply to the bacteria present within the biofilm is made possible through the utilization of this mushroom-like assemblage. As the cells increase, they produce polysaccharide intercellular adhesion polymers which facilitate stronger attraction between adjacent cells [26, 27].

2.3.5 Final stages of biofilm development

As the bioburden increases, organisms enter into the stationary phase of growth cycle where the rate of cell division equals the rate of cell death. At this level of high population density, the bacteria cells interact with each other via signaling mechanisms called quorum sensing, a good explanation for the role of auto inducers in the stimulation of the mechanical and enzymatic procedures through genetic expression.

Death phase entails the collapse of the formed biofilm. The bacteria culture produce enzymes which catalyze the catabolism of the structural polysaccharides within the biofilm leading to exposure of the bacteria within the matrix to colonize new substrates [26, 28].

2.4 Antibiotic resistance due to biofilm formation

Inside biofilms, organisms resist antibiotic action by multicellular strategies, rather than the known genetic processes that involve plasmids, transposons and mutations that make individual cells resistant [29]. Biofilms are the root cause of many persistent chronic infections due to bacteria. Several organisms have been known to produce biofilms. In *P. aeruginosa*, it has been shown that the gene (*alg*C) that controls phosphomannomutase involved in alginate (exopolysaccharide) synthesis is up-regulated within minutes of adhesion to a solid surface. It has also been shown that *alg*D, *alg*U, *rpo*S and the genes controlling polyphosphokinase synthesis are all up-regulated during biofilm formation [22]. Multi-system that includes poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute the organisms' resistance to antibiotics in biofilms [29]. Organisms that have shown resistance to antibiotics as a result of biofilm production include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Pseudomonas pseudomallei*, and *Streptococcus sanguis* [22].

Susceptibility tests with *in vitro* biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture indicates a state of a high resistance to antibiotics [30]. Antibiotics used for the treatment of such bacterial infections suppress symptoms

of infections by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells still embedded in the biofilm. The biofilm, thus, offers protection to the organisms from the effects of the antibiotics. After the treatment course of the infection, the biofilm can act as a microbial repository for recurrence of infection. Biofilm infections can linger on for months, years, or even a lifetime as long as the colonized surface is not removed from the body [29].

In biofilms, the familiar mechanisms of antibiotic resistance such as efflux pumps, modifying enzymes, and target mutations do not account for the resistance of the organism and protection of bacteria in a biofilm. Even sensitive bacteria that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm. The fact that all these antibiotic resistance mechanisms are inherently multicellular helps to explain why bacteria dispersed from biofilms rapidly revert to a susceptible phenotype [31]. Although they are less common, fungal biofilms are also found on implanted medical devices. *Candida albicans* biofilms are drastically very resistant to most antifungal drugs, and are a major cause of morbidity in blood-stream infections [32, 33].

2.5 Other effects of biofilms

Biofilms can be both beneficial and detrimental. They are beneficial in the degradation of environmental hazardous substances in the soil, in a bioreactor and as bio-flocculants in the separation of coal particles from associated mineral matter. They are detrimental on food and slaughterhouse equipment, ship hulls, biomaterials implants, and in the oral cavity [20]. They are implicated in otitis media [34], otolaryngologic infections [35], osteomyelitis [36], bacterial endocarditis [37], cystic fibrosis and nonhealing wounds [38].

When organisms are in biofilms, they tend to cause infections with similar features although there could be significant variations in the causative organisms. Importantly, bacterial biofilms tend to bypass host defense mechanism and can withstand drug treatment irrespective of the competence of the individual's immune system. Actually, tissues adjacent to the biofilm might undergo collateral damage by immune complexes and invade the neutrophils [29]. Biofilms have also been documented as the major sources of infection by *Candida albicans*, specifically in view of the vast number of biomaterials that are now being used in the medical industry. Biomaterials serve as ideal substrates for microbial adhesion and eventual biofilm formation. Such materials include stents, catheters, and orthopedic joints [39].

2.6 Biofilm formation inhibitors

Prevention of biofilm formation or countering the resistance mechanisms due to biofilms may simplify the treatment of infections caused by biofilm producing organisms and bring back the usefulness of antibiotics that are out of use due to biofilm resistance [29]. Many substances, both natural and synthetic, have been found to inhibit biofilm formation. For example, silver nanoparticles (AgNPs) effectively prevent the formation of biofilms and kill bacteria in established biofilms produced by clinical strains including *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus mutans*. Anti-biofilm formation activity of AgNPs was more pronounced on Gram-negative than Gram-positive bacteria although both groups exhibit equal antibacterial activity to the substance. AgNPs also inhibit biofilm production by *Candida albicans* [40]. The helical human cathelicidin LL-37 exhibits effective antimicrobial, anti-attachment, and anti-biofilm activity against *Staphylococcus aureus* [41]. Some plants have also been found to inhibit biofilm formation. Extracts of the sticks of *Salvadora persica*

has been reported for its antibacterial and anti-biofilm activities against *Streptococcus mutans* [42]. Essential oils of lemon grass have biofilm inhibition activity against *S. aureus* [43] while the essential oils of Boswellia spp. (*B. papyrifera* and *B. rivae*) are active against staphylococcal and *C. albicans* biofilms [44].

2.7 Detection of biofilms

Various methods have been used to determine the presence of biofilms produced by bacteria. The methods include tissue culture plate method (TCP), tube method (TM), Congo red agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination. In the tube method a loopful of overnight culture of the test organism is inoculated into trypticase soy broth containing 1% glucose and incubated. The bacterial cultures are poured out of the solution and the tubes washed using phosphate buffer saline and dried. The dried tubes are stained with 0.1% crystal violet with the excess stained washed with deionized water. The tubes are dried while inverted with observation made for biofilm formation which confirmed by the presence of visible film lining the wall as well as the bottom of the tube. If there is an observed ring-like development at the liquid interface it does not indicate biofilm formation [45]. In the Congo red agar method, the test organism is grown in red Congo agar on Brain heart infusion broth containing Congo red dye (0.8 g/L). The appearance of black colonies with a dry crystalline consistency indicated biofilm formation [45, 46]. In the tissue culture plate method a 24-h broth culture of the test organism is inoculated into trypticase soy broth (TSB). This primary inoculum is then inoculated into TSB with 1% glucose prepared in different dilutions (1:20, 1:40, 1:80 and 1:100) and loaded into 96 wells flat bottom microtiter plate, which is an abiotic surface. The plate is then incubated at 37°C for 24 h. The content of the wells are decanted and washed with phosphate buffer, fixed with methanol for 15 min and decanted. They are then stained with crystal violet (0.5%) for 20 min, decanted and washed with distilled water. Glacial acetic acid is then added to extract the crystal violet and the optical density determined at 490 nm using an ELISA plate reader [45].

3. Antibacterial natural products

3.1 Introduction

Natural products play a significant role in the discovery of lead compounds for the development of drugs for the treatment of human diseases. The importance to medicine of natural product molecules lies not only in their pharmacological or chemotherapeutic effects but also in their role as template molecules for the production of new drug substances. Nature in one way or another continues to influence the design of small molecules and most of the antibacterial drugs in clinical usage are naturally derived.

Natural products have the ability to provide diversity, complexity, novelty and new scaffolds with various chiral centers, rings, bridges and functional groups in the molecule [47]. They differ from synthetic compounds by having more oxygen atoms and stereochemical elements such as polycycle (often bridged) carbon skeletons [48, 49]. Some of the most valuable products and promising leads in oncology were naturally derived or naturally inspired. For instance paclitaxel a chemically established drug came from natural sources. Doxorubicin, camptothecins, and tamoxifen derived from natural product leads, steroid hormones. Most of the promising pipeline candidates in oncology all arose from natural products screening followed by synthetic modifications.

3.2 Examples of antibacterial natural products

Antibacterial natural compounds include secondary metabolites isolated from plants, bacteria, fungi, marine organisms and algae. These compounds are categorized based on their chemical type as terpenes (sesquiterpenes, diterpenes, sesterterpenes, and triterpenes), steroids (sterols), alkaloids (indole, quinoline, pyridoacridone, and amine alkaloids), aromatics (flavonoids, chalcones, coumarins, lignans, xanthones, anthracenes, anthraquinones, naphthalene), polyketides (acetylenic fatty acids, polycyclic esters and quinones), and peptides. Sometimes, the categorization tends to put together structurally relevant natural products with low bioactivity and also those synthetic analogues with remarkable antibacterial activity [47].

Since there is a continual need for a pipeline of new agents to combat multidrug-resistant bacteria, it is important the search goes on especially from plant materials. Microbially derived products, of which there are many first class drug examples which can be readily fermented with few re-supply issues [50]. The value of natural products as a screening resource has recently been highlighted and it is likely the focus is on plants, microbes and marine organisms [51].

Examples of antibacterial natural products include cranberry juice *Vaccinium macrocarpon* (family: Ericaceae) which is used in the management of urinary tract infections and the prevention of recurrent cystitis. Berberine has antibacterial activity against various strains of methicillin resistant *Staphylococcus aureus* (MRSA). Thymol is used as an antiseptic. Bearberry, *Arctostaphylos uva-ursi* L. (family: Ericaceae) is an antimicrobial agent used particularly for urinary tract infections against several organisms including *Bacillus subtilis, Escherichia coli, Mycobacterium smegmatis, S. aureus* and *Shigella* spp. and the antibacterial activity is attributed to arbutin. Lemon balm, *Melissa officinalis* (family: Lamiaceae) and tea tree, *Melaleucae alternifolia* (family: Myrtaceae) are taken as herb teas and tea tree oil is applied as ointment [51]. Garlic, *Allium sativum* (family: Alliaceae) have antimicrobial and antiseptic properties and is used for respiratory tract infections [48, 49] .

Tannins and resins produced by plants have antimicrobial and wound-healing properties. The essential oil constituents of plants also possess antimicrobial activity. Plants produce phytoalexins in response to infections caused by fungi viruses and bacterial that may infect them. Resveratrol is an example of an antifungal phytoalexin, which has anticancer, antioxidant and cardioprotective benefits for humans. Plant metabolites with antibacterial properties include anti-staphylococcal activities of the acylphloroglucinols and terthiophenes [47].

The quest for newer and potent antimicrobial agents has ventured into studies on plants in this research driven direction. It, however, makes a cogent argument since plants are known to produce varied chemicals for defense purposes against microorganisms. Also they produce cytotoxic compounds some of which have been successfully utilized as chemotherapeutic agents and hence give a laudable reason to continue with search for new treatment protocols for man. There is also an ecological rationale for the production of natural products that modify microbial resistance. Plants may have evolved compounds which evade MDR mechanisms and that plant antimicrobials might be developed into broad-spectrum antibiotics in combination with inhibitors of MDR [52]. These MDR proteins are commonly found in nature as efflux pumps for foreign toxic substances, as they are in clinical isolates of resistant pathogens.

In the food industry, herbs, spices and essential oils are chiefly employed as preservatives of foods. A number of plants and spices are used as antimicrobial agents for killing and decreasing pathogenic bacterial load in foods hence improving its quality. These plant-based antimicrobials are produced from various extraction

techniques using the flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots of various plants [50, 51].

4. Non-medicinal antibacterial chemicals

An ideal antibacterial agent (medicinal or non-medicinal) tends to have characteristics, which include ability to effectively inhibit or kill bacteria. Also it must have appreciable solubility either in water or any suitable solvent [53]. Antibacterial chemicals should be stored for a reasonable period of time with no significant loss of antibacterial action [54]. Such preparation must also be homogeneous with the active ingredients present in each application of the non-medicinal chemical against bacteria. An antibacterial chemical should be minimally inactivated when exposed to extraneous material such as proteins and other organic materials found in substances they are being utilized in treating. This decreases the amount of the agent the bacteria are exposed to [55, 56]. Antibacterial chemicals should not necessarily require elevated temperatures beyond that of the environments they are being used [57]. Again, such ideal antibacterial chemicals do possess cleansing properties, must be either odorless or have pleasant odor [58] and should be physically safe on surfaces they are used on.

The antimicrobial activities of these chemicals are often strongly influenced by the biocide affinity for the structural or molecular components of the cell, which can, in turn, depend on the attraction of dissimilar charges or hydrophobic interactions [53, 59]. It is henceforth convenient to consider the modes of action based on biocides target on the bacteria. Some probable drug target to be exploited include the cell wall, cell membrane and the cytoplasm. The precise mechanism of antimicrobial action of some of these agents will be discussed in this section as well as unraveling the concentration-dependent multiplicity in action of some of these agents [57].

4.1 Alcohols

Two water-soluble alcohols, i.e., ethyl alcohol and isopropyl alcohol, are normally employed as disinfectants due to their high germicidal activity [60]. They have rapid bactericidal and bacteriostatic activity against vegetative forms of bacteria. Although not active against bacterial spores, their tuberculocidal, fungicidal and virucidal activity are also apparent. Their cidal activity drops sharply when diluted below 50% concentration, and the optimum bactericidal concentration is 60–90% v/v solutions in water [60, 61]. Protein denaturation is the most likely cause of the alcohol action. This assertion is affirmed by the realization of the reduced bactericidal activity of absolute ethyl alcohol when compared with hydroalcohol due to the requirement of water to facilitate the protein denaturation process [62, 63]. This observation is also in conformation with the observed destruction of dehydrogenases of *Escherichia coli* by alcohol [64] and also the enhancement of the lag phase of *Enterobacter aerogenes* by alcohol [65] which is however reversed with some amino acids. Blockade of metabolites required for cell division has been linked to the bacteriostatic action of alcohol.

The role of hydrophobic alkyl groups of long-chain alcohols in antibacterial activity against *Staphylococcus aureus* and *Propionibacterium acnes* has been established [66]. The maximum activity was found to be dependent on the chain length from the hydrophilic hydroxyl group, and also the test bacteria. The antimycobacterial activities of alcohols with chain length ranging from C_5 to C_{13} against *Mycobacterium smegmatis* mc^2155 and *M. tuberculosis* $H_{37}R_v$ have been established, with best activity found with alcohol with C_{10} chain length [67]. This bactericidal activity is attributed

to decanol's potential to harm the robust and complex cell envelope of *Mycobacteria* and its ability to reduce biofilm formation by *M. smegmatis* [67].

4.2 Aldehydes

The aqueous solutions of some aldehydes, such as formaldehyde and glutaraldehyde, have been found to be bactericidal, tuberculocidal, fungicidal, virucidal, and sporicidal [68–70]. Formaldehyde inactivates microorganisms by alkylating the amino and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases [71]. The biocidal activity of glutaraldehyde results from its alkylation of sulfhydryl, hydroxyl, carboxyl, and amino groups of microorganisms, which alters RNA, DNA, and protein synthesis [72].

Studies have established that different concentrations of formaldehyde have deleterious effect on a lot of microorganisms. It has been realized that 8% formalin can inactivate poliovirus in 10 min although other viruses are inactivated by as low as 2% formalin [68]. Tuberculocidal potential can be seen with 4% formalin with the agent being able to inactivate 10^4 *Mycobacterium tuberculosis* cells with 2 min [69]. Also, about 10 million *Salmonella typhi* cells can be inactivated by 2.5% formaldehyde within 10 min even in the presence of organic matter [70]. However, formaldehyde has significantly reduced sporicidal activity than glutaraldehyde as seen from experimental studies [69].

Some aliphatic saturated and unsaturated aldehydes are produced by enzymatic cleavage of unsaturated fatty acids when plants undergo microbial attack; thus they may be one of the multichemical defense mechanisms used by several fruits to resist invasion by microorganisms [73]. In this direction, Trombetta and colleagues established the mechanism of the antimicrobial activity of aliphatic α,β-unsaturated aldehydes which included (E)-2-hexenal **(1)**, (E)-2-eptenal **(2)**, (E)-2-octenal **(3)**, (E)-2-nonenal **(4)**, (E)-2-decenal **(5)** and (E,E)-2,4-decadienal **(6)** (**Figure 1**). Their findings suggested that the 2E-alkenals tested elicit, very likely, a gross perturbation of the lipidic fraction of plasma membranes and are able to penetrate into bacterial cells.

The mechanisms of antimicrobial action of other aldehydes, such as *o*-phthalaldehyde **(7)**, are likely to involve interaction with the cytoplasmic membrane and increase in its permeability [74, 75]. *o*-Phthalaldehyde also appears to kill spores by blocking the spore germination process [76]. Although membrane functional proteins are generally supposed to be the potential targets toward which aldehydic antimicrobial agents are directed, other mechanisms of action/interaction can help explain their antimicrobial activity [73].

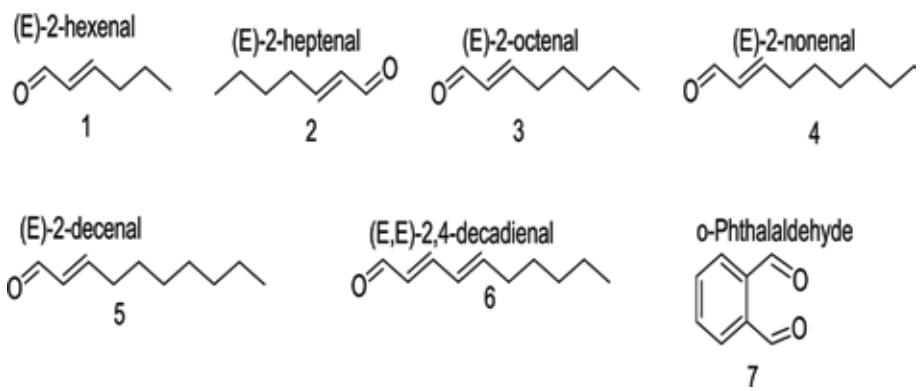

Figure 1.
Aldehydes with antibacterial properties.

4.3 Halogens

Over the years, tinctures and solutions of iodine have been used mainly as antiseptic agents on skin surfaces and other tissues. However, iodophors such as povidone iodine, are employed mainly as antiseptics and disinfectants. Such iodophors tend to possess germicidal ability but generally do not stain surfaces as well as do possess less toxic and irritant potential [77]. Iodine has the potential to penetrate the cell wall of microorganisms rapidly and tend to compromise proteins and nucleic acids as well as the synthesis of proteins.

Chlorine and products of chlorine do have broader spectrum of activity but they also leave behind toxic residues. They however are not affected by hardness of water, are less expensive and do possess faster onset of action [78]. They tend to possess activity on organisms fixed to surfaces and biofilms [79] with accompanied low risk of toxicity [80]. Its mechanism of antimicrobial action is associated with hypochlorous acid (HOCl) it yields. When HOCl dissociates, it produces hypochlorite ion (OCl^-) which has reduced antimicrobial activity and the process is pH-dependent [81]. It has not been mechanistically established how chlorine specifically kills microorganisms. However, chlorine tends to cause oxidation of sulfhydryl enzymes and amino acids leading to loss of intracellular contents with reduced nutrition uptake by cells. Also, chlorine tends to reduced adenosine triphosphate production as well as inhibition of DNA synthesis and increased DNA destruction [81]. A number of these process may account for the mechanism of antimicrobial action of chlorine [82].

4.4 Oxidizing agents

Peroxyacetic acid **(1)**, chlorine dioxide **(2)** (**Figure 2**), and hydrogen peroxide have similar mechanism of antimicrobial action (chemical oxidation of cellular components), but they do vary greatly in their efficacy against microorganisms [83, 84]. Biochemically, there is significant variations in these agents with resultant variation in their outcomes on macromolecules. This attests to the variations in their biocidal activity, most especially between liquid and gas peroxide [84].

Administration of hydrogen peroxide results in the production of hydroxyl free radicals which attack lipid membranes, DNA and essential cell components leading to cell death. Aerobic organisms and facultative anaerobes tend to produce catalase and these enzymes offer protective benefits from hydrogen peroxide by catalyzing its conversion to water and oxygen. Such protective benefits are compromised by the concentrations of oxidizing agents administered as disinfectant [85]. Peracetic acid, which has rapid action on all microorganisms, does not yield any harmful products when it is broken down and is easily cleared from organic materials without leaving behind any residues [86]. In addition to its potent activity in the presence of organic matter, it retains sporicidal activity even at low temperatures.

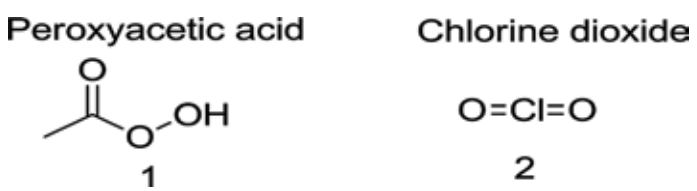

Figure 2.
Oxidizing agents with antibacterial activity.

However, its specific mechanism of antimicrobial action has not been unraveled although postulated to be working in the same manner as other oxidizing agents. Hence possibly through oxidation of sulfhydryl and sulfur bonds in proteins, enzymes and other cellular metabolites [85].

4.5 Phenolic compounds

Phenolic compounds are produced from structural modification of phenol by addition of alkyl, phenyl, benzyl groups, and halogen as substituent on the aromatic ring. *o*-Phenylphenol **(1)** and *o*-benzyl-*p*-chlorophenol **(2)** are commonly employed phenol derivatives in disinfectants. Phenolic compounds tend to have enhanced antimicrobial potential than phenol [87].

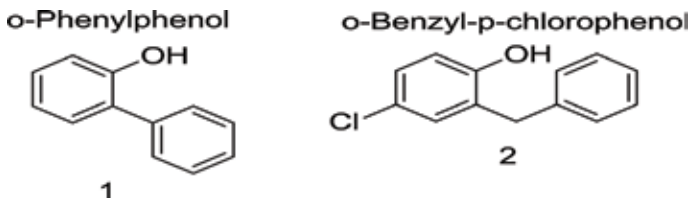

Figure 3.
Phenolic antibacterial chemicals.

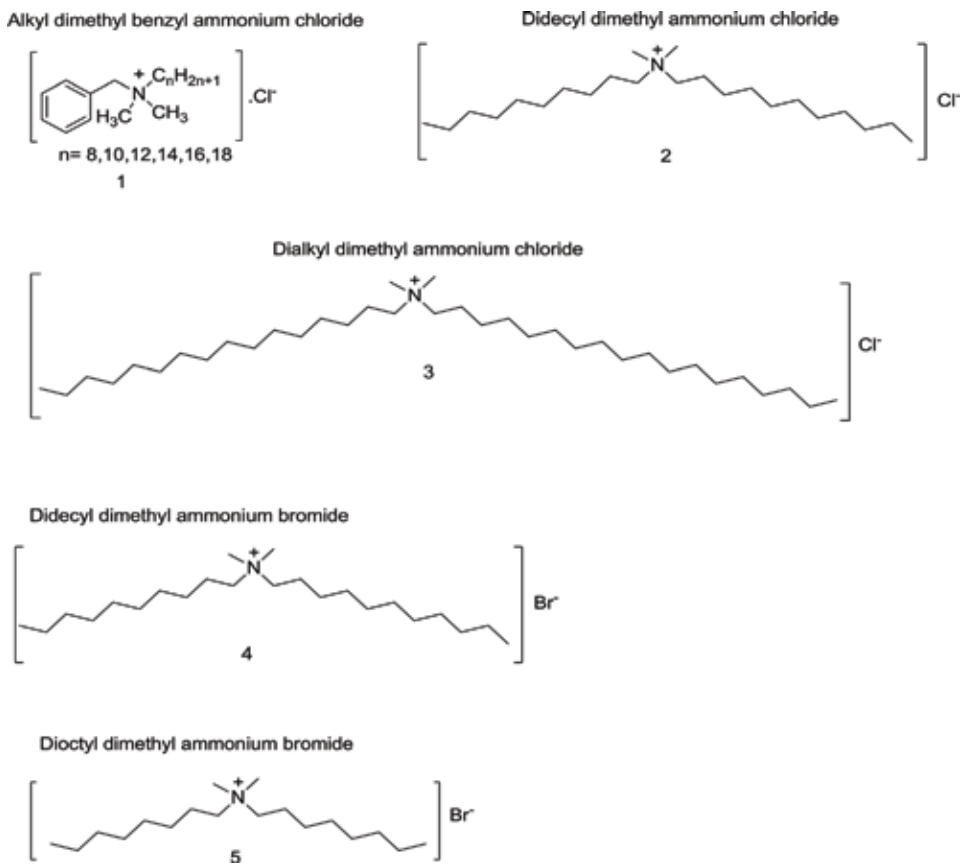

Figure 4.
Quaternary ammonium compounds with antibacterial activity.

Phenol acts as a penetrating and disrupting molecule on cell walls of organisms as well as increase precipitation in important proteins within cells leading to cell death at high concentrations. However, phenol and high molecular weight phenolic compounds tend to kill bacteria cells via enzyme inactivation and compromising of cell wall at lower concentration (**Figure 3**) [88].

4.6 Quaternary ammonium compounds

One of the most used disinfectants is the quaternary ammonium compounds. When contaminated quaternary ammonium compounds are employed in patient-care supplies, they contribute to significant proportion of its associated infections [89]. Gram-negative bacteria are documented to survive in this group of disinfectants [90].

The quaternary compounds are ammonium-derived moieties with nitrogen atom with a valence of 5. There are four alkyl or heterocyclic radical substitutes and a fifth halide, sulfate or similar radical substitute in the structure [91]. Some of these quaternaries include alkyl dimethyl benzyl ammonium chloride **(1)**, alkyl didecyl dimethyl ammonium chloride **(2)**, and dialkyl dimethyl ammonium chloride **(3)** (**Figure 4**). The innovative quaternary ammonium compounds (i.e., fourth generation), referred to as twin-chain or dialkyl quaternaries (e.g., didecyl dimethyl ammonium bromide **(4)** and dioctyl dimethyl ammonium bromide **(5)** (**Figure 4**)), have been reported to be active even in hard water and can withstand anionic residues [92]. Quaternaries are believed to be bactericidal due to their ability to inactivate energy-producing enzymes and also denature and disrupt cell proteins and membrane respectively [91, 92].

5. Conclusion

A multidisciplinary approach to antimicrobial drug discovery, involving the generation of novel molecular diversity from natural product sources, combined with total and combinatorial synthetic methodologies, and including the manipulation of biosynthetic pathways will continue to provide the best approach to antibiotic discovery and development and also overcome the challenges associated with antimicrobial resistance.

Conflict of interest

Authors declare no conflict of interest.

DOI: http://dx.doi.org/10.5772/intechopen.82560

Author details

Yaw Duah Boakye[1], Newman Osafo[2], Cynthia Amaning Danquah[2], Francis Adu[1] and Christian Agyare[1*]

1 Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

2 Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

*Address all correspondence to: cagyare.pharm@knust.edu.gh

References

[1] Becker DE. Antimicrobial drugs. Anesthesia Progress. 2013;**60**(3):111-123

[2] Bergand T. Pharmacokinetics of beta lactam antibiotics. Scandinavian Journal of Infectious Diseases. Supplementum. 1984;**42**:83-98

[3] Kuriyama T, Karasawa T, Nakagawa K, Yamamoto E, Nakamura S. Incidence of β-lactamase production and antimicrobial susceptibility of anaerobic Gram-negative rods isolated from pus specimens of orofacial odontogenic infections. Oral Microbiology and Immunology. 2001;**16**(1):10-15

[4] Mehta D, Sharma AK. Cephalosporins: A review on imperative class of antibiotics. Inventi Rapid/Impact: Molecular Pharmacology. 2016;**1**:1-6

[5] Laudano JB. Ceftaroline fosamil: A new broad-spectrum cephalosporin. Journal of Antimicrobial Chemotherapy. 2011;**66**(3):11-8

[6] Cluck D, Lewis P, Stayer B, Spivey J, Moorman J. Ceftolozane-tazobactam: A new-generation cephalosporin. 2015;**72**:2135-2146

[7] Reynolds P. Structure biochemistry and mechanism of action of glycopeptide antibiotics. European Journal of Clinical Microbiology & Infectious Diseases. 1989;**8**(11):943-950

[8] Stein G. Oritavacin: A long-half life lipoglycopeptide. Clinical Infectious Diseases. 2015;**61**(4):627-632

[9] Karrine D, Ryback J. Oritavancin: A new lipoglycopeptide antibiotic in the treatment of Gram-positive infection. Infectious Disease and Therapy. 2016;**16**:1-15

[10] Saravolatz LD, Stein GE, Johnson LB. Telavancin: A novel lipoglycopeptide. Clinical Infectious Diseases. 2009;**49**(12):1908-1914

[11] Chen AY, Zervos MJ, Vazquez JA. Dalbavancin: A novel antimicrobial. International Journal of Clinical Practice. 2007;**5**(May):853-863

[12] Fernandes P, Martens E. Antibiotics in late clinical development. Biochemical Pharmacology. 2017;**133**:152-163

[13] Burdette SD, Trotman R. Tedizolid: The first once-daily oxazolidinone class antibiotic. Agents. 2018;**61**(June):1315-1321

[14] Mullane K, Gorbach S. Fidaxomicin: First-in-class macrocyclic antibiotic. Expert Review of Anti-Infective Therapy. 2011;**9**(7):767-778

[15] Gupta A. Plazomicin: A step toward next generation aminoglycosides. Review. Asian Journal of Research in Pharmaceutical Sciences. 2017;**7**(3):1-8

[16] Paukner S, Riedl R. Pleuromutilins: Potent drugs for resistant bugs—Mode of action and resistance. Cold Spring Harbor Perspectives in Medicine. 2017;**7**(1).pii:a027110

[17] Greer ND. Tigecycline (Tygacil): The first in the glycylcycline class of antibiotics. Proceedings (Baylor University, Medical Center). 2006;**19**(2):155-161

[18] Ackermann G, Rodloff AC. Drugs of the 21st century: Telithromycin (HMR 3647)—The first ketolide. The Journal of Antimicrobial Chemotherapy. 2003;**51**:497-511

[19] Mogle BT, Steele JM, Thomas SJ, Bohan KH, Kufel WD. Clinical review of delafloxacin: A novel anionic fluoroquinolone. The Journal

of Antimicrobial Chemotherapy. 2018;**73**(6):1439-1451

[20] Bos R, Van Der MHC, Busscher HJ. Physico-chemistry of initial microbial adhesive interactions its mechanisms and methods for study. FEMS Microbiology Reviews. 1999;**23**(2):179-230

[21] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;**358**(9276):135-138

[22] Donlan RM, Costerton JW, Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. 2002;**15**(2):167-193

[23] Reid G, Cook L, McGroarty JA, Angotti R. Lactobacillus inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Canadian Journal of Microbiology. 1988;**34**(3):344-351

[24] Simm R, Morr M, Kader A, Nimtz M, Römling U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Molecular Microbiology. 2004;**53**(4):1123-1134

[25] Hickman JW, Tifrea DF, Harwood CS. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**(40):14422-14427

[26] Banerjee P, Singh M, Sharma V. Biofilm formation: A comprehensive review. 2015;**3**(2):556-560

[27] Hall-Stoodley P. Developmental regulation of microbial biofilms. Current Opinion in Biotechnology. 2002;**13**:228-233

[28] Dunne WM. Bacterial adhesion: Seen any good biofilms lately? 2002;**15**:155166

[29] Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. International Journal of Medical Microbiology. 2002;**292**(2):107-113

[30] Ceri H, Olson ME, Stremick C, Read RR, Morck D. The calgary biofilm device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. Journal of Clinical Microbiology. 1999;**37**(6):1771-1776

[31] Walsh C. Molecular mechanisms that confer antibacterial drug resistance. Nature. 2000;**406**:775-781

[32] Douglas LJ. Candida biofilms and their role in infection. Trends in Microbiology. 2003;**11**(1):30-36

[33] Wesenberg-Ward KE, Tyler BJ, Sears JT. Adhesion and biofilm formation of Candida albicans on native and pluronic-treated polystyrene. Biofilms. 2005:63-71

[34] Bakaletz LO. Biofilms in otitis media: Evidence and relevance. The Pediatric Infectious Disease Journal. 2007;**26**(10):S17-S19

[35] Post JC, Stoodley P, Hall L, Ehrlich GD. The role of biofilms in otolaryngologic infections. Current Opinion in Otolaryngology & Head and Neck Surgery. 2004;**12**(3):185-190

[36] Brady RA, Leid JG, Calhoun JH, Costerton JW, Shirtliff ME. Osteomyelitis and the role of biofilms in chronic infection. FEMS Immunology and Medical Microbiology. 2008;**52**(1):13-22

[37] Høiby N. Understanding bacterial biofilms in patients with cystic fibrosis: Current and innovative approaches to potential therapies. Journal of Cystic Fibrosis. 2002;**1**:249-254

[38] James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J,

et al. Biofilms in chronic wounds. Wound Repair and Regeneration. 2008;**16**(1):37-44

[39] Ramage G, Saville SP, Wickes BL, López-ribot JL. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. Applied and Environmental Microbiology. 2002;**68**(11):5459-5463

[40] Martinez-Gutierrez F, Boegli L, Agostinho A, Sánchez EM, Bach H, Ruiz F, et al. Anti-biofilm activity of silver nanoparticles against different microorganisms. Biofouling. 2013;**29**(6):651-660

[41] Dean SN, Bishop BM, Van HML. Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. BMC Microbiology. 2011;**11**:114

[42] Al-sohaibani S, Murugan K. Anti-biofilm activity of *Salvadora persica* on cariogenic isolates of *Streptococcus mutans*: *In vitro* and molecular docking studies. Biofouling. 2012;**28**(1):29-23

[43] Adukwu EC, Allen SCH, Phillips CA. The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. Journal of Applied Microbiology 2012;**113**(5):1217-2

[44] Schillaci D, Arizza V, Dayton T, Camarda L, Stefano VD. *In vitro* anti-biofilm activity of Boswellia spp. oleogum resin essential oils. Letters in Applied Microbiology. 2008;**47**(5):433-438

[45] Deka N. Comparison of tissue culture plate method , tube method and congo red agar method for the detection of biofilm formation by coagulase negative Staphylococcus isolated from non-clinical isolates. International Journal of Current Microbiology and Applied Sciences. 2014;**3**(10):810-815

[46] Kaiser TD, Pereira EM, Dos Santos KR, Maciel EL, Schuenck RP, Nunes AP. Modification of the Congo red agar method to detect biofilm production by *Staphylococcus epidermidis*. Diagnostic Microbiology and Infectious Disease. 2013;**75**(3):235-239

[47] Gibbons S. Anti-staphylococcal plant natural products. Natural Product Reports. 2004;**2**:263-277

[48] Danquah CA, Kakagianni E, Khondkar P, Maitra A, Malkinson J, Bhakta S, et al. Analogues of disulfides from *Allium stipitatum* demonstrate potent anti-tubercular activities through drug efflux pump and biofilm inhibition. 2018, 1150;**8**(1):18

[49] O'Donnell G, Poeschl R, Zimhony O, Gunaratnam M, Moreira JBC, Neidle S, et al. Bioactive pyridine-N-oxide disulfides from *Allium stipitatum*. Journal of Natural Products. 2009;**72**(3):360-365

[50] Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of Natural Products. 2012;**75**(3):311-335

[51] Heinrich M, Barnes J, Gibbons S, Williamson EM. Fundamentals of Pharmacognosy and Phytotherapy. Edinburgh: Churchill Livingstone/ Elsevier; 2012

[52] Tegos G, Mylonakis E, editors. Antimicrobial Drug Discover: Emerging Strategies. Wallingford: CABI; 2012

[53] McDonnell G, Russel AD. Antiseptics and disinfectants: Activity, action, and resistance. . 1999;**12**(1):147179

[54] Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews. 2010;**74**(3):417-433

[55] Marriott NG. Principles of Food Sanitation. P. 143. Gaithersburg, MD: Aspen Publishers, Inc; 1999. p. 364

[56] Cords BR, Burnett SL, Hilgren J, Finley M, Magnuson J. Sanitizers: Halogens, surface-active agents, and peroxides. In: Davidson PM, Sofos J, Branen AL, editors. Antimicrobials in Food. 3rd ed. Boca Raton: Taylor and Francis; 2005

[57] Kahrilas GA, Blotevogel J, Stewart PS, Borch T. Biocides in hydraulic fracturing fluids: A critical review of their usage, mobility, degradation, and toxicity. Environmental Science & Technology. 2015;**49**(1):16-32

[58] Lambert D. Topical deodorant. In: Baran R, Maibach HI, editors. Textbook of Cosmetic Dermatology. 2nd ed. London, UK: Martin Dunitz Ltd; 1998. p. 172

[59] Denyer SP, Hodges NA, Gorman SP. Non-antibiotic antibacterial agents: Mode of action and resistance. In: Hugo and Russell's Pharmaceutical Microbiology. 7th ed. Oxford, UK: Blackwell Publishing Company; 2008. p. 309

[60] Spaulding EH. Alcohol as a surgical disinfectant: Pros and Cons of a much dicussed topic. AORN Journal. 1964;**2**:67-71

[61] Morton HE. The relationship of concentration and germicidal efficiency of ethyl alcohol. Annals of the New York Academy of Sciences. 1950;**53**(1):191-196

[62] Ali Y, Dolan MJ, Fendler EJ, Larson EL. Alcohols. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 229-254

[63] Morton HE. Alcohols. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lea & Febiger; 1983. pp. 225-239

[64] Sykes G. The influence of germicides on the dehydrogenases of Bact. coli. Part I. The succinic acid dehydrogenase of Bact. coli. (London). 1939;**39**(4):463-469

[65] Dagley S, Dawes EA, Morrison GA. Inhibition of growth of Aerobacter aerogenes: The mode of action of phenols, alcohols, acetone and ethyl acetate. Journal of Bacteriology. 1950;**60**:369-378

[66] Kubo I, Muroi H, Himejima M, Kubo A. Antibacterial activity of long-chain alcohols: The role of hydrophobic alkyl groups. Bioorganic & Medicinal Chemistry Letters. 1993;**3**(6):1305-1308

[67] Mukherjee K, Tribedi P, Mukhopadhyay B, Sil AK. Antibacterial activity of long-chain fatty alcohols against mycobacteria. FEMS Microbiology Letters. 2013;**223**(2):177-183

[68] Klein M, DeForest A. The inactivation of viruses by germicides. Chemical Specialties Manufacturers Association Proceedings. 1963;**49**:116-118

[69] Rubbo SD, Gardner JF, Webb RL. Biocidal activities of glutaraldehyde and related compounds. Journal of Applied Microbiology. 1967;**30**:78-87

[70] McCulloch EC, Costigan S. A comparison of the efficiency of phenol, liquor cresolis, formaldehyde, sodium hypochlorite and sodium hydroxide against *Eberthella typhi* at various temperatures. The Journal of Infectious Diseases. 1936;**59**:281-284

[71] Favero MS, Bond WW. Chemical disinfection of medical and surgical materials. In: Block SS, editor. Disinfection, Sterilization, and

Preservation. Philadelphia: Lea & Febiger; 1991. pp. 617-641

[72] Scott EM, Gorman SP. Glutaraldehyde. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 361-381

[73] Trombetta D, Saija A, Bisignana G, Arena S, Caruso S, Mazzanti G, et al. Study on the mechanisms of the antibacterial action of some plant α,β-unsaturated aldehydes. Letters in Applied Microbiology. 2002;**35**:285-290

[74] Simons C, Walsh SE, Maillard JY, Russell AD. A note: Ortho-phthalaldehyde: Proposed mechanism of action of a new antimicrobial agent. Letters in Applied Microbiology. 2000;**31**:299-302

[75] Tsuchiya H. Biphasic effects of acetaldehyde-biogenic amine condensation products on membrane fluidity. The Journal of Pharmacy and Pharmacology. 2001;**53**:121-127

[76] Cabrera-Martinez RM, Setlow B, Setlow P. Studies on the mechanisms of the sporicidal action of ortho-phthalaldehyde. Journal of Applied Microbiology. 2002;**92**:675-680

[77] Gottardi W. Iodine and iodine compounds. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 159-184

[78] Rutala WA, Weber DJ. Uses of inorganic hypochlorite (bleach) in health-care facilities. Clinical Microbiology Reviews. 1997;**10**:597-610

[79] Merritt K, Hitchins VM, Brown SA. Safety and cleaning of medical materials and devices. 2000;**53**:131-136

[80] Heidemann SM, Goetting MG. Treatment of acute hypoxemic respiratory failure caused by chlorine exposure. 1991;**7**:87-88

[81] Dychdala GR. Chlorine and chlorine compounds. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 135-157

[82] Gerba CP, Rusin P. Relationship between the use of antiseptics/disinfectants and the development of antimicrobial resistance. In: Rutala WA, editor. Disinfection, Sterilization and Antisepsis: Principles and Practices in Healthcare Facilities. Washington, DC: Association for Professional in Infection Control and Epidemiology; 2001. pp. 187-194

[83] Denyer SP, Stewart GSAB. Mechanisms of action of disinfectants. International Biodet and Biodegrad. 1998;**41**:261-268

[84] Finnegan M, Linley E, Denyer SP, McDonnel G, Simons C, Maillard J-Y. Mode of action of hydrogen peroxide and other oxidizing agents: Differences between liquid and gas forms. The Journal of Antimicrobial Chemotherapy. 2010;**65**(10):2108-2115

[85] Block SS. Peroxygen compounds. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 185-204

[86] Tucker RC, Lestini BJ, Marchant RE. Surface analysis of clinically used expanded PTFE endoscopic tubing treated by the steris process. ASAIO Journal. 1996;**42**:306-313

[87] Kahn G. Depigmentation caused by phenolic detergent germicides. Archives of Dermatology. 1970;**102**:177-187

[88] Prindle RF. In: Block SS, editor. Phenolic compounds, Disinfection, Sterilization, and Preservation. Philadelphia: Lea & Febiger; 1983. pp. 197-224

[89] Ehrenkranz NJ, Bolyard EA, Wiener M, Cleary TJ. Antibiotic-sensitive *Serratia marcescens* infections complicating cardiopulmonary operations: contaminated disinfectant as a reservoir. Lancet. 1980;**2**:1289-1292

[90] Rutala WA, Cole EC. Antiseptics and disinfectants—Safe and effective? Infection Control. 1984;**5**:215-218

[91] Sykes G. Disinfection and Sterilization. London: E & FN Spon Ltd; 1965

[92] Merianos JJ. Surface-active agents. In: Block SS, editor. Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 283-320

Chapter 6

The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents

Sahra Kırmusaoğlu

Abstract

Biofilm producer microorganisms cause nosocomial and recurrent infections. Biofilm that is a sticky exopolysaccharide is the main virulence factor causing biofilm-related infections. Biofilm formation begins with attachment of bacteria to biotic surface such as host cell or abiotic surface such as prosthetic devices. After attachment, aggregation of bacteria is started by cell-cell adhesion. Aggregation continues with the maturation of biofilm. Dispersion is started by certain conditions such as phenol-soluble modulins (PSMs). By this way, sessile bacteria turn back into planktonic form. Bacteria embedded in biofilm (sessile form) are more resistant to antimicrobials than planktonic bacteria. So it is hard to treat biofilm-embedded bacteria than planktonic forms. For this reason, it is important to detect biofilm. There are a few biofilm detection and biofilm production methods on prosthetics, methods for screening antibacterial effect of agents against biofilm-embedded microorganism and antibiofilm effect of agents against biofilm production and mature biofilm. The aim of this chapter is to overview direct and indirect methods such as microscopy, fluorescent in situ hybridization, and Congo red agar, tube method, microtiter plate assay, checkerboard assay, plate counting, polymerase chain reaction, mass spectrometry, MALDI-TOF, and biological assays used by antibiofilm researches.

Keywords: mature biofilm, biofilm-embedded bacteria, antibiofilm methods, detection of biofilm gene expression, biofilm detection, MALDI-TOF, CLSM, checkerboard assay, microtiter plate assay, Congo red agar method

1. Introduction

1.1 The structure and pathogenesis of microbial biofilms

Microbial biofilms that are sticky exopolymeric substances (EPS) causing adherence of microorganism to biotic surfaces such as host cells or abiotic surfaces such as medical devices cause antimicrobial resistance, due to its molecular contents such as eDNA and exoenzymes (β-lactamase, toxins, etc.), limited diffusion of antimicrobials through the biofilm matrix, persister cell content, and limited nutrient and oxygen. Surface proteins and polysaccharide intercellular adhesions (PIA) play a role in the biofilm production and development. It is hard to treat

biofilm-embedded bacteria than planktonic forms. Biofilm producer microorganism causes biofilm-related infections such as indwelling and medical device-related infections such as endocarditis, urinary tract infections, septic arthritis, chronic rhinosinusitis, ocular infections, wound infections, etc. The results of biofilm produced on indwelling medical devices are recurrent, untreatable infections and failure of medical device. To overcome chronic and recurrent infections, it is important to detect biofilms of microorganisms, maturation and dispersion, and determine antibiofilm and antibacterial activity of agents against biofilm and bacteria within biofilm, respectively [1]. Identification of genes involved in biofilm formation and measurement of gene expression as a result of antibiofilm and antibacterial activity of agents can be advantageous with carrying out high-throughput screens using microtiter plate assay system.

1.2 Techniques used to study biofilms and biofilm-embedded microorganisms

The standard antimicrobial susceptibility tests such as broth macrodilution and microdilution methods that are routinely used in laboratories and published by Clinical Laboratory Standards Institute (CLSI), National Committee for Clinical Laboratory Standards (NCCLS), and European Committee on Antimicrobial Susceptibility Testing (EUCAST) could never yield accurate results in biofilm producer microorganisms, due to being appropriate for the detection of antimicrobial activity of agents against planktonic microorganism [2].

There are several methods which have been used by clinical microbiologist for detection and measurement of microbial biofilms in response to agents (**Tables 1–3**). Several instruments as model system have been improved such as modified Robbins device, Calgary biofilm device, disk reactor, Centers for Disease Control (CDC) biofilm reactor, perfused biofilm fermenter, and model bladder. Model systems help to define susceptibility of antimicrobial agents against biofilm producer microorganisms by providing information about biofilm mechanisms. Substratums of modified Robbins device, Calgary biofilm device, disk reactor, CDC biofilm reactor, and perfused biofilm fermenter are silastic disks, plastic pegs, Teflon coupons, plastic needleless connectors, and cellulose acetate filters, respectively, whereas substratum of model bladder is urinary catheters (UCs). Medical devices of which dimensions are adjusted to appropriate sizes can also be used as a substratum (abiotic surfaces) for biofilm production by adapting and modifying to related methods by some biofilm researchers. The methods of modified Robbins device and Calgary biofilm device are based on viable counting. In Calgary biofilm device, pegs are sonicated before counting. The methods of disk reactor and CDC biofilm reactor based on direct and viable counting,

Method	Action of application	Aim
Roll plate	Extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Sonication, vortex, and plate counting	Intraluminal and extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Acridine orange staining	Extraluminal biofilm detection	Direct investigation of biofilm produced on catheter by microscopy
Streak plating of alginate swab	Investigation of biofilm produced on indwelling catheter	Growth of biofilm-embedded bacteria

Table 1.
The methods used for detection and measurement of biofilms produced on medical devices.

Method	Aim
Tube method (TM)	Qualitative detection by observing biofilm lined on bottom and walls of tube
Congo red agar (CRA)	Qualitative detection by observing colony color change
Microtiter plate (MtP)	Quantitative detection of biofilm by microplate reader (microELISA)
Real-time PCR	Detection of biofilm genes
Conventional PCR	
Multiplex PCR	

Table 2.
The methods used for detection of biofilm.

Method	Application	Target
Microtiter plate (MtP)	Measurement of biofilm produced on walls of wells in response to agent	Measures the effect of agents against biofilm production
Microtiter plate (MtP) (MBEC)	Measurement of biofilm remained on walls of wells in response to agent and detecting MBEC of agents	Measures the effect of agents against mature biofilm formed on walls of wells
Vortex and plate counting	Plate counting of biofilm-embedded bacteria and detecting bMBC of agents	Screens antimicrobial activity of agents against biofilm-embedded bacteria
Checkerboard assay	Plate counting of biofilm-embedded bacteria and FIC indexes are calculated	Screens antimicrobial activity of combination of agents
Sonication, vortex, and plate counting	Plate counting of biofilm-embedded bacteria and detecting bMBC of agents	Screens antimicrobial activity of agents against biofilm-embedded bacteria
Quantitative PCR	Measurement of specific biofilm gene expression	Monitors expression of biofilm genes in response to agents
Mass spectrometry (MS)	Measurement of exoenzymes located in biofilm matrix	Monitors expression of bacterial proteins in response to agents

Table 3.
The screening methods for antibiofilm and antimicrobial activity of agents against biofilm producer bacteria.

after substratums, are sonicated, vortexed, and homogenized. In perfused biofilm fermenter, viable counting is done, after filters are shaken in sterile distilled water, whereas in model bladder, UCs are examined directly by scanning electron microscopy (SEM) or transmission electron microscopy (TEM) or by chemical analysis [2]. Rate of biofilm formed on model system can be adjusted by parameters such as composition of medium that can contain glucose, iron, antimicrobial agents, multivalent cations such as Ca^{2+} and Mg^{2+} supporting adhesion of bacteria by cross-linking anionic groups on bacteria and substratum, shear force, retention time, flow rate, roughness, and chemistry of substratum and species of organisms (**Table 4**) [2, 3].

The aim of this chapter is to overview certain methods used by biofilm detection and antibacterial and antibiofilm researches such as tube method (TM), Congo red agar (CRA) method, microtiter plate (MtP) assay, plate counting of biofilm-embedded bacteria (sessile bacteria), PCR, mass spectrometry (MS), confocal laser scanning microscopy (CLSM), etc.

Instruments	Culture dynamics	Substratum	Method
Modified Robbins device	Batch culture	Silastic disks	Viable counting
Calgary biofilm device	Batch culture	Plastic polycarbonate pegs	Viable counting, after pegs are sonicated
Disk reactor	Batch culture	Teflon coupons	Direct or viable counting, after coupons are sonicated, vortexed, and homogenized
CDC biofilm reactor	Continuous culture	Plastic connectors	
Perfused biofilm fermenter	Continuous culture	Cellulose-acetate filters	Viable counting, after filters are shaken in sterile distilled water
Model bladder	Continuous culture	Urinary catheters	Examining directly by SEM or TEM or analyzing chemically
Flow cell	Continuous culture	Chambers with transparent surfaces	Examining by confocal laser scanning microscopy

CDC biofilm reactor, Centers for Disease Control biofilm reactor; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Table 4.
Instruments used to produce biofilm and examine biofilm process.

2. Detection of biofilm producer microorganism

2.1 Direct observation

Complexity and dynamics of biofilms can be observed by biofilm imagining optical technology including light microscopy, SEM, TEM, and CLSM. These techniques are used to visualize 3D structure and check the existence of biofilm [4].

2.1.1 Light microscope

Light microscopy is the easiest, cheapest, most simple, convenient and fastest method to quantitatively observe the morphology of microorganisms adhered to surfaces and to semiquantitatively estimate the amount of microorganism attached on surface (exist, absent, abundant, rare, etc.). Microorganisms including *Candida albicans*, *E. coli*, *Pseudomonas*, and *Staphylococcus epidermidis* adhered on acrylic sheets of polymethacrylate films, glass cover slips, and polystyrene petri dishes have been observed by light microscope, respectively. Observation with light microscopy that requires clear, transparent, and planar surfaces on which microorganisms attach does not create 3D vision of biofilm. Dyes can be used such as epifluorescence and fluorescent to enhance image clarity of microorganisms. The observation with the light microscope enables researchers to compare morphologies of sessile form and planktonic form of microorganism required by making smear and centrifuging of sample, respectively [3].

2.1.2 Transmission electron microscope (TEM)

Images of cells and cell structures such as protein and nucleic acid are obtained by electrons at high magnification and resolution. Monitoring of components of cell can be done directly in TEM by negative staining. Due to photons and electrons penetrating cells poorly, thin section of cell cut is stabilized and stained by certain chemicals with the treatment of osmic acid, permanganate, uranium, lanthanum, or lead salts. These

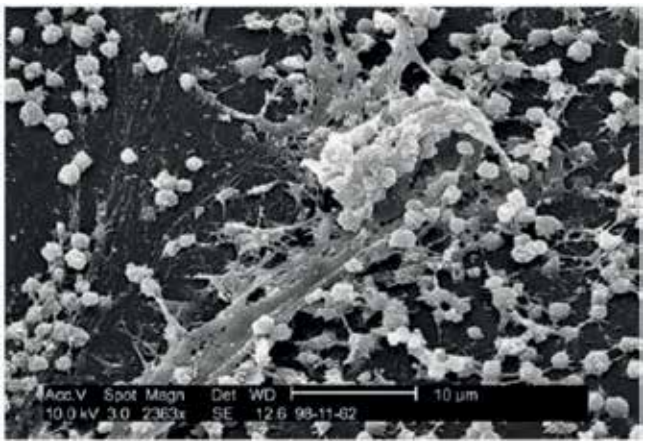

Figure 1.
The SEM image of S. aureus embedded in biofilm colonized on intravenous catheter [6].

stains contain high atomic weight. Due to stains having high atomic weight, contrast is accelerated by electron dispersion from sample. If observation of outer structure of cells will be done, it is not important whether the section of cell is thin or thick.

Due to inadequate stabilization of polysaccharides is done by the conventional fixatives such as aldehydes, glutaraldehyde, paraformaldehyde, and osmium tetroxide, water content of biofilm is eliminated by graded dehydration with alcohol after this postfixation step. After sample is infiltrated with resin, sample is embedded in gelatin capsule and headed for polymerization. Then, thin section taken is poststained with uranyl acetate and lead citrate.

Exopolysaccharide constituents are not observed with its own electron dense and staining poststains such as uranyl acetate and lead citrate with TEM, due to not only having high electron translucent, but also contrast is not developed by conventional poststains. According to the studies, glycocalyx of *Staphylococcus hominis* and *Staphylococcus epidermidis* can be stabilized by the usage of certain cationic reagent combinations including ruthenium red, alcian blue, lysine, lysine monohydrochloride, or lysine acetate and paraformaldehyde [5]. After all these steps are done, sample is observed in TEM (**Figure 1**).

2.1.3 Scanning electron microscope (SEM)

To visualize 3D images of cell sample is coated with heavy metals such as gold. Electrons released from metal coating of sample are caught by SEM for image

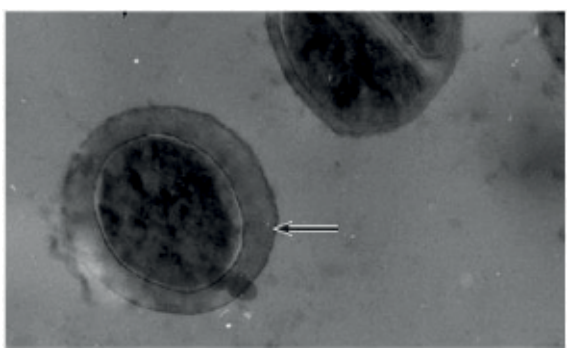

Figure 2.
The TEM image of Staphylococcus spp. surrounded by glycocalyx [6].

production. The procedure of SEM is similar to TEM except for some additional chemicals (gold), lacking infiltration, embedment in resin, polymerization, and thin section staining with lead citrate and uranyl acetate [5]. As in the steps of TEM method, postfixation and dehydration steps of SEM are similar to TEM. The step is applied after dehydration step is drying and coating sample with gold in the processing for SEM, rather than infiltration with resin, embedment in gelatin capsule, and staining with lead citrate and uranyl acetate in the processing for TEM. After dehydration process with graded alcohol, sample is dried and coated with gold palladium [5]. After all these steps are done, sample is observed in SEM (**Figure 2**).

2.1.4 Florescent tagging of biofilm

2.1.4.1 Confocal laser scanning microscopy (CLSM)

Biofilms formed on flow cells of which surface are transparent can be observed by confocal laser screening microscopy (CLSM). Three-dimensional (3D) morphology and physiology of biofilms can be screened by CLSM [2]. Thick samples such as biofilms and microorganisms localized in the depth such as biofilm-embedded microorganisms need to be observed by CLSM (**Figure 3**).

For observation of biofilm with confocal microscopy and related methods, biofilm must be fluorescent as a result of fluorescent molecules such as green fluorescent protein (GFP) that is fluorescent protein expressed by biofilm producer microorganism within biofilm (gene of cell interested is tagged by gene cassette encoding GFP) or staining components of heterogeneous mass of biofilm with fluorescence or fluorescence-labeled dyes [2]. Stains such as lectins target extracellular matrix, whereas certain fluorophores target extracellular DNA (eDNA) to visualize eDNA content of biofilm matrix [2, 7].

By scanning laser light across the sample, deep penetration of excitated energy is provided. As a result of fluorescence of biomolecules such as GFP or chlorophyll that are intrinsic fluorophores or molecules signed by exogenous probes such as fluorescent-labeled antibodies detected by photomultiplier, 3D digital image is formed. Observation of biofilms that are multilayered and have complex 3D structures requires additional resolution [2]. Images of each layers of biofilm obtained are combined by computer for construction of digital 3D images of whole biofilm.

Biofilm producer microorganisms can be manipulated genetically by tagging of microbial gene of interest by gene cassette encoding GFP as a reporter gene

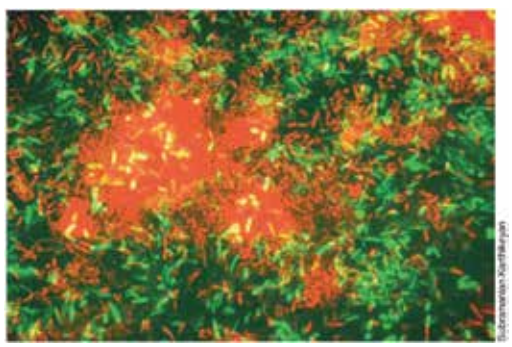

Figure 3.
Bacterial community embedded in a biofilm matrix visualized by CLSM. Each bacterium observed with a distinct color located at different depths of biofilm [11].

(*gfp* genes) to monitor gene expression and metabolic physiology activity in biofilms and determine location of microorganism within biofilm [2, 8, 9].

Idea about gene activity in biofilms is given by confocal microscopy applied to 3D localization of nonenzyme reporter systems such as GFP. Growth phase and activity of bacteria embedded in biofilms can be defined by promoter-reporter systems that is designed and fluoresced just in living dividing cells. In situ cellular growth activity of bacteria embedded in biofilms is determined by measuring ribosome-hybridization-signal intensity, due to synthesis rate and content of ribosome correlated with the growth rate (especially in exponential phase). Expression cassette that is active only in growing cells, labeled by GFP and controlled by rRNA promoter, can be constructed to monitor growth phase and activity of bacteria within biofilm [2, 10].

2.1.4.2 Fluorescent in situ hybridization (FISH)

Specific microorganisms present in a heterogeneous biofilm community can be identified by the probes of fluorescent in situ hybridization (FISH) method. GFP that is translated enables procedure not to require fixation or staining. Fluorescent-labeled microorganism within biofilm can also be examined by FISH. DNA probes designed to hybridize 16S rRNA of microorganism integrated to either fluorescent dye such as FITC or Rhodamine or enzyme such as horseradish peroxidase. The advantage of probes conjugated with horseradish peroxidase is not to destroy microorganism within biofilm. The growth rate of microorganism within biofilm can be determined by FISH method, due to the amounts of ribosomes existing in a microorganism that is directly proportional to growth activity of microorganism. Probe must be designed to label conserved region of only a single species (**Figure 4**) [2, 12].

2.2 Indirect observation

2.2.1 Roll plate method

Roll plate method is applied for the detection of possible microbial colonization having a potential to develop indwelling device-associated infection on the outer surface of cylindrical materials such as catheters and vascular grafts. Microorganism colonize on external surface of catheter is detected by roll plate method, instead of microorganism colonize on intraluminal site of catheter. Material is touched and rolled on the surface of medium [3].

Figure 4.
Fluorescence image of 28 distinct E. coli strains labeled by fluorophore-conjugated oligonucleotides complementary to 16S rRNA of E. coli [11].

2.2.2 Congo red agar (CRA) method

Congo red agar (CRA) method that is a qualitative assay for detection of biofilm producer microorganism, as a result of color change of colonies inoculated on CRA medium, is described by Freeman et al. The CRA medium is constructed by mixing 0.8 g of Congo red and 36 g of sucrose to 37 g/L of Brain heart infusion (BHI) agar. After incubation period that was 24 h at 37°C, morphology of colonies that undergone to different colors is differentiated as biofilm producers or not. Black colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink are non-biofilm producers (**Figure 5**) [13].

2.2.3 Tube method (TM)

Tube method (TM) that is a qualitative assay for detection of biofilm producer microorganism, as a result of the occurrence of visible film, is described by Christensen et al. [14]. Isolates are inoculated in polystyrene test tube which contained TSB and incubated at 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of polystyrene test tube are stained with safranine for 1 h, after planktonic cells are discharged by rinsing twice with phosphate-buffered saline (PBS). Then, safranine-stained polystyrene test tube is rinsed twice with PBS to discharge stain. After air drying of test tube process, the occurrence of visible film lined the walls, and the bottom of the tube indicates biofilm production (**Figure 6**) [14].

2.2.4 Detection of biofilm production by microtiter plate assay

Microtiter plate (MtP) assay is a quantitative method to determine biofilm production by microplate reader. Bacterial suspension is prepared in MHB supplemented with 1% glucose and adjusted to 0.5 McFarland (1.10^8 cfu/ml). This bacterial suspension is 20-fold (1/20) diluted to reach 5.10^6 cfu/ml. Then 180 μl of Mueller-Hinton Broth (MHB) supplemented with 1% glucose [15] and 20 μl of bacterial suspensions are inoculated into 96-well flat-bottomed sterile polystyrene microplate to obtain 5.10^5 cfu/ml as a final concentration (tenfold dilution (1/10)). Microplates are incubated at 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of wells of microplate are stained with only 150 μl of safranine for 15 min, after planktonic cells in wells of microplate are discharged by washing twice with phosphate-buffered saline (PBS) (pH 7.2) and wells are dried at 60°C for

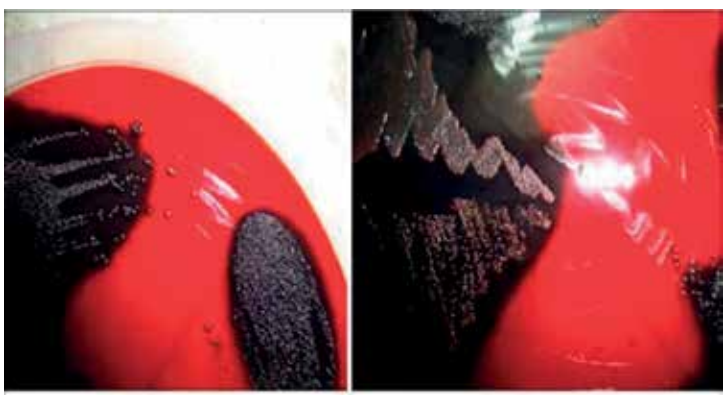

Figure 5.
CRA method applied on CRA medium. Black crystalline colonies of biofilm producer cell and pinkish-red colonies of biofilm nonproducer cell.

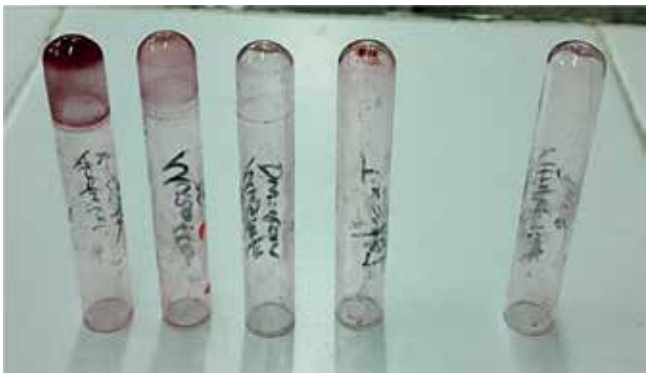

Figure 6.
Tube method. The first two polystyrene test tubes from the left indicate biofilm production. Other test tubes rather than the first two polystyrene test tubes from the left indicate lacking of biofilm production.

1 h [14]. Before staining with safranine, fixation of biofilms can be done by either subjecting to 150 μl of methanol for 20 min or drying at 60°C for 1 h. Then safranine-stained wells of microplates are washed twice with PBS to discharge safranine stain. After air drying process of wells of microplate, dye of biofilms that lined the walls of the microplate is resolubilized by 150 μl of 95% ethanol or 33% glacial acetic acid or methanol. Then microplate is measured spectrophotometrically at 570 nm by a microplate reader [15, 16]. The studies are repeated in triplicates. Uninoculated wells containing sterile MHB supplemented with 1% glucose that are considered to be the negative controls are used as blanks. The blank absorbance values are used to identify whether biofilm formation of isolates exists or not. The wells of isolates of which OD values are higher than blank well are considered to be biofilm producers. Cut off value (ODc) can provide categorization of isolates as biofilm producer or not.

$$\text{Odc: Average OD of negative control} + (3 \times \text{standard deviation (SD) of negative control}) \quad (1)$$

$$\text{OD}_{\text{isolate}}\text{: Average OD of isolate} - \text{ODc} \quad (2)$$

Negative value obtained from this formula and represented as zero indicates lack of biofilm production, whereas positive value indicates biofilm production (**Figure 7**).

Figure 7.
Microtiter plate assay indicating biofilm production.

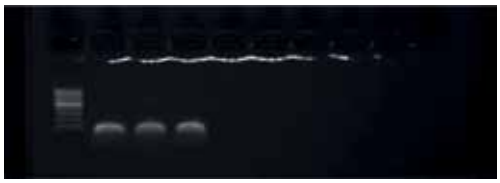

Figure 8.
Image of mecA gene on agarose gel. First sample is DNA size marker (ranging from 250 to 10,000 kb), second sample is ATCC 43300 methicillin-resistant Staphylococcus aureus (MRSA) positive control, third and fourth samples are mecA gene-positive isolates, and fifth sample is ATCC 29213 methicillin-sensitive Staphylococcus aureus (MSSA) as a negative control.

To interpret results, categorization can be done as no biofilm production (0), weak (+ or 1), moderate (++ or 2), and strong biofilm production (+++ or 3) by the calculation of cutoff value (ODc) shown below [15]:

$OD \leq ODc$ no biofilm production

$ODc < OD \leq 2 \times ODc$ weak biofilm production

$2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm production

$4 \times ODc < OD$ strong biofilm production.

2.2.5 Detection of biofilm-associated genes by PCR

PCR techniques is used for not only identification of pathogens by amplifying species-specific nucleic acid sequences but also detection of virulence factors by amplifying target virulence genes such as biofilm genes with the usage of gene-specific primers, even in the uncultured pathogen present in the sample.

Forward and reverse primers of biofilm-associated gene are designed. Firstly, multiple alignments were done in the NCBI to find oligonucleotide sequences specific to the species. Then primer pair of biofilm-associated gene is designed by using Primer3Plus verified by FASTA analysis checking specificity of primers for microbial sequences in the database [17, 18].

Genomic DNA of microorganism is extracted by extraction kits of which protocols can vary according to species and Gram-positivity or Gram-negativity of microorganisms. DNA of microorganism is measured spectrophotometrically by microplate spectrophotometry reader to determine the amount of DNA extracted as microgram per microliter.

Biofilm-associated gene is amplified by PCR such as qualitative real-time PCR, multiplex and conventional PCR that is used to detect whether biofilm-associated gene is present or not in microorganism. If conventional and multiplex PCR protocols are applied to detect biofilm gene, rather than qualitative real-time PCR, PCR product isolated is visualized on an agarose gel containing a DNA-intercalating dye such as ethidium bromide to confirm the presence of amplified gene (**Figure 8**). Only in qualitative real-time PCR, the amplicon is detected by fluorescence using a pair of specific hybridization probes labeled with fluorescence dye [11].

3. Detection of antimicrobial efficacy of agents against biofilm production

3.1 Detection by microtiter plate assay

Microtiter plate (MtP) assay is a qualitative assay to detect efficacy of agent against biofilm production by microplate reader.

Bacterial suspension is prepared in MHB supplemented with 1% glucose and adjusted to 0.5 McFarland (1.10^8 cfu/ml). This bacterial suspension is 20-fold (1/20) diluted to reach 5.10^6 cfu/ml.

A 180 μl of agent doses and 20 μl of bacterial suspension are dispersed to each well of microplate to obtain 5.10^5 cfu/ml as a final concentration (tenfold dilution (1/10)). After incubation at 37°C for 24 h, ongoing processes are done according to MtP assay as mentioned previously for the determination of effect of agent against biofilm production [14, 16].

4. Detection of antimicrobial efficacy of agents against mature biofilms

4.1 Determination of MBEC by microtiter plate assay

Biofilms remained after eradication by agent are measured by this technique. Biofilms of bacteria that line the walls of wells are formed according to MtP method.

After the content of microplate is discharged, 200 μl of each dose of agents is dispersed to each well of microplate of which the walls are lined with biofilm. A 200 μl of distilled water is added into a well of microplate of which the walls are lined with biofilm as a control. Then the effect of agent against mature biofilm is determined according to MtP assay as mentioned previously. Minimum concentration of agent eradicating mature biofilm that is named as minimum biofilm eradication concentration (MBEC) can be determined by this modified plate assay. MBEC50 and MBEC90 indicate the minimum concentrations of agents inhibiting 50 and 90% of mature biofilm formed.

5. Detection of antimicrobial efficacy of agents against biofilm-embedded bacteria

5.1 Determination of bMIC and bMBC by plate counting

In summary, biofilm formation process on abiotic surfaces by bacteria is done. Quantification of sessile biofilm-embedded bacteria lined on abiotic surface and sessile biofilm-embedded bacteria remained on abiotic surface after addition of agent on abiotic surface on which mature biofilms formed is determined by plate counting. Bacterial suspension is prepared and adjusted to 0.5 McFarland (1.10^8 cfu/mL) in Mueller-Hinton Broth (MHB) supplemented with 1% glucose [15]. This bacterial suspension is 200-fold (1/200) diluted to gain 5.10^5 cfu/mL. Kirschner wire orthopedic implants are placed into each test tube containing 5.10^5 cfu/mL isolate and incubated at 37°C for 24 h to lead bacteria to produce biofilm on Kirschner wire. After incubation, Kirschner wires on which biofilms are produced are discharged and rinsed with PBS (pH 7.2) and then transferred into each test tubes containing agent concentrations. After incubation at 37°C for 24 h, Kirschner wires are discharged and placed into test tubes containing 1 mL of sterile MHB and sonicated at 42 kHz for 2 min after vortexed for 5 min. Then 100 μl samples of each test tube sonicated and vortexed are inoculated on Mueller-Hinton agar (MHA) and incubated at 37°C for 24 h [19].

The lowest concentration of agent in which bacterial growth is below or equal to control is determined as biofilm minimum inhibitory concentration (bMIC) for biofilm. bMIC50 and bMIC90 indicate the minimum concentrations of agent inhibiting 50 and 90% of biofilm-embedded bacteria. After incubation, the lowest concentration of agent in which colonies of biofilm-embedded bacteria are not grown is determined as biofilm minimum bactericidal concentration (bMBC) of agent for biofilm [19].

5.2 Determination of combination effects of agents against biofilm

Checkerboard assay is used for the determination of combination effects of two different agents. A 250 μl twofold dilutions of each agent from the stock solutions are dispersed to each row and column to obtain final varying concentrations by starting at fourfold of zero MIC for each isolate. So each well contains distinct combination of concentrations of two agents. First wells of rows and columns are left behind for sole treatments of each dose of agents. One well is used for bacterial control (**Figure 9**). Kirschner wires on which bacterial biofilm is produced are dispersed to each well. This microplate is incubated at 37°C for 24 h. After incubation, Kirschner wires are discharged and sonicated at 42 kHz for 2 min after vortexed for 5 min. The lowest concentration of agent in which bacterial growth that is not observed is determined as biofilm minimum inhibitory concentration (bMIC) of agent for biofilm. Then 100 μl samples of each test tube sonicated and vortexed are inoculated on MHA and incubated at 37°C for 24 h. After incubation, the lowest concentration of agent in which colonies of biofilm-embedded bacteria are not grown is determined as biofilm minimum bactericidal concentration (bMBC) of agent for biofilm. For the determination of whether the synergism is present between agents or not, fractional inhibitory concentrations (FICs) index that are calculated for each agent are summed up according to formula written below:

$$\text{FIC (A)}: \text{MIC A}\left(\text{A in the presence of B (A + B)}\right)/\text{MIC A (A alone)} \quad (3)$$

$$\text{FIC (B)}: \text{MIC B}\left(\text{B in the presence of A (A + B)}\right)/\text{MIC B (B alone)} \quad (4)$$

$$\Sigma\text{FIC}: \text{FIC (A)} + \text{FIC (B)} \quad (5)$$

When ∑FIC is equal and lesser than 0.5, between 0.5 and 1, equal to 1, higher than 1 and equal and lesser than 4, and higher than 4, it is interpreted that the effect between agents in combination is synergistic, partial synergistic, additive, indifferent, and antagonistic, respectively [20].

The wells having the highest synergy rates of two agents that constitute the combinations are determined by taking the average and standard deviation of FIC indexes calculated of the wells with the lowest drug combination without bacterial growth in each row and column (**Figure 9**).

5.3 Measurement of biofilm-associated gene expression by quantitative real-time PCR

Measurements of biofilm genes repressed or induced by agent are done by quantitative real-time PCR (qPCR). So efficacy of agent against biofilm-associated genes can be detected by qPCR.

Complementary DNA (cDNA) is copied from RNA by enzyme reverse transcriptase. Gene expression in pathogen is monitored by qPCR copying cDNA from RNA of target gene. Amplified cDNA probed for identification. Fluorescent probes such as dye SYBR Green are used to indicate double-stranded DNA, consequently amplification. Accumulation of PCR amplicons labeled fluorescently is monitored through the qPCR processes (**Figure 10**). Visualization of amplicon on agarose gel is not needed to confirm amplification in qPCR [11].

Bacterial control	Agent X Dose 1	Agent X Dose 2	Agent X Dose 3	Agent X Dose 4	Agent X Dose 5	Agent X Dose 6	Agent X Dose 7
Agent Y Dose 1							
Agent Y Dose 2							
Agent Y Dose 3							
Agent Y Dose 4							
Agent Y Dose 5							
Agent Y Dose 6							
Agent Y Dose 7							

Figure 9.
The schematization of checkerboard assay.

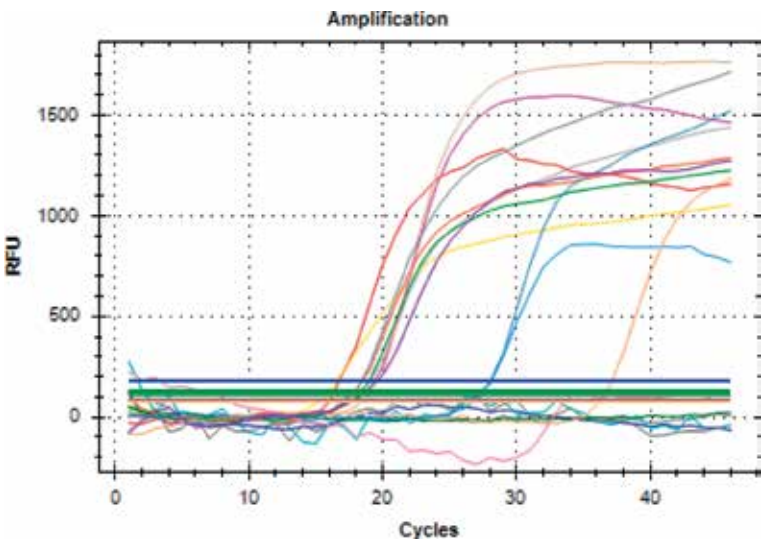

Figure 10.
Quantitative real-time polymerase chain reaction (qPCR) of atl (light blue), 16S RNA (red), mecA (purple), and nuc (light pink) genes of sample; icaA (gray), icaD (plato), atl (pink), mecA (blue), and nuc (green) genes of ATCC 43300 methicillin-resistant Staphylococcus aureus (MRSA); and icaA (orange), 16S RNA (yellow), and nuc (grayish blue) genes of ATCC 29213 methicillin-sensitive Staphylococcus aureus (MSSA). Expressions of all these genes are monitored by qPCR except nuc (light pink) gene sample. Lines below threshold monitored by qPCR indicate the negativity of genes such as icaA and icaD genes samples and icaD (turquoise), atl (plato), and mecA (light pink) genes of ATCC 29213 MSSA. RFI, relative fluorescence intensity.

Total RNA of microorganism is isolated according to protocols of RNA isolation kits. Kit protocols can vary according to the species of microorganism. Total RNA of microorganism is measured spectrophotometrically by microplate spectrophotometry reader to determine the amount of total RNA isolated as microgram per microliter. Then cDNA is synthesized from total RNA with qPCR using primer pair of the biofilm-associated gene, which is designed using Primer3 and verified by FASTA analysis, which controls the specificity of the primers for microbial sequences in the data system, after multiple alignments were done in the NCBI to find oligonucleotide sequences specific to the species [17, 18].

5.4 Mass spectrometry

Extracellular polymeric substances (EPS) not only contain polysaccharides but also contain proteins such as extracellular enzymes. These expressed proteins located in the matrix of EPS can be detected and characterized by mass spectrometry (MS) [1]. Large biologic molecules can be also detected and characterized in complex biologic structures such as EPS by MS. Chemicals involved in biofilm process are examined in detail by MS. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the types of MS [2]. In time-of-flight (TOF) mass spectrometer, mass is analyzed by ions desorbed in vacuum chamber. These two technics are combined and called MALDI-TOF.

Sample is ionized and vaporized by laser. Ions generated from sample by laser pass through the column of MALDI-TOF device toward TOF detector by an electric field. Depending on the mass/charge ratio of molecule, measurements are done by TOF. If this ratio is smaller, ions move faster (**Figure 11**).

Bacteria are identified, expression of bacterial proteins such as surface proteins and exoenzymes like β-lactamase in response to antimicrobials can be monitored, and growth of bacteria is measured by applications of MALDI. MS has high sensitivity and requires minimum amount of sample [2].

5.5 Biological assay

Biofilm-embedded bacteria can be estimated by biologic assays that is an indirect assay. Biological assays that measure production of microbial product give an opinion about estimation of the number of microorganism within biofilm. Amount of biologic product is correlated with biofilm-embedded microorganism producing the product by standardization of planktonic microorganism. Biologic products produced by planktonic microorganism are similar to biologic products produced

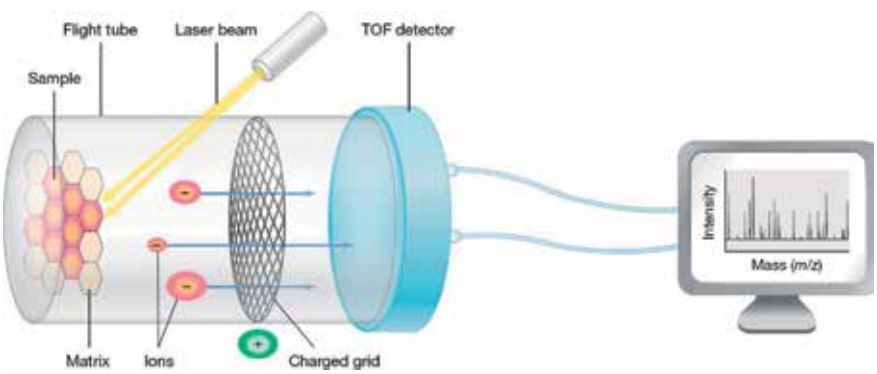

Figure 11.
MALDI-TOF mass spectrometry device [11].

by biofilm-embedded bacteria. Standardization curves of each microorganism tested need to be formed. Measurement of total protein at the absorbance is 550 or 950 nm; tryptophan fluorescence, endotoxin [2], ATP production via bioluminescence caused by luciferin and luciferase, urease production to estimate number of attached microorganism, and electron transport via the production of formazan are done by biological assays [3].

6. Conclusion

Biofilms cause resistance to many antimicrobial agents. The results of biofilm produced on indwelling medical devices are recurrent, untreatable infections and failure of medical device. To overcome chronic and recurrent infections, it is important to detect biofilms of microorganisms, determine antibiofilm activity of agents against biofilm, and determine antibacterial activity of agents against biofilm-embedded microorganism with the appropriate methods by clinical microbiologist and biofilm researcher microbiologist. Identification of genes involved in biofilm formation and measurement of gene expression as a result of antibiofilm and antibacterial activity of agents can be advantageous in biofilm studies.

Author details

Sahra Kırmusaoğlu
Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Haliç University, Istanbul, Turkey

*Address all correspondence to: kirmusaoglu_sahra@hotmail.com

References

[1] Kırmusaoğlu S. Staphylococcal biofilms: Pathogenicity, mechanism and regulation of biofilm formation by quorum sensing system and antibiotic resistance mechanisms of biofilm embedded microorganisms. In: Dhanasekaran D, Thajuddin N, editors. Microbial Biofilms—Importance and Applications. Croatia: Intech; 2016. pp. 189-209. DOI: 10.5772/62943

[2] Shunmugaperumal T. Biofilm Eradication and Prevention: A Pharmaceutical Approach to Medical Device Infections. New Jersey: John Wiley & Sons, Inc.; 2010. pp. 116-151

[3] Christensen GD, Simpson WA, Anglen JO, Gainor BJ. Methods for evaluating attached bacteria and biofilms. In: An YH, Friedman RJ, editors. Handbook of Bacterial Adhesion: Principles Methods, and Applications. Totowa: Humana Press; 2000. pp. 213-233

[4] Roy R, Tiwari M, Donelli G, Donelli V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 2018;**9**(1):522-554

[5] Fassel TA, Edmiston CE. Evaluating adherent bacteria and biofilm using electron microscopy. In: Yuehuei H, Friedman RJ, editors. Handbook of Bacterial Adhesion: Principles, Methods, and Applications. New Jersey: Humana Press Inc; 2000. pp. 235-248

[6] Willey JM, Sherwood LM, Woolverton CJ. Prescott, Harley, and Klein's Microbiology. 9th ed. New York: The McGraw-Hill Companies, Inc; 2013

[7] Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Molecular Microbiology. 2006;**59**:1114-1128

[8] Lambertsen L, Sternberg C, Molin S. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environmental Microbiology. 2004;**6**:726-732

[9] Sternberg C, Christensen BB, Johansen T, Na T, Andersen JB, Givskov M, et al. Distribution of bacterial growth activity in flow-chamber biofilms. Applied and Environmental Microbiology. 1999;**65**:4108-4117

[10] Lee N, Nielsen P, Andreasen K, Juretschko S, Nielsen J, Schleifer KH, et al. Combination of fluorescent in situ hybridization and microautoradiography, a new tool for structure-function analyses in microbial ecology. Applied and Environmental Microbiology. 1999;**65**:1289-1297

[11] Madigan MT, Martinko JM, Bender KS, Buckley DH, Stahl DA. Brock Biology of Microorganisms. 14th ed. The United States of America: Pearson Education, Inc; 2015

[12] Stahl DA, Amann R. Development and application of nucleic acid probes. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. New York: John Wiley & Sons, Inc; 1991. pp. 205-248

[13] Freeman J, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. Journal of Clinical Pathology. 1989;**42**:872-874

[14] Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology. 1985;**22**:996-1006

[15] Stepanović S, Vuković D, Hola V, Bonaventura GD, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007;**115**(8):891-899

[16] Tang HJ, Chen CC, Ko WC, Yu WL, Chiang SR, Chuang YC. In vitro efficacy of antimicrobial agents against high-inoculum or biofilm-embedded methicillin-resistant *Staphylococcus aureus* with vancomycin minimal inhibitory concentrations equal to 2 μg/mL (VA2-MRSA). International Journal of Antimicrobial Agents. 2011;**38**:46-51

[17] http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. 2018 [Accessed: 28-11-2018]

[18] http://www.ncbi.nlm.nih.gov/GenBank [Accessed: 28-11-2018]

[19] Wayne PA. Performance standards for antimicrobial susceptibility testing. In: Clinical and Laboratory Standards Institute. Twenty-Third Informational Supplement CLSI Document M100-S23. 2013

[20] Moody J. Susceptibility tests to evaluate synergism. In: Isenberg HD, editor. Clinical Microbiology Procedures Handbook. Washington: ASM Press; 2004

Section 3

Antibiotic Production

Chapter 7

Streptomyces as a Source of Antimicrobials: Novel Approaches to Activate Cryptic Secondary Metabolite Pathways

Ángel Manteca and Paula Yagüe

Abstract

Streptomyces is the most important bacterial genus for bioactive compound production. These soil bacteria are characterized by a complex differentiation cycle. *Streptomyces* is extremely important in biotechnology, producing approximately two thirds of all antibiotics, as well as many compounds of medical and agricultural interest. Drug discovery from streptomycetes became challenging once the most common compounds were discovered, and the system was basically abandoned by industry. Simultaneously, antibiotic resistance is increasing dramatically, and new antibiotics are required. Screening from nature is being resumed (exploring new environments, looking for elicitors, metagenome, etc.). Secondary metabolism is conditioned by differentiation; although the relationship between both has long remained elusive, differentiation as a trigger for antibiotic production remains basically unexplored. Most cultures used in screening campaigns for new bioactive molecules have been performed empirically, and workflow was extremely productive during the so-called golden age of antibiotics; however, currently there is a bottleneck. *Streptomyces* is still the most important natural source of antibiotics, and it also harbors many cryptic secondary metabolite pathways not expressed under laboratory conditions. In this chapter, we review strategies based on differentiation, one of the keys improving secondary metabolite production and activating cryptic pathways to face the challenges of drug discovery.

Keywords: *Streptomyces*, screening, antibiotics, secondary metabolism, differentiation, elicitors, morphology, liquid cultures

1. Introduction

1.1 *Streptomyces* development

1.1.1 Solid cultures

The complex *Streptomyces* life cycle on solid-grown cultures is made up of different phases.

Microorganisms on soils are exposed to different stress factors: chemical, physical and/or biological. Of these variable conditions, sometimes nutrients can

be limited, preventing growth [1]. A good survival strategy of some bacteria and fungi is the latency stage of spores. Under this stage, cells stop growing and their metabolism is mainly inactive [2]. Spores are very resistant to stress conditions. In Streptomycetes, they are formed by thick hydrophobic covers that preserve genetic information during unfavorable conditions. Spores are dispersed by wind, water or insects and remain in latency until germination in favorable situations [3]. Besides the thickness of the cell wall, their resistance to extreme temperatures and other physicochemical factors is because of their low water content [4]. In this dehydrated environment, molecules have inactive conformations (denaturalized proteins, etc.) that are stabilized and protected by the sugar trehalose [2]. From this null or limited metabolism, spores are able to germinate, as the macromolecules needed have already been synthesized before the latency stage [2]. For germination, optimal hydration conditions are required. However, it can be faster and more successful in the presence of nutrients and specific stimuli. Spores also need enough levels of intracellular trehalose [5], and polyphosphates [6] that allow metabolic activity to commence before cells are able to detect and assimilate an external source of energy [2].

Despite the fact that germination is a very important stage in the *Streptomyces* life cycle, it has remained largely unstudied, because it is not an industrially important stage. When environmental conditions are prosperous, spores on soils/laboratory conditions (petri plates with solid culture medium) germinate. Germination of *Streptomyces* spores is a sequential process, divided into at least four stages: darkening, swelling, emission of the germination tube [7] and reduced swelling [8].

Darkening is the stage in which spores allow the entry of water into the cell losing their impermeability and heat resistance. After a few minutes, spores change their optical characteristics and the cells are able to reactive their metabolism. Darkwelling requires bivalent cations such as Ca^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2} and Fe^{+2} as well as energy reserves of the spore [7, 9, 10]. Degradation of the cover leads to the reactivation of the cell wall hydrolases. These enzymes, like Rpf (Resuscitation-promoting factors), are involved in cell wall degradation, facilitating peptidoglycan remodeling by hydrolyzing glycosylic bonds B-1,4 between cell wall components, N-acetylglucosamine and N-acetylmuramic acid, which in turn, allow the access to external nutrients [2].

Swelling is the stage in which spores increase its volume due to the entrance of water. This increases the levels of glucose, as a result of trehalose hydrolysis [11]. This seems to be an essential step in germination since only when trehalose concentrations decrease and proteins able to recover their functional conformations [12, 13]. Ribosomes are inactive inside the spores [14, 15]. However, only few minutes after germination starts (30–60 min), they are fully functional and new proteins can be translated [6]. During this stage, spores are metabolically active and are able to use trehalose as main energy source [11].

Emission of the germination tube: This stage begins at the same time as the first DNA replication, when spores are able to detect external sources of nutrients and adjust their metabolic pathways as a result [2]. The accurate point from which the germination tube is emitted is indicated by the accumulation of the protein, SsgA [3]. SsgA is not essential for germination; nevertheless, its absence significantly reduces the number of germ tubes in each spore, and its overexpression has the opposite effect [3].

Reduced swelling: Once the germ tube emerges, the spore starts to gradually reduce its swelling. This stage depends on the quantity of peptidoglycan cross-link that involves a carboxypeptidase (which corresponds to the SCO4439 gene in the *Streptomyces coelicolor* genome www.strepdb.com). A deletion of this

carboxypeptidase causes spores to continue swelling until 5 μm diameter (2.5 times more than the wild-type strain), after the emission of the germ tube [8]. The existence of this stage demonstrates that the regulation of water entering into the spores is complex and highly regulated.

During germination, several important morphological changes occur, including the degradation of the spore cover, which is mainly formed of peptidoglycan, and causing a huge increase of metabolic activity [2]. On the other hand, shortly after the emission of the germ tube, the expression of the protein DivIVA starts [6]. This protein plays a critical function in the vegetative growth and is located at the apex of the hyphae [16–18]. After germination, genes involved in cell division (like *ftsZ*) and proteins involved in cellular growth (like FilP) are expressed [2]. The expression of these genes is considered as the final of the germination phase [2, 6].

Curiously, during germination, three different secondary metabolites are produced: albaflavenone (a terpenoid) with an antibacterial effect against *Bacillus subtilis*, giving an important advantage in competitive environments, and two polyketides (germicidin A and chancone) with a germination inhibitory effect. These compounds are *de novo* generated during germination and are good examples of compounds produced during vegetative growth [19].

After germination, the *Streptomyces* mycelium starts to grow apically (regulated by *div*IVA gene) as a very transitory, multinucleated stage called first mycelium (MI), which is multinucleated [20]. This is a very transitory stage in which dead and living segments alternate in the same hyphae [21]. There are two kinds of septa separating segments during the MI stage: one of them is composed of a cell membrane and cell wall (with peptidoglycan) and the other, recently discovered, is a 1-μm spacing cross-membrane-based septa, without peptidoglycan [20]. *Fts*Z participates in this septation, but it is not essential, as cross-membranes were observed in the *Fts*Z null mutant. Later, the dead segments are completely disintegrated and living segments continue apical growth. In this phase, the growth is especially branched [18].

After this early stage of branching growth, there is a morphological differentiation into a second mycelium (MII), a sort of multinucleated mycelium with sporadic and apparently randomly positioned septa (formed of cell membrane and cell wall), which is the secondary metabolite producer [22]. This mycelium has two different populations: one of which remains as a branching mycelium growing on surfaces and is called substrate mycelium and the other develops hydrophobic covers and starts to grow into the air without branching (aerial mycelium) [18]. This is known as the "sky-pathway," which regulates the expression of genes encoding proteins forming hydrophobic proteins (rodlins, chaplins and ramS) that cover the aerial hyphae. Some of the so-called "bald" (*bld*) mutants among others (defective in aerial growth) regulate the activation of this pathway [23]. At the end of the cycle, aerial hyphae undergo extensive synchronous septation (cell division) to create up to 100 uninucleoid compartments, which finally differentiate to create chains of spores [24]. At the end of the cycle, the "white" (*whi*) genes are activated (whose mutants are defective in the formation of mature gray spores on the fluffy aerial mycelium) and the hyphae septate and sporulate [25].

1.1.2 Liquid cultures

Streptomyces differentiation in liquid cultures has hardly been studied, mainly due to the fact that most strains do not sporulate under these conditions. However, the fact that *Streptomyces venezuelae* is capable of sporulation in liquid cultures has made it a new study model in recent years [26].

The applications of novel methodologies (confocal microscopy, proteomics, transcriptomics) to the study of *Streptomyces* biology have extended the knowledge regarding the development during the phases preceding sporulation in liquid cultures [27–31].

In these conditions, in most *Streptomyces* strains, aerial mycelium formation and sporulation are blocked [28]; so, there is no hydrophobic cover formation, or sporulation, but there is a first exponential growth phase corresponding to the compartmentalized MI morphology, and then there is a growth arrest phase corresponding to programmed cell death (PCD). After that, the hyphae, which remain alive, continue growing in a second exponential growth phase in which the morphology of the hyphae becomes MII multinucleated mycelium, which corresponds to the antibiotic production [27, 30].

1.2 Correlation between *Streptomyces* life cycle and antibiotic production

Most industrial processes for secondary metabolite production are performed in liquid (flasks or bioreactors). This fact makes the knowledge of the *Streptomyces* behavior essential in these conditions. The optimization of *Streptomyces* liquid cultures has been empiric for decades; however, the end of the so-called "golden age of antibiotics," which meant fewer and fewer compound discoveries or the advances in genome sequences revealing the existence of cryptic biosynthetic pathways that are not expressed under laboratory conditions, calls the attention of the scientific community.

Work on *Streptomyces* differentiation in liquid cultures has largely focused on the analysis of mycelial macroscopic morphology (pellets/clumps formation), media composition and bioreactor design [31]. The differentiation of the hyphae during the life cycle is essential for secondary metabolism [30]; however, there is still some controversy over which is the best morphology for production. Some authors affirm that pellets are better for production, while others report about clumps or even disaggregated growth. Hence, these conditions seem to be strain dependent. For instance, in *Streptomyces olidensis* (retamycin) [32], *Streptomyces tendae* (nikkomycins) [33], *Streptomyces lividans* (hybrid antibiotics) [34] and *Streptomyces coelicolor* (Undecylprodigiosin, Actinorhodin) [30], pellet formation is essential for good production. However, in *Streptomyces noursei* (nystatin) [35] and *Streptomyces fradiae* (tylosin) [36], formation of pellets leads to worse antibiotic production than disaggregated growth. The lack of a reliable developmental model in streptomycetes liquid cultures has hindered the precise identification of reliable phenotypes for use in the analysis and optimization of industrial fermentations. It has been demonstrated that antibiotics are produced by the substrate mycelium at the end of the proliferation phase, i.e., second mycelium morphology (after PCD process) [27, 30, 37]. However, despite the fact that there is a distinctive mycelium producing antibiotics (MII) [27, 30] (hence there is not secondary metabolite production until the differentiation of MII), antibiotic production has additional regulations, and each *Streptomyces* strain does not display its entire potential secondary metabolism at a specific developmental condition.

1.3 Screening for new bioactive compounds and drug discovery approaches

Most antibiotics and other bioactive compounds (antitumorals, immunosuppressors, etc.) were discovered from actinomycetes, but this source of drugs became challenging once the most common compounds were discovered. New antibiotic scaffolds are required, and resuming screening from nature is mandatory.

During the past 30 years, only two new classes of antibiotics have been brought to the clinic for treatment systemic infections [38]. At the same time, microbial resistance to existing antibiotics has increased dramatically, rendering some microbial infections that are extremely hard to treat. The current scenario looks gloomy, and there is a concrete risk of humanity returning to the pre-antibiotic era, with a high mortality from routine surgical or chemotherapeutic procedures because of infections by antibiotic-resistant pathogens. There is a general consensus that new antibiotics are urgently required and are the best chance in the fight against resistance.

Only a fraction of secondary metabolite pathways is active in actinomycete laboratory cultures, and there are a large number of strains whose potential to produce bioactive compounds remains unexplored [39]. When whole genome sequencing became available at the start of the twenty-first century, the existence of many silent or cryptic biosynthetic gene clusters in actinomycetes was revealed, which may encode antibiotic-like substances but are not, or only poorly, expressed under laboratory growth conditions. Each *Streptomyces* strain harbors 20–50 biosynthetic pathways for natural products, for which only some products have been identified [39]. The big challenge is to find ways to activate these pathways so as to allow screening for new secondary metabolites from nature to resume.

2. Strategies in drug discovery

Once the most common antibiotics and bioactive compounds were discovered, drug discovery has become more challenging, and industry had mostly discontinued the search for new drugs, with *Streptomyces* as the source. Nevertheless, the urgent necessity of new antibiotics in the clinic has caused screening from nature to be resumed [39]. New environments are being explored and some new actinomycete strains have been discovered [40]; the potential of the marine environment has been highly explored during last decade, as well as some, *a priori* hostile soils, such as high mountains, deserts or icy places. The scientific community is also paying more attention to symbiotic relationships.

Thinking of natural conditions of growth is another important point of view. Laboratory/industrial conditions in which microorganisms are typically grown are very different conditions from nature, which imply the dormancy of most of the genetic pathways involved in antibiotic production, since these substances are related to stress conditions or defense against niche competitors. Furthermore, it is necessary to take into account that the majority of environmental bacteria are not easily culturable or are even nonculturable in laboratory conditions. This broad view is now possible, thanks to the access to the metagenome in different environments [41].

Another current strategy is to look for elicitors. Elicitors are small molecules, which are able to induce *Streptomyces* differentiation as well as trigger cryptic antibiotic pathways.

Additionally, secondary metabolism is conditioned by differentiation, so it is another aspect that requires special attention. As mentioned above, the life cycle stages and the mycelium morphology are keys for antibiotic production.

At present, different complementary strategies are being developed to improve secondary metabolite production and active cryptic biosynthetic gene clusters. One way to classify them is by unselective and selective methods [42], considering unselective methods are used in the improvement of screening of new activities and selective methods are used in the improvement of production of known molecules.

2.1 Methods for searching for new bacteria/compounds

2.1.1 Exploring new environments

The actinomycetes are widely distributed in the environment; they were discovered at the end of the nineteenth century as a group of soil-living microorganisms. However, their importance started in 1943 with the discovery of streptomycin, the first effective treatment against tuberculosis by the most important actinomycetes genus, *Streptomyces*. Researchers have isolated different *Streptomyces* as soil inhabitants during decades as the most important source of antibiotics and bioactive compounds in nature. Clavulanic acid (*Streptomyces clavuligerus* [43]), neomycin (*S. marinensis* [44]), chloramphenicol (*Streptomyces venezuelae* [45]), the insecticide avermectin (*Streptomyces avermitilis* [46]), the immunosuppressant tacrolimus (*Streptomyces tsukubaensis* [47]), kanamycin (*Streptomyces kanamyceticus* [48]) and potent antitumoral platenolides (*Streptomyces platensis* [49]) are only few examples of the 12,400 bioactive compounds produced by the genus *Streptomyces* that society is routinely using in the clinic or agriculture. The rest of the phylum produces 3600 bioactive compounds (being 3400 antibiotics) [50]. Until the 1980s, new bioactive compounds were discovered relatively easily; however, since 1985, only three new compounds have been discovered, with the last one, platensimycin (*S. platensis* [40]), in 2006.

Once the most common compounds are discovered and producer strains are isolated, one of the strategies for founding new activities is the exploration of new environments, under-exploited habitats, generally difficult to access. Marine environment are an important niche of new species of *Streptomyces* [51]. Besides marine actinomycetes, they have been isolated from Himalaya Mountains [52], several islands [53], Atacama Desert [54], Antarctica [55] and several extremophile habitats [56–58].

Promising results and new species are being discovered from these unexplored areas, in combination with next-generation genome sequencing, metagenomics and new ways of bacterial culture [59], being one of the most important approaches in the new age of bioactive compound research.

2.1.2 Symbiosis relationships

As mentioned above, *Streptomyces* are ubiquitous in soils. However, it has been discovered that they are not only free-living soil bacteria but many have also evolved to live in symbiosis with plants, fungi and animals [60]. Their secondary metabolism is a consequence of these complex interactions [60].

There are many and very diverse symbiotic relationships, which involve *Streptomyces*. One of the first discovered is its relationship with plant roots providing a natural defense against fungal infections [61]; it is very common to find bacteria of the *Streptomyces* genus in the rhizosphere. This fact raises the possibility, currently discussed, about its use as biofertilizers in crops [62, 63]. Furthermore, genome mining is now allowing us to know on one hand which genes are involved in this interaction and on the other hand the discovery of potential novel molecules produced only in these conditions [64].

Other important interactions of *Streptomyces* genera take place between bacteria and insects. For instance, in the case of Beewolf digger wasps, *Streptomyces* provides protection against pathogens to the larval cocoon producing a mixture of nine antibiotic compounds [65, 66].

In *Streptomyces* isolates from colonies of the South African termite *Macrotermes natalensis*, a number of interesting novel compounds including natalamycin A

[67] and the potent antitumor geldanamycin [67] have been discovered. Another *Streptomyces* isolate from the same termite produces a novel cyclic analogue of dentigerumycin [68], the antifungal from the fungus-growing ant system. Finally, a series of glycosylated isoflavonoid compounds, including termisoflavone A [69], were recovered from a third *Streptomyces* isolate.

In 2012, 30 *Streptomyces* strains were isolated from a different South African fungus-growing termite, *Microtermes* sp., and two novel polyketides, microtermolides A and B [69], were identified.

Two *Streptomyces* strains, isolated from *Dendroctonus frontalis* beetles, inhibit the antagonistic *Ophiostoma* fungus, but not the mutualistic fungus *Entomocorticium*, suggesting a defensive role that supports the beetle-fungus mutualism. The molecules responsible for this selective antifungal activity are the polyketide mycangimycin [70] and the polycyclic tetramate macrolactams, frontalamides A and B [71, 72].

Other important symbiotic relationship takes place between ants and *Streptomyces*. In recent years, new strains and compounds have been isolated from ant head, legs and nests [73–76].

2.1.3 Nonculturable microorganisms

Since the beginning of microbiology, it was known that many microorganisms do not grow under laboratory conditions and we cannot cultivate them. The new techniques of massive sequencing have revealed the existence of a huge amount of nonculturable microorganisms. Nonculturable microorganisms are, in fact, a majority, and they represent an important challenge in the screening for new secondary metabolites. Next generation sequencing (NGS) has revealed the big pharmacological potential of uncultured bacteria; hence, approaches to improve cultivation-based methods, such as isolation chip (iChip), which is a method of culturing bacteria within its soil environment, or co-cultures, culturing actinomycetes with other bacteria (generally species with which they naturally coexist), are being used successfully [70–72]. The combination of iChip and unexplored ecosystems or symbiotic relationships, mentioned above, is promising as well.

2.2 Methods for screening improvement in a known microorganism (unselective)

Unselective methods include classical strategies for improving the production of a certain compound by modifying the growth of the *Streptomyces* strain. This strategy used to be empiric.

2.2.1 Changing media components

Streptomyces grow well in rich culture mediums; nevertheless, antibiotic production often needs some specific nutrients. Sometimes defined medium can be better for determined compounds. Optimization of nutritional requirements has been one of the most useful changes for secondary metabolite activities [73–76].

2.2.2 Inducing stress response

Stressful situations are one of the reasons why microorganisms start to produce and export bioactive compounds. For this reason, the induction of cultures with several treatments of stress is one of the improvement strategies for synthesis of secondary metabolite compounds in bacteria. Heat and ethanol shock treatments,

high salt conditions, increased hydrostatic pressure, acidic pH shock or feed limitations are some examples of this classical strategy (reviewed in [77]).

2.2.3 Random mutagenesis

There are different random mutagenesis methods used in *Streptomyces*: chemical mutagenesis, in which are used (e.g., by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), or nitrous acid (NA)) and physical mutagenesis (e.g., by ultraviolet (UV) light or X-rays)) that remain being robust methods to generate very high-producing strains [78–80].

On the other hand, there is transposon mutagenesis, a powerful tool for random mutagenesis of bacterial genomes and insertion of foreign DNA. A plasmid containing the enzyme transposase and the transposon (small fragment of DNA with transposase target regions) is extracted and inserted randomly into the host chromosome. Transposition creates single mutations that can be identified and mapped by plasmid rescue and DNA sequencing, generating a direct link between the higher yield (phenotype) and the transposon insertion (genotype) [81, 82].

2.2.4 Ribosomal engineering

The fact that some antibiotic resistances trigger some cryptic pathways of secondary metabolites has been recently used as another strategy in the search for new compounds.

It has been demonstrated that this occurs in strains affected by antibiotics, which target a ribosomal protein (for instance, streptomycin, paromomycin and gentamicin [42]), so the effects of the mutations at this level have had very good results, for example, in *Streptomyces coelicolor*, for improving secondary metabolite production [83]. The mechanism of this strategy is still unknown, but it has been proved that there is activation of the positive regulators like *act*II-*orf*4 gene at least in *S. coelicolor* [42]. Through this approach, the antibacterial piperidamycins family compounds have been discovered [84].

2.2.5 Elicitors

Elicitors are small diffusible molecules, which are being used as signals for improving secondary metabolite production or even activating cryptic antibiotic pathways [85].

Elicitors are also present in *Streptomyces* natural environments; hence one of the strategies for stimulating bioactive compound production is the co-culture of different bacteria [85, 86]. Co-cultures usually include species that have symbiotic relationships with *Streptomyces* in nature [87, 88] or pathogenic partners to activate the production of antimicrobial compounds against them [71, 89, 90].

Other kinds of elicitors are also useful, for instance, fungal elicitors (a complex mix of fungal cell walls and filtered fungi cultures) can positively affect the production of natamycin [91]; small molecules, such as GlcNac or phosphate, can activate differentiation and antibiotic production in *S. coelicolor* through the activation of *actII*-ORF4/*red*Z genes in some conditions [92].

At the end of the 1980s, the discovery of new compounds reached a bottleneck, and screening processes often led to the rediscovery of compounds already known. To avoid rediscovery, Pimentel-Elardo et al. [93] developed a workflow based on the use of chemical elicitors combined with activity-independent screening [93]. Activity-independent screening is based on the low-abundant

compounds found in biological samples. The use of elicitors increases the production of these low-abundant compounds, distinguishing them from the most abundant ones. The elicitor "CI-ARC" has been identified as being responsible for triggering several cryptic biosynthetic genes [93].

2.2.6 Differentiation strategies based on morphology

2.2.6.1 Streptomyces behavior in liquid cultures

Large-scale antibiotic production is mostly performed in liquid cultures. Macroscopic morphology of the mycelium (pellets and clumps) correlates with the production of secondary metabolites [30]. The genes controlling mycelium aggregation have been characterized in the *S. coelicolor mat* gene cluster [94], and the *cslA, glxA* and *dtpA* genes [95–97] are responsible for pellet formation. This knowledge can be used for controlling and improving morphology in industrial fermentations.

Since spores are inoculated in liquid medium, there is an aggregation trend [98]. Germlings determine the macroscopic morphology (pellets and clumps) of the culture, which involves the highly conserved genes *matA, matB* [94, 98] and the *cslA/glxA* operon [98].

In liquid cultures, there are some strains able to sporulate [99], but even those which aren't able to sporulate in flasks can sporulate in bioreactors, beacuse of the stess conditions inside the fermenter, such as lack of oxygen, strong agitation, etc. [100]. When *Streptomyces* bacteria sporulate, all the metabolic efforts are focused on that, which means that the secondary metabolism stops by. Therefore, one of the main things for taking into account during a screening for new secondary metabolites is to avoid sporulation in the industrial fermentations. Hence, it is very important to look out the cultures for keeping secondary metabolism as much as possible [100].

Morphology in liquid cultures can be monitored by several tools. Laser diffraction has been used to measure pellet size, an improvement over image analysis, which is trickier and requires more time [101], and a technique based on flow cytometry has been used to establish the pellet size distribution of a culture population [102, 103]. Recently, a useful algorithm has been developed as a plugin for the open-source software ImageJ to characterize the morphology of submerged growing cells [104]. On the other hand, mathematical models have been performed to predict the behavior of *Streptomyces* liquid cultures based on pellet/clump morphology [105, 106].

Furthermore, it has been reported that the oxygen transfer rate (OTR) and oxygen uptake rate (OUR) are crucial for the development and production in liquid cultures [100]. Controlling these rates, which directly affects morphology, was described as being crucial for scaling up production to industrial conditions [107]. Biophysical parameters, including pH, viscosity, agitation, dissolved oxygen levels and surface tension, directly affect mycelium morphology. The optimization of these factors is another factor to consider [31].

2.2.6.2 Programmed cell death and MII differentiation

As mentioned in the introduction of this chapter, programmed cell death (PCD) is a key event, triggering the differentiation of antibiotic producer mycelium (MII) in liquid and solid cultures [30]. However, the specific signals derived from cell death are not yet known. The N-acetylglucosamine resulting from

peptidoglycan dismantling accelerates the development and antibiotic production [108, 109] and might be one of the signals released during PCD. A simple methodology based on fluorimetric measures was designed to quantify PCD in liquid cultures [37], allowing prediction of the efficiency of antibiotic production based on the level of PCD.

PCD and MII differentiation can be modulated by modifying culture conditions and morphology. Modifying developmental conditions to enhance PCD and MII differentiation leads to the improvement of secondary metabolite production. This approach was recently applied in a study combining the heterologous expression of plant flavonoids in *Streptomyces albus* with a strategy to enhance PCD and MII differentiation [110]. The same strategy was successful at improving mTGase production from *Streptomyces mobaraensis* [111]. Therefore, PCD and MII differentiation were demonstrated to be crucial for the streamlining of secondary metabolite production in bioreactors [100].

2.3 Methods for production improvement in a known compound (selective)

Selective methods with a specific target include regulatory engineering, heterologous expression, genome mining and combinatorial biosynthesis.

2.3.1 Regulatory engineering

Each active compound, for example, antibiotic or antitumor, etc., has several molecules synthesized first in the pathway, and sometimes these molecules are a limited source for the compound of interest. In this sense, one strategy widely used is the addition of these precursors, previously synthetized from an external source. In the same way, it is possible to enhance the production of a specific compound through the overexpression of its positive regulators. Inversely, knocking out the suppressors (negative regulation) of the gene of interest has also had good results increasing the final compound production [112, 113].

2.3.2 Heterologous expression

In microbiology, this molecular genetic technique is based on the expression of a gene or a group of genes (gene cluster) in a host microorganism that does not have this particular gene or gene cluster in its own genome. This procedure, performed by recombinant DNA technology, is very useful when hosts are bacteria with simple developmental cycles, such as *E. coli* or *Bacillus* [114]. However, in the case of *Streptomyces,* due to its complex pathways to produce antibiotics, heterologous expression usually works only among *Streptomyces* strains [115, 116], not solving the problem of its tricky growth.

Nevertheless, good results have been achieved in activating cryptic metabolites, for example, by using the widely conserved *Streptomyces coelicolor* pleiotropic regulator *Afs*Q, which activates the production of new compounds in several streptomycetes [117]. Despite the fact that heterologous expression in *Streptomyces* is a challenge, this technique continues to be improved [118].

2.3.3 Genome mining

Genome mining is defined as the obtaining of information about an organism, based on genome analysis. In the postgenomic era, the combination of easier genome sequencing and bioinformatics analyses allows researchers to uncover the

biosynthetic potential of the microorganisms, i.e., the gene clusters for producing secondary metabolites into actinomycetes strains that are in the genome, but silent. Genome mining is nowadays one of the more powerful tools in the screening for new antibiotics [119–122].

2.3.4 Combinatorial biosynthesis

Combinatorial biosynthesis can be defined as the application to modify biosynthetic pathways to natural products to produce new and altered structures using nature's biosynthetic machinery [123]. The chemical modification of existing chemical scaffolds is challenging and sometimes provides only a temporary solution against resistant organisms [124]. Combinatorial biosynthesis has been largely developed over the last 20 years, and it has been enhanced, thanks to genome mining and synthetic biology [125–127].

3. Conclusions

Humanity faces the great challenge of fighting antibiotic resistance, which is growing at a faster rate than our capacity to find new antimicrobials and new strategies to solve this problem.

The *Streptomyces* genus is still a huge source of natural bioactive compounds, but we need to elaborate new multidisciplinary strategies to avoid rediscovering the same compounds. Therefore, we need to invest efforts into developing alternative strategies to resume screening from natural actinomycetes.

There is not a "magic" methodology to activate cryptic pathways and improve the discovery of new bioactive compounds, but multidisciplinary approaches combining the methodologies discussed in this chapter will play a key role in the improvement of screening for new bioactive compounds from streptomycetes.

Acknowledgements

We thank the Spanish "Ministerio de Economía y Competitividad" (MINECO; BIO2015-65709-R) and the "Marie Curie cofund Clarin" Grant for financial support and Proof-Reading-Service.com for proofreading the final manuscript.

Conflict of interest

There is no conflict of interest in this work.

Author details

Ángel Manteca[1] and Paula Yagüe[2*]

1 Oviedo University, Oviedo, Spain

2 Leiden University, Leiden, Netherlands

*Address all correspondence to: p.yague.menendez@biology.leidenuniv.nl

IntechOpen

References

[1] Challis GL, Hopwood DA. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(Suppl 2):14555-14561

[2] Bobek J, Smidova K, Cihak M. A waking review: Old and novel insights into the spore germination in *Streptomyces*. Frontiers in Microbiology. 2017;**8**:2205

[3] Noens EE, Mersinias V, Willemse J, Traag BA, Laing E, Chater KF, et al. Loss of the controlled localization of growth stage-specific cell-wall synthesis pleiotropically affects developmental gene expression in an ssgA mutant of *Streptomyces coelicolor*. Molecular Microbiology. 2007;**64**(5):1244-1259

[4] Kalakoutskii LV, Agre NS. Comparative aspects of development and differentiation in actinomycetes. Bacteriological Reviews. 1976;**40**(2):469-524

[5] Ranade N, Vining LC. Accumulation of intracellular carbon reserves in relation to chloramphenicol biosynthesis by *Streptomyces venezuelae*. Canadian Journal of Microbiology. 1993;**39**(4):377-383

[6] Strakova E, Bobek J, Zikova A, Vohradsky J. Global features of gene expression on the proteome and transcriptome levels in *S. coelicolor* during germination. PLoS One. 2013;**8**(9):e72842

[7] Hardisson C, Manzanal MB, Salas JA, Suarez JE. Fine structure, physiology and biochemistry of arthrospore germination in *Streptomyces antibioticus*. Journal of General Microbiology. 1978;**105**(2):203-214

[8] Rioseras B, Yague P, Lopez-Garcia MT, Gonzalez-Quinonez N, Binda E, Marinelli F, et al. Characterization of SCO4439, a D-alanyl-D-alanine carboxypeptidase involved in spore cell wall maturation, resistance, and germination in *Streptomyces coelicolor*. Scientific Reports. 2016;**6**:21659

[9] Grund AD, Ensign JC. Properties of the germination inhibitor of *Streptomyces viridochromogenes* spores. Journal of General Microbiology. 1985;**131**(4):833-847

[10] Salas JA, Guijarro JA, Hardisson C. High calcium content in *Streptomyces* spores and its release as an early event during spore germination. Journal of Bacteriology. 1983;**155**(3):1316-1323

[11] McBride MJ, Ensign JC. Effects of intracellular trehalose content on *Streptomyces griseus* spores. Journal of Bacteriology. 1987;**169**(11):4995-5001

[12] Nwaka S, Holzer H. Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. Progress in Nucleic Acid Research and Molecular Biology. 1998;**58**:197-237

[13] Singer MA, Lindquist S. Multiple effects of trehalose on protein folding in vitro and in vivo. Molecular Cell. 1998;**1**(5):639-648

[14] Mikulik K, Janda I, Weiser J, Stastna J, Jiranova A. RNA and ribosomal protein patterns during aerial spore germination in *Streptomyces* granaticolor. European Journal of Biochemistry. 1984;**145**(2):381-388

[15] Bobek J, Halada P, Angelis J, Vohradsky J, Mikulik K. Activation and expression of proteins during synchronous germination of aerial spores of *Streptomyces* granaticolor. Proteomics. 2004;**4**(12):3864-3880

[16] Flardh K. Essential role of DivIVA in polar growth and morphogenesis in *Streptomyces coelicolor* A3(2). Molecular Microbiology. 2003;**49**(6):1523-1536

[17] Flardh K. Growth polarity and cell division in *Streptomyces*. Current Opinion in Microbiology. 2003;**6**(6):564-571

[18] Flardh K, Richards DM, Hempel AM, Howard M, Buttner MJ. Regulation of apical growth and hyphal branching in *Streptomyces*. Current Opinion in Microbiology. 2012;**15**(6):737-743

[19] Cihak M, Kamenik Z, Smidova K, Bergman N, Benada O, Kofronova O, et al. Secondary Metabolites Produced during the Germination of *Streptomyces coelicolor*. Frontiers in Microbiology. 2017;**8**:2495

[20] Yague P, Willemse J, Koning RI, Rioseras B, Lopez-Garcia MT, Gonzalez-Quinonez N, et al. Subcompartmentalization by cross-membranes during early growth of *Streptomyces hyphae*. Nature Communications. 2016;**7**:12467

[21] Manteca A, Fernandez M, Sanchez J. A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*. Microbiology. 2005;**151**(Pt 11):3689-3697

[22] Yague P, Rodriguez-Garcia A, Lopez-Garcia MT, Martin JF, Rioseras B, Sanchez J, et al. Transcriptomic analysis of *Streptomyces coelicolor* differentiation in solid sporulating cultures: First compartmentalized and second multinucleated mycelia have different and distinctive transcriptomes. PLoS One. 2013;**8**(3):e60665

[23] Claessen D, de Jong W, Dijkhuizen L, Wosten HA. Regulation of *Streptomyces* development: Reach for the sky! Trends in Microbiology. 2006;**14**(7):313-319

[24] Flardh K, Buttner MJ. *Streptomyces morphogenetics*: Dissecting differentiation in a filamentous bacterium. Nature Reviews. Microbiology. 2009;**7**(1):36-49

[25] Kaiser BK, Stoddard BL. DNA recognition and transcriptional regulation by the WhiA sporulation factor. Scientific Reports. 2011;**1**:156

[26] Schlimpert S, Flardh K, Buttner J. Fluorescence time-lapse imaging of the complete *S. venezuelae* life cycle using a microfluidic device. Journal of Visualized Experiments. 2016;**108**:53863

[27] Yague P, Rodriguez-Garcia A, Lopez-Garcia MT, Rioseras B, Martin JF, Sanchez J, et al. Transcriptomic analysis of liquid non-sporulating *Streptomyces coelicolor* cultures demonstrates the existence of a complex differentiation comparable to that occurring in solid sporulating cultures. PLoS One. 2014;**9**(1):e86296

[28] Manteca A, Jung HR, Schwammle V, Jensen ON, Sanchez J. Quantitative proteome analysis of *Streptomyces coelicolor* nonsporulating liquid cultures demonstrates a complex differentiation process comparable to that occurring in sporulating solid cultures. Journal of Proteome Research. 2010;**9**(9):4801-4811

[29] Rioseras B, Shliaha PV, Gorshkov V, Yague P, Lopez-Garcia MT, Gonzalez-Quinonez N, et al. Quantitative proteome and phosphoproteome analyses of *Streptomyces coelicolor* reveal proteins and phosphoproteins modulating differentiation and secondary metabolism. Molecular & Cellular Proteomics. 2018;**17**(8):1591-1611

[30] Manteca A, Alvarez R, Salazar N, Yague P, Sanchez J. Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. Applied

and Environmental Microbiology. 2008;**74**(12):3877-3886

[31] van Dissel D, Claessen D, van Wezel GP. Morphogenesis of *Streptomyces* in submerged cultures. Advances in Applied Microbiology. 2014;**89**:1-45

[32] Giudici R, Pamboukian CR, Facciotti MC. Morphologically structured model for antitumoral retamycin production during batch and fed-batch cultivations of *Streptomyces olindensis*. Biotechnology and Bioengineering. 2004;**86**(4):414-424

[33] Vecht-Lifshitz SE, Sasson Y, Braun S. Nikkomycin production in pellets of *Streptomyces tendae*. The Journal of Applied Bacteriology. 1992;**72**(3):195-200

[34] Sarra M, Casas C, Godia F. Continuous production of a hybrid antibiotic by *Streptomyces lividans* TK21 pellets in a three-phase fluidized-bed bioreactor. Biotechnology and Bioengineering. 1997;**53**(6):601-610

[35] Jonsbu E, McIntyre M, Nielsen J. The influence of carbon sources and morphology on nystatin production by *Streptomyces noursei*. Journal of Biotechnology. 2002;**95**(2):133-144

[36] Stratigopoulos G, Gandecha AR, Cundliffe E. Regulation of tylosin production and morphological differentiation in *Streptomyces fradiae* by TylP, a deduced gamma-butyrolactone receptor. Molecular Microbiology. 2002;**45**(3):735-744

[37] Yague P, Manteca A, Simon A, Diaz-Garcia ME, Sanchez J. New method for monitoring programmed cell death and differentiation in submerged *Streptomyces* cultures. Applied and Environmental Microbiology. 2010;**76**(10):3401-3404

[38] Procopio RE, Silva IR, Martins MK, Azevedo JL, Araujo JM. Antibiotics produced by *Streptomyces*. The Brazilian Journal of Infectious Diseases. 2012;**16**(5):466-471

[39] Genilloud O. The re-emerging role of microbial natural products in antibiotic discovery. Antonie Van Leeuwenhoek. 2014;**106**(1):173-188

[40] Takahashi Y, Omura S. Isolation of new actinomycete strains for the screening of new bioactive compounds. The Journal of General and Applied Microbiology. 2003;**49**(3):141-154

[41] McMahon MD, Guan C, Handelsman J, Thomas MG. Metagenomic analysis of *Streptomyces lividans* reveals host-dependent functional expression. Applied and Environmental Microbiology. 2012;**78**(10):3622-3629

[42] Craney A, Ahmed S, Nodwell J. Towards a new science of secondary metabolism. Journal of Antibiotics (Tokyo). 2013;**66**(7):387-400

[43] Reading C, Cole M. Clavulanic acid: A beta-lactamase-inhiting beta-lactam from *Streptomyces clavuligerus*. Antimicrobial Agents and Chemotherapy. 1977;**11**(5):852-857

[44] Waksman SA, Lechevalier HA. Neomycin, a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. Science. 1949;**109**(2830):305-307

[45] Shapiro S, Vining LC. Nitrogen metabolism and chloramphenicol production in *Streptomyces venezuelae*. Canadian Journal of Microbiology. 1983;**29**(12):1706-1714

[46] Ikeda H, Nonomiya T, Usami M, Ohta T, Omura S. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. Proceedings of the National Academy

of Sciences of the United States of America. 1999;**96**(17):9509-9514

[47] Karlsson H, Truedsson L, Nassberger L. The immunosuppressive agent FK506 inhibits in vitro expression of membrane-bound and soluble interleukin-2 receptors on resting but not on activated human lymphocytes. Immunology Letters. 1991;**30**(1):129-132

[48] Kharel MK, Subba B, Basnet DB, Woo JS, Lee HC, Liou K, et al. A gene cluster for biosynthesis of kanamycin from *Streptomyces kanamyceticus*: Comparison with gentamicin biosynthetic gene cluster. Archives of Biochemistry and Biophysics. 2004;**429**(2):204-214

[49] Sakai T, Sameshima T, Matsufuji M, Kawamura N, Dobashi K, Mizui Y. Pladienolides, new substances from culture of *Streptomyces platensis* Mer-11107. I. Taxonomy, fermentation, isolation and screening. Journal of Antibiotics (Tokyo). 2004;**57**(3):173-179

[50] Hamedi J, Poorinmohammad N, Wink J. The role of actinobacteria in biotechnology. In: Wink J, Mohammadipanah F, Hamedi J, editors. Biology and Biotechnology of Actinobacteria. Cham: Springer; 2017. pp. 269-328. DOI: 10.1007/9783319603391

[51] Lam KS. Discovery of novel metabolites from marine actinomycetes. Current Opinion in Microbiology. 2006;**9**(3):245-251

[52] Bhattacharjee K, Banerjee S, Joshi SR. Diversity of *Streptomyces* spp. in eastern Himalayan region–computational RNomics approach to phylogeny. Bioinformation. 2012;**8**(12):548-554

[53] Charousova I, Medo J, Halenarova E, Javorekova S. Antimicrobial and enzymatic activity of actinomycetes isolated from soils of coastal islands. Journal of Advanced Pharmaceutical Technology & Research. 2017;**8**(2):46-51

[54] Idris H, Labeda DP, Nouioui I, Castro JF, Del Carmen Montero-Calasanz M, Bull AT, et al. *Streptomyces aridus* sp. nov., isolated from a high altitude Atacama Desert soil and emended description of *Streptomyces noboritoensis* Isono et al. 1957. Antonie Van Leeuwenhoek. 2017;**110**(5):705-717

[55] Sanyal A, Antony R, Samui G, Thamban M. Microbial communities and their potential for degradation of dissolved organic carbon in cryoconite hole environments of Himalaya and Antarctica. Microbiological Research. 2018;**208**:32-42

[56] Bonilla JO, Kurth DG, Cid FD, Ulacco JH, Gil RA, Villegas LB. Prokaryotic and eukaryotic community structure affected by the presence of an acid mine drainage from an abandoned gold mine. Extremophiles. 2018;**22**(5):699-711

[57] Ben Abdallah M, Karray F, Kallel N, Armougom F, Mhiri N, Quemeneur M, et al. Abundance and diversity of prokaryotes in ephemeral hypersaline lake Chott El Jerid using Illumina Miseq sequencing, DGGE and qPCR assays. Extremophiles. 2018;**22**(5):811-823

[58] Bull AT, Idris H, Sanderson R, Asenjo J, Andrews B, Goodfellow M. High altitude, hyper-arid soils of the Central-Andes harbor mega-diverse communities of actinobacteria. Extremophiles. 2018;**22**(1):47-57

[59] Stewart EJ. Growing unculturable bacteria. Journal of Bacteriology. 2012;**194**(16):4151-4160

[60] Seipke RF, Kaltenpoth M, Hutchings MI. *Streptomyces* as symbionts: An emerging and widespread theme?

FEMS Microbiology Reviews. 2012;**36**(4):862-876

[61] Schrey SD, Tarkka MT. Friends and foes: Streptomycetes as modulators of plant disease and symbiosis. Antonie Van Leeuwenhoek. 2008;**94**(1):11-19

[62] Rey T, Dumas B. Plenty is no plague: *Streptomyces* symbiosis with crops. Trends in Plant Science. 2017;**22**(1):30-37

[63] Vurukonda S, Giovanardi D, Stefani E. Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. International Journal of Molecular Sciences. 2018;**19**(4)

[64] Ceapa CD, Vazquez-Hernandez M, Rodriguez-Luna SD, Cruz Vazquez AP, Jimenez Suarez V, Rodriguez-Sanoja R, et al. Genome mining of *Streptomyces scabrisporus* NF3 reveals symbiotic features including genes related to plant interactions. PLoS One. 2018;**13**(2):e0192618

[65] Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, et al. Symbiotic Streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nature Chemical Biology. 2010;**6**(4):261-263

[66] Engl T, Kroiss J, Kai M, Nechitaylo TY, Svatos A, Kaltenpoth M. Evolutionary stability of antibiotic protection in a defensive symbiosis. Proceedings of the National Academy of Sciences of the United States of America. 2018;**115**(9):E2020-E2029

[67] Kim KH, Ramadhar TR, Beemelmanns C, Cao S, Poulsen M, Currie CR, et al. Natalamycin A, an ansamycin from a termite-associated *Streptomyces* sp. Chemical Science. 2014;**5**(11):4333-4338

[68] Wyche TP, Ruzzini AC, Beemelmanns C, Kim KH, Klassen JL, Cao S, et al. Linear peptides are the major products of a biosynthetic pathway that encodes for cyclic depsipeptides. Organic Letters. 2017;**19**(7):1772-1775

[69] Kang HR, Lee D, Benndorf R, Jung WH, Beemelmanns C, Kang KS, et al. Termisoflavones A-C, isoflavonoid glycosides from termite-associated *Streptomyces* sp. RB1. Journal of Natural Products. 2016;**79**(12):3072-3078

[70] Kealey C, Creaven CA, Murphy CD, Brady CB. New approaches to antibiotic discovery. Biotechnology Letters. 2017;**39**(6):805-817

[71] Sung AA, Gromek SM, Balunas MJ. Upregulation and identification of antibiotic activity of a marine-derived *Streptomyces* sp. via co-cultures with human pathogens. Marine Drugs. 2017;**15**(8)

[72] Onaka H, Mori Y, Igarashi Y, Furumai T. Mycolic acid-containing bacteria induce natural-product biosynthesis in *Streptomyces* species. Applied and Environmental Microbiology. 2011;**77**(2):400-406

[73] Vastrad BM, Neelagund SE. Optimization of medium composition for the production of neomycin by *Streptomyces* fradiae NCIM 2418 in solid state fermentation. Biotechnology Research International. 2014;**2014**:674286

[74] Alduina R, Sosio M, Donadio S. Complex regulatory networks governing production of the glycopeptide A40926. Antibiotics (Basel). 2018;**7**(2)

[75] Jakeman DL, Graham CL, Young W, Vining LC. Culture conditions improving the production of jadomycin B. Journal of Industrial Microbiology & Biotechnology. 2006;**33**(9):767-772

[76] Saudagar PS, Singhal RS. Optimization of nutritional requirements and feeding strategies

for clavulanic acid production by *Streptomyces clavuligerus*. Bioresource Technology. 2007;**98**(10):2010-2017

[77] Yoon V, Nodwell JR. Activating secondary metabolism with stress and chemicals. Journal of Industrial Microbiology & Biotechnology. 2014;**41**(2):415-424

[78] Baltz RH. Genetic manipulation of secondary metabolite biosynthesis for improved production in *Streptomyces* and other actinomycetes. Journal of Industrial Microbiology & Biotechnology. 2016;**43**(2-3):343-370

[79] Khaliq S, Akhtar K, Afzal Ghauri M, Iqbal R, Mukhtar Khalid A, Muddassar M. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of *Streptomyces fradiae* NRRL-2702. Microbiological Research. 2009;**164**(4):469-477

[80] Korbekandi H, Darkhal P, Hojati Z, Abedi D, Hamedi J, Pourhosein M. Overproduction of clavulanic acid by UV mutagenesis of *Streptomyces clavuligerus*. Iranian Journal of Pharmaceutical Research. 2010;**9**(2):177-181

[81] Fedashchin A, Cernota WH, Gonzalez MC, Leach BI, Kwan N, Wesley RK, et al. Random transposon mutagenesis of the *Saccharopolyspora erythraea* genome reveals additional genes influencing erythromycin biosynthesis. FEMS Microbiology Letters. 2015;**362**(22)

[82] Xu Z, Wang Y, Chater KF, Ou HY, Xu HH, Deng Z, et al. Large-scale transposition mutagenesis of *Streptomyces coelicolor* identifies hundreds of genes influencing antibiotic biosynthesis. Applied and Environmental Microbiology. 2017;**83**(6)

[83] Wang G, Hosaka T, Ochi K. Dramatic activation of antibiotic production in *Streptomyces coelicolor* by cumulative drug resistance mutations. Applied and Environmental Microbiology. 2008;**74**(9):2834-2840

[84] Hosaka T, Ohnishi-Kameyama M, Muramatsu H, Murakami K, Tsurumi Y, Kodani S, et al. Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. Nature Biotechnology. 2009;**27**(5):462-464

[85] Onaka H. Novel antibiotic screening methods to awaken silent or cryptic secondary metabolic pathways in actinomycetes. Journal of Antibiotics (Tokyo). 2017;**70**(8):865-870

[86] Marmann A, Aly AH, Lin W, Wang B, Proksch P. Co-cultivation--a powerful emerging tool for enhancing the chemical diversity of microorganisms. Marine Drugs. 2014;**12**(2):1043-1065

[87] Piel J. Metabolites from symbiotic bacteria. Natural Product Reports. 2004;**21**(4):519-538

[88] Yu J, Liu Q, Chen C, Qi X. Antifungal activity change of *Streptomyces rimosus* MY02 mediated by confront culture with other microorganism. Journal of Basic Microbiology. 2017;**57**(3):276-282

[89] Perez J, Munoz-Dorado J, Brana AF, Shimkets LJ, Sevillano L, Santamaria RI. *Myxococcus xanthus* induces actinorhodin overproduction and aerial mycelium formation by *Streptomyces coelicolor*. Microbial Biotechnology. 2011;**4**(2):175-183

[90] Wang Y, Wang L, Zhuang Y, Kong F, Zhang C, Zhu W. Phenolic polyketides from the co-cultivation of marine-derived *Penicillium* sp. WC-29-5 and *Streptomyces fradiae* 007. Marine Drugs. 2014;**12**(4):2079-2088

[91] Wang D, Yuan J, Gu S, Shi Q. Influence of fungal elicitors on biosynthesis of natamycin by *Streptomyces natalensis* HW-2. Applied Microbiology and Biotechnology. 2013;**97**(12):5527-5534

[92] Liu G, Chater KF, Chandra G, Niu G, Tan H. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. Microbiology and Molecular Biology Reviews. 2013;**77**(1):112-143

[93] Pimentel-Elardo SM, Sorensen D, Ho L, Ziko M, Bueler SA, Lu S, et al. Activity-independent discovery of secondary metabolites using chemical elicitation and cheminformatic inference. ACS Chemical Biology. 2015;**10**(11):2616-2623

[94] van Dissel D, Claessen D, Roth M, van Wezel GP. A novel locus for mycelial aggregation forms a gateway to improved *Streptomyces* cell factories. Microbial Cell Factories. 2015;**14**:44

[95] Chaplin AK, Petrus ML, Mangiameli G, Hough MA, Svistunenko DA, Nicholls P, et al. GlxA is a new structural member of the radical copper oxidase family and is required for glycan deposition at hyphal tips and morphogenesis of *Streptomyces lividans*. The Biochemical Journal. 2015;**469**(3):433-444

[96] Petrus ML, Vijgenboom E, Chaplin AK, Worrall JA, van Wezel GP, Claessen D. The DyP-type peroxidase DtpA is a Tat-substrate required for GlxA maturation and morphogenesis in *Streptomyces*. Open Biology. 2016;**6**(1):150149

[97] Petrus ML, Claessen D. Pivotal roles for *Streptomyces* cell surface polymers in morphological differentiation, attachment and mycelial architecture. Antonie Van Leeuwenhoek. 2014;**106**(1):127-139

[98] Zacchetti B, Willemse J, Recter B, van Dissel D, van Wezel GP, Wosten HA, et al. Aggregation of germlings is a major contributing factor towards mycelial heterogeneity of *Streptomyces*. Scientific Reports. 2016;**6**:27045

[99] Geneviève Girard BAT, Sangal V, Mascini N, Hoskisson PA, Goodfellow M, van Wezel GP. A novel taxonomic marker that discriminates between morphologically complex actinomycetes. Open Biology. 2013;**3**(10):130073

[100] Rioseras B, Lopez-Garcia MT, Yague P, Sanchez J, Manteca A. Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: Programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production. Bioresource Technology. 2014;**151**:191-198

[101] Ronnest NP, Stocks SM, Lantz AE, Gernaey KV. Comparison of laser diffraction and image analysis for measurement of *Streptomyces coelicolor* cell clumps and pellets. Biotechnology Letters. 2012;**34**(8):1465-1473

[102] van Veluw GJ, Petrus ML, Gubbens J, de Graaf R, de Jong IP, van Wezel GP, et al. Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach. Applied Microbiology and Biotechnology. 2012;**96**(5):1301-1312

[103] Petrus ML, van Veluw GJ, Wosten HA, Claessen D. Sorting of *Streptomyces* cell pellets using a complex object parametric analyzer and sorter. Journal of Visualized Experiments. 2014;**84**:e51178

[104] Willemse J, Buke F, van Dissel D, Grevink S, Claessen D, van Wezel GP. SParticle, an algorithm for the analysis of filamentous microorganisms in submerged cultures. Antonie Van Leeuwenhoek. 2017;**111**(2):171-182

[105] Celler PC. K, van Loosdrecht MC, van Wezel GP., Structured

morphological modeling as a framework for rational strain design of *Streptomyces* species. Antonie Van Leeuwenhoek. 2012;**102**(3):409-423

[106] Nieminen L, Webb S, Smith MC, Hoskisson PA. A flexible mathematical model platform for studying branching networks: Experimentally validated using the model actinomycete, *Streptomyces coelicolor*. PLoS One. 2013;**8**(2):e54316

[107] Ha S, Lee KJ, Lee SI, Gwak HJ, Lee JH, Kim TW, et al. Optimization of Herbicidin A Production in Submerged Culture of *Streptomyces* scopuliridis M40. Journal of Microbiology and Biotechnology. 2017;**27**(5):947-955

[108] Rigali S, Nothaft H, Noens EE, Schlicht M, Colson S, Müller M, et al. The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links N-acetylglucosamine metabolism to the control of development. Molecular Microbiology. 2006;**61**(5):1237-1251

[109] Rigali S, Titgemeyer F, Barends S, Mulder S, Thomae AW, Hopwood DA, et al. Feast or famine: The global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. EMBO Reports. 2008;**9**(7):670-675

[110] Marin L, Gutierrez-Del-Rio I, Yague P, Manteca A, Villar CJ, Lombo F. De novo biosynthesis of apigenin, luteolin, and eriodictyol in the actinomycete *Streptomyces albus* and production improvement by feeding and spore conditioning. Frontiers in Microbiology. 2017;**8**:921

[111] LuciaTreppiccione AO, Luongo D, Maurano F, Manteca Á, FelipeLombó MR. Development of gluten with immunomodulatory properties using mTG-active food grade supernatants from *Streptomyces mobaraensis* cultures. Journal of Functional Foods. 2017;**34**

[112] Yi JS, Kim M, Kim EJ, Kim BG. Production of pikromycin using branched chain amino acid catabolism in *Streptomyces venezuelae* ATCC 15439. Journal of Industrial Microbiology & Biotechnology. 2018;**45**(5):293-303

[113] Razmilic V, Castro JF, Andrews B, Asenjo JA. Analysis of metabolic networks of *Streptomyces leeuwenhoekii* C34 by means of a genome scale model: Prediction of modifications that enhance the production of specialized metabolites. Biotechnology and Bioengineering. 2018;**115**(7):1815-1828

[114] Ece S, Lambertz C, Fischer R, Commandeur U. Heterologous expression of a *Streptomyces cyaneus* laccase for biomass modification applications. AMB Express. 2017;**7**(1):86

[115] Novakova R, Nunez LE, Homerova D, Knirschova R, Feckova L, Rezuchova B, et al. Increased heterologous production of the antitumoral polyketide mithramycin A by engineered *Streptomyces lividans* TK24 strains. Applied Microbiology and Biotechnology. 2018;**102**(2):857-869

[116] Kawahara T, Izumikawa M, Kozone I, Hashimoto J, Kagaya N, Koiwai H, et al. Neothioviridamide, a polythioamide compound produced by heterologous expression of a *Streptomyces* sp. cryptic RiPP biosynthetic gene cluster. Journal of Natural Products. 2018

[117] Daniel-Ivad M, Hameed N, Tan S, Dhanjal R, Socko D, Pak P, et al. An engineered allele of afsQ1 facilitates the discovery and investigation of cryptic natural products. ACS Chemical Biology. 2017;**12**(3):628-634

[118] Mevaere J, Goulard C, Schneider O, Sekurova ON, Ma H, Zirah S, et al. An orthogonal system for heterologous expression of actinobacterial lasso peptides in *Streptomyces* hosts. Scientific Reports. 2018;**8**(1):8232

[119] Yu Y, Tang B, Dai R, Zhang B, Chen L, Yang H, et al. Identification of the streptothricin and tunicamycin biosynthetic gene clusters by genome mining in *Streptomyces* sp. strain fd1-xmd. Applied Microbiology and Biotechnology. 2018;**102**(6):2621-2633

[120] Liu W, Sun F, Hu Y. Genome mining-mediated discovery of a new avermipeptin analogue in *Streptomyces actuosus* ATCC 25421. ChemistryOpen. 2018;**7**(7):558-561

[121] Gran-Scheuch A, Trajkovic M, Parra L, Fraaije MW. Mining the genome of *Streptomyces leeuwenhoekii*: Two new type I Baeyer-Villiger Monooxygenases from Atacama Desert. Frontiers in Microbiology. 2018;**9**:1609

[122] Ye S, Molloy B, Brana AF, Zabala D, Olano C, Cortes J, et al. Identification by genome mining of a type I polyketide gene cluster from *Streptomyces argillaceus* involved in the biosynthesis of pyridine and piperidine alkaloids argimycins P. Frontiers in Microbiology. 2017;**8**:194

[123] Floss HG. Combinatorial biosynthesis--potential and problems. Journal of Biotechnology. 2006;**124**(1):242-257

[124] Genilloud O. Actinomycetes: Still a source of novel antibiotics. Natural Product Reports. 2017;**34**(10):1203-1232

[125] Olano C, Mendez C, Salas JA. Post-PKS tailoring steps in natural product-producing actinomycetes from the perspective of combinatorial biosynthesis. Natural Product Reports. 2010;**27**(4):571-616

[126] González A, Rodríguez M, Braña AF, Méndez C, Salas JA, Olano C. New insights into paulomycin biosynthesis pathway in *Streptomyces* albus J1074 and generation of novel derivatives by combinatorial biosynthesis. Microbial Cell Factories. 2016:15

[127] Baltz RH. Synthetic biology, genome mining, and combinatorial biosynthesis of NRPS-derived antibiotics: A perspective. Journal of Industrial Microbiology & Biotechnology. 2017;**45**(7):635-649

Edited by Sahra Kırmusaoğlu

To prevent bacterial adherence, invasion and infection, antimicrobials such as antibiotics are being used and vastly researched nowdays. Several factors such as natural selection, mutations in genes, the presence of efflux pumps, impermeability of the cell wall, structural changes in enzymes and receptors, biofilm formation, and quorum sensing cause microorganisms to develop resistance against antimicrobials. Isolates that synthesize extended spectrum-β-lactamases (ESBL), induced β-lactamases (IBL), carbapenamases, metallo-β-lactamases (MBLs), and New Delhi metallo-β-lactamases (NDM) have emerged. Determining virulence factors such as biofilms and the level of antimicrobial activities of antimicrobial agents alone and in combination with appropriate doses against microorganisms is very important for the diagnosis, inhibition, and prevention of microbial infection. The goal of this book is to provide information on all these topics.

Published in London, UK

IntechOpen

ISBN 978-1-83962-103-1
9 781839 621031