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Progress in Understanding Cystic Fibrosis

Edited by Dinesh Sriramulu

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Notice

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Meet the editor

Dinesh Sriramulu graduated from the Technical University of Braunschweig, Germany, with his doctorate degree in Medical Microbiology. He started his research career at the Helmholtz Centre for Infection Research, Braunschweig, Germany, in collaboration with the Karolinska Institutet, Stockholm, Sweden. His area of expertise is on the adaptation of bacteria toward diverse niches, ranging from the human lung to the cattle rumen. He also worked on tumor microenvironment in the case of esophageal and breast cancers. He continued his research work at various reputed institutions worldwide: University College Cork, Ireland; University of Medicine and Dentistry of New Jersey, USA; Food and Drug Administration, Rockville, USA; University of Southern California, Los Angeles, USA; University of Trento, Italy; and University of Cape Town, South Africa. He has published his research findings in various international peer-reviewed journals and presented his works at international conferences. He has been serving as an editorial board member, a peer reviewer, and an expert referee for scientific journals and research funding agencies.

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Preface

The Open Access Initiative has been making its stride toward liberal dissemination of scientific knowledge to the world community through unrestricted access to full-text articles, chapters, and other contents. Since the inception of virtual media science publishing has been adopting a constant fast-track update of information as and when it occurs. In this line, InTech has been making its footprint all along by identifying and openly involving eminent scientists worldwide. The book, *Progress in Understanding Cystic Fibrosis*, is the successor of *Cystic Fibrosis – Renewed Hopes Through Research*.

Cystic fibrosis (CF), also known as mucoviscidosis, is an autosomal recessive multisystem genetic disorder that occurs predominantly among Caucasians. Though found highest among Irish population, the incidence of CF is on the rise among other populations including the least-affected ones. The interdisciplinary approach toward better understanding the CF condition and the development of sensitive early diagnostic methods have contributed toward efficient diagnosis, treatment, and management of the disease. The CF condition is characterized by abnormal transport of chloride and sodium across the epithelium that leads to thickening of secretions especially in the lungs, pancreas, liver, and intestine. The complex nature of this disease involving multiple organs and subsequent secondary infections by microbes is the basis for mortality in CF population. Decades of research by scientists worldwide has narrowed down the cause of CF to a single target gene. But the complexity of the disease is the most challenging impediment to finding a single-shot cure. This book is a simple collection of chapters on CF-related cellular biochemistry, diabetes, microbiome, and immunotherapy that highlight the progress in CF research. From the information contained in the chapters of this book, it is obvious that only with the help of interdisciplinary research, better understanding and management of the CF disease condition would be possible and this approach has certainly increased the level of life expectancy among CF patients. In addition, a cohort- or patient-specific treatment strategy supported by intense bench-to-bedside research flow seems to be a feasible option to reduce morbidity and mortality in CF population.

I thank InTech for appointing me as the editor of this book and for providing me the opportunity to contribute to the scientific community. I thank the authors of the chapters for their valuable contributions. I thank Helmholtz Centre for Infection Research, Braunschweig, Germany, which served as the knowledge center for me to gain expertise in this field.

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Chapter 1

CFTR Involvement in Cell Migration and Epithelial Restitution

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Additional information is available at the end of the chapter

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Abstract

Over the past decade, research has shown that *c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator (CFTR) plays an important role in epithelial cell migration and wound healing. Experiments with airway epithelium, ovarian epithelial cells, placental epithelium and epidermal keratinocytes demonstrated that CFTR function is necessary to achieve maximum migration rates during restitution and in certain cancer cells, CFTR activity contributes to tumor cell invasion. Multiple mechanisms appear to underlie the motility-promoting actions of CFTR, and although many details remain to be established, our present understanding indicates that processes such as electrotaxis (galvanotaxis), integrin-mediated cell adhesion and lamellipodia protrusion are dependent on normal CFTR function. In this chapter, the role of CFTR in epithelial cell migration and its implications in cystic fibrosis (CF) will be reviewed with emphasis on the underlying mechanisms that may explain observations made in various epithelial tissues, particularly in airways. Ultimately, a better understanding of CFTR involvement in epithelial repair may lead to new therapeutic approaches to improve barrier function and reduce airway infection and inflammation associated with CF.

Keywords: cystic fibrosis, CFTR, wound healing, collective migration, barrier function, inflammation

1. Introduction

1.1. Ion channels and cell motility

The role of ion channels and membrane transporters in cell migration has been the subject of several recent reviews [1–4], so only a few examples will be highlighted in this section to provide

the reader with an appreciation of their importance in cell motility. Ion channels and other membrane transport pathways participate in multiple housekeeping functions within cells that include regulation of membrane potential, intracellular [Ca^{2+}], cytoskeletal assembly, integrin-mediated signaling, cell volume regulation, as well as the maintenance of intracellular and extracellular pH. Each of these housekeeping functions can influence cell migration. For instance, changes in ion channel activity produces changes in membrane potential that can facilitate uptake of Ca^{2+} from the extracellular media. A recent example involves the slow calcium wave that develops approximately 1 h after wounding of corneal endothelial cells [5]. The rise in intracellular [Ca^{2+}] is associated with plasma membrane depolarization of cells along the margin of the wound and serves to increase the rate of cell migration. This depolarization has been attributed to increased expression and activity of epithelial Na^+ channels (ENaC) within cells that boarder the wound, resulting in elevated intracellular [Na^+]. The combined effect of depolarization and increased Na^+ load drives Na^+/Ca^{2+} exchange (NCX) activity in reverse mode to produce Ca^{2+} uptake, which propagates from the border of the wound into the epithelium. There may also be an additional role for transient receptor potential (TRP) channels in this process since inhibition of NCX activity does not completely block Ca^{2+} uptake, whereas inhibition of both NCX and TRP activity abolishes the increase in intracellular [Ca^{2+}].

Membrane hyperpolarization can also stimulate Ca^{2+} uptake and enhance the rate of cell migration. Differentiated intestinal epithelial cells with increased expression of voltage-gated K^+ channels (Kv1.1/Kv1.5) exhibit membrane hyperpolarization and increased intracellular [Ca^{2+}] as a result of a greater driving force for electrogenic Ca^{2+} uptake across the plasma membrane [6]. The elevation in intracellular [Ca^{2+}] was shown to augment formation of myosin II containing stress fibers necessary for efficient cell migration. Similarly, ionotropic $P2X_7$ receptors have also been shown to contribute to changes in intracellular [Ca^{2+}] and cell migration. During injury of corneal epithelial cells, $P2X_7$ receptors redistribute to the leading edge of cells that border the wound [7]. Adenosine triphosphate (ATP) is released from the damaged cells leading to activation of these receptors and subsequent uptake of Ca^{2+} from the extracellular solution. The increase in intracellular [Ca^{2+}] induces actin cytoskeletal rearrangements that facilitate the formation of branched dendritic networks of actin within lamellipodia, promoting the dynamic regulation of focal adhesions within cells at the wound margin.

ATP release and $P2X_7$ receptor activation have also been shown to be initiated in response to ligand-activated $\alpha_V\beta_3$ integrin and syndecan-4 engagement leading to increased formation of focal adhesions and an enhanced rate of migration in astrocytes [8]. The mechanism of ATP release involved activation of PI3K, PLCγ and IP_3 receptors following integrin activation. This resulted in opening of Cx43/Panx-1 hemichannels in the plasma membrane, facilitating ATP release, transactivation of $P2X_7$ receptors and ultimately, an increase in intracellular [Ca^{2+}]. Furthermore, enhanced expression of both $\alpha_6\beta_4$ integrin and TRPV1 receptors at the leading edge of keratinocytes after wounding has also been linked to increases in intracellular [Ca^{2+}]. Evidence appears to support a model where TRPV1-mediated increases in intracellular [Ca^{2+}] trigger the activation of transcription factors such as nuclear factor of activated T cells (NFAT) and cAMP response element binding protein (CREB) to stimulate expression of β_4 integrins in cells at the margin of the wound leading to an increase in directional migration [9]. Direct

coupling between the β_1 integrin and $K_{Ca}3.1$ channel expression has been demonstrated in alveolar type II cells grown on a fibronectin matrix and inhibition of channel activity was shown to decrease the rate of migration [10]. This inhibitory effect may be due in part to reducing Ca^{2+} uptake through TRPC4 channels which were also shown to participate in migration during wound repair. TRP channel-associated Ca^{2+} uptake has also been shown to be stimulated by mechanical stretch of the plasma membrane associated with tension and cell shape changes occurring during migration. A specific example involves activation of TRPM7 which mediates transient and highly localized increases in intracellular [Ca^{2+}] known as Ca^{2+} flickers that take place within lamellipodia in response to mechanical forces linked to contraction [11]. This initial Ca^{2+} response is amplified by localized Ca^{2+}-induced Ca^{2+} release from internal stores leading to transactivation of protein kinase A (PKA) through stimulation of Ca^{2+}-sensitive adenylyl cyclases. PKA is known to have multiple cell migration-associated targets including components of the cytoskeleton and the focal adhesion proteome that can have both positive and negative effects on migration depending on intracellular localization.

An interesting example of enhanced cell migration linked to K^+ channel regulation has been reported in glioblastoma cells [12]. In astrocytes and oligodendrocytes from normal brain tissue, the $\alpha_9\beta_1$ integrin is not expressed; however, expression has been shown to increase with glioma grade and appears to be critical for sustaining increased migration rates following exposure to urokinase receptor (uPAR), agonists. A unique feature of the α_9 subunit is that its cytoplasmic domain specifically interacts with spermidine/spermine-N-acetyl transferase (SSAT), which catalyzes the breakdown of higher-order polyamines (spermidine and spermine) to putrescine. Spermidine and spermine are known to regulate the rectification properties of Kir channels by binding to negatively charged residues within the channel pore, significantly reducing K^+ efflux from the cell. In contrast, putrescine is a much less effective blocker of outward K^+ current in Kir channels. In glioma cells, the α_9 subunit colocalizes with Kir4.2 and silencing of the channel inhibits uPAR-enhanced cell migration. A proposed mechanism to explain the increase in migration rate involves activation of SSAT in response to uPAR-dependent $\alpha_9\beta_1$ integrin activation, which produces a localized decrease in the [spermidine/spermine] ratio ultimately leading to reduced rectification, increased K^+ efflux and membrane hyperpolarization.

1.2. Airway inflammation and epithelial damage

Loss of CFTR function in the airways of CF patients leads to reduced anion secretion, enhanced Na^+ absorption and a decrease in the depth of airway surface liquid that ultimately impairs mucociliary clearance and the removal of pathogens from the lungs [13–15]. Reduced pathogen clearance facilitates infection that induces neutrophilic inflammation, leading to progressive epithelial damage within the conducting airways [16–19]. Over time, a recurrent cycle of intense inflammation, epithelial injury and airway remodeling produce irrevocable damage that dramatically compromises lung function [20–22]. Mounting evidence from in vitro studies and animal models of CF indicate that CFTR malfunction appears to alter the innate immune response of the airways leading to increased release of proinflammatory mediators evoking an amplified, yet less effective inflammatory reaction that is unable to

eliminate airway pathogens [17, 18]. In some cases, elevated cytokine levels, neutrophil infiltration and neutrophil elastase (NE) concentrations within the bronchial alveolar lavage (BAL) fluid have been reported in infants without signs of infection, although other studies support the concept that infection is necessary to initiate inflammation [17, 23–25]. Neutrophils represent the major inflammatory leukocyte recruited into CF airways where they release a variety of mediators including oxidants and proteases such as neutrophil elastase (NE), which possess bacteriocidal properties [26, 27]. Moreover, NE catalytic activity is also known to damage the epithelium and reduce structural integrity of the airways leading to bronchiectasis and deteriorating lung function [28–31]. Furthermore, the airways of CF patients encounter various reactive oxygen species (ROS) derived from bacterial pathogens or from the environment [32]. ROS production can exceed the endogenous oxidative defense capacity of the airways leading to oxidative stress and additional injury. In adults, the concentration of reduced glutathione (GSH), a major ROS scavenger present in the airway surface liquid, is significantly reduced in CF patients [33, 34]. This condition may be directly related to the loss of CFTR function since the channel is known to transport GSH in addition to anions in normal airways [35].

Decreases in CFTR channel activity also result in acidification of airway surface liquid coupled to an increase in intracellular pH, which reduces antimicrobial function of the airway surface liquid, promoting bacterial infection [36–41]. Intracellular alkalinization also appears to enhance the accumulation of ceramide, a metabolite of sphingomyelin, within lysosomes [42–44]. Ceramide is thought to amplify the inflammatory response by triggering tumor necrosis factor (TNF)α signaling pathways involving mitogen-activated protein kinases (MAPK), IκB-kinase degradation [an inhibitory regulator of necrosis factor (NF)-κB] and NF-kB nuclear localization [45–47]. Additionally, for class II CFTR mutations, the accumulation of misfolded CFTR protein within the endoplasmic reticulum (ER) induces stress and stimulates what is known as an unfolded protein response, which involves activation of signaling pathways that mitigate ER stress [48–51]. For the most common class II mutation, retention of misfolded ΔF508 CFTR within the ER causes an unfolded protein response that stimulates inflammation by activating NF-κB and inducing cytokine secretion that can result in apoptosis.

1.3. Evidence for defective epithelial regeneration in CF

In an earlier investigation, a humanized airway xenograph model was created by inoculation of CF and non-CF airway epithelial cells onto epithelium-deleted rat trachea that was then subcutaneously implanted into nude mice over a period from 4 to 35 days [52]. This model was then used to investigate the process of reepithelialization following injury and to determine if remodeling of CF epithelium is a consequence of defective epithelial regeneration independent of infection. The results showed that CF epithelial cells exhibited enhanced proliferation along with continuous expression of IL-8, matrix metalloproteinases (MMP7, MMP9) and tissue inhibitor of metalloproteinase (TIMP)-1. Moreover, regeneration was delayed and final restitution resulted in a remodeled epithelium that appeared to be a product of aberrant regeneration unrelated to bacterial contamination. A relationship between

abnormal regeneration and loss of CFTR function was not identified in this study, although it was concluded that it might be a consequence of altered MMP/TIMP/IL-8 expression observed in CF epithelium. In a subsequent study, wound healing experiments using immortalized normal (NuLi-1 cells) and CF (CuFi-1 cells) human airway epithelial cells revealed that CuFi-1 cell migration was significantly delayed relative to NuLi-1 cells [53, 54]. This difference in migration activity was attributed to defective epidermal growth factor (EGF)/epidermal growth factor receptors (EGFR) signaling and reduced K^+ channel expression. Interestingly, no significant effect on migration was reported in the presence of the CFTR inhibitor, $CFTR_{inh}$-172 [53]. In subsequent investigations described below, loss of CFTR function was shown to directly contribute to delayed epithelial repair in CF airways and that expression of normal CFTR augments epithelial restitution.

2. Anion channels, cell migration and epithelial restitution

2.1. Volume-sensitive anion channels in cell migration and invasion

Earlier electrophysiological studies of human glioma cells showed that they express voltage-sensitive Cl^- channels that were blocked by chlorotoxin (Ctx), a peptide isolated from scorpion venom as well as tamoxifen, an estrogen receptor modulator [55–57]. Furthermore, hypotonic solutions were also shown to activate tamoxifen and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB)-sensitive, outwardly rectifying Cl^- currents carried by channels that were shown to contribute to the resting Cl^- conductance under isotonic conditions [55]. Treatment with either Ctx or NPPB inhibited glioma cell migration and invasiveness in transwell migration assays. Similarly, osmotically activated cell swelling and regulatory volume decrease (RVD) were also blocked by Ctx and tamoxifin indicating a role in the regulation of cell volume that contributes to migration and tumor cell invasion [56]. Simultaneous time lapse imaging and patch clamp recording of glioma cells demonstrated detectable changes in cell shape and movement that was associated with activation of volume-sensitive Cl^- currents. Changes in cell shape and motility were attributed to Cl^- efflux coupled to K^+ and water movement across the plasma membrane resulting in cell shrinkage, which appeared to be localized at the leading edge of the cell. Consequently, cell flattening at the leading edge was proposed to facilitate protrusion through restricted extracellular spaces required for tumor cell invasion [58].

Experiments with murine primary microglial cells or a microglial (BV-2) cell line demonstrated that exposure to hypotonic saline or an elevated extracellular $[K^+]$ produced localized swelling and protrusion of lamellipodia at the leading edge of these cells [59]. Blockade of volume-activated Cl^- channels or inhibition of K-Cl co-transporters (KCC) effectively inhibited lamellipodia formation. The migratory response induced by localized increases in extracellular $[K^+]$ may likely result from cell death caused by injury. Ischemia for example, has been shown to increase extracellular $[K^+]$ by more than 20 fold [60]. Such increases in $[K^+]$ would provide a favorable driving force for KCl uptake by KCC leading to cell swelling and produce membrane depolarization. This would establish conditions for electrogenic Cl^- influx through

volume-activated anion channels which also contributes to localized swelling. Furthermore, signaling proteins such as the chemokine ligand CCL21 is released by damaged neurons and is known to induce a chemotaxis response in microglia which is inhibited by Cl^- channel blockers [61]. This response was not dependent on activation of the canonical CCL21 receptor CCR7, but instead was shown to stimulate CXCR3 receptors. Short-duration exposure (30 s) to CCL21 or the selective CXCR3 ligand CXCL10 in either brain slice preparations or microglial cells in culture produced a sustained increase in Cl^- channel activation that appears to represent an initial trigger for stimulating directed cell migration in response to neuronal injury.

2.2. CFTR and epithelial wound repair

The first direct evidence of a role for CFTR in cell migration was obtained from studies of airway epithelial cells [62]. Experiments using Calu-3 cells, a human airway adenocarcinoma cell line and normal human bronchial epithelial cells revealed that inhibition of CFTR channel activity with the selective CFTR blocker, $CFTR_{inh}$-172 or silencing CFTR expression by RNAi significantly slowed cell migration and epithelial restitution (see **Figure 1**). Moreover, CFTR channel inhibition or silencing also reduced the extent of lamellipodia protrusion during migration. These results demonstrated that the ion transport activity of CFTR was necessary for airway epithelial cells to achieve a maximum rate of migration during wound closure and that lamellipodia protrusion was at least one aspect of the migration process that was affected by the loss of CFTR function. Following publication of this initial investigation, Sun et al. (2011) showed that epithelial wound repair in a tracheal preparation from rhesus monkeys was delayed following treatment with $CFTR_{inh}$-172 [63]. Experiments employing the use of a noninvasive vibrating probe demonstrated that inhibition of CFTR activity inhibited the spontaneous outward current induced by wounding and that treatment with aminophylline, a phosphodiesterase inhibitor and CFTR activator, stimulated this outward current. These results suggested that CFTR activity contributes to the wound current that serves as a guidance cue for directed migration and that inhibition of CFTR activity disrupts the process of electrotaxis, thus delaying wound closure. Further support for the importance of CFTR in airway cell migration and epithelial restitution was provided by a set of rescue experiments involving (i) expression of wild-type CFTR into CF airway epithelial cell lines to restore the normal rate of wound closure and (ii) treatment with VRT-325, a CFTR corrector molecule that facilitates apical membrane localization of CFTR with the ΔF508 mutation in CFBE-ΔF508 cells and in primary bronchial epithelial cells obtained from CF patients [64].

Involvement of CFTR in cell migration has also been observed in other epithelial cell types besides airways. For example, in human trophoblast (BeWo) cells, CFTR activation with forskolin increased cell migration into the wound and subsequent addition of $CFTR_{inh}$-172 significantly inhibited the response to forskolin [65]. Poor trophoblast migration/invasiveness and associated spiral artery remodeling represent early recognizable pathologies that underlie preeclampsia and previous studies demonstrated that CFTR expression is reduced in preeclamptic placentas [66]. Thus, changes in CFTR function not only appear to have consequences on placental ion and fluid transport but may also contribute to altered trophoblast invasion in preeclampsia. Another example based on experiments with human ovarian carcinoma cells

showed that CFTR silencing by RNAi significantly reduced cell migration and invasion under in vitro conditions and that the tumorigenic potential of these cells in vivo was suppressed compared to controls [67]. This result was consistent with the observation that CFTR expression in ovarian cancer was higher relative to normal ovarian epithelial cells or benign ovarian tumors and that enhanced CFTR expression was associated with advanced International Federation of Gynecology and Obstetrics (FIGO) staging and poor histopathology grade. Lastly, CFTR was also shown to play a role in cutaneous wound healing, where ΔF508*cftr*$^{-/-}$ mice that lack plasma membrane localization and normal CFTR channel function exhibited delayed wound closure compared to wild-type mice [68].

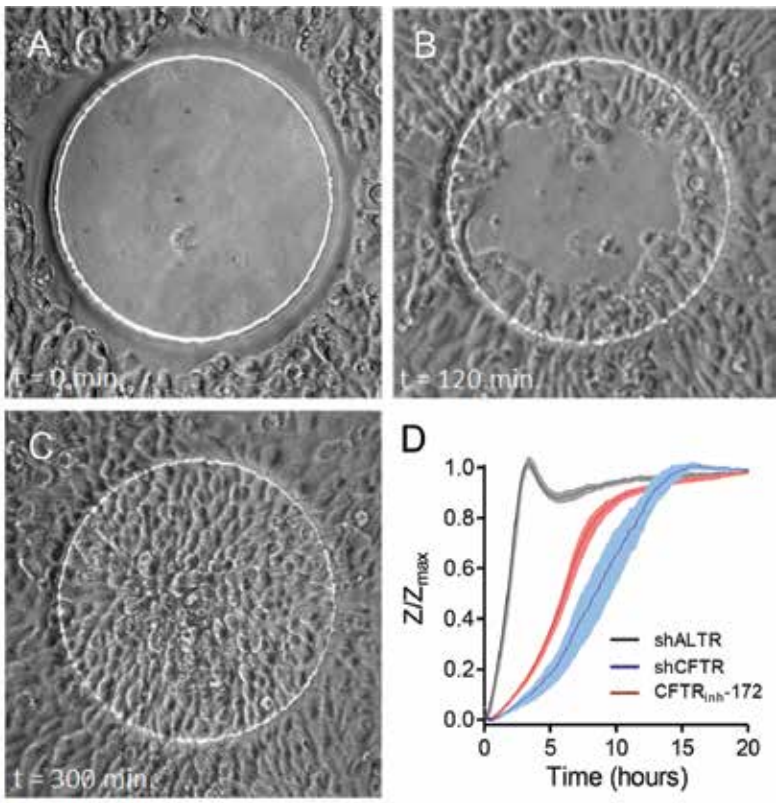

Figure 1. Inhibition of CFTR channel activity or silencing expression by RNAi delay airway epithelial restitution. (**A**)–(**C**) Impedance-sensing arrays were used to track the process of Calu-3 cell migration over the surface of a 250 μm diameter electrode following wounding by electroporation. Images show the extent of Calu-3 cell confluence at three time points (0, 120 and 300 min). **(D)** Normalized impedance (Z/Z_{max}) measurements as a function of time for Calu-3 cells expressing shRNAs designed to selectively target CFTR (shCFTR cells) or have an altered sequence that no longer recognizes CFTR mRNA (shALTR cells). Note that as cells reach confluence on the electrode surface, the normalized impedance value approaches 1, which indicates complete epithelial restitution. For these experiments, shALTR cells were used as controls where the black line represents the mean Z/Z_{max} values and the shaded grey area corresponds to the SEM (n = 8). The blue line (mean) and light-blue-shaded area (SEM) shows the effects of silencing CFTR on wound closure, where the slope provides a measure of the average rate of cell migration into the wound (n = 8). Finally, the red line (mean) and pink-shaded area (SEM) are the results from shALTR cells treated with 20 μM $CFTR_{inh}$-172, a selective inhibitor of CFTR channel activity, throughout the process of restitution (n = 8). Images were adapted from Ref. [114].

Exceptions to the migration-promoting actions of CFTR can be found in studies of non-small cell lung cancer (NSCLC) cells and human keratinocytes [69]. Experiments with NSCLC cells showed reduced CFTR expression which correlated with an advanced stage of the cancer, lymph node metastasis and enhanced malignant behavior which manifested as an increase in epithelial-mesenchymal transition, invasion and migration. In contrast, overexpression of CFTR reduced cancer progression and metastasis, supporting the observation that in some types of cancer, CFTR appears to function as a tumor suppressor. Similarly, CFTR silencing by RNAi in human keratinocytes was shown to promote cell migration and inhibit differentiation, whereas overexpression inhibited migration and stimulated differentiation [68]. The effects of manipulating CFTR expression on migration appeared to be related to its role in the formation of epithelial junctions since silencing the channel downregulated adhesion molecule (E-cadherin, ZO-1 and β-catenin) expression and intercellular junction formation while overexpression promoted junction formation.

2.3. ANO1, cell migration and cystic fibrosis

TMEM16A/ANO1 is one of the 10 known members of the anoctamin family (TMEM16A-K) of proteins, some of which function as anion channels. Certain members of this family, such as ANO1, ANO2 and ANO6, can be activated by increases in intracellular [Ca^{2+}] and are classified as Ca^{2+}-activated chloride channels (CaCCs) [70–72]. CaCCs exhibit voltage dependence, outward rectification and are perhaps best known for their role in Ca^{2+}-dependent Cl^- secretion in various epithelial tissues. Compounds including T16Ainh-A01, CaCCinh-A01 and NS3728 block channel activity to varying degrees depending on cell type [73]. Prior to the discovery of its anion channel activity, ANO1 was regarded as either a tumor cell marker or as an oncogene in human cancers with poor prognosis [74, 75].

In prostate cancer (LNCaP and PC-3) cells, ANO1 is highly expressed and these cells exhibit large CaCC currents in response to increases in cytosolic [Ca^+] [76]. Silencing ANO1 by RNAi in PC-3 cells significantly inhibited cell proliferation and migration/invasion. Studies using Ehrlich Lettre ascites (ELA) cells revealed that they express both ANO1 and ANO6 [71]. Interestingly, silencing ANO1 expression was shown to alter directionality of ELA migration while knockdown of ANO6 was shown to cause a ~40% decrease in the overall rate of migration. Although the mechanism responsible for ANO1-dependent control of directionality is not understood, it is likely that some contribution to outward current associated with wounding may be important in electrotaxis. Various pancreatic ductal adenocarcinoma cells have also been shown to have increased expression of ANO1 and enhanced CaCC activity. Knockdown of ANO1 or inhibition by CaCC blockers including CaCCinh-A01, and NS3728 delay migration in BxPC-2 cells, however, T16Ainh-A01 exhibited no effect [77]. The authors speculated that activation of ANO1 was important for cell volume changes necessary to control cell shape and that the channel may serve as a potential target for reducing the metastatic potential of pancreatic tumor cells.

Investigations of bronchial epithelial cell repair in cystic fibrosis (CF) demonstrated that the expression of ANO1 and CaCC channel activity were significantly reduced in CF cells compared to bronchial epithelial cells from normal subjects [78]. Consequently, epithelial

restitution in wound healing assays was delayed in CF cells relative to non-CF cells. Moreover silencing ANO1 expression in non-CF cells reduced the rate of migration, whereas overexpression of ANO1 in CF cells partially restored cell motility, although complete recovery was not achieved. To establish whether ANO1 channel function was necessary for supporting cell migration, primary non-CF cells were treated with T16Ainh-A01 which produced a significant delay in wound closure. These findings indicate that reduced rates of cell migration in bronchial epithelial cells from CF patients may be attributed to an overall decrease in apical membrane Cl^- conductance resulting from loss of both CFTR and ANO1 anion channel activity.

3. Mechanisms of CFTR-dependent cell migration and epithelial repair

Although the molecular mechanisms underlying the contribution of CFTR to the processes of cell migration and epithelial restitution remain to be fully characterized, the data collected so far have identified three important aspects of migration that merit further investigation. These include the process of lamelliopdia protrusion, electrotaxis and the dynamics of integrin-mediated adhesion, each of which are discussed in more detail below.

3.1. Lamellipodia protrusion

Lamellipodia are actin-containing, sheet-like structures that protrude from the leading edge of migrating cells [79]. They are capable of sensing environmental cues and are necessary for sustained directional migration. A key force contributing to the protrusion of lamellipodia is provided by the extension of actin filaments at the leading edge of the cell. Within lamellipodia, actin forms networks of branched filaments with highest density near the membrane at the leading edge, where the barbed (positive) ends of the filaments are directed toward the plasma membrane to form brush-like assemblies [80, 81]. Elongation occurs primarily at junctions formed by a multiprotein structure known as the Arp2/3 complex, which functions as a nucleation site for new actin monomers to attach to the sides of existing actin polymers to create a branched arrangement of fibers [82]. As these monomers add to the growing meshwork at the barbed end, cleavage and dissociation of monomers takes place at the pointed (minus) end of filaments located in the more proximal regions of the lamellipodium. ATPase activity associated with actin filaments facilitates accumulation of ADP-actin at the pointed ends as filament disassembly takes place. This dynamic process of simultaneous actin monomer addition to the barbed end and dissociation at the pointed end of the filament is known as treadmilling and is controlled by several actin-regulatory proteins [82, 83] as well as intracellular pH [84, 85]. Previous studies have shown that during polarization along the axis of movement, a redistribution of the Na^+-H^+ exchanger (NHE1) occurs, which localizes toward the leading edge of the cell. Redistribution of NHE1 results in the development of a steady-state pH gradient extending from the front of the cell, which becomes more alkaline, to the rear, developing a more acidic pH relative to the leading edge [86–88]. A key regulator of polarization is Cdc42, a small guanosine-5'-triphosphatase (GTP)ase that accumulates at the leading edge where it stimulates actin polymerization. Cdc42 activation is pH sensitive, requiring NHE1 activation and proton efflux to produce localized alkalinization of the

cytoplasm to enhance its activity [86]. Moreover, alkalinization also promotes F-actin cleavage by cofilin, an actin binding protein that facilitates treadmilling by causing depolymerization at the pointed ends of actin filaments [87]. Other acid extruding or base loading transport mechanisms could potentially contribute to this alkalinization process, including CFTR and its ability to conduct bicarbonate ions, provided that a favorable electrochemical driving force exists.

As previously mentioned localized osmotic swelling can also contribute to the force that powers lamellipodia protrusion [59]. Solute uptake serves as a driving force for fluid uptake into the cell, often involving electroneutral transporters that couple cation uptake with Cl^- transport (e.g. KCC or NKCC cotransporters). It is also possible that if the plasma membrane is depolarized to a voltage that is more positive than the reversal potential of anion channels such as CFTR or ANO1, then the inwardly directed Cl^- concentration gradient would facilitate influx, setting up a favorable osmotic gradient for fluid uptake and lamellipodia protrusion. Whether Cl^- influx or efflux is occurring at the leading edge may not be predicable, since this would depend on the activity of multiple electrogenic transport pathways or conditions associated with injury. It is worth emphasizing that depending on the electrochemical gradient for Cl^-, CFTR could contribute instead to retraction taking place at the rear of the cell. In this case, efflux of Cl^-, perhaps coordinated with K^+ channel activity, would enable localized solute and fluid exit at the trailing edge of the cell, promoting forward movement [3].

3.2. Electrotaxis

Epithelia engaged in active electrolyte transport typically generate spontaneous transepithelial potentials (TEP) that provide an electrical driving force for paracellular ion movement across the epithelium [89]. Following wounding, the TEP at the site of the wound collapses as laterally oriented electric fields develop with the cathode (negative pole) located at the center of the wound. In dermal, corneal and airway epithelia, for example, outward current can be detected using a noninvasive technique that employs a self-referencing vibrating probe [90]. Many epithelial cell types respond to wound-induced electric fields by migrating toward the cathode although some cell types exhibit anodal migration in response to electric field stimulation [91, 92]. In fact, changing the polarity of the field will reverse the direction of migration. In experiments with primate tracheobronchial epithelial cells, an applied electric field with a threshold intensity of 23 mV/mm was effective at stimulating migration with a displacement speed that increased with field strength [63]. The displacement speed reflected greater migration efficiency and in the case of tracheobronchial epithelial cells, the increase in speed primarily resulted from improved directionality, which was quantitatively expressed using a directedness parameter for the migrating cells. Directedness was expressed as the angle (θ) that individual cells moved relative to the electric field vector, where cosine θ was defined as the directedness value. Cells moving randomly in the absence of an electric field have an average directedness value near zero, whereas those that move entirely along the electric field lines toward the cathode have a value approaching 1. Experiments with tracheobronchial epithelial cells showed that directedness increased with increasing field strength such that when the voltage achieved 90 mV/mm, a number of the cells migrated directly toward the

cathode. The finding that inhibition of CFTR reduces the electric field evoked by wounding and that CFTR inhibition or silencing decreases lamellipodia protrusion [62], strongly suggests that CFTR activity plays an important role in sustaining directed migration in airway epithelial cells.

The cellular mechanisms underlying the increase in directed migration induced by wound-evoked electric fields are complex and cell type dependent. Studies of corneal epithelial cells and keratinocytes, for instance, revealed changes in the localization of epidermal growth factor receptors (EGFR) toward the cathode-facing borders of migrating cells, and in at least one study, EGFRs appeared to be activated by the electric field independently of ligand binding to the receptor [93–97]. Moreover, inhibition of EGFR-MAPK signaling was shown to alter the actin cytoskeleton at the leading edge and diminish directed migration of epithelial cells, demonstrating an important role for EGFR in detecting and initiating the epithelial response to electric field stimulation [96]. Additionally, electric fields can redistribute and activate PI3K/Akt signaling in a polarized manner at the cathode-oriented leading edge of the cell [98]. When PI3K is activated, membrane protrusion and lamellipodia formation is initiated at that site, facilitating directed migration toward the cathode. Pharmacological inhibition of PI3K activity or selective disruption of the PI3K-γ isoform has been shown to block electrotaxis in wound healing assays and organ cultures [98, 99]. Furthermore, in keratinocytes, deletion of phosphatase and tensin homolog (PTEN), a phosphatase that functions as a negative regulator of PI3K, resulted in increased Akt phosphorylation and enhanced electrotaxis. Other kinases linked to the control of cell motility such as extracellular signal-regulated kinase (ERK) have also been shown to be involved in electric field-evoked migration [97, 100, 101]. In experiments with glioma and fibrosarcoma cells, electric field stimulation induced NADPH oxidase activation, resulting in the production of reactive oxygen species (ROS) [100]. Intracellular accumulation of ROS stimulates ERK phosphorylation/activation which leads to reorganization of the cytoskeleton and an increase in directed migration [101]. Based on these observations, it appears likely that further studies will uncover additional molecular targets and signaling pathways involved in the detection and regulation of directionality by injury-induced electric fields.

3.3. Dynamics of integrin-mediated adhesion

A recent study examining the consequences of CFTR silencing on cell migration and epithelial repair in human airway (Calu-3) epithelial cells demonstrated a ~60% reduction in GM1 ganglioside content within the plasma membrane of CFTR deficient cells compared to controls [102, 103], which was restored following expression of wild-type CFTR. Similarly, treatment of cells with the selective CFTR blocker, $CFTR_{inh}$-172, also produced comparable reductions in GM1 content in cells expressing wild-type CFTR. These observations were consistent with earlier studies showing reduced levels of sialylated gangliosides in cells expressing CFTR with the ΔF508 mutation [104, 105]. Furthermore, previous investigations have also shown that gangliosides are capable of regulating integrin signaling and cell migration [106, 107]. Experiments with Calu-3 cells revealed that CFTR knockdown did not directly affect β_1 integrin surface expression; however, the level of activated β_1 integrin was

significantly lower than observed in CFTR expressing control cells [102]. β_1-integrin activation could be completely recovered by incubating CFTR deficient cells with exogenous GM1, but not with GM3 gangliosides, confirming that integrin activation was dependent on GM1 and CFTR expression. Reduced β_1 integrin phosphorylation was associated with lower levels of focal adhesion kinase (FAK) and Crk-associated substrate (CAS) phosphorylation which was

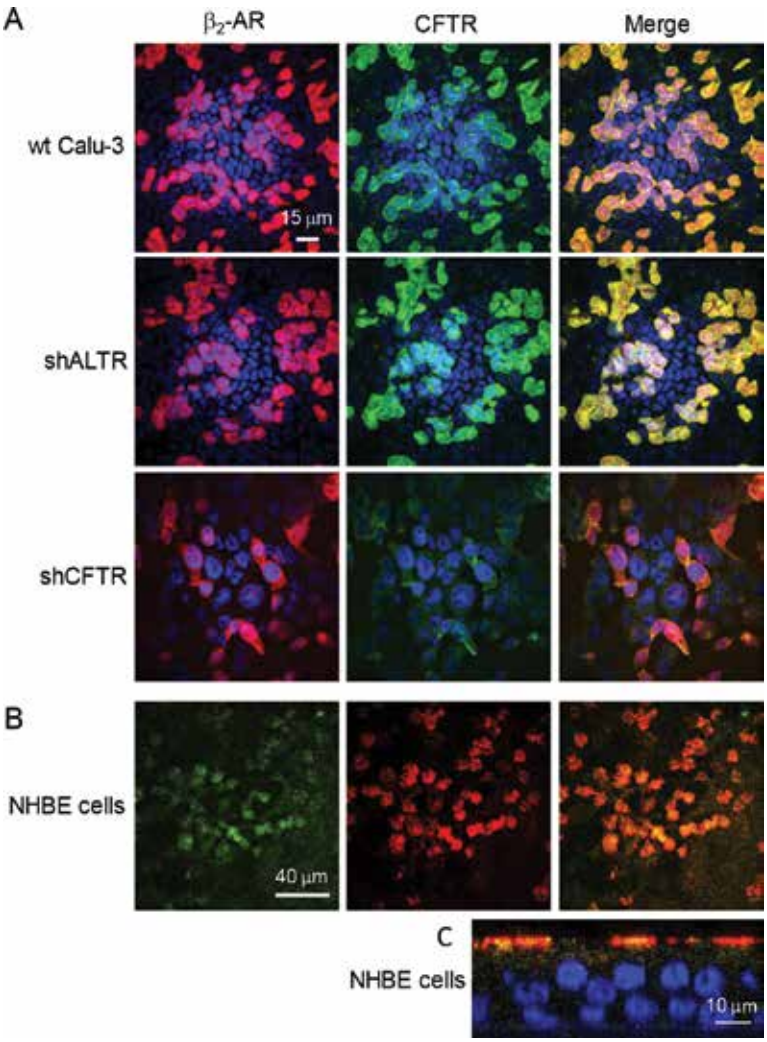

Figure 2. Colocalization of CFTR and the β_2-adrenergic receptor (β_2-AR) in the apical membrane of Calu-3 cells and in cilia of differentiated primary normal human bronchial epithelial (NHBE) cells grown under air-liquid interface conditions. (**A**) Antibody labeling of the β_2-AR, CFTR and merged images (where yellow represents colocalization) collected from wild-type Calu-3 cells (wt Calu-3), Calu-3 cells expressing shRNA that does not recognize CFTR (shALTR) and CFTR-deficient cells were CFTR expression was silenced by RNAi (shCFTR). (**B**) Labeling of the β_2-AR and CFTR within the cilia of differentiated primary NHBE cells. Yellow-orange represents colocalization of the receptor and channel. (**C**) Cross section of differentiated, pseudostratified primary NHBE cells showing colocalization of the β_2-AR and CFTR within the cilia. Note the layering of nuclei reflecting pseudostratification. Images were adapted from Refs. [114] and [115].

also restored by incubation with exogenous GM1 ganglioside. A possible explanation for the reduction in β_1 activation may be related to loss of localization to specific membrane microdomains that function as integrin signaling platforms. This would be consistent with GM1 localization within lipid raft domains where it is known to associate with CFTR [108, 109]. Moreover, recovery of FAK and CAS phosphorylation along with β_1 integrin activation with GM1 repletion also produced partial restoration of cell migration, suggesting that reduced integrin engagement with the extracellular matrix, presumably at the leading edge of the cell, accounts for at least part of the effect that loss of CFTR expression or inhibition of channel activity has on epithelial restitution.

More recently, stimulation of β_2-adrenergic receptors (β_2-AR) expressed on the apical membrane of normal human bronchial epithelial cells and Calu-3 cells caused a significant delay in cell migration and wound closure. This effect could be reproduced using carvedilol, a β_2-AR agonist that functions as a bias ligand to activate cAMP-independent, β-arrestin-dependent signaling cascades [110]. The inhibitory effects of β_2-AR agonists could be blocked if cells were pretreated with an inhibitor of PP2A phosphatase, indicating that PP2A activation was a critical step in regulating cell migration [111, 112]. Interestingly, in airway epithelial cells, β_2-ARs form a tightly coupled signaling complex with CFTR in the apical membrane (see **Figure 2**) such that receptor activation by endogenous ligands, such as epinephrine, or by selective β_2-AR agonists, like salbutamol, stimulate CFTR channel activity [113]. The reduced rate of migration following β_2-AR activation was associated with a reduction in lamellipodia protrusion, similar in magnitude to the effect produced by CFTR channel inhibition with $CFTR_{inh}$-172 or silencing by RNAi. Furthermore, β_2-AR agonists, including carvedilol, decreased β_1-integrin activation, and in CFTR-deficient Calu-3 cells, β_2-AR activation had no effect on cell migration [114]. These findings suggested a model where exposure to β_2-AR agonists stimulates PP2A phosphatase

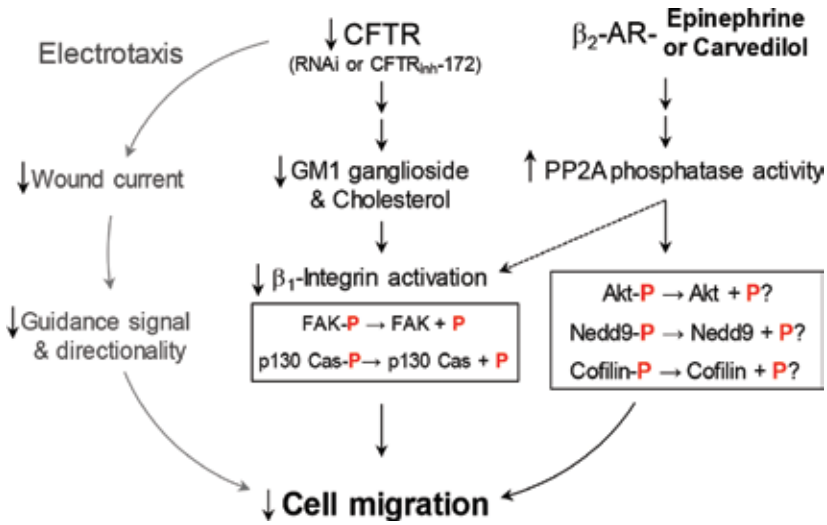

Figure 3. Summary of proposed interactions and pathways accounting for the decrease in cell migration and epithelial repair associated with loss of CFTR function and β_2-AR activation in airway epithelial cells. This model was adapted from Ref. [114].

activity to produce dephosphorylation of multiple proteins involved in the control of cell motility (see **Figure 3**). Moreover, CFTR inhibition or silencing and β_2-AR stimulation appear to converge on a common control point involving the activation of β_1 integrin, which is thought to be the reason why CFTR silencing and β_2-AR activation do not produce additive effects on cell migration.

4. Conclusions

This chapter has focused on the impact of CFTR dysfunction on cell migration and epithelial repair that has direct relevance to airway barrier function. This role for CFTR constitutes an important intrinsic deficit of the CF epithelium that contributes to disease progression. Other intrinsic deficits such as those linked to altered innate immune function appear to underlie abnormal regeneration and remodeling of the CF epithelium that occurs in the absence of infection. Delayed wound repair exacerbates intrinsic inflammation by providing opportunities for pathogen access to the airway submucosa, augmenting inflammation and tissue damage. Furthermore, dysregulation of the repair process establishes a chronic cycle of injury and inadequate restitution that intensifies remodeling, ultimately leading to deterioration of lung function. Further investigation is required to more clearly understand the molecular and cellular mechanisms by which CFTR expression and function affect cell motility. Results from these studies should aid in identifying pathways that could be targeted for development of novel pharmacotherapies to reduce airway infection and inflammation in CF.

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Chapter 2

Cystic Fibrosis–Related Diabetes

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Additional information is available at the end of the chapter

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Abstract

Cystic fibrosis–related diabetes (CFRD) results in significant morbidity and mortality for patients with cystic fibrosis (CF). It is the endpoint of a spectrum of progressive insulin deficiency with resulting abnormalities of glucose tolerance. The consequence of glycaemic abnormalities in CF is poorer nutritional status, an increase in respiratory exacerbations with decline in lung function and ultimately greater morbidity and mortality. CFRD can be diagnosed by the standard oral glucose tolerance test (OGTT) usually performed from 10 years of age. However, this may miss early glycaemic abnormalities which appear to be clinically important. Early recognition of CFRD and treatment have been shown to improve outcomes in CF. Novel diagnostic methods such as 30-min sampled OGTT and continuous glucose monitoring (CGM) may prove to be useful in screening for this disorder and in the early identification of glycaemic abnormalities.

Keywords: cystic fibrosis–related diabetes, glucose, insulin, abnormal glucose tolerance, indeterminate glycaemia, impaired glucose tolerance, oral glucose tolerance test, continuous glucose monitoring

1. Introduction

Cystic fibrosis (CF) is the most common life-limiting autosomal recessive genetic condition seen in the Caucasian population, affecting approximately 1/2500 live births in Australia [1]. It is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, located on the long arm of chromosome 7 [1] and expressed in the epithelial cells of lungs, pancreas and sweat glands and other organs. Cystic fibrosis–related diabetes (CFRD) is one of the most important complications of the disease as it is known to have a significant impact on morbidity and mortality [2]. Patients with CF ultimately die from recurrent respiratory tract infections

and respiratory failure which may be hastened by abnormalities of glucose tolerance affecting respiratory function and nutrition.

2. Pathophysiology

The pathophysiology of CFRD is likely multifactorial and complex. Historically CFRD was thought to be the result of progressive pancreatic destruction by secretions of the exocrine pancreas, pancreatic autodigestion and replacement with nonfunctioning fatty tissue, amyloid deposits or fibrotic tissue [3, 4]. This theory was supported by ultrasound findings in patients with CF of an "echogenic" and atrophied pancreas which progresses with age. MRI has also been used to study the pancreas of patients with CF. Sequeiros et al. attempted to determine the pancreatic volume of patients with CF using MRI and compare with Type 1 diabetic patients and controls. In over 70% of patients with CF, the pancreas could not be visualised and this was irrespective of glycaemic status [5]. Pancreatic tissue on autopsies of patients with CF has also noted to have fewer islet cells and replacement with fibrotic tissue. Histologically, patients with CFRD have a relative decrease in the number of islet cells and insulin-containing cells within the islets, relative to the non-CFRD cohort [4, 6].

However, recent information supports the theory that destruction of the physical pancreas does not entirely explain the glycaemic abnormalities in patients with CF. Insulin deficiency has been shown to occur in young children and infants with CF [7], and even infants have been reported to have CFRD [8]. This has also been demonstrated in animal models of CF. In both the pig and ferret CF models, the animals demonstrate abnormal insulin secretion from birth, suggesting that CFTR may play a more direct role in insulin secretion [9, 10]. In the pig model, newborn pigs were noted to develop hyperglycaemia even when there was no significant islet cell destruction [10]. Recent studies of the CFTR potentiator ivacaftor (Kalydeco™), which improves gating defects and thus should not have any impact on fatty or fibrotic tissue, have demonstrated an improvement in glucose abnormalities [11, 12]. This suggests that the intrinsic abnormality in the CFTR protein may play a role in glycaemic control in CF.

The timeframe during which patients with CF develop glycaemic abnormalities and CFRD has significant variability, and the specific CFTR class abnormality does not entirely account for this unpredictability. Non-CFTR genetic modifiers may play a key role in determining this risk. Patients with CF who have a family history of Type 2 diabetes are known to have an increased risk of CFRD [13, 14]. Polymorphisms in *TCF7L2*, a "susceptibility gene" for Type 2 diabetes, are more common in patients with CFRD. The pathophysiology also appears to have similarities. Couce et al. noted that islet cell amyloidosis, which is characteristic of pancreatic histology of patients with Type 2 Diabetes mellitus, is also present in CF patients with CFRD and "borderline diabetes" but not in nondiabetic CF patients or controls [3]. Other genetic modifiers have been shown to modify CF phenotypes, including *SLC26A9* which has been demonstrated to be more common in patients who develop CFRD [15].

In normal insulin physiology, insulin secretion occurs in two phases—the first phase results from exocytosis of preformed insulin granules which is the result of a voltage-dependent

calcium channel being triggered by blood glucose elevations [16–18]. The second phase requires maturation of insulin granules and lasts minutes to hours [19, 20]. Oral glucose ingestion results in a limited and delayed first-phase insulin peak when compared with intravenous administration [21, 22]. Overall, the amount of insulin secreted appears to be amplified when glucose is given orally, rather than intravenously. Incretins (glucagon-like peptide and gastric inhibitory peptide) are secreted from neuroendocrine cells of the gastrointestinal system and increase insulin secretion and decrease glucagon secretion. The secretion of incretins is hypothesised to be the result of the action of oral glucose within the gastrointestinal tract [21]. The role of incretins in CFRD has not yet been fully elucidated, and it is unclear whether or not patients with CFRD have abnormal levels of incretins. However, the diet of CF patients may play a role in the development of CFRD. In patients with Type 2 diabetes mellitus (DM), those prescribed orlistat, a lipase inhibitor, had diminished fat digestion which resulted in greater postprandial hyperglycaemia [23]. In a randomised crossover trial, Perano et al. demonstrated that adolescent patients with CF, who did not take appropriate pancreatic enzyme supplementation, experienced amplified postprandial hyperglycaemia [24]. Barrio postulates in her review that inadequate enzyme supplementation in patients with CF results in fat malabsorption, which may hasten gastric emptying, thereby inhibiting the normal augmentation of insulin response by the neuroendocrine cells [25]. Exogenous incretin therapy has proven beneficial in patients with Type 2 DM, but it has also been associated with weight loss in this cohort, an undesirable outcome for patients with CF [26]. Hyperglycaemia is known to promote beta-cell apoptosis, and as such, postprandial hyperglycaemia from dysfunctional incretin secretion in CF may potentiate the glycaemic abnormalities demonstrated and hasten the progression to CFRD.

CFRD is distinct from both Type 1 and Type 2 diabetes. CFRD is not an autoimmune condition like Type 1 DM and is not associated with autoantibodies found in Type 1 DM. Moreover, Type 2 DM is primarily a disorder of insulin resistance, whereas glucose abnormalities in CF are primarily the result of insulin deficiency, which is present even in CF patients with normal glucose tolerance on oral glucose tolerance test (OGTT) [7]. One of the features of CFRD that differentiates it from other forms of diabetes is the variation in glucose tolerance demonstrated over time [27]. Although abnormalities of glucose tolerance are known to progress and the complications of diabetes increase in the degree of abnormal glycaemia, some patients with the diagnosis of CFRD will have OGTT results that normalise [27]. The role of insulin resistance has been less well defined although there is emerging evidence of its importance. Ahmad et al. illustrated that patients with CF actually had an increase in peripheral insulin sensitivity compared to healthy controls matched for age and body mass index. They concluded that this increase in peripheral sensitivity in CF patients was a metabolic compensation for insulin deficiency [28]. Moran et al. replicated these findings in exocrine-insufficient CF patients without diabetes. However, once CFRD had developed, there was an increase in peripheral insulin resistance [29]. The mechanism by which this may occur could be the result of a downregulation of GLUT-4 insulin-sensitive channels secondary to chronic hyperglycaemia [30] (“glucose toxicity”). Insulin resistance is also thought to vary over time which could explain the variability of glucose tolerance seen in patients with CF, including a normalisation of previously abnormal glucose tolerance on OGTT. It is often cited that glucose abnormalities worsen during pulmonary exacerbations (due

to cytokine and stress hormone release), but the data to support this suggestion is limited and was not found in the study by Widger et al. [31]. This group performed OGTT in patients with a pulmonary exacerbation and then repeated the OGTT when well. Although the sample size was small, 8/9 patients remained within their glycaemic category even when recovered from their pulmonary exacerbation. However insulin resistance is known to increase during periods of corticosteroid usage, overnight feeds [32, 33], pregnancy and during puberty [34–36]. In the latter case, insulin resistance is thought to increase as a result of a physiological elevation in growth hormone [34], and this may account for the increased detection of CFRD in this age group.

Chronic inflammation may play a key role in the development of glucose abnormalities in CF. Bismuth et al. demonstrated in their cohort of patients with CF that the erythrocyte sedimentation rate (ESR), a marker of inflammation, positively correlated with HbA_{1c} and the area under the curve (AUC) for glucose in patients undergoing OGTT [37]. Significant and ongoing oxidative stress is one mechanism hypothesised to result in an inflammatory state and beta-cell apoptosis [38, 39]. One review postulated that the imbalance in inflammatory T-cell lymphocytes known to play a role in the development of other forms of diabetes may contribute to lung inflammation and thereby a chronic inflammatory states resulting in glucose abnormalities [40]. T-helper 17 (Th-17) lymphocyte cells secrete a pro-inflammatory cytokine-IL-17 known to be involve in pulmonary inflammation in CF and is known to be present in higher levels compared to controls in patients with Type 2 diabetes. Furthermore, studies also suggest that IL-17 may play an important role in the development of Type 1 diabetes [41] and may contribute to β-cell destruction. It has also been postulated that cytokines such as TNF-α may act directly on the insulin receptor by inducing insulin resistance, thereby inhibiting the potential action of insulin [42].

The pathophysiology of CFRD is likely to be multifactorial but ultimately resulting from progressive insulin deficiency secondary to islet cell destruction and defective beta-cell secretion, combined with stressors that intermittently increase insulin resistance resulting in a further deterioration of glycaemic status. Certain patients may be more at risk if non-CFTR genetic modifiers are present [13, 14], and perhaps these patients are unable to compensate for the degree of histological pancreatic destruction and defective beta-cell functioning.

3. Epidemiology

3.1. Prevalence of glycaemic abnormalities in CF

CFRD is known to occur in up to 50% of patients with CF by the age of 30 years [43] and the prevalence increases with age. CFRD can occur in young children with CF but is rare [8]. Recent studies suggest that CFRD affects approximately 9% in the 5–9 year age group [44] and a smaller proportion of children under 5 may also meet the CFRD diagnostic criteria. Yi et al. recently reported a series that suggested 5% of their cohort between 6 months and 5 years had CFRD [45]. Although a small proportion of young children have CFRD, the average age of onset is 20 years [46]. CFRD occurs more commonly in females with a prevalence of 17% in young female adults compared with 12% in males previously described [47].

Children with CF are known to be insulin deficient from birth. Milner et al. demonstrated that children with CF in the first year of life had lower insulin levels than controls [7]. Insulin deficiency will progress over time and results in a gradual deterioration of glucose tolerance. As such, impaired glucose tolerance is much more common than CFRD and can affect up to 41% of children in the 6–9 year age group [48], compared with only 10% of this group being classified as CFRD. The risk of early CFRD is much higher in children with abnormal glucose tolerance on OGTT [48].

3.2. Screening

The prevalence of identified CFRD has been shown to increase after the introduction of screening [49]. Unlike Type 1 or Type 2 diabetes which are often symptomatic, CFRD does not often present with symptoms of hyperglycaemia although this can occur in approximately one third of patients. Symptoms can include polyuria and polydipsia, but CFRD is more likely to present insidiously with the catabolic complications of insulin deficiency such as nutritional deterioration or decline in pulmonary function. When routine screening was introduced in Australia, the incidence of CFRD increased from 2.0 to 22.1 per 1000 person years between 2000 and 2008, which represents a tenfold increase [50]. A decline in the age of diagnosis has also been demonstrated after the introduction of routine screening; Noronha et al. reported a reduction in the mean age of diagnosis from 22.3 years to 13.5 years [49]. Routine screening from at least 10 years of age with an OGTT is recommended by most guidelines [51, 52].

3.3. Risk factors for CFRD

The risk factors for the development of CFRD are closely linked to the specific CFTR genotype and the severity of the CFTR protein dysfunction [53]. CFTR mutations are classified according to the resulting functional deficit [54]. Class 1 and class 2 mutations result in the total or partial absence of CFTR protein at the surface membrane due to defective/non-functional protein (Class 1, e.g. stop codon mutations) or due to defective transfer of the protein to the cell membrane, i.e. defective "trafficking" (Class 2, e.g. F508) [25]. Classes 3, 4, 5 and 6 have irregularities in regulation, conductance, prevalence and stability of CFTR at the membrane [55]. Of the latter, 4 classes, all except class 3, which is known as a gating mutation, have partial function. Those classes with no action have a more severe phenotype and are associated with a greater risk of CFRD, such as homozygous F508 patients [46].

CFRD generally occurs in patients with pancreatic insufficiency. There have been reports of CFRD in patients who are pancreatic sufficient, but the diagnostic criteria for exocrine pancreatic function do not appear to be robust [47]. Some of these patients were classified as pancreatic sufficient because they were not taking replacement enzymes, but had not undergone any formal diagnostic testing such as faecal elastase or 3-day fat stool sampling. More recent studies have demonstrated that the degree of pancreatic exocrine function appears to correlate with the development of CFRD. Soave et al. demonstrated a causal relationship between the level of serum trypsinogen on the newborn screen (a marker of exocrine pancreatic function used to diagnose CF) and the development of CFRD over time [15]. Trypsinogen is an inactive pancreatic enzyme precursor required for protein digestion and absorption. It is

converted to trypsin when secreted into the small intestine, but this process is inhibited in CF and results in an elevated serum trypsinogen. A significant elevation in the blood levels of immunoreactive trypsinogen (IRT) on newborn screening is used to identify neonates with CF. The IRT level is known to decline rapidly over Time with ongoing pancreatic destruction. Soave et al. postulated that patients with CF who had more significant pancreatic disease at birth would have IRT levels that had already started to decline and would be relatively lower than the rest of the CF cohort [15]. They also demonstrated that those children with relatively low IRT amongst the CF cohort had an increased risk of CFRD, thus confirming the relationship between exocrine pancreatic function and endocrine disease.

The presence of CF liver disease appears to be a significant risk factor in the development of CFRD. Leung et al. examined over 700 liver ultrasounds of patients with CF and found that patients with the features of heterogenous or cirrhotic liver disease on ultrasound were more likely to have abnormalities of glucose tolerance, including CFRD, than those with normal liver ultrasounds [56]. The relationship between liver disease and CFRD remains unclear. It could be a result of the more severe genotypes causing CFRD also increasing the risk of liver disease, or it could be the result of a non-CFTR genetic modifier.

Abnormal glucose tolerance is a known risk factor for progression to CFRD. CF patients with glucose abnormalities are up to 11 times more likely to develop early CFRD than other 6–9-year-old patients [48].

4. The clinical impact of glucose abnormalities in CF

Glucose abnormalities in CF are associated with significantly increased morbidity and mortality [2]. Prior to the introduction of routine screening for CFRD, less than 25% of CFRD patients survived to age 30, compared with 60% of patients without diabetes [57]. When Moran et al. examined female CF cohorts with and without CFRD in the 1990s and compared them with cohorts after the introduction of routine CFRD screening, mortality rates had halved: 6.9 per 100 patients years in patients with CFRD versus 3.2 per 100 patient years in CF without diabetes, with similar results seen in men were reported [58]. Although mortality rates for patients with CFRD have seen a marked improvement, a significant difference between CF patients with and without diabetes persists [59].

CFRD leads to a significant increase in respiratory exacerbations, increased infection with CF pathogens [60] (including *Pseudomonas aeruginosa*) and poorer lung function [57, 61]. The mechanism by which insulin deficiency resulting in CFRD has such a negative impact on lung function in CF is probably multifactorial. The hyperglycaemic environment is said to create a "pro-inflammatory" environment, optimal for bacterial growth and colonisation that allows CF pathogens to thrive [52]. In vitro studies have demonstrated an amplification of bacterial growth, in particular *Staphylococcus aureus* and *P. aeruginosa* with increasing glucose concentrations [62], and this evidence supports the hypothesis of glycaemic abnormalities playing a significant and direct role in the infection and colonisation of patients with CF.

Blood glucose levels >8 mmol/L correlate with increased airway glucose levels in patients with CF [62]. In non-CF patients, elevated airway glucose has been demonstrated to be a risk factor for respiratory infections, including MRSA (based on studies in patients intubated due to critical illness in the intensive care unit [63]). When Brennan et al. examined the airway glucose of patients with CF, they demonstrated that even patients with normal glucose tolerance on OGTT had glucose in their airway for longer periods of time than the control population. The duration of time spent with airway glucose levels >8 mmol/L correlated with the degree of glucose abnormality [62]. The level at which glucose appears in the airway is much lower than the 2-h OGTT glycaemic threshold for CFRD and also appears to be very close to the level of blood glucose level (BGL) which correlates with significant nutritional and respiratory decline [64].

Respiratory tract infections may not entirely account for the deterioration in lung function seen in patients with CF. Patients with diabetes mellitus from other causes have also been demonstrated to have poorer lung function than matched controls, even in the absence of respiratory disease [65, 66]. It is unclear whether this is a direct result of glucose in the airways or an indirect result of inflammation from relative insulin deficiency.

Nutrition in CF has a direct correlation with survival [67], and insulin, an anabolic hormone, plays an integral role in maintaining weight and building muscle [18]. When CF patients are insulin deficient, this manifests as poorer nutritional status. Multiple studies have demonstrated the impact of CFRD and insulin deficiency on nutrition and growth [37]. The data of over 8000 CF patients on the epidemiologic study of cystic fibrosis (ESCF) was analysed in 2005 and confirmed a greater impairment in nutrition in the CFRD group when compared with the nondiabetic group [47]. The CFRD cohort had statistically lower height for age percentiles, weight for age percentiles and BMI ($p < 0.001$ for all three parameters). A statistically significant difference in body weight and BMI has also been demonstrated in the "prediabetic" CF patients when compared with CF patients with normal glucose tolerance [61]. This decline was detected by Lanng et al. in some patients 4 years prior to the diagnosis of CFRD being. Given the insidious nature of glycaemic abnormalities and the inherent difficulties with nutrition in patients with CF, particularly those with exocrine pancreatic insufficiency, the impact of insulin deficiency is often not recognised until CFRD is diagnosed on routine screening.

5. The spectrum of glucose abnormalities in CF

Insulin deficiency is progressive and results in a deterioration of glucose tolerance over time. CFRD lies at the end of a spectrum of glucose abnormalities. Glycaemic categories in CF are determined based on the results of the oral glucose tolerance test (OGTT) [51]. To perform an OGTT, a glucose load of 1.75 g/kg (maximum 75 g) is consumed after fasting. Classically the blood glucose level (BGL) is measured at 0 and 120 min [68]. Additional information about glucose tolerance is gained by also checking the BGL at 30 min, 60 min and 90 min, i.e. a 30-min sampled OGTT [64].

The diagnosis of CFRD is made based on the American Diabetic Association (ADA) criteria [51] (see **Table 1**). CFRD is diagnosed when the 2-h OGTT level is ≥ 11.1 mmol/L and can

Category	Fasting level	Midpoint peak (1 h)	2-h plasma level
Normal glucose tolerance	<7 mmol/L	<11.1 mmol/L	<7.8 mmol/L
Indeterminate glycaemia (INDET)	<7 mmol/L	≥11.1 mmol/L	<7.8 mmol/L
Impaired glucose tolerance (IGT)	<7 mmol/L		≥7.8 and < 11.1 mmol/L
CFRD without fasting hyperglycaemia	<7 mmol/L		≥11.1 mmol/L
CFRD with fasting hyperglycaemia	≥7 mmol/L		≥11.1 mmol/L

Table 1. Classification of abnormalities of glucose tolerance in cystic fibrosis on OGTT.

occur with or without fasting hyperglycaemia (fasting BGL ≥ 7.0 mmol/L is defined as fasting hyperglycaemia). Fasting hyperglycaemia can also be considered diagnostic of CFRD, if still abnormal when repeated. One fasting BGL ≥ 7.0 mmol/L and another non-fasting level ≥ 11.1 mmol/L can also make a diagnosis of CFRD. If a patient is sick and glycaemic abnormalities persist for two days, then the diagnosis can also be made. Most guidelines recommend the OGTT/BGL is repeated before the diagnosis is confirmed. Some guidelines subclassify CFRD based on the fasting BGL, but this distinction does not alter management, as insulin treatment is recommended for those with and without fasting hyperglycaemia.

Additional criteria have been published to subclassify the patients into glycaemic categories based on 30-min samples (see **Table 1**) [1]. Patients with normal glucose tolerance have fasting BGL <7.0 mmol/L and 2-h level <7.8 mmol/L. Indeterminate glycaemia (INDET) is defined as normal fasting and 2-h levels with a midpoint level ≥ 11.1 mmol/L. Impaired glucose tolerance (IGT) is defined by a 2-h level <11.1 mmol/L but ≥7.8 mmol/L.

Children with abnormal glucose tolerance and CF may fluctuate between glycaemic categories because of increasing insulin requirements at times of illness or because of variable levels of resistance. In one study 18% of CF patients with abnormal glucose tolerance had glycaemic abnormalities that improved over time. Twenty-two percent of patients had a deterioration in their glucose tolerance [27]. This variability was replicated by Lanng et al. who saw a normalisation of the patient OGTT in 58% of adult patients with CF when followed up after 5 years [69]. Yi et al. examined glucose tolerance in young children (<6 years) and found that some of these children with abnormal glucose tolerance that normalised, including those that met CFRD criteria [45]. This variability adds to the difficulty seen in managing patients with CFRD, particularly younger children.

6. Issues with the OGTT in CFRD

The OGTT was not designed to diagnose diabetes in the CF population. The test was designed to determine the treatment threshold for Pima Native American population with Type 2 diabetes based on their risk of developing microvascular complications [70].

Although microvascular diabetes complications can occur in CF, the major concern for CFRD is its impact on nutrition and lung function. Complications from chronic intermediate hyperglycaemia may also result in microvascular disease prior to the patient meeting the criteria for CFRD [71]. More practical goals would include an initiation of treatment at a time that would alleviate significant respiratory morbidity such as recurrent infections and respiratory function decline. The drop in nutritional status and weight, or poor growth in younger children because of insulin deficiency catabolism, would be a more relevant CF-specific outcome to guide diagnostic targets.

The decrease in lung function and nutrition seen in CFRD actually precedes the diagnosis by several years and is often insidious. Lanng et al. noted that a decline was present up to four years prior to the OGTT 2-h criteria being met [61]. Furthermore, insulin therapy has been demonstrated to reverse some of the nutritional decline seen in patients with abnormal glycaemia [72, 73]. However, once patients meet the criteria for CFRD, recovery of lung function is not always possible. Widger et al. postulate that by waiting until the patient meets the CFRD criteria to start insulin, the conceded progression from abnormal glucose tolerance to CFRD allows irreversible structural remodelling of the lungs that cannot be corrected with insulin therapy [74].

Further evidence for insulin therapy at an earlier stage of the glycaemic spectrum is warranted, and initial data has highlighted which patients may benefit most. Schmid et al. demonstrated that in 1000 patients with CF, patients with midpoint level ≥11.1 mmol/L (INDET) were predictive for later development of CFRD [75]. Brodsky et al. were able to establish that the 1-h level on the OGTT correlated with poorer lung function [76]. They examined 101 patients with CF and these patients with higher 1-h levels had poorer respiratory status even when corrected for nutritional status. The 2-h "diagnostic" level in this group did not correlate with BMI or lung function. The findings of Coriati et al. [77] confirm that waiting for the 2-h BGL to be diagnostic of CFRD may be too late. Their cohort of patients with indeterminate glycaemia already had significant loss of lung function, equivalent to the lung function of patients with newly diagnosed CFRD. The criteria to start insulin in the future may be determined by the patient's own risk of developing CFRD or by early clinical signals in lung function and intermediate glucose abnormalities.

Hameed et al. used a 30-min sampled OGTT and found that a peak BGL ≥ 8.2 mmol/L was reliably predictive of a decline in lung function and nutrition in the preceding year [64]. Based

Proposed new criteria	Peak blood glucose (BG_{max}) mmol/L	Blood glucose at 120 min mmol/L
CFID1	≥8.2	<11.1
CFID2	≥11.1	<11.1
CFID3		≥11.1 Without fasting hyperglycaemia
CFID4		≥11.1 With fasting hyperglycaemia

CFID = cystic fibrosis insulin deficiency.

Table 2. Proposed new staging criteria for insulin deficiency and early glucose abnormalities in CF, based on the OGTT with samples every 30 min.

on these results, this group proposed a new staging criteria to identify insulin deficiency and early glucose abnormalities in patients with CF (see **Table 2**) [78]. Cystic fibrosis insulin deficiencies (CFID) 4 and 3 correspond to existing CFRD categories with and without fasting hyperglycaemia, respectively. CFID 1 and 2 are earlier stages of insulin deficiency that are distinct from impaired glucose tolerance (IGT) because they are based on the peak glucose level and have 2-h levels < 11.1. CFID 1 is defined by a midpoint peak glucose level ≥8.2 mmol/L, and CFID2 has a midpoint glucose peak ≥11.1 mmol/L.

7. Continuous glucose monitoring in CF

Continuous glucose monitoring (CGM) has been used for several years in the management of Type 1 diabetes although it is not licenced for use as a diagnostic device. CGM uses a small probe inserted into the subcutaneous space where it measures interstitial glucose levels. Inserting the device is a relatively simple procedure that can be done within a few minutes in a clinic environment. It is easy to remove at home by the patient or carer, without any specific medical training. The device averages the glucose readings every five minutes and can be worn for several days whilst the patient continues to participate in normal activities and consumes their normal diet. The CGM device has been validated in CF and non-CF populations and shown to correlate with plasma glucose measurements [79, 80]. When compared with OGTT, CGM appears to be reproducible and a reliable assessment of glycaemic abnormalities. When used in Type 1 diabetes, Bergenstal was able to demonstrate that children and adults on insulin pumps had improved glycaemic control, as measured by HbA_{1c} than those who did not use CGM [81].

CGM may be particularly useful in managing cystic fibrosis. CF patients frequently demonstrate early postprandial hyperglycaemia [79, 82, 83], reflected by elevations in readings on a 30-min sampled OGTT in the setting of a normal 2-h level. This intermittent postprandial hyperglycaemia may be reflected in the poor correlation of HbA_{1c} (glycated haemoglobin) with early glycaemic abnormalities in CF. HbA_{1c} represents an index of the average of blood glucose concentrations in the preceding 2–3-month period, and the result is influenced by the half-life of the red cells [84]. When measured in CF, it is a poor indicator of glycaemic abnormalities as it is often still normal by the time a diagnosis of CFRD has been made. The poor sensitivity of the test may result from the intermittent nature of hyperglycaemia in patients with CF, which is not revealed in the HbA_{1c} level when the glucose levels are "averaged", as well as increased red cell turnover in CF.

CGM provides a useful tool to guide insulin treatment once the diagnosis of CFRD has been made [79], but it may also offer a potential opportunity to capture the moments of postprandial hyperglycaemia in CF in the screening and diagnostic phase. In CF patients with normal glucose tolerance on OGTT, abnormalities on CGM have been detected [79, 82, 83]. This could reflect the fact that patients with CF undergo a period of fasting prior to their glucose load in the OGTT which will only measure two values. When a CGM is worn, patients can be at home and may consume their normal CF diet including a carbohydrate load that may exceed the glucose level consumed during an OGTT. In the same way that HbA_{1c} may not reflect a true

picture of glycaemic abnormalities in CF, so too may the OGTT underestimate the hyperglycaemia in these patients, particularly in the early phase of glucose abnormalities.

CGM may be a useful device in predicting which children with CF will develop glycaemic abnormalities. Schiaffini et al. performed OGTT and CGM on children with CF and then repeated the OGTT after 2 years. Children who had diabetic excursions on CGM at baseline, even those with normal glucose tolerance on OGTT, developed impaired glucose tolerance or CFRD when the OGTT was repeated 2 years later [83]. Initial data on CGM does appear to suggest that this tool may be useful in identifying clinically significant glucose abnormalities in CF. Leclercq et al. demonstrated, in a CF population with normal OGTT, that patients who recorded glucose levels in the diabetic range (≥11.1 mmol/L) on CGM had poorer lung function and greater colonisation with CF respiratory pathogens such as *P. aeruginosa* [85].

Glycaemic abnormalities are known to have a significant impact on nutrition in patients with CF. CGM may provide an opportunity to highlight which children are at risk of nutritional decline secondary to abnormities of glucose tolerance as described in the study by Hameed et al. [64]. In this study of 25 children with CF undergoing CGM, if ≥ 4.5% of the study duration was spent with an interstitial glucose reading >7.8 mmol/L, this was predictive of a decline in weight standard deviation score. This CGM criterion had a sensitivity of 89% and a specificity of 86% in detecting this nutritional decline. CGM abnormalities do appear to be clinically significant, but there are not studies as yet demonstrating a benefit from treatment based on CGM recordings in CF, and the device is not yet licenced to make a diagnosis of diabetes.

8. Management of CFRD

The main aim of CFRD treatment is to correct the hyperglycaemia and its downstream effects on respiratory function and infections, in addition to reversing significant protein catabolism secondary to insulin deficiency. Optimal management has been shown to improve lung function and morbidity [72]. Although a drop in mortality from late CFRD diagnosis has been seen, the risk of early mortality is still higher in this population. The mainstay of treatment is exogenous insulin therapy, but studies are underway examining the benefits of dietary changes and the use of oral hypoglycaemic agents in CF.

8.1. Insulin

Insulin plays a major role in the management of CFRD. Insulin replacement by subcutaneous injection in CFRD has been shown to improve lung function and reduce pulmonary exacerbation frequency [86]. It has also been shown to benefit the nutritional status of the patient, with an improvement in growth seen in children with CF [73]. Recent studies have also demonstrated that insulin therapy in the prediabetic phase may also play a valuable role in the management of patients with CF. Hameed et al. were able to replicate previous studies demonstrating a benefit of insulin therapy on lung function and nutrition in patients with CF and revealed an improvement in weight standard deviation score ($p = 0.003$) and lung function (FEV1 improvement $p = 0.004$) with once daily insulin injections (detemir, Levemir™) [73].

Insulin is given via subcutaneous injection. Unlike Type 1 diabetes, a once daily dose of long-acting insulin may be all that is required to demonstrate a benefit for this population [73]. Insulin doses vary with each patient, but because of the important anabolic role insulin plays in growth and nutrition, the highest tolerated dose without hypoglycaemia or other side effects is generally recommended [52] (taking into account patient-specific factors such as ability to recognise hypoglycaemic symptoms). The dose prescribed may vary over time with increasing requirements during times of relative increase in insulin resistance such as with glucocorticoid use or during periods of growth and pregnancy. Given the progressive nature of insulin deficiency in CF, increasing requirements may be seen over time, particularly in the paediatric population with CF that have age- and weight-based doses.

Insulin pumps that continuously deliver a small amount of insulin into the subcutaneous space have been used in patients with CFRD [87] although the uptake in CF has been poor when compared with other forms of diabetes. When wearing a pump, patients are currently required to undertake much more intensive finger-prick blood glucose testing than that required with a once daily insulin injection. This may prove to be too onerous for patients with CFRD who already have a significant treatment burden with multiple oral and nebulised medications and physiotherapy. Future insulin pump devices may include closed loop systems, in which interstitial glucose levels measured by CGM calibrate the rate and amount of insulin secreted by the pump [88]. These devices are currently under investigation for Type 1 DM, but there are no data published about their use in CFRD to date.

8.2. Nutrition

Nutritional education and support are of utmost importance for patients with a diagnosis of CFRD. Children with CF require a higher caloric intake (may need up to 200% of usual recommendations [89]) to achieve optimal nutritional and growth targets. If nutritional targets are not met, there may be significant consequences as a lower BMI has been associated with increased mortality in CF [67]. These additional calories are best taken from fat and protein-based meals, but a significant proportion is taken from carbohydrates [90]. Patients with abnormalities of glucose tolerance and CFRD will be required to recognise carbohydrates in their diet, as the carbohydrate load will affect the glucose level and the resulting insulin requirements. This is usually done by educating the family and patient about carbohydrate-insulin ratios.

There are very limited data regarding the dietary management of CFRD. This is of particular significance given that hyperglycaemia has been demonstrated to worsen glycaemic abnormalities in CF, possibly by potentiating beta-cell apoptosis. As such, glycaemic control in CFRD needs to be tight, and diets that perpetuate postprandial hyperglycaemia may have a negative impact on glycaemic abnormalities in CF and increase insulin requirements. A low glycaemic diet is often recommended in Type 1 and Type 2 diabetes to optimise control of hyperglycaemia and has been shown to decrease insulin requirements and improve glucose homeostasis, without having a significant impact on quality of life for these patients. Whereas weight loss due to change in diet may be beneficial in Type 2 DM, this may have serious negative consequence in CF. There is not enough information in the literature to recommend any dietary changes that might improve glycaemic control or prevent or delay progression to CFRD if instituted at an earlier stage.

8.3. Oral hypoglycaemic agents

Oral agents do not play a role in the management of patients with CFRD. Many agents target insulin resistance (e.g. metformin), which is not a major feature in the early glycaemic abnormalities of CF where insulin deficiency plays the key role and as such will not be of significant benefit to CF patients. Significant side effects from oral hypoglycaemic agents such as hepatotoxicity are a serious complication for the CF population where a significant proportion may develop CF liver disease [91]. Insulin therapy in states of insulin deficiency such as Type 1 diabetes has been shown to preserve insulin secretion and "rest" the residual beta cells. Conversely, agents that stimulate insulin secretion may potentially hasten beta-cell loss. For example, agents such as repaglinide may be useful in the short term but ultimately have a negative long-term impact.

8.4. The role of potentiators in CFRD

Evidence for the use of potentiators in CFRD is limited, but a few pilot studies have been published that suggest a benefit on glucose homeostasis in CF. In a single pair of CF siblings with abnormal glucose tolerance (one with CFRD) and gating mutations, a reduction in the glucose AUC and an improvement in the insulin secretion profile was demonstrated after the introduction of ivacaftor (Kalydeco™). Bellin et al. also demonstrated improvements in glucose homeostasis after the introduction of ivacaftor. In this group of five CF patients with glucose abnormalities, four of five demonstrated improvements in insulin secretion. The patient whose insulin secretion did not improve had long-standing CFRD, whereas the others had earlier glycaemic abnormalities. Theoretically, the patient with long-standing CFRD could already have undergone such significant pancreatic destruction that the abnormalities of glucose tolerance could not be corrected at the level of the CFTR.

9. Complications of CFRD

Long-standing hyperglycaemia and insulin deficiency will result in an increase in respiratory exacerbations and morbidity and poorer nutrition. It will also result in complications from chronic hyperglycaemia seen in other forms of diabetes. Historically the life-limiting nature of CF and in particularly those with CFRD meant that CF patients were unlikely to live long enough to develop end-organ dysfunction from the macrovascular and microvascular complications seen in other forms of diabetes. With an improvement in life expectancy, these long-term issues need to be addressed, and routine screening needs to be a part of CF clinical care. This will include examination for neuropathy and retinopathy and urine screening for microalbuminuria. In one study, 10 years after the diagnosis of CFRD has been made, subjects with fasting glycaemia demonstrated rates of microalbuminuria of approximately 14%, retinopathy 16%, neuropathy 55% and autonomic gastropathy 50% [51]. Gilchrist et al. reported retinopathy in three patients with abnormal glucose tolerance but not meeting criteria for CFRD [71] which further supports the proposition that the OGTT may not be the ideal test for significant glycaemic abnormalities in patients with CF.

10. Conclusion

Cystic fibrosis–related diabetes continues to pose a significant risk of increased morbidity and mortality to the CF population. However, CFRD lies at the endpoint of spectrum of glucose abnormalities, and increasing evidence implies that earlier glycaemic abnormalities may also be clinically significant. The standard OGTT does not appear to be sensitive enough to pick up early, clinically significant abnormalities of glucose tolerance secondary to insulin deficiency and the dysregulation of insulin secretion detected in CF patients. Hyperglycaemia in CF affects lung function, risk of respiratory pathogens, nutrition and growth in young children, and treating teams need to be proactive in the screening and diagnosis of glycaemic abnormalities that may be insidious and potentially irreversible if recognised late. Early recognition of hyperglycaemia in CF is required to prevent significant morbidity. Novel techniques such as continuous glucose monitoring may play a role in screening and early identification of at risk patients, as they have been shown to be predictive of significant glucose abnormalities in the future such as CFRD, but there is not enough evidence as yet to recommend their routine use in diagnosis. Future directions may include the use of potentiators and correctors in CF which appear to have potential to correct abnormalities of glucose tolerance but may be limited if instituted late and once significant pancreatic destruction has occurred.

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Chapter 3

The Cystic Fibrosis Airway Microbiome and Pathogens

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Additional information is available at the end of the chapter

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Abstract

Cystic fibrosis (CF) is an autosomal recessive genetic disorder resulting from genetic defects in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR dysfunction in patients with CF leads to a number of pleiotropic manifestations with the prime pathology being mucus plugging in the airways and paranasal sinuses. Patients with CF are prone to polymicrobial infections and the airway microbiome in such patients changes continuously and evolves over time. The composition of the airway microbiome in CF patients is dependent on a number of factors including geographic variation, type of genetic mutation (e.g., ΔF508), antibiotic exposures, and chronic infection with certain pathogenic bacteria (e.g., *Pseudomonas aeruginosa*). Proteomic and genomic approaches to understanding the microbiome of patients with CF have provided new insights into the pathogenesis of this disease. High-throughput pyrosequencing, Sanger sequencing, and phylogenetic microarray analysis have enabled the recognition of multiple lineages and clonal populations of a single bacterial species within the same patient. This provides a unique opportunity to explore novel therapeutic approaches to this disease (for instance, use of probiotics and environmental manipulation) and potentially translate them into bedside clinical interventions.

Keywords: cystic fibrosis, microbiome, dysbiosis, *Pseudomonas aeruginosa*, burkholderia cenocepacia

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene [1]. CF is most prevalent in the Caucasian population and is a common life-limiting disease [2]. CFTR is expressed on the apical surface of epithelial cells of the respiratory, gastrointestinal, pancreatic and reproductive tracts, and sweat glands [3]. The prime function of CFTR ion channel is to transport chloride ions across epithelial surfaces in order to maintain the osmotic gradient. Chloride ions are actively

pumped out into the luminal side of the gastrointestinal and respiratory tracts, which decrease water potential on the luminal side. Subsequently, water molecules move from a higher osmotic potential to a lower osmotic potential (down the osmotic gradient) and combine with mucin glycoproteins to keep them adequately hydrated. This in turn helps to maintain the thin consistency of the mucus layer, which is essential for optimal mucociliary function [4]. Thick and viscid mucus caused by a defect in chloride-conducting transmembrane channel results in stagnation of mucus. Moreover, CFTR channel also plays an important role in regulating the transepithelial transport of sodium and bicarbonate ions [5]. Defective CFTR functioning leads to an increase in pH of the mucus layer, which compromises the innate immune system and promotes inflammation. Defects in innate immunity and chronic inflammation predispose patients to recurrent pulmonary infections, which result in permanent lung damage—the prime cause of morbidity and mortality [6]. Pulmonary system is not the only organ-system affected in CF; endocrine, gastrointestinal, and reproductive systems are also involved in this multisystem disorder [3].

The human microbiome project aims to identify and characterize microbial flora of healthy and diseased individuals [7]. Understanding the role of infectious pathogens in the pathogenesis of CF in general and pulmonary exacerbations and lung damage in particular has enabled the scientific community to devise new treatment modalities for CF patients, which can potentially improve outcomes and survival in such patients. In patients with CF, different bacteria inhabit different parts of the lung at various stages of the disease and persistent inflammation in the lungs can change and modify the composition of the microbiome [8]. For instance, methicillin-sensitive *Staphylococcus aureus* (MSSA) and *Hemophilus influenzae* are common pathogens early in life of such patients [9]. As the disease progresses, more virulent pathogens—such as *Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MRSA)—invade the lung and cause pulmonary damage [10]. By understanding the evolution of the CF microbiome, we can gain further insights into the natural course of CF. This in turn can have important implications for developing interventions that can halt or reverse the course of progressive pulmonary damage and prolong survival and quality of life in CF patients [11]. In the following pages, we discuss the CF microbiome, its evolution and heterogeneity in CF patients, interaction between different bacteria within the CF lung and the factors that potentially affect the CF microbiome.

2. The microbiome

As mentioned previously, the human microbiome project aims to identify and characterize microbial flora of healthy and diseased individuals [7]. There is a diversity of microbes in every single human being i.e., diversity being defined as the number and distribution of a particular type of organism in a body habitat. Every human has particular and distinct microbes; dysbiosis (alteration in composition and balance) of these microbes is now thought to underlie the pathogenesis of many diseases, such as inflammatory bowel disease, *Clostridium difficile* (CD) colitis, bacterial vaginosis, obesity, and CF [12]. The human microbiome plays a very important role in human biology, defense mechanisms, metabolic processes (such as

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digestion, absorption, and assimilation) and even pathogenesis of acute and chronic diseases [13]. For instance, CD colitis is a disease that arises as a consequence of interaction of bacterial virulence factors, host immune mechanism and the intestinal microbiome [14]. Research studies have shown that variability in the innate host response may also impact upon the severity of CD colitis, and this variation may be accounted for by alterations in the gut microbiota [15]. Based on improved understanding of the pathogenesis of CD colitis, fecal microbiota transplantation (FMT) and other novel types of bacteriotherapy have become potentially effective treatment options for this deadly disease [16].

Another example of a disease where microbiota plays a major role in pathogenesis is Crohn's disease. The exact cause of Crohn's disease is unknown; however, evidence suggests that microbiota contribute to the underlying pathology and disease development [17]. No single bacterium has been convincingly shown to contribute to the overall pathogenesis of Crohn's disease. Instead, dysbiosis (bacterial imbalance) is more widely accepted as a leading factor in the disrupted host immune system cross-talk that results in subsequent intestinal inflammation [18]. Depletion of symbiont (beneficial) microbes (including Firmicutes, Bifidobacteriaceae, and Clostridia) in conjunction with an increase in pathobiont (harmful) microbes (such as Bacteroidetes and Enterobacteriaceae) is a striking feature observed in Crohn's disease. No single factor has been definitely identified as driving this dysbiosis; instead, a host of environmental factors—such as the diet, antibiotic exposures and possible early life infections—in the presence of underlying genetic susceptibilities may contribute to the overall pathogenesis of Crohn's disease [17].

In CF patients, composition of the microbiome of pulmonary and gastrointestinal tracts changes over time, presumably as a consequence of inflammation [19]. Most research studies have demonstrated the influence of inflammation in negatively selecting against potential pathogens. Moreover, some bacterial species may also have the ability to exploit inflammatory byproducts for their benefit, which may promote their natural selection in inflamed habitats [20]. Reactive nitrogen species produced during inflammatory responses can be exploited by pathogens for their growth. Moreover, inflammatory mediators can provide an environment for some bacteria to grow and use these inflammatory mediators for their survival [21]. Examples of such bacteria include *Escherichia coli* and *P. aeruginosa* in the gastrointestinal and respiratory tracts of CF patients, respectively. *P. aeruginosa* uses nitric oxide produced in the process of inflammation for its anaerobic respiration and promotes its growth in inflammatory environments. Likewise, *E. coli* uses increased nitrate in the environment for its anaerobic respiration and enhances its growth in the inflamed gut of CF patients [19].

3. Heterogeneity of the CF airway microbiome

Due to defects in innate immunity, CF patients are prone to polymicrobial infections and their airway microbiome changes continuously and evolves over time. The primary cause of death in CF patients is respiratory failure due to persistent and recurrent pulmonary infections with different pathogenic organisms [22]. Over the past decade, the median survival for such

patients stands at 37 years despite increases in life expectancy [23]. MSSA and *H. influenzae* are one of the most common pathogens cultured from sputum samples of affected children. *P. aeruginosa* has been associated with increased morbidity as most strains of this organism are multidrug resistant. Infections with bacteria of the Bukholderia cepacia complex (BCC) are associated with a worse prognosis [24]. Likewise, other multidrug resistant organisms, such as *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia*, can also be isolated from CF patients with end-stage pulmonary disease [25]. Nontuberculous mycobacterium (NTM) has also been identified as emerging causes of infections in patients with CF and their incidence may have been underestimated in the past [26]. More recently, research studies have shown that when sputum samples obtained from adults with CF are cultured, a significantly high density of anaerobic bacteria can be isolated—the most common of which are *Streptococcus milleri*, *Prevotella* spp., *Actinomyces*, and *Veillonella* [27].

Microbes of the lower airways in all humans exist in a dynamic state. Literature published on microbiome of CF patients has shown a complex and dynamic interaction between different organisms in the airways of such patients [28]. Organisms within a single patient are genetically and phenotypically diverse and heterogeneity is detectable even in different parts of the same lung. Over a period of time, community diversity of bacteria declines in CF patients as pulmonary function declines and lung disease progressively worsens. Studies have shown that diversity of microbial communities correlates positively with pulmonary function and outcome [29]. Such diversity was previously unrecognized as most studies relied solely on culture-based methods of culturing bacteria. However, novel state-of-the-art molecular techniques (such as Sanger sequencing of clone libraries, terminal restriction fragment length polymorphism [RFLP] analysis and microarray hybridization) have enabled the detection of subtle molecular diversity among seemingly similar bacterial species [30]. This diversity may be influenced by a number of factors including the patient's age, sex, type of CFTR mutation, antibiotic exposures, environmental factors, and extent and severity of lung disease. In a study by Zhao et al., sputum samples were collected from six CF patients over a period of 10 years. Of a total of 126 sputum samples, 662 operational taxonomic units (OTU) were identified and each patient had 5–114 different OTUs [29]. Similarly, in another observational study, sputum samples of patients with acute infective exacerbation of non-CF related bronchiectasis were collected. Sputum cultures from each patient contained large quantities of multiple bacterial species with a single predominant pathogenic species [31]. In one study, polymerase chain reaction (PCR)-temporal temperature gel electrophoresis (PCR-TTGE) was used to evaluate intraspecific and intragenomic 16S rDNA variability among commonly isolated respiratory pathogens from CF patients [32]. Significant discordance in intraspecific and intragenomic variability was noted among different bacterial species with *H. influenzae* displaying the highest level of intraspecific variability.

4. Composition of the CF microbiome and its determinants

The composition of the airway microbiome in CF patients is dependent on a number of factors including geographic variation (more common in white population), type of genetic

mutation (e.g., ΔF508), antibiotic exposures, and chronic infection with certain pathogenic bacteria (e.g., *P. aeruginosa*) [8]. Fetal lungs are sterile, just like fetal gastrointestinal tract, but they soon become colonized after birth. Fetal skin becomes colonized with microbes present in maternal reproductive and gastrointestinal tracts and lungs become colonized from gut flora of the child [33]. The common phyla found in healthy lungs include Bacteroides, Firmicutes, and Proteobacterium. Other genera include Prevotella, Veillonella, Streptococcus and Pseudomonas [34]. Many techniques have been used for the detection of microbes in CF patients. Some of these techniques include terminal RFLP profiling, microarray analysis, clone library sequencing, and pyrosequencing. The most frequently used samples from CF patients for analysis are expectorated sputum, tracheal aspirates, bronchial washings, and bronchoalveolar lavage (BAL).

The microbiome in patients with CF evolves as patients grow older, and this is a consequence of the wide adaptability of pathogenic bacteria. Clustering of phylogenetically similar bacterial communities and loss of the architectural diversity of the airway microbiome is a key feature of late-stage CF airway disease. Moreover, the type of bacterial species predominating at a particular age group is also of immense importance. In one study, phylogenetic diversity of CF airway microbiota in patients of different age groups was studied using microarray analysis [35]. *S. aureus* was detected in 65% of sputum samples and was more common in the pediatric population (72% of the pediatric sample). *Pseudomonas* spp. was found in 73% of samples and were most common in adults (91% of the adult sample). In the same study, older CF patients had reduced airway bacterial diversity and aggregation of relatively similar organisms; this process occurred in conjunction with a progressive decline in pulmonary function. *H. influenzae* was most prevalent in the pediatric population when the bacterial diversity was highest. Conversely, *P. aeruginosa* was most common in older individuals with a lower level of bacterial diversity. Likewise, members of the Mycobacteriaceae family and obligate intracellular pathogens (such as Chlamydia and Mycoplama spp.) were more prevalent in younger CF patients. Certain known or potential pathogens of CF patients, such as members of the Burkholderiaceae and Thermoactinomycetaceae families, were almost exclusively observed among adult patients.

In another study [29], CF patients with progressive lung disease were noted to have a decrease in bacterial diversity with increasing age, but the total bacterial density remained stable over time. Antibiotic exposures in conjunction with recurrent pulmonary exacerbations were proposed as a possible contributing factor toward this observation. In a study by Tunney et al., several anaerobic species (including a number of Veillonella and Prevotella species) constituted a significant portion of the CF airway microbiota [36]. In a unique study, next generation sequencing was used to study the microorganisms of gastric juice among patients with CF and non-CF controls [37]. CF gastric juice was noted to have an abundance of Pseudomonas spp. and a relative paucity of normal gut bacteria (such as Bacteroides and Faecalibacterium), which was in contrast with normal gastric juice samples. These results suggest that CF patients possess a unique aerodigestive microbiome that is inter-related. This explanation seems plausible as the factors that influence the airway microbiome (for instance, antibiotic exposures) are also likely to influence the microbiota of gut and other organ-systems of the body [38].

In patients with CF, different bacterial colony morphotypes can be isolated from a single sputum sample. There is some evidence to suggest that these different morphotypes arise from a single bacterial strain [39]. Microbes in the lungs of CF patients are capable of constantly adapting to selection pressures. Some of the mechanisms that enable the evolution of microbes include motility, type III secretion systems, lipopolysaccharide, plasmids (encoding for antibiotic resistance), biofilm formation, small colony variants, quorum sensing, and hypermutability. As a consequence of these mechanisms, different phenotypes arise from a single bacterial species and, over time, a single bacterial strain with dominating features may evolve [40]. Given that different bacterial strains have differing capacities to evolve, multiple lineages of bacterial colonies evolve and coexist [41]. Some studies have shown that complexity of bacterial communities inversely correlates with patient age, antibiotic exposures, and presence of *P. aeruginosa* [42]. In one study, heterozygosity for the ΔF508 mutation and presence of mutations other than the ΔF508 was associated with relative preservation of airway bacterial diversity over time [35]. This shows that apart from environmental exposures (such as antibiotic pressures), patients' genotype (type of mutation) also plays an important role in determining the composition of the CF airway microbiome. In terms of environmental exposures, antibiotic use has been shown to be the prime factor that adversely affects microbial diversity among CF patients [29]. Loss of bacterial diversity (under the selection pressure of antibiotics) has been associated with an increased risk of pneumonia in mechanically ventilated patients colonized with *P. aeruginosa* [43]. Smith et al. studied this further by performing whole genomic analysis of a single species of *P. aeruginosa* isolated from a patient with CF. Whole genomic sequencing was repeated multiple times during the course of the patient's illness, which enabled the detection of an overwhelming number of mutations. Based on these analyses, it was found that the strain of *P. aeruginosa* that inhabits patients with advanced CF differs significantly from wild-type *P. aeruginosa* [40].

The interaction among different bacterial colonies has also become a subject of intense research and genomic and proteomic approaches are currently being used to understand their interrelationships. In an experimental study, production of 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) by a strain of *P. aeruginosa* enhanced the aminoglycoside resistance of *S. aureus* [44]. This study provided some evidence of how bacterial interspecies interaction can alter the airway microbiome by selecting for resistant strains of a bacterial species. Previous studies have shown that HQNO is detectable in the sputum of infected CF patients. Therefore, an interaction between *P. aeruginosa* and *S. aureus* may account for the increased incidence of small colony variant (SCV) of *S. aureus* species in CF patients with advanced lung disease.

In the recent literature, an increasing number of unusual microbes have been reported as the cause of infective exacerbations of CF. Such bacteria include multidrug resistant pathogens like *S. maltophilia*, multidrug resistant *P. aeruginosa*, MRSA, *Burkholderia cenocepacia* and even NTM [45]. The emergence of such bacteria as members of the CF airway microbiome can have important implications for management and prognosis for patients. For instance, studies have shown that in CF patients with an acute exacerbation, there is discordance between the results of microbial sensitivity testing and response to antibacterial therapy [46]. Polymicrobial infections and presence of fastidious organisms may account for this observation. Moreover, such pathogenic bacteria can interact with other less virulent bacterial species and lead to

architectural distortion of the entire CF microbiome. In the following lines, we discuss common members of the CF airway microbiome, some of which are commonly implicated in infective exacerbations.

4.1. Methicillin-sensitive *Staphylococcus aureus*

S. aureus is a common colonizer of the anterior nares of adolescent and adult patients [47]. Among patients with CF, MSSA is one of the most common pathogens isolated from sputum samples obtained for culture and sensitivity testing. In the CF Foundation (CFF) patient registry (Bethesda, Maryland, USA), *S. aureus* was most commonly isolated from children and adolescents accounting for approximately 51% of the total samples. Moreover, the overall prevalence of *S. aureus* has been increasing over the past few decades. Infection with *S. aureus* has been associated with increased bronchial inflammation and decreasing pulmonary function [48]. Moreover, when coinfection with *P. aeruginosa* and MSSA occurs, mortality is increased manifold. Interestingly, studies have shown that MSSA is associated with more severe disease in children as compared to adults.

With the widespread use of antistaphylococcal antibiotics, incidence of Gram-negative infections among CF patients has increased and MSSA has become less common among adult patients. Overall, the most common cause of chronic lung infections in CF patients is *P. aeruginosa*, an oxidase-positive Gram-negative bacillus. Moreover, as CF patients grow older, MRSA becomes a more frequent cause of infective exacerbation than MSSA. Over the past few years, the incidence of MRSA infections has been steadily increasing, owing to increasing use of antistaphylococcal penicillins (such as oxacillin and nafcillin) [49]. More recently, a subtype of *S. aureus* species (viz. small colony variant) has been isolated more frequently from CF patients. The small colony variant of *S. aureus* species is fastidious and slow-growing, and it has also been associated with rapid decline in pulmonary function. As mentioned previously, selection of small colony variant species is promoted by HQNO—a product synthesized and secreted by *P. aeruginosa* species [44]**.** Increasing use of broad-spectrum antibiotics that select for multidrug resistant pathogens can explain this distortion in the composition of the airway microbiome in patients with CF.

4.2. Methicillin-resistant *Staphylococcus aureus*

S. aureus is typically the first bacterial pathogen to invade the pulmonary parenchyma in patients with CF. Chronic infection with this organism can persist in the airways of CF patients for several years. Acquisition of mecA gene mediates methicillin resistance in community-acquired MRSA by encoding for a mutated penicillin binding protein-2A (PBP-2A) [50]. The prevalence of MRSA has increased substantially over the past several years from an estimated 7.3% in 2001 to 22.6% in the year 2008 and 25.7% in 2012 [10]. This increase in prevalence of MRSA was noticed across CF patients of all age groups with the highest increase being in the adolescent age bracket. This increase in the prevalence of MRSA in CF patients has been directly linked to the increase in overall incidence of community-acquired MRSA in the general population [51]. In a study by Glikman et al., 22 of 34 (64.7%) MRSA isolates from patients with CF contained the gene SCCmec II—a typical feature of health-care associated

MRSA strains. On the other hand, 9 of 34 (26.5%) MRSA strains harbored the SCCmec IV gene, which characterizes them as community-acquired MRSA strains. Most patients with community-acquired MRSA were newly colonized with the strain. Additionally, children with CF were more likely to harbor MRSA isolates that were resistant to clindamycin and ciprofloxacin compared with strains from non-CF patients [52]. Other studies have reported persistent infections in CF patients with both hospital-acquired and community-acquired MRSA strains (including Panton-Valentine leukocidin-positive strains) with an overall prevalence of 7.8% [53]. In these studies, persistence was due to presence of different clones over time or identical clones that underwent minor modifications in their toxin content. Moreover, isolation of MRSA from CF patients aged 7–24 years has been associated with an increased severity of the disease. Alarmingly, some of these strains may be vancomycin-intermediate *S. aureus* (VISA), which implies that treatment with glycopeptides (such as vancomycin) may also be ineffective. Highly virulent strains, such as vancomycin-resistant *S. aureus* (VRSA), have also been reported to cause necrotizing pneumonia in a small number of CF patients [54]. Persistent infection with virulent strains of *S. aureus* has been associated with a rapid decline in pulmonary function [55]. In a case-control study, CF patients who were colonized with MRSA had a significantly higher rate of decline in FEV_1 (forced expiratory volume in first second) as compared to those who were not colonized with MRSA [56]. Moreover, MRSA-infected CF patients have been shown to have longer hospital stays than age- and sex-matched controls [57]. Serious manifestations of MRSA infections have also been described in various reports. Cavitary lesions have been described in two CF patients infected with Panton-Valentine leukocidin-positive MRSA strains [54]. This observation was consistent with other reports of serious pulmonary manifestations of community acquired MRSA infection [54, 58]. In a cohort study of longitudinal data, risk of death among CF patients who had at least one culture positive for MRSA was 1.27 times greater than for CF patients in whom MRSA was never detected [55]. In a meta-analysis of 76 studies, a clear and strong association was noted between exposure to antibiotics and isolation of MRSA [59]. The risk of acquiring MRSA was increased by 1.8-fold in patients who had taken antibiotics as compared to others. The risk ratios for quinolones, glycopeptides, cephalosporins, and other beta-lactam antibiotics were 3, 2.9, 2.2, and 1.9, respectively.

4.3. Hemophilus influenzae

H. influenzae is a facultative, anaerobic, Gram-negative bacillus. In many patients, this organism begins to colonize the upper respiratory tract since infancy. Approximately 20% of infants with CF are colonized by the end of first year of life and the rate is even higher for patients of older ages [60]. By the age of 5–6 years, more than 50% of children are colonized with this bacterium [61]. *H. influenzae* is a common pathogen of chronic lung infections and is frequently implicated in infective exacerbations of CF [62]. In children with CF, about 32% are colonized with this microorganism. However, as these patients grow older and are exposed to a wide range of broad-spectrum antibiotics, more virulent bacteria inhabit their respiratory tracts. Consequently, in adults with CF, the rate of colonization with *H. influenzae* is reported to be only 10–15%. Having said this, the prevalence of *H. influenzae* has increased from 10.3% in the year 1995 to 16.3% in the year 2008.

Similar to the general population, colonization of the upper respiratory tract of CF patients with *H. influenzae* is quite a dynamic process. Children will typically carry multiple strains of this bacterium simultaneously, whilst adults will be colonized with only one strain [63]; again, this is a natural consequence of the loss of microbial diversity induced by antibiotic selection pressures. Even in most healthy adults, the upper airway is colonized with *H. influenzae*; most strains in such healthy subjects are nontypeable. In particular, the nasopharynx is an area of the respiratory tract that serves as a potential reservoir of this bacterium. Eventually, the organism may spread from the nasopharynx to the lower respiratory tract and cause an infection of the pulmonary parenchyma [64]. Studies have shown that most CF patients are cocolonized with two or more distinct strains of *H. influenzae* [65].

H. influenzae is not considered a virulent pathogen in patients with CF. Interestingly, some studies have shown that colonization with *H. influenzae* is associated with a relatively preserved lung function. This is in sharp contrast to other microorganisms like *P. aeruginosa* and MRSA, whose colonization of the pulmonary parenchyma is strongly associated with a rapid decline in lung function [66]. In a prospective study, 27 patients with CF (under the age of 12 years) and 27 matched patients with asthma were followed up for 1 year [67]. The isolation rate of noncapsulated (nontypeable) strains of *H. influenzae* was significantly higher in the CF group as compared to that of the asthma group. During exacerbations, the isolation rate of *H. influenzae* in the CF group was significantly greater than at other times, whereas there was no significant difference in the control group. The distribution of biotypes of *H. influenzae* and *Hemophilus parainfluenzae* was similar in the two groups. In the CF group, biotype I was commonly detected and was associated with infective exacerbations of CF. In contrast, biotype V was more common in the asthma group, although it had no association with the development of infective exacerbations [67].

4.4. Pseudomonas aeruginosa

P. aeruginosa is an obligate aerobic, oxidase-positive, nonlactose fermenting Gram-negative rod. *P. aeruginosa* is the most common organism implicated in infective exacerbations in patients with CF. In the CFF patient registry (Bethesda, Maryland, USA), more than half of the patients (52.5%) were reported to be infected with *P. aeruginosa* in 1995. The risk of chronic infection with *P. aeruginosa* increased proportionately with increasing age. Moreover, the incidence of *P. aeruginosa* has been reported to be increasing in infants. Despite changes in the management of patients with CF, the frequency of persistent infection with *P. aeruginosa* has remained relatively stable over time [68]. In a study based on the CFF patient registry, prevalence of colonization with *P. aeruginosa* was 60% in 1995 and 56.1% in 2005 [69]. However, recent data suggest that the prevalence of P. aeruginosa is slowly decreasing over time and has been estimated to be 30.4% in the year 2015 [70].

The main reservoir of *P. aeruginosa* is the environment surrounding CF patients. It has been thought that among siblings with CF, prolonged exposure of young children to their older siblings with CF is a potential risk factor for acquisition of *P. aeruginosa*. A study published in 1991 reported that *P. aeruginosa* may be acquired by patients at CF recreation camps, clinics, and/or rehabilitation centers [71]. Studies on genotypes of *P. aeruginosa* performed using

conventional pyocin typing and DNA probe analysis reported that most CF patients harbored a persistent strain of *P. aeruginosa* in their lungs [72]. These studies suggested that cross-colonization possibly could occur among patients. Another study showed that 59% of CF patients harbored a clonal strain of *P. aeruginosa* and the dominant pulsotype was indistinguishable from nonclonal strains with respect to both colony morphology and resistance patterns [73]. Wolz et al. used DNA probe amplification assays and demonstrated that 46% of CF patients (who were initially uninfected) acquired P. aeruginosa infection at the end of a CF recreation camp [74]. Clear evidence of a cross-infection among patients attending a CF clinic was published in 2001 [75]. In this study, 22 of 154 patients attending an adult CF clinic were chronically infected with similar isolates (based on pyocin typing and pulsed-field gel electrophoresis [PFGE] analysis) of *P. aeruginosa* that shared unusual phenotypic features: lack of motility and pigmentation along with a remarkable resistance to many antibiotics. In another study from a large pediatric CF clinic from Australia, 65 patients (55%) were found to be infected with a similar strain of *P. aeruginosa*. These patients were more likely to have been hospitalized in the preceding 1 year for respiratory exacerbations [76]. On the other hand, a study conducted by Speert et al. in Vancouver (Canada) reported a low rate of transmission of *P. aeruginosa* from one CF patient to the other [77]. In this study, a total of 157 genetic types of *P. aeruginosa* were identified, of which 123 were unique to individual patients. These apparently conflicting findings may be accounted for by the highly adaptable nature of *P. aeruginosa* and its ability to evolve. In a study by Mahenthiralingam et al., different strains of *P. aeruginosa* were studied using genomic fingerprinting and random DNA amplification assays [78]. A total of 385 isolates from 20 patients were grouped into 35 random amplified polymorphic DNA (RAPD) strain types. Secretion of mucoid exopolysaccharide, loss of expression of RpoN-dependent surface factors and acquisition of serum-susceptible phenotypes in Pseudomonas were shown to be a specific adaptation to infection, rather than being acquired from a new bacterial strain. This explanation is also in congruence with observations from other studies that found different strains of *P. aeruginosa* in unrelated CF patients and identical or closely related strains among siblings [79]. The presence of distinct strains of *P. aeruginosa* in these studies reflects an absence of nosocomial transmission of organisms at respective CF centers [80]. This may be a consequence of strict hygiene measures and microbiologic surveillance instituted at most CF centers across the world following reports of nosocomial spread [75, 76].

The effects of *P. aeruginosa* infection on the CF lung are deleterious. In one observational study, outcomes of CF children colonized with *P. aeruginosa* were compared with those of noncolonized patients. Children colonized with *P. aeruginosa* had a worse outcome and experienced rapid decline in pulmonary function as measured by FEV_1 and FEF_{25} (forced expiratory flow at 25% of vital capacity) [81]. In another longitudinal observational study, the temporal relationship between *P. aeruginosa* infection and pulmonary damage (as measured by FEV_1 and Wisconsin additive chest radiograph score) was explored. Acquisition of *P. aeruginosa* was independently associated with a worsening pulmonary status in children with CF [82]. Moreover, in these studies, decline in pulmonary function after colonization with *P. aeruginosa* was observed to be gradual. This decline in pulmonary function associated with *P. aeruginosa* infection is noted across all age groups. In another study, acquisition of mucoid

strains of *P. aeruginosa* was associated with an unfavorable prognosis [83]. From a pathologic perspective, *P. aeruginosa* causes repeated airway infections with eventual progression to chronic airway infection. This organism can also lead to necrotizing pneumonia, chronic bronchopneumonia, and chronic parenchymal lung disease. While the aggressive use of antipseudomonal antibiotics has been shown to delay the onset of chronic infection, prevalence rates of *P. aeruginosa* colonization have remained relatively stable over the past two decades [84, 85].

The CF airway provides a pathological milieu and a scaffold for chronic infection with resistant organisms, the most notable of them being *P. aeruginosa*. A number of virulent factors enable this resilient organism to establish it within the CF airways. One such virulence factor—overproduction of alginate slime capsule—characterizes the mucoid type of *P. aeruginosa*, which allows it to adhere firmly to the airway epithelium. Being encoded by the AlgT gene, alginate negatively regulates flagella, fimbriae, and quorum sensing. TTSS (injectosome) positively regulates alginate production indirectly through heat shock, osmotic, and oxidative stress responses [86]. In the inflamed CF airway, polymorphonuclear leukocytes (PMN) lead to the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) [87]. Moreover, mutated CF epithelial cells are unable to efflux glutathione (a potent free radical scavenger) and unable to absorb other dietary antioxidants. Production of ROS and RNI by PMN leads to DNA damage, lipid peroxidation and denaturation of proteins. At the same time, RNI and ROS lead to upregulation of alginate production by *P. aeruginosa*. The alginate slime capsule enables the bacterium to adhere firmly to the airway epithelial cells and results in persistence of this organism within the airways. At the same time, other virulence factors produced by *P. aeruginosa* (such as exotoxins) incur progressive pulmonary damage and help it to evade the (already impaired) host immune response. Over time, ROI and RNI lead to loss of microbial diversity and disruption of the airway microbiota. Simultaneously, such an environment favors the survival and selection of *P. aeruginosa* within the CF airway and leads to persistent infection with this organism [88, 89]. Moreover, antibiotic exposures select for multidrug resistant variants of the organism and allow them to predominate and colonize the airways [24, 90]. Alarmingly, recent reports from CF centers across the world have described certain strains of *P. aeruginosa* that exhibit resistance to all clinically relevant classes of antimicrobials ("pan-resistant" *P. aeruginosa*) [91]. This can explain the worse prognosis associated with this organism in most studies of CF patients.

4.5. Burkholderia cepacia complex

More than 60 species belonging to the genus Burkholderia are not pathogenic to humans, but some of the remaining species are implicated in serious infections in CF patients. Using 16S rDNA and recA gene analysis, 17 species of this genus have been grouped together as the Burkholderia cepacia complex (BCC). BCC is a group of virulent pathogens that are frequently implicated in infective exacerbations in CF patients with end-stage lung disease. Colonization with BCC in CF patients indicates a poor prognosis and has been shown to be associated with a requirement for lung transplantation. This worse prognosis is due to the inherent antibiotic resistance

possessed by these organisms and their ability to rapidly spread from patient to patient. In some cases, infection with BCC can lead to the development of cepacia syndrome—a rapid fulminating pneumonia that often leads to bacteremia and sepsis. Given their virulent nature, strict infection control measures are essential to prevent outbreaks of BCC in CF clinics and centers [92]. A report of rapid spread and outbreak of BCC infection was reported in a CF center in Toronto [93]. This center reported the development of cepacia syndrome in many patients, being characterized by rapidly deteriorating pulmonary function, fever, leukocytosis, elevated markers of inflammation, and BCC bacteremia. Furthermore, in another report, cepacia syndrome occurred in approximately 20% of infected patients and had a case fatality rate of 62% [93].

Outside of the BCC group, a few other species of the Burkholderia genus are also implicated in infective exacerbations. These species include *Burkholderia gladioli*, *Burkholderia fungorum*, *Burkholderia multivorans* and *Burkholderia pseudomallei* [94]. Of these, *B. gladioli* now accounts for a significant proportion of Burkholderia infections in CF patients [95]. In the United States, *B. multivorans* and *B. gladioli* together account for more than 50% of Burkholderia infections in CF patients.

Most infected CF patients harbor genotypically distinct strains of the BCC. Strains of Burkholderia spp. that are shared by multiple CF patients are very uncommon. This suggests that most Burkholderia infections in CF patients result from acquisition of strains from the natural environment [92, 96]. In this regard, *B. gladioli* and *B. cepacia* have been described as recognized plant pathogens. In one study, multilocus sequence typing of Burkholderia spp. revealed that more than 20% of CF isolates were identical to strains recovered from the environment [97].

In the CFF patient registry, prevalence of BCC was reported to have declined from 9% in 1985 to 4% in 2005. Incidence of BCC was also found to be reduced from 1.3% in 1995 to 0.8% in 2005 [69]. This has not changed significantly over the past decade as shown by data published in 2016 [70]. Ramette et al. analyzed 285 confirmed isolates of BCC using restriction analysis of recA and identified seven different BCC species in the environment [98]. Healthcare-associated outbreaks of BCC infections as a consequence of contaminated medical devices and products (such as mouthwashes, ultrasound gels, skin antiseptics, and medications) have been reported previously. While most of these outbreaks have generally involved non-CF patients, the potential for developing such outbreaks among CF patients remains a hazard [99]. Infection of the respiratory tract with BCC species in CF patients often results in a chronic persistent infection [100]. In most such cases, a single strain of Burkholderia spp. colonizes the respiratory tract.

Infection with BCC species has been associated with a worse prognosis. In one study, CF patients who were infected with *Burkholderia dolosa* had a rapid decline in FEV_1 over time [101]. In another study, patients colonized with *B. cenocepacia* had a worse outcome in terms of body mass index (BMI) and FEV_1 as compared to those colonized with *P. aeruginosa* or *B. multivorans* [102].

4.6. Anaerobic bacteria

Anaerobic bacteria have been described in the airways of people with healthy lungs and are generally not considered to be pathogenic. In patients with CF, anaerobic bacteria are persistent members of the lower airway community as the anaerobic conditions (and steep oxygen gradients) in the lower airways provide an ideal environment for their growth [88, 103].

However, in the CF lung, anaerobic bacteria can produce virulence factors and damage the lung parenchyma (perhaps as a consequence of impaired innate immunity), which may worsen pulmonary function and exacerbate the inflammatory response. Short-chain fatty acids produced by anaerobic bacteria can increase production of interleukin-8 (IL-8) by upregulating expression of the short-chain fatty acid receptor GPR41 [104]. Moreover, in the CF microbiome, anaerobic bacteria can interact with other established pathogens and lead to progressive pulmonary damage [105]. Previously, anaerobic bacteria were thought to be an infrequent cause of CF exacerbation; however, with the advent of novel (culture-independent) microbial detection methods [106–109], anaerobes have been isolated from more frequently. In one study, 23.8% of sputum specimens from CF patients grew more than 10^5 colony forming units (CFU) per milliliter of anaerobic bacteria [110]. In another study, 15 genera of obligate anaerobes were identified in 91% of CF patients with counts (CFU/ml) being comparable to that of *P. aeruginosa* and *S. aureus* [111]. The most common anaerobes were *Staphylococcus saccharolyticus* and *Peptostreptococcus prevotii*. Some studies suggest that patients with lower aerobic and anaerobic bacterial load have worse pulmonary function and higher levels of inflammatory markers [112]. From a biological standpoint, lower quantity of aerobes and anaerobes may reflect disruption of the CF microbiota. Studies have shown that antibiotic therapy directed against *P. aeruginosa* during acute exacerbations does not affect anaerobes [111]. This observation could be explained by considering the resistance patterns of anaerobes. In 58% of patients, obligate anaerobes detected during acute infective exacerbations were resistant to antibiotics used for treatment. The chief obligate anaerobes in such cases were *Bacteroides* spp., *Porphyromonas* spp., *Prevotella* sp., *Veillonella*, *anaerobic Streptococcus* spp., *Proprionibacterium*, *Actinomyces*, *S. saccharolyticus* and *P. prevotii* [36, 111, 113]. Interestingly, infection with *P. aeruginosa* significantly increases the likelihood of isolating anaerobic bacteria from CF patients [36]. Some of these anaerobic bacteria (such as S. milleri) are now known to be associated with worse clinical outcomes. Furthermore, new anaerobic organisms have been detected for the first time from samples of CF patients. Such bacteria, for instance Gemella and Rothia mucilaginosa, have been found to be associated with dismal pulmonary outcomes. Most such patients are often coinfected with *P. aeruginosa* as well [114, 115].

4.7. Nontuberculous mycobacteria

Traditionally, the frequency of CF patients infected with NTM has been reportedly low. In the CFF patient registry, the prevalence of NTM infections among CF patients has been estimated to be 2.2%. Nevertheless, the prevalence of NTM has been increasing slowly over the past few decades. The prevalence of NTM infection in 1999 among CF patients was 0.85%, which increased to 2.18% in 2008 [116]. More recent data published in 2016 shows that the prevalence of NTM may be as high as 11.9% [70]. The most common NTM species have been reported to be Mycobacterium avium-intracellulare (MAI) complex and *Mycobacterium abscessus*. Factors associated with a culture positive for NTM are older age, greater FEV_1, higher frequency of MSSA colonization and lower frequency of *P. aeruginosa* infection [117]. In most patients, unique strains of NTM are detected by molecular typing, which suggests that neither person-to-person transmission nor nosocomial acquisition is implicated. In one study, the prevalence of NTM infection among 385 patients in three Parisian centers was 8.1%. *M. abscessus*

was isolated in all age groups. About 4.1% (16/385) of the study cohort met the American Thoracic Society (ATS) criteria for NTM-related lung disease [118]. In another multicenter study done in Israel [119], prevalence of NTM-related lung disease (as defined by the 2007 ATS criteria) was 10.8%. This study further suggested that the incidence of NTM infections is increasing over time. Other studies have demonstrated that the incidence of MAI complex infections in CF patients is decreasing with time, while that of *M. abscessus* complex is increasing [120]. Alarmingly, infection with *M. abscessus* complex has been associated with a worse impact on pulmonary function. Some researchers have proposed that eradication of *M. abscessus* complex may provide a significant improvement in terms of pulmonary outcome [121]. However, *M. abscessus* is difficult to manage, commonly affects younger children, and requires prolonged courses of intravenous antibiotics [122].

4.8. *Stenotrophomonas maltophilia*

S. maltophilia is a Gram-negative bacillus that is commonly implicated in nosocomial infections in non-CF patients. However, in patients with CF, *S. maltophilia* has been recognized as a cause of acute infective exacerbation. The medical importance of this pathogen is that it is inherently resistant to a wide range of broad-spectrum antibiotics (most notably carbapenems). The prevalence of infection with this organism has increased from 1 to 4% over a period of 20 years (1985–2005) [68]. In the CFF patient registry, the prevalence of *S. maltophilia* increased from 4.0% in 1996 to 12.4% in 2005 [69]. From 2005 till 2015, the prevalence of *S. maltophilia* seems to have plateaued [70]. *S. maltophilia* infections of the respiratory tract in CF patients tend to be acute and, in most cases, the organism does not persist in the lower airways (although recurrent infections can occur). Most isolates of this organism have been shown to be transmitted from patient-to-patient, especially among siblings, or those who are otherwise epidemiologically linked [123]. One-third of CF patients who experience recurrent infections with *S. maltophilia* harbor more than one strain of the organism [124]. The most important risk factors for acquiring *S. maltophilia* infections are therapy with carbapenems and central venous catheterization [125]. In one study, history of treatment with imipenem was 10 times more frequent among cases (who contracted *S. maltophilia*) than among controls [125]. Furthermore, all fatal infections with *S. maltophilia* occurred in patients who had received imipenem. Based on these results, it is advisable to cover *S. maltophilia* empirically in CF patients who develop super-infection while receiving imipenem therapy. In a report by Sanyal and Mokaddas [126], most strains of *S. maltophilia* were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole. Moreover, some evidence shows that CF patients infected with *S. maltophilia* were more likely to have been hospitalized for many days in the past one year [127]. Other factors associated with *S. maltophilia* acquisition were more than two courses of intravenous antibiotics, isolation of *Aspergillus fumigatus* or *P. aeruginosa* in sputum and oral steroid use [128]. *S. maltophilia* is also more common among CF patients who develop allergic bronchopulmonary aspergillosis (ABPA) [129]. While chronic infection with *S. maltophilia* is infrequent, it can occur in certain patients and requires repeated courses of antibiotics [130]. Chronic infection with *S. maltophilia* confers a threefold higher risk of mortality or the need for lung transplantation [131].

4.9. *Achromobacter xylosoxidans*

A. xylosoxidans has been recognized as a pathogen and cause of infective exacerbation in patients with CF [132]. In the CFF patient registry, the prevalence of *A. xylosoxidans* infection was 1.9% in 1995 [69]. In 2015, the prevalence had increased almost three-folds to 6.1% [70]. *A. xylosoxidans* is a ubiquitous organism that occurs widely in natural habitats. This organism is an opportunistic pathogen that affects only immunocompromised patients and those with CF. *A. xylosoxidans* is mostly implicated in nosocomial infections, such as hospital acquired pneumonia, catheter-associated urinary tract infection, and wound infections. Lung infections with this fastidious organism are difficult to eradicate. Most patients respond to antipseudomonal penicillins (such as piperacillin–tazobactam) and third- or fourth-generation cephalosporins [133]. In one report, two cases of *Achromobacter ruhlandii* developed after indirect contact between CF patients [134]. Another study from a French CF center reported that most isolates of Achromobacter spp. were resistant to fluoroquinolones and carbapenems [135]. In a retrospective study, CF patients who were chronically infected with *A. xylosoxidans* were more likely to have impaired pulmonary function. Additionally, the frequency of hospitalization was higher among such patients than others [136].

5. Implications for further research

Cystic fibrosis is a monogenetic multisystem disorder, but, pulmonary disease is the leading cause of morbidity and mortality. Recurrent pulmonary infections with pathogenic bacteria can lead to progressive pulmonary damage and eventually lead to death. Therefore, understanding the CF airway microbiome has immense importance for understanding the overall pathology of the disease. Disruption of the CF airway microbiome under the influence of environmental factors and antibiotic exposures is a crucial step in the development of end-stage pulmonary disease in such patients [40]. Colonization of the lower airways with pathogenic bacteria, such as *P. aeruginosa* [82] and *B. cenocepacia* [101], has been associated with end-stage pulmonary disease.

As the CF airway microbiome evolves under the influence of antibiotic exposures, microbes undergo a number of mutations and changes in their genome [137]. While these genetic mutations are an evolutionary mechanism for microorganisms (for instance, to acquire resistance to antibiotics), they create potential vulnerabilities that may be exploited in unique therapeutic approaches. Traditionally, the approach to management of CF pulmonary exacerbations has been through employment of antibiotics. While antibiotics are useful in the short run, multidrug resistant microbes eventually evolve and become a challenge to tackle. In view of this, novel approaches to the management of CF pulmonary disease have been proposed, which involve manipulating patients' microbial consortia [8]. From a theoretical perspective, such an approach aims to maintain the architecture of the CF airway microbiome and avoids the use of antimicrobials, thereby circumventing the problem of destroying the community structure of a patient's microbiome. Such a novel treatment approach is based on

the principles of personalized medicine and aims to tailor treatment according to each patient's individual microbiome [138]. By manipulating and restoring the structure of a patient's airway microbiome, the complex metabolomic profile of the patient's sputum (and other body fluids) can be altered, which may have long-lasting and pleiotropic consequences [139].

Novel treatment approaches for the treatment of CF patients hold theoretical promise, but their practical applicability and clinical efficacy remains to be established [140]. A recent pilot study compared the use of a probiotic (*Lactobacillus* spp.) versus placebo in pediatric CF patients. Patients receiving the probiotic demonstrated a significant reduction in hospitalization for pulmonary exacerbation and a beneficial effect on the gut in terms of reducing gastrointestinal inflammation [141]. Another clinical trial examined the efficacy of enteric probiotics in reducing the frequency and severity of pulmonary exacerbations in CF patients. Both studies reported that the use of enteric probiotics provided a significant reduction in the frequency of pulmonary exacerbations when compared to the placebo group [142]. Larger randomized controlled studies are needed to more fully evaluate the effect of probiotics on hard clinical endpoints [143]. Other treatment options based on these novel concepts need to be developed further, and they may help to improve the overall outcomes of patients with CF [144].

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BCC	Burkholderia cepacia complex
CD	Clostridium difficile
CF	Cystic fibrosis
CFF	Cystic Fibrosis Foundation
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
FAFLP	Fluorescent amplified fragment length polymorphism
FEF_{25}	Forced expiratory flow at 25% of vital capacity
FEV_1	Forced expiratory volume in first second
FMT	Fecal microbiota transplantation
HQNO	4-Hydroxy-2-heptylquinoline-N-oxide
IL-8	Interleukin-8
MAI	Mycobacterium avium-intracellulare
MRSA	Methicillin-resistant Staphylococcus aureus
MSSA	Methicillin-sensitive Staphylococcus aureus

NTM	Non-tuberculous mycobacteria
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PBP-2A	Penicillin binding protein-2A
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocyte
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
VISA	Vancomycin-intermediate Staphylococcus aureus

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Chapter 4

Burkholderia cepacia Complex Infections Among Cystic Fibrosis Patients: Perspectives and Challenges

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Additional information is available at the end of the chapter

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Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacterial species that emerged in the 1980s as the etiological agents of severe and often lethal respiratory infections among cystic fibrosis (CF) patients. After several outbreaks in CF centers in Europe and North America, segregation measures were introduced to avoid patient-to-patient transmission. Presently, the prevalence of Bcc infections among CF patients worldwide is below 5% in the majority of CF centers, although exceptions are registered in some European countries. Infections by these pathogens remain problematic due to the high resistance to antimicrobials, the easy patient-to-patient transmission, and the unpredictable outcome of infections that range from asymptomatic carriage to the cepacia syndrome, a fulminating pneumonia often associated with septicemia that can lead to the decease of patients within a period of time as short as 1 week. In this chapter, we review the evolving epidemiology of Bcc infections in CF patients, the virulence traits and mechanisms used by these bacteria, and the recent developments in vaccine and vaccine components research to prevent Bcc infections.

Keywords: *Burkholderia cepacia* complex, emerging species, evolving epidemiology, virulence determinants, immunoreactive proteins, vaccine development

1. Introduction

The *Burkholderia cepacia* complex (hereafter referred to as Bcc) is a group of closely related bacteria that emerged in the 1980s as problematic pathogens to cystic fibrosis (CF) patients [1]. Infections by Bcc are particularly feared due to (1) the easy patient-to-patient transmission of

specific strains; (2) the ability to resist to multiple antibiotics; and (3) the unpredictable outcome of infections, which ranges from asymptomatic carriage to the so-called cepacia syndrome, an often lethal necrotizing pneumonia accompanied with septicemia [1, 2]. Initially described in the 1950s by Burkholder [3] as the cause of soft rot in onions, the species then named *Pseudomonas cepacia* was moved into the new genus *Burkholderia* after the work of Yabuuchi and colleagues in 1992 [4]. However, the most impressive developments on the taxonomy of this group of bacteria have been achieved after the seminal work of Vandamme and colleagues who proposed the division of the species into distinct genomovars [5]. Presently, the Bcc comprises 20 species (**Table 1**), and the genome sequence of several strains is publicly available in databases such as the Burkholderia Genome DB and the Integrated Microbial Genomes & Microbiomes [6, 7].

Bcc species	Genome sequence availability	Reference
B. ambifaria	4 complete genomes (strains AMMD, MC40-6, MEX-5, IOP-120)	[8]
B. anthina	In progress	[9]
B. arboris	In progress	[10]
B. cenocepacia	18 complete genomes (strains J2315, H111, AU1054, B1, MCO-3, PC184, HI2424, DDS 22E-1, DWS 37E-2, ST32, 842, 895, MSMB384 WGS, 6, 7, CEIB, 869T2, TAtl-371)	[11]
B. cepacia	8 complete genomes (strains 383, AMMD, ATCC 25416; Bu72, DDS 7H-2, GG4, JBK9, LO6)	[4]
B. contaminans	1 complete genome (strain MS14)	[12]
B. diffusa	In progress	[10]
B. dolosa	1 complete genome (strain AU0158)	[13]
B. lata	1 complete genome (strain 383)	[12]
B. latens	In progress	[10]
B. metallica	No information	[10]
B. multivorans	3 complete genomes (ATCC17616, ATCC BAA-247, DDS 15A-1)	[5]
B. pseudomultivorans	In progress	[14]
B. pyrrocinia	1 complete genome (strain DSM 10685)	[9]
B. seminalis	In progress	[10]
B. stabilis	No information	[15]
B. stagnalis	In progress	[16]
B. territorii	In progress	[16]
B. ubonensis	1 complete genome (strain MSMB22)	[17]
B. vietnamiensis	3 complete genomes (strains G4, LMG10929, WPB)	[18]

Databases were assessed by the end of July 2016.

Table 1. *Burkholderia cepacia* complex species names and genome sequence availability in the databases Burkholderia Genome DB and Integrated Microbial Genomes & Microbiomes [6, 7].

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2. Evolving epidemiology of Bcc infections

All Bcc species are virtually potential pathogens to CF patients. However, epidemiology studies have shown an uneven geographical and regional distribution of clinical isolates among the Bcc species, with the predominance of *Burkholderia cenocepacia*, followed by *Burkholderia multivorans*. Early studies performed during the 1980s and 1990s have shown that in addition to cases of chronic infection due to specific strains, many outbreaks reported in Europe and North America were due to the spread of particularly virulent strains that easily disseminated within a given CF center [1]. Although the environment is thought to be the natural reservoir of these strains, a definitive proof is still lacking.

A few particularly epidemic strains became notorious for the worst reasons. Perhaps, the best-known strain is the Edinburgh-Toronto lineage also known as the ET12 clone, an intercontinental clone responsible for several infections and fatalities in CF centers in the UK and Canada [19]. The best-known representative strain of this highly transmissible clone is the *B. cenocepacia* J2315 strain, the first Bcc strain with its genome sequence publicly available (**Table 1**) and one of the best studied Bcc strains [20]. Another example of a strain that disseminated within centers and even among centers is the PHDC strain. The strain, responsible for almost 20% prevalence in one CF center in the USA, was later found in another CF center, where an increase in Bcc prevalence was experienced. The dissemination of the strain was associated with the transfer of an infected patient from the initial center to the second one [21]. A later study by Coenye et al. [22] showed that the PHDC strain was also present in European patients (namely in France, Italy, and the UK), concluding that the PHDC strain was the second-identified Bcc transatlantic clone. Interestingly, both intercontinental clones belong to the *B. cenocepacia* species, although the ET12 belongs to subgroup IIIA and the PHDC belongs to subgroup IIIB. The *B. cenocepacia* species includes other clones that spread among CF centers, namely the Midwest American clone and the CZI Czech epidemic clone [23, 24]. Evidence of transmission of particularly epidemic strains of *B. cenocepacia* led to the introduction of segregation measures in CF centers in Europe and America, with a significant reduction of prevalence of infections [1, 25–27]. However, these segregation policies had a devastating impact on patients infected with Bcc due to social isolation and stigma and negative psychological impacts [28]. Although effective in interrupting strain transmission, segregation measures do not prevent new acquisitions. Nevertheless, these measures led to a reduction of prevalence of Bcc infections from more than 20% in several centers to less than 5% both in the USA and the majority of European countries [29, 30]. However, prevalence of chronic Bcc infections is still ranging 5–10% in Denmark, Portugal, Slovak Republic, Russian Federation, and Latvia, reaching values of 15 and 23% in Serbia and Lithuania, respectively [30].

Although the Bcc strains responsible for the vast majority of infections both in Europe and North America belong to the *B. cenocepacia* species, recent evidence indicates a changing epidemiology. *B. multivorans* emerged as the dominant species in France by 2004 and as the second most important species in the USA [31, 32]. Recent reports also indicate *Burkholderia contaminans* as an emerging Bcc species associated with CF infections. Early reports of a high incidence of the species among CF patients came from Portugal and Argentina [33–35].

Interestingly, in the case of the Portuguese CF population, two *B. contaminans* clones infecting CF patients were found as indistinguishable from two *B. contaminans* strains isolated from nonsterile nasal saline solutions of commercial origin during routine surveillance by the Portuguese Medicines and Health Products Authority [36]. A recent work by Medina-Pascual and colleagues on the surveillance of Bcc infections in Spanish CF patients also reported a *B. contaminans* overall incidence of 36.5% in the period 2008–2012, surpassing the previously dominant species *B. cenocepacia* and *B. multivorans* [37]. The emergence of *B. contaminans* among Spanish CF patients was hypothesized to be due to unspecified ecological advantages that enable the species to increase its presence in hospitals or in the environment [37]. In the case of Swiss CF-patients, *B. cenocepacia* was the most frequently isolated species in the period 1998–2013, but *B. multivorans* and *B. contaminans* emerged during the last years of the study period [38]. A 30-year study of Bcc infections among CF patients from British Columbia (Canada) evidenced a major impact of segregation measures in Bcc epidemiology; while *B. cenocepacia* was dominant before the introduction of these measures, *B. multivorans* strains became dominant after implementation of novel infection control measures in 1995 [39]. This study and others highlight the impact of infection control measures on Bcc species recovered from CF patients. It is now apparent that while epidemic *B. cenocepacia* strains dominated in early years, nonclonal *B. multivorans* and *B. contaminans* strains are emerging.

3. Bcc virulence factors and traits

Over the last 20 years, substantial progress has been achieved on the knowledge of Bcc virulence factors and determinants, although the exact contribution of some of them to the success of infection remains to be fully understood. It is currently accepted that Bcc virulence does not rely on a single virulence factor, being multifactorial. Bacterial structures such as flagella, the cable pili, and the 22-kDa adhesin are considered virulence factors since they play important roles in the initial steps of interaction with the host cell, promoting the adherence to the lung surface and the invasion of lung epithelial cells [39–41]. In addition, the majority of *B. cenocepacia* strains are able to survive and replicate intracellularly in airway epithelial cells and macrophages, evading the primary cellular defense mechanisms of the lung and avoiding clearance. The factors involved in this ability, exopolysaccharide (EPS) biosynthesis, biofilm formation, resistance to antibiotics, and oxidative stress resistance, as well as the iron acquisition ability are also among virulence determinants described for Bcc [20, 42, 43]. Some of these virulence factors are further detailed below.

3.1. Alternative sigma factors

RpoE and RpoN are two alternative sigma factors involved in the regulation of the ability of intracellular *B. cenocepacia* to delay phagolysosomal fusion in murine macrophages [44, 45]. RpoE is the extra-cytoplasmic stress response regulator required by *B. cenocepacia* to grow under conditions of high osmolarity and high temperature [44]. RpoN, or sigma factor σ^{54}, is best known for its involvement in nitrogen-related gene regulation. In *B. cenocepacia,* σ^{54} is involved

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in motility and biofilm formation [45]. Results from the mapping of σ^{54} regulon and the characterization of a *B. cenocepacia* H111-derived σ^{54} mutant suggest that this alternative sigma factor plays an important role in the control of nitrogen metabolism, in the metabolic adaptation of *B. cenocepacia* H111 to stressful and nutrient-limited environments and in virulence toward the nematode *Caenorhabditis elegans* [46]. In addition, it was also reported that RpoN regulates genes involved in exopolysaccharide production, biofilm formation, motility, and virulence [46]. A *B. cenocepacia* mutant defective in a gene encoding a putative σ^{54}-related transcription regulator (BCAL1536) was found as attenuated in the rat agar bead infection model [47].

3.2. Lipopolysaccharides and extracellular polysaccharides

One of the central components of the outer membrane in Gram-negative bacteria is the lipopolysaccharide (LPS), a complex molecule composed by the lipid A, the core oligosaccharide, and the O-antigen moieties (reviewed in Ref. [48]). The genes involved in LPS production by *B. cenocepacia* are located in chromosome I, organized in three main clusters, one for each LPS component (lipid A: *BCAL1929* to *BCAL1935*; core: *BCAL2402* to *BCAL2408*; O antigen: *BCAL3110* to *BCAL3125*) together with additional genes encoding sugar modification enzymes [49, 50]. Bcc bacteria LPS differs from other Gram-negative bacteria LPS due to the complete lack of negatively charged residues and the presence of the heterodimeric disaccharide D-glycero-D-talo-oct-2-ulosonic acid-(2–4)-3-deoxy-D-manno-oct-2-ulosonic acid (Ko-(2–4)-Kdo) in the core region; the presence of a 4-amino-4-deoxyarabinose (Ara4N) residue, either in the core or in lipid A; and the structure of O-antigen [50, 51]. This particular composition changes the bacterial surface charge, inhibiting the binding and successful action of antibiotics, contributing to the persistence of bacterial infection [51]. Recently, it was demonstrated that although L-Ara4N modifications do not affect recognition, they are critical for the establishment of infection [52]. Several studies have demonstrated that when neutrophils interact with Bcc LPS, the expression of CD11b on their surface increases, stimulating neutrophil respiratory burst response [53]. In addition, macrophages and human blood cells are also stimulated by Bcc LPS, producing pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 [54, 55].

B. cenocepacia J2315 is unable to produce the O-antigen. In this particular strain, this is due to an interruption in the *wbcE* gene-encoding BCAL 3125 [56]. The expression of O-antigen by Bcc strains was demonstrated to reduce phagocytosis by macrophages without interfering with the intracellular survival of bacteria [56].

The production of exopolysaccharides (EPSs) was described for several *Burkholderia* species. EPS production by Bcc is regarded as playing an important role in the chronicity of Bcc infections [57–62]. Cepacian is the most common EPS produced by Bcc and non-Bcc species, both from clinical and environmental sources [59, 63]. Cepacian interferes with phagocytosis by human neutrophils, facilitating the bacterial persistence in a mouse model of infection [64, 65]. The EPS was shown to inhibit the production of ROS by neutrophils and to scavenge reactive oxygen species (ROS), playing a role in the survival of cepacian-producing strains in different environments [64–67]. As a result of a frameshift mutation in the *bceB* gene (*BCAM0856*) encoding a putative

glycosyltransferase, Cepacian is not produced by the *B. cenocepacia* ET12 representative strain J2315 [49, 62].

3.3. Biofilms

Bcc bacteria were found to persist in biofilms *in vitro*. Biofilm formation and maturation depend on many factors, including EPS production, motility, iron availability, and multiple gene regulatory systems, such as quorum sensing, alternative sigma factors, or global regulators such as the ShvR and AtsR [45, 58, 68–73]. In addition, Bcc can form small colony variants *in vitro*, a colony morphology that is associated with enhanced biofilm formation, antibiotic resistance, and persistence [74].

Several studies have been performed to understand the importance and relevance of biofilm formation in Bcc biology. Bcc bacteria growing in biofilms are usually more tolerant to multiple antibiotics, although similar susceptibilities were reported for plancktonic and biofilm cells to the antibiotics kanamycin, amikacin, and ciprofloxacin [75, 76]. Recently, Bcc biofilms were shown to contain persister cells that are able to survive in the presence of high concentrations of antibiotics by avoiding production of reactive oxygen species [77]. In addition, using neutrophil-like dHL60 cells, it was shown that the presence of these immune system cells enhanced biofilm formation that protected Bcc bacteria against neutrophils by inducing their necrosis, acting as a barrier to the migration of neutrophils, and masking the bacteria from being recognized by neutrophils [78]. Although some evidence suggests that biofilm formation plays a role in bacterial persistence in the CF airways, this topic needs to be further studied.

3.4. Quorum sensing

Quorum sensing is a mode of regulation of gene expression that is dependent on the density of the bacterial population. Bcc bacteria have at least four quorum sensing systems. The CepIR quorum sensing system is homologous to the LuxIR system of *Vibrio fischeri* (reviewed in Ref. [79]). The CepIR system positively regulates the virulence of *B. cenocepacia* toward model organisms like *C. elegans*, *Galleria mellonella*, rodents, zebrafish, alfalfa, and onions [80–83]. In addition to the CepIR, *B. cenocepacia* encodes the CciIR, the CepR2, and the BDSF quorum sensing systems [84, 85]. While the CepIR and CciR quorum sensing systems rely on acyl homoserine lactones as signaling molecules, the BDSF system uses cis-2-dodecenoic acid as the signaling molecule, and the CepR2 is an orphan quorum sensing system [85]. An arsenal of genes regulated by quorum sensing in Bcc bacteria was described, including the negatively regulated siderophore synthesis and the positively regulated expression of the genes encoding zinc metalloproteases (Zmps), swarming motility and biofilm formation, all thought to have an impact when the bacterium is infecting the CF patient [71, 80, 86, 87].

3.5. Protein secretion systems

Both Gram-negative and positive bacteria use protein secretion systems to secrete toxins or other proteins, either directly into the environment or into host cells. These systems are

particularly well studied in the CF pathogens Bcc and *Pseudomonas aeruginosa*. For instance, Bcc strains of the ET12 lineage and *Burkholderia vietnamiensis* harbor type I and II secretion systems (T1SS, T2SS) implicated, for instance, in the secretion of hemolytic proteins [88, 89]. The T2SS is also involved in *B. cenocepacia* secretion of two zinc metalloproteases, ZmpA and ZmpB, which play a role in virulence [80, 90]. Two T4SSs are encoded by *B. cenocepacia*; the T4SS-1 encoded in a plasmid, and the T4SS-2 encoded in chromosome 2 [91]. Until now, only the T4SS-1 was identified in *B. cenocepacia* strains as necessary for virulence in onions and intracellular survival in phagocytes [92].

In a mouse agar bead infection model, the T3SS has been shown to be important for bacterial pathogenesis [93]. Although the precise mechanism is still not clear, T3SS seems to play no role in intracellular survival of *B. cenocepacia* [94].

Four type V secretion systems are encoded within the genome of *B. cenocepacia* J2315 [49]. Proteins transported by this type of transporters contain pertactin and hemagglutinin domains and are thought to play a role in bacterial adhesion [49].

B. cenocepacia also encodes a T6SS, which was shown to affect the actin cytoskeleton of macrophages and the assembly of the reduced nicotinamide adenine dinucelotide phosphate (NADPH) oxidase complex in *B. cepacia*-containing vacuoles (BcCV's) by inactivation of Rac1 and Cdc42 [73, 95, 96]. *B. cenocepacia* was found to efficiently activate the inflammasome by a yet uncharacterized T6SS effector [97]. Consequently, monocytes and THP-1 cells release IL-1β in a pyrin-, Asc-, and T6SS-dependent manner [97]. The T6SS also enhances caspase-1 activation, negatively regulated by the sensor kinase-response regulator AtsR [73]. In addition, a recent paper suggests that the T6SS might be important for the secretion of T2SS effectors into the host cytoplasm, such as ZmpA and ZmpB, revealing an unanticipated role for type II secretion systems in intracellular survival and replication of *B. cenocepacia* [96]. Although membrane vesicles cannot be considered a canonical secretion system, they can effectively allow the secretion of several hydrolytic enzymes and toxins [98]. **Table 2** summarizes and compares the most relevant information available about secretion systems of Bcc bacteria and their counterparts in the major CF pathogen *P. aeruginosa*.

3.6. Iron uptake

In order to carry out iron chelation and uptake, members of the Bcc can produce up to four distinct siderophores: ornibactin, pyochelin, cepabactin, and cepaciachelin [122]. Ornibactin appears to be the most important and abundant siderophore produced by *B. cenocepacia* strains [123, 124]. The pathways and regulatory mechanisms of ornibactin synthesis and uptake are relatively well known [87, 125–127]. The requirement of this siderophore for *B. cenocepacia* virulence was demonstrated in different infection models, including the rat agar bead, *G. mellonella,* and *C. elegans* [82, 125, 127].

The competition for available iron by Bcc bacteria and other CF lung colonizing organisms such as *P. aeruginosa* was reported to occur in the CF lung, although it is not completely clear how Bcc organisms acquire iron from host proteins [128, 129].

Secretion system	*Burkholderia cepacia* complex	*P. aeruginosa*
T1SS	Hemolytic proteins [88, 89]	HasAp (heme-binding) [99]; AprA and AprX (alkaline proteases) [100, 101]
T2SS	ZmpA and ZmpB [80, 90]	LasB (Major extracellular protease) [102], Staphylolysin LasA [102], Aminopeptidase PaAP [103], Protease IV [104], Lipases LipA, LipC, phospholipase C, PlcH, and PlcN [105, 106], CbpD Chitin-binding protein CbpD [107]; Exotoxin A [108]
T3SS	No effector described yet, plays a role in evasion of the host immune system [93, 94]	GTPase-activator ExoS and ADP-ribosyltransferase ExoT [109], adenylate cyclase ExoY [110], phospholipase A2 ExoU and ExoS [111]
T4SS	T4SS-1: Plant cytotoxic proteins, T4SS-2: Plasmid mobilization [91]	Integrative and conjugative elements (ICEs): ICE*clc* [112], Pathogenicity islands: pKLC102 (includes the type IV sex pili-encoding pil cluster and the *chvB* gene encoding a virulence factor) [113], and PAP-I (includes several virulence factors, such as CupD type fimbriae, and the PvrSR/RcsCB regulatory system) [114]
T5SS	Four T5SS: two containing pertactin domains involved in adhesion, other two contain haemagglutinin repeats [49]	Autotransporter: EstA (esterase activity) [115]; Two-partner secretion systems LepA/LepB [116] and CupB [117], and the PdtA/PdtB system [118]
T6SS	Hcp and VgrGs [73, 95, 96]	Hcp and VgrGs [119, 120]
Membrane vesicles (MV)	MV-associated (metallo)proteases, (phospho)lipases, peptidoglycan-degrading enzymes [98]	Multiple virulence factors: Alkaline phosphatase, hemolytic phospholipase C; the Cif toxin that inhibits CFTR-mediated chloride secretion in the airways [121]

Table 2. Summary of secretion systems from Bcc and the respective counterparts from the CF major pathogen *P. aeruginosa*.

3.7. Resistance to antimicrobials

Difficulties in eradicating Bcc infections mainly result from their intrinsic resistance to multiple antibiotics, including polymyxins, aminoglycosides, and most β-lactams. In addition, these bacteria have the ability to develop *in vivo* resistance to virtually all classes of antibiotics [20, 130, 131]. Antibiotics administration to CF patients was also reported to affect resistance profiles of Bcc bacteria [132]. Various mechanisms involved in the resistance of Bcc to multiple antibiotics have been described and include enzymatic inactivation (β-lactamases, aminoglycoside-inactivating enzymes, dihydrofolate reductase), alteration of drug targets, integrons, cell wall impermeability, and active efflux pumps [88, 133–140]. However, major contributions to intrinsic and acquired multidrug resistance by Bcc seem to be due to efflux pumps of the resistance nodulation cell division (RND) family. In fact, the *B. cenocepacia* J2315 genome encodes at least 16 efflux systems of the RND family [141]. At least six of these RND efflux pumps were implicated in drug resistance—RND-1, RND-3, RND-4, RND-8, RND-9, and RND-10 [138–140, 142,

http://dx.doi.org/10.5772/67712

143]. RND-3 and RND-4 efflux pumps were described as being involved in the resistance to various antimicrobial drugs including tobramycin and ciprofloxacin; the RND-3, RND-8, and RND-9 efflux systems protect biofilm-grown cells against tobramycin; the RND-8 and RND-9 efflux pumps are not involved in ciprofloxacin resistance; and RND-10 efflux pump seems to confer resistance to chloramphenicol, fluoroquinolones, and trimethoprim [140, 143]. It was suggested that mutations in the RND-3 regulator-encoding gene may be responsible for the prevalent overexpression of this efflux pump in clinical Bcc isolates, contributing to their high levels of antibiotics resistance [144].

3.8. Motility

Genes involved in the synthesis and assembly of *B. cenocepacia* flagella are located in chromosome I, distributed within five clusters, with two additional genes found on chromosomes 2 and 3 [49]. These genes were found as being upregulated when the organism was incubated in CF sputum, contributing to its virulence in a murine agar bead infection model [145, 146]. More recently, flagellin expression and flagellar morphology of *B. cenocepacia* grown in a medium mimicking the CF sputum was analyzed [147]. Those nutritional conditions led to increased motility and flagellin expression, by inducing the synthesis of multiple flagella on the cell surface of *B. cenocepacia* K56-2 [147]. A link between the loss of bacterial motility and the development of the cepacia syndrome was recently established based on a transcriptomics analysis comparing the *B. cenocepacia* ST32 CF isolates recovered from bloodstream, at the time of cepacia syndrome, with their sputum counterparts, recovered prior to the development of this syndrome, revealing that flagellar genes were downregulated in isolates recovered from the bloodstream [148].

3.9. Intracellular survival

Infection assays using free-living amoeba demonstrated that *B. cenocepacia* can survive in an acidified intracellular compartment [94, 149]. These bacteria were also demonstrated to have the ability to delay the maturation of phagolysosomes in murine macrophages [94–96, 150]. Although the *B. cenocepacia* containing vacuoles (BcCVs) progress normally to the early phagosomal stage, the fusion of the BcCV's with late endosomes and subsequent maturation is significantly delayed comparing with vacuoles containing heat-killed bacteria [94]. In contrast to heat-killed bacteria that ended up in phagolysosomes with a pH of 4.5, BcCVs did not acidify normally maintaining a luminal pH around 6.4 [94]. This ability of *B. cenocepacia* to alter the acidification of the vacuole seems to be correlated with the delay in recruitment or assembly on the BcCV membrane of both the 16-kDa subunit of the phagosomal vacuolar ATPase (vATPase) and the NADPH phagocyte oxidase [96, 151]. In contrast, Al-Khodor and colleagues demonstrated that *B. cenocepacia* J2315 only transiently interacts with the endocytic pathway, event after which the bacterium is able to rapidly escape to the cytosol [152]. Escaped bacteria are afterward targeted by the host autophagy pathway, through the recruitment to the bacterial vicinity of the ubiquitin conjugation system, the autophagy adaptors p62 and NDP52, and the autophagosome membrane-associated protein LC3B. However, apparently, this host cell control through autophagy ultimately fails in a high proportion of infected cells,

being *B. cenocepacia* able to block the autophagosome completion and replicate in the cytosol of the host cell [152].

To better understand the intracellular behavior of *B. cenocepacia* in CF infected patients, studies have also been performed in Cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages. Remarkably, the delayed maturation arresting of BcCV's is more exaggerated in CFTR-defective macrophages than in normal macrophages and is specific to live *B. cenocepacia* [153]. Although it is not clear how the CFTR defect enhances the *B. cenocepacia* intracellular survival, there is evidence of a link between the defective CFTR with autophagy deficiency and decreased clearance of protein aggregates and inflammation [154]. The elucidation of these survival details, especially the ability of *B. cenocepacia* to synergize with the CFTR defect and its consequences on the mechanism of autophagy will provide new avenues to explore novel therapeutic approaches for CF patients [155].

4. Toward a vaccine to prevent Bcc infections

No objective guidelines for eradication strategies are available for Bcc infections, as these pathogens are intrinsically resistant to the majority of the clinical available antimicrobials [156]. Currently, no immunotherapeutic strategy to protect CF patients from Bcc infections is available. Several studies on the immune response elicited by Bcc species in CF patients have been performed; however, they are challenging due to the ability of this bacteria to modulate and overcome the host immune responses and the ability to survive intracellularly in phagocytes and epithelial cells [157, 158].

An important aspect to consider during vaccine design is the optimal balance of Th1 and Th2 responses required for effective pathogen clearance. For example, a Th1 bias elicits a cell-mediated response, while Th2 induces a humoral immune response [159]. In the case of CF, their immune phenotype appears to be skewed toward Th2 responses [160]. In the case of Bcc, the type of host response necessary to clear the pathogen is still not fully understood, making it difficult to develop a protective vaccine (**Table 3**). Recently, BALB/c mice immunized intraperitoneally with the proteins Linocin and OmpW showed a significant reduction of *B. cenocepacia* and *B. multivorans* cells in the lung and lower dissemination of bacteria to the spleen [161]. While Linocin led to a robust Th1 response, the OmpW led to a mixed Th1/Th2 response [161]. The protection achieved with these proteins was greater against *B. cenocepacia* infection, and OmpW immunization was more efficient in reducing the lung bacterial load [161].

Nonpurified outer membrane proteins (OMP) from *B. multivorans*, supplemented with the mucosal adjuvant adamantylamide dipeptide (AdDP) that promotes a robust Th2 response, were tested for immunization of BALB/c mice [162]. A statistically significant increase in IgG and in mucosal IgA OMP-specific antibodies was observed, together with a reduction of *B. multivorans* burden and lung pathology, but only a moderate cross protection to *B. cenocepacia* was reported. The specificity of the immune response was found to be against

90, 72, 66, and 60 kDa proteins. Elicitation of specific IgA antibodies by mucosal immunization was also reported to be important to prevent the colonization of the respiratory tract by Bcc bacteria. In another study, the intranasal immunization of CD-1 mice with outer membrane proteins (OMP) from *B. cenocepacia* was described to originate a Th2-biased response with the maintenance of the bacterial burden, while mice immunized with OMP and the noninflammatory mucosal adjuvant nanoemulsion (NE) elicited a Th1/Th2-balanced response that led to a significant reduction of the *B. cenocepacia* cell burden [163]. The serum derived from mice vaccinated with OMP-NE could also inhibit *B. multivorans* growth by 80.1%, showing that induction of cross-reactive antibodies occurred after mice immunization. Additionally, a highly conserved 17-kDa OmpA-like protein was recently identified as a new immunedominant epitope in mucosal immunization [163].

Metalloproteases are also considered as potential effective candidates for vaccine development [90]. It was demonstrated that immunizations of rats using a conserved zinc metalloprotease peptide 15 (PSCP) decreased the severity of *B. cenocepacia* infection and the lung damage was reduced by 50% upon challenge with a *B. cenocepacia* strain after immunization [90].

In 2012, it was shown that the bacterial surface polysaccharide poly-β-(1-6)-N-acetyl-glucosamine (PNAG) confers protective immunity against Bcc infection in a lethal peritonitis mice model [164]. In this study by Skurnik and colleagues using opsophagocytic assays, it was observed that goat-raised antibodies against PNAG could kill Bcc strains (>80%) of the *B. ceno-*

Antigen	Immune response	Bcc animal model	*In vitro* models	References
OmpW	Mixed Th1/Th2	BALB/c mice immunosuppressed with cyclophosphamide	Spleen cells from mice	[161]
Linocin	Th1	BALB/c mice immunosuppressed with cyclophosphamide	Spleen cells from mice	[162]
OMP plus NE	Mixed Th1/Th2	CD-1 mice	Murine splenocytes	[163]
OMP plus AdDP	Higher IgG and IgA titers	BALB/c mice immunosuppressed with cyclophosphamide	ND	[162]
PNAG	ND	FVB/N mice	Opsonophagocytic assay	[164]
Zinc metalloprotease peptide 15 (PSCP)	Higher IgG and IgA titers	Sprague-Dawley rat agar bead model	ND	[90]
FliC	ND	ND	T cell hybridoma assays	[165]
BCAL2958	High IgG titers in human CF serum samples	ND	Human neutrophils	[166]

ND—Not determined.

Table 3. Summary of vaccine development against Bcc infections.

cepacia, *Burkholderia dolosa* and *B. multivorans* species. Furthermore, bacterial killing was found to depend of the presence of the complement [164].

Other proteins of putative immunogenic activity have been reported as potential vaccine candidates. However, studies in a Bcc infection animal model are still lacking (**Table 3**). One of these promising antigens is the OmpA-like BCAL2958 protein that was shown to be highly conserved in Bcc, to elicit IgG antibodies in CF patients and to elicit an increase of TNFα, elastase, NO, and MPO in neutrophils [166].

Musson and colleagues have shown that T-cell hybridomas against the *Burkholderia pseudomallei* flagellar protein FliC epitope cross-reacted with orthologous FliC sequences from *B. multivorans* and *B. cenocepacia* [165]. FliC epitopes were accessible for processing and presentation from live or heat-killed *B. cenocepacia* bacteria, demonstrating that flagellin enters the HLA class II Ag presentation pathway during infection of macrophages with *B. cenocepacia*.

Studies referred above revealed that subunit vaccines that only produce an antibody response cannot fully prevent an infection caused by Bcc bacteria [157, 161, 164]. Therefore, Bcc vaccines containing multiple antigens that elicit a balanced Th1 and Th2 response are expected to be effective in preventing Bcc infections. With this aim, immunoproteomics approaches have been performed. For instance, Mariappan and colleagues identified 18 immunogenic proteins from culture supernatants of *B. cepacia* that reacted with mice antibodies raised against inactivated *B. cepacia* whole cells [167]. More recently, the analysis of the imunoproteome of two clinical relevant strains of *B. cenocepacia* and *B. multivorans* revealed 15 common immunoreactive proteins that reacted with CF human serum samples [168].

5. Concluding remarks

An overview of Bcc infections in CF from early 1980s until the more recent available data was presented. The prevalence of Bcc species in CF patients worldwide is still evolving, most probably as a result of infection control measures and segregation policies. Many virulence factors have been identified, and the resulting wealth of information prompted the establishment of new research lines envisaging the development of novel protective strategies and products, namely vaccines and vaccine components.

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Chapter 5

Pseudomonas aeruginosa Extracellular Secreted Molecules Have a Dominant Role in Biofilm Development and Bacterial Virulence in Cystic Fibrosis Lung Infections

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Additional information is available at the end of the chapter

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Abstract

Cystic fibrosis (CF) is a genetic disorder that predominantly affects Caucasian populations. *Pseudomonas aeruginosa* is the most important Gram-negative pathogen that persists in CF patients' lungs. By evading host defence mechanisms and persisting, it is ultimately responsible for the morbidity and mortality of about 80% of CF patients worldwide. *P. aeruginosa* is also responsible for infections in burns, wounds, eyes, nosocomial patients and HIV patients. Prevalence and progression of infection by *P. aeruginosa* in the host is dependent on secretion of numerous extracellular molecules such as polysaccharides, proteases, eDNA, pyocyanin and pyoverdine. These molecules have multiple roles in facilitating *P. aeruginosa* colonisation and virulence. Pyocyanin is one of the major factors dictating progression of infection and biofilm formation. Pyocyanin is a potent virulence factor causing host cell death in CF patients. In this chapter, we have outlined the roles of various extracellular molecules secreted by *P. aeruginosa* and specifically focused on the role of pyocyanin in inducing eDNA production, binding to eDNA via intercalation and facilitating biofilm promoting factors, whilst inducing oxidative stress to host cells via production of reactive oxygen species. In line with this, we have described the current challenges in treatment of CF infections and the development of new strategies to control *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, polysaccharides, protease, pyoverdine, pyocyanin, eDNA, glutathione, biofilm

1. Introduction

Cystic fibrosis (CF) is a genetic disorder whose effects are felt from birth. It predominantly affects Caucasian populations; however, it is also present in non-Caucasians [1]. The prevalence of CF varies around the globe; however, extensive evidence suggests that in the USA, Canada, Australia, New Zealand and European countries the ratio of newborns with CF is 1:2000–3000 [2]. CF is induced by mutations (amino acid deletions/substitutions) in the cystic fibrosis transmembrane conductance regulator (CFTR), with a loss of the phenylalanine at position 508 (ΔF508) leading to the most severe outcome. The dysfunctional CFTR leads to greatly reduced transport of ions across epithelial cells and membranes, resulting in dehydration of the mucus in the host respiratory tract/lungs and the digestive pathway, reduced mucus clearance and severe breathing problems [1, 2]. The slow-moving mucous facilitates the growth of microbes, including potentially life-threatening bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae* and *Burkholderia cenocepacia*, as well as fungal species such as *Candida*, *Aspergillus* and *Malassezia* spp. and viruses [3]. Chronic microbial infections and concomitant airway inflammation induced by the bacterial are primarily responsible for respiratory failure in about 95% of CF patients [1]. In spite of intensive antibiotic therapy and other associated therapy (chest physical therapy, pure oxygen therapy) and finally lung transplantation to combat the effects of CF, the mean life expectancy of CF sufferers is still shorter than that of non-CF people, ranging between 35 and 50 years [2].

P. aeruginosa is the most important Gram-negative pathogen that persists in CF patients' lungs, and this persistence is achieved primarily by evading host defence mechanisms through a shutdown of potential trigger genes. *P. aeruginosa* is ultimately responsible for the morbidity and mortality of about 80% of CF patients worldwide [2]. Clinical research has shown that during a CF patient's infancy and childhood more infections are caused by *S. aureus* and *H. influenzae*, whereas in adulthood, the severity of infection is accelerated by *P. aeruginosa* colonisation [4]. *P. aeruginosa* is the most prevalent Gram-negative pathogen in CF patients' lungs by adolescence, by which time the strains isolated from patients are usually multidrug resistant. Evidence suggests that *P. aeruginosa* and its associated infections are more persistent and dominant in CF patients aged over 18 years (91%) than in patients less than 18 years (39%) [5]. In addition to CF-related infections, *P. aeruginosa* is also primarily responsible for airway infections in bronchiectasis, infection of burns and wounds, surgery-associated infections, eye infections due to contact lens contamination and nosocomial infections such as pneumonia and urinary tract infections in the immunocompromised [6]. In CF and bronchiectasis patients, *P. aeruginosa* infection results in chronic airway inflammation, lung tissue damage, declining lung function, respiratory failure and premature death [1, 6].

Persistence of bacterial infections in the host is due to the bacterium's ability to form biofilms via secretion of numerous extracellular biopolymers, collectively known as extracellular polymeric substances (EPS) and small molecules [7, 8]. Different extracellular biopolymers and small molecules conjugate with each other through physico-chemical interactions to form a highly complexed and structurally integrated matrix [7]. This matrix represents a critical interface between bacterial cells and the host or its environment. Extracellular biopolymers

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(EPSs) play a primary role in immobilising planktonic cells (cell adhesion) and cell-cell communication (aggregation), leading to colonisation and biofilm formation on both biotic and abiotic surfaces. It also provides bacterial cells/biofilms with inherent protection against physical stress, traditional antibiotic therapy and host immune defences, thus making eradication extremely difficult [7, 9]. Potentially all biopolymers (e.g. proteins, polysaccharides, eDNA) in EPS serve as an excellent source of nutrients and specifically eDNA promotes horizontal gene transfer between cells within the biofilm [7].

P. aeruginosa EPS plays multiple roles in bacterial adhesion, colonisation, biofilm formation and pathogenesis of *P. aeruginosa* infections [7]. EPS primarily consists of bio-polymers such as polysaccharides (alginate, lipopolysaccharides), proteins (protease, elastase), nucleic acids such as extracellular DNA (eDNA) and RNA, and small molecules such as siderophores and metabolites (phenazines/pyocyanin) [8, 9]. Secretion of EPS and metabolites (phenazines) by *P. aeruginosa* is regulated by the quorum sensing (QS) system. With QS, bacterial cells communicate with each other via small molecules comprising N-acyl homoserine lactones (AHL) and the *Pseudomonas* quinolone signal (PQS). These AHL and PQS promote *P. aeruginosa* biofilm formation through activation of numerous genes expressing extracellular molecules at different stages of the bacterial growth phase [7, 8], with roles in virulence and biofilm development (**Figure 1**).

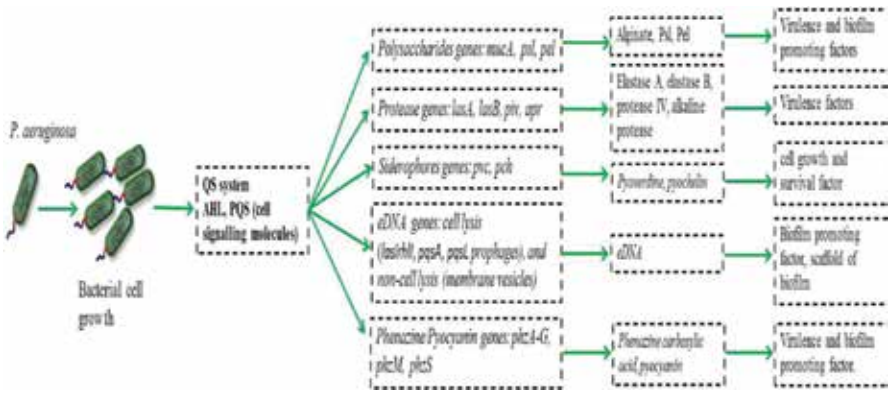

Figure 1. Schematic diagram showing quorum-sensing-mediated production of various extracellular molecules (polysaccharides, protease, pyoverdine, eDNA, pyocyanin) by *P. aeruginosa* and their potential roles in biofilm development and virulence.

Of the many extracellular molecules secreted by *P. aeruginosa*, phenazine-pyocyanin stands out as a molecule that has numerous functions including assistance in growth and multiplication of the cell population, biofilm promotion and virulence. Pyocyanin is a small metabolite with oxidant properties that act as a virulence factor by producing reactive oxygen species (ROS) and generating oxidative stress in the host [10]. Pyocyanin is also a key metabolite in strengthening the e-DNA backbone of the *P. aeruginosa* biofilm [10]. The major focus of this chapter will be on pyocyanin in its role as a *P. aeruginosa* virulence factor. This will involve a review of the literature in the field as well as our work in understanding pyocyanin's role in strengthening

the *P. aeruginosa* biofilm and inducing virulence in the host. In addition, we will briefly review the role of other essential molecules such as polysaccharides, protease, e-DNA and pyoverdine, secreted by *P. aeruginosa* in establishment of the biofilm and progression of infection. This chapter will also address various developments in therapeutic treatment that involves these extracellular metabolites and biopolymers, and our development of new approach disrupts *P. aeruginosa* biofilms in vivo using a combined antioxidant/DNase-I/antibiotic approach.

2. Role of *P. aeruginosa* secreted extracellular molecules in development of biofilm and pathogenesis

2.1. Polysaccharides

Alginate (capsular polysaccharide) is acknowledged as a virulence factor responsible for mucoidal *P. aeruginosa* infection in CF lung [11]. Transformation from initial non-mucoid *P. aeruginosa* colonies occurs after a mutation in the negative regulator of mucoidy, *mucA*, leads to expression of the alginate biosynthesis operon [12] and extracellular secretion of alginate, the basis of the robust mucoid phenotype. Alginate is also partly responsible for the pathogenicity of *P. aeruginosa* infection and has been shown to enhance the resistance of biofilms against antibiotics and the host immune response, by scavenging reactive oxygen species (ROS) released by host immune cells [13, 14]. In line with this, studies have shown that mucoid *P. aeruginosa* biofilms treated with alginate lyase demonstrated enhanced efficacy to antibiotic treatment [15]. However, evidence suggests that alginate is not essential for *P. aeruginosa* biofilm development since *P. aeruginosa* wild-type alginate-producing and alginate deficient strains form morphologically and structurally similar biofilms [16].

Other polysaccharides that are essential and partly associated with biofilm formation include Psl and Pel (coded by the *psl* and *pel* gene clusters, respectively) [16]. Interestingly, studies show that *P. aeruginosa* laboratory strains that do not produce detectable amounts of alginate (UCBPP-PA14 (PA14) and PAO1), still form robust biofilms through expression of Psl, indicating that biofilm formation is independent of alginate production [16]. Psl and Pel polysaccharides are distinct biochemically and play different roles in the establishment of *P. aeruginosa* biofilms. Psl is a mannose and galactose-rich polysaccharide and is essential for initiation of *P. aeruginosa* cell surface adhesion and aggregation (cell-cell interactions) and maintenance of the structural integrity of established biofilms [17]. In respect to the host, Psl plays a significant role in initiating *P. aeruginosa* adhesion to mucin-coated surfaces, airway epithelial cells and biotic surfaces, thus triggering colonisation of CF lung [18]. Pel is a glucose-rich matrix polysaccharide that is essential for pellicle formation and biofilm structure in *P. aeruginosa* [11]. Studies with Pel-deficient mutants concluded that Pel only influences morphological changes in *P. aeruginosa* colonies and does not influence biofilm initiation [19].

2.2. Proteases

P. aeruginosa secretes several protease enzymes identified as important virulence factors, such as alkaline protease (AP), elastase (Ela) B, elastase A (LasA protease), toxin A, phospholipase

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C and protease IV [20, 21]. Through their activity, these proteases contribute to the pulmonary damage seen in CF patients [21]. Interestingly, studies have shown that both environmental (soil and water) and clinical *P. aeruginosa* isolates produce similar concentrations of toxin A, phospholipase C, AP and Ela and have similar levels of elastolytic activity [22]. Protease production in *P. aeruginosa* is triggered through the QS system and numerous genes including *lasA* (elastase A/LasA protease), *lasB* (elastase B), *piv* (protease IV) and the *apr* (alkaline protease) operon are involved [23]. A significant amount of AP, Ela and protease IV has been detected in bronchial secretions from the lungs of CF patients [23]. These bacterial proteases can significantly influence a broad range of biological functions including the infection process, by hydrolysing peptide bonds and degrading proteins essential for basic biological functions in the host. They are also active against the host's humoral immunity system [23]. For example, AP and Ela cleave the major human immunoglobulins IgA and IgG in the respiratory tract [24]. In infected CF lung, protease has been shown to induce a severe inflammatory response, with increased interleukin-8 (IL-8) and interleukin-6 (IL-6) cytokine levels in the airways [25]. *P. aeruginosa* protease secretions in infected burn and wounds patients have been shown to induce sepsis, leading to an increased mortality rate [26]. However, the effectiveness of proteases is limited, as studies have shown that chronically infected CF patients produce specific antibodies against proteases and that these antibodies provide a defensive mechanism for the host by inhibiting protease-mediated cleavage of secretory immunoglobulins [27].

P. aeruginosa-secreted elastase B degrades human elastin, and over time, the decreased levels of elastin and increased levels of collagen in lung tissue result in lung fibrosis [25]. Elastase A cleaves glycine-containing proteins and interestingly influences the activity of several other host elastolytic proteases, including human leukocyte elastase, human neutrophil elastase [28]. *P. aeruginosa* protease IV potentially cleaves IgG and fibrinogen (required for blood clotting). Low levels of fibrinogen lead to haemorrhaging, which is a characteristic of *P. aeruginosa* CF infection [29, 30]. In vitro studies demonstrated that secretion of *P. aeruginosa* proteases is significantly affected by antibiotic (ciprofloxacin) treatment [31]. Biofilms of *P. aeruginosa* PA1159 and PA1230 when treated with 64 μg/ml ciprofloxacin(twice the minimum inhibitory concentration (MIC)) showed up to a 65% decrease in total proteolytic activity [31]. However, the remaining *P. aeruginosa* population displayed increased resistance to ciprofloxacin compared to their planktonic counterparts when grown in fresh medium [31].

2.3. Pyoverdine

Iron is an important cofactor required for bacterial metabolism, growth and survival and also essential for induction of infection in host by various pathogenic bacteria including *P. aeruginosa* [32]. Various iron-binding proteins (a class of ferritin) secreted by mammalian systems reduce the bioavailability of free iron essential for progress of infection and growth by pathogens, thus ferritin acts as an innate immunity molecule against bacterial infection [32]. Under iron limitation conditions, bacteria secrete siderophores (iron-chelating molecules) to acquire iron from the host [32]. *P. aeruginosa* secretes two types of siderophores: pyoverdine (the predominant siderophore) and pyochelin, with high and low affinity for Fe^{3+} ions, respectively [33, 34]. Pyoverdine production is encoded mainly bythe *pvc* gene cluster and

pyochelin production by the *pch* gene cluster [35]. Pyoverdine is more efficient in releasing iron from human ferritin and also has high affinity for Fe^{2+} ions [33, 34]. Studies have demonstrated that pyoverdine is more important than its counterpart pyochelin for the development of *P. aeruginosa* biofilm and infection, whereas mutants that produce pyochelin but are deficient in pyoverdine production are significantly hampered in their biofilm-forming ability [34]. In line with this, a study using an animal model (immunosuppressed mice) showed that pyoverdine predominantly contributes to *P. aeruginosa* virulence and infection [36].

Various factors influence the bioavailability of iron for *P. aeruginosa* and other pathogens in the host; in vitro studies show mutations in the CFTR gene trigger increased release of extracellular iron from lung epithelial cells in comparison to healthy epithelial cells, while elevated iron levels in CF patients directly correlated with an increase in the *P. aeruginosa* population [34]. The proteolytic activity of *P. aeruginosa* protease degrades human ferritin so that it cannot bind iron, thus allowing pyoverdine to scavenge iron and triggering *P. aeruginosa* pathogenicity [34]. Tate et al. showed that iron acquisition by *P. aeruginosa* in CF also occurs through the heme uptake (FeoABC and EfeU) pathways, which are independent of regular siderophore uptake pathways [37]. The presence of an elevated concentration of haem in CF sputum due to haemolysis resulting from pulmonary exacerbations provides bacteria in general with an excellent source of iron. Studies have also demonstrated that under oxygen-deficient conditions in *P. aeruginosa* biofilms or in CF airways,iron exists as Fe^{2+} ions and *P. aeruginosa* takes up Fe^{2+} via the FeoABC and EfeU pathways [37].

Interestingly, mammalian biological systems have an innate defence strategy against siderophores, a neutrophil-gelatinase-associated lipocalin (NGAL). NGAL functions as a scavenger by directly binding with siderophores, blocking *P. aeruginosa's* ability to sequester iron and thereby inhibiting bacterial growth and infection [32]. However, studies have reported that pyoverdine does not bind to NGAL and consequently is able to assist *P. aeruginosa* growth, as demonstrated by biofilm formation and chronic infection in CF lung in spite of elevated amounts of NGAL in lung secretions and bronchoalveolar lavage fluid [32].

2.4. Role of eDNA

eDNA is currently recognised as an essential constituent of EPS and plays a pivotal role in the various processes of biofilm formation in numerous medicallyrelevant Gram-negative and Gram-positive bacteria [8, 9]. In *P. aeruginosa*, eDNA is recognised as an essential molecule in facilitating biofilm formation, including assisting initial bacterial adhesion to surfaces, cell-to-cell interaction (aggregation), microcolony formation and enhancement of biofilm strength and stability [38–41]. eDNA, which is similar to chromosomal DNA in its primary structure [42], is not only released by many bacterial species, predominantly through cell-lytic, but also partly through non-lytic mechanisms [9, 43, 44]. In cell-lytic release, various cell lysing agents such as prophages, autolysin proteins, enzymes and phenazines lyse bacterial cells and trigger eDNA release [8, 38]. Non-lytic eDNA release occurs through the lysis of bacterial outer membrane blebs/vesicles that contain large amounts of DNA [44, 45]. In *P. aeruginosa,* both lytic and non-lytic eDNA releases have been recorded [38, 43, 44]. Studies show that mutants deficient in eDNA production are significantly hampered in biofilm formation. In the same

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vein, biofilm treatment with DNase I, an enzyme that non-specifically cleaves DNA via hydrolysis of phosphodiester bonds in DNA, significantly inhibits biofilm formation and dispersal of mature biofilms [39, 40, 43].

eDNA also serves as a nutrient source (an excellent source of carbon,phosphate and nitrogen), facilitates horizontal gene transfer through Type IV pili and competence stimulating peptides and helps maintain the structural integrity of the biofilm by binding to various extracellular molecules (proteins, polysaccharides, metabolites) in the biofilm matrix [7, 8]. Recent investigations have revealed that eDNA protects bacterial cells in biofilm from physical challenges such as shear stress by increasing biofilm viscosity, and from chemical challenges by antibiotics and detergents. For example, eDNA binds to various positively charged antibiotics (aminoglycosides) thus shielding *P. aeruginosa* in biofilms against their action [46]. eDNA at sub-MIC concentrations creates a cation-limited atmosphere by chelating divalent cations such as Ca^{2+}. This results in the induction of genes involved in resistance to cationic antimicrobial peptides [47]. Swartjeset al. demonstrated that continuous exposure of bacterial cells (*P. aeruginosa* and *S. aureus*) to a DNase I-coated surface inhibits biofilm formation [40]. Treating biofilms with DNase I alters the biofilm architecture leading to penetration by antibiotics, thus promoting the efficacy of antibiotics in killing mature biofilms [48]. It is important to note that *P. aeruginosa*-infected CF lung secretions and bronchitis sputum contain a significant amount of eDNA (3–14 mg/ml), compared to none in non-CF patients [49]. eDNA aids bacterial viability by inducing antibiotic resistance [48] and it also contributes tothe high viscosity of CF sputum [49].

While eDNA is well-recognised as one of the prime factors in the establishment of *P. aeruginosa* biofilms [39, 43], it has also been demonstrated to have such a role in other biofilm-forming bacteria [50, 51]. eDNA initiates biofilm formation by binding with bacterial extracellular biomolecules such as polysaccharides, peptides/enzymes/proteins and other bacterial cell surface structures. In *Listeria monocytogenes* (a food-borne pathogen), Harmsen et al. demonstrated that eDNA binds with peptidoglycan *(N*-acetyl glucosamine), and this molecular interaction initiates adhesion by *L. monocytogenes* to surfaces [50]. In *Caulobacter crescentus* (environmental freshwater bacterium) biofilms, eDNA binds to polar adhesive structure called 'hold-fast' that is present on the tip of the stalk cell (a part of the cell wall that is essential for *C. crescentus* adherence to surfaces), while eDNA from lysed cells masking the adhesive properties of hold-fast, inhibit swarmer cell adherence to the same surface [52]. Rather than acting as an essential structural element of the biofilm, this unusual role for eDNA means that it functions as a regulatory component assisting in the escape of cells from the biofilm and thus promoting development of new, independent colonies [52]. Peptide-eDNA interactions have also been found to be an essential factor promoting biofilm growth of *Streptococcus mutans* (an oral pathogen responsible for dental plaque). In *S. mutans*, uptake of eDNA is triggered through a competence-stimulating peptide, whereas bacterial cell-to-cell interaction and biofilm formation are initiated through the DNA-binding protein ComGB [51]. In *P. aeruginosa*, Das et al. were the first to discover that the phenazine metabolite (pyocyanin) binds with DNA to facilitate *P. aeruginosa* biofilm formation [53].

2.5. Role of pyocyanin

2.5.1. Pyocyanin production in P. aeruginosa

Pyocyanin, a member of the phenazine class, is a molecule only known to be expressed by *P. aeruginosa*, and thus distinguishes it from other pathogens. Up to 95% of *P. aeruginosa* isolates synthesise pyocyanin [54]. It is a bluish-green-coloured extracellular metabolite that is secreted in copious quantities both in vitro and in vivo. In *P. aeruginosa*, phenazine production is regulated through the bacterium's complex QS mechanism. The primary QS molecules, AHL and PQS, trigger the induction of the phenazine operon (*phz A-G*) to produce phenazine-1-carboxylic acid (PCA). Seven genes have been identified as having a role in pyocyanin synthesis, namely *phzCDEFGMS*. Amongst these, *phzM* and *phzS* are central to the conversion of PCA to pyocyanin in a two-step reaction. First, PCA is converted to 5-methylphenazine-1-carboxylic acid betaine (encoded by *phzM*) and then to pyocyanin (encoded by *phzS*) [54, 55]. PCA is also converted in much lower ratios to other types of phenazines, including phenazine-1-carboxamide (PCN, encoded by *phzH*) and 1-hydroxyphenazine (1-OHPHZ, encoded by *phzS*) [54].

In chronic CF lung infection, up to 85 μM of pyocyanin has been recorded in *P. aeruginosa*-infected CF lung secretions and up to 130 μM in bronchitis sputum [56]. In vitro measurement of pyocyanin production by *P. aeruginosa* in both clinical CF and laboratory reference strains showed, in most cases, the expression of large amounts of pyocyanin within 24 h of growth in Luria-Bertani (LB) medium. Amongst CF isolates, the Liverpool Epidemic Strain LESB58 and the Australian Epidemic Strain-2 (AES-2) produced close to 100 μM pyocyanin, as did the laboratory reference strain DKN-370 (a pyocyanin overproducing strain), while the laboratory reference strain PA14 and the Australian epidemic strain-1 isolate AES-1R produced 70–80 μM pyocyanin. Conversely, the chronic infection isogen of AES-1R (AES-1M) produced less than 5 μM pyocyanin indicating expression is reduced as the strain adapts to the CF lung [11]. Evidence suggests that many factors activate pyocyanin production, including low iron [57] and phosphate depletion [58].

2.5.2. Pyocyanin facilitates eDNA release

Pyocyanin is a redox molecule and electrochemically active (has potential to accept and donate electrons as a shuttle) with a multitude of biological activities [59]. Recent investigations have demonstrated that pyocyanin facilitates eDNA release in *P. aeruginosa*. Comparison of eDNA release by *P. aeruginosa* PA14 wild-type and a phenazine/pyocyanin-deficient PA14 mutant (Δ*phzA-G*) showed up to 50% increase in eDNA release by the wild-type under laboratory growth conditions in LB. In line with this, the Δ*phzA-G* mutant showed a significant increase in eDNA release when grown in the presence of exogenous pyocyanin, with the rate of eDNA release directly correlated to the concentration of pyocyanin [38]. Pyocyanin-mediated eDNA release is induced through cell lysis due to hydrogen peroxide (H_2O_2) expression. In PAO1 and PA14 planktonic growth cultures, pyocyanin has been shown to donate electrons to molecular oxygen to form H_2O_2 and initiate an increase of up to 14% in cell lysis under laboratory growth conditions [38]. Interestingly, the surviving *P. aeruginosa* population is protected from H_2O_2 by

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catalase, whose expression is upregulated by *P. aeruginosa* as a self-defence mechanism against its own and host-released H_2O_2 molecules [60]. H_2O_2-mediated eDNA release has also been documented in other bacterial species including *Streptococccus sanguinis*, an oral bacterium responsible for dental disease. In this species, pyruvate oxidase activity by *S. sanguinis* induces a ca. 10% increase in cell death in its own population and consequently facilitates eDNA release [61].

2.5.3. Pyocyanin and eDNA intercalate in biofilms

Pyocyanin's intercalation with DNA has been demonstrated using various bio-physical techniques (circular dichroism, Fourier transform infrared spectroscopy, fluorescence and UV-Vis spectroscopy) [53]. In a preliminary study using fluorescence emission spectroscopy, it was shown that pyocyanin displaces ethidium bromide bound to dsDNA, indicating pyocyanin is an intercalating agent. Fluorescence emission spectroscopy data were further complemented using the UV-Vis spectra of the DNA-pyocyanin complex. Results indicated a significant shift (from 259 to 253 nm) and increase in absorbance intensity in the DNA peak. This marked change in the DNA peak from 259 nm indicates effective intercalation of pyocyanin molecules between the nitrogenous base-pairs of DNA [53]. Meanwhile, the circular dichroism spectra of the DNA-pyocyanin mixtures confirmed that pyocyanin binds to the sugar-phosphate backbone of DNA and strongly intercalates with the nitrogenous bases of DNA, consequently creating local perturbations in the DNA double helix structure [53]. This type of interaction is a typical characteristic feature of all intercalating molecules. In the same study, Das et al. also discovered that pyocyanin significantly increased the viscosity of DNA solutions, and that by intercalating with DNA pyocyanin-facilitated electron transfer [53]. These results are in line with previous studies concluding that in order to remain viable in biofilms, *P. aeruginosa* exploits redox-active metabolites such as pyocyanin, where direct access to electron acceptors such as oxygen or nitrate is diffusion-limited [59].

2.5.4. Pyocyanin-eDNA binding influences biofilm formation via physico-chemical interactions

Molecules that bind to both biological and non-biological surfaces are known to influence hydrophobicity, charge and the physico-chemical properties that assist or resist interactions. Previous studies have demonstrated that in both bacteria and fungi, the presence of such bio-molecules (eDNA or proteins) plays a significant role in dictating cell surface hydrophobicity and physico-chemical interactions [41]. In *P. aeruginosa*, the presence of eDNA has been shown to increase cell surface hydrophobicity. Water contact angle measurements on DNase I-treated *P. aeruginosa* PA14 and PAO1 reduced the angle from 50 to 34° and 46 to 31°, respectively. Interestingly, the PA14 phenazine deficient mutant (Δ*phzA-G*) had a water contact angle similar to DNase I-treated PA14, and DNase I treatment of Δ*phnzA-G* did not show any further reduction in cell surface hydrophobicity [41], indicating that pyocyanin-DNA binding is an essential factor influencing *P. aeruginosa* cell surface hydrophobicity. eDNA-mediated modulation in cell surface hydrophobicity has also been reported in other pathogenic strains, including *Staphylococcus epidermidis* and *S. aureus* [62].

Analysis of bacteria-to-bacteria and bacteria-to-substratum physico-chemical interactions (Lifshitz-Van der Waals interactions forces, acid-base interactions forces) has revealed that the presence of pyocyanin and eDNA facilitates attractive physico-chemical interactions [41]. Removal of eDNA from the *P. aeruginosa* wild-type cell surface or absence of pyocyanin in the *ΔphzA-G* strain showed significant impact, that is, resulted in non-attractive interaction, especially on the short-range acid-base interactions, which include electron donating and electron accepting parameters. However, the long-range Lifshitz-Van der Waals interactions remained unaffected between wild-type and *Δphz*A-G regardless of DNase I treatment [42]. Similarly, the effect of eDNA on physico-chemical forces between *S. epidermidis* cells has been reported, and results suggest that eDNA triggers *S. epidermidis* cell-to-cell interactions [62]. Similarly, adhesion force analysis in *S. mutans* using atomic force microscopy and phase-contrast microscopy imaging and quantification indicates that in the presence of eDNA, *S. mutans* has a stronger adhesion force and adheres to surfaces in significantly higher cell numbers [63].

It should be noted, however, that physico-chemical interactions do not explain bacterial interaction in all cases, since bacterial cell structures (pili, fimbriae) and bio-polymers (polysaccharides, proteins, eDNA) extend up to hundreds of nanometres from the bacterial cell surface and can affect other interaction types [64]. These cell structures and bio-polymers initiate hydrogen bonding and ionic interactions by colliding with bio-molecules anchored on the bacterial cell surface to stabilise the biofilm matrix and also to its adjacent cells and thereby help bacterial cells to overcome the physico-chemical energy barrier and promote bacterial cell-to-cell interactions and biofilm formation [7, 64]. Confocal laser scanning microscopy (CLSM) analysis revealed that the intercalation of pyocyanin with eDNA facilitates *P. aeruginosa* PA14 wild-type biofilm formation while the absence of pyocyanin significantly inhibits biofilm formation [65]. To investigate this further, Klare et al. grew the CF *P. aeruginosa*AES-1 isolate R (isolated at the acute stage of infection)in an artificial sputum media (ASMDM+) that mimics CF sputum, and found it formed robust biofilms in comparison to its isogenic counterpart AES-1M (isolated at chronic infection). AES-1M which produces about 15 times less pyocyanin than AES-1R, and the exogenous addition of pyocyanin to AES-1M cultures facilitated enhanced biofilm formation [65] (**Figure 2**).

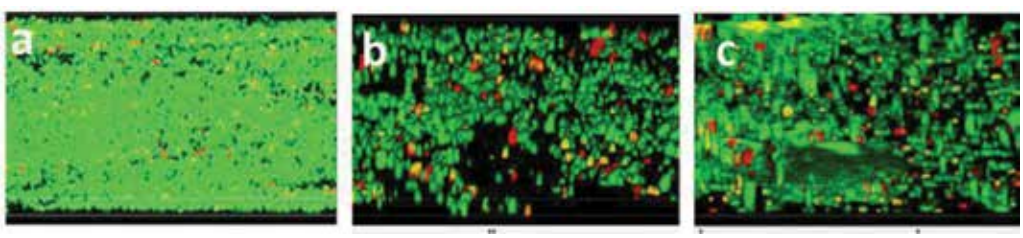

Figure 2. Biofilm formation by *P. aeruginosa* CF isogens in ASMDM+ medium (a) AES-1R, (b) AES-1M and (c) AES-1M grown in the presence of exogenous pyocyanin. The biofilm architecture of (c) indicates pyocyanin facilitates/enhances biofilm formation. Images taken with permission from Ref. [65].

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2.5.5. Pyocyanin as a virulence factor

Pyocyanin was formerly recognised only as a bacterial secondary metabolite, but has recently gained significant attention for its involvement in a variety of crucial roles in microbial ecology, specifically correlated with the severity of *P. aeruginosa* pathogenicity in plants and humans [66]. **Figure 3** is a schematic representation of pyocyanin-induced H_2O_2 production and toxicity on bacterial, fungal and human cells. Pyocyanin also has antibacterial and antifungal activity that is toxic to other pathogenic bacteria and fungi. Pyocyanin-mediated bactericidal activity occurs through production of H_2O_2, which consequently depletes oxygen supply to cells and disables electron flow and metabolic transport processes [67]. Studies suggest that pyocyanin potentially kills *Staphylococcus* sp. and other species in the CF lung environment; and that it also has anti-*Escherichia coli* activity [67, 68] (**Figure 3**). The inhibitory effect of pyocyanin on the growth of fungi such as *Aspergillus fumigatus* and *Candida albicans* isolated from the sputum of CF patients has also been reported earlier [69] (**Figure 3**). These results could be interpreted as a pyocyanin-mediated modulation of the microbial community in the CF lung by *P. aeruginosa*, resulting in its predominance [70].

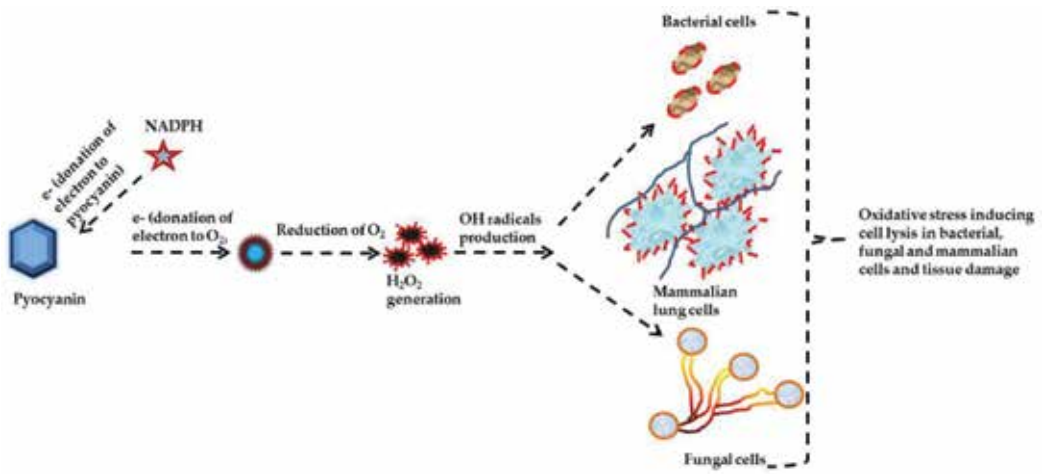

Figure 3. Schematic diagram of pyocyanin induced H_2O_2 production and toxicity on bacterial, fungal and human cells.

In the host, pyocyanin appears to participate in a reduction mechanism, which is capable of reducing and releasing the iron from transferrin in host cells to stimulate the growth of *P. aeruginosa* [71]. Previous research concluded that a direct correlation exists between pyocyanin concentration in CF sputum and severity of infection [71]. Studies using *P. aeruginosa*-infected bronchiectasis airways in a mouse model of lung infection demonstrated that pyocyanin rapidly inhibited lung function and caused cell hyperplasia and metaplasia (abnormal changes in cell or tissue morphology), airway fibrosis and alveolar airspace destruction [71]. Harmer et al. analysed the difference between *P. aeruginosa* epidemic and non-epidemic isogenic strains

that were collected 5–8 years apart from five chronically infected adult CF patients, this study suggested that epidemic (FCC) strains are more virulent and more efficient in killing *Caenorhabditis elegans* than their non-epidemic counterparts [72]. The isogens collected early in the infection produced more virulence factors including elastase, pyoverdine and pyocyanin. Over the course of chronic infection, the isogens undergo a significant downregulation in virulence factors *lasB*, *rsaL*, *lecB* and *oprG*, with a significant decrease in elastase and pyoverdine production, however, pyocyanin production increased in three out of five strains and so did biofilm production [72]. Fluctuations in pyocyanin concentration observed among different CF strains are probably due to adaptation of a particular strain to the host and time of acquisition of sample, for example, at exacerbation (when the patient is seriously ill and hospitalised). At exacerbation, the pyocyanin levels may be switched on by the *P. aeruginosa* strain as a protective mechanism against host defences, and this leads to the increased lung damage seen at that time [72]. If the sample was taken when the patient was not in exacerbation, the pyocyanin expression may be very low or negligible [72]. Other phenazine-like PCA molecules secreted by *P. aeruginosa* were also shown to be highly toxic, killed *C. elegans* and caused serious cell damage in a mouse model of lung infection [73].

Pyocyanin has also been extensively studied due to its electrochemical and redox activity. The diffusible nature and small size of pyocyanin means it can easily pass through the host cell membrane and undergo redox reactions with other molecules [74]. For example, it accepts electrons from NADH and subsequently donates electrons to molecular oxygen to form reactive oxygen species (ROS) such as H_2O_2 [74] (**Figure 3**). Pyocyanin-mediated ROS cause oxidative stress and affect calcium homeostasis while also obstructing cellular respiration and depleting intracellular cAMP and ATP levels [75]. Pyocyanin significantly alters human protease activity, inhibits nitric oxide production and consequently influences blood flow, blood pressure and immune functions. It also modulates the host immune response to support evasion of the host immune system and establish chronic infection [75]. In CF, pyocyanin-mediated ROS oxidise host intracellular and extracellular reduced glutathione (GSH) to form glutathione disulphide or oxidised glutathione (GSSG) [76]. Depleted GSH levels during the chronic stage of CF infection lead to widespread epithelial cell death and consequent lung damage and leading to respiratory failure and death [75, 76]. Pyocyanin also inhibits catalase activity in airway epithelial cells, thus increasing oxidative stress in these cells and initiating pulmonary tissue damage [77]. In a recent study, Rada et al. showed that pyocyanin promotes neutrophil extracellular trap (NET) formation [78]. NET formation is an important innate immune mechanism initiated by neutrophils to trap and kill pathogens, however, the aberrant NET release triggered by pyocyanin-mediated intracellular ROS production directly damages host tissues and has been linked to the severity of many diseases including CF [78].

3. Treating *P. aeruginosa* infections

Substantial research over many decades has led to a good degree of understanding of the mechanisms *P. aeruginosa* utilises to cause infection and colonisation. In brief, *P. aeruginosa* has been shown to evade the host's innate defence system through production of various

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extracellular molecules and render antibiotics ineffective through several efflux pump mechanisms [6, 8, 79]. This research has particular implications for CF, burns and wounds patients, particularly as *P. aeruginosa* antibiotic resistance is a serious concern. This in turn has given impetus to the development of new therapeutic methods. Prominent amongst the extracellular molecules available to *P. aeruginosa* are the previously discussed eDNA, protease, pyocyanin and pyoverdine.

3.1. Current antibiotic treatment and challenges against *P. aeruginosa* infections in CF patients

Many antibiotics developed in recent decades such as aminoglycosides, ticarcillin, ureidopenicillins, ceftazidime, cefepime, aztreonam, the carbapenems, ciprofloxacin and levofloxacin display anti-pseudomonal activity. However, the choice of best antibiotic to use in a particular case remains a major challenge as *P. aeruginosa* can readily adapt by mutation or horizontal gene transfer to acquire resistance in a portion of remaining cells, leading to consequent treatment failure.

Antibiotics commonly used to treat *P. aeruginosa* infection in CF patients include tobramycin, colistin, aztreonam, ciprofloxacin and azithromycin. Administration methods include nebulised, dry powder inhalation, oral or intravenous, or a combination of different strategies [2, 80]. Studies have shown the size of inhaled antibiotic particles is very important in determining whether they will reach deep infection sites. Particles of 1–5 μm diameters are more effective in reaching deep lung tissue efficiently [81]. However, one of the major concerns in inhalation therapy is that most antibiotics are trapped in the thick viscous mucus that covers both the large respiratory zone (respiratory bronchioles, alveolar ducts and alveolar sacs) and the conductive zone (trachea, bronchi and terminal bronchioles) [81, 82]. With intravenous or oral therapy, antibiotics are readily transported through the bloodstream mainly reaching the respiratory zone but not effectively reaching the conductive zone. A combination of both strategies has been shown to enhance the access of antibiotics to infection sites at both the conductive zone and respiratory zones [82].

Other serious challenges with nebuliser treatment (in comparison to dry powder inhalation) strategies are that the antibiotic particles do not reach infection sites at a faster rate, but even with dry powder inhalation does not provide immediate relief to CF patients [83]. For example, studies with CF patients demonstrated that inhaled tobramycin is effective in reducing *P. aeruginosa* density from the lower airways but has no effect in reducing lung inflammation, and consequently certain infection loci and disease symptoms remain [84]. Azithromycin has been shown to improve lung function (lung inflammation, exacerbations and cough) in CF patients compared to other antibiotics and lead to a reduction in *P. aeruginosa* colonisation [85]. However, azithromycin or the macrolide class of antibiotics has significant side effects, including a significant increase in macrolide resistant *S. aureus* and *H. influenzae* strains in CF sputum [85]. In general, many antibiotics are known to cause adverse side-effects in patients, targeting the central nervous system, gastrointestinal tract and urinary tract leading to kidney failure [86, 87]. With the increase in antibiotic resistance, there is an urgent need to develop

novel therapeutic approaches to disrupt bacterial biofilms and eradicate the causative bacteria in the host.

3.2. Current non-antibiotic strategies against CF lung infection

Non-antibiotic treatment strategies that have shown potential to reduce the severity of respiratory symptoms in CF patients and bacterial associated infections have largely centred on the use of aerosolised recombinant human DNase I (rhDNase I (Pulmozyme)) in a nebuliser [88]. Earlier studies showed DNase I reduced the viscosity of CF sputum by cleaving DNA present in sputum and thus leading to increased pulmonary function [49]. As noted above, eDNA is an essential biofilm promoting factor in many pathogenic bacterial species, is the backbone of the *P. aeruginosa* biofilm matrix, which by its impenetrable structure constitutes a defence strategy against antibiotics [46–48]. In line with this studies have shown that DNase I inhalation reduces the prevalence of bacterial strains in CF patients [88].

3.3. New non-antibiotic treatments

A new potential treatment strategy involves the use of reduced GSH to bind to pyocyanin and prevent its intercalation with eDNA. Intracellular GSH levels in mammalian cells are in the millimolar (mM) range, and lower concentrations are found in some bacterial cells. However, in CF patients, GSH levels in whole blood, blood neutrophils lymphocytes and epithelial lung fluid are markedly decreased [89]. Replenishment of GSH levels in CF has thus been investigated in a number of human studies using either inhaled GSH [90, 91] or oral N-acetylcysteine, a GSH precursor [92]. These studies demonstrated the feasibility of successfully delivering GSH to human lung, with a significant improvement in lung function (FEV1), especially in patients with moderate lung disease. The GSH therapy was well tolerated by CF patients with no noticeable side effects [91].

GSH, being a thiol antioxidant, will donate electrons/protons to pyocyanin directly through the –SH group from cysteine [53, 76], thereby interfering in the pyocyanin oxidation process by inhibiting H_2O_2 generation [76]. The antioxidant properties of GSH make it a potential inhibitor of pyocyanin toxicity. GSH binding to pyocyanin tends to modulate pyocyanin's structure, and this has been confirmed using nuclear magnetic resonance (NMR) spectroscopyand mass spectrometry [53, 93]. This structural change consequently inhibits the intercalation of pyocyanin with DNA, confirmed using circular dichroism [53]. In line with this, Muller and Merrett concluded that GSH forms a cell-impermanent conjugate with pyocyanin and consequently inhibits pyocyanin entry into host cells, thus preventing pyocyanin-mediated lung epithelial cell lysis [93].

Recent studies in the Manos laboratory by Klare et al. have demonstrated the excellent utility of GSH in disrupting *P. aeruginosa* biofilms. It was demonstratedusing CLSM that GSH-mediated inhibition of pyocyanin-DNA binding modulates *P. aeruginosa* biofilm architecture, significantly decreases biofilm biomass, surface coverage and leads to a significant increase in the percentage of dead bacterial cells [65]. GSH alone was shown to have a significant effect on disruption of mature 72-h-old biofilms of the epidemic isolate AES-1R grown in ASMDM+,

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while the combined treatment with GSH and DNase I of biofilms from a range of CF isolates showed greater disruption and significantly increased susceptibility to ciprofloxacin killing. GSH-treated biofilms were also shown by RNA-sequencing to display a transcriptomic profile that was distinctly different from those of both mature biofilms and dispersed cells, including those resulting from dispersal agents such as NO [65]. In contrast to dispersed cells, GSH-disrupted biofilm cells significantly upregulated cyclic-di-GMP synthesis genes (*siaA* and *siaB*), and there was no concomitant induction of flagellar biosynthesis genes. Cyclic-di-GMP gates the transition from sessile to motile lifestyle, and its expression prevents this transition [94]. GSH-disrupted cells also significantly upregulated the pyoverdine biosynthesis operon, in contrast to the downregulation of pyoverdine shown by dispersed cells. The active expression of pyoverdine is essential for biofilm structure formation [95]. CF sputum and ASMDM+ both have low levels of iron, and this may have triggered increased pyoverdine expression to sequester iron for processes required to re-form the disrupted biofilm.

In comparison to other techniques, GSH treatment has a distinct advantage, being an intrinsic and essential antioxidant for host cells that not only has antibiofilm properties but has also been proven to enhance lung epithelial growth and increase pulmonary function in CF patients [91].

3.4. Development of new antibacterial agents

Several new antibacterial agents are being developed and undergoing stringent testing both in vitro and in vivo (animal models) against *P. aeruginosa* and other CF pathogens. QS-inhibiting molecules against *P. aeruginosa* biofilms, such as furanone-based compounds (naturally secreted by the alga *Delisea Pulchra*) and synthetic furanone compounds have high affinity and compete with the cognate AHL signal for the LuxR receptor site in *P. aeruginosa* [96]. Thus by binding with and controlling the LuxR mechanism, these furanone molecules significantly alter biofilm architecture and enhance the efficiency of the antibiotic tobramycin against planktonic cells and biofilms [96]. Most interestingly, furanones have been shown to repress numerous QS-regulated virulence genes and production of concomitant virulence factors, including LasA protease and elastase B (encoded by *lasA* and *lasB*, respectively), rhamnolipid (encoded by the *rhlAB* operon) and phenazine biosynthesis (encoded by the *phzA-G* operon) [96].

Other antibiofilm agents under investigation include nitric oxide (NO) which has recently been discovered to induce dispersal of *P. aeruginosa* biofilms by mediating an increase in bacterial phosphodiesterase activity and a decrease in intracellular levels of the secondary messenger cyclic di-GMP, thereby inhibiting signal transduction in bacteria [97]. NO was shown to disperse released cells, and the remaining biofilms displayed enhanced sensitivity towards antibiotics [97]. A recent study by Kimyon et al. showed that prodigiosin (a heterocyclic bacterial secondary metabolite secreted by *Serratia sp.*) induces biofilm disruption and exhibits bactericidal activity against *P. aeruginosa* [98]. Prodigiosin-mediated *P. aeruginosa* biofilm disruption occurs by the release of H_2O_2 and generation of hydroxyl radicals in the presence of copper ions that consequently cleaves/damages eDNA and alters *P. aeruginosa* cell surface

hydrophobicity. Prodigiosin also induces bacterial cell lysis as a consequence of the oxidative stress generated by H_2O_2 [98].

4. Conclusions

Extracellular molecules released by bacteria form a scaffold for biofilm formation. In *P. aeruginosa,* polysaccharides, eDNA and pyocyanin are major factors that integrate the biofilm matrix and provide defence against cationic antibiotics by binding to it [8]. On the other hand, molecules such as pyoverdine help promote bacterial growth and prevalence in the host by chelating iron [34]. Increased resistance to antibiotic therapies and the persistence of bacterial colonisation within the CF lung is associated with bacteria-secreted extracellular molecules. Novel treatment strategies seek to act on molecules that are essential for bacterial persistence such as biofilm constituents. Biofilm matrix disruption is associated with increased antibiotic susceptibility and clearance of bacteria. Current antibiotics strategies target growth inhibition without cleaving the biofilm matrix, whereas other strategies including DNase I and GSH cleave or disrupt biofilm matrix constituents, but have no bactericidal activity. In CF patients, the severity of disease due to *P. aeruginosa* infection is the leading cause of death, so there is an urgent need to develop new strategies that could disrupt bacterial biofilm matrix and facilitate bactericidal activity, ultimately allowing for repair and re-growth of lung epithelial tissue. The combination of biofilm-disrupting agents with traditional antibiotics could serve as a new line of therapy for CF patients in the near future.

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Chapter 6

Structure-Function Relationships of Rhamnolipid and Exopolysacharide Biosurfactants of *Pseudomonas aeruginosa* as Therapeutic Targets in Cystic Fibrosis Lung Infections

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Additional information is available at the end of the chapter

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Abstract

Chronic *Pseudomonas aeruginosa* lung infection is the cause of much morbidity and most of the mortality in cystic fibrosis (CF) patients. The high prevalence of *P. aeruginosa* infections in CF is related to the microbe's large genome and mechanisms of adaptation to the CF lung environment, the host immune system and antibiotic resistance. Among a wide range of *P. aeruginosa* metabolites involved in infection development in CF, the biosurfactant compounds, rhamnolipids (RLs) and exopolysaccharides (EPSs), have important roles in the early stages of *P. aeruginosa* infection in CF. RLs and EPSs are involved in bacterial adhesion, biofilm formation, antibiotic resistance, and impairment of host immune system pathways, as well as in processes such as biofilm maintenance and the mucoid phenotype of *P. aeruginosa*, which lead to development of chronic infection. Due to the proposed roles of RLs and EPSs and the importance of prevention and treatment of *P. aeruginosa* respiratory infections in CF, these compounds are promising targets for patient therapy. In the future, impairment of *P. aeruginosa* quorum sensing (QS) pathways and modification of host respiratory mucus epithelial membranes should be considered as potential approaches in preventing respiratory infections caused by this microbe in CF patients.

Keywords: cystic fibrosis, *Pseudomonas aeruginosa*, biosurfactant, rhamnolipid, exopolysaccharide, biofilm

1. Introduction

Cystic fibrosis is a congenital, recessively inherited disorder. The genetic background of CF development is >1500 mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) on chromosome 7, which lead to malfunction of the chloride channel in CF patients. CF affects a large number of organs and tissues (airways, pancreas, the small intestine, liver, the reproductive tract and sweat glands), resulting in numerous clinical symptoms (viscid mucus, respiratory infections, intestinal malabsorption of fat, diabetes mellitus, meconium ileus, impaired liver function, male infertility and salt loss) [1].

The malfunction of the chloride channel in CF patients leads to impairment of the non-inflammatory defense mechanism of the lower respiratory tract. Therefore, CF patients, from early childhood, suffer recurrent and chronic respiratory tract infections caused by *P. aeruginosa*, *Burkholderia cepaci*, *Achromobacter xylosoxidans*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, nontuberculous *Mycobacteria* and *Aspergillus fumigatus*. In spite of the host inflammatory response and intensive antibiotic therapy, infections persist and lead to respiratory failure requiring lung transplantation or death [1].

Chronic *P. aeruginosa* lung infection is the cause of much of the morbidity and most of the mortality in CF patients. Chronic infection is considered as growth of *P. aeruginosa* from multiple respiratory cultures over a 6-month period [2]. About 80% of adults with CF have chronic *P. aeruginosa* infection [3]. *P. aeruginosa* is able to survive and persist for several decades in the respiratory tract of CF patients, in spite of the defense mechanisms of the host and intensive antibiotic therapy. However, the microbe has adaptive mechanisms, which explain why it can survive in the hostile CF lung for so long. These include phenotype splitting due to mutations (into nonmucoid or mucoid), their different distributions in respiratory and conductive zones in the lungs and switching to a biofilm mode of growth—mucoid phenotype [4–7].

Recent research indicates that chronic *P. aeruginosa* infections are caused by the ability of bacteria to organize themselves into microcolonies regarding to formation of biofilms. In this state, the bacteria are incorporated in a self-produced protective matrix, often with surrounding inflammatory cells, which is very well protected against antibiotics and the host defense [4]. The biosurfactant compounds (RLs and EPSs), due to their structures and physicochemical properties, as well as their interactions and correlation with other metabolites, significantly contribute to colonization, motility and biofilm formation [8–10]. Additionally, the mucoid colony morphology of *P. aeruginosa* is highly correlated with overproduction of alginate (a type of EPS) [8]. Therefore, it is important to consider these biosurfactants and their biosynthetic pathways as possible targets and approaches for CF therapy in order to impair *P. aeruginosa* mechanisms of pathogenicity. Furthermore, cell-to-cell communication and QS signaling pathways together with their genetic aspects, closely related to RL and EPS biosynthesis, are the most significant targets for new therapy approaches in CF treatment [10–13].

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2. *P. aeruginosa* infection in CF

2.1. *P. aeruginosa*

Pseudomonas is ubiquitously present worldwide, being an extremely diverse bacterial genus. Pseudomonads are frequently closely associated with animals and plants; they are common and numerous in a wide range of environmental habitats. Their ability to adapt genetically, producing varying physiological advantages as a response to their pervasiveness, is the subject of much scientific speculation and study. *P. aeruginosa*, as all species that belong to the genus *Pseudomonas*, due to its metabolic diversity, has potential for adaptation, survival and growth in a wide range of environmental conditions [14, 15].

P. aeruginosa produces an arsenal of secondary metabolites, including EPSs, RLs, enzymes (elastase, alkalne protease, exoenzyme S, phospholipase C and hemolysins), pigments and toxins (exotoxin A), using these virulence factors for infecting and colonizing a wide range of hosts (animals, plats, insect and nematodes) and surfaces [12–24]. The major biosurfactant compounds produced by *P. aeruginosa*, RLs and EPSs, are involved in bacterial adherence, biofilm formation and maintenance, which all are necessary for respiratory infection establishment, development and progression in CF patients [4, 8, 12, 13, 16].

2.2. Pathogenesis of *P. aeruginosa* infection in CF

Despite constant exposure to a wide range of microorganisms, CF patients are predisposed to infection by only specific groups of microorganisms [8]. The proximal event in development of CF is mutation of the CFTR gene (see Introduction), but still, it remains unclear how this primary step causes particular infections in CF patients. However, numerous proposed mechanisms are related to CFTR gene mutation, defective CFTR channels and infection development [8]: (1) reduced ion transport; (2) modified salt content in the airway surface liquid; (3) increased levels of acylated glycolipids on the surface of CF airway epithelial cells; (4) defective CFTR exposed on airway epithelial cell membranes become receptors; (5) low levels of antimicrobial compounds (inducible nitric oxide synthase and nitric oxide); and (6) intrinsic hyperinflammation of airways (**Table 1**) [25–36].

The first step in infection of CF airways by *P. aeruginosa* is microbe acquisition [8]. Due to the abundance of *P. aeruginosa* in many natural environments, most individuals acquire *P. aeruginosa* through casual contact with natural bacterial sources, while some individuals acquire *P. aeruginosa* directly or indirectly from other CF patients. Transmission data and genotype/phenotype properties of clinical and environmental *P. aeruginosa* isolates indicate that characterizing the ecology of *P. aeruginosa* originating from natural environments would lead to a better understanding CF epidemiology [8].

Initially, infection of *P. aeruginosa* in CF is usually the result of an alternating series of acquisitions of different isolates and in the first stage of infection, most of the isolates are nonmucoid and highly antibiotic sensitive [8, 37–39]. Eventually, one or two isolates establish themselves and, due to their genetic, phenotypic and physiological changes, develop chronic infection [13, 16, 40].

Mechanism	Effect
Decreased ion transport, which results from defective CFTR channels enhances fluid absorption in the airways	Lowered airways surface liquid and impaired ciliary transport of the mucous layer, which results in defects in microbial clearance
Altered salt content in the airway surface liquid	Inactivation of immune system defenses pathways; defected neutrophils activity
Increased levels of acylated glycolipids on the surface of CF airway epithelial cells due to defective CFTR molecules	Modified glycolipids are receptors for *P. aeruginosa* adherence
Binding of *P. aeruginosa* to defective CFTR molecule exposed on airway epithelial cells membranes	Internalisation of *P. aeruginosa*
Lowered level of antimicrobial compounds	Propensity of individuals to lung infection
Istrinical hyperinflammation of airways	Damage of host cells and disruption of effective microbe clearance

Table 1. Proposed mechanisms of *P. aeruginosa* in development of respiratory infection in CF airways.

2.2.1. P. aeruginosa quorum sensing systems and biofilm

One of the most important factors which facilitate *P. aeruginosa* to colonize and persist in acute and chronic lung infection in CF patients is the ability of this microbe to grow as a biofilm, assembly of which is regulated by a QS system [13, 30, 40].

QS is the mechanism by which bacteria engage in cell-to-cell communication using diffusible molecules based on a critical cell density [41]. QS molecules manage and regulate diverse physiological processes, some of which are interrelated. In *P. aeruginosa*, expression, production and/or secretion of many virulence factors, such as EPSs, RLs, enzymes, pigments production, biofilm formation and antibiotic resistance, are controlled by QS [10, 13, 42]. *P. aeruginosa* possesses two interrelated QS systems, the *las* and *rhl* systems. The *las* system comprises the transcriptional regulatory protein, LasR and its cognate autoinducer, N-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL). The *rhl* system comprises the RhlR transcription regulator protein (also known as R-protein) and N-butyryl homoserine lactone (C_4-HSL), its cognate autoinducer [13]. Additionally, these two systems are not independent but are interlinked in a hierarchical manner (the *las* system directs the *rhl* system). They are linked by a third signal molecule, 2-heptyl-3-hydroxy-4-quinolone, known as the *Pseudomonas* quinolone signal (PQS). PQS is produced under the control of the *pqs* system, which is considered as the third distinct QS system [11, 42]. Interestingly, transcriptome analyses have revealed that between 6 and 10% of the *P. aeruginosa* genome is regulated by the *las* and/or *rhl* systems [13].

Biofilms are matrix-enclosed microbial accretions that adhere to biological or nonbiological surfaces [43]. *P. aeruginosa* biofilms are related to development of different acute and chronic infections, not only in CF patients [16, 44, 45]. Formation of *P. aeruginosa* biofilm occurs in stages: bacterial attachment and irreversible adhesion to surface, microcolony development, biofilm maturation and dispersion of bacterial cells from the biofilm [46]. Heterogeneous microenvironments due to oxygen and nutrient diffusion limitations occur in biofilms and

they lead to physiological and phenotype diversity [47, 48]. Suggested mechanisms of *P. aeruginosa* biofilm formation involve QS signaling, which coordinates and protects biofilm assembly and maintenance [44, 49–52]. The *las I*, which encodes the biosynthetic pathway for 3O-C_{12}-HSL, is critical for biofilm maturation [50]. Heterogeneity of the bacterial population in biofilm is an important characteristic of *P. aeruginosa* pathogenicity and contributes to the microbe's resistance to antimicrobial therapy. In laboratory conditions, *P. aeruginosa* can form two types of biofilm, "flat" and "structured", and alginate-producing isolates (the mucoid phenotype) form complex structured type of biofilm which is resistant to tobramycin [53]. Additionally, the QS system is involved in regulation of several genes such as *rhlA*, *rpoS*, *sad A* and genes in the denitrification pathways. These genes are important for all stages of biofilm development, maintenance, or dispersion: (1) biosynthesis of the biofilm matrix (EPSs, extracellular DNA); (2) biosynthesis of RLs; and (3) other metabolic pathways (not discussed here) [13, 42].

2.2.2. Adaptation mechanisms of P. aeruginosa in CF lungs

The CF lungs are an unfriendly and varied environment for invading bacteria due to the presence of stressors such as osmotic stress of viscous mucus, oxidative and nitrosative stresses of the host responses, sublethal concentrations of antibiotics and other microbes presence. Regarding to the environment of CF lungs, it is a great challenge of *P. aeruginosa* populations to overcome these stressors and persist [54].

It is believed that mechanisms that allow *P. aeruginosa* to cause persistent chronic infection are related to its remarkable potential for adaptation to environmental changes [8, 15]. *P. aeruginosa* adaptations in CF patients' lungs are dynamic and generate subpopulations of bacteria with differing phenotypes [8]. It is thought that primary infection is related to the large *P. aeruginosa* genome, while development of persistent infection is dependent on spontaneous mutations [55, 56]. Mutations are multiple due to different factors such as the presence of hypermutable strains, development of biofilm and downregulation of antioxidant enzymes [57–59]. Environmental conditions in CF airways then further favor specific *P. aeruginosa* phenotypes. This set of adaptations finally leads to development of the subpopulations of bacteria (mentioned above) within the same respiratory tract, which are relatively similar, but which carry unique groups of genes [56, 60, 61]. Some commonly and intensively studied *P. aeruginosa* adaptation mechanisms present during respiratory infections in CF involve: transition to mucoid phenotype, antibiotic resistance, alterations in lipopolysacharride (LPS), loss of type III secretion and motility, auxotrophy, small-colony variants, defects in the QS system and hypermutability [8, 54].

3. Biosurfactants of *P. aeruginosa*—rhamnolipids and exopolysaccharides

Biosurfactants are a group of amphiphilic compounds, comprise a hydrophobic and a hydrophilic moiety and are produced by a range of microorganisms [9, 62]. *Pseudomonas* spp. are the most common producers of biosurfactants [63], with *P. aeruginosa* being the preeminent RL and EPS biosurfactant-producing species [9, 63]. Up to date, a variety of biosurfactants

have been studied, but RLs (glycolipid biosurfactants) and EPSs (polymeric biosurfactants) are currently attracting the most attention, as they are relevant in medicine, environmental protection, food and the pharmaceutical industry [15, 24, 64–66].

3.1. *P. aeruginosa* rhamnolipids

Rhamnolipids comprise one or two L-rhamnose units and one or two units of 3-hydroxy fatty acid. Variations in lipid components contribute to the biodiversity of RLs [9, 67]. Due to their chemical composition, RLs are classified into four homologue groups (**Figure 1**): RL1—mono-rhamno-di-lipidic, RL2—mono-rhamno-mono-lipidic, RL3—di-rhamno-di-lipidic and RL4–di-rhamno-mono-lipidic structures. RL1 and RL3 are usually classified as principal—common RLs, while RL2 and RL4 are classed as atypical–uncommon RLs [68]. The development of sensitive, high throughput analytical techniques, such as soft ionization mass spectrometry, has led to the further discovery of a wide diversity of RL congeners and homologues (about 60) produced in different concentrations by various *Pseudomonas* spp. and other bacteria [9].

3.1.1. Diversity of rhamnolipid structures

RL biosurfactants are always produced as mixtures of different RL congeners, as observed with various *P. aeruginosa* isolates [15, 69–74]. The complexity of the RL mixtures produced depends on various factors such as bacterial isolate origin, type of carbon substrate, culture conditions and isolation procedure and age of the culture and of course, the *P. aeruginosa* isolate itself [15, 23, 63, 72, 75–80]. Despite the number of such factors reported, some particular RL congeners are predominant in all *P. aeruginosa* producer isolates. These are classified as the major RL structures (Rha-C_{10}-C_8, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12:1}$, Rha-Rha-C_{10}-C_8, Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-C_{12} and Rha-C_{10}-$C_{12:1}$) [23, 72, 81–84]. Other RLs, produced only sometimes or in low abundance, are the minor RL structures [23, 72, 81–84]. Both the major and the minor RL congeners contribute to the complete profile of RLs. In all studies of RL mixtures produced by various *P. aeruginosa* isolates, mono-rhamnolipid Rha–C_{10}–C_{10} and di-rhamnolipid Rha–Rha–C_{10}–C_{10} were the predominant congeners, in spite of the varying compositions produced [23, 72, 81–84].

The presence of different functional groups in RL molecules (the hydrophobic, lipid part and the hydrophilic and carbohydrate part) gives RLs important physicochemical properties. Due to their amphipathic structure, RLs behave as wetting agents, surface active compounds, emulsifiers and detergents. These RL functional groups are, therefore, utilized in enhancing and facilitating bacterial movement, adhesion and contact with surfaces, as well as substrate uptake, or solubilization.

3.1.2. Rhamnolipid biosynthesis and quorum sensing

Biosynthesis of RLs requires three rhamnosyltransferases. The fatty acid dimer moiety in RLs and free 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) are both synthesized by the rhamnosyltransferase RhlA. Next, dTDP-L-rhamnose is transferred to HAA by the rhamnosyltransferase RhlB, or to a previously generated mono-RL by the rhamnosyltransferase RhlC [85]. HAA precursors are derived from the FASII cycle (bacterial fatty acid synthesis system), while activated L-rhamnose is derived from the glucose moiety of deoxythymidine

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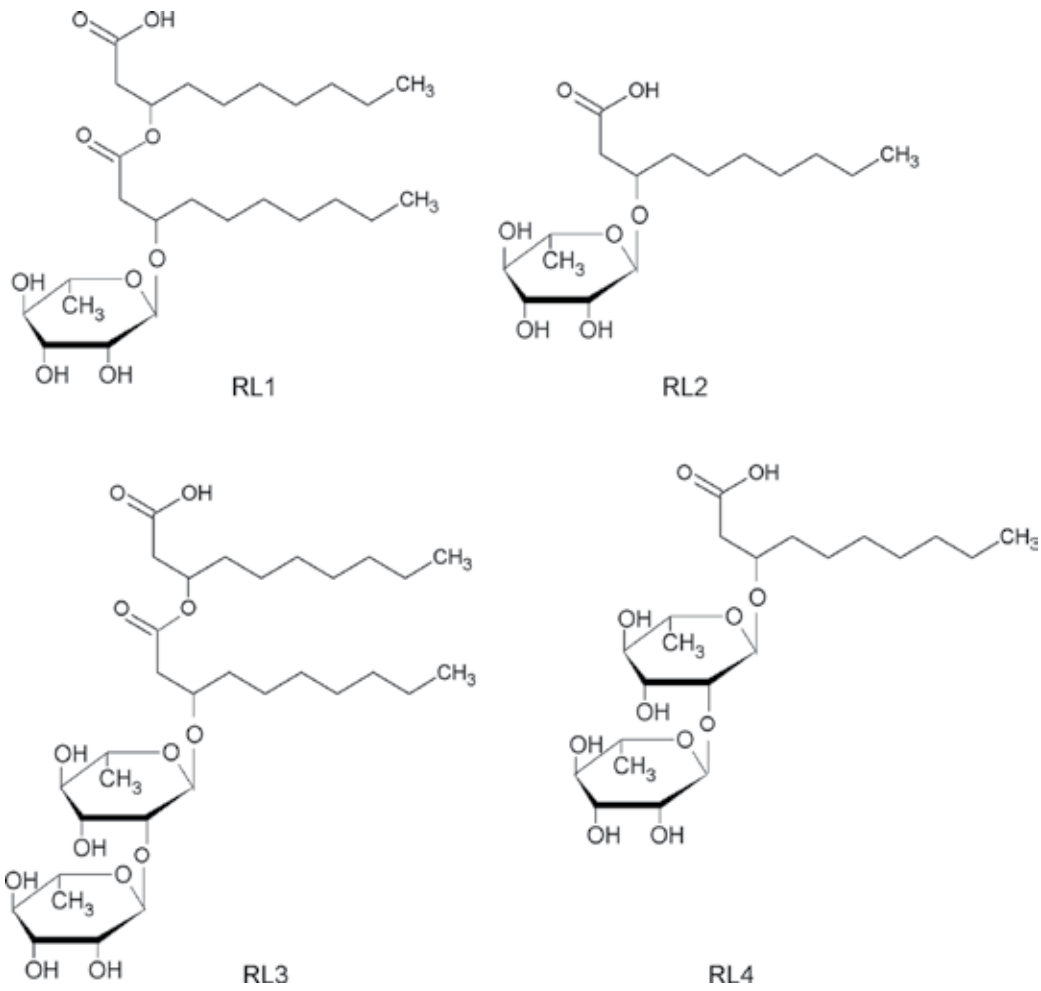

Figure 1. Structure of rhamnolipid congeners: RL1 (mono-rhamno-di-lipidic), RL2 (mono-rhamno-mono-lipidic), RL3 (di-rhamno-di-lipidic) and RL4 (di-rhamno-di-lipidic).

di-phospho (dTDP)-L-rhamnose through several reactions catalyzed by four enzymes that, in *P. aeruginosa,* belong to single operon, *rmlBDA* [11]. dTDP-L-rhamnose has an important role in the regulation of RL biosynthesis, as it is an allosteric regulator for RmlA (which catalyzes transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate and is a sensor enzyme in this metabolic pathway). Also, this molecule is a precursor for other L-rhamnose containing molecules (LPSs and EPSs). dTDP-L-rhamnose affects the production of mono-RL through coexpression of the operons *rmlBDAC* and *rhlAB*, which are responsible for expression of rhamnosyltransferases RhaA and RhaB [86, 87]. However, in *P. aeruginosa,* the QS system has an essential role in regulation of the *rhlAB* operon and, therefore, in RL biosynthesis.

In Section 2.2.1, we stated that *P. aeruginosa* QS has two interrelated systems, *las* and *rhl*, which are linked by the PQS molecule and that their relationship influences the biosynthesis

of various metabolites. Production of RLs is governed by three *QS* molecules: *Pseudomonas* autoinducer 1 (PAI-1, also known as 3O-C_{12}-HSL), *Pseudomonas* autoinducer 2 (PAI-2, also known as C_4-HSL) and PQS. In *P. aeruginosa*, the *las* operon consists of two transcriptional activator proteins, LasR and LasI, which direct the synthesis of PAI-1. The production of RLs is regulated by the *rhl* system. The synthesis of RLs takes place under the coordinated guidance of the *rhlAB* genes. The *rhl* system consists of the transcriptional activator proteins, RhlR and RhlI, which regulate the synthesis of PAI-2. The transcriptional activator RhlR activates the transcription of *rhlAB* operon and gene *rhlC* (encoding RhlC) [10, 11].

The *rhlAB* operon is clustered on *P. aeruginosa* DNA together with *rhlR* and *rhlI*, which together direct the synthesis of all proteins required for RL production (the rhamnosyltransferases and the transcriptional activators, RhlR and RhlI) [10]. RL synthesis is upregulated and promoted at transcriptional level, related to the QS system, by the Vfr (global virulence regulation) and the *pqs* systems through activation of RhlR expression and *rhlRI* operon, respectively [11]. RasL (repressor of *las* system) and AlgR (biofilm formation) downregulate RL synthesis by repression of LasI and *rhlAB*/*rhlI*, respectively [11]. For instance, increasing bacterial cell density induces the *las* system, resulting in an increased concentration of PAI-1 that binds to the transcriptional activator site LasR and forms the LasR–PAI-1 complex. The LasR–PAI-1 complex induces genes controlled by the *rhl* system, including the regulator gene *rasL*, *rhlR* and *pqsH*, required for PQS production. PQS acts as a link between the *las* and *rhl* systems. The activity of these signals depends on their ability to dissolve in and freely diffuse through aqueous solution [10]. PQS induces the *rhlI* gene, which directs the production of PAI-2 that binds to and activates RhlR (RhlR–PAI-2 complex). The RhlR–PAI-2 complex induces genes for RL production, which are controlled by the *rhl QS* system (operons *rhlAB*, *rhlC*, *rhlI*, *rhlR* and *rhlG*). The RLs produced enhance the solubility of PQS in aqueous solutions and promote cell-to-cell communication. This is important because of the role PQS plays in the *P. aeruginosa* stress response, in conditions related to the CF lung environment (oxidative stress and antimicrobial agents) [88].

In conclusion, in the complex QS network, there is a hierarchy between *las* and *rhl* systems in RLs biosynthesis. Furthermore, RL biosynthesis is regulated at the transcriptional level according to nutritional and environmental conditions, as well as at the posttranscriptional level [11, 42]. However, most of the regulatory mechanisms are not completely understood [11, 42].

3.2. *P. aeruginosa* exopolysaccharides

Pseudomonads have the potential to produce various types of EPSs such as alginate, levan, marginalan and cellulose, as well as different heteropolysaccharides and protein polysaccharides complexes [89]. Nearly all *Pseudomonas* isolates, including *P. aeruginosa*, *Pseudomonas putida* and *Pseudomonas fluorescens* can produce alginate as the main acidic EPS [90–92]. Alginate is composed of β-1,4-D-mannuronic and L-guluronic acids linked via β-1,4-glycosidic bonds [93]. Alginates are also produced by *Azotobacter* isolates and some genera of brown and red algae. In comparison to algal alginates, bacterial alginates are O-acetylated at some of the C-2 and C-3 carbons of the mannuronic acid residues and acetylation occurs

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during transport through the periplasm. A high degree of O-acetylation increases the viscosity and flexibility of alginate, as well as its ability to bind water [94].

3.2.1. Diversity of exopolysaccharide structures

P. aeruginosa has the genetic ability to produce at least three polysaccharides: alginate, Psl (polysaccharide synthesis locus) and Pel (pellicle formation locus). Alginate and Psl have different chemical structures (**Figure 2a**) although they have similar biosynthetic mechanisms [89]. In comparison to alginate, a highly O-acetylated linear polymer of 1,4-linked mannuronic acid (M) and guluronic acid (G), Psl is a helicoid polysaccharide composed of a repeating pentamer containing D-mannose, L-rhamnose and D-glucose (**Figure 2b**). The structure of Pel is not completely characterized and it is supposed that it differs from alginate and Psl

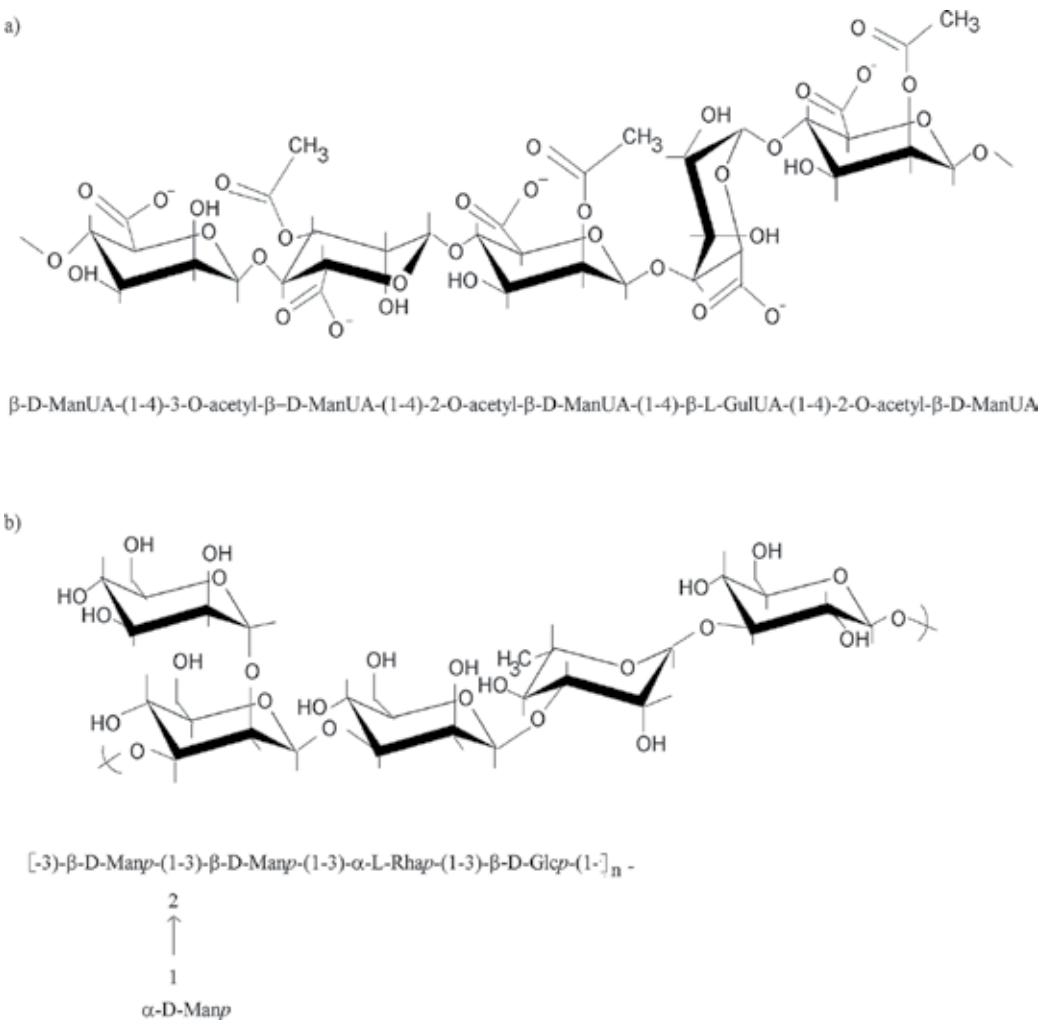

Figure 2. Structures of extracellular polysaccharides produced by *P. aeruginosa*: (a) alginate and (b) exopolysaccharide Psl.

[95]. Pel is proposed to be a glucose-rich polysaccharide, different to cellulose [96]. Each EPS has distinct physiological properties, affecting the cells and the biofilm matrix. While alginate is secreted into the surrounding medium without covalently linking to the cell surface, Psl has helical distribution around the cell surface with a key role in cell-to-cell and cell-to-surface interactions during biofilm formation. Pel forms a connecting matrix allowing it a structured assembly at the air-liquid interface connecting the cells. This matrix could also contain O-antigen-LPS and cyclic glucans [95]. The diversity of EPSs produced by bacterial biofilm subpopulations is one of the proposed *P. aeruginosa* survival strategies for adaptation to environmental changes, as related to the conditions in CF lungs.

3.2.2. Exopolysaccharide biosynthesis and quorum sensing

EPS biosynthesis requires sugar-nucleotide precursors and for alginate production, this is GDP-mannuronate. The enzymes required for GDP-mannuronate production include: (1) the bifunctional enzyme, AlgA which exhibits phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activity; (2) AlgC, a phosphomannomutase; and (3) AlgD, which is a GDP mannose dehydrogenase [97–99]. AlgD catalyzes the first step in alginate biosynthesis, which is responsible for the mucoid phenotype often observed in clinical *P. aeruginosa* from chronically infected CF patients [13].

Alginate is first synthesized as a linear homopolymer of D-mannuronic acid residues. The polymer is then modified in the periplasm through selective O-acetylation by the concerted action of AlgI, AlgJ and AlgF and epimerized by AlgG [100, 101]. Alginate has a reasonably random structure (**Figure 2a**). This differentiates alginate from Psl and numerous *E. coli* capsule polysaccharides, the structures of which are more regular, with repeating subunits (**Figure 2b**). The randomness of alginate's structure occurs because during polymerization, AlgG converts D-mannuronic acid residues to L-guluronic acid and critically, either the C-2 and/or C-3 carbons can have acetylated hydroxyl functional groups, which become available for linking the residues.

AlgC appears to be crucial for general EPS biosynthesis, not just alginate, as it is also required for precursor synthesis of Psl, as well as LPSs and RLs [102, 103]. The LasR from the *las* system might, to some extent, regulate expression of *algC* and *algD*, confirming the correlation of QS systems with EPS production [13].

4. Physiological role of *P. aeruginosa* biosurfactants in CF infection

4.1. Physiological role of rhamnolipids and exopolysaccharides

Among proposed functions of RL biosurfactants, related to their physicochemical properties (surface activity, wetting ability, detergency and other amphipathic-related properties), are promotion of the uptake and biodegradation of poorly soluble substrates, immune modulators and virulence factors [9, 15]. Additionally, these molecules are involved in the process of swarming, as surface wetting agents and chemotaxis stimuli and in *P. aeruginosa* biofilm structuring, maturation (the formation of water channels in mature biofilms) and dispersion

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[10]. Probably because they do not present the profile of typical or traditional virulence factors, RLs are sometimes not considered significant members of the virulence arsenal of *P. aeruginosa* [9]. However, published data strongly demonstrate their importance as virulence determinants and their significant role in infection establishment and persistence [8, 9].

Physicochemical properties of EPSs, such as surface activity, viscosity, flexibility of molecule, as well as its ability to bind water, protect the microbe from dehydration in the unique CF microenvironment following the switch from nonmucoid to mucoid phenotype [94]. In this regard, the *P. aeruginosa* mucoid phenotype is the most studied adaptation in patients with CF and it is directly proportional to overproduction of EPSs, which is widely considered to be a marker for the transition to chronic infection [8, 54]. Alginates are well studied as compounds associated with biofilm formation and invasion of pathogenic microorganisms. The alginate-containing matrix of mucoid *P. aeruginosa* is thought to allow the formation of protected microcolonies and provide increased resistance to opsonization, phagocytosis and destruction by antibiotics [104]. Alginates also have a protective role in *P. aeruginosa* infection because they scavenge free radicals released by activated macrophages *in vitro*, prevent phagocytic clearance and protect the microorganism from the host defense system [13].

4.2. Rhamnolipids and exopolysaccharides in *P. aeruginosa* biofilm formation

Swarming motility is the rapid and coordinated movement of a bacterial population across a surface, which often results in characteristic flowery, dendritic colony shapes on agar plates [105]. This type of colony movement is related to the production of an extracellular slime layer, mainly composed of EPSs and surface active compounds, which is a pivotal feature of swarming cells, acting as a wetting agent that reduces the surface tension [106]. Several studies suggest that *P. aeruginosa* expresses swarming motility and that it requires flagella and the production of wetting agents (RLs and its lipidic precursors HAAs) [85, 107–109]. Also, HAAs and di-RLs actually modulate the swarming process, as di-RLs and HAAs behave as self-produced chemotactic attractants with opposite activity, while mono-RLs seem to be act solely as wetting agents [107, 109]. Additionally, swarming motility is clearly related to biofilm formation [105].

The importance of swarming motility for biofilm formation indicates that RLs are involved in the process of biofilm formation. Indeed, it was shown that RLs enhance adhesion of planktonic cells in the early stages of biofilm development, when an initial microcolony is formed (**Figure 3**). Proposed mechanisms for RL effects on cell adhesion include regulation of cell-surface hydrophobicity and modification of adhesive interactions, especially when nutritional conditions are changed [85, 110–112]. Also, RLs are involved in later differentiation of the biofilm structure, the detachment and dispersion of *P. aeruginosa* cells, where RLs behave as mediators which disturb cell-to-cell and cell-to-substratum interactions and maintenance of open channels inside the biofilm [111, 113]. Furthermore, regulation of RL production by *P. aeruginosa* is regulated not only in temporal terms, but also in quantifiable terms, because overproduction of RLs disrupts biofilm structure or impedes biofilm formation [113].

EPSs also play an important role in biofilm formation and invasion of pathogenic microorganisms. During biofilm maturation, *P. aeruginosa* begin to excrete EPSs, such that the

bacteria in the mature biofilm are encased in a matrix of EPSs that they have produced [114]. Overproduction of alginate is the main indicator of *P. aeruginosa* converting to the mucoid phenotype and is responsible for the notable microbial resistance to antibiotics as well as defense from the host immune system of CF patients (**Figure 3**). The mucoid phenotype of *P. aeruginosa* produces a great amount of alginate as a result of several genes, including *algD,* which encodes GDP-mannose dehydrogenase, responsible for synthesis of alginate precursor [8, 94]. The alginate-containing matrix of the mucoid phenotype allows the formation of protected microcolonies and provides increased resistance to opsonization, phagocytosis and antibiotics, resulting in persistent infection and a worsening prognosis for CF patients [104].

In the context of immune system pathways, polymorphonuclear leukocytes (PMNs) are considered as the central line of defense in innate immunity and they are produced as a predominant response to infection, especially in CF lungs [115]. When PMNs phagocytose bacteria, the host cells produce highly reactive oxygen species, which kill *P. aeruginosa* or induce mutations in the microbial *mucA* gene. However, the alginate produced by mucoid phenotype *P. aeruginosa* is also an oxygen radical scavenger, helping to protect this pathogen against host inflammatory defense mechanisms [116]. Airway epithelial cells play a crucial role during establishment of respiratory infection because *P. aeruginosa* attaches to and enters respiratory epithelia, producing an immune response in the lung by activating lymphocytes at the site of infection [117].

Surfactant protein A (SP-A) is involved in prevention of alginate-induced *P. aeruginosa* invasion of lung epithelial cells. SP-A plays a part in the innate immunity in the lung, with a

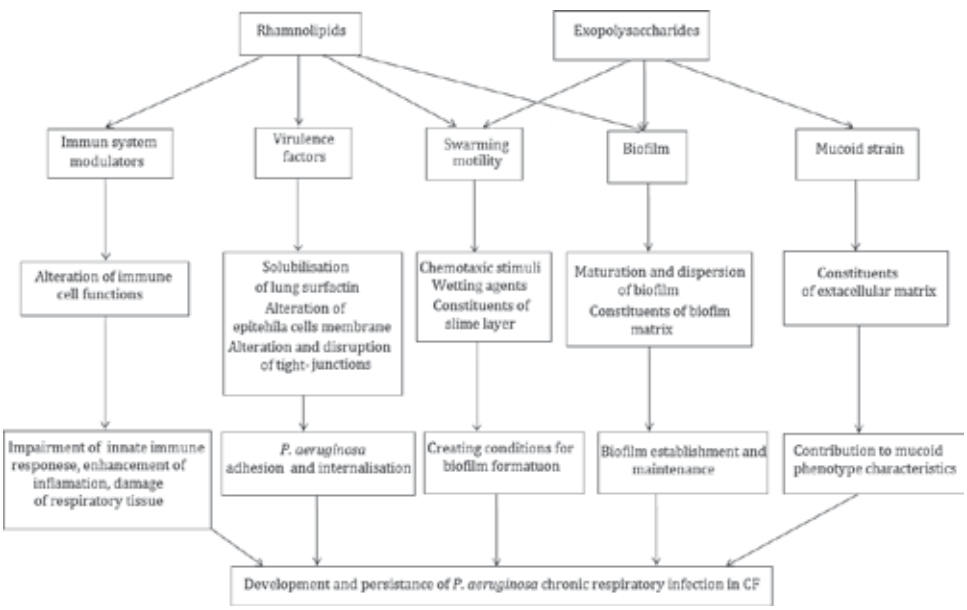

Figure 3. Proposed roles, relations and effects of *P. aeruginosa* biosurfactants RLs and EXPs in development and persistence of chronic respiratory infection in CF patients.

direct role in bacteria opsonization and killing, as well as impairment of bacterial membrane permeabilization [117]. Alginate is surface exposed and levels of SP-A could be crucial in modulating the interaction of *P. aeruginosa* with the epithelial barrier.

4.3. Effect of *P. aeruginosa* rhamnolipids and exopolysaccharides

Respiratory mucosa protects host airways from microbial infection. *P. aeruginosa* and other microbial species capable of causing lung infections have developed mechanisms to overcome this barrier, such as alteration of the apical membrane of epithelial cells or alteration and disruption of tight junctions (TJ) [118]. Proposed mechanisms involve alterations of respiratory epithelial ion transport, inhibition of transcellular ion transport and interference with the normal tracheal ciliary function. Bacterial adherence to the basolateral domain of epithelial cells and internalization are suggested as a potential mechanism of *P. aeruginosa* pathogenicity (**Figure 3**). The physiological pathways of these processes are not still completely clarified, but reports indicate involvement of virulence factors, production of which is controlled by the type III secretion (cytotoxic proteins) and the *las* and *rhl* QS (RLs, elastase) systems [119, 120].

RLs concentration of up to 8 μg/ml was found in the sputum of CF patients infected by *P. aeruginosa* [120], while secretions from a lung removed contained 65 μg/ml RLs [121]. These concentrations of RLs are likely adequate for promotion of *P. aeruginosa* epithelial cell infiltration. Furthermore, this indicates link between elevated levels of RLs and worsening of patient clinical status.

RLs produce damage to the bronchial epithelium and inhibit ciliary function [122–124]. Damage to the bronchial epithelia is related to impairment of the protective layer of lung surfactant in CF patients. Phospholipase C and RLs produced by *P. aeruginosa* can act synergistically to break down lipids and lecithin from lung surfactant [12]. It is believed that RLs, due to their detergency, solubilize the phospholipids in lung surfactant, making them more accessible to cleavage by phospholipase C [12].

The effects of *P. aeruginosa* RLs on the respiratory epithelia function were studied in several animal models [122]. RLs caused ciliostasis and cell membrane damage to rabbit tissue were a secretagogue in cats and inhibited epithelial ion transport in sheep tissue. Additionally, the authors investigated the effect of RLs on mucociliary transport in the anesthetized guinea pig and guinea pig and human respiratory epithelia *in vitro* [122]. Reduction of tracheal mucus velocity (TMV) *in vivo* occurred depending on the applied RL concentration (10 μg of RLs caused cessation of TMV without recovery; 5 μg of RLs reduced TMV by 22.6% over a period of 2 h and 2.5 μg of RLs caused no overall change in TMV). RLs (10 μg) did not disrupt the ultrastructure of guinea pig tracheal epithelium. RL (250 μg/ml) stopped ciliary beating of guinea pig tracheal. Treatment with RL concentration of 100 μg/ml caused immediate slowing of the cilliar beat frequency (CBF) of human nasal brushings, as well as CBF of human nasal turbinate organ culture. Mono- and di-RL had equivalent effects [122]. In addition, RLs stimulate the release of mucus glycoconjugates from feline trachea or human bronchial mucosa [125, 126].

In vitro reconstructed respiratory epithelium was exposed to several *P. aeruginosa* isolates with alterations in genotype: wild type, CF isolates and strains with altered QS system expression

[118]. The authors found that only RL-producing *P. aeruginosa* (those that expressed the *rhl* QS system) was able to infiltrate the epithelia by modulating the permeability of the tissue. The early stages of infection did not correlate with type III secretion and elastase activity [118], in contrast to previous reports [127, 128]. The effect of exogenously applied purified RLs on the epithelial barrier was also studied [118]. The authors used JBR 515, which is commercial mixture of 50% w/v Rha-C_{10}-C_{10} and 50% w/m Rha-Rha-C_{10}-C_{10}. RLs produced by bacteria *in situ* or purified. The applied RLs caused loss of epithelial cell polarity by: incorporation in first, the apical and later, the basolateral epithelial membranes (due to chemical structure); cilia loss; ezrin displacement; and alterations of TJ. The final result was a decrease of transepithelial resistance and higher permeability of respiratory epithelia, without affecting cell viability [118]. After disruption of TJ, paracellular invasion by some *P. aeruginosa*, involving RL deficient strains, was observed, but they were not internalized [118]. This was in contrast to previous reports [129, 130], perhaps due to the *in vitro* conditions used in the studies as difference. Altogether, the importance of RL biosurfactant and the QS system in *P. aeruginosa* invasion of respiratory epithelium is acknowledged, but the exact mechanisms of cell polarity and structure alterations remain unclear.

The effect of RLs on immune system pathways with direct impairment and modulation of immune cell activity is well known [9] (**Figure 3**). RLs are reported to have hemolytic activity on various erythrocyte species; induce direct neutrophil chemotactic activity [130]; enhance the oxidative burst response of monocytes; stimulate and release inflammatory mediators from mast cells and platelets; induce lysis of PMNs; stimulate both chemotaxis and chemokinesis of PMNs (depending on concentration); and enhance production of several interleukins produced by granulocyte-macrophage and nasal epithelial cells (at noncytoxic levels) [131–135]. Furthermore, RLs, especially di-RLs, are cytolytic for human monocyte-derived macrophages and at lower concentrations, they inhibit the phagocytic response of macrophages [136].

The response of *P. aeruginosa* mutants (PAO1 and QS, *rhlA* and *pqsA* deficient) to the presence of PMNs was studied [115]. Previously reported data showed that *in vitro,* PMNs performed their immune function and eliminated QS-deficient *P. aeruginosa* biofilms, although they were incapable of eliminating QS-proficient biofilms [51]. Additionally, purified RLs induced necrosis in PMNs [134]. In biofilm, *P. aeruginosa* (PAO1 wild type) produced increased levels of various virulence factors in response to PMNs, while *P. aeruginosa rhlA* mutant was eliminated by PMNs [115]. Additionally, 2000-fold higher levels of RLs from *P. aeruginosa* PAO1 occurred in biofilm than in surrounding fluid, indicating that RL molecules were grouped around biofilm [115]. Similarly, a *P. aeruginosa rhlA* mutant was cleared more quickly than the wild strain from two *in vivo* mouse models of lung infection [137]. Also, microscopic analysis showed that there were no intact PMNs in close contact with outer layers of biofilm. This correlated with microscopic investigations of *P. aeruginosa* infected *ex vivo* tissues samples from CF lungs, where PMNs were located peripherally [115]. The RLs isolated in this study were a mixture of mono- and di-RL congeners (Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12\Delta}$ and respective di-RL derivates) [137]. Van et al. [137] proposed that RLs have a role as a protective mechanism in biofilm resistance to phagocytosis and supported a "launch a shield" model, where

RLs surround the biofilm and on contact destroy PMNs. This study [137], in correlation with previous reports about QS regulation of bacterial response to PMNs [50, 134] showed that *P. aeruginosa pqsA* mutant was unable to respond to exposure to PMNs by increasing RL production and that there was impairment of the QS hierarchy. These studies show that RLs probably contribute to the inflammatory-related tissue damage observed in lungs of CF patients, which involves complex and tight regulation by the QS system. RL production, though, is not continued because it affects all host cells, not only immune cells and high levels of RL may create conditions (due to inflammation and host tissue damage) which are not favorable for *P. aeruginosa* persistence [137]. This study supports a model by which cross-kingdom-based communication contributes significantly to immunomodulation and evasion and which is one reason studying the infective properties of *P. aeruginosa* is so fascinating.

Modification of membrane LPSs in *P. aeruginosa* is also an important mechanism in the development of chronic infection in CF patients [138–140]. Membrane LPSs in *P. aeruginosa* are composed of three parts: highly acylated lipid A; a central core oligosaccharide bound to lipid A and O-antigen; and a variable polysaccharide composed of repeated units located out from the core [138, 140]. It is not surprising that the structure of LPSs is modified in *P. aeruginosa* isolated from CF patients because of their direct interface position with the pulmonary environment [8]. Compared to normal lipid A, that from CF patients contains more hexa- and hepta-acylated moieties as well as added aminoarabinose, a cationic amino sugar residue which is responsible for resistance to antimicrobials [140]. Acylation levels of lipid A are responsible for LPS recognition by the host and induction of the proinflammatory response, so their modification causes *P. aeruginosa* to be less visible to the host immune system [141]. Also, in CF isolates, O-antigen is lost, due to mutations in genes responsible for O-antigen production. This loss can facilitate chronic persistence in respiratory tracts of CF patients [138–140]. Modification of LPS can directly correlate with overproduction of alginate, which is typical for the mucoid phenotype. Alginate might interact via the carboxylic groups in polyguluronic acid units with modified membrane LPSs in *P. aeruginosa*, across cationic amino sugar aminoarabinose residues. This likely enhances polymerization and facilitates release of EPSs from the membrane. Thus, study of factors that influence increased production of EPSs and RLs, as well as the structure-function relationships of these compounds would likely be of great importance for improved therapy of CF patients [8].

Figure 3 summarizes the proposed roles, relationships and effects of the biosurfactant RLs and EPSs produced by *P. aeruginosa* in the development of chronic respiratory CF infection.

5. Rhamnolipids and exopolysaccharides as targets—current and future perspectives

The importance of biofilm formation and maintenance for the establishment and persistence of *P. aeruginosa* chronic respiratory infection in CF has been discussed in Section 2.2.1. The complex regulation of biofilm development includes the QS network, swarming motility and production of extracellular metabolites and involves significant roles for RLs and EPSs.

Agents	Type	Strategy	Resistance	References
Ticarcillin, Piperacillin Cefrazidime, Cefepime Imipenem, Meropenem Aztreonam	β-Lactams	Impairment of biofilm structure and *QS* inhibitors	Antibiotic cleavage by β-lactamase enzymes, antibiotic expulsion by encoded efflux mechanisms and reduced drug uptake due to loss of outer membrane porin proteins	[16, 155]
Ciprofloxacin	Fluoroquinolones	*QS* inhibitors	Mutations by DNA gyrase and topoisomerase IV enzymes and efflux systems	[155, 156]
Tobramycin, Gentamicin, Amikacin	Aminoglycosides	Impairment of biofilm structure	Aminoglycoside-modifying enzymes AMEs and rRNA methylases as well as efflux mechanisms	[16, 155, 157]
Patulin, penicillin acid, cis-2 decanoic acid	Bacterial metabolites	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 158]
Solenopisin A	Fire ant venom	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 154]
Salicylic acid and 4-nitro-pyridine oxide (4-NPO)	Synthetic compounds	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 152, 154]
Garlic extract	Natural mixture	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 152, 159]
Halogenated furanones from algae *D. pulchra*, Furanone C-30	Synthetic or modifies natural derived furanones	*QS*-inhibitor and *P. aeruginosa* elimination in combination with antibiotics	No resistance	[16, 160]

Table 2. Antibiofilm approaches in therapy of P. *auruginosa* infection of CF patients.

Therefore, a logical approach in preventing and treating chronic *P. aeruginosa* infection in CF patients is focused on antibiofilm strategies. Antibiofilm strategies can take two differing approaches, one common, related to antibiotic therapy and the other novel, related to interruption of QS (**Table 2**). Furthermore, vaccination is proposed as a modern approach to prevent *P. aeruginosa* infection in CF, where virulence factors, such as alginate, have been used as the antigen. However, most vaccines are still in the clinical research phase and have not reached the market [142].

Traditional antibiotic therapy is related to the early colonization period, the only possible phase when *P. aeruginosa* can be eradicated from CF airways [143, 144]. The effectiveness of antibiotics later is significantly reduced due to microbe adaptation mechanisms (membrane changes, efflux system changes, production of various virulence factors and EPS-containing extracellular matrix, mutation and modification of enzymes) [16] (**Table 2**). Furthermore, tobramycin (an aminoglycoside) is the most common antibiotic for *P. aeruginosa* therapy

choice in CF lungs [145]. This is in spite of the fact that alginates produced by the microbe decrease, the movement of aminoglycosides, cationic antimicrobial peptides and quaternary ammonium compounds through *P. aeruginosa* biofilms [27, 146] (**Table 2**). To overcome obstacles related to antibiotic resistance and increase the antimicrobial effects, an inhaled version of tobramycin, as well as liposomal-encased current antibiotics are available. These antibiotic formulations have improved delivery times and provide higher drug concentrations at the site of infection. Additionally, the importance of biofilm formation as having a crucial role in the antibiotic resistance of *P. aeruginosa* (as well as other CF pathogens) is now being recognized. Recent research trends include analysis of biofilm formation in terms of *P. aeruginosa* antibiotic resistance/susceptibility and the potential for antibiotics as efficient therapy agents for biofilm impairment [147–150].

A more novel antibiofilm strategy, QS interruption, is a promising approach for treating CF respiratory infections. In this strategy, the QS system is targeted, due to its regulation of the biosynthesis of RLs and EPSs [151–153]. The QS impairment approach involves identification of molecules which can interrupt QS pathways. Generally, these compounds have one of following mechanisms of activity: blocking production of QS signal molecules, degradation of QS signal molecules or prevention of microbe recognition and response to QS stimuli [16]. Various natural compounds inhibited QS or directly impaired biofilm (**Table 2**) (e.g., garlic extract, metabolites from *Penicillium* spp., salicylic acid, the *P. aeruginosa* metabolite *cis*-2-decanoic acid). Furanones are QS blockers and the furanone produced by *Delisea pulchra and* synthetic furanones, enhanced *P. aeruginosa* elimination in combination with antibiotic therapy [16]. Furanone C-30 repressed 77% of *P. aeruginosa* genes induced by exposure to PMNs [50]. The great advantage of using QS inhibitors in CF therapy is that they are not expected to induce bacterial resistance, because their activity is not closely related to bacterial growth [154].

In the context of the physiological roles of RLs and EPSs discussed in Section 4, these compounds are also promising targets for future strategies in CF therapy related to specific modulation of respiratory mucus [118].

6. Conclusion

RLs and EPSs, biosurfactant molecules, play significant roles in bacterial acquisition, biofilm development and establishment of chronic *P. aeruginosa* infections in CF patients. Specifically, RLs and EPSs are, due to their amphipathic structures and physicochemical properties, involved in processes of respiratory mucus alteration, modulation of immune system defense pathways, biofilm development and maintenance and the *P. aeruginosa* mucoid phenotype. These compounds are responsible for antibiotic resistance and survival and general persistence of *P. aeruginosa* in the specific, dynamic environmental conditions in CF patients' lungs. Consequently, RLs and EPSs are the direct or indirect cause of bad outcomes and high mortality rates among these patients. Currently, therapy generally based on application of antibiotics fails to prevent and treat chronic *P. aeruginosa* infection. Therefore, RLs and EPSs are interesting novel targets for dealing with respiratory infection in CF patients. In addition, the

P. aeruginosa QS system is an important aspect of CF lung infection, as it regulates synthesis of the biosurfactants and other virulence factors, as well as biofilm formation. Future perspectives to prevent and treat *P. aeruginosa* respiratory infections in CF certainly should involve impairment of QS pathways. Finally, further study of potential approaches to modify host respiratory mucus epithelial membranes is required.

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Chapter 7

Anti-PcrV Immunization for *Pseudomonas aeruginosa* Pneumonia in Cystic Fibrosis

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Additional information is available at the end of the chapter

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Abstract

Propagation of multidrug-resistant *Pseudomonas aeruginosa*, which causes endemic nosocomial infections, has become a major concern in various parts of the world. In patients with cystic fibrosis, a major cause of death is respiratory tract infections with antibiotic-resistant *P. aeruginosa*. This condition has prompted medical research aimed at developing effective prophylaxis and treatments that do not rely on conventional antimicrobial agents. The pathogenesis that results in cytotoxicity and mortality in immunocompromised patients infected with *P. aeruginosa* is associated with the type III secretion system of this bacterium. Clinical isolates that are cytotoxic and drug-resistant are involved in acute exacerbation of chronic infectious diseases. The *P. aeruginosa* V-antigen PcrV, a *Yersinia* V-antigen LcrV homolog, is involved as an indispensable component in the translocational process of type III secretory (TTS) toxins. Vaccination against PcrV ensures survival of infection-challenged mice and decreases lung inflammation and injury. Furthermore, anti-PcrV IgG can inhibit translocation of TTS toxins. These observations support the hypothesis that anti-PcrV strategies have the potential as nonantibiotic immune strategies for preventing aggravation of *P. aeruginosa* infections in patients with cystic fibrosis.

Keywords: cystic fibrosis, exoenzyme, PcrV, *Pseudomonas aeruginosa*, type III secretion system, V-antigen

1. Introduction

Propagation of multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) has become a serious concern in various parts of the world [1–4]. Recent outbreaks of extensively drug-resistant

P. aeruginosa (XDR-PA) are threatening to increase XDR-PA colonization of immunocompromised and artificially ventilated patients because efficacious antimicrobial choices against XDR-PA are limited [3–5]. In patients with cystic fibrosis (CF), a major cause of death is respiratory tract infections with *P. aeruginosa*. This condition has led to medical research on the development of effective prophylaxis and treatments that do not rely on conventional antimicrobial agents [6]. Furthermore, recent reports have suggested that XDR-PA strains appear to have a greater ability to promote bacteremia and sepsis [7]. Therefore, the pathogenic mechanisms that are responsible for this infection are important for protecting patients from the lethal consequences of these infections.

The pathogenesis responsible for mortality in *P. aeruginosa* pneumonia is associated with the development of septic shock and multiple organ failure. This is because certain *P. aeruginosa* strains have the ability to cause necrosis of the lung epithelium and disseminate into the circulation [8]. Advances in genomic analysis and cellular microbiology have shown that damage to the lung epithelium is associated with expression of toxins. These toxins are directly translocated into eukaryotic cells through the type III secretion system (TTSS) of *P. aeruginosa* [9]. Unlike classic type I and type II secretion systems, the newly identified TTSS, through which bacteria directly transfer their toxins from the bacterial cytosol to the eukaryotic cell cytosol, were discovered in most pathogenic Gram-negative bacteria (**Figure 1**) [10]. Four type III secretory (TTS) toxins, called ExoS, ExoT, ExoU, and ExoY, have been identified in *P. aeruginosa* [11, 12]. ExoS is the 49-kDa form of exoenzyme S, which is a bifunctional toxin that has ADP-ribosyltransferase and GTPase-activating protein (GAP) activities [13]. ExoS disrupts endocytosis,

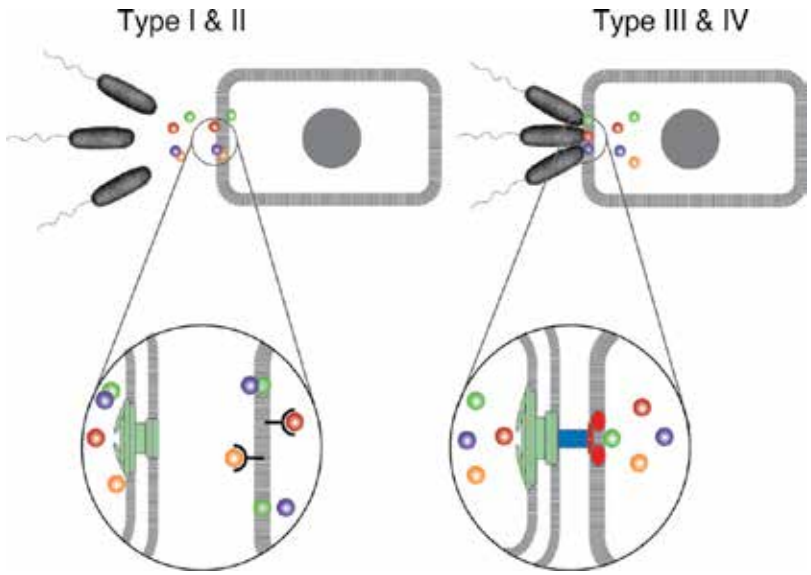

Figure 1. Toxin secretion systems in Gram-negative bacteria. In the types I and II secretion systems, bacteria secrete toxins into the extracellular space (left side of figure). As one example, secreted toxins are captured by surface receptors on the eukaryotic cell membrane and are then transferred to the cytosol. In the types III and IV secretion systems, bacteria secrete toxins directly into the cytosol of target eukaryotic cells through their secretion apparatus (right side of the figure). The mechanism whereby the secreted toxins are transferred to the eukaryotic cell cytosol is called translocation.

the actin cytoskeleton, and cellular proliferation. ExoT, which is a 53-kDa form of exoenzyme S with 75% sequence homology to ExoS, also exerts GAP activity and interferes with cell morphology and motility [13–15]. ExoY is a nucleotidyl cyclase that increases intracellular levels of cyclic adenosine and guanosine monophosphates, resulting in the formation of edema [12]. ExoU exhibits phospholipase A_2 activity activated by host cell ubiquitination after translocation. ExoU is a major pathogenic cytotoxin that causes alveolar epithelial injury and necrosis of macrophages [16–19].

In this review, we summarize the TTSS of *P. aeruginosa* and its association with CF. We also review the development of immune therapies, including passive and active immunization against the TTSS-associated virulence of *P. aeruginosa*.

2. Cytotoxic or invasive *P. aeruginosa* strains

In most acute clinical manifestations of *P. aeruginosa* infection, severe pneumonia occurs in patients under ventilatory management and in immunocompromised patients [2, 3]. Patients with severe *P. aeruginosa* pneumonia frequently develop sepsis and subsequent multiorgan failure [7].

In the mid-1990s, researchers investigating *P. aeruginosa* reported that the strains expressing ExoS (49-kDa type exoenzyme S) had low cytotoxicity to eukaryotic cells, whereas the strains that did not express ExoS had strong cytotoxicity [20, 21]. Therefore, at that time, *P. aeruginosa* strains were classified as either a cytotoxic *exoS*$^-$ type or an invasive *exoS*$^+$ type, depending on the genotypes of the 49-kDa exoenzyme S [20]. The 53-kDa exoenzyme S (ExoT) was discovered as a gene product that was distinct from ExoS [14, 15]. However, because cytotoxic *exoS*$^-$ type- and invasive *exoS*$^+$ type-strains both possessed ExoT, the mechanism for cytotoxic virulence could not be explained solely on the basis of possessing the ExoT gene. Therefore, the mechanism whereby the cytotoxic characteristics of *exoS*$^-$*exoT*$^+$ type-strains develop is unknown. In 1997, a new cytotoxin, ExoU, was identified [16]. *P. aeruginosa* strains that do not have *exoU* always possess *exoS*. Therefore, the genotype of cytotoxic strains is *exoS*$^-$*exoT*$^+$*exoU*$^+$ and the genotype of invasive strains is *exoS*$^+$*exoT*$^+$*exoU*$^-$ [20, 21]. While searching for major cytotoxins in *P. aeruginosa*, *P. aeruginosa* exoenzymes were found to translocate directly into the eukaryotic cytosol through the TTS mechanism [22]. Among the four TTS cytotoxins, ExoS, ExoT, ExoU, and ExoY, *P. aeruginosa* clinical isolates that cause severe sepsis and mortality express TTS ExoU [16, 23–25]. After discovery of ExoU, studies showed the enzymatic action of phospholipase A_2, which is activated by a eukaryotic cell factor after translocation into the eukaryotic cell cytosol [17–19].

3. Genomic analyses of *P. aeruginosa* strains

The whole genome sequencing project by the *Pseudomonas* Genome Project was completed for the *P. aeruginosa* strain PAO1 in 2000 [26]. Since this time, comparative genomics is currently

on-going between the PAO1 reference strain and various clinical isolates of which the characteristics differ from PAO1 (**Figure 2**). In this research field, the major question is how much do the strains differ from each other at the gene level, especially between strains isolated from patients with acute infections and strains isolated from chronically infected patients with CF. The genetic mechanisms underlying multidrug resistance are also of major interest in these comparative genomic studies.

The clinical isolate UCBPP-PA14 is cytotoxic and is similar to the laboratory strain PA103 UCBPP-PA14 that has the TTS phenotype ExoS$^-$ExoT$^+$ExoU$^+$ExoY$^-$, whereas invasive PAO1 is ExoS$^+$ExoT$^+$ExoU$^-$ExoY$^+$ [16, 20]. Therefore, genomic analysis of UCBPP-PA14 was conducted to identify their phenotypic differences. As a result, two pathogenicity islands that do not exist in PAO1 were discovered in the UCBPP-PA14 genome [27]. Thereafter, researchers found that clinical strains containing *P. aeruginosa* pathogenicity island-2 (PAPI-2) and *exoU* display the cytotoxic phenotypes that are responsible for acute lung injury in animal models [16, 27]. Concurrently, strains harboring PAPI-2 and *exoU* were found to have deletional mutation of the *exoS* gene, which creates the *exoS*$^-$ genotype in PA103 and UCBPP-PA14 [27].

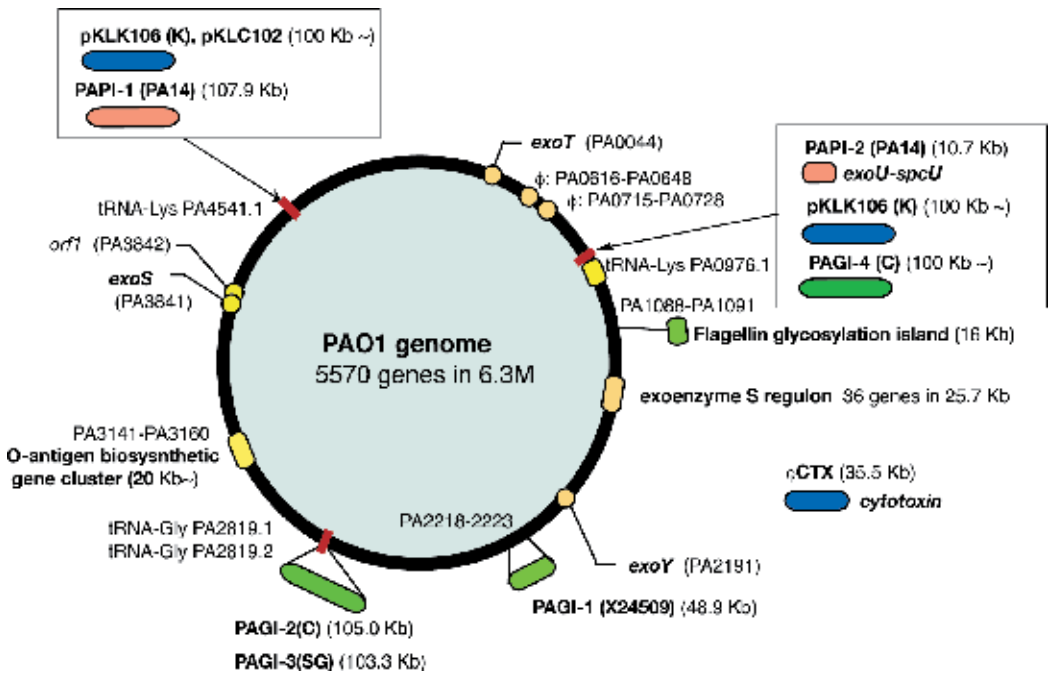

Figure 2. The PAO1 reference strain genome and its pathogenic gene configuration. The *P. aeruginosa* strain PAO1 possesses 5570 genes in its 6.3-Mb circular genome. The exoenzyme S regulon is a gene cluster (25.7 kb) for type III secretion. Type III secretory toxin genes, such as *exoS*, *exoT*, and *exoY*, are scattered throughout the genome. The *P. aeruginosa exoU* gene, which is located in an insertional pathogenic gene cluster in pathogenicity island-2 (PAPI-2), was discovered in the UCBPP-PA14 virulent clinical strain.

4. The type III secretion system of *P. aeruginosa*

4.1. Components of the type III secretion system and the exoenzyme S regulon

The TTSS is composed of the following: (1) secretion apparatus (injectisome), (2) translocators, (3) a set of secreted toxins, and (4) regulatory components [28]. In the *P. aeruginosa* genome, a pathogenic gene cluster called the exoenzyme S regulon encodes the genes for regulation, secretion, and translocation of the TTSS [9, 11] (**Figure 3**). In the exoenzyme S regulon, the *exsCBA* operon encodes the transcriptional activator protein ExsA, which regulates expression of exoenzyme S and its co-regulated proteins (**Figure 4**) [11]. Four TTS toxins, ExoS, ExoT, ExoU, and ExoY were identified in *P. aeruginosa* (**Table 1**) [11, 12, 16]. The genes for these TTS toxins are scattered in the genome [26]. In contrast, *exoU*, which is located in the insertional gene pathogenic cluster PAPI-2, is present with its chaperone protein gene *spcU* only in the genomes of cytotoxic strains, such as PA103 and UCBPP-PA14 [16, 27, 29].

4.2. The *pcrGVHpopBD* translocation operon

One operon in the exoenzyme S regulon, called *pcrGVHpopBD*, encodes five proteins associated with translocational processes for TTS toxins [11]. These proteins are PcrV, PopB, and PopD, and their chaperones are PcrG and PcrH. PcrV, which is the *P. aeruginosa* V-antigen, is a cap structure component on the tip of the injection needle formed by PscF in the secretion apparatus [30] (**Figure 5**). The genetic organization for the exoenzyme S regulon shares

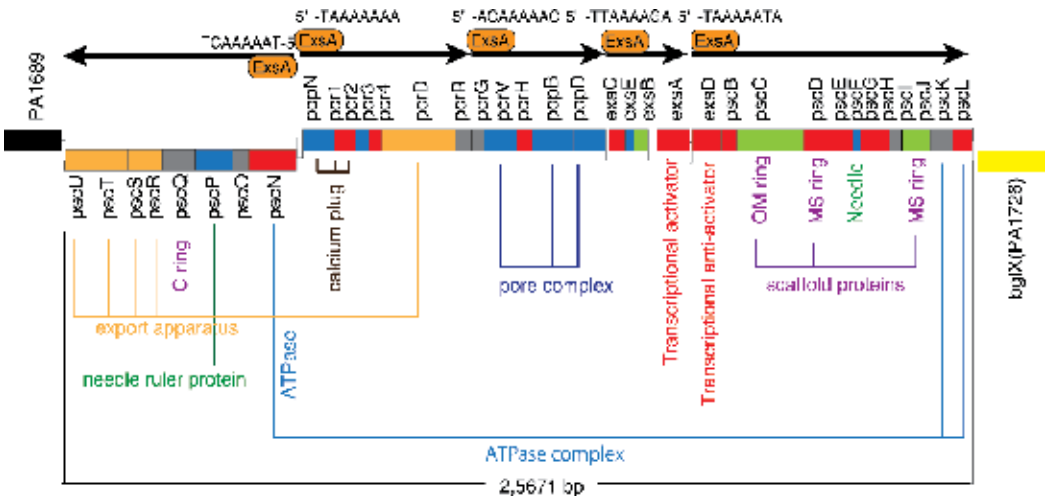

Figure 3. Genomic structure of the exoenzyme S regulon. The type III secretion regulatory region (25.5 kb), found as a gene cluster, was named the exoenzyme S regulon. The exoenzyme S regulon comprises five operons, including 36 genes for transcription (*exsA-exsD*), genes encoding the secretion apparatus (*pscB-pscU*), and others encoding translocation-related proteins (*pcrGVHpopBD*). The *exsCBA* operon encodes the transcriptional activator ExsA protein, which regulates expression of exoenzyme S and co-regulated proteins.

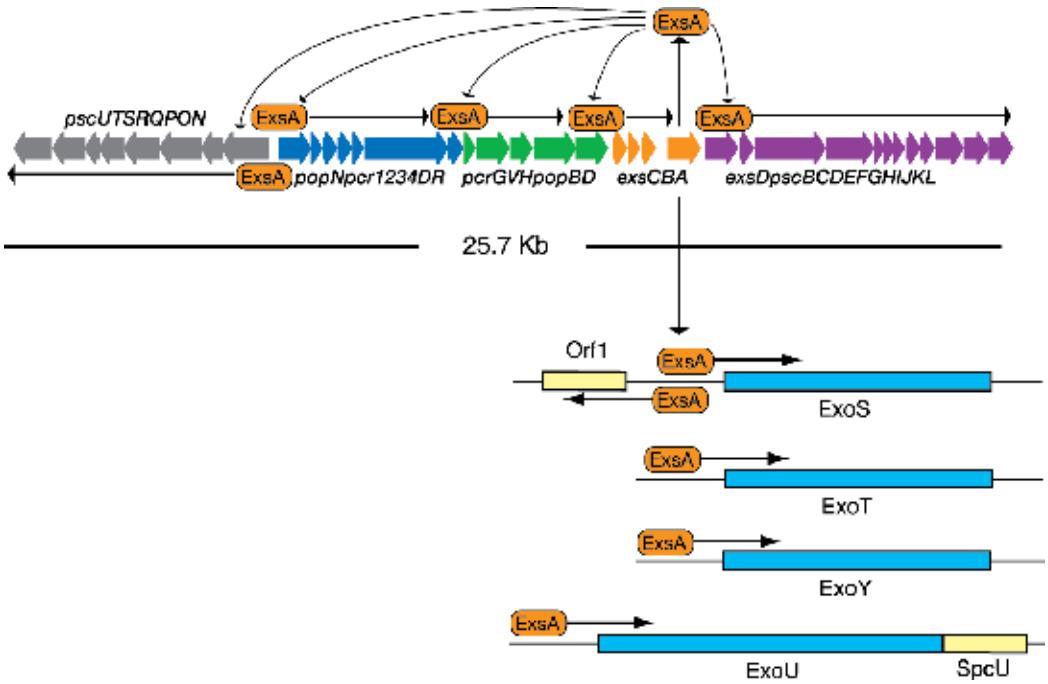

Figure 4. Type III secretion regulation in *P. aeruginosa* by the ExsA transcriptional activator. The ExsA transcriptional activator protein activates five operons in the exoenzyme S regulon via five ExsA binding motifs "TxAAAAxA". ExoT, ExoU, and ExoY were identified in *P. aeruginosa*. Genes for ExoS, ExoT, and ExoY are scattered in the genome. The *orf1* gene, which encodes a chaperone protein for ExoS, is next to *exoS*. The *exoU* gene, which is located in the insertional gene pathogenic cluster PAPI-2, is present with its chaperone protein gene *spcU* only in the genomes of cytotoxic strains, such as PA103 and UCBPP-PA14.

Toxins	Size (kDa)	Enzymatic activity	Action
ExoS	49	FAS-dependent ADP-ribosyltransferase	Antiphagocytosis, inhibition of endocytosis
ExoT	53	Small GTPase activating protein activity	Inhibition of tissue repair
ExoU	74	Phospholipase A2	Cytotoxin, lipid degradation
ExoY	42	Adenylate cyclase	Edema formation, anti-inflammatory

Table 1. Type III secretory toxins in P. aeruginosa.

the most homology with the *Yersinia* Yop virulon [31]. *Yersinia* LcrV has been reported to be a molecular target competing with TTSS virulence. Similarly, *P. aeruginosa* PcrV also is a molecular target that can compete with TTSS virulence in *P. aeruginosa* [32, 33]. This competition with TTSS virulence will be discussed later in this review.

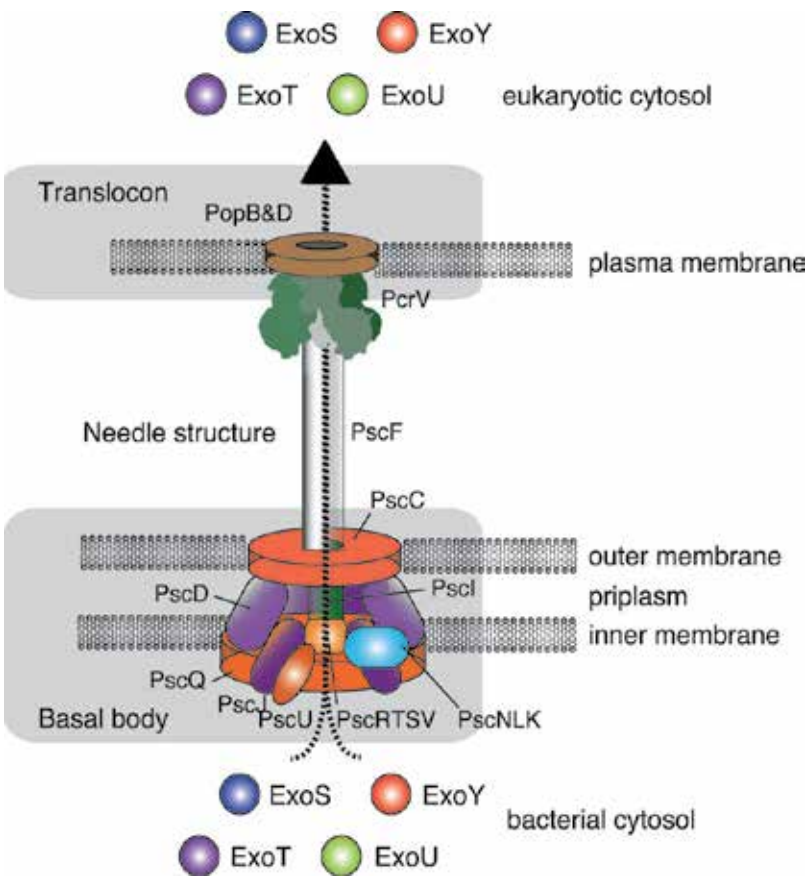

Figure 5. The type III secretory apparatus of *P. aeruginosa.* The *P. aeruginosa* type III secretory apparatus comprises many protein components as follows: a cap component, PcrV; a needle component, PscF; an outer ring component, PscC; and basal components, including PscJ, ATPase PscN, and others. Four type III secretory toxins, ExoS, ExoT, ExoU, and ExoY, are injected directly into the cytosol of target eukaryotic cells through the type III secretory apparatus. Translocated toxins are activated by specific eukaryotic cell cofactors. Following activation, ADP-ribosyltransferase activity is shown by ExoS, whereas ADP-ribosyltransferase and GTPase-activating protein activity is shown by ExoT. Activated ExoU has phospholipase A_2 activity, and ExoY exhibits adenylate cyclase activity.

4.3. *Yersinia* V-antigen LcrV and *P. aeruginosa* PcrV

Historically, *Yersinia* LcrV has been referred to as the *Yersinia* V-antigen. Approximately 50 years ago in the UK, Burrows et al. reported that *Yersinia* V-antigen was an antigen substance associated with the pathogenic toxicity [34–38] of this bacterium. They found that only *Y. pestis*, with the antigenic factor they called the V-antigen, induced immunity in a mouse model of infection [34–38]. In 1986, the gene encoding the LcrV V-antigen, *lcrV*, was cloned from the Low-calcium response (LCR) operon of the *Y. pestis* pCD1 plasmid [39]. A genetic mutation experiment then showed that LcrV is essential for translocation of the toxin [32]. Additionally, antibodies against LcrV were reported to be capable of blocking transfer of the toxin [32]. As well as *Yersinia* LcrV in the TTSS, *P. aeruginosa* PcrV is essential for transition of the TTSS toxin, and the antibody against PcrV can block transition of the TTS toxin [33]. PcrV might play a role

in connecting the needle rod (composed of PscF) to the pore (formed with PopB/PopD) on the eukaryotic cell membrane. Indeed, electron microscopy has successfully visualized V-antigens as cap structures on the mushroom needle tip portion of the protein [30]. Specific blocking antibodies against the V-antigen also block translocated toxin from binding to the top part of the cap structure [32, 40].

5. *P. aeruginosa* type III secretory toxins

5.1. ExoS and ExoT

In the late 1970s, *P. aeruginosa* exoenzyme S was discovered as an adenosine diphosphate ribosyltransferase that was distinct from exotoxin A [41, 42]. However, in the mid-1990s, exoenzyme S activity was determined to be the result of two highly homologous toxins, termed ExoS (49-kDa exoenzyme S) and ExoT (53-kDa exoenzyme S), which are encoded in two separate portions of the *P. aeruginosa* genome [14, 15]. ExoS and ExoT were also found to be secreted by the TTS mechanism [15, 22].

5.1.1. ADP-ribosyltransferase activity

ExoS and ExoT are two immunologically indistinguishable proteins that co-fractionate with exoenzyme S activity [14]. ExoS and ExoT encode proteins of 457 and 453 amino acids, respectively, and share 75% amino acid identity. ExoT possesses approximately 0.2% of the ADP-ribosyltransferase activity of ExoS [14, 15]. ExoT diminishes motility of macrophages and phagocytosis, at least in part through disrupting the eukaryotic cellular actin cytoskeleton, and also blocks wound healing [43, 44]. The ExoS carboxyl terminal catalyzes transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of different proteins, including the intermediate filament protein, vimentin [45–47].

5.1.2. GTPase-activating protein activity

The amino terminal domains of ExoS and ExoT have been characterized as GAPs of Rho GTPases [48]. The Rho GAP activity of ExoS stimulates reorganization of the actin cytoskeleton by inhibiting Rac and Cdc42, and induces formation of actin stress fibers by inhibiting Rho [49]. These domains, which include catalytic arginines, share sequence homology with not only *Yersinia* YopE and *Salmonella* SptP, but also with mammalian Rho GAP proteins, such as hsp-120GAP, hsNF1, dmGAP1, and sclRA1. Biochemical studies have shown that ExoT possesses GAP activity for RhoA, Rac1, and Cdc42, and interferes with Rho signal transduction pathways, which regulate actin organization, exocytosis, cell cycle progression, and phagocytosis [50, 51].

5.2. ExoU

A specific isogenic mutant of the cytotoxic *P. aeruginosa* strain PA103, which does not have ExoS and is genetically modified to lack ExoT, is still cytotoxic *in vitro.* This mutant causes epithelial injury *in vivo*, indicating that another cytotoxin is responsible for the observed pathology [16, 17]. In 1997, ExsA-activated ExoU was discovered to be a major virulence factor causing lung injury and the *exoU* gene was cloned from the cytotoxic strain PA103 [16]. A region downstream

of *exoU* encodes a small 15-kDa protein named SpcU, which functions as a chaperone for ExoU [29]. ExoU is a TTS protein of *P. aeruginosa* that is necessary for epithelial cell cytotoxicity *in vitro* and virulence in a mouse model of pneumonia [16].

5.2.1. Patatin-like phospholipase A_2 activity

ExoU contains a potato patatin-like phospholipase A (PLA) domain [17]. Patatin is a member of a multigene family of vacuolar storage glycoproteins with lipid acyl hydrolase and acyl transferase activities. Alignment of ExoU, potato patatin, and human PLA2 shows three highly conserved regions in the ExoU amino acid sequence as follows [17]: (1) a glycine-rich nucleotide binding motif, GXGXXG/A (position 111–116 in ExoU); (2) a serine-hydrolase motif, which includes a serine active site for cPLA2, GXSXG/S (position 140–144 in ExoU); and (3) an active site motif containing aspartate for cPLA2, DGG/A (position 344–347 in ExoU) (**Figure 6**).

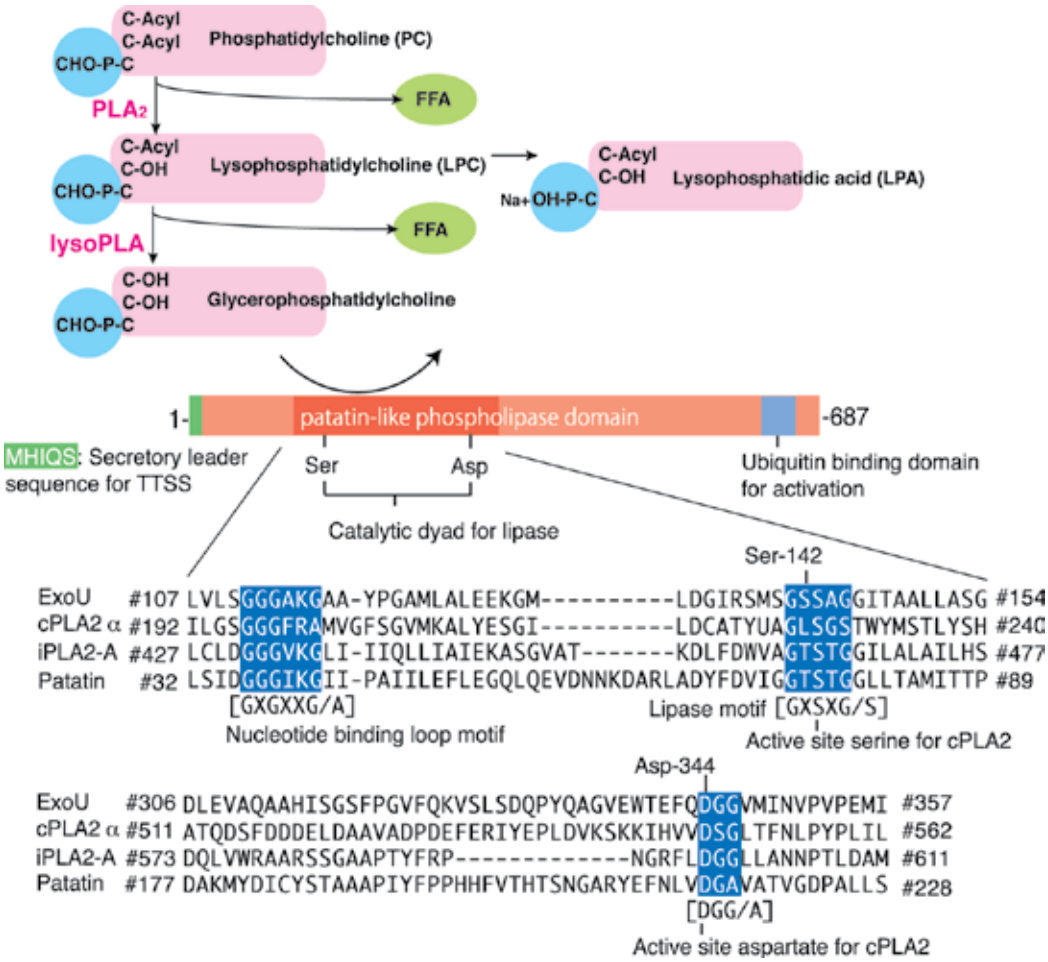

Figure 6. Enzymatic activity and consensus motifs in ExoU. *P. aeruginosa* ExoU, a major factor causing cytotoxicity and epithelial injury in the lungs, contains a patatin domain that catalyzes membrane phospholipids through its phospholipase A_2 activity. Homology in the amino acid sequence, with a catalytic dyad in the primary structure, is found among patatin, mammalian phospholipase A_2 (cPLA$_2$-α and iPLA$_2$), and ExoU. FFA: free fatty acids.

5.2.2. Phospholipase A_2 activity and acute lung injury

Site-directional mutations in the predicted catalytic site of ExoU cause a loss of lysophospholipase A activity [52]. Airspace instillation of virulent *P. aeruginosa* expressing ExoU causes acute lung injury and death in infected mice [53]. However, airspace instillation of isogenic mutants secreting catalytically inactive ExoU is non-cytotoxic and this does not cause acute lung injury or death in these mice [53]. Therefore, virulent *P. aeruginosa* causes acute lung injury, with concomitant sepsis and mortality, via cytotoxic activity derived from the patatin-like phospholipase domain of ExoU. Cells targeted by ExoU through the TTSS are not only epithelial cells, but also macrophages [54]. Through the TTSS, ExoU is activated after its translocation into the cytosol of eukaryotic cells [55–57]. Ubiquitin and ubiquitin-modified proteins are associated with the activation of ExoU [18, 19].

5.3. ExoY

ExoY has adenylate cyclase activity and is secreted by the TTS mechanism [12]. The primary ExoY sequence shares homology with sequences of the extracellular adenylate cyclases of *Bordetella pertussis* (CyaA), *Bacillus anthracis* (EF), and *Y. pestis* insecticidal toxin [12]. An unknown eukaryotic cell factor, distinct from calmodulin, enhances recombinant ExoY catalysis. Infection of eukaryotic cells with *P. aeruginosa* that produce catalytically active ExoY results in an elevation of intracellular cAMP and morphological changes in cells. ExoY increases the permeability of lung endothelial cells and alters Chinese hamster ovary cell morphology but does not result in acute cytotoxic responses. Ninety percent of clinical isolates that are tested show the presence of the *exoY* gene in DNA hybridization experiments [12]. ExoY production may play a role in protecting the bacterium from local phagocytic cells [58].

6. Cystic fibrosis and *P. aeruginosa* type III secretion

6.1. *P. aeruginosa* pneumonia and cystic fibrosis

Respiratory infections with *P. aeruginosa* are the major causes of morbidity and mortality in individuals with CF. *P. aeruginosa* isolates from newly infected patients with CF resemble those from acutely infected non-CF patients, and have a number of virulence factors including flagella, pili, pyocin, pyoverdin, and the TTSS [59, 60]. Expression of these virulence factors is considered to be essential for successful development of infection at an early stage of infection in patients with CF. However, at the chronic stage of infection, triggered by high selective pressure in CF lungs and by antibiotic treatments, *P. aeruginosa* gradually generates genotypes and phenotypes that are specially adapted to the lungs in CF. These include overproduction of alginate (mucoid phenotype), loss of lipopolysaccharide O-antigen components, loss of motility, resistance to antibiotics, virulence factor loss, and adapted metabolism [61]. These changes might be essential for *P. aeruginosa* to facilitate evasion of the host defense mechanisms and immune surveillance [62].

6.2. Epidemiological studies of isolates from patients with cystic fibrosis

In our epidemiological study that analyzed clinical isolates, there was a subset of isolates that displayed the TTS phenotype ExoS$^-$ExoU$^-$ with extensive drug-resistant characteristics [63]. Most of these isolates were from chronic infections in patients with CF. Therefore, clinical isolates of *P. aeruginosa* are classified into three subgroups depending on their ExoS and ExoU phenotypes. ExoS$^+$ExoU$^-$ strains are invasive and cause infections in burns tissues, whereas ExoS$^-$ExoU$^+$ strains are cytotoxic and cause acute pneumonia and sepsis. Most strains isolated from chronic infections in CF patients are ExoS$^-$ExoU$^-$ (**Figure 7**). *P. aeruginosa* strains that are isolated from acutely infected patients show positive phenotypes for TTS proteins (ExoS, ExoU, and PcrV) and the positive O-antigen phenotype. However, strains that are isolated from chronic infections of patients with CF are frequently the O-antigen phenotype (−), TTS protein phenotypes (−), and the mucoid phenotype (+) with increased antibiotic resistance (**Figure 8**). Recent studies have shown that TTSS production, as well as other virulence factors, such as flagella, pili, pyocin, and pyoverdine, are attenuated in many isolates from chronically infected patients with CF [64–66]. The results of several studies that investigated the relationship between CF clinical isolates and the TTSS in *P. aeruginosa* are shown in **Table 2** [61, 67–71]. Two of the six studies were longitudinal and followed the same patients with CF. Additionally, four studies performed genotype analysis on strains, and five others performed immunoblot analysis of TTS proteins. Findings from these epidemiological studies suggest that CF isolates from children are more virulent with a positive TTSS phenotype than isolates that are recovered from adults. These studies also suggest that isolates from initial infections are more virulent than isolates from subsequent infections. The ratio between ExoS$^+$ExoU$^-$ and ExoS$^-$ExoU$^+$ differed in each study. However, a more recent report from Hu et al. showed that 7 isolates among 40 in total from subsequently occurring infections were ExoU$^+$ [71]. These findings suggest the potential pathogenic involvement of ExoU-associated virulence, even in patients with CF.

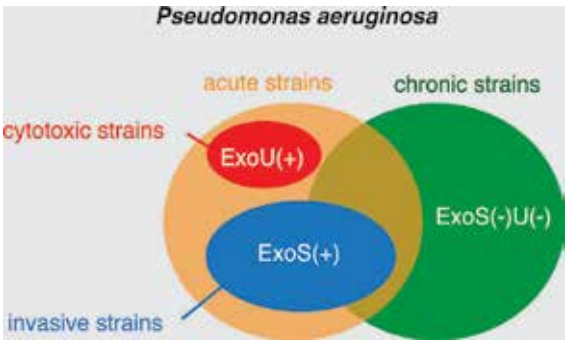

Figure 7. Type III secretory toxin phenotypes in *P. aeruginosa* clinical isolates. *P. aeruginosa* clinical isolates can be classified into three subgroups depending on their ExoS and ExoU phenotypes. ExoS(+)ExoU(−) stains are invasive and cause infections in burnt tissue, whereas ExoS(−)ExoU(+) strains are cytotoxic and cause acute pneumonia and sepsis. Most strains that are isolated from chronic infections in patients with cystic fibrosis are ExoS(−)ExoU(−).

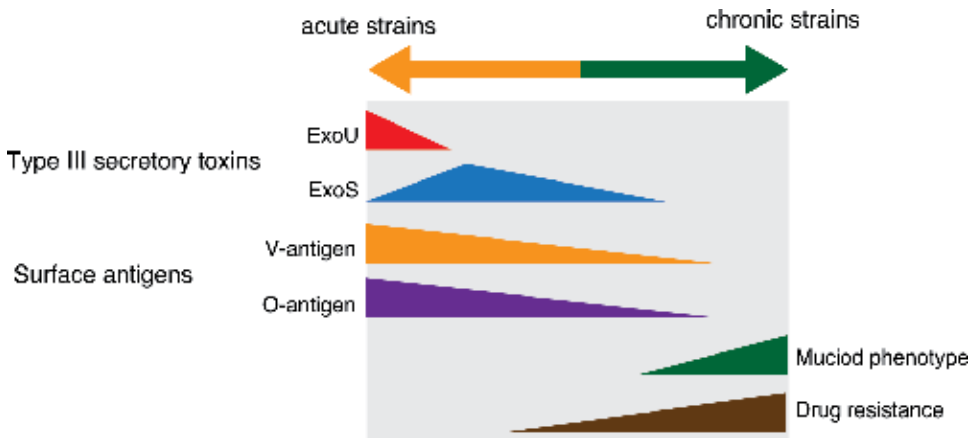

Figure 8. Phenotypic variation in *P. aeruginosa* clinical isolates. *P. aeruginosa* strains that are isolated from acutely infected patients are positive for type III secretory proteins, such as ExoS, ExoU, and PcrV, and are O-antigen positive. In contrast, strains that are isolated from chronic infections in patients with cystic fibrosis are frequently O-antigen-negative and type III secretory protein-negative, but are mucoid with increased antibiotic resistance.

6.3. Type III secretion and cystic fibrosis isolates

Most studies have reported that the proportion of *P. aeruginosa* strains secreting TTS proteins decreases over the duration of *P. aeruginosa* infection. Jain et al. showed a significant inverse correlation between the percentage of TTS proteins and the duration of *P. aeruginosa* infection [67]. They also reported an association between the proportion of TTS protein-secreting isolates and a decline in the rate of forced expiratory volume in 1 s in patients who still harbor at least some TTS-positive isolates. Other reports that investigated the genotype and phenotype of the TTSS showed that all *P. aeruginosa* strains harbor at least some TTSS genes (*exoS*, *exoT*, *exoU*, *exoY*), regardless of the expression of TTS proteins (ExoS, ExoT, ExoU) [66, 69–71]. This suggests that the TTSS regulon may remain intact and the expression of TTSS can be reversible. There are other variants called rough small-colony variants in *P. aeruginosa*, and these have been isolated from chronically infected patients with CF [72, 73]. These variants are hyperpiliated and hyperadherent, and differ from the mucoid phenotype in their secretion of TTS proteins. Their remarkably high resistance to several antibiotic classes enables their persistence in the lungs in CF.

6.4. Comparative genome studies on recent *P. aeruginosa* isolates

Comparative genomics on the reference PAO1 strain and isolates from patients with CF are on-going. In 2003, two comparative studies between CF isolates and PAO1 were reported. These studies demonstrated that clinical strains do not express TTSS, whereas most of them that are isolated from chronic infections possess this gene cluster [74, 75]. Additionally, these studies show that 10% of genes in CF isolates do not exist in the PAO1 genome, and half of them are newly identified genes.

Year	Author	Reference	CF clinical isolates, *n*	TTSS secretion (+), *n* (%)	Exoenzyme genotype, *n*				Exoenzyme phenotype, *n* (% positive by genotype)			
					S	*T*	*Y*	*U*	S	T	Y	U
2000	Dacheux et al.	[69]	29	8 (27.5%)	28	28	28	3	8 (28.6)	8 (28.6)	NA	0 (0)
2004	Jain et al.	[61]	235 (CI, children)	40 (18%)	NA	NA	NA	NA	35 (88)	40 (100)	NA	2 (5)
			200 (CI, adults)	8 (4%)					8 (100)	8 (100)	NA	0 (0)
			35 (NI)	17(49%)					17 (100)	17 (100)	NA	0 (0)
2005	Lee et al.	[68]	7	5 (71.4%) (initial)	7	NA	NA	0	5 (71.4)	NA	NA	0 (0)
				3 (42.8%) (subsequent)	6	NA	NA	1	2 (28.6)	NA	NA	1 (14.3)
2007	Wareham et al.	[70]	75	39 (52%)	66	75	74	12	NA	NA	NA	9 (75)
2008	Jain et al.	[67]	1299 (CI, children)	29.1%	NA	NA	NA	NA	NA	NA	NA	NA
			1217 (CI, adult)	11.5%								
			135 (NI)	45.2%								
2013	Hu et al.	[71]	52 (initial)	43 (82.7%)	44	NA	NA	8	36 (82)	NA	NA	7 (88)
			40 (subsequent)	26 (65%)	33	NA	NA	7	19 (58)	NA	NA	7 (100)

Table 2. Studies on the relationship between cystic fibrosis and type III secretion in *P. aeruginosa*.

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Recent reports have indicated that the combination of carbapenem and fluoroquinolone resistance and the presence of the gene encoding the TTSS ExoU effector in *P. aeruginosa* are the strongest predictors of development of pneumonia [76–78]. Further investigations have suggested that the fluoroquinolone-resistant phenotype and the *exoU*$^{+}$ genotype of *P. aeruginosa* cause poor clinical outcomes in patients with *P. aeruginosa* pneumonia [76–79]. Several genome sequence analyses of small colony variants of *P. aeruginosa* have been reported recently [80–85]. These studies showed multifactorial antibiotic-resistance mechanisms, such as overexpression of efflux mechanisms, LPS modification, and a drastic downregulation of the *Pseudomonas* quinolone signal quorum-sensing system. These reports suggest that, over the last 15 years, wide-spread global carbapenem and fluoroquinolone use has rapidly enhanced propagation of virulent and drug-resistant *P. aeruginosa* strains.

7. Anti-PcrV strategies in *P. aeruginosa* infections

Recent outbreaks of XDR-PA are threatening to increase colonization by MDR-PA in immunocompromised patients because efficacious antimicrobial choices are extremely limited. Therefore, this situation requires development of new prophylactic or therapeutic strategies that do not rely on conventional antimicrobial agents [86, 87].

7.1. Active and passive immunization against *P. aeruginosa* PcrV

The first experimental trial on immunotherapy against the TTSS of *P. aeruginosa* was performed using *E. coli*-derived recombinant PcrV protein to actively immunize mice [33]. In this experiment, the immunized mice survived lethal challenge infections with *P. aeruginosa* pneumonia. Together with the active immunization trial, passive immunization was carried out in mice with a purified protein A binding γ-globulin fraction, which was separated from the sera of rabbits that were actively immunized with recombinant PcrV [30]. In this series, the immunized mice survived pulmonary administration of a lethal dose of *P. aeruginosa*. A correlation between the survival rate of the mice and the dose of the polyclonal anti-PcrV antibody was found. The effects of this polyclonal anti-PcrV antibody were later tested in various animal models of burns and chronic bacterial pneumonia [88, 89].

The mechanism responsible for the positive effect of the polyclonal anti-PcrV antibodies, in terms of whether the effect depends on the Fc-portion of the antibody, was investigated. The anti-PcrV polyclonal antibody $F(ab)'_2$ was tested in a rabbit model, and the same effect as whole IgG was confirmed [90]. This finding strongly suggests that the prophylactic and therapeutic effects of anti-PcrV polyclonal antibodies are derived by blocking the action involved in the pathogenicity of the antigen. Monoclonal antibody screening on normal mouse hybridomas was then performed and the clone mAb166 was discovered as the strongest TTSS blocker [40]. The clone mAb166 displayed equivalent therapeutic and prophylactic effects to those of the anti-PcrV polyclonal antibody [40, 91, 92]. The mAb166 Fab fragment also conferred the same therapeutic effect as the original whole IgG in *P. aeruginosa* pneumonia [85]. In particular, mAb166 exerted a strong therapeutic effect following airway administration of *P. aeruginosa* in a pneumonia model in rats [93]. By using this mAb166 antibody as a template with the

bacteriophage gene shuffling recombination technology, the US venture company KaloBios Inc. started a project to create a humanized anti-PcrV antibody. Consequently, KB001-A was developed as a humanized monoclonal antibody [94]. This antibody underwent phase I and phase II clinical trials in the USA and France [95, 96].

7.2. Immunization against PcrV in immunocompromised models

Active immunization with PcrV was examined in immunocompromised mice that were pretreated with cyclophosphamide [97]. Cyclophosphamide treatment induced immunosuppression in the mice, decreased immunity against *P. aeruginosa*, and decreased the lethal dose of *P. aeruginosa*. In this study, five truncated PcrV fragments and full-length-PcrV were tested as vaccine candidates in a mouse model of *P. aeruginosa* pneumonia. Acute systemic infection was introduced by intraperitoneal injection of a lethal dose of *P. aeruginosa* in this mouse model [97]. This study showed that active immunization with either full-length $PcrV_{1–294}$ or $PcrV_{139–294}$, both of which contain the $_{PcrV144–257}$ blocking epitope region of monoclonal anti-PcrV IgG mAb166, successfully protected the immunocompromised mice from lethal *P. aeruginosa* infection. This finding suggested that the anti-PcrV strategy might be effective in neutropenic conditions in which human patients frequently develop *P. aeruginosa* infections.

The intravenous immunoglobulin (IVIG) was recently shown to confer significant protection against lethal infection with virulent *P. aeruginosa* [98, 99]. The effect of administrating 2.5 mg of IVIG was comparable with that of administrating 10 μg of specific anti-PcrV polyclonal IgG. The mechanism of protection is likely to involve the synergic action of anti-PcrV titers and some surface antigen to block the TTSS-associated virulence of *P. aeruginosa* [98]. There is considerable variation in anti-PcrV titers in adult subjects without any obvious history of infection with *P. aeruginosa* [100]. IVIG extracted from high anti-PcrV titer human sera confers protective effects in a mouse model of lethal *P. aeruginosa* pneumonia [101]. These results suggest that, not only monoclonal strategies against PcrV, but serum-derived immunoglobulin therapy with specific titers against PcrV also has great potential as effective immunotherapeutic tool against lethal *P. aeruginosa* infections.

8. Conclusions

In this review, we summarize the current status of research on the pathogenesis and treatment of *P. aeruginosa* infections from the viewpoint of acute and chronic infections. First, there are two phenotypes of *P. aeruginosa* strains: one causes acute types of infection, whereas the other causes chronic types of infection. Genomic level differences exist between these two phenotypes. In the course of evolution, acquisition of virulence gene cassettes, especially PAPI-2, created subtypes with increasing toxicity. Second, exposure to antibiotics enhances their drug resistance together with a loss of cytotoxicity and antigenicity that can be targeted by host immunity. Third, some mutant cytotoxic and drug-resistant *P. aeruginosa* strains may be involved in acute exacerbation of chronic infectious diseases. The lifespan of patients with CF has improved via various medical advances. Rather than focusing on eradication of infectious pathogens, prophylaxis against lethal pathogenic factors to avoid acute exacerbation during

the chronic infection state should probably be given more priority at present. Currently, the monoclonal antibody strategies that are used against bacterial infections have not yet reached the level of practical application that is found in cancer therapy. The on-going challenge for anti-PcrV immunotherapy is realizing its potential to improve the clinical outcome of *P. aeruginosa* infections occurring in patients with CF.

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Abbreviations

CF	cystic fibrosis
GAP	GTPase-activating protein
IVIG	intravenous immunoglobulin
MDR-PA	multidrug-resistant *Pseudomonas aeruginosa*
PAPI-2	*P. aeruginosa* pathogenicity island-2
PLA	Phospholipase A
TTS	Type III secretory
TTSS	Type III secretion system
XDR-PA	extensively drug-resistant *P. aeruginosa*

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Congenital defects in humans are of greater concern, and in that line, cystic fibrosis (CF) has been one of the most complex diseases posing treatment challenge till date. Though it is a chronic condition, CF is closely associated with dysfunction of various organ systems of the human body, which in turn results in secondary infections by microbes. Decades of research by scientists worldwide has narrowed down the cause of CF to a single target gene. But the complexity of the disease is the prime impediment to finding a single-shot cure. Fortunately, the multidisciplinary approach toward understanding and management of the CF condition has certainly increased the level of life expectancy among CF patients. In particular, the "omics" and the "systems biology" approach have greatly widened the focal area for better understanding of the disease. This book includes a collection of interesting chapters contributed by eminent scientists around the world who have been striving to improve the life of those affected by CF.

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