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**).

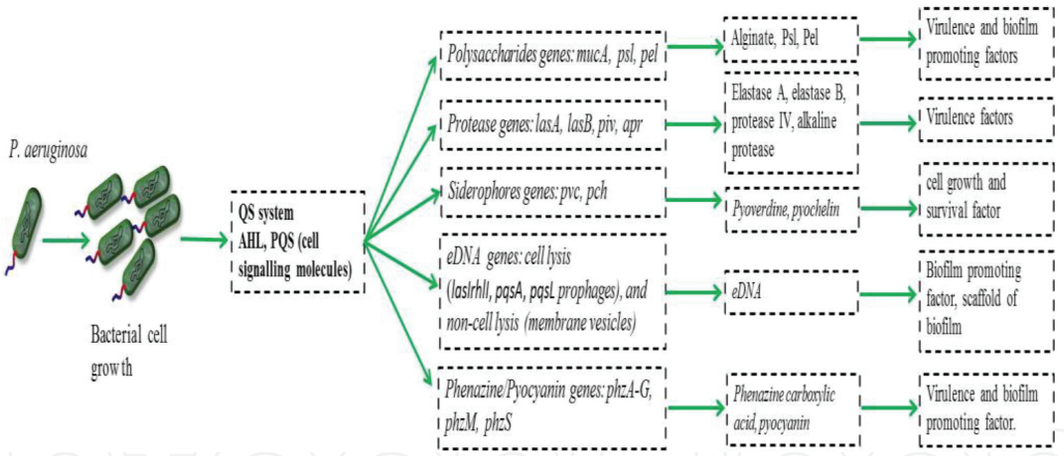

Figure 1. Schematic diagram showing quorum-sensing-mediated production of various extracellular molecules (polysaccharides, protease, pyoverdine, eDNA, pyocyanin) by *P. aeruginosa* and their potential roles in biofilm development and virulence.

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

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

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**).

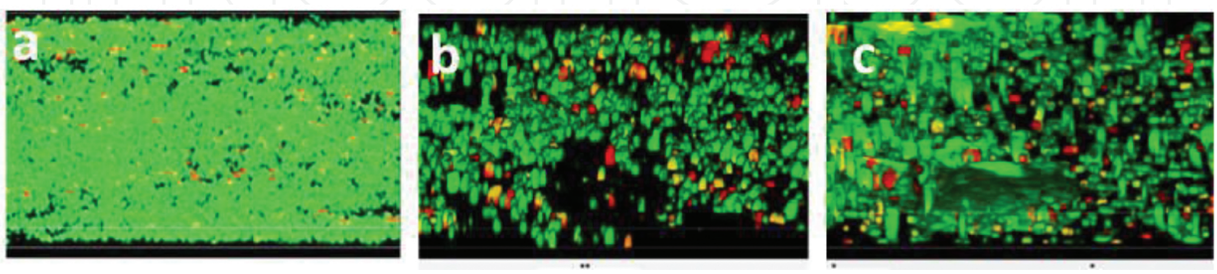

Figure 2. Biofilm formation by *P. aeruginosa* CF isogens in ASMDM+ medium (a) AES-1R, (b) AES-1M and (c) AES-1M grown in the presence of exogenous pyocyanin. The biofilm architecture of (c) indicates pyocyanin facilitates/enhances biofilm formation. Images taken with permission from Ref. [65].

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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].

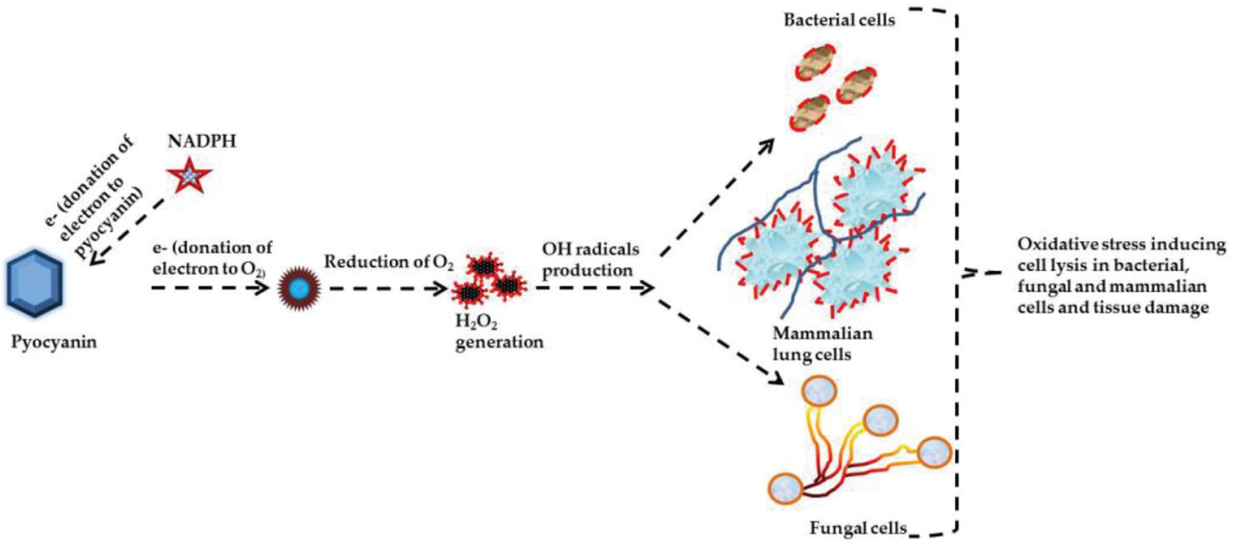

Figure 3. Schematic diagram of pyocyanin induced H_2O_2 production and toxicity on bacterial, fungal and human cells.

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+,

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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