TRANSLATIONAL REVIEW

Pseudomonas aeruginosa Biofilms: Host Response and Clinical Implications in Lung Infections

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Abstract

Pseudomonas aeruginosa is a major health challenge that causes recalcitrant multidrug-resistant infections, especially in immunocompromised and hospitalized patients. *P. aeruginosa* is an important cause of nosocomial and ventilator-associated pneumonia characterized by high prevalence and fatality rates. *P. aeruginosa* also causes chronic lung infections in individuals with cystic fibrosis. Multidrug- and totally drug-resistant strains of *P. aeruginosa* are increasing threats that contribute to high mortality in these patients. The pathogenesis of many *P. aeruginosa* infections depends on its ability to form biofilms, structured bacterial communities that can coat mucosal surfaces or invasive devices. These biofilms make conditions more favorable for bacterial persistence, as embedded bacteria are inherently more difficult to eradicate than planktonic bacteria. The molecular mechanisms that underlie *P. aeruginosa* biofilm pathogenesis and the host response to *P. aeruginosa* biofilms remain to be fully defined. However, it is known that biofilms offer protection from the host immune response and are also extremely recalcitrant to antimicrobial therapy. Therefore, development of novel therapeutic strategies specifically aimed at biofilms is urgently needed. Here, we review the host response, key clinical implications of *P. aeruginosa* biofilms, and novel therapeutic approaches to treat biofilms relevant to lung infections. Greater understanding of *P. aeruginosa* biofilms will elucidate novel avenues to improve outcomes for *P. aeruginosa* pulmonary infections.

Keywords: *Pseudomonas aeruginosa*; biofilms; ventilator-associated pneumonia; cystic fibrosis; anti-infective agents

Pseudomonas aeruginosa is among the most virulent of opportunistic pathogens and is a leading cause of a variety of acute infections, including ventilator-associated pneumonia (VAP). There continues to be a high rate of antibiotic failure in *P. aeruginosa* VAP and a high mortality rate, despite adequate antibiotic treatment (1, 2). In addition, *P. aeruginosa* can cause chronic lung infections in patients with cystic fibrosis (CF) and non-CF bronchiectasis. Acquisition of *P. aeruginosa* is associated with increased morbidity and mortality in patients with CF, and is an important factor in the development and progression of CF respiratory disease (1).

The pathogenic profile of *P. aeruginosa* is related to its complex genome and a large and variable arsenal of virulence factors (1). In particular, the capacity to form biofilms provides the bacteria an enormous advantage to establish infections, including VAP and CF lung infections, within susceptible hosts. Biofilms, which are structured communities of sessile bacteria encapsulated within an extracellular polymeric substance (EPS) matrix, provide homeostasis and stability in the face of fluctuating and harsh environmental conditions, including those within the human host (3, 4). Biofilms can protect bacteria from both host defenses and antimicrobial therapy through numerous adaptive mechanisms (3, 5, 6). Consequently, development of alternative strategies specifically targeted toward biofilm biology is an active area of research and has the potential to significantly improve clinical practice. Others have previously reviewed the development and dispersal of *P. aeruginosa* biofilms (7). Here, we review the host response to *P. aeruginosa* biofilms, clinical importance, and novel therapies under development.

Biofilm Composition, Function, and Regulation

Biofilms are highly structured communities of bacterial cells encased within an extracellular matrix that adhere to abiotic or

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biological surfaces. The bacteria, which account for less than 10% of the biofilm biomass, produce the EPS matrix, which makes up the more than 90% of the biofilm (8). The matrix is primarily composed of polysaccharides, proteins, extracellular DNA (eDNA), and lipids (9). Biofilm development can be affected by both the genetic makeup of the *P. aeruginosa* isolates and the environmental conditions, as well as the interplay between the two (10). These factors include hydrodynamic conditions, nutrient concentrations, pH, temperature, bacterial motility, intercellular communication, and host-derived factors (11). The EPS matrix allows the microbes to function synergistically as a community by maintaining close contact via intercellular communication pathways and sharing group resources (8, 12). The formation and dispersal of biofilms is regulated by several mechanisms, including quorum sensing (QS), bis-(3′-5′) cyclic diguanosine monophosphate (c-di-GMP) signaling, and regulation of small RNAs (13).

QS is an intercellular signaling pathway that allows bacteria to coordinate gene transcription and group activity in response to population density. In *P. aeruginosa*, there are two primary interconnected QS systems that rely on acylhomoserine lactone (AHL) signaling molecules: the Las system, which uses N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and the Rhl system, which uses N-butanoyl homoserine lactone. Two additional QS systems that do not involve AHL molecules act as redundant accessory pathways: *Pseudomonas* quinolone signal system and the integrated quorum sensing system (IQS) (14). QS directs the generation of essential biofilm components, namely eDNA and the biosurfactant glycolipid, rhamnolipid, and is therefore necessary for the development of mature biofilms (10, 15). c-di-GMP signaling is also an important regulator of biofilm formation. High levels of c-di-GMP promote synthesis of EPS component polysaccharides and extracellular proteins. Alternatively, low levels of c-di-GMP promote biofilm dispersal (13, 16).

Host Response against *P. aeruginosa* Biofilms

The host response against *P. aeruginosa* infections is complex and involves the coordinated activity of a variety of cell types comprising both the innate and adaptive immune systems (Figure 1). A clear understanding of the *in vivo* host response to *P. aeruginosa* biofilms in the lungs is made more challenging by the difficulty of developing animal models that mimic biofilm infections in humans. In fact, the majority of immune research investigating *P. aeruginosa* has used infection models with the bacteria in the planktonic state. Nevertheless, a growing body of literature supports a role for both the innate and adaptive immune systems in the response to *P. aeruginosa* biofilm infections (17, 18). We will discuss the roles of various aspects of host immunity in response to *P. aeruginosa* infections with a particular focus on their role in biofilm infections.

Epithelium

The epithelial barrier provides the first line of defense against *P. aeruginosa* lung infections. Patients with a damaged or disrupted epithelium, such as intubated patients, are at an increased risk of developing *P. aeruginosa* infections. The epithelial barrier provides a physical barrier to bacterial invasion through the

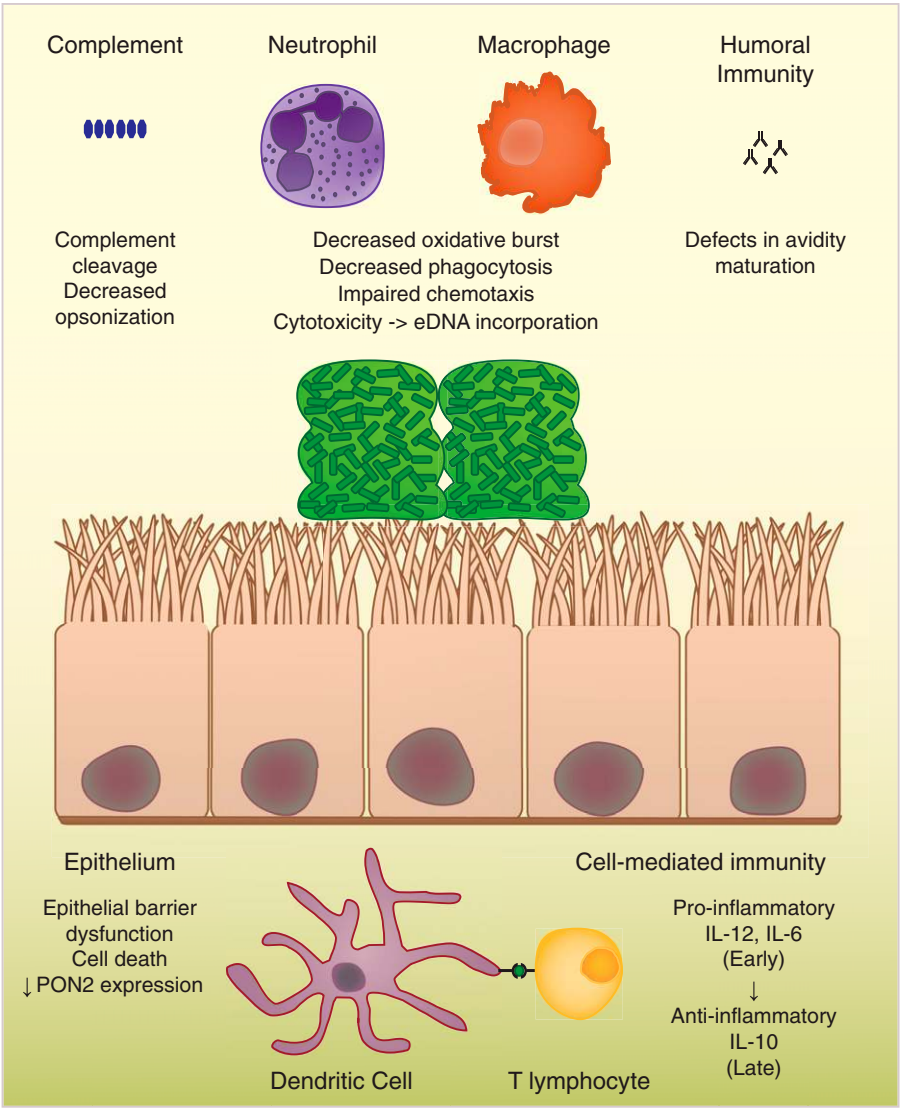

Figure 1. Disruption of host response to *Pseudomonas aeruginosa* biofilms. The host response to *P. aeruginosa* biofilms depends on the coordinated action of various cells of the innate and adaptive immune systems. *P. aeruginosa* biofilms disrupt this immune response through various actions on host cells detailed here. eDNA = extracellular DNA; PON2 = paraoxonase 2.

network of cell–cell contacts, including tight junctions. In addition, mucociliary clearance in the upper respiratory tract prevents the establishment of *P. aeruginosa* infection (19).

Epithelial cells also secrete a variety of antimicrobial peptides, including lactoferrin, a ubiquitous component of external secretions that is known to sequester iron. Lactoferrin has been shown to block *P. aeruginosa* biofilm development by stimulating twitching motility in planktonic bacteria and thereby preventing attachment and microcolony formation (20). Short palate, lung, and nasal epithelium clone (SPLUNC) 1 is a surfactant-like protein secreted by the respiratory epithelium that helps to maintain surface tension of airway fluids and also has direct antimicrobial properties. SPLUNC1 has been shown to inhibit *P. aeruginosa* pulmonary infections and *P. aeruginosa* biofilm formation in the lung (21). Cell surface receptors on the apical membranes of epithelial cells, such as asialo-ganglioside M1 (asialoGM1) and Toll-like receptors can recognize *P. aeruginosa* and activate signal transduction pathways that result in production of inflammatory cytokines and chemokines (17). Epithelial cells also express paraoxonases (PONs) that inactivate AHL QS molecules, and thereby inhibit biofilm maturation (22–24). Finally, chemical factors, including normoxia, low iron levels, and neutral pH, all contribute to innate immunity against *P. aeruginosa* (19, 25).

Neutrophils and Macrophages

There is significant evidence demonstrating the importance of innate immune cells in the host response to *P. aeruginosa* biofilm infections (17, 26). *In vivo* models of biofilm infections have demonstrated the abundance of activated neutrophils surrounding biofilms (27). The addition of *P. aeruginosa* biofilms to isolated primary human macrophages and neutrophils results in a robust antibacterial response (28, 29). Furthermore, microscopic analysis of the airways and sputum from patients with CF demonstrates an intense accumulation of neutrophils in close association with biofilms (30), and these neutrophils appear to be metabolically active (31).

However, despite some activity against biofilms, the function of neutrophils and macrophages is significantly attenuated by *P. aeruginosa* biofilms as a mechanism to evade host defenses and establish infection. The oxidative burst generated by neutrophils against *P. aeruginosa* biofilms was only 25% of the response observed against planktonic bacteria (32). In addition, human neutrophils exposed to mature biofilms become immobilized and unpolarized (28), and undergo necrotic cell death (33). Bacteria in biofilm form are also more resistant to phagocytosis by neutrophils via a mechanism mediated by rhamnolipids within the extracellular matrix (34). Moreover, rhamnolipids are directly cytotoxic to neutrophils and impair their chemotaxis (33). Interestingly, *P. aeruginosa* has the capability to respond to the presence of nearby neutrophils by upregulating rhamnolipid expression to form a shield that protects the biofilm bacterial community (35).

Alternative bacterial strategies at evading neutrophil detection and killing have also been elucidated. Loss of flagella, or, more fundamentally, flagellar motility, which is frequently observed in mucoid colonies from patients with CF, results in a resistance to phagocytosis by neutrophils and macrophages that is independent of Toll-like receptor signaling (36, 37). The alginate exopolymeric matrix may provide additional protection from phagocytosis (38). Neutrophils employ a defense strategy by releasing neutrophil extracellular traps made up of DNA and granule proteins, such as neutrophil elastase, myeloperoxidase, and cathepsin G. Mucoid strains of *P. aeruginosa*, however, appear to be partially resistant to neutrophil extracellular trap–mediated killing (39). In addition, evidence suggests that incorporation of eDNA and actin from necrotic neutrophils into the biofilm matrix protects *P. aeruginosa* from antimicrobial peptides and promotes biofilm maturation (40).

Alveolar macrophages, which are important mediators of the innate immune response to *P. aeruginosa* lung infections, are responsible for the phagocytosis of bacteria and recruitment of additional immune cells. An understanding of their specific response to biofilms is less well known. *In vitro* studies have demonstrated that alginate protects against macrophage killing (29). In addition, *P. aeruginosa* biofilms, as compared with the planktonic bacteria, appear to stimulate a stronger proinflammatory cytokine response from macrophages. However, growth of the biofilm and production of bacterial virulence factors were enhanced when the biofilms were cultured in media enriched with macrophage secretory products (41). This suggests that the presence of human host macrophages promotes biofilm growth (17). Similar results have also been demonstrated with neutrophils (40) and human epithelial cells (22), highlighting the important interplay between bacterial biofilm colonies and host cells. These studies suggest that biofilm formation evades host cell defense mechanisms, allowing *P. aeruginosa* to establish infection.

Complement

The role of the complement system in response to biofilm infections is not completely understood. There is evidence that *P. aeruginosa* can evade the complement system in several ways. *P. aeruginosa* inactivates the complement system through cleavage of complement components by alkaline protease and elastase (both bacterial-derived and neutrophil elastase) (1, 42). In addition, O-acetylation of alginate protects against complement-mediated opsonization in mucoid strains (43), whereas expression of the Psl polysaccharide and O-glycosylation of type IV pili protected against opsonization in other strains (44, 45). Furthermore, the lectin pathway of complement activation is suppressed by *P. aeruginosa* (46).

Adaptive Immunity

The adaptive or acquired immune response, which encompasses cell-mediated T helper (Th) 1 immunity and humoral-mediated Th2 immunity, is also activated in parallel with the innate immune system to respond to chronic biofilm infections. Interestingly, strains of mice have disparate T cell responses that correspond to susceptibility to *P. aeruginosa* infections: a Th1-dominant response predominates in resistant strains, whereas a Th2-dominant response predominates in susceptible strains (47). Similarly, studies have suggested that chronic *P. aeruginosa* infections in patients with CF are typically associated with a Th2-predominant response, but a Th1-predominant response is associated with improved outcomes (48). This improved outcome in Th1-dominated responses may be mediated by the enhanced

ability of the alveolar macrophage to clear apoptotic and necrotic neutrophils from the airways and thereby prevent persistent inflammation (17).

Previous studies have reached differing conclusions regarding the importance of humoral immunity against *P. aeruginosa* biofilms. During an 11-year follow-up of patients with CF with chronic *P. aeruginosa* infection, all patients developed increasing numbers of antibodies, but, interestingly, there was no maturation in the avidity of the antibodies as would typically be expected (49). This failure of affinity maturation results in decreased humoral immunity against *P. aeruginosa* and an increased susceptibility to immune complex deposition and subsequent tissue injury (17). However, there has also been evidence suggesting a role for protective antibody responses to *P. aeruginosa* biofilms. In particular, antibodies against bacterial β-lactamase have been shown to improve outcomes in a rat model of *P. aeruginosa* chronic lung infection (50).

Additional T lymphocyte subsets have also been implicated in the host response to *P. aeruginosa* infections. Th17 cells, which are characterized by the production of IL-17, can promote inflammation and neutrophil recruitment. Higher levels of IL-17 were observed during exacerbations among patients with CF colonized with *P. aeruginosa*, and these levels decreased with antibiotic therapy (51). In addition, chronic infection with *P. aeruginosa* in stable patients is associated with higher expression of IL-17 (52), suggesting that Th17 cells are involved in the persistent inflammatory state seen in chronic CF *P. aeruginosa* infections. In a prospective observational trial of children with CF, the presence of a Th2 and Th17 cytokine profile was associated with an increased risk of developing chronic *P. aeruginosa* infection (53).

The action of dendritic cells has also been demonstrated in a mouse model of chronic *P. aeruginosa* lung infection. Activated dendritic cells can be observed in the lung by Day 2 and in the regional lymph nodes by Day 7 after infection. Moreover, an initial burst of the proinflammatory cytokines, IL-12 and IL-6, is supplanted by a predominant antiinflammatory IL-10 response. This suggests that dendritic cells play a role in repressing a prolonged inflammatory state (54). However, it remains to be seen whether this immune modulation is beneficial or detrimental to the host.

Bacterial Subversion of Host Response

As discussed previously here, there is a dynamic relationship between *P. aeruginosa* and host response, with increasing awareness that *P. aeruginosa* can hijack aspects of host response to promote virulence (14). For example, Wu and colleagues (55) demonstrated that the *P. aeruginosa* outer membrane protein, OprF, is able to bind the human cytokine, IFN-γ, and subsequently activate the Rhl QS system to direct production of important virulence factors. Similarly, host natriuretic peptides can activate QS signaling in *P. aeruginosa*, and thereby promote virulence (56). LL-37, a host antimicrobial peptide produced by phagocytes and epithelial cells previously shown to inhibit biofilm formation, can, when used at physiologic concentrations, paradoxically promote virulence factor production in *P. aeruginosa* and decrease susceptibility to antibiotics (57).

Numerous studies have demonstrated that *P. aeruginosa*–produced QS molecules can modulate host response through numerous mechanisms that may contribute to its capacity to promote host tolerance and cause chronic infections (18, 58). These mechanisms include induction of cell death in a variety of host cells (59), disruption of epithelial barrier integrity (60), inhibition of T cell activation and proliferation (61), promotion of an antiinflammatory rather than proinflammatory cytokine profile released by activated macrophages (62), epigenetic reprogramming of immune cells (63), upregulation of regulatory T cells (64), and inhibition of the inflammasome pathway (65).

Clinical Importance of *P. aeruginosa* Biofilm Infection

Bacterial biofilms are a significant cause of human infections, particularly chronic infections (3). The ability of *P. aeruginosa* to form biofilms is a critical factor that allows it to cause severe and recalcitrant infections associated with significant morbidity and mortality (66). Biofilms provide *P. aeruginosa* an enormous advantage by promoting survival on artificial materials, evasion from the immune system, and tolerance to antimicrobial therapy (5, 10, 26, 66). We discuss subsequently here the importance of *P. aeruginosa* biofilms in device-related and CF pulmonary infections, and then discuss the recalcitrance of *P. aeruginosa* biofilms to standard antimicrobial therapies.

Device-related Infections

The insertion of artificial devices into humans in a variety of clinical contexts has become commonplace in modern medicine. Common medical devices include endotracheal tubes (ETTs), urinary catheters, vascular catheters, peritoneal catheters, orthopedic implants, prosthetic joints, and prosthetic cardiac valves. These abiotic devices can quickly become coated with a conditioning film of host proteins that can then support bacterial attachment, which can occur as early as 1 day after insertion. Biofilm bacteria can subsequently undergo dispersal, allowing the planktonic forms of the bacteria to potentially cause disseminated infection. Although antibiotics can be effective against the planktonic forms of the bacteria, device removal is often the only effective way to completely eliminate the source of infection (10, 26).

After tracheal intubation, a bacterial biofilm rapidly forms on polyvinyl-chloride ETTs and represents a significant source of bacterial inoculation into the lungs. Intubated patients on mechanical ventilation are at a high risk for bacterial colonization leading to the development of VAP, a major health threat that carries an attributable 60-day mortality of 1.5% among critically ill patients (67). In the majority of cases of VAP, the same organism isolated from the ETT biofilm matches the causative organism identified on respiratory cultures. Furthermore, the production of the QS-dependent virulence factor, rhamnolipids, by colonizing *P. aeruginosa* isolates was found to be associated with the development of VAP (68).

CF Lung

CF is caused by defective function of the CF transmembrane conductance regulator in airway epithelium and submucosal glands. The mutation results in decreased chloride transport across the epithelial barrier, reduced periciliary fluid, increased sputum viscosity, disrupted airway anatomy, acidification of the airway surface liquid layer, and impaired mucociliary clearance. These conditions predispose to a repetitive cycle of respiratory infections with several microbial pathogens (1). Patients with CF will typically acquire recurrent intermittent infections with *P. aeruginosa* by adolescence or early adulthood. Despite aggressive

antibiotic treatments, the infection will often become chronic and recalcitrant and characterized by bacterial biofilm development and a robust host inflammatory response (Figure 2). The acquisition of chronic *P. aeruginosa* infection is associated with worsened disease progression and increased mortality (69).

There is good evidence that the chronic *P. aeruginosa* infections seen in CF are characterized by the bacteria in the biofilm form based on microscopic analyses of sputum samples and explanted lung tissue from patients with CF (30) and isolation of QS molecules in the sputum of patients with CF (70).

The bacterial strains isolated from patients with CF with chronic *P. aeruginosa* infections have several other key differences from more typical strains, likely owing to the fact that these bacteria are under significant selective pressure from a hostile host environment and repeated antibiotic treatments (10, 71). CF-associated isolates are commonly characterized by inactivation of the MucA gene, which encodes a negative transcriptional regulator that represses bacterial stress pathways. This results in a mucoid conversion characterized by increased production of the extracellular polysaccharide, alginate. In addition, inactivation of MucA promotes a stress response that represses bacterial metabolism, motility, and virulence. This mucoid conversion is associated with poorer prognosis in patients with CF (72–74). Upregulation of the nonalginate polysaccharide, Psl (75), and loss of flagellin and type IV pili are also commonly observed, and may result in decreased immune recognition (36, 76, 77). The presence of anoxic and hypoxic microenvironments within the CF lung also selects for bacterial isolates with enhanced microaerobic and anaerobic metabolic pathways (78). Furthermore, in CF, there is selection for *P. aeruginosa* small colony variants that have an increased ability to form biofilms and an enhanced resistance to antibiotics (79). In the late stages of CF disease, defects in the master QS regulator, lasR, also develop (77, 80). However, many of these mutants still have functional LasR activity, and others have uncoupled the Rhl QS system from LasR regulation (81). In addition, there is emergence of hypermutable strains of *P. aeruginosa* (82). Finally, there is a selection for antibiotic resistance genes that encode multidrug efflux pumps (77).

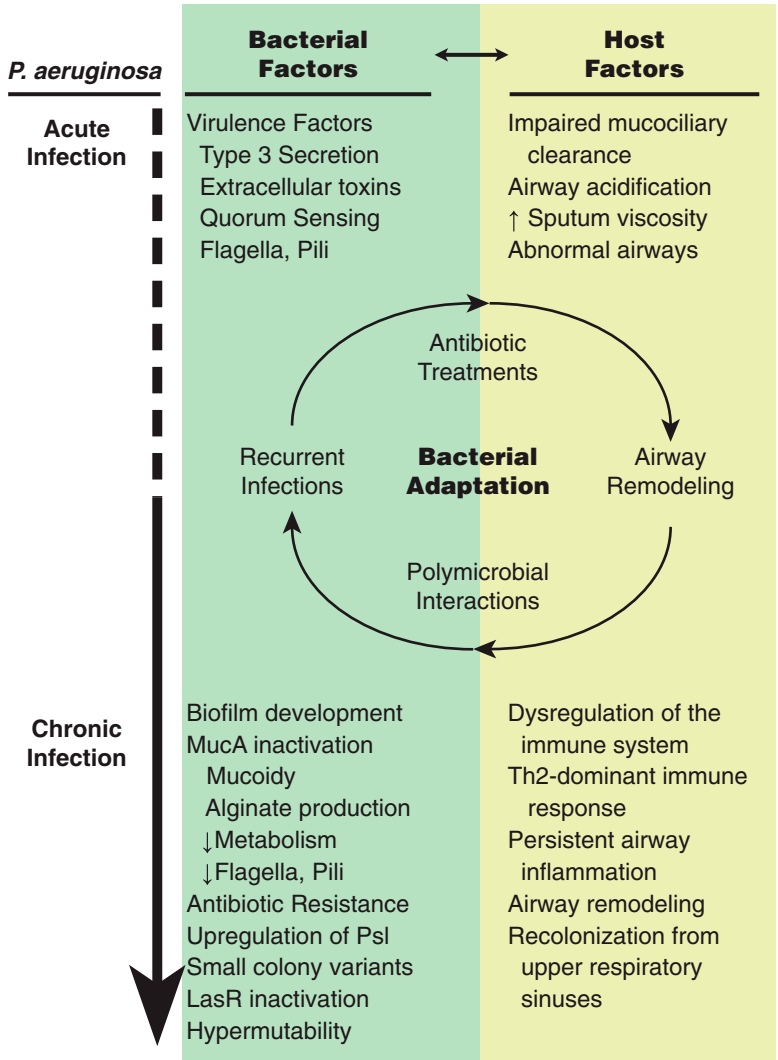

Figure 2. Bacterial and host factors that contribute to acute and chronic *Pseudomonas aeruginosa* lung infections in cystic fibrosis (CF). The relationship between bacterial and host factors that contribute to the development of chronic *P. aeruginosa* infections is complex. Patients with CF will typically develop recurrent acute *P. aeruginosa* infections early in life that are treated aggressively with antibiotic therapy. Under significant selective pressure to survive in a hostile host environment, bacteria develop several important adaptations. Similarly, there are aspects of susceptible host biology that also promote persistent infections. Psl = polysaccharide synthesis locus; Th2 = T-helper type 2.

As discussed previously here, both the cellular and humoral immune responses to *P. aeruginosa* biofilm infections are dysregulated, and this likely contributes to the ongoing tissue injury and airway remodeling in CF. Subversion of the host response is the result of several bacterial factors, including biofilm development, decreased virulence and motility, and alteration of the extracellular polysaccharide matrix; however, it is difficult to weigh the relative impact of these factors, given the challenges of developing *in* vivo models of biofilm infections that mimic the complex milieu observed in the CF lung (17).

The situation is more complicated in CF, because extrapulmonary sites, the upper respiratory sinuses, are hypothesized to serve as a microenvironment more protected from antimicrobial therapy, from which recolonization of the lower respiratory tract can repeatedly occur after antibiotic treatment courses (74).

Biofilm Recalcitrance to Antimicrobial Therapy

The principal concern with biofilm-related infections involves the difficulty in fully

eradicating the infection, despite aggressive antimicrobial therapy. There are two primary mechanisms that have been illustrated to explain how bacterial pathogens can evade antimicrobial therapy: resistance and tolerance. Resistance, which is measured by the minimum inhibitory concentration, refers to the ability of bacteria to multiply despite treatment with an antimicrobial compound. Resistance is typically inherited from mother bacteria or acquired from horizontal gene transfer, which is facilitated by the close proximity of bacterial cells within a biofilm. Resistance can be mediated by several processes, including drug inactivation or modifications, alteration of the binding site, reduced drug accumulation through increased efflux or decreased entry, and use of alternative metabolic pathways.

Tolerance, by comparison, is defined as the capability of a microbe to survive despite treatment with a bactericidal antibiotic to which it is susceptible based on the minimum inhibitory concentration value. However, the recalcitrance to antimicrobial therapies that characterizes biofilms is more complex, and is incompletely explained by the concepts of resistance and tolerance (5).

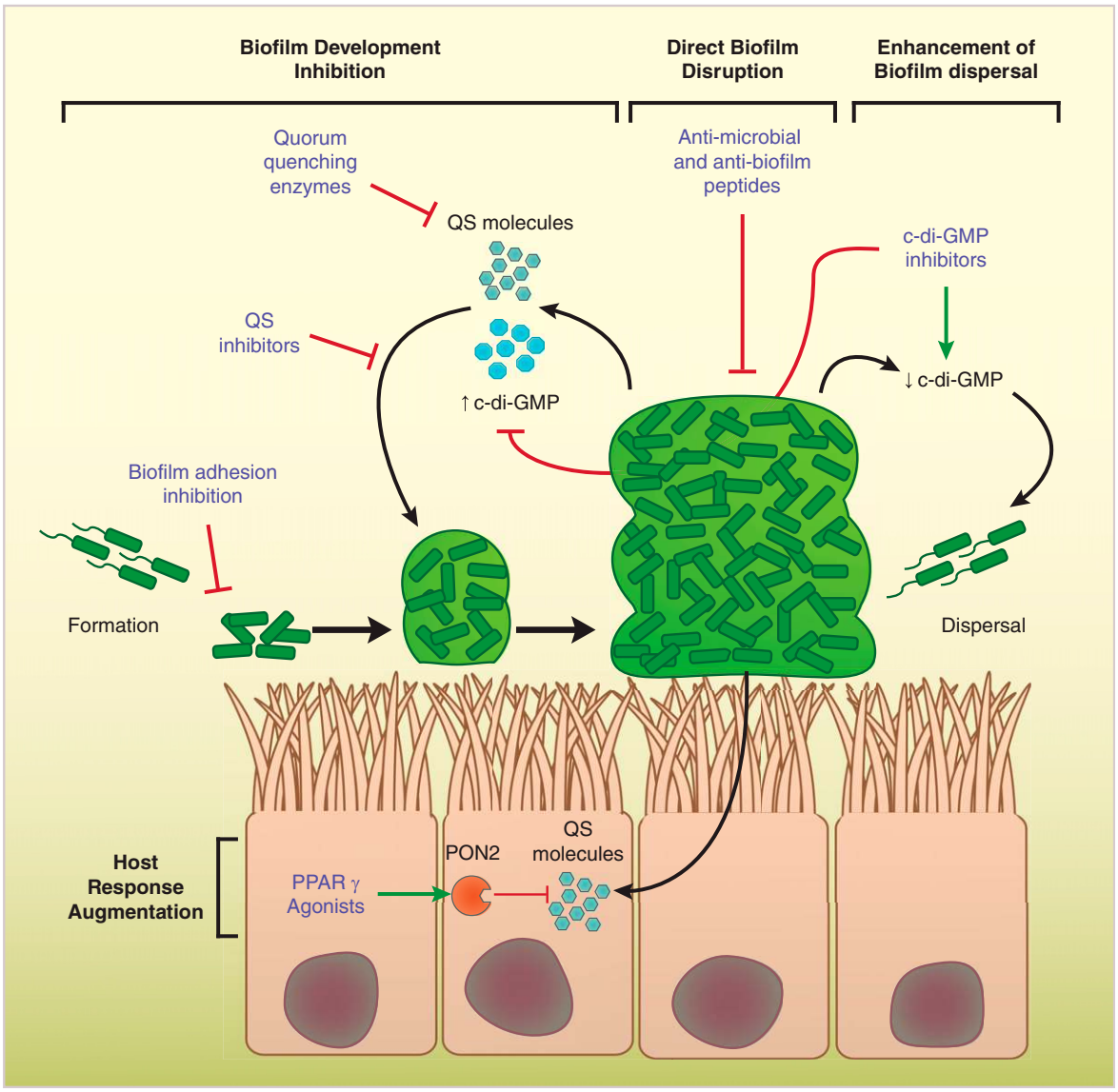

Figure 3. Biofilm development and dispersal and site of action of various biofilm-directed therapies. Biofilm formation begins with the attachment of planktonic bacteria to biotic or abiotic surfaces. These bacterial colonies, under the regulation of cell-signaling molecules, including quorum sensing (QS) molecules and bis-(3′-5′) cyclic diguanosine monophosphate (c-di-GMP), secrete components of the extracellular polymeric substance matrix that form the bulk of the biofilm. QS molecules also rapidly diffuse into host cells, where they can be degraded by the enzyme PON2. Biofilm dispersal is induced by downregulation of c-di-GMP signaling. Within this dynamic process, there are multiple possible therapeutic targets that have clinical promise. These include biofilm adhesion inhibition, QS inhibition, quorum quenching, antibiofilm peptides, c-di-GMP inhibition, and activation of the host response. PPARγ = peroxisome proliferator–activated receptor γ.

Inability of antibiotics or antiseptics to kill biofilm bacteria has been classically attributed to a decreased ability of these agents to penetrate biofilms due to the mechanical and chemical properties of the extracellular matrix. For example, the positively charged aminoglycoside antibiotic, tobramycin, exhibited reduced diffusion through *P. aeruginosa* biofilm models (83). Variable results have been demonstrated for other antibiotics. The incorporation of DNA into biofilms may also limit antibiotic penetration into biofilms. This is supported by both *in vitro* data showing that DNase, in combination with antibiotics, reduced biofilm biomass as compared with antibiotics alone (84) and clinical studies that have demonstrated better outcomes in patients with CF treated with inhaled DNase. However, even antibiotics that fully penetrate the biofilm matrix do not kill all of the susceptible biofilm bacteria. Therefore, a reduction in antibiotic penetration cannot completely account for the recalcitrance of biofilms to antimicrobial therapy (5).

The particular microenvironment within *P. aeruginosa* biofilms also likely contributes to recalcitrance to antibiotics. The low metabolic activity of bacteria and hypoxic conditions within the interior biofilm is associated with increased tolerance of *P. aeruginosa* biofilms to tobramycin and ciprofloxacin. Similarly, the efficacy of β-lactam antibiotics, which are effective against only actively replicating bacteria, is inversely related to the metabolic activity of biofilm bacteria (85).

As discussed previously here, phenotypic variation also represents another mechanism of antibiotic tolerance. Drenkard and Ausubel (79) found that these small colony variants, which have an enhanced propensity to form biofilms, are more tolerant to antibiotic therapy.

Finally, the concept of bacterial persistence, in which a small fraction of the larger bacterial population displays tolerance to antibiotics, significantly contributes to biofilm recalcitrance to antibiotics in *P. aeruginosa*. These bacterial persisters can remain dormant in bacterial biofilms shielded from the brunt of the host immune response. Then, upon cessation of the antibiotic therapy, they can resume replication and repopulate the biofilm (86). Analyses of *P. aeruginosa* strains have directly implicated bacterial persistence as a major cause of biofilm antimicrobial recalcitrance in patients with CF (87).

Biofilm-directed Therapies

Both the CDC and the World Health Organization have highlighted the seriousness of the health threat of antibiotic-resistant *P. aeruginosa* and urged the development new antimicrobial therapies for *P. aeruginosa*, given the high rates of antibiotic resistance. Furthermore, given the issues of tolerance and recalcitrance of *P. aeruginosa* observed in biofilm infections, urgent, novel antibiofilm treatments are needed. Here, we review the blossoming research field investigating several alternative strategies that aim to target biofilms (5, 10, 88, 89). These strategies employ both natural and synthetic compounds, and use varied mechanisms (Figure 3, Table 1).

Biofilm Adhesion Inhibition

Given the importance of medical device–related biofilm infections, an attractive strategy to prevent biofilm infections involves the engineering of materials more resistant to biofilm colonization. This strategy could involve impregnation of devices with antimicrobial compounds, use of nanoparticle-embedded material, or modulation of the topographic

Table 1. Biofilm-directed Therapies

Therapeutic Strategy	Mechanism of Action	Representative Examples (References)
Biofilm adhesion inhibition	Engineering artificial devices to be more resistant to bacterial attachment and biofilm development	Silver-coated ETTs (90, 91) Impregnation of antimicrobial nanoparticles (88) Nanoscale topographic alterations (88)
QS inhibition	Inhibition of bacterial QS signaling	Halogenated furanones (natural and synthetic) (10) Garlic; garlic compound ajoene (96, 97) Patulin, a mold metabolite (99) Iberin found in horseradish (100) Eugenol found in clove extract (101) Ellagic acid (fruit extract) (102) Macrolides (e.g., azithromycin) (103–106)
Quorum quenching	Enzymatic degradation of AHL QS molecules (lactonases, acylases)	SsoPox-1 lactonase (108) Aiid (acylase) (110) PvdQ (acylase) (111)
	Enzymatic inactivation of AHLs (oxireductases)	bpiB09 (NADP-dependent oxidoreductase) (109)
Augmentation of host response	Activation of host innate immune effectors targeted toward antibiofilm response	PPARγ agonists (22, 114)
Inhibition of c-di-GMP signaling	Attenuation of c-di-GMP signaling to decrease biofilm formation and promote dispersal	Phosphodiesterase-mediated c-di-GMP degradation (115) Doxorubicin (116)
Antibiofilm peptides and molecules	Compounds with direct antibacterial activity or activity against EPS components	LL-37, hCAP-18 (118, 119) TP359 (120) Peptide 1018, DJK-5, DJK-6 (121) DNase (84)

Definition of abbreviations: AHL = acylhomoserine lactone; c-di-GMP = bis-(3′-5′) cyclic diguanosine monophosphate; EPS = extracellular polymeric substance; ETT = endotracheal tube; hCAP-18 = human cathelicidin antimicrobial peptide, 18 kDa; NADP = nicotinamide adenine dinucleotide phosphate; PPARγ = peroxisome proliferator–activated receptor γ; QS = quorum sensing.

nanostructure of materials to make them less susceptible to biofilm attachment (88).

Silver coating of ETTs is one such strategy that has been demonstrated to have efficacy in preventing *P. aeruginosa* infection. In an experimental canine model of *P. aeruginosa* VAP, use of ETT coated with an antimicrobial silver hydrogel coating, as compared with an uncoated ETT, resulted in significantly less *P. aeruginosa* colonization and inflammation (90). Furthermore, in a large, prospective, single-blind, randomized, controlled study, patients who received a silver-coated ETT had a statistically significant reduction in the incidence of VAP and delayed time to VAP occurrence compared with those receiving an uncoated tube (91). These data suggest that antibacterial coating of ETTs may prevent development of biofilms and reduce the incidence of VAP.

QS Inhibition

As discussed previously here, QS is an important interbacterial communication system necessary for the development of mature biofilms. Consequently, numerous approaches to inhibit *P. aeruginosa* QS signaling have been explored. The first bacterial QS inhibitors identified were halogenated furanones made by the macroalga, *Delisea pulchra*. Although these did not have activity against *P. aeruginosa* QS, synthetic furanones have subsequently been developed (Figure 4). Several of these synthetic halogenated furanone compounds have been tested and been shown to significantly reduce biofilm biomass, increase the susceptibility of *P. aeruginosa* biofilms to tobramycin *in vitro*, and decrease pathogenicity of *P. aeruginosa in vivo* in mouse pulmonary infection models (92, 93). Since these initial studies, the field has rapidly expanded to develop novel synthetic furanones through a variety of biochemical screens (94, 95).

A variety of natural compounds has also been investigated for their properties inhibiting QS. For example, garlic extract, and specifically the chemical ajoene found in garlic, have been shown to inhibit QS-mediated virulence factors, increase the susceptibility of *P. aeruginosa* to antibiotics, and decrease the pathogenicity in a murine lung infection model (96, 97). A clinical study using garlic in patients with CF with chronic *P. aeruginosa* was unfortunately underpowered to detect any significant difference (98). Administration of patulin, a metabolite produced by the mold, *Penicillium*,

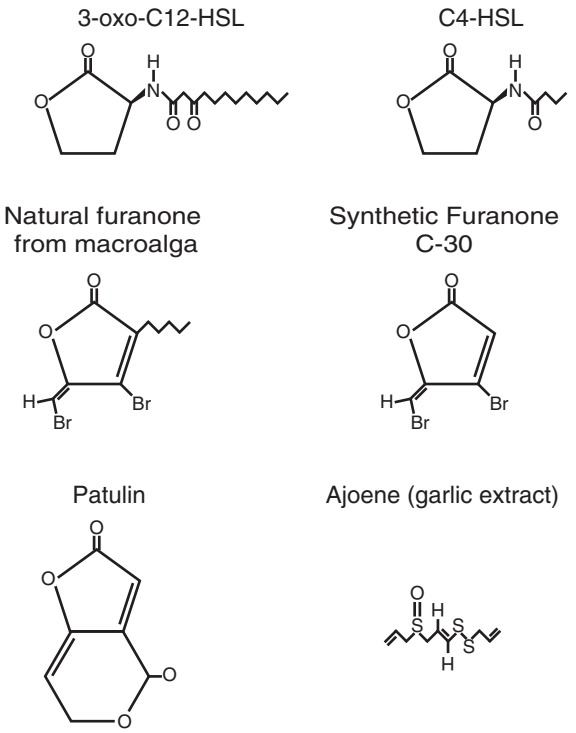

Figure 4. Chemical structures of QS molecules and selected QS inhibitors. The primary QS molecules used by *Pseudomonas aeruginosa* are the acylhomoserine lactones (AHLs), N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl homoserine lactone (C4-HSL). These differ in the length of the acyl chain and the 3-oxo substituent. It has been found that natural halogenated furanones, which share a similar structure with AHLs, can inhibit QS signaling. Subsequently, synthetic furanones, such as C-30, have also been developed. In addition, a variety of natural chemicals, including patulin and ajoene, which have differing levels of structural similarity to QS molecules, have also been shown to exhibit QS inhibitory properties.

decreased *P. aeruginosa* virulence *in vitro* and resulted in increased bacterial clearance in a mouse pneumonia model (99). Numerous other natural compounds have also been tested (100–102) using *in vitro* systems, including iberin found in horseradish (100), eugenol found in clove extract (101), and ellagic acid from *Terminalia chebula* fruit (102), but further investigation using preclinical models is needed.

Finally, macrolide antibiotics, such as azithromycin and erythromycin, have also been studied for their QS inhibition effects (103, 104). In a mouse model of chronic *P. aeruginosa* pulmonary infection, azithromycin decreased airway inflammation (105). In addition, azithromycin improves clinical outcomes in patients with CF with chronic *P. aeruginosa* infection (106).

Quorum Quenching

In addition to these strategies targeting the bacterial perception of QS molecules, therapies using quorum quenching enzymes, such as acylases and lactonases, which degrade AHLs, or oxireductases, which inactivate AHLs by modification, have also been investigated (107). The lactonase, SsoPox-1, attenuated QS signaling, virulence factor production, and biofilm formation *in vitro*, and administration of an inhaled form in a rat *P. aeruginosa* model decreased lung injury and reduced mortality from 75% to 20% (108). AHL acylases, such as AiiD acylase and PvdQ, and the oxioreductase, Bpi09, have also had favorable results in *in vitro* systems and in *P. aeruginosa* nematode infection models (109–111).

Augmentation of Host Response

PONs are a family of enzymes with lactonase activity that are expressed by host cells. These enzymes, particularly PON-2, hydrolyze AHL molecules and thereby inhibit QS and downstream virulence and biofilm formation (23, 24). PON-2–deficient mice had a marked impairment in their ability to hydrolyze

3-oxo-C12-HSL and decreased clearance of *P. aeruginosa* (112). In addition, lower PON-2 expression in patients with CF was associated with a higher incidence of *P. aeruginosa* infections (113). Furthermore, *P. aeruginosa* can directly modulate the host response through attenuation of PON-2 mediated by the QS molecule, 3-oxo-C12-HSL (22, 114).

We have recently shown that peroxisome proliferator–activated receptor (PPAR) γ agonists induce PON-2 in host alveolar macrophages and epithelial cells and enhance clearance of *P. aeruginosa* from the lungs. Most importantly, PPARγ agonists significantly reduce the biomass of *P. aeruginosa* biofilms grown in association with human epithelial cells by a mechanism that is mediated by upregulation of PON-2 (22, 114). These results identify a potential novel therapy for *P. aeruginosa* biofilm infections aimed at boosting the host response to infection through PPARγ agonism.

Inhibition of c-di-GMP Bacterial Signaling

Suppression of c-di-GMP signaling has been proposed as a novel therapy against biofilms due to the importance of c-di-GMP signaling in biofilm formation and dispersal. This was supported by a study using a foreign body infection model of *P. aeruginosa*, in which levels of c-di-GMP can be reduced via induction of a phosphodiesterase. The attenuation of bacterial c-di-GMP signaling resulted in greater clearance of the infection (115). However, a pharmacologic approach using doxorubicin, which was identified to be a potent c-di-GMP inhibitor in a large screen, paradoxically caused an increase in biofilm size (116). Therefore, further studies are needed to ascertain whether suppression of c-di-GMP signaling represents a promising potential therapy.

Antimicrobial and Antibiofilm Peptides and Molecules

Antimicrobial peptides are naturally occurring peptides produced by organisms from all classes of life. Numerous synthetic antimicrobial peptides have also been developed (117). A subset of these peptides has been shown to more effective against biofilms than planktonic bacteria (118). Many of these peptides have demonstrated promising results using *in vitro* systems, but further research is needed before these advances can be translated into clinical studies (119–122). Furthermore, as discussed previously here, the enzyme, DNase, which has direct action against eDNA, a critical component in biofilms, is already used as an adjunctive therapy in patients with CF.

Conclusions

The increasing risk of multidrug- and totally drug-resistant *P. aeruginosa* strains has dramatically complicated treatment options. Furthermore, many *P. aeruginosa* infections rely on biofilms, which are inherently recalcitrant to host defenses and antimicrobial therapies. Therefore, development of novel therapeutics that block virulence mechanisms, inhibit biofilm formation or stability, or enhance host cell immunity are urgently needed to complement the existing antibacterial arsenal. Over the past few decades, there has been a vast growth in the knowledge of *P. aeruginosa* biofilms and the clinical implications of *P. aeruginosa* biofilms formation in disease states. There is an increased understanding of both the bacterial factors that influence biofilm development, maturation, and dispersal, and also the host factors that contribute to the immune response to biofilm infections. However, a more thorough understanding of biofilm biology and host immune response to biofilms is necessary to design new classes of therapeutics. Strategies currently under investigation include those that directly block biofilm attachment and formation, as well as those that promote dispersal and degradation. In addition, a novel avenue of potential therapies could act by augmenting host immune response against biofilms. These avenues appear very promising, and will hopefully provide complementary approaches along with antibiotics for treatment of patients with recalcitrant *P. aeruginosa* infections. ■

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