Published in final edited form as:
Microb Ecol. 2014 July ; 68(1): 1–12. doi:10.1007/s00248-013-0297-x.

Pseudomonas aeruginosa biofilms in disease

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Abstract

Pseudomonas aeruginosa is a ubiquitous organism that is the focus of intense research because of its prominent role in disease. Due to its relatively large genome and flexible metabolic capabilities, this organism exploits numerous environmental niches. It is an opportunistic pathogen that sets upon the human host when the normal immune defenses are disabled. It's deadliness is most apparent in cystic fibrosis patients, but it also is a major problem in burn wounds, chronic wounds, chronic obstructive pulmonary disorder (COPD), surface growth on implanted biomaterials, and within hospital surface and water supplies where it poses a host of threats to vulnerable patients [1,2]. Once established in the patient, *P. aeruginosa* can be especially difficult to treat. The genome encodes a host of resistance genes, including multidrug efflux pumps [3]and enzymes conferring resistance to beta-lactam and aminoglycoside antibotics [4], making therapy against this gram-negative pathogen particularly challenging due to the lack of novel antimicrobial therapeutics [5]. This challenge is compounded by the ability of *P. aerugionsa* to grow in a biofilm, which may enhance its ability to cause infections by protecting bacteria from host defenses and chemotherapy. Here we review recent studies of *P. aeruginosa* biofilms with a focus on how this unique mode of growth contributes to its ability to cause recalcitrant infections.

Keywords

Biofilm; Tolerance; Persister; Antibiotic; Resistance; Wound; Infection

Infectious Biofilms

A long-standing problem in patient care is the ability of bacteria, including *P. aerugionsa*, to form biofilms on implanted and indwelling devices. These localized infections can be difficult to detect with routine clinical microbiology and very frequently fail to resolve with aggressive antibiotic therapy [6]. Electron micrographs of an artificial hip removed from a patient with a chronic infection revealed the presence of extensive *P. aeruginosa* biofilms, which were confirmed by clinical microbiology [7]. This patient's infection failed to resolve despite two courses of aggressive antibiotic therapy ultimately resulting in surgical intervention [7]. This is an undesirable outcome due to the burden it places on the patient and the costs associated with otherwise unanticipated surgery.

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P. aeruginosa is a major cause of nosocomial infections which affect more than 2 million patients every year and are accounted for around 90,000 deaths annually [8]. Many of these infections are associated with catheterization and intubation, with urinary tract infections being the leading nosocomial infection [8]. Biofilms have been shown to form readily on catheters and ventilator tubes and represent a major risk to patients [9–11]. It has been demonstrated that bacteria found on the ventilator tubes correspond to strains causing respiratory infection, particularly ventilator associated pneumonia (VAP) [12]. This represents a serious challenge as the act of enabling critical patients to breathe is exposing them to a potentially deadly biofilm infection. *P. aeruginosa* and *S. aureus* are known to be major pathogens associated with VAP, but as culture independent diagnostic methods are being utilized, it is becoming clear that many clinical biofilms are polymicrobial in nature [13,14]. Indwelling urinary catheters removed directly from patients show robust biofilm formation on these surfaces and culture independent methods indicate that these biofilms are polymicrobial as well [9,14]. The polymicrobial nature of biofilms presents another potential complication for the attending clinician.

Cystic fibrosis patients most frequently succumb to a chronic infection of the lungs with *P. aeruginosa* [15]. The patients suffer from a relapsing cycle of infection, inflammatory response, and airway obstruction that causes continual damage to the airways. Intensive culture and culture-independent methods have demonstrated that CF airway infections are polymicrobial in nature as well [16–20]. Improvements in antimicrobial therapy have resulted in increased longevity and health of patients with CF. Aerosolized antibiotics, particularly tobramycin, has revolutionized care by allowing high doses of antibiotics to be delivered to the site of infection in CF patients [21]. This aggressive therapy often fails to eradicate the infection, despite clinical microbiology evidence indicating that the pathogen should be susceptible to the high doses of administered antibiotic [22]. This paradox has been explained in a number of ways. *P. aeruginosa* has been shown to form biofilm-like microcolonies in the lungs of CF patients [23,24]. Singh *et al.* found that quorum sensing production signals found in patients lungs were only produced by isolated strains when they were grown in biofilms [23]. These two lines of evidence suggested that *P. aerugionsa* forms biofilms in the CF lung, possibly explaining the difficulty of treating this infection.

P. aeruginosa biofilms potentially play a role in clinical outcomes of patients with chronic wounds. Patients with these types of wounds fall into many categories, but a major group are diabetic patients with non-healing ulcers on their lower extremities. Due to problems with circulation, nervous malfunction, and possibly other causes, a large percentage of diabetic patients develop chronic wounds, with up to 25% of these patients requiring amputation to deal with the problem [25]. These types of wounds are ideal for bacterial colonization due to loss of skin and the poor circulatory conditions that minimize the immune response and healing. James and colleagues examined samples from patients with chronic or acute wounds and found visual evidence of biofilm-like formations showing densely clustered cells [26]. Overall there was a higher prevalence of biofilm-like formations in chronic wounds. This was the first evidence suggesting that biofilms are present in chronic leg wounds. Follow up studies suggest that while wound infections may be polymicrobial, the distribution of bacteria within wounds favors monospecies biofilms [27]. *P. aerugionsa* cells

are found further from the surface of wounds when compared to infections caused by *S. aureus.* This finding has implications for treatment as it may hinder clinical detection of the causative infecting microbes and prevent topical treatments from reaching the intended target. Indeed, the authors suggest that this distribution could lead to under detection of *P. aeruginosa* in chronic wounds and thus less successful design of antimicrobial therapy [27,28].

The clinical observation of biofilms in a host of infectious diseases raises the natural question of what makes biofilms unique from planktonic cells? Evidence from laboratory studies has guided much of our thinking of what is happening in the clinic and perhaps leads to some confusion about the nature of biofilms as well.

Antimicrobial penetration of biofilms

Several hypotheses have been formed to explain the reduced susceptibility of biofilms to antimicrobials. The penetration limitation hypothesis posits that only the surface layers of a biofilm face exposure to lethal levels of antibiotics due to physical, mechanical, or biochemical mechanisms that limit the transport of antibiotics into the biofilm substratum [29–33]. This hypothesis implies that antimicrobials may be consumed, deactivated, or adsorbed by components of the biofilm extracellular matrix. Furthermore, the penetration of antimicrobials through the biofilm may be retarded as these agents meet diffusional resistance [34]. Indeed this mechanism has been shown to protect biofilms from killing by reactive oxygen species such as hydrogen peroxide and hypochlorite [34]. However, there are conflicting reports in the literature about the ability of clinically relevant antibiotics to effectively penetrate biofilms, as well as there exist differences in the diffusion parameters for different antibiotic classes [29,35].

Anderl *et al.* tested the ability of ciprofloxacin and ampicillin to penetrate *Klebsiella pneumoniae* biofilms in order to determine if penetration limitation was the cause of increased tolerance to these antimicrobial agents. Ciprofloxacin was shown to fully penetrate biofilms quickly, whereas the penetration of ampicillin was hindered by the production of a β-lactamase [29]. However, ampicillin was fully able to penetrate the biofilms of a β-lactamase deficient mutant, and these biofilms were also highly tolerant to ampicillin treatment [29]. Flouroquinolones were also shown to fully and rapidly diffuse through biofilms of *P. aeruginosa* as measured by Fourier transform infrared spectroscopy [36]. In the biofilms of uropathogenic *E. coli*, Stone *et al.* developed a fluorescence-based method for visualizing cells exposed to tetracycline. All cells, both biofilm and planktonic, developed maximal fluorescence with the same kinetics, with no layers or pockets of the biofilm that were suggestive of reduced tetracycline exposure. These data demonstrated that tetracycline penetration is not affected by biofilm growth [37]. Taken together, these data suggest that the high level of antibiotic tolerance exhibited by biofilms cannot be accounted for by penetration limitation.

Organisms upregulate β-lactamase synthesis in response to antibiotic exposure, and these enzymes can accumulate in the biofilm matrix, deactivating β-lactam antibiotics on the surface layers of the biofilm before they can diffuse into the substratum [38,39]. As

discussed above, penetration of ampicillin into the *K. pneumoniae* biofilm substratum was impeded in the presence of a β-lactamase, though this may be of little significance, as this did not account for the increase in tolerance [29]. In the case of positively charged aminoglycosides, certain negatively charged matrix components slow diffusion through the biofilm, giving additional time for bacteria to mount a stress response. This has been shown for the alginate component of *P. aeruginosa* biofilms [40]. Aminoglycosides also require a proton motive force to be taken up into the cell. It stands to reason that the oxygen-limited, slowly metabolizing cells of the biofilm substratum would take up aminoglycosides at a reduced rate, even if there were no obstacle to the penetration of the antibiotic itself. However, if *P. aeruginosa* has access to alternative electron acceptors such as nitrate, anaerobic killing by aminoglycosides is not problematic [41]

The presence of DNA, a major matrix component of the biofilms of various unrelated gram negative and gram positive bacteria, can alter antibiotic penetration [32]. Tetz *et al.* showed that the addition of DNase I to the biofilms of various bacteria could decrease the biofilm biomass by as much as 40% without changing the number of viable cells within the biofilm itself [32]. They then show that DNaseI treatment of bacterial biofilms, when combined with antibiotics, led to a greater degree of biofilm killing than with antibiotics alone. This held true for all of the major classes of antibiotics. They concluded that the destruction of the extracellular DNA component of bacterial biofilms permits the increased penetration of antibiotics. However, the authors did not provide any experimental evidence to show that there was any difference in the diffusion profiles of antibiotics between normal and DNase I-treated biofilms. For most classes of antibiotics the more likely scenario for the increased killing seen in the study is that the altered biofilm morphology observed after DNase I treatment, which led to large decreases in biomass, enabled greater access of nutrients and other factors conducive to bacterial growth deeper into the biofilm layers. This, in turn, would lead to increased bacterial metabolism, likely leading to greater antibiotic killing as bacterial metabolism increases and drug targets are available for corruption by antibiotics.

Owing to their positive charge, aminoglycosides are capable of being bound by DNA through electrostatic interactions [42], and experimental evidence demonstrated that the presence of extracellular DNA delayed penetration of aminoglycosides across *P. aeruginosa* biofilms [43]. DNA can therefore act as a sink to sequester positively charged antibiotics and antimicrobial peptides. This delayed penetration phenomenon was short lived however, as extracellular DNA was found to be saturated with antibiotic after constant, fresh delivery [43]. Nonetheless, the extracellular DNA-mediated delayed penetration of aminoglycosides in biofilms may play an important role *in vivo*. For example, an infected CF lung could contain zones with limited access to aerosolized aminoglycosides, preventing saturation of the biofilm matrix [43]. However, it remains unclear just how much biofilm mass is composed of components that capture positively charged antibiotics.

Protection from the immune system

Biofilms are difficult to eradicate due to the inherent protection they provide from host defenses. One method of protection relates to the ability of biofilms to evade the humoral host response. Precisely how biofilm cells avoid this immune response is the subject of

intense study. Whatever the precise mechanisms, it is likely that the biofilm provides an immune privileged environment for drug tolerant persister cells to survive. Studies have been performed to measure the diffusion coefficient of fluorescently labeled antibodies across *K. pneumoniae* and *Pseudomonas aeruginosa* biofilms. These studies have determined that antibodies lack the ability to effectively penetrate biofilms due to binding to the biofilm extracellular matrix [44]. However, other studies have found that large macromolecules such as antibodies are capable of rapid penetration of biofilms [45,46]. This indicates that there are differences both in the means of measuring such penetration and most likely in how biofilms are cultivated *in vitro.*

Other components of adaptive immunity are affected by chronic *P. aeruginosa* infection. The T-cell response to chronic lung infection in cystic fibrosis patients is dominated by a Th2 response versus CF patients that are not yet chronically infected, who demonstrated higher levels of IFN-γ, greater lung function, and a more balanced Th1/Th2 response [47]. The tipping of the Th1/Th2 balance towards a less effective Th2 response has been reported in chronic CF patients by others as well [48,49]. By mechanisms not completely understood, the presence of *P. aeruginosa* biofilms causes remodeling of the host immune response to favor biofilm survival. The clinical implications of this are that a change to the Th1-type immune response might improve lung function in CF patients. Indeed this has been shown to be true in rodents, where a Th1-dominated response against *P. aeruginosa* infection was correlated with greater lung function and reduced disease sequellae [50–52]. This is primarily thought to be mediated by increased stimulation of alveolar macrophages that results in resolution of pulmonary inflammation and reduced chemoattraction of neutrophils, as well as reduced formation of immune complexes that lead to tissue damage [53,54].

Established biofilms also offer protection from the innate immune system. Researchers have observed the innate response to *P. aeruginosa* biofilms in early mouse lung infection. These studies have demonstrated that activated neutrophils and macrophages in the airways are a major component of innate immunity [55–57]. The neutrophil response has undergone a great deal of study, as the collateral damage caused by these effector cells is believed to be high, and the long sustained presence of these cells throughout the course of chronic infection is likely responsible for major disease sequellae [58]. Though neutrophils and macrophages can accumulate at the surface of *P. aeruginosa* biofilms, they can no longer engulf the pathogen, undergoing a phenomenon known as “frustrated phagocytosis [59].” This phenomenon can leave these activated phagocytic cells in a state of secreting toxic compounds and damaging nearby healthy host tissues. The exopolysaccharide alginate, which is an important component of the *P. aeruginosa* extracellular matrix, has been implicated in frustrated phagocytosis. Researchers showed that alginate production did not play a significant role in bacterial attachment, biofilm formation, or biofilm development, however, *P. aeruginosa* strains deficient in alginate production were far more susceptible to phagocytosis compared to their isogenic parental strains [59]. The molecular mechanism of this alginate-mediated inhibition of phagocytosis is still unclear, although production of alginate is under control of the σ-22 extracytoplasmic stress response system, which responds to cell wall stress. This suggests that the inhibition of neutrophil function is a targeted response by *P. aeruginosa* [60].

Other mechanisms to prevent phagocytosis of biofilms by effector cells of the immune system also exist. *P. aeruginosa* growing in a biofilm show increased production of rhamnolipids compared to their planktonic counterparts [56]. Rhamnolipids are powerful detergents that can cause cellular necrosis and neutrophil elimination [58]. Evidence suggests that *P. aeruginosa* utilize rhamnolipids to form a biofilm-encompassing shield that eliminates phagocytes upon contact [56,61]. van Gennip *et al.* recently used confocal and electron microscopy to visualize *P. aeruginosa* biofilm development *in vivo* following intraperitoneal inoculation of mice with bacteria growing on silicone tubes [62]. This group showed that wild type *P. aeruginosa* strains caused cytotoxic effects on PMNs and a failure to protect against biofilm growth. However, a *rhlA* mutant, which is defective in rhamnolipid production, was actively phagocytosed by infiltrating PMNs [62]. Together, alginate and rhamnolipids play an important role in evading the phagocytic cells of the innate immune system.

Other factors of innate immunity, including complement and antimicrobial peptides, are also subject to immune evasion during biofilm growth. Production of alkaline protease and elastase by *P. aeruginosa*, both of which are upregulated during biofilm growth, is capable of inactivating complement directly [63,64]. It was also found that the O-acetylated alginate component of the *P. aeruginosa* extracellular matrix prevents activation of the alternative pathway of complement, protecting cells from antibody-independent phagocytosis [65]. Positively charged antimicrobial peptides may bind to the extracellular DNA component of the biofilm matrix, acting as a sink and preventing binding to the bacterial cell surface [66]. The *pmr* genes of *P. aeruginosa* are upregulated in the presence of extracellular DNA, and a subset of these genes are responsible for the covalent addition of aminoarabinose to the 1- and 4′-phosphates of lipid A [66,67]. This positively charged moiety masks the bacterial outer membrane from antimicrobial peptides [67]. Similarly, upregulation of PmrAB-controlled spermidine synthase genes resulted in increased surface associated spermidine, whose positive charge also masks the cell surface charge [66].

Biofilm Tolerance to Antimicrobial Agents

Early biofilm researchers found *P. aeruginosa* biofilms to be difficult to kill with extremely high concentrations of antibiotics [68]. While striking, it is common to compare growing planktonic cultures to well developed biofilms. This is a flawed comparison because of the well-known correlation between growth rate and efficacy of bactericidal antibiotics [69–71]. The susceptibility of biofilms is now quantified as the minimal biofilm eradication concentration (MBEC) and is a method accepted as part of CLSI standards [72]. In the MBEC test, biofilms grown on plastic surfaces are exposed to antibiotics for a predetermined period of time, after which they are transferred to fresh medium [73]. The MBEC value is the lowest concentration of antibiotic that prevents regrowth in the fresh media. It is possible for a single surviving cell from a biofilm to return a result of resistance in this test, which is beyond the stringency of the standard CLSI MBC assay of 99.9% killing. Thus the MBEC is assessing the ability of a given antibiotic concentration to completely sterilize an *in vitro* culture, which is extremely difficult to accomplish given that all bacterial strains form drug tolerant persisters [74].

Attempts have been made to link the efficacy of antibiotic treatment *in situ* to biofilm susceptibility testing. Moskowitz and colleagues developed a clinically feasible assay designed to test the biofilm inhibitory concentration (BIC) [75]. The BIC differed from the MBEC assay in the recovery time allowed in fresh media after antibiotic treatment. In the BIC, growth of surviving cells is detected six hours after placing surface attached biofilms into fresh media. Thus very low surviving numbers of cells are unlikely to result in detectable growth as measured by spectrophotometer. While significant differences in BIC were found for beta-lactam antibiotics, only two fold differences were found for the aminoglycosides tobramycin and gentamicin [75,76]. The results of this and a preliminary *in vitro* study lead to the utilization of the BIC method in treatment of CF patients in a controlled clinical trial [76]. The results suggested that BIC testing added no valuable information guiding clinical treatment. However, the authors noted that follow up studies with a different cohort of patients might prove that BIC testing is useful. The BIC may not provide useful information for the clinician for a variety of reasons. Clinical biofilms may not behave like *in vitro* biofilms, polymicrobial biofilms may complicate therapy in unanticipated way, or drug tolerant persister cells cause antibiotic therapy to fail.

We found that the resistance of biofilms to killing is largely attributable to the formation of drug tolerant persister cells [77]. Indeed, stationary phase planktonic cultures and biofilm cultures demonstrate similar tolerance to a range of bactericidal antibiotics [77]. We suggested then that drug tolerant persister formation explains the ability of biofilms to survive antimicrobial treatment. The importance of persisters in biofilm recalcitrance is now generally accepted [78–81]. Moreover, these drug tolerant cells are not detected in clinical microbiology labs, meaning their contribution to therapeutic failure is largely overlooked.

Experimental evidence *in vitro* shows that *P. aeruginosa* biofilms form persisters tolerant to a host of antibiotics under a variety of growth conditions. While no two studies are alike, a common theme emerges, biofilm researchers universally detect tolerant populations in their biofilms and a variety of mechanisms tip cells into a dormant, drug-tolerant persister state. Recent evidence suggested that the stringent response plays a role in antimicrobial tolerance of *P. aeruginosa* biofilms [82]. In this study, simulated nutrient deprivation increased persister formation, while genetic inactivation of the stringent response decreased persister formation. The authors found that stringent response mutants have increased reactive oxygen species and production of 4-hydroxy-2-alkylquinoline molecules (HAQs), which they propose as the mechanism of decreased tolerance. However, a subsequent study in *E. coli* found that in aging biofilms, it is the SOS response rather than the stringent response that is key to increased persister formation [83]. This agrees with our studies demonstrating a connection between SOS induced toxin-antitoxin expression and persister formation [84]. In addition, our findings and other findings have demonstrated that ROS formation is not a universal mechanism behind antibiotic lethality [41,85]. Thus target inactivation remains the most important means to cause persister formation.

One of the great challenges of treating any *P. aeruginosa* biofilm infection is the intrinsic antibiotic resistance of this pathogen. *P. aeruginosa* encodes for multiple MDR efflux pumps and antibiotic inactivating enzymes and a recent review highlights recent work details the increasingly complex “resistome” that makes treating this pathogen quite difficult

[86]. Further complicating the resistance picture, Liao and Sauer identified a biofilm specific transcriptional regulator dubbed *brlR* (for biofilm resistance locus regulator) that specifically alters susceptibility of *P. aerugionsa* to multiple classes of antibiotics and biocides [87]. BrlR overexpression also alters the susceptibility of planktonic cells when overexpressed. Mutants lacking b*rlR* had no defects in biofilm formation and no altered susceptibility in planktonic growth. Gene expression analysis and CHIP assays demonstrate that BrlR directly promotes expression of the multi-drug efflux pumps MexAB-OprM and MexEF-OprN [88]. These MDR pumps are responsible for resistance to a wide range of antibiotics suggesting that *brlR* regulates a biofilm specific program of increasing antibiotic resistance. This resistance would not be detected in clinical MIC assays as cells are grown planktonically. However, the previously mentioned BIC might be capable of detecting this resistance, which may explain the differences in BIC and MIC values in that study [75]. This intriguing, biofilm-specific regulatory mechanism suggests a reason for why *P. aeruginosa* biolfilms are so difficult to treat clinically, though it remains to be seen if this occurs *in vivo*. Interestingly, we have found that sufficient antibiotic concentrations can overcome the ability of efflux pumps to protect cells from antibiotic killing [89]. If *brlR* is active *in vivo*, it may still be possible to treat infections with very aggressive chemotherapy.

P. aerugionsa biofilms grown *in vitro* have spatial heterogeneity of metabolically active vs. inactive cells [90]. The metabolically inactive cells tolerate multiple classes of antibiotics, likely due to the downregulation and reduced activity of the molecular targets, such as the ribosome. Catabolite Repression Control (CRC) is a global, posttranscriptional regulatory mechanism that involves translational inhibition of select mRNAs in order to fine tune metabolism [91]. O'Toole and colleagues found that CRC is necessary for robust biofilm formation [92]. Mutants lacking *crc* are able to attach to plastic surfaces, but not expand beyond a simple monolayer. Linares *et al.* found that *crc* mutants are hyper-susceptible to fluoroquinolones, aminoglycosides, beta-lactams, rifampin and fosfomycin [93]. Importantly, *crc* mutants had no defect in growth rate, indicating that the hypersusceptibility was not related to growth rate. Further testing with both an MDR pump inhibitor and transcriptional analysis indicated that the hypersusceptibility of *crc* mutants was not due to defects in MDR pump levels. The conclusion of the study is that *crc* mutants were hypersuscetible due to a host of changes in the cell envelope and expression of metabolic genes important in antimicrobial susceptibility. Zhang *et al.* hypothesized that CRC may control the phenotypic heterogeneity of biofilm cells in response to differential access to nutrients. They grew biofilms of wild-type and *crc* mutants in flow cells and first found that *crc* mutants have an early, but not a late biofilm forming defect [94]. After four days, *crc* mutants formed biofilms that were equal in robustness to wild type biofilms. Using a metabolic dye, the authors found that cells lacking *crc* form biofilms that are generally more metabolically active, likely due to their inability to repress metabolic function when faced with nutrient limitation. Live-dead staining and confocal microscopy revealed that *crc* mutant biofilms were hypersusceptible to ciprofloxacin and the authors conclude that this is due to their increased metablic activity. It remains to be determined how these *crc* mutants affect tolerance quantitatively, as only an indirect measure detecting living cells was used in the aforementioned study. However, these studies demonstrate the important interplay

between metabolism and antimicrobial tolerance. Given that *crc* mutants have reduced virulence, it may be difficult to assess how this global regulator affects tolerance *in vivo*.

To get a global picture of genetic loci that contribute to biofilm specific susceptibility/tolerance to tobramycin, Amini *et al* grew a complete transposon library of PA14 in a biofilm, exposed the biofilm to bactericidal concentrations of tobramycin, and compared the ratio of input mutants to tobramycin survivors [95]. They compared this to planktonically grown cells and they identified genes that specifically contributed to susceptibility in a given growth state. One important observation from these studies was that an increased ability to form biofilm did not correspond with increased tolerance to tobramycin, indicating that ability to form biofilm itself is not sufficient to cause recalcitrance to antimicrobial therapy.

Persister formation in *P. aeruginosa*

Our research on persisters causing so-called biofilm resistance questioned the importance of drug tolerance in clinical infections. Given that persisters are phenotypic variants with a transient phenotype, their isolation and characterization from clinical infections is challenging. Patients with CF often suffer from chronic and clonal infections with *P. aerugionsa*. Genomic studies of isolates taken from such patients have demonstrated that *P. aerugionsa* adapts to the CF lung environment through a variety of mutations [96,97]. While persisters are phenotypic variants, it is well known that genomic mutations can increase or decrease the numbers of persisters formed by a given strain [74]. We reasoned that if persister formation is the reason that susceptible chronic infections fail to resolve with antibiotic therapy, then high persister (hip) mutants should emerge in chronic CF infections. Isolates from 15 CF patients with chronic and clonal infections where early and late isolates were available were tested for *in vitro* multi-drug tolerance [98]. In at least 11 patients, hip mutants emerged during the course of the patient's life. In seven cases, hip mutants had no change in antimicrobial susceptibility, suggesting that persister formation was the principal reason for therapeutic failure. It also suggests that these cells were exposed to therapeutic agent, as it is extremely unlikely this phenotype would be selected in the absence of antibiotic exposure. These results strongly suggest that persister formation is a primary reason why clinical treatment fails and argues for further investigation into the mechanisms of persister formation in *P. aeruginosa*, which are not well understood.

Screening of an incomplete transposon library (5,000 mutants total) identified insertions in dinG, spuC, PA14_17880 and PA14_66140 that exhibited a low persister phenotype and insertions in algR, pilH, ycgM, pheA and PA14_13680 that exhibited a high persister phenotype [99]. The changes in persister levels of these mutants however were modest, and little work on these genes has followed since publication. Moker and colleagues found that pyocanin, paraquot and the acyl-homoserine lactone 3-OC12-HSL slightly increased persister formation in exponentially growing cutlures of *P. aeruginosa*, but not in *E. coli* or *S. aureus* cultures [100]. De Groote *et al.* discovered that strains with fosfomycin resistance correlated with decreased tolerance to fluoroquinolones [101]. The reasons for this correlation are still unclear, but a simple assumption is that fosfomycin resistance has a fitness cost on cells that affects susceptibility or tolerance to other antibiotics. TA genes have been shown to play a major role in forming drug tolerant persister cells in *E. coli* [102].

There are three TA loci in the *P. aerugionsa* genome. As of yet, these loci have not been implicated in persister formation in *P. aeruginosa*, and a triple TA knockout displayed no phenotype following antibiotic challenge (Vincent Isabella, unpublished data). In *E. coli*, at least 6 TA loci had to be disrupted before an effect on persister formation was observed [102], and it is possible that more, as of yet unidentified, TA cassettes exist in *P. aeruginosa* that mask a potential role for TA systems in *Pseudomonas* persister formation. In any case, in order to better understand how to tackle the persister problem of chronic infections, we must gain a better understanding of the mechanisms of persister formation in *P. aerugionsa.*

Recent *in vivo* biofilm models

Several in vitro biofilm models have been widely adopted. While these can be important for understanding the mechanisms behind biofilm formation and differentiation, continual work is required for many models to faithfully replicate clinical infections. Recent progress suggests that more relevant models are emerging. Several animal models of biofilm infection with *P. aerugionsa* have been developed and warrant attention for their potential to guide clinical treatments. Watters *et al.* developed a murine model of chronic diabetic wounds to examine important questions related to infections and wound healing [103]. Mice were treated with Streptozotocin (STZ) to destroy insulin producing beta-cells and thus induce diabetes, followed by administration of a 1.5×1.5 cm surgical wound. The wounds were hold open by and covered with vapor and moisture permeable OPSITE dressings (Smith & Nephew, St. Petersburg, FL). These dressings also kept environmental bacteria out of the wounds allowing the investigators to control the species of bacteria present in each infection. Animals were infected with *P. aeruginosa*, and followed for approximately two and a half weeks. Diabetic, insulin treated diabetic, and non-diabetic mice were compared for their wound healing progress, biofilm formation, and bacterial load. Non-diabetic mice healed significantly faster than diabetic and insulin treated diabetic mice with more rapidly reduced bacterial burden in the wound. The incidence of biofilm-like aggregates was also lower in non-diabetic mice. Importantly, even though insulin treatment improved the overall health of diabetic animals, it provided no benefit to wound healing. The authors indirectly tested the tolerance of bacteria in wounds by removing a gauze carrying bacteria from wounds of diabetic and non-diabetic mice and found increased tolerance in bacteria from non-diabetic mice. The significance of this finding is questionable though as it involves *ex vivo* treatment and no direct treatment of wounds with antibiotics was attempted in this study. Regardless, the model is promising and should allow investigators to better understand how bacterial pathogens impede wound healing. It also raises the possibility of testing the significance of *in vivo* biofilm formation by allowing for infection with biofilm deficient strains, though these strains will have to be carefully selected to avoid strains with significantly reduced virulence.

Seth *et al.* developed a rabbit model useful for studying non-healing subcutaneous wounds [104]. The wounds are formed on the rabbit ears using a wound punch mechanism to damage the skin. In their initial experiments, GFP labeled *S. aureus* was used to cause and track infections. They found that *S. aureus* formed a biofilm within 24 hours and caused a prolonged, low-grade inflammatory immune response that inhibited wound healing. These results were extended in a follow up study where wound biofilms were observed with *S.*

aureus, *Klebsiela pneumoniae*, and *P. aeruginosa* [105]. Importantly, these authors observed that infection with *P. aeruginosa* lead to the greatest inflammatory response and delay of wound healing. *P. aeruginosa* biofilms in this wound model formed from small clusters of cells, with extensive amounts of extracellular polymeric substance (EPS) observed by scanning electron microscopy. This observation lead them to test EPS deficient *P. aeruginoas* mutants in this model, and they found that wound healing improved due to lower induction of inflammation. Interestingly, these EPS deficient mutants still formed biofilm like clusters, and cell numbers found in the wound were similar to infections with wild-type. This model provides a good platform for testing new antibiotic regimens aimed at treating biofilm infections.

Numerous studies have used animal models to study the mechanisms behind airway infections of the CF lung. Despite genetic manipulations and controlled conditions, mice and other animal models failed to replicate the spontaneous chronic infections that develop in CF patients. The development of a porcine model of cystic fibrosis has held more promise [106]. Newborn CF pigs spontaneously develop airway infections that are maintained for longer periods of time. In follow up studies, Pezzulo *et al.* found that a decrease in the pH of airway secretion liquid in CF pigs allowed bacteria to establish spontaneous infections due to decreased efficacy of host antimicrobial defenses [107]. This important study suggests that biochemical manipulation of the CF lung may inhibit or completely prevent bacterial colonization of the airways. Whether the porcine model will prove useful for studying the chronic biofilm-like infection that occurs in CF patients remains to be seen.

Therapeutic Research

Besides understanding what makes biofilms unique from planktonic cells, it is critical to develop therapeutic interventions to treat biofilm infections. As discussed, indwelling medical devices are excellent sites for development of biofilm infections. Catheterization is necessary for a host of patients and presents a challenge to clinicians owing to the ability of microbes to colonize the devices. This can then lead to blood-born infections and may require removal of the catheter, placing extra burdens on an already unhealthy patient. For many types of catheter infections, antibiotic lock therapy (ALT) is the clinically recommended course of action [108]. A high concentration of antibiotic is left within the catheter in the hopes of eradicating what is frequently a biofilm infection. This has limited success for certain pathogens and often can require that the ALT be conducted for as long as two weeks in conjunction with systemic antibiotic administration. Chauhan and colleagues demonstrated that combinations of high concentrations of gentamicin and EDTA are very effective at eradicating *in vivo* biofilm infections formed by Gram-negative as well as Gram-positive pathogens [109]. In several instances, the therapy is capable of killing all bacteria growing on the catheter with a single ALT dose, and is useful against *P. aeruginosa*, which was previously not recommended for ALT therapy. These animal data are promising and thus warrant consideration for clinical usage in treating catheter infections.

Sharp debridement of wounds is known to improve chronic wound healing [110]. One hypothesis for why debridement works is that sharp debridement breaks up biofilms that are preventing healing and wound closure. Given that biofilms are known to be highly tolerant

to antibiotics due to the presence of drug tolerant persisters Wolcott, *et al.* decided that antibiotic treatment should occur in the time periods shortly after debridement when they believed cells would resume growth to cover freshly exposed territory in the wound [111]. Actively growing cells are inherently more susceptible to antibiotics. Combining the results of tests of *P. aeruginosa* as well as *S. aureus* biofilms (*in vitro*, in a pig skin model of infection, a mouse model of infection, and in patients with wounds predominantly infected with *P. aeruginosa*) a rather remarkable discovery was made: appropriate treatment applied within a 24-hour time period after wound debridement results in highly effective killing of bacterial cells. In a follow up clinical trial applying this principal to the actual treatment of patients with chronic wounds, Dowd *et al*. found that sharp debridement and antibiotic treatment shortly thereafter led to the best outcomes of wound healing by a wide margin over other treatments [112]. Thus, chronic wounds in diabetics are difficult to treat because of an overwhelming presence of drug-tolerant persisters in the wounds, and the authors have found a simple and effective method to eradicate those persisters and improve wound healing by simply stimulating growth of the dormant persister cells present in the wound followed by killing those cells with antibiotics. This is not unlike a therapeutic course we previously recommended, where pulse dosing antibiotics would be used to eliminate persisters that had resuscitated [113].

The treatment of CF airway infections continues to evolve with newer therapies passing through the discovery pipeline. Antimicrobial therapy advances chiefly consist of new methods of delivering existing antibiotics to the CF lung and rational combination therapies. In the pre-clinical stages there is a new tobramycin formulation, a liposomal amikacin, aerosolized fluoroquinolones, and a combination therapy of fosfomycin and tobramycin aimed at targeting gram-positive, gram-negative, and anaerobic microbes [114]. Another ongoing clinical trial aims to utilize IV gallium nitrate (Ganite) to treat airway infections. Ganite starves infectious microbes of iron rendering them inactive and it is also known to disrupt biofilm formation *in vitro* [115]. The phase I trial results for this therapy have yet to be published [114]. Miller and colleagues demonstrated that a nitric oxide has antimicrobial efficacy at dosage concentrations and schedules that should not be harmful to patients with lung infections [116]. A phase I clinical trial demonstrated that doses of NO that are effective *in vitro* and in animals models of infection are well tolerated by patients[117]. These emerging therapies make it clear that clinicians will have an increased arsenal of antimicrobial therapies to combat the chronic CF airway infection in the near future.

The FDA recently approved the small molecule ivacaftor (Kalydeco), which potentiates the function of mutated cystic fibrotis transmembrane conductance regulator (CFTR) protein [118]. While this therapy only works in 5% of CF patients that carry a specific CFTR mutation (G551D), there is promise that additional advancements will expand the number of patients treated by this strategy. Vertex Pharmaceuticals is conducting a phase 2 clinical trial with combination therapy of Kalydeco and VX-809, which enables misfolded CFTR to reach the cell surface in a functional state [119]. This combination therapy would target a greater proportion of the CF community. Both these and other approaches would aim to restore airway function so that infections do not get a chance to take hold, though it is possible that antimicrobial therapy will be required along with these treatments as well.

Conclusions and outlook

The biofilm infection presents a host of barriers to successful treatment. The presence of drug tolerant persister cells is a major problem that may require altered chemotherapeutic practice or new antimicrobial agents capable of targeting dormant cells. Also of concern is the biofilm specific increase in multi-drug resistance that is mediated by *brlR*. A pressing question is what role this regulator plays in clinical infections. If active, it may indicate that prescribed doses of antibiotic are ineffective *in vivo*. Clinicians are making advances in using physical debridement and carefully timed antimicrobial therapy to advance the care of chronic wounds. A host of strategies is aimed at preventing *P. aeruginosa* from establishing an infection in the first place, either by restoring the health of the patient or by preventing its sessile growth on indwelling devices. It is encouraging to see basic and clinical research leading to progress in treatment of chronic biofilm infections.

Future research must focus on understanding the nature of *in vivo* tolerance and resistance. Gene expression and proteomic studies of bacteria recovered directly from clinical wounds will be critical in this regard. This requires new technological breakthroughs, as at present we are limited to genomic analysis of the microbes present in a wound, not the functional genes that are important in maintaining that wound.

Acknowledgments

This work was supported by National Institutes of Health grant T-R01AI085585-01 and by Army Research Office grants W9911NF-09-1-0265 and 55631-LS-RIP.

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