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Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. A short review

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Abstract

The CF lung is chronically inflamed and infected by *Pseudomonas aeruginosa*, which is a major cause of morbidity and mortality in this genetic disease. Although aerosolization of Tobramycin into the airway of CF patients improves outcomes, the lungs of CF patients, even those receiving antibiotic therapy, are persistently colonized by *P. aeruginosa*. Recent studies suggest that the antibiotic resistance of *P. aeruginosa* in the CF lung is due to the formation of drug resistance biofilms, which are defined as communities of microbes associated with surfaces or interfaces, and whose growth is facilitated by thick and dehydrated mucus in the CF lung. In this review, we discuss some of the current models used to study biofilm formation in the context of biotic surfaces, such as airway cells, as well as the contribution of host-derived factors, including DNA, actin and mucus, to the formation of these microbial communities. We suggest that better *in vitro* models are required, both to understand the interaction of *P. aeruginosa* with the host airway, and as models to validate new therapeutics, whether targeted at bacteria or host.

Keywords

Pseudomonas; biofilm; cystic fibrosis

1. Introduction and overview of biofilms and CF

The cystic fibrosis (CF) lung is in a chronic inflammatory state and responds poorly to antimicrobial therapy. CF patients typically suffer from persistent and recurrent lung infections caused by *Pseudomonas aeruginosa*, the dominant pathogen in CF airways, and by late adolescence, 80% of CF patients are chronically infected [1,2]. According to the National Nosocomial Infections Surveillance System, *P. aeruginosa* is the third most common pathogen associated with all hospital acquired infections [3] and several studies have suggested that CF patients could become infected by *P. aeruginosa* after visiting hospitals or CF clinics [4–7]. Chronic *P. aeruginosa* infections are associated with a declining clinical status of CF patients and a worsened prognosis, and once established, current antibiotic regimens are unable to eradicate *P. aeruginosa* infections in CF airways [8]. This lack of treatment efficacy is believed to be due in part to the formation of antibiotic resistant biofilms forming in the CF lungs [9]. The ability of *P. aeruginosa* to grow and establish drug resistant biofilms in the lungs of CF

patients is thought to be facilitated by the hypersecretion of a viscous mucus layer in the CF airway, which provides a low oxygen environment and the presence of DNA and actin in the CF airway resulting from the necrosis of neutrophils that are recruited into the CF lung as part of the innate immune response [10,11].

2. Biofilms

Biofilms are intricate bacterial communities found attached to living or abiotic surfaces and surrounded by a bacteria-produced extracellular matrix composed of exopolysaccharides, DNA and proteins [12–14]. Biofilms develop in a complex and well-coordinated manner that involves sensing and responding to cues, such as bacterial cell density, nutrient availability and energy sources present in the environment. The switch toward the biofilm mode of growth is often considered to be a survival strategy for bacteria [15].

A limited number of studies support the hypothesis that biofilms form in the CF lung *in vivo*. Clusters of *P. aeruginosa* cells have been retrieved from post-mortem CF lung [16], have been observed in the endobronchiolar region of the lung in immunostaining studies performed on preserved CF lung tissues [17], and were observed in freshly excised lung sections [10,15], lung abscess [15] and sputum [18] of CF patients. These clusters of *P. aeruginosa* might be analogous to the microcolonies of this microbe commonly observed when biofilms are grown *in vitro* on abiotic surfaces [19]. Furthermore, the mucus environment in which *P. aeruginosa* is detected is considered a microaerophilic or anaerobic environment [10,20–22]. Taken together, these data have been interpreted to mean that *P. aeruginosa* resides in a low oxygen and/or anaerobic mucus layer overlying airway epithelial cells in the CF airway.

The presence of quorum-sensing molecules in CF sputum, used by *P. aeruginosa* and other bacteria for cell to cell communication *in vitro* [23], has been cited as evidence that *P. aeruginosa* in the CF lung form biofilms [18]. The ratio of the two main quorum sensing molecules of *P. aeruginosa* found in CF sputum was more similar to the ratio observed for *in vitro* biofilm grown, rather than planktonic grown bacteria. These data established a correlation between one well-known property of *in vitro* grown biofilms and the *P. aeruginosa* populations found in the CF lung. Recent studies performed by Hoiby and colleagues show that treatment with azithromycin, which interferes with quorum sensing signaling, as well as alginate production, *in vitro* helps clear *P. aeruginosa* from a mouse model of chronic lung infection [24]. These data are consistent with a role for biofilm formation in the CF lung.

Despite this intriguing work, studies to date have not unequivocally established that the drug resistance of *P. aeruginosa* in the CF airway is due to the biofilm phenotype. This is due in part to the technical difficulties of studying biofilms in the context of animal and human lungs. In addition, recent studies, described below, suggest that studies of biofilms that form on abiotic surfaces are different in several important ways from biofilms that form either on human airway epithelial cells or form on the products secreted or released by airway epithelial cells. Thus, several research groups have developed new experimental models to elucidate biofilm formation in the context of the CF lung and to identify new therapeutics, and these systems will be reviewed in the next sections.

3. Mucus

As noted above, in the CF lung, clusters of *P. aeruginosa* are found in the thick layer of mucus overlying airway epithelial cells. To elucidate the role of mucus in facilitating the growth of *P. aeruginosa* in the CF lung several groups have developed *in vitro* approaches that mimic the thick layer of mucus typically thought to accumulate in the CF lung. For example, an artificial growth medium, containing porcine mucin, DNA, salts and amino acids, was shown to support the formation of *P. aeruginosa* microcolonies [25]. The authors tested the effects

of each component individually and uncovered a potential role for amino acids in the expression of OprF, a porin required for the anaerobic respiration and biofilm formation on abiotic surfaces by *P. aeruginosa* [21].

Another study reported the growth and development of antibiotic resistant *P. aeruginosa* biofilms on a layer of mucin covalently attached to glass surfaces [26]. These authors suggested that the attachment of bacteria to the host mucin could be one of the steps leading to increased antimicrobial resistance associated with biofilms.

In order to analyze the relationship between water content of mucus and biofilm formation, Matsui and colleagues collected the mucus layer secreted by human bronchial epithelial cells grown at an air-liquid interface [27] and concentrated it to reflect a normal or CF-like concentration. The restricted motility of *P. aeruginosa* in the CF-like mucus layer apparently resulted in the formation of bacterial cell clusters, in contrast with the normal, hydrated mucus layer, whose larger mesh pore size likely prevented the accumulation of quorum-sensing molecules and the formation of biofilms.

Thus, the use of an artificial mucus layer has then proven extremely helpful in providing first hand evidence of biophysical interactions between bacteria and some of the CF airways components. The rheological properties of CF sputum could be analyzed and placed in perspective with bacterial motility and biofilm formation. However, these *in vitro*, reconstituted systems might lack the contribution of yet to be identified mucus components, especially given that the ΔF508-CFTR mutation is known to lead to the disregulation of more than 1,000 genes [28,29], many of which have not yet been characterized. It is also important to note that the analysis of the physicochemical properties of sputum has shown that the water content of CF sputum is variable, and furthermore in a recent study on 27 CF patients, that the presence of *P. aeruginosa* is not linked to the dehydration level of the CF sputum [30]. Therefore, clinical studies do not uniformly support a model linking the dehydration level of the CF sputum with susceptibility to infection.

4. DNA and actin

DNA and actin are two other common components of the CF lung sputum. Whitchurch and colleagues showed several years ago that DNA facilitates the formation of biofilms [31]. In these studies, DNAse I was shown to block initial biofilm formation and dissolve preformed biofilms, suggesting that DNA is involved in both establishing the biofilm and as a component of the matrix. Furthermore, the source of DNA does not seem to be important for its activity, suggesting that it is the chemical properties of DNA that is important rather than a specific sequence. In subsequent years, DNA has been established as a component of the biofilm matrix in a number of other studies of *P. aeruginosa* [13,32], as well as other Gram-negative and Gram-positive organisms [33]. Another host component, actin, also may serve as a site of *P. aeruginosa* attachment. A study by Walker and colleagues indicated that this microbe might use F-actin as an attachment site [34] and thus could promote biofilm formation *in vivo*. However, more recent work using actin-derivatized glass indicated no role for this protein in bacterial attachment [26].

5. Epithelial cells

Several cell culture systems have been used to analyze the pathogenicity of *P. aeruginosa* on live epithelial cells. Studying the direct interaction between human airway cells and *P. aeruginosa* has the added benefit of taking into account the potential crosstalk between bacteria and human cells. Most of these earlier systems utilized multi-well plates with epithelial cells grown on a glass or plastic substratum. In these environments, the use of oxygen during metabolism and the production of secondary metabolites by *P. aeruginosa* resulted in the death

of airway cells in approximately 3–4 hours. Furthermore, the host cells typically do not fully differentiate when grown on these substrata. These studies reveal only dispersed attachment by *P. aeruginosa* to the airway cells, rather than the clusters of bacterial cells often observed in the context of CF sputum and lung samples.

In an effort to bypass the de-differentiation of cells grown in traditional flat monolayers, Carterson and colleagues [35] optimized the growth of A549 lung epithelial cells in a rotating-wall vessel bioreactor. They successfully established 3-D aggregates of polarized cells able to produce mucoglycoproteins and capable of mounting a proinflammatory defense in response to *P. aeruginosa* infection. The 3-D aggregates did not support bacterial attachment likely making them inappropriate for the study of biofilm formation, however further characterization of this system should be considered in light of recent data regarding the growth of *P. aeruginosa* as clusters in mucus (see below).

Some have argued that *P. aeruginosa* forms bioflms only in mucus and that in the CF lung *P. aeruginosa* is not in contact with epithelial cells [10]. Although there are compelling data supporting this view (outlined above), other studies are at odds with this model. For example, several studies suggest that *P. aeruginosa* grows directly on epithelial cells or inside these host cells [36–39]. What might be the clinical or physiological relevance of such interactions?

A recent study showed that *P. aeruginosa* was able to develop biofilm-like structures inside mouse airway epithelial cells [38]. These intracellular bacteria were resistant to antibiotic killing and displayed a change in gene expression also observed in *in vitro P. aeruginosa* biofilms. In human epithelial respiratory cells, the internalization of *P. aeruginosa* has been shown to depend on both cell polarity [40] and CFTR protein expression at the apical membrane [41–43].

Alternatively, another recent study examined a *P. aeruginosa* mutant lacking the ability to synthesize neuraminidase. The strain lacking neuraminidase is unable to expose the asialoGM1 receptor at the surface of airway CF cells and was shown to be defective in its ability to form a biofilm in the respiratory tract of a mouse model of infection [44]. This result, together with the fact that the neuraminidase gene is highly expressed in *P. aeruginosa* strains isolated from CF patients [45], was interpreted by the investigators to indicate that a direct physical contact between *P. aeruginosa* and the epithelium cell surface might be required, at least in the very early stages of airway colonization.

Consistent with the studies above, Henke and co-workers used antibodies to the major airway gel-forming mucins, MUC5AC and MUC5B, as a surrogate marker for mucin production. Comparison of the sputum of patients with and without CF revealed a 70% and 93% decrease in MUC5AC and MUC5B levels (vol/vol), respectively, in the sputum from patients with stable established CF lung disease who are colonized with *P. aeruginosa* [46,47]. This relative decrease in MUC5AC and MUC5B levels may be due to an overall increase in sputum volume or a defect in MUC5AC and MUC5B production. Confocal studies of sputum from CF patients did indicate increased amounts of DNA [46]. Interesting, following a pulmonary exacerbation, the concentrations of MUC5AC and MUC5B increase to levels found in the mucus of normal subjects [48]. These data beg the question of whether *P. aeruginosa* exploits mucus as a colonization site in the CF lung, or whether initial colonization of the airway surface by *P. aeruginosa* stimulates mucus production and provides a secondary niche wherein *P. aeruginosa* can establish a chronic infection. Additional investigation is required to resolve these issues.

6. Conclusion

Although it is not yet possible to study biofilms in the lungs of CF patients, several new models have provided compelling data regarding *P. aeruginosa* biofilms. These data reveal that numerous factors in the CF lung facilitate biofilm formation, which may include the presence of a layer of mucus that provides an anaerobic or microaerophilic environment conducive to *P. aeruginosa* growth, a reduction in the secretion of bactericidal compounds into the CF airway, increased levels of DNA and actin that contribute colonization sites and/or biofilm matrix components, and secretions by airway cells. These studies indicate that specific components of the host likely play a key role in the formation of biofilms in the context of the CF lung. While a great deal has been learned regarding factors that contribute to biofilm formation in the context of abiotic surfaces, biofilm formation on and in the context of the host is much more poorly characterized. As described above, however, it is clear that host factors do contribute to the formation of biofilm *in vivo*, and thus in the context of the clinic, these host determinants do need to be considered when assessing the efficacy of new treatments targeted at CF. Future studies to identify new therapeutics to treat the host defects in CF and the associated bacterial infections may be more relevant and powerful if conducted on polarized human airway epithelial cells.

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6. References

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