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Microreview

Evolving concepts in biofilm infections

Luanne Hall-Stoodley[1,2]* and Paul Stoodley[1,2]
[1]*Center for Genomic Sciences, Allegheny-Singer Research Institute, Pittsburgh, PA 15212, USA.*
[2]*Department of Microbiology and Immunology, Drexel University College of Medicine, Allegheny Campus, Pittsburgh, PA 15212, USA.*

Summary

Several pathogens associated with chronic infections, including *Pseudomonas aeruginosa* in cystic fibrosis pneumonia, *Haemophilus influenzae* and *Streptococcus pneumoniae* in chronic otitis media, *Staphylococcus aureus* in chronic rhinosinusitis and enteropathogenic *Escherichia coli* in recurrent urinary tract infections, are linked to biofilm formation. Biofilms are usually defined as surface-associated microbial communities, surrounded by an extracellular polymeric substance (EPS) matrix. Biofilm formation has been demonstrated for numerous pathogens and is clearly an important microbial survival strategy. However, outside of dental plaques, fewer reports have investigated biofilm development in clinical samples. Typically biofilms are found in chronic diseases that resist host immune responses and antibiotic treatment and these characteristics are often cited for the ability of bacteria to persist *in vivo*. This review examines some recent attempts to examine the biofilm phenotype *in vivo* and discusses the challenges and implications for defining a biofilm phenotype.

Introduction

Bacteria in most environments form organized communities of aggregated cells embedded in a hydrated matrix of extracellular polymeric substances (EPS) called biofilms (Costerton *et al*., 1999; Donlan and Costerton, 2002). Biofilm development is an ancient prokaryotic adaptation (Hall-Stoodley *et al*., 2004) and represents a mode of growth that allows bacteria to survive in hostile environments and to colonize new niches by various dispersal mechanisms (Hall-Stoodley and Stoodley, 2005; Purevdorj-Gage *et al*., 2005; Mai-Prochnow *et al*., 2008). Biofilm bacteria demonstrate coordinated behaviour with the formation of complex three-dimensional structures (Fig. 1) and functionally heterogeneous bacterial communities (Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004). This phenotypic heterogeneity, or localized specialization in biofilms is striking. For example, populations of bacteria within biofilms exhibit differences in the expression of surface molecules, antibiotic resistance, nutrient utilization and virulence factors (Bagge *et al*., 2004; Vuong *et al*., 2004; Pearson *et al*., 2006; Jurcisek and Bakaletz, 2007; Lenz *et al*., 2008; Zhang and Mah, 2008). Bacteria in biofilms also coordinate behaviour by cell–cell communication using secreted chemical signals. Cell signalling allows bacteria to sense and phenotypically respond to their environment, for example, by assessing cell density (also called quorum sensing or QS) or environmental cues. In *Pseudomonas aeruginosa* QS has been linked with biofilm formation and the production of virulence factors (Jensen *et al*., 2007). This extraordinary ability to adapt to and modify different microniches allows bacteria in biofilms to weather diverse and stressful conditions facilitating survival at a population level.

Biofilm infections are recalcitrant to antibiotic treatment and host defences

Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence in spite of sustained host defences. Also a metabolically heterogeneous bacterial population created by multiple microniches (Hall-Stoodley *et al*., 2004; Lenz *et al*., 2008) differs markedly from a flask or chemostat culture. In a biofilm the development of gradients can create anoxic and acidic zones in the interior of biofilm clusters (de Beer and Stoodley, 1995; de Beer *et al*., 1997; Stoodley *et al*., 2008a). Nutrient-depleted zones can result in a stationary phase-like dormancy within the biofilm, which may be responsible for the general resistance of biofilms to antibiotics (Walters *et al*., 2003; Fux *et al*., 2004). Therefore, limited penetration of nutrients, rather than restricted antibiotic diffusion, may

Received 11 February, 2009; revised 30 March, 2009; accepted 30 March, 2009. *For correspondence. E-mail lstoodle@wpahs.org; Tel. (+1) 412 359 5016; Fax (+1) 412 359 6995.

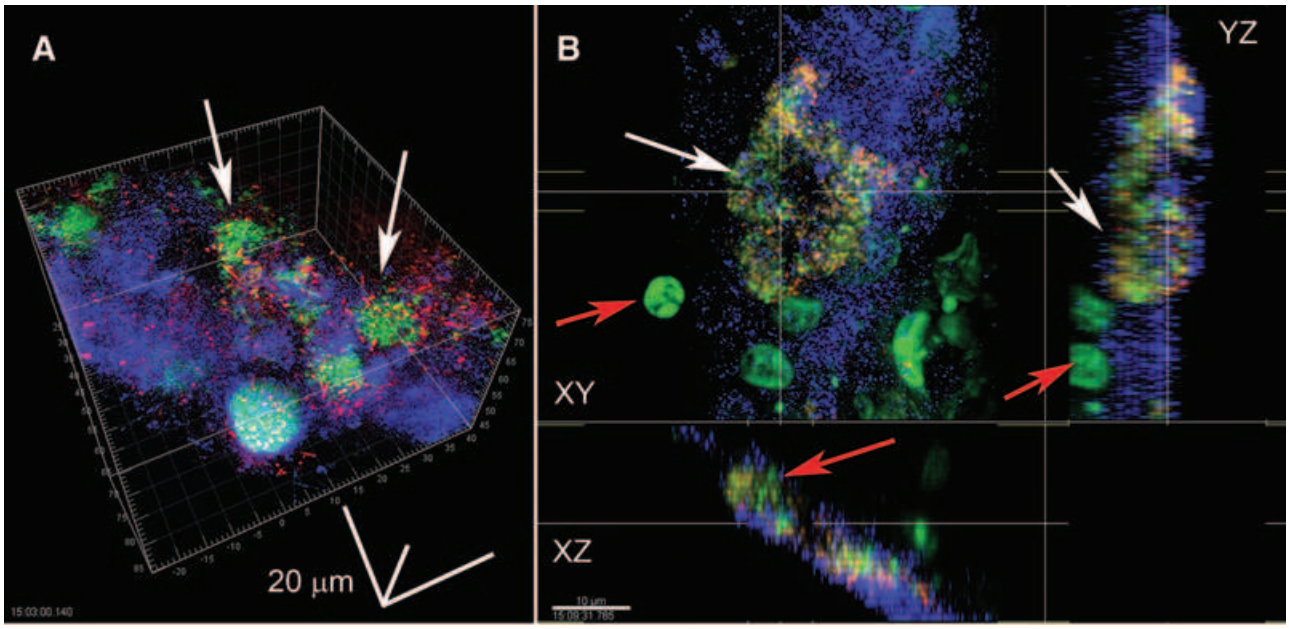

Fig. 1. Confocal micrographs showing biofilm clusters (white arrows) consisting of rods and cocci on the mucosa of a paediatric adenoid taken at two locations (A and B) on the adenoid. The adenoid was removed as part of the routine treatment of recurrent otitis media. The specimens were stained with the nucleic acid Molecular Probes Live/Dead kit in which live bacteria stained green and dead bacteria stained red. Host inflammatory cells (red arrows) were also stained but were readily distinguished on the basis of size and nuclear morphology. The mucosal surface was imaged using reflected light (blue). (B) shows an *X–Y* plan view and the *X–Z* and *Y–Z* sagittal cross-sections. Scale bar = 20 μm in (A) and 10 μm in (B) (L. Nistico and P. Stoodley, unpubl. image).

contribute to a generalized resistance or tolerance to antibiotics (Brown *et al*., 1988; Anwar *et al*., 1992; Stewart and Costerton, 2001; Borriello *et al*., 2004; 2006; Shah *et al*., 2006).

Although the matrix may not inhibit the penetration of antibiotics into the biofilm altogether, it may retard the rate of penetration enough to induce the expression of genes within the biofilm that mediate resistance (Jefferson *et al*., 2005). Also, the charge of polymers (Walters *et al*., 2003) and antibiotic-degrading enzymes (Bagge *et al*., 2004) in the matrix may lead to binding and/or deactivation. Naturally dead cells within the biofilm may even 'dilute' antibiotics on a per cell basis (Mai-Prochnow *et al*., 2008). Gillis *et al*. (2005) showed that unlike planktonic cells, *P. aeruginosa* in biofilms were resistant to azithromycin (AZM) due to an efflux pump, which was only expressed in the presence of the antibiotic. Different genes in the efflux pump operon were expressed in different parts of the biofilm and disruption of two gene clusters was required to induce AZM tolerance indicating that *P. aeruginosa* has redundant mechanisms for AZM resistance. Furthermore the efflux pump did not affect the ability of *P. aeruginosa* to resist other antibiotics such as tobramycin. However, tolerance to tobramycin, gentamicin and ciprofloxacin was recently linked to another efflux pump expressed to a greater extent in biofilms (Zhang and Mah, 2008). Thus, there appear to be multiple mechanisms for antibiotic recalcitrance in biofilm cells, depending on the type of bacterium and the type of antibiotic.

In addition to reduced antibiotic susceptibility, biofilms exhibit resistance to host phagocytic defences. Early evidence of resistance came from transmission electron micrographs of clinical specimens, which showed biofilms surrounded, but not penetrated, by antibodies and inflammatory cells (Lam *et al*., 1980). Several recent studies support the resistance-to-phagocytes hypothesis. Specifically, leukocytes penetrated channels, but not bacterial cells within the EPS matrix in *Staphylococcus aureus* biofilms and exhibited an unstimulated morphology with no evidence of motility or phagocytosis (Leid *et al*., 2002). In *Staphylococcus epidermidis* biofilms, protection from phagocytosis by polymorphic neutrophils (PMNs) and innate immune mechanisms was mediated by the intracellular adhesin, PIA, controlled by the *ica* locus (Vuong *et al*., 2004). PMNs were also immobilized in the EPS matrix in *P. aeruginosa* biofilms, and although phagocytosis occurred, PMNs exhibited diminished oxidative potential measured by the generation of H_2O_2 (Jesaitis *et al*., 2003). This effect may be QS-dependent, since PMNs penetrated a QS-deficient *P. aeruginosa* biofilm but did not penetrate a wild-type biofilm (Bjarnsholt *et al*., 2005). Furthermore, alginate was shown to protect *P. aeruginosa* in biofilms from phagocytosis by IFN-γ-activated macrophages (Leid *et al*., 2005). Recently QS-induced *P.*

aeruginosa production of a PMN toxin, rhamnolipid B, was shown to kill neutrophils (Jensen *et al*., 2007). Moreover, when biofilms of *P. aeruginosa* and *S. aureus* were observed in chronic wounds, host phagocytic cells did not penetrate bacterial aggregates, with *P. aeruginosa* distinctly sequestered from host cells (Kirketerp-Møller *et al*., 2008). Interestingly, the presence of PMNs augmented *P. aeruginosa* biofilm development and both DNA and actin polymers facilitated binding and matrix cohesion likely mediated by type IV pili binding to DNA (Walker *et al*., 2005; Allesen-Holm *et al*., 2006). These findings suggest that *P. aeruginosa* may opportunistically take advantage of 'neutrophil extracellular traps' (NETs), which are thought to trap bacterial cells (Brinkmann *et al*., 2004). PMNs also enhanced the ability of *Candida albicans* to form biofilms, while planktonic cells were vulnerable to this host response (Chandra *et al*., 2007). In *Escherichia coli*, Antigen 43, a surface protein that mediates aggregation, regulated both uptake by PMNs and its intracellular survival (Fexby *et al*., 2007).

Moreover the contribution of antibody in the protection against biofilm aggregates may be inadequate. Diffusion studies showed that IgG remained bound to the periphery of biofilm clusters, and failed to penetrate the EPS matrix (de Beer *et al*., 1997) or failed to mediate opsonic killing of biofilm cells (Cerca *et al*., 2006). However, antibody to an outer membrane protein in *P. aeruginosa* was shown to inhibit biofilm formation by interfering with adhesion to the surface (Tashiro *et al*., 2008), suggesting that antibody may play a role in preventing biofilm development. Antibodies also inhibited biofilm formation by the yeast *Cryptococcus neoformans* (Martinez and Casadevall, 2005).

Finally, in a report that studied polymicrobial interactions and innate immunity, exposure to H_2O_2 produced by *Streptococcus gordonii* induced an outer membrane protein on another oral pathogen, protecting it from killing by human serum during co-culture (Ramsey and Whiteley, 2009). Co-culture also protected *Streptococcus pneumoniae* against β-lactam antibiotics in a polymicrobial biofilm containing a β-lactamase-producing strain of *Moraxella catarrhalis* (Budhani and Struthers, 1998). The polymicrobial nature of oral biofilms associated with dental plaque and periodontitis has made oral biofilms a pioneering model of interspecies interactions and highlights yet another level of complexity in biofilm research (Kuramitsu *et al*., 2007).

The *in vitro* study of biofilms is important because it allows the investigation of complex bacterial phenotypes that are not readily interpreted in an *in vivo* system, including how specific molecular mechanisms affect biofilm development and how biofilms resist killing. Although clinical specimens rarely demonstrate a specific mechanism, they remain important in the examination of biofilm infections in the context of human disease. For example, fluorescent *in situ* hybridization (FISH) was used to determine the spatial distribution of *P. aeruginosa* in the sputum of cystic fibrosis (CF) patients (Yang *et al*., 2008). Patients colonized with mucoid variants demonstrated actively growing cell clusters encased in an extracellular matrix in the airway mucus while sputum from patients colonized with non-mucoid strains indicated that *P. aeruginosa* was distributed as dispersed cells. This study indicates that biofilms may not always be attached to a surface within the host, but may be associated with a surrounding mucus layer or fluid. For example, in a case history reporting evidence of biofilms in a failed elbow arthroplasty, the most extensive *S. aureus* cell clusters were found in aggregates in the joint fluid (Stoodley *et al*., 2008b). It was not possible to tell whether the biofilm aggregates had grown in the fluid or had detached during surgery from a surface-associated biofilm. Similar detached biofilm aggregates were observed from biofilms of a methicillin-sensitive strain of *S. aureus* (MSSA) in an *in vitro* catheter model (Fux *et al*., 2004). The decreased susceptibility to oxacillin of these aggregates suggested that bacteria exhibited a biofilm phenotype, and in terms of the development of gradients of consumed nutrients and waste products creating microniche environments, as well as gradients of secreted diffusible signal molecules, bacterial aggregates will almost certainly behave functionally as attached biofilms. Thus aggregates of bacteria seen in clinical specimens may have different origins: growth of aggregates in a fluid, or as naturally detached emboli from biofilms. In support of this, *P. aeruginosa* formed biofilm macrocolonies more readily in dehydrated mucus and mucin stimulated aggregation and biofilm formation (Landry *et al*., 2006; Matsui *et al*., 2006). It is possible that aggregation, uncoupled from attachment *per se*, may influence host–pathogen interactions. For example *C. neoformans* exited macrophages as biofilm-like aggregates, which may protect against dissemination, but increase the potential for localized inflammatory damage (Alvarez *et al*., 2008).

Biofilms in human disease and the criteria for biofilm infections

The complexity of biofilm research resulting from the diversity of microorganisms in different adaptive environments and the intricacies of biofilms at the host–pathogen interface defies a simple restrictive definition. Nevertheless, biofilm formation is now recognized as causing or exacerbating numerous chronic infections. These include periodontitis, device-related infections, CF pneumonia, chronic urinary tract infections (UTI), recurrent tonsillitis, chronic rhinosinusitis, chronic otitis media (OM) and chronic wound infections. Thus, biofilm infections add a further level of complexity to the existing multifactorial

process of biofilm development and the disease process itself. As with any infectious disease, biofilm infections reflect the interplay between pathogen and host genetic programmes. Many biofilm infections occur at epithelial sites within the host and it is useful to first draw a distinction between biofilm infections and colonization. Biofilm infections are associated with pathogenic or opportunistic bacteria coupled with a chronic condition (i.e. the infection is recurrent or long-lasting in spite of host immune responses and antibiotic therapy). Organisms such as *P. aeruginosa*, *Haemophilus influenzae*, *S. pneumoniae* and *S. aureus* may be found in asymptomatic hosts and finding adherent organisms *in vivo* is commonly referred to as a colonization, rather than an infection. Typically colonization (microbial presence) is differentiated from an infection by evidence of a pathological process in the host such as an overt inflammatory response in the case of infection and its absence in colonization. This issue is particularly challenging, in view of some studies that suggest that biofilms are also present in control specimens (e.g. chronic rhinosinusitis). One problem with clinical specimens is defining an appropriate control group, since it is rarely possible to obtain these from persons *without disease*. Another problem is the paucity of animal colonization models. Therefore, determining corollaries for the presence of opportunists in normal carriage versus a disease process remains difficult. However, an interesting possibility is that biofilm formation may result from the carriage state, making biofilm development a 'persistence factor' as opposed to a virulence factor *per se*.

Biofilms are also troublesome to diagnose because culture, although generally sufficient in acute disease, is not an accurate predictor of chronic biofilm infection. While diagnostic parsimony may indicate no infectious aetiology with culture-negative results, recurrent signs and symptoms of infection argue otherwise. For example in chronic OM with effusion, detection of bacteria by culture is poorly correlated with infection compared with acute OM (Post *et al*., 1995; Rayner *et al*., 1998). Detection of bacteria by culture methods was positive for OM pathogens in children undergoing tympanostomy tube (TT) placement less than 30% of the time, compared with polymerase chain reaction (PCR) and FISH which were positive ~80–90% of the time (Hall-Stoodley *et al*., 2006). Similarly there was no correlation between culture methods and the detection of *P. aeruginosa* by FISH in chronic wounds, in spite of demonstrated biofilm aggregates in the wound (Kirketerp-Møller *et al*., 2008). Exactly why culture is less reliable in biofilm disease may be due to inadequate sampling, inadequate incubation times or inadequate nutrient composition in the media.

The difficulty of identifying biofilms infections *in vivo* has led to the outline of specific criteria for diagnosing biofilm infections from clinical specimens by Parsek and Singh and others (Fux *et al*., 2003; Parsek and Singh, 2003) (see Box 1). Briefly, the 'Parsek–Singh criteria' include (1) surface-associated, (2) microbial aggregates, (3) localized infections that are (4) intractable to antibiotic treatment despite the demonstrated susceptibility of planktonic bacteria. Fux *et al*. further suggested that diagnosis based on culture results alone was insufficient without giving some thought to sampling techniques since localized bacteria in a biofilm may be missed in a conventional blood sample or aspirate. Consistent with this last point, multiple consecutive lavage samples demonstrated that the bacterial burden of biofilm associated non-typeable *H. influenzae* (NTHi) was much greater than that shown by single middle-ear effusion samplings in the chinchilla model of OM (Leroy *et al*., 2007). Moreover, Tunney *et al*. associated biofilms with orthopaedic implant infections by observing bacterial aggregates by immunostaining and 16S reverse transcription polymerase chain reaction (RT-PCR) (Tunney *et al*., 1999) and diagnosis of orthopaedic infection was markedly improved by direct sampling of the prosthesis surface and sonication (Trampuz *et al*., 2007).

Since antibiotic susceptibility of planktonic cultures can only be tested when clinical specimens result in a positive culture, we suggest a modification of criteria '4'. In the absence of culture, resistance to antibiotic therapy might be inferred by demonstrating the presence of live aggregated bacteria using methods such as direct viability staining coupled with molecular methods such as RT-PCR or FISH (Stoodley *et al*., 2008b). We also propose a sixth criterion for biofilm-associated infections: ineffective host clearance indicated by the location of bacterial cell clus-

Box 1. Diagnostic criteria for biofilm infections.

Diagnostic criteria for biofilm infections based on the Parsek–Singh criteria (Parsek and Singh, 2003):

(1) Pathogenic bacteria are associated* with a surface.
(2) Direct examination of infected tissue demonstrates aggregated cells in cell clusters encased in a matrix, which may be of bacterial and host origin.
(3) Infection is confined to a particular site in the host.
(4) Recalcitrance to antibiotic treatment despite demonstrated susceptibility of planktonic bacteria**.
(5) Culture-negative result in spite of clinically documented high suspicion of infection (since localized bacteria in a biofilm may be missed in a conventional blood sample or aspirate).
(6) Ineffective host clearance evidenced by the location of bacterial cell clusters (macrocolonies) in discrete areas in the host tissue associated with host inflammatory cells.

Notes:
*Note the use of the term 'associated' allows the inclusion of aggregates not necessarily firmly attached.
**We propose the following modification of criteria '4', since antibiotic susceptibility of planktonic cultures can only be tested upon positive culture from a clinical specimen. We suggest that in the absence of culture, recalcitrance to antibiotic therapy may be inferred from the presence of live bacterial cells in the biofilm from *in situ* viability staining or reverse transcription polymerase chain reaction (RT-PCR).

ters in discrete areas in the host tissue associated with host inflammatory cells. Evidence of PMNs and macrophages surrounding aggregated bacteria *in situ* considerably increases the suspicion of infection. However, in the absence of biofilm-specific markers and appropriate animal models for many biofilm infections, fulfilling 'Koch's postulates' for biofilm infections remains a challenge.

Biofilms are demonstrated locally associated with tissues such as oral, respiratory or urogenital epithelium

Biofilms have now been associated with several chronic infections in the respiratory tract by examining clinical samples or biopsies. Sputum and bronchiolar lavage samples and lung biopsies (BAL), often in conjunction with *in vitro* experiments, have been useful in examining the distribution of bacterial aggregates in CF by *P. aeruginosa* and NTHi in chronic bronchitis in order to examine the distribution of bacterial *in situ* in the lung during chronic infections (Lam *et al*., 1980; Singh *et al*., 2000; Worlitzsch *et al*., 2002; Starner *et al*., 2006; Martínez-Solano *et al*., 2008; Yang *et al*., 2008). These studies have observed aggregated bacteria, suggesting a biofilm infection. *P. aeruginosa* was found in bacterial aggregates enclosed in a matrix within O_2-depleted mucus from CF patients, although not necessarily adherent to the airway epithelium (Worlitzsch *et al*., 2002). The role that insufficiency of mucociliary clearance plays as a contributing factor in biofilm infections needs to be further explored since a stagnant mucin-rich effusion that promotes bacterial proliferation has been demonstrated in the chinchilla model of OM (Miyamoto and Bakaletz, 1997) and mucin (MUC) gene polymorphism in patients with chronic OM may contribute to the inadequate host clearance of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (Kerschner, 2007; Ubell *et al*., 2008).

Chronic OM has been identified as a biofilm infection from studies using both animal models and clinical specimens. NTHi formed mucosal biofilms demonstrated by electron and confocal microscopy in the *Chinchilla laniger* model of OM, whereas no biofilm was found in sham-infected controls (Ehrlich *et al*., 2002). Moreover, biofilm aggregates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were present on the epithelium of the middle-ear mucosal biopsies in children undergoing TT placement for the treatment of chronic or recurrent OM, but not on middle-ear mucosal biopsies obtained from patients undergoing surgery for cochlear implantation (Hall-Stoodley *et al*., 2006). Antibiotic resistance was inferred since children had been treated for multiple OM infections and pharmacokinetic penetration studies of antibiotics commonly used in OM indicate that bactericidal concentrations are readily achievable in the middle-ear space and sufficient to kill planktonic pathogenic bacteria (Rosenfeld and Post, 1992).

Biofilm formation by *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* is now well documented *in vitro* and *in vivo* (Swords *et al*., 2004; Allegrucci *et al*., 2006; Moscoso *et al*., 2006; Oggioni *et al*., 2006; Pearson *et al*., 2006; West-Barnette *et al*., 2006; Hong *et al*., 2007a,b; Jurcisek *et al*., 2007; Leroy *et al*., 2007; Luke *et al*., 2007). Biofilm formation *in vivo* by NTHi has been specifically investigated by several groups resulting in various mechanisms putatively associated with biofilm development. For example, NTHi were demonstrated in biofilm aggregates within a matrix of lipooligosaccharide (LOS) and outer membrane proteins (Swords *et al*., 2004; Jurcisek *et al*., 2005). The persistence of NTHi biofilms *in vivo* has been linked to increased phosphorylcholine (Pcho) and sialylation in LOS (Swords *et al*., 2004; West-Barnette *et al*., 2006). Binding of Pcho via the plasminogen activating factor receptor has been shown to result in increased adherence to epithelium (Swords *et al*., 2000). Similarly, NTHi phenotypic variants within biofilms expressing surface associated sialic acid and Pcho correlated with increased biofilm formation *in vitro* and decreased host inflammatory responses in both the gerbil and chinchilla model (Hong *et al*., 2007a,b). NTHi biofilms in the chinchilla middle ear were associated with DNA and pilin proteins in an extracellular matrix (Jurcisek and Bakaletz, 2007). The demonstration of DNA in biofilms of NTHi mirrors *P. aeruginosa* biofilms (Walker *et al*., 2005) and more recently *S. pneumoniae* (Hall-Stoodley *et al*., 2008), and suggests that DNA may be an important component of the biofilm matrix in other respiratory pathogens. These studies highlight the value of multiple *in vitro* and animal models in the investigation of biofilm pathogenesis.

These studies also suggest a primary role for the epithelium in the defence against biofilm infections. While the ability of pathogens to exploit opportunities that arise in deficient host defences is likely to be multi-factorial, the association of pathogenic bacteria with epithelial tissues raises an interesting question about a fundamental criterion of biofilm and whether aggregated bacteria must be attached to a surface. The evidence with *P. aeruginosa* argues for aggregates of bacteria within the mucus layer overlaying the epithelium being an important determinant for a biofilm infection particularly in concert with impairment of multiple components of innate immunity (Matsui *et al*., 2006; Döring and Gulbins, 2009). Since non-attached aggregates of bacteria retain the antibiotic recalcitrance seen in biofilms, cell aggregation may be a key indicator of a biofilm infection (Fux *et al*., 2004). Nevertheless, the examination of several chronic infections in the upper respiratory tract such as recurrent tonsillitis and chronic rhinosinusitis in human clinical specimens suggests that both attachment and aggregated bacteria are present

(Chole and Faddis, 2003; Psaltis *et al*., 2007; Prince *et al*., 2008). Chole and Faddis used electron microscopy and culture to show that biofilms were associated with the mucosal epithelium of tonsils in 73% of tonsils removed for tonsillitis and 75% of those tonsils removed due to hypertrophic tonsils alone. These results suggest that biofilms are present associated with mucosal epithelium in disease and in hypertrophic lymphoid tissues.

More recently molecular *in situ* techniques have demonstrated that biofilms, formed by pathogens (sometimes in polymicrobial communities), also occur on the mucosal surface of human adenoids, associated with epithelial cells (Kania *et al*., 2008; Nistico *et al*., 2009). Sections from the same adenoid biopsies that demonstrated bacterial pathogenic biofilms associated with the epithelium by FISH showed increased resistance to azithromycin and the presence of a carbohydrate matrix *in situ* (L. Nistico, P. Stoodley, and L. Hall-Stoodley, unpubl. obs.). Therefore, using an algorithm of tests in the case of clinical samples which includes specific probes to determine the distribution of pathogenic bacteria and whether they are aggregated will be more effective in diagnosis of a biofilm infection. FISH in conjunction with pathogen-specific 16S probes represents a particularly good method for detecting specific pathogens *in situ* compared with electron and light microscopy. However pathogen-specific antibody staining is also compatible with transmission electron microscopy or immunofluorescence on clinical samples. The ability of FISH to be used in conjunction with antibodies to specific human cell markers can also give specific information about the location of the biofilm in tissue (Nistico *et al*., 2009). The real value of these studies is that it is possible to see the microbial host interface *in situ*, which may be an important guide for future exploration of specific mechanisms used by pathogens in biofilm infections.

Adherence of pathogens to host cells and intracellular bacteria in biofilm infections

Recently one of the best examples of the evolution of the concept of a biofilm infection has come from the investigation of recurrent UTI. Work by Hultgren and others has demonstrated that uropathogenic *E. coli* (UPEC) adhere to and invade the bladder epithelium during the early stages of infection in a mouse model of UTI (Anderson *et al*., 2003; Justice *et al*., 2006). Prior to this work, UPEC was thought to reside extracellularly on the epithelial surface of the urinary tract and, clinically, the recurrence of UTI was thought to be due to re-infection from the gastrointestinal tract. However the observation that UPEC existed in large aggregates of bacteria in mouse bladder epithelial cells suggested another mechanism for chronic recurrent infection of the bladder epithelium (Anderson *et al*., 2003). Remarkably, intracellular UPEC undergo a complex developmental process into biofilm aggregates within the epithelial cell cytoplasm (Justice *et al*., 2006). These aggregates exhibit biofilm ultrastructure (large cell clusters of *E. coli* surrounded by a matrix) and resistance to antibiotic and host killing. Recently another UTI pathogen, *Klebsiella pneumoniae*, was shown to develop intracellular biofilms linked to the expression of type 1 pili (Rosen *et al*., 2008) and intracellular UPEC exhibiting the same phenotype in epithelial cells present in human UTI infections has been described (Rosen *et al*., 2007).

Interestingly, intracellular *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus in situ* is observed in adenoids from children undergoing adenodectomy for the treatment of chronic OM or hypertrophic adenoids using FISH (Forsgren *et al*., 1994; Nistico *et al*., 2009). *H. influenzae* and intracellular *S. pneumoniae* have also been found in the bronchial biopsies in patients with chronic bronchitis and in middle-ear mucosal biopsies in children with chronic OM (Bandi *et al*., 2001; Coates *et al*., 2008). *H. influenzae* and *S. pneumoniae* have been shown to invade epithelial cells by numerous molecular mechanisms (Swords *et al*., 2000; St Geme, 2002; Hammerschmidt *et al*., 2005; Pracht *et al*., 2005). *S. aureus* is found intracellularly in the nasal mucosal of patients with recurrent sinusitis (Clement *et al*., 2005). Thus biofilm aggregates attached to the epithelial surface, residing in the mucus layer or sequestered in intracellular aggregates may all be mechanisms for persistence in mucosal infections. It is interesting to speculate that pathogens such as *S. aureus*, *S. pneumoniae*, *H. influenzae* reside extracellularly in an aggregated biofilm phenotype that protects bacteria from the host response and antibiotic therapy, but also as intracellular sequestered aggregates in respiratory epithelial cells. Both extracellular and intracellular aggregation would facilitate the persistence of pathogens at epithelial interfaces. The phenotypic heterogeneity exhibited by bacteria in biofilms may result in an invasive phenotype under certain conditions in the host (Garcia-Medina *et al*., 2005; Li *et al*., 2005) and could influence the induction of adaptive host immune mechanisms. However, the role of epithelial cells in the protection against infection is only beginning to be investigated in the human host.

Conclusions

Biofilm development is an important component of bacterial survival. However understanding how it contributes to infectious disease in the human host is not easily investigated by a reductionistic approach. This is due to the multifaceted nature of biofilm research which examines biofilm development as a process of intrinsic genomic programmes and adaptive plasticity exhibited by each organism in response to different host environments. At the host level, we are only beginning to understand how

biofilm infections caused by well-known pathogens affect the host response. Moreover, we have barely considered the further complexity that polymicrobial infections may present in terms of both the responses to other organisms and in the overall host response. By investigating the distribution of bacterial pathogens as they are found in various chronic infections we can better understand the relevant microenvironments and the limitations of the host responses to various pathogens. We think that assessing putative biofilm infections using clinical specimens and biopsies *ex situ* will be useful towards understanding the relationship and interplay between biofilm bacteria and the inflammatory and innate immune response relevant to a specific tissue. These studies will help build a more comprehensive and coherent model of biofilm-related pathogenesis and provide data for the refinement of existing treatment strategies for biofilm infections as well as novel treatment strategies.

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