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Mucin–*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance

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Summary

***Pseudomonas aeruginosa* is an opportunistic pathogen that causes chronic lung infections in people suffering from cystic fibrosis (CF). In CF airways, *P. aeruginosa* forms surface-associated communities called biofilms. Compared with free-swimming cultures, biofilms resist clearance by the host immune system and display increased resistance to antimicrobial agents. In this study we developed a technique to coat surfaces with molecules that are abundant in CF airways in order to investigate their impact on *P. aeruginosa* biofilm development. We found that *P. aeruginosa* biofilm development proceeds differently on surfaces coated with the glycoprotein mucin compared with biofilm development on glass and surfaces coated with actin or DNA. Biofilms formed on mucin-coated surfaces developed large cellular aggregates and had increased tolerance to the antibiotic tobramycin compared with biofilms grown on glass. Analysis of selected mutant backgrounds in conjunction with time-lapse microscopy revealed that surface-associated motility was blocked on the mucin surface. Furthermore, our data suggest that a specific adhesin–mucin interaction immobilizes the bacterium on the surface. Together, these experiments suggest that mucin, which may serve as an attachment surface in CF airways, impacts *P. aeruginosa* biofilm development and function.**

Introduction

Cystic fibrosis (CF) is the most common, lethal inherited disorder in Caucasian populations. Impaired innate pulmonary defences render people suffering from CF susceptible to chronic *Pseudomonas aeruginosa* lung infections, which cause most of the morbidity and mortality in CF patients (Gilligan, 1991; Govan and Deretic, 1996). These chronic infections involve the formation of surface-associated communities called biofilms (Costerton *et al*., 1999; Boucher, 2002; Parsek and Singh, 2003). Although the initial steps in chronic colonization are unclear, once the disease is established high numbers of *P. aeruginosa* can be found in airway secretions. These bacteria manifest many characteristics of biofilms. Microscopic analysis of sputum and lung tissue from CF patients reveals the presence of *P. aeruginosa* in aggregates encased in an extracellular matrix (Lam *et al*., 1980; Singh *et al*., 2000). *P. aeruginosa* cannot be eradicated from colonized individuals with aggressive long-term antibiotic therapy, even though they become antibiotic sensitive when cultured planktonically *ex vivo* (Mendelman *et al*., 1985). Finally, *P. aeruginosa* produces quorum sensing signal patterns in CF sputum similar to those produced by laboratory biofilms, indicating that the organism is in a biofilm-like physiological state (Singh *et al*., 2000). The role of biofilms in CF pathogenesis is of key importance for several reasons. Biofilm bacteria are more resistant to antimicrobial agents than planktonic bacteria (Costerton *et al*., 1999; Stewart, 2002), and are also thought to inhibit host-clearance by preventing phagocytosis and penetration of immune cells (Leid *et al*., 2002; Jesaitis *et al*., 2003). These factors probably contribute to the persistence of *P. aeruginosa* in CF airways.

The understanding that CF airway infections are caused by biofilms has generated increased interest in the basic biology of biofilm development with the hope that a strategy specifically directed towards biofilms may lead to a treatment of chronic *P. aeruginosa* infections. Thus, a number of laboratories have begun to examine the molecular and environmental determinants that govern biofilm formation on abiotic surfaces (O'Toole and Kolter, 1998; Hentzer *et al*., 2001; Vallet *et al*., 2001; Davey *et al*., 2003; Klausen *et al*., 2003a). An important point of emphasis derived from these studies is that environmental conditions have a significant influence on what molecular factors are needed to build a biofilm. For example, flagella mutants of *P. aeruginosa* have an attachment defect when either glucose, glutamate or casamino acids are the sole

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carbon source (Klausen *et al*., 2003b). However, the mutants have an attachment phenotype identical to the wild-type when grown on citrate as the sole carbon source.

An environmental parameter that can have a profound effect on biofilm development and function is the attachment surface (Gilbert *et al*., 1991; Bakker *et al*., 2004). For example, host surfaces present in the oral cavity, such as enamel and subgingival tissue, are colonized by distinct subpopulations of the oral microbiotic flora (Kolenbrander, 2000). Although the attachment surface for biofilm formation in CF is unclear, a number of candidate surfaces have been suggested. These surfaces include host-derived polymers present in CF airways, such as DNA, actin, and mucin, as well as damaged airway epithelium (Prince, 1992; 2002; Pier *et al*., 1997; Pier, 2002; Worlitzsch *et al*., 2002). Mucin, the major protein component of mucus, is of particular interest, because it is one of the most abundant host polymers in CF airways and *P. aeruginosa* has mucin-specific adhesins that mediate bacterium–mucin interactions (Carnoy *et al*., 1994; Arora *et al*., 1996; 1998; 2000; Ramphal and Arora, 2001). In particular, a known mucin adhesin, the flagellar cap protein, FliD, appears to engage in a highly effective, specific interaction with mucin (Arora *et al*., 1998).

In this study we begin to examine the effect of host polymers found in CF airways on *P. aeruginosa* biofilm development and function. We report the development of a generalized method utilizing surface chemistry to uniformly coat glass surfaces with polymers relevant to CF airways. DNA, actin, and mucin were covalently linked to glass surfaces and *P. aeruginosa* biofilm formation and function were evaluated. Here we show that biofilms formed on mucin display distinct, large cellular aggregates that are significantly more resistant to the antibiotic tobramycin than a comparable biofilm grown on glass. Analysis of selected mutants show that surface-associated motility is blocked on mucin surfaces and that the flagellar cap adhesin, FliD, serves to anchor *P. aeruginosa* to the mucin surface. These results suggest that components of CF airways have the potential to directly influence the formation of *P. aeruginosa* biofilm communities.

Results

Mucin-coated surfaces have a profound effect on biofilm development

To immobilize biological macromolecules on a solid support, we generated a surface with terminal aldehyde groups, which react readily with primary amines present on biological macromolecules to form a Schiff base linkage (Fig. 1A). We used several techniques to characterize and validate these surfaces, including atomic force microscopy (AFM), a lectin binding assay and water contact angle measurements. AFM images of mucin-derivatized slides revealed a confluent and relatively smooth film (Fig. 1B) without breaks in the mucin coating. These results were compared with an actin-derivatized surface. The mucin surface height fluctuated by only a few nanometres, while the actin surface was an order of magnitude rougher (Fig. 1B). An AFM image of a glass coverslip surface was included in Fig. 1 as a reference. The TRITC-labelled peanut agglutinin (PNA) lectin assay confirmed the presence of a uniform layer of mucin on the substratum (data not shown).

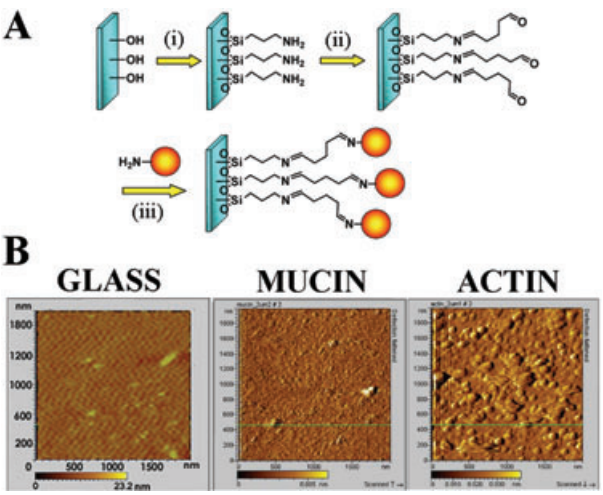

Fig. 1. A. Schematic for surface derivatization. (i) Initial incubation with APTMS (3-aminopropyltrimethoxysilane), acetone, 50°C, 12 h; (ii) Incubation with 1% (v/v) glutaraldehyde, 4°C, 1 h; (iii) Final incubation with protein, 5 h.
B. AFM topographical images of a glass surface and surfaces derivatized with mucin and actin.

Biofilm development of wild-type *P. aeruginosa* on mucin-, actin- and DNA-derivatized surfaces was compared with the corresponding untreated glass surface. Biofilm formation on glass began with an even distribution of cells on the surface, as visualized at initial attachment (Fig. 2A). This pattern continued over the course of the experiments (Fig. 2), until the biofilm completely covered the substratum with a fairly homogeneous distribution of biomass. This flat, undifferentiated biofilm is characteristic for PAO1 using these growth conditions. Biofilms grown on actin and DNA derivatized surfaces showed a pattern of biofilm development identical to that on glass (data not shown).

In contrast, biofilms grown on mucin feature a dramatically different developmental pattern from those grown on glass, actin or DNA. There was a reduced initial attachment to the mucin surface compared with glass (Fig. 2A, $t = 0$, Fig. 3), although the initial distribution of attached cells was uniform on the surface. However, *P. aeruginosa* cells attached to the mucin surface grew

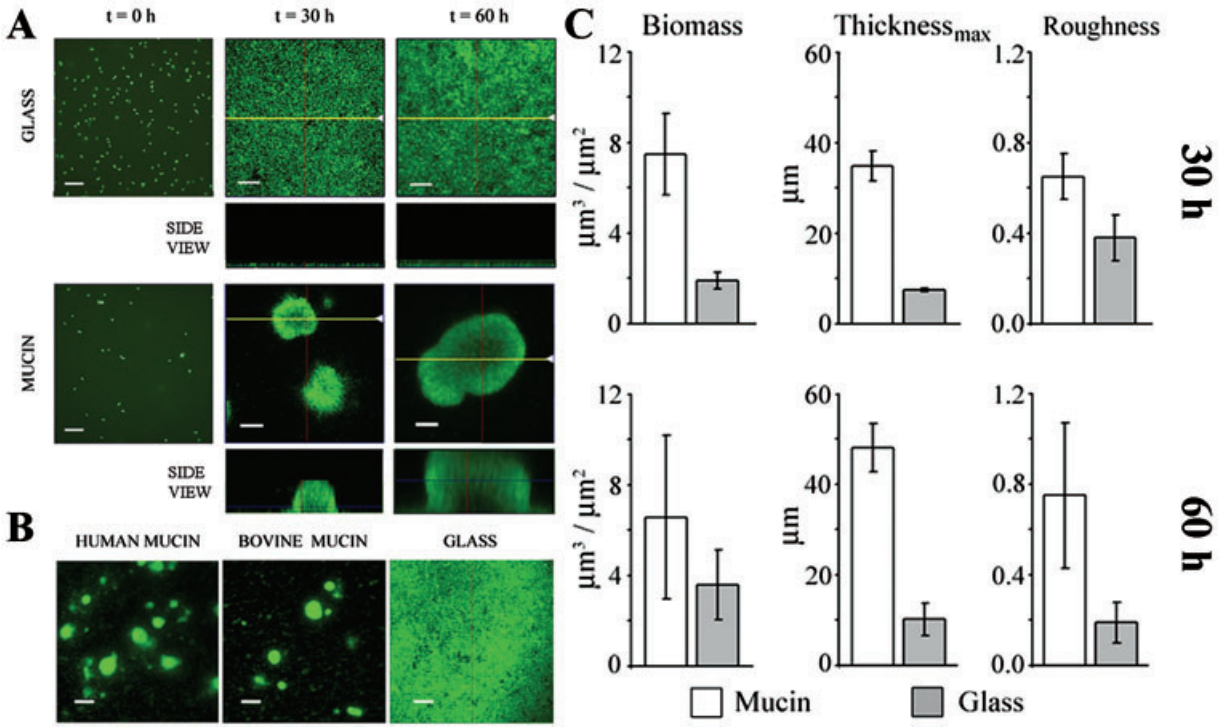

Fig. 2. A. Epifluorescence and SCLM micrographs of the wild-type *P. aeruginosa* biofilm formed on mucin and glass over time. The strains are constitutively producing GFP. Biofilms were grown in a once flow-through flow cell and imaged at several time points, t = 0, 30, 60 h. Images at t = 30 h and 60 h contain side views in addition to top-down views. The side views of the biofilms are indicated on the figure. Bars = 20 μm.
B. A micrograph showing a top-down view of comparable wild-type *P. aeruginosa* biofilms at 30 h on a human respiratory mucin-coated surface, bovine mucin-coated surface, and a glass surface. Bars = 50 μm.
C. COMSTAT analysis of PAO1 biofilms grown on glass and mucin-coated surfaces. The image analysis software COMSTAT was used to quantify total biomass, maximum thickness and surface roughness at two time points, 30 h (top three panels) and 60 h (bottom three panels). Biofilms grown on mucin are indicated by the open bars and the filled bars represent biofilms grown on glass.

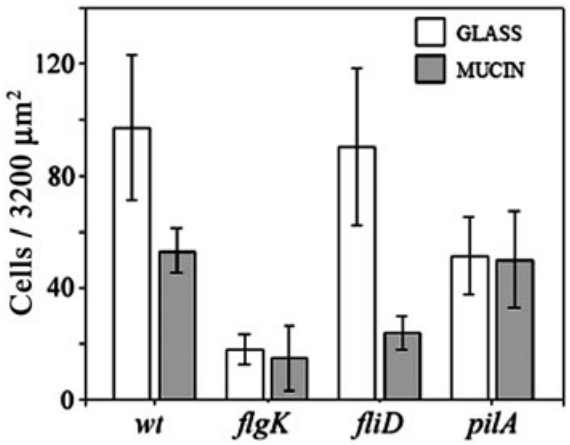

Fig. 3. Initial attachment of wild-type and mutant strains to glass and mucin-coated surfaces. The number of bacteria attached following inoculation of the flow cell system was determined by microscopic direct counts.

almost exclusively in discrete microcolonies (Fig. 2A, t = 30 h). These microcolonies continued to mature into a biofilm characterized by a high degree of roughness and low surface coverage. Biofilm architecture was measured using the image analysis software COMSTAT (Heydorn *et al.*, 2000) (Fig. 2C). The results show that the PAO1 biofilms grown on mucin exhibited at least two times more biomass compared with the glass-grown biofilms. The maximum thickness of the mucin-grown biofilm far exceeded the value for the biofilm grown on glass due to the large cell aggregates. At the end of the experiment, bacteria were dislodged and the mucin coating was determined to be intact using the lectin binding assay (data not shown).

Mucin is a high molecular weight glycoprotein. Because mucin can vary structurally depending upon the source, we determined if the same biofilm phenotype was observed on glass derivatized with human airway mucins as on bovine submaxillary mucins. Biofilms grown on human respiratory mucins purified from Calu-3 cells exhibited the same pattern of development as seen on the bovine submaxillary mucins (Fig. 2B).

Biofilm development on mucin surfaces is characterized by limited surface motility

Initial microscopic observations led us to propose the motility hypothesis. This hypothesis predicts that, under these culturing conditions, biofilm formation on glass is characterized by significant surface-associated motility, while the large cell aggregate morphology seen for mucin-grown biofilms is the result of limited surface movement and clonal growth of attached cells.

To initially test the motility hypothesis, time-lapse microscopy was performed on developing biofilms. Images were acquired every 2 min of the same field of view over a 6 h time period shortly after inoculation of a flow cell reactor. During the experiment, flow cell reactors were subjected to continuous flow and were incubated at 30°C on the microscope stage in an environmental chamber. Continuous flow ensured that observed bacteria were directly associated with the surface.

This study revealed markedly different degrees of surface-associated motilities on the two surfaces. On glass, attached cells moved freely across the surface (Movie S1). When the parent cell divided, the daughter cell also continued in motion on the surface or was released into the overlying liquid medium. Although not readily apparent from the movie, bacteria released into the overlying medium were observed to wash away in the direction of flow. The average surface speed of a bacterium on glass was estimated to be ~2.2 μm min^{-1} ($n = 20$). This is much slower than the estimated swimming speed of bacteria in a liquid environment (~10–40 μm s^{-1}) (Johansen *et al*., 2002). In contrast, bacteria attached to the mucin surface remain relatively stationary (Movie S2). Upon division, most daughter cells remained near the site of parental division, rapidly forming large cell aggregates. However, not all attached bacteria gave rise to microcolonies. The average surface speed of a bacterium on mucin was estimated to be ~0.1 μm min^{-1} ($n = 20$). Figure 4 displays tracings of individual cells depicting relative surface-associated motility over time.

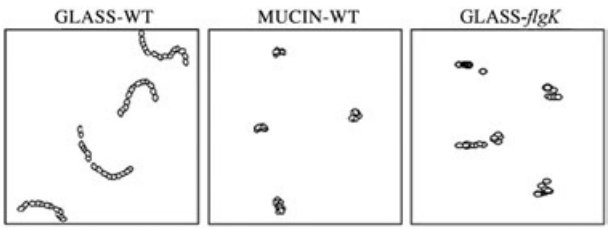

Fig. 4. Tracings of individual cells of a wild type and FlgK mutant strain over a 26 min period showing relative surface motility on glass and mucin-coated surfaces. Each panel depicts the relative motility of four individual cells on the two surfaces. An oval is drawn at the exact location of a bacterium at 2 min intervals during a 26 min time period.

An analysis of key mutants confirms a limited role for surface motility on mucin-coated surfaces

Time-lapse microscopy observations led us to predict that mutations affecting surface motility would greatly impact biofilm development on a glass surface, but not on mucin-coated surfaces. Therefore, we further tested the motility hypothesis using *P. aeruginosa* motility mutants. *P. aeruginosa* is known to utilize three different types of motility, traditional flagellar-mediated swimming motility and two types of surface motility, swarming and type IV pili-mediated twitching motility. Initially a *flgK* mutant strain (*flgK* encodes a flagellar hook-filament junction protein) that does not produce a flagellum and is swimming motility deficient and swarming motility impaired was examined. Over the course of 3 days, biofilm development was monitored via microscopy and images were acquired. The *flgK* mutant had an attachment-deficient phenotype on both the mucin and glass surfaces (Fig. 3). This mutant ultimately produced a heterogeneous biofilm with large cell aggregates on both glass and mucin surfaces, similar to PAO1 grown on mucin (Fig. 5). A *flgK* mutant was then shown to exhibit reduced surface motility on the glass surface using time-lapse microscopy, similar to PAO1 on the mucin surface. Tracings of individual *flgK* cells on the glass are shown in Fig. 4. This mutant was complemented *in trans* by plasmid-encoded *flgK* (pDA3). The complemented strain restored the wild-type biofilm swimming motility phenotypes (data not shown).

A *pilA* mutant which does not produce type IV pili was examined next. This strain is twitching motility deficient and shows reduced swarming motility. This gene encodes the pilin subunit of the type IV pilus. The *pilA* mutant was slightly deficient in attachment to glass and mucin surfaces (Fig. 3). At 72 h, loosely associated cell aggregates were observed on both mucin and glass surfaces (Fig. 5). This trend continued over the course of the experiment, with many small cell aggregates spread across the surface. This mutant was complemented by plasmid-encoded *pilA* (pDA2) and a wild-type biofilm phenotype was restored on glass and mucin surfaces (data not shown).

Unlike the wild-type strain whose biofilm architecture is substratum dependent, the *flgK* and *pilA* mutants formed biofilms on both surfaces with an architecture characterized by large cellular aggregates, similar to the wild-type strain grown on mucin-coated surfaces.

A specific adhesin–mucin interaction immobilizes P. aeruginosa *on the mucin surface*

Why doesn't *P. aeruginosa* move on a mucin-coated surface? We predicted that a specific interaction between the

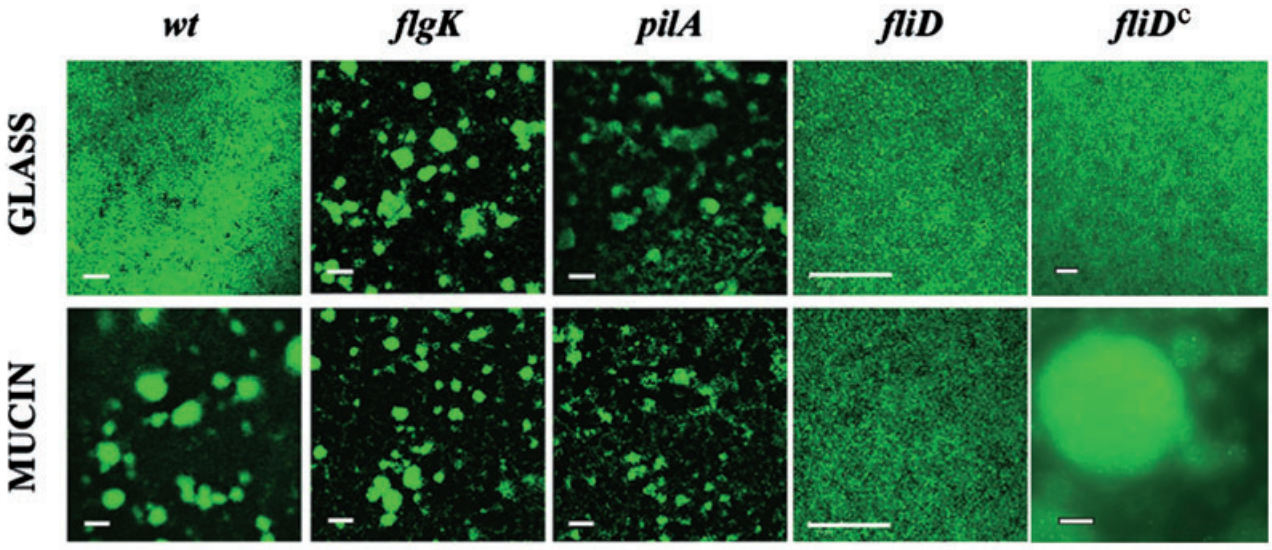

Fig. 5. Biofilm development on glass and mucin surfaces of wild-type (wt) and isogenic mutant strains. Biofilms were imaged at ~3 days of development. Wild-type *P. aeruginosa*, PAO1, develops as a homogenous, flat, thin biofilm on glass compared with heterogeneous biofilm dominated by microcolonies observed on the mucin surface. Both the *P. aeruginosa flgK* and the *pilA* mutants eventually form heterogeneous biofilms on both glass and mucin derivatized surfaces, similar to the biofilm formed on mucin by wt. In contrast, the *P. aeruginosa fliD* mutant forms a homogenous, flat, thin biofilm similar to wt on glass with reduced initial attachment to the mucin surface. The superscript 'c' on fliD in the last column indicates complementation of this mutant with the plasmid pPZ375D. All scale bars represent 50 μm.

cell and the surface may immobilize the bacterium. *P. aeruginosa* engages in specific interactions with mucin through proteins known as adhesins. Therefore, we examined a strain harbouring a mutation in the gene encoding a mucin adhesin, *fliD*. This gene encodes the flagellar cap protein located at the distal end of the helical flagellar filament. The *fliD* mutant does not produce a functional flagellum and is impaired for swimming motility, but did not harbour a defect in swarming motility (data not shown). Not surprisingly, the *fliD* mutant displayed reduced attachment to the mucin surface in comparison with the wild-type strain (Fig. 3). This attachment-deficient phenotype on mucin is gradually overcome and the mutant ultimately produced flat biofilms on both glass and mucin (Fig. 5). A COMSTAT analysis of these data revealed that the *fliD* mutant biofilms on glass and mucin were very similar structurally to each other and to a PAO1 biofilm grown on glass (Fig. 6). This analysis also indicated that these biofilms contained a similar number of cells (biofilm biomass, Fig. 6). Complementation of the *fliD* mutant with a plasmid harbouring the *fliD* gene (pPZ375D) restored the wild-type biofilm phenotype on mucin (Fig. 5).

These data indicate that adhesin–mucin interactions can dictate biofilm development. The relative high abundance of mucins in CF airways also suggests that this interaction may be relevant to biofilm development in CF lung infections.

Biofilms grown on mucin surfaces are more tolerant to tobramycin than biofilms grown on glass

A key aspect of CF biofilms is their increased tolerance to antimicrobial agents. Therefore the relative tobramycin tolerance of biofilms grown on glass and mucin-coated surfaces was tested. Wild-type PAO1 biofilms were grown for 36 h on glass and mucin surfaces, then exposed to 2.0 μg ml^{-1} of tobramycin for an additional 24 h. Biofilms were then evaluated using viability staining (Fig. 7). Staining allowed determination of total biofilm biomass before and after treatment, as well as the relative percentage of live and dead cells. Images were analysed

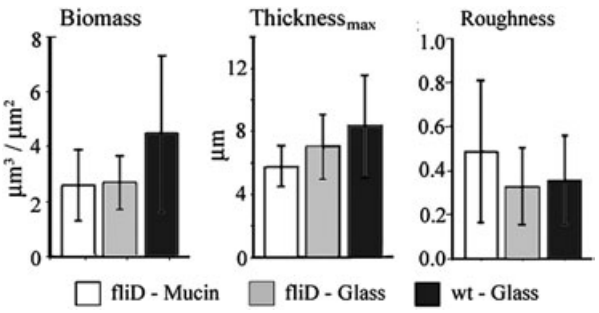

Fig. 6. COMSTAT analysis of PAO1 *fliD* biofilms grown on mucin and glass. Surface roughness, biofilm thickness, and biofilm biomass measurements indicate that at 60 h PAO1 biofilms grown on glass and *fliD* mutant biofilms grown on mucin and glass are structurally similar.

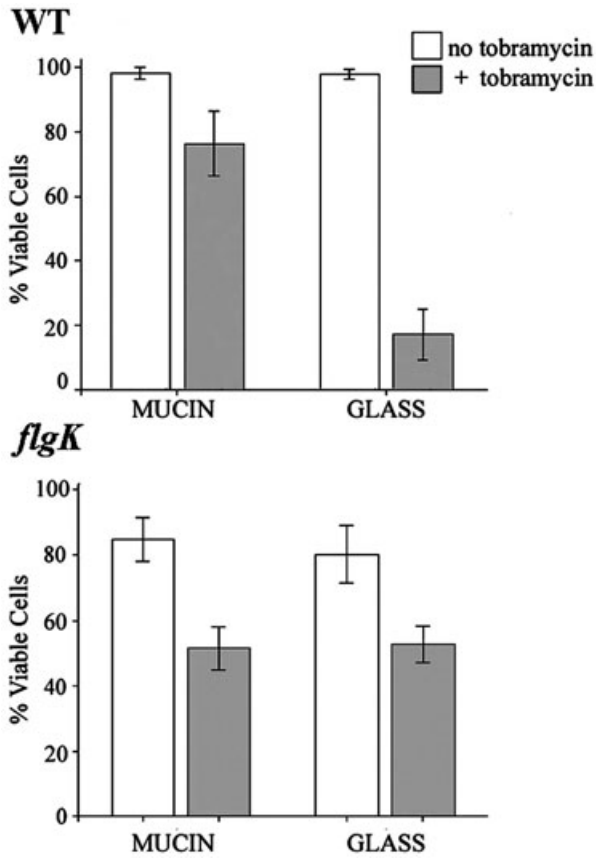

Fig. 7. Tobramycin sensitivity of biofilms grown on mucin and glass. Biofilms were grown for 24 h then subjected to tobramycin treatment for another 24 h. The amount of living and dead biomass was imaged using SCLM and viability staining and quantified using COMSTAT. The open bars represent the untreated control and the closed bars represent the corresponding tobramycin treated biofilm.

using COMSTAT to establish quantitatively the extent of killing. Biofilms grown on glass experienced a dramatic reduction in viable biomass (83 ± 8% death). This is striking when compared with the treated mucin biofilms, which exhibit a relatively low degree of killing (24 ± 10% death). Tobramycin treatment did not result in the loss of biomass by sloughing or shedding of cells for either the glass or mucin-grown biofilms (data not shown). Untreated control biofilms showed little or no reduction in viable biomass.

We hypothesized that the large cell aggregates characteristic of biofilms grown on mucin contributed to its increased tobramycin tolerance. There are two potential reasons for this: First, tobramycin may inefficiently penetrate the aggregates and second, cells within the nutrient-starved interior of the aggregates may have an antibiotic tolerant slow growth phenotype. An alternative hypothesis that is independent of the biofilm structure is that specific bacterium–mucin interactions promote increased tobramycin tolerance in biofilms. To investigate further, the tobramycin sensitivity of *flgK* mutant biofilms grown on glass and mucin was determined. This strain formed biofilms characterized by large cell aggregates on both surfaces, similar to the wild-type biofilm grown on mucin (Fig. 5). The planktonic minimum inhibitory concentration (MIC) of the *flgK* strain was identical to the PAO1 parent (data not shown). The tobramycin sensitivities of the *flgK* biofilms grown on glass and mucin were nearly identical, showing about 30% killing of the biofilm biomass (Fig. 7). This is similar to the 24% killing observed for the PAO1 biofilm grown on mucin. These data support the first hypothesis that changes in biofilm structure explain the increased tobramycin tolerance of PAO1 biofilms grown on mucin.

Discussion

Environmental conditions such as the type of growth medium, the amount of available nutrients, and the liquid flow rate have all been shown to have effects on *P. aeruginosa* biofilm development and structure (O'Toole and Kolter, 1998; Stoodley *et al*., 2001; Singh *et al*., 2002; Klausen *et al*., 2003a). It is also known that the physicochemical properties of the substratum have the potential to influence the attachment of individual cells and their maturation into a biofilm community (Gilbert *et al*., 1991; Bakker *et al*., 2004). This study shows that surfaces coated with mucin, a glycoprotein that is a major CF airway component, impact *P. aeruginosa* biofilm development. Our results suggest that a specific adhesin–mucin interaction blocked surface motility resulting in a highly structured, heterogeneous biofilm that had increased tolerance to tobramycin.

An analysis of biofilm formation using select mutant backgrounds and time-lapse microscopy indicated that significant surface motility is a feature of the biofilms grown on glass. This is not surprising, as mutations affecting flagella and type IV pili have previously been shown to influence biofilm development on abiotic surfaces for *P. aeruginosa* and a number of other species (O'Toole and Kolter, 1998; Klausen *et al*., 2003b). The interesting observation in this study is that mutations affecting surface motility cause biofilms grown on glass to form biofilms with the heterogeneous structure seen with the wild-type strain grown on the mucin-coated surface (Fig. 5).

A systematic analysis of the biofilms formed by the mutant strains (Fig. 5) is informative when considering the underlying mechanism for biofilm formation on glass and mucin surfaces. A *pilA* mutant is severely impaired for surface motility. This strain cannot move by twitching motility and is severely impaired for swarming motility. Therefore, the motility hypothesis would predict that cells would attach and form biofilms composed primarily of cellular

aggregates on glass and mucin surfaces (which is observed experimentally, Fig. 5). A FliD–mucin interaction might still occur in this strain; however, in the absence of surface motility, its only impact on biofilm development would be at the attachment stage. The *flgK* mutant does not produce a functional flagellum, and hence swarming and swimming motility is impaired. However, this strain does produce a type IV pilus and can move on surfaces by twitching motility (data not shown). This strain also forms biofilms containing large cellular aggregates on both glass and mucin surfaces. On mucin this is not surprising, because the strain still produces FliD, which may still associate with the defective hook of this strain and function to anchor the bacterium to the surface. On glass surfaces, the *flgK* strain should still be free to move by twitching motility. However, time-lapse microscopy revealed that this strain is not moving on the glass surface and forms biofilms characterized by large cell aggregates (Fig. 3). Why this strain does not move by twitching motility under these conditions is unclear, although the motility hypothesis holds true in explaining biofilm architecture for this strain under these conditions. Interpretation of the *fliD* data is fairly straightforward. In the absence of FliD, *P. aeruginosa* is no longer immobilized and is free to move about the mucin-coated surface (Fig. 5). Although the *fliD* strain has an attachment deficiency on the mucin-coated surface (Fig. 3), this strain eventually forms a flat biofilm on both surfaces, similar to PAO1 biofilms on glass (Figs 5 and 6). Our findings are fairly consistent with Klausen *et al*. (2003b) who reported that *P. aeruginosa* 'flat' biofilms grown on glass are characterized by extensive surface motility.

An attractive model, illustrated in Fig. 8, summarizes our data. This model involves immobilization of the bacterium via a FliD–mucin interaction, with subsequent clonal growth accounting for the observed cellular aggregates on mucin surfaces. Consistent with this model, we find that in the absence of *fliD*, bacterial cells freely navigate the mucin surface and ultimately produce biofilms having the homogeneous flat morphology of wild-type biofilms on glass, actin and DNA surfaces. Numerous other *P. aeruginosa* mucin adhesins (such as outer membrane proteins) have been identified, and it is possible that not every strain will employ FliD as a primary adhesin. With that in mind, subsequent work will focus on validation of the immobilization model presented in Fig. 8, and assessment of its generality with respect to other *P. aeruginosa* strains. A feature of *P. aeruginosa* adaptation to the CF lung is loss of motility functions. It is thought that loss of surface antigens may protect the organism from the host immune response. Recent results reported by Wolfgang *et al*. (2004) suggest that *P. aeruginosa* specifically responds to CF airway liquid by downregulating expression of genes involved in production of flagella. Our results are consistent with these observations, suggesting that motility plays a limited role in the formation of biofilms in CF airways.

Equally significant may be the strongly enhanced antibiotic tolerance of biofilms formed on mucin surfaces in comparison with biofilms grown on abiotic (glass) surfaces. The most pronounced observed differences

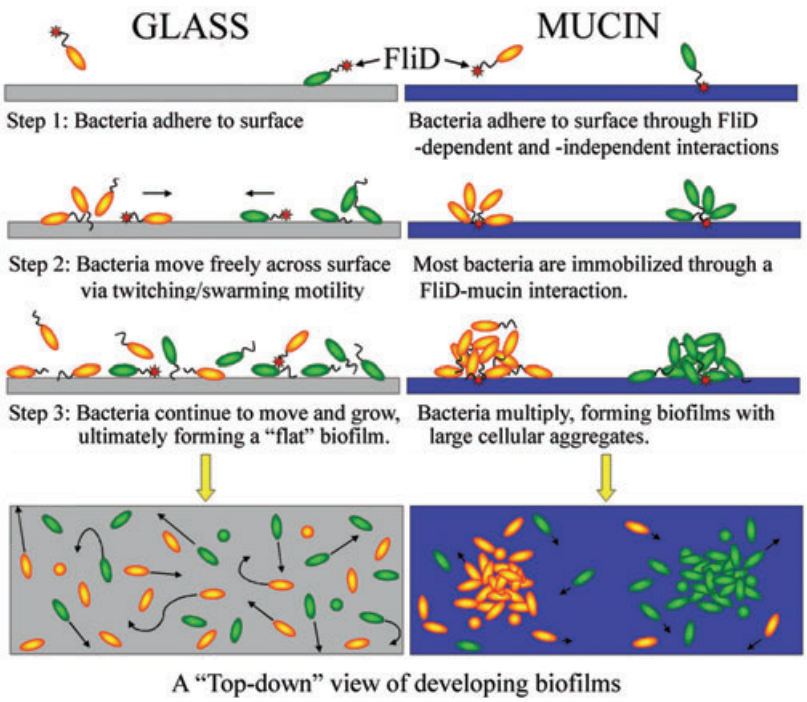

Fig. 8. Model of *P. aeruginosa* biofilm development on glass and mucin surfaces. Bacteria attach to both the glass and mucin-coated surfaces. The bacteria on the mucin-coated surfaces are immobilized via the FliD protein, represented by the red star. Motion is limited for these cells immobilized on the mucin-coated surface and they develop into microcolonies by clonal growth. On the glass surface, bacteria exhibit extensive surface-associated motility. This results in the formation of a homogenous, flat, thin biofilm. The different coloured cells are meant to indicate bacteria of different parental lineages.

between the two systems are the increased biomass associated with the mucin-supported biofilms and the strikingly different morphologies. Both may well be relevant here. For the large, mucin-supported bacterial aggregates one could envision, for example, a substantial core region of tobramycin tolerant cells characterized by significantly reduced metabolism. Alternatively, or additionally, the aggregates might comprise a less permeable structure than the thin flat structure of colonies formed on glass surfaces. The analysis of the *flgK* biofilms supports this view. This strain formed biofilms characterized by large cellular aggregates on both glass and mucin surfaces. These biofilms were equally tolerant of tobramycin, and comparable with the tobramycin-tolerant PAO1 biofilms grown on mucin. Regardless of the explanation, the experiments highlight a potentially profound functional consequence of biofilm formation on mucin, i.e. very substantial antimicrobial tolerance can be engendered by host polymer induced effects on biofilm architecture.

Much recent work in the field has focused on identifying molecular determinants and environmental conditions that contribute to and affect *P. aeruginosa* biofilm development. These studies have revealed that biofilm development for this organism is surprisingly complex. *P. aeruginosa* can form distinct biofilm communities in response to numerous environmental conditions. Perhaps this is not surprising, given that this organism can thrive in soil and aquatic environments and interacts with a variety of eukaryotic species. One unique environment in which *P. aeruginosa* biofilms can thrive is the CF lung. Our results suggest that a specific component of the CF lung, mucin, can affect the structure and function of *P. aeruginosa* biofilm communities. These results highlight the importance of the environmental context in which *P. aeruginosa* biofilm development is studied.

Experimental procedures

Substratum derivatization and characterization

To immobilize biological macromolecules on a solid support, we generated a surface with terminal aldehyde groups, which react readily with primary amines present on biological macromolecules to form a Schiff base linkage. Glass coverslips ($50 \times 30 \times 1$ mm) (Fisher Scientific) were cleaned with a acid solution (70% H_2SO_4 : 30% H_2O_2) for 10 min followed by 5 min soaking in 2 M hydrochloric acid. The coverslips were then rinsed with water and dried with nitrogen. Once dry, the coverslips were submerged immediately in a 4% (v/v) solution of aminopropyltrimethoxysilane (APTMS) in dry acetone and heat treated at 50°C for 12 h. The slides were then rinsed with agitation in acetone followed by water and submerged in a 1% (v/v) solution of electron microscopy grade glutaraldehyde for 1 h. The coverslips were placed immediately into the mucin protein solution (100 μg ml^{-1}) for several hours. Before affixing to a sterile flow cell, the slides were rinsed with 0.1 M phosphate buffered saline (PBS). All chemicals were obtained from Sigma-Aldrich.

Atomic force microscopy images of mucin-derivatized slides revealed a confluent and relatively smooth film without breaks in the mucin coating. These results were compared with an actin-derivatized surface. The mucin surface height fluctuated by only a few nanometres, while the actin surface was an order of magnitude rougher. AFM provides topographical information about a surface by rastering a tip across the surface. Derivatized slides were examined using a Molecular Imaging PicoSPM AFM (Tempe, AZ). The TRITC-labelled PNA lectin assay confirmed the presence of a uniform layer of mucin on the substratum. For the lectin assay, TRITC conjugated lectin from *Arachis hypogaea*, or PNA, was used according to standard staining techniques (Brooks *et al*., 1997). Briefly, the lectin was introduced at a concentration of 10 μg ml^{-1} to a coplin jar containing both mucin derivatized slides and glass controls and incubated for 1 h. The specimens were rinsed vigorously with running deionized water for 15 min and examined using epifluorescent microscopy with a set exposure. The slides were also routinely verified using a water contact angle measurement device. This device measures the wettability of a surface and allows for comparison between different substrates.

Strains, plasmids and media

The *P. aeruginosa* strains used were PAO1 derivative chromosomally tagged with a mini-Tn7 site-specific construct bearing the green fluorescent protein (GFP) to facilitate microscopy (Lambertsen *et al*., 2004). Additionally, several mini-Tn7 GFP-tagged mutants of PAO1 were used: PAO1-*flgK* (O'Toole and Kolter, 1998), PAO1-*pilA* (Jacobs *et al*., 2003) and PAO1-*fliD* (Jacobs *et al*., 2003). All mini-Tn7 insertions were confirmed by polymerase chain reaction (PCR). Bacteria were routinely grown in Luria–Bertani (LB) broth and LB agar at 30°C, as well as, Jensen's media with appropriate antibiotics when necessary. A 1450 bp PstI-XbaI fragment harbouring the entire *pilA* gene and 1 kb of sequence upstream of the translational start site was cloned into the broad host range plasmid pUCP18, expressed from the *lac* promoter to give pDA2. The *flgK* and *flgL* genes were cloned together as a 3403 bp HindIII-SmaI fragment into the plasmid pEX1.8 oriented to be expressed in tandem from the *tac* promoter, to give pDA3. The plasmid pPZ375D was obtained from R. Ramphal and contained a functional copy of the *fliD* gene on the plasmid pGEM3Z (Arora *et al*., 1998). Swimming, swarming and twitching motility assays on solid medium were used to verify complementation of the *flgK, fliD* and *pilA* strains respectively (Kohler *et al*., 2000; Rashid and Kornberg, 2000; Beatson *et al*., 2002).

Biofilm cultivation

A once-through, continuous culture biofilm reactor was assembled as described by Christensen *et al*. (1999). Biofilms were grown at 30°C in flow cells with individual channel dimensions of $1 \times 4 \times 40$ mm and were supplied with a 0.067 ml min^{-1} flow of Jensen's media as previously described (Hentzer *et al*., 2001). The substrata were either

glass coverslips or chemically derivatized glass coverslips affixed to the flow cell.

Antimicrobial resistance of biofilms

Biofilms were grown in the flow cell system described above for 24 h. The medium was then supplemented with 2 μg ml^{-1} tobramycin for an additional 24 h. Bacterial viability was evaluated using the LIVE/DEAD *Bac*light bacterial viability staining kit (Molecular Probes) as previously described (Teitzel and Parsek, 2003). The biofilms were then visualized by scanning confocal laser microscopy (SCLM) and analysed by COMSTAT with a modification designed to compile amounts of live and dead cells (Heydorn *et al*., 2000; Teitzel and Parsek, 2003).

Human mucin isolation and purification

Mucus secretions were purified from Calu-3 cells, a mucin overproducing immortalized human airway epithelial cell line (Berger *et al*., 1999). Calu-3 cells were cultured as polarized monolayers on filter supports at the air–liquid interface as described previously (Karp *et al*., 2002). After 14 days of growth, the apical surface of each monolayer was gently washed twice with 250 ml of PBS. This procedure allowed collection of mucus without disruption to cell-to-cell junctions. The isolation of large gel forming mucins from secretion samples was accomplished using a protocol published by Davies and Carlstedt (2000). The resulting fractions were further separated on a sepharose 4B-CL column several times and dialysed versus PBS. Mucin purity was assessed using SDS-PAGE.

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Supplementary material

The following supplementary material is available for this article online:

Movies S1 and S2. Time-lapse microscopy of biofilm development on glass (Movie S1) and mucin (Movie S2). Images were captured every 2 min for 6 h.

This material is available as part of the online article from http://www.blackwell-synergy.com

