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Mucin inhibits *Pseudomonas aeruginosa* biofilm formation by significantly enhancing twitching motility

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Abstract

In *Pseudomonas aeruginosa*, type IV pili (TFP)-dependent twitching motility is required for development of surface-attached biofilm (SABF), yet excessive twitching motility is detrimental once SABF is established. In this study, we show that mucin significantly enhanced twitching motility and decreased SABF formation in PAO1 and other *P. aeruginosa* strains in a concentration-dependent manner. Mucin also disrupted partially-established SABF. Our analyses revealed that mucin increased the amount of surface pilin and enhanced transcription of the pilin structural gene, *pilA*. Mucin failed to enhance twitching motility in *P. aeruginosa* mutants defective in genes within the pilin biogenesis operons *pilGHI/pilJK-chpA-E*. Furthermore, mucin did not enhance twitching motility and reduce biofilm development by chelating iron. We also examined the role of the virulence factor regulator Vfr in the effect of mucin. In the presence or absence of mucin, PAO *vfr* produced a significantly reduced SABF. However, mucin partially complemented the twitching motility defect of PAO *vfr*. These results suggest that mucin interferes with SABF formation at specific concentrations by enhancing TFP synthesis and twitching motility, that this effect, which is iron-independent, requires functional Vfr, and only part of the Vfr-dependent effect of mucin on SABF development occurs through twitching motility.

Keywords

biofilm; mucin; pilin; *Pseudomonas aeruginosa*; twitching motility

INTRODUCTION

Cystic fibrosis (CF), an inherited disorder that affects approximately 30,000 Americans of primarily Northern European descent, is caused by a mutation in the gene that encodes the cystic fibrosis transmembrane conductance regulator (Gibson et al., 2003; Rommens et al., 1989). Mutations in this protein cause an insufficiency in chloride secretion with resultant

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accumulation of thick, stagnant mucus within the lung alveoli of patients (Baltch, 1994; Burns et al., 1993; Gibson et al., 2003; Jiang et al., 1993; Rommens et al., 1989). Nutrients in the thick mucus facilitate the colonization of various bacterial pathogens, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus* (Baltch, 1994; Gibson et al., 2003; Hassett et al., 2002). Colonization by these pathogens leads to decimation of the lung tissue and, eventually, death from respiratory failure (Burns et al., 1993; Gibson et al., 2003; Pier and Ramphal, 2010). *P. aeruginosa* is one of the significant pathogens involved in the chronic lung infections of CF patients (Gibson et al., 2003; Lyczak et al., 2002).

Among the different factors that contribute to the virulence of *P. aeruginosa* is its ability to form a biofilm. Biofilms are defined as microbial communities in which bacteria are attached to a substratum or to each other (Donlan and Costerton, 2002). Within a biofilm, bacteria are surrounded by an extracellular polysaccharide matrix which protects them from the effects of the host immune system and a wide variety of antibiotics (Leid et al., 2005; Matsukawa and Greenberg, 2004; Stewart and Costerton, 2001). A number of *P. aeruginosa* infections are associated with biofilm formation, including chronic wound infections, otitis media, endocarditis, and chronic lung infections in CF patients (Costerton et al., 1999; Donlan and Costerton, 2002). Specific bacterial factors are required at each stage of biofilm development. For instance, during the initial (attachment) stage of biofilm formation, bacteria depend on both the flagellum-mediated swimming motility and the pilum-mediated twitching motility (Sauer et al., 2002). However, twitching motility is detrimental to an established biofilm (Hammond et al., 2010).

We previously demonstrated that in the artificial sputum medium ASM+, which resembles the thick mucus that accumulates in the CF lung, *P. aeruginosa* does not form a biofilm on a surface, but produces biofilm-like structures (BLS) (Haley et al., 2012). The concentration of mucin, a major component of secreted mucus, varies depending on the pathophysiologic condition within the lungs of the CF patient (Henke et al., 2007). In this study, we examined the role of mucin in biofilm development. Our results showed that mucin interferes with biofilm development by significantly enhancing twitching motility. This effect, which is iron-independent, requires functional Vfr.

Materials and methods

Bacterial strains, plasmids, and media

Bacterial strains utilized in this study are described in Table 1. All strains were routinely grown overnight in Luria Bertani (LB) broth under shaking conditions at 37 °C prior to inoculation into different assays. To examine the effect of iron, strains were grown in chelated trypticase soy broth dialysate (TSB-DC) with and without FeCl₃ 20 µg/mL (Ohman et al., 1980). Type 3 porcine mucin (Sigma-Aldrich, St. Louis, Missouri, USA) was added to LB broth or agar at a concentration of 5 mg/mL (LBM broth or agar or TSB-DC). The ASM⁺ medium was prepared and utilized as previously described (Haley et al., 2012). To allow visualization of the bacteria, we utilized *P. aeruginosa* PAO1 strains containing pMRP9-1 from which the gene for green fluorescent protein (GFP) is constitutively expressed (Davies et al., 1998). LB and LBM broth and agar were supplemented with

antibiotics were required to maintain plasmids or transposons: carbenicillin 300 µg/mL, tetracycline 60 µg/mL, and gentamicin 60 µg/mL for PAO1 strains and 15 µg/mL for PA14 strains.

Swimming and twitching motility

The swimming motility assay was done as previously described (Deziel et al., 2001). Swimming plates (0.3% agar [w/v] with or without mucin 5 mg/mL) were incubated for 16 h at 32 °C. The twitching motility assay was also done as previously described with slight modifications (Deziel et al., 2001). Briefly, bacteria were stab-inoculated to the bottom of twitching plates (1 % agar [w/v] with or without mucin 5 mg/mL). The plates were incubated at 37 °C for 48 h and then at room temperature for an additional 48 h. The agar was removed from the plate and the twitching zone was visualized by staining with 1.0 % crystal violet (w/v) (Deziel et al., 2001). All experiments were performed in triplicate and diameters of the twitching zones were measured and reported in mm.

Pilin preparation

Bacteria from an overnight culture (150 µL) were spread on six freshly prepared 1.8 % LB agar plates and six LBM agar plates and grown overnight at 37 °C. Bacteria were then harvested and prepared as previously described with slight modifications (Giltner et al., 2011). Briefly, bacteria were scraped from the agar surface with a sterile glass rod and resuspended in 4.5 mL of 1X PBS, pH 7.4. Cells from different experimental groups were adjusted with 1X PBS to the same optical density at 600 nm (OD₆₀₀). Pilin was removed from the cells by vortexing and recovered by centrifugation at 11,688 × g and precipitation in 5M NaCl and 30% polyethylene glycol (MW 8000) (w/v). Protein pellets were resuspended in 100 µL of protein loading buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [w/v], 10% glycerol [v/v], 5% - mercaptoethanol [v/v], 0.001% bromophenol blue [w/v]) and the proteins were separated by 15% SDS-PAGE. Pilin proteins were visualized by silver stain.

qRT-PCR analysis of pilin genes

Overnight cultures of PAO1 were spread on four LB agar plates and four LBM agar plates and incubated at 37 °C for 16 h. Cells were harvested from the surface of the plates with a sterile glass rod and resuspended in 2 mL of 1X PBS. The cell suspension was mixed with twice its volume of RNAProtect Bacteria Reagent (QIAGEN, Valencia, California, USA) for 5 min at room temperature. The cells were pelleted and stored at -80 °C. Bacterial pellets were first lysed with lysozyme and proteinase K for 15 min at room temperature. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations. The RNA was digested with the RNase-free DNase Set (QIAGEN). RNA was purified from DNase by the RNA cleanup protocol (QIAGEN) with the exception that on-column DNase digestion was applied to eliminate any remaining traces of genomic DNA. Purified RNA was quantified by NanoDrop® spectrophotometer (NanoDrop Products, Wilmington, Delaware, USA) and utilized as a template to synthesize cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN). A 200-ng aliquot of cDNA was mixed with SYBR Green PCR Master Mix (Life Technologies, Carlsbad,

California, USA) and 250 nM of specific primers for *pilA* (forward 5'-AACCTGAACCTGGACTGTGG-3'; reverse 5'-TTGCCTTCGCCATCTTTT-3'). Amplification and detection of the product was conducted using StepOne Plus real-time PCR system (Life Technologies). Three independent biological replicates were used for each experiment. The quantity of cDNA in different samples was normalized using 30S ribosomal RNA (*rplS*) as an internal standard. Gene expression analysis was performed using StepOne Plus software version 2.2.2 (Life Technologies).

Microtiter plate culture system for development of the biofilms

Biofilms were developed utilizing the microtiter plate culture system as previously described (Hammond et al., 2010). Briefly, plastic cell-culture treated coverslips (Sarstedt, Newton, North Carolina, USA), 13 mm diameter × 0.2 mm thick, were placed into wells of sterile 24-well polystyrene plates (Falcon; Becton Dickinson, Franklin Lakes, New Jersey, USA). Overnight cultures of *P. aeruginosa* strains were inoculated into wells containing LB or LBM broth to an initial OD₆₀₀ of 0.02-0.03. Uninoculated wells containing LB or LBM broth were used as sterility controls. The plates were covered and incubated at 37 °C for various time points with slight shaking on a plate shaker (Lab-line Instruments, Melrose Park, Illinois, USA). For analysis of biofilm disruption, PAO1/pMRP9-1 was grown in three sets of microtiter wells containing only LB broth to allow initiation of biofilm formation. One set of wells was examined at 8 h to confirm biofilm initiation. Mucin was added to one set of wells to a final concentration 5 mg/mL from a 0.0625 g/mL stock solution, and the remaining two sets of wells were incubated for an additional 24 h (32 h total).

Crystal violet assay

The assay was conducted as previously described with minor modifications (Hammond et al., 2010). Coverslips were removed from the wells of the microtiter plate, rinsed gently with distilled H₂O, and placed into the corresponding wells of a new plate containing 1 mL 1.0 % crystal violet solution. After incubation at room temperature for 30 min, the crystal violet solution was discarded. The coverslips were rinsed, and placed in corresponding wells of a new plate containing 1 mL of 95 % ethanol. The plates were incubated at room temperature for 1 h and the amount of extracted crystal violet, which is proportional to the biofilm biomass, was measured at an absorbance of 595 nm (A_{595}).

Visualization of the biofilms

Plastic coverslips were removed from wells, gently rinsed in 1X PBS, and placed in a Petri plate for visualization by confocal laser scanning microscopy (CLSM) using an IX71 Fluoview 300 confocal laser scanning microscope (Olympus America, Melville, New York, USA). All images were obtained through a 203/0.40 Ph1 NA objective utilizing an argon laser (510-530 nm). Three-dimensional (3D) image reconstructions were performed using NIS-Elements 2.2 (Nikon Instruments, Melville, New York, USA) to visualize the architecture of biofilms. All instrument settings were consistent for each set of experimental conditions tested. Images of the BLS formed by PAO1 and PAO *vfr* strains were acquired on a Nikon Ti-E microscope with A1 confocal and STORM super resolution, and 3D reconstructions were done as above.

Quantitative structural analysis of the biofilms

The number of images obtained in a single stack was based on the greatest depth of the structures formed under the test conditions and was the same for all strains/conditions within an experiment. Each experiment was done in duplicate. Two 10-image stacks were obtained from random positions within each biofilm for a total of four 10-image stacks analyzed for each strain and/or condition. The image stacks were analyzed using the COMSTAT program (Heydorn et al., 2000) for structural features of the biofilm: biovolume, estimates the biomass of the biofilm; mean thickness, a measure of spatial size of the biofilm; roughness coefficient, a measure of how much the thickness of the biofilm varies, or the heterogeneity of the biofilm; total surface area, space occupied in each image stack; and surface to biovolume ratio, estimates the portion of the biofilm exposed to nutrients (biovolume divided by the surface area of the substratum).

Beta-galactosidase assays

Beta-galactosidase assays were performed as previously reported (Gaines et al., 2005; Miller, 1972; Stachel et al., 1985). To determine the level of β -galactosidase activity produced by the *chpC::lacZ* fusion strain within the BLS, the strain was grown in LBM broth as described above. The BLS were collected, resuspended in LB broth, and gently vortexed to disperse the BLS. The calculation of relative units of β -galactosidase activity accounts for the level of growth.

Statistical analyses

Statistical analyses of the results were done using GraphPad InStat 3.06 (GraphPad Software, San Diego, California, USA). One-way ANOVA with the Tukey-Kramer multiple comparisons post-test was used to determine significant differences among groups of strains or treatments. The two-tailed *t*-test was used to compare two strains or two treatments.

Results

Mucin prevents surface-attached biofilm formation by *P. aeruginosa* and enhances its twitching motility

We previously showed that upon their growth in ASM+, different *P. aeruginosa* strains failed to develop surface-attached biofilms (SABF), instead forming free-floating BLS (Haley et al., 2012). Since a main constituent of ASM+ is mucin, we examined whether mucin itself inhibits biofilm formation and promotes the production of BLS. *P. aeruginosa* strain PAO1/pMRP9-1, which expresses GFP to allow visualization, was grown in LB or LBM broth in the modified microtiter plate culture system (Haley et al., 2012). The concentration of mucin used in LBM broth (5 mg/mL) is the same as that in the ASM+ we used previously (Haley et al., 2012; Sriramulu et al., 2005). PAO1 formed a mature and well-developed SABF in LB broth (Fig. 1A). However, similar to our observation in ASM+, PAO1 failed to develop SABF when grown in LBM broth (Fig. 1B). Instead, it produced a well-developed BLS (Fig. 1C).

The flagellar-mediated swimming motility and the pilin-mediated twitching motility are essential in the initial establishment of *P. aeruginosa* biofilms. Mutants defective in either

motility form architecturally dissimilar biofilms when compared to wild-type strains (Klausen et al., 2003). However, once the biofilm is established the swimming and/or twitching motility is detrimental for the maintenance of biofilms. The presence of factors that stimulate either motility would prevent the development of a *P. aeruginosa* biofilm and reduce an already established one. We previously showed that serum, which significantly enhances twitching motility, inhibited biofilm development by PAO1 and significantly reduced established biofilms (Hammond et al., 2010). Therefore, similarly to serum, the presence of mucin in LB agar may enhance PAO1 swimming and/or twitching motility and prevent the maintenance of a stable SABF. We examined the effect of mucin on the swimming and twitching motility of PAO1 using previously described assays (Deziel et al., 2001). Mucin was added to standard swimming or twitching plates and the motilities were examined in the presence and absence of mucin. We detected no significant alteration in the swimming motility (data not shown). However, mucin enhanced PAO1 twitching motility significantly (Fig. 2A-2B).

Since mucin enhanced PAO1 twitching motility and prevented the establishment of a mature SABF, the addition of mucin to a partially-developed biofilm should enhance twitching motility leading to disruption of the SABF. PAO1 was grown in three sets of microtiter plate wells without mucin for 8 h to allow initiation of biofilm formation. At 8 h, one set of wells was examined for SABF development. Mucin (5 mg/mL) was added to one of the two remaining sets of wells and incubation was continued for 24 h (32 h total). At 8 h, PAO1 had formed a partially-developed biofilm that covered most of the surface area (Fig. 3A). By 32 h, PAO1 incubated without the addition of mucin produced a dense biofilm with characteristic features of high biomass, thickness and surface area (Fig. 3B; Table 2). In contrast, the addition of mucin resulted in significant reduction of the partially-developed SABF with decreases in biomass, thickness, and surface area covered and increases in roughness and surface to volume ratio (Fig. 3C; Tables 2 and 3). These results strongly support our hypothesis that mucin prevents the establishment of *P. aeruginosa* SABF by enhancing twitching motility.

The effect of mucin on SABF formation and twitching motility is concentration-dependent

We previously showed that variations in the amount of mucin in ASM+ altered the development of BLS (Haley et al., 2012). When the concentration of mucin was raised or lowered (2.5 or 10 mg/mL), the formation of BLS was significantly reduced (Haley et al., 2012). Therefore, at certain concentrations (2.5 or 10 mg/mL), mucin may favor the formation of SABF at the expense of BLS. To examine this possibility, we analyzed biofilm development by PAO1 in the presence of different concentrations of mucin. Biofilms were developed for 24 h and examined by CLSM. In the mucin-free medium, PAO1 produced a robust SABF (Fig. 4A). At mucin concentrations of 2.5, 5 and 10 mg/mL, PAO1 produced variable BLS (Haley et al., 2012). At these same concentrations, PAO1 still failed to develop a SABF (Fig. 4B-4D). The biomass, mean thickness and total surface area of these biofilms were significantly reduced when compared with those of a PAO1 biofilm developed in mucin-free medium (Tables 2 and 3). Interestingly however, when we decreased the amount of mucin farther to 0.625 mg/mL, PAO1 developed SABF that closely resemble those produced in mucin-free medium (Fig. 4E; Tables 2 & 3). These results

suggest that mucin concentration plays a critical role in the development of PAO1 biofilm, with lower mucin concentrations (less than 2.5 mg/mL) favoring development of SABF.

If mucin inhibits the development of PAO1 biofilm by enhancing the twitching motility, we expected that a decrease in mucin concentration would also limit twitching motility allowing the development of SABF. To test this possibility, we examined the effect of 2.5, 1.25 and 0.625 mg/mL mucin on PAO1 twitching motility. Compared with the standard 5 mg/mL concentration, 2.5 mg/mL produced a similar enhancement in PAO1 twitching motility (Fig. 4F). However, further decrease in the concentration of mucin to 1.25 or 0.625 mg/mL led to significant reductions in the enhancement of PAO1 twitching motility compared with that observed at 5 mg/mL (Fig. 4F). Thus, the effect of mucin on twitching motility is concentration dependent. At 0.625 mg/mL, mucin still enhanced twitching motility but did not inhibit SABF development (Fig. 4E-4F). This strongly suggests that only part of the mucin effect on SABF development occurs through the twitching motility.

Mucin enhances pilin production and *pilA* expression

Twitching motility in *P. aeruginosa* occurs through the coordinated extension, retraction and tethering of the surface filamentous appendages known as type IV pili (TFP). TFP consist of the 15 kDa major subunit protein, pilin, which is encoded by the *pilA* gene (Paranchych et al., 1985). Several additional *P. aeruginosa* proteins assemble the synthesized pilin on the outer surface of *P. aeruginosa*. Therefore, mucin may enhance PAO1 twitching motility by increasing TFP surface pilin. We compared the level of sheared surface pilin produced by PAO1 cells that were grown on LB or LBM agar. As a control, we used the pilin deficient mutant PAO *pilA* (Table 1). As shown in Fig. 5A, PAO1 grown on LBM agar produced a higher amount of sheared surface pilin than PAO1 grown on LB agar, while PAO *pilA* produced no detectable pilin. To determine if the increase is due to the efficient processing, rather than synthesis, of pilin protein, we tried to compare the intracellular pilin in PAO1 grown in LB and LBM by immunoblotting experiments using pilin polyclonal antibody. However, the antibody cross reacted with other cellular proteins (data not shown). We then analyzed the level of *pilA* transcription in PAO1 grown on either LB or LBM agar using qRT-PCR. In PAO1 grown on LBM agar, the level of *pilA* transcription was higher (1.2 fold) than that of PAO1 grown on LB agar (Fig. 5B). For the analysis of pilin protein and *pilA* transcripts, the cultures were adjusted to a comparable OD₆₀₀. Thus, the less dramatic increase in surface pilin and *pilA* expression suggests that multiple mechanisms contribute to the mucin-induced enhancement in PAO1 twitching motility.

Mucin requires functional pilin protein to reduce SABF formation

Besides *pilA*, *P. aeruginosa* contains more than 40 genes that function in the assembly and biogenesis of the TFP (Mattick, 2002). Together with FimL, the peptidoglycan-binding protein FimV plays a role in pilus assembly (Semmler et al., 2000; Wehbi et al., 2011; Whitchurch et al., 2005). Additionally, the pilin biogenesis gene cluster, encompassed by the *pilGHI/pilJK-chpA-E* operons, encodes chemotaxis-like chemosensory signal transduction systems that control *P. aeruginosa* TFP production and twitching motility (Mattick, 2002). To examine the role of these genes in the observed effect of mucin, we utilized *P. aeruginosa* PA14 transposon mutants defective in each of these genes (Liberati et al., 2006).

Therefore, we first determined if mucin affects twitching motility in other *P. aeruginosa* strains. As with PAO1, mucin significantly enhanced the twitching motility of *P. aeruginosa* strains PA14, PAK, and PA103 (Fig. 6).

We then selected mutants defective in *fimV*, *pilG*, *pilH*, *pilI*, *pilJ*, *pilK*, and *chpA* for examination. These mutants produce three specific phenotypes: (1) mutants defective in *pilG*, *pilI*, *pilJ*, *chpA* or *fimV* synthesize pilin protein, but fail to assemble pili and are defective in their twitching motilities; (2) the *pilH* mutant synthesizes pilin protein and assembles it on its outer surface, but is defective in twitching motility; and (3) in contrast, the mutant defective in *pilK* is piliated and displays twitching motility comparable to its parent strain (Fulcher et al., 2010). Except for PA14 *pilK*, all mutants produced twitching motilities that were significantly lower than that produced by PA14 when grown in the absence of mucin (Fig. S1). The addition of mucin to the twitching medium enhanced the twitching motility of PA14 *pilK* only (data not shown). Additionally, in LB broth and compared with PA14, all mutants including PA14 *pilK* produced significantly reduced SABF (Fig. S2). However, in the presence of mucin, all mutants showed a 1.7- to 3.5-fold increase in SABF formation except PA14 *pilK*, which showed a 2.3-fold decrease (Fig. 7). These results suggest that, depending on the functional status of the pilin protein, mucin affects SABF in *P. aeruginosa* differently. In the presence of functionally active pilin and twitching motility as in PA14 and PA14 *pilK*, mucin reduces biofilm development. However, in the presence of nonfunctional pilin (twitching motility defective), mucin increases SABF formation. The nonfunctional pilin may be synthesized but not assembled, as in PA14 *pilG*, PA14 *pilI*, PA14 *pilJ*, PA14 *chpA* and PA14 *fimV*; or synthesized and assembled, as in PA14 *pilH*.

Based on the results of our previous study (Haley et al., 2012) and our current analysis (Figures 5 & 7), mucin may require functional pilin biogenesis genes to reduced SABF formation and enhance BLS development. In this study, we compared the twitching motility of PA14 and different mutants in the pilin biogenesis genes using the twitching motility plate assay (Fig. S1). Therefore, we examined the effect of mucin on the expression of one of the pilin biogenesis genes, *chpC* (as a representative of the *pilGHI/pilJK-chpA-E* operon), within the BLS. This was done using PAO1 strain PW1762 that carries an in-frame *chpC::lacZ* chromosomal fusion. We grew PW1762 in LBM broth for 16 h, and collected pellets of the BLS and planktonic cultures for β -galactosidase assay. The level of β -galactosidase activity produced within the BLS was significantly higher than that within the planktonic cultures (16.5 ± 0.625 [SEM] units vs. 4.3 ± 0.612 units; $P < 0.001$). These results suggest that within the BLS, the expression of pilin biogenesis genes is increased.

Mucin does not enhance *P. aeruginosa* twitching motility by chelating iron

Singh et al. (2002) previously suggested that at concentrations lower than those preventing bacterial growth, lactoferrin chelates iron and stimulates *P. aeruginosa* twitching motility. As a result of their continuous movement, *P. aeruginosa* bacteria fail to form stable biofilms (Singh, 2004; Singh et al., 2002). Since lactoferrin exists in sufficient amounts in surface secretions, including airway secretions (Abe et al., 1999; Thompson et al., 1990), mucin may inhibit the development of SABF through the lactoferrin iron-chelating activity. To

examine this possibility, we determined the effect of mucin, iron, or both on the twitching motility of PAO1 that was grown in the iron-deficient medium TSB-DC. TSB-DC medium, in which iron has been chelated, is a standard medium for the analysis of different *P. aeruginosa* iron-regulated genes. As shown in Figure 8A, PAO1 twitching motility was reduced in TSB-DC containing iron but was significantly enhanced in TSB-DC containing mucin or mucin plus iron. Additionally, using a *pvdS-lacZ* transcriptional fusion plasmid, we determined the effect of mucin, iron, or both on the expression of the PAO1 iron-regulated gene *pvdS*, which codes for an alternative sigma factor (Vasil and Ochsner, 1999). Iron represses the expression of *pvdS* (Vasil and Ochsner, 1999). However, *pvdS* expression was significantly reduced in the presence of mucin, iron, and mucin plus iron (Fig. 8B). This suggests that, although lactoferrin is one of the components of mucin, mucin as a whole does not chelate iron. Rather, it functions like iron and represses the production of *P. aeruginosa* iron-regulated factors.

Mucin requires functional Vfr to affect twitching motility and biofilm development in *P. aeruginosa*

The virulence factor regulator, Vfr, regulates several *P. aeruginosa* virulence systems including the TFP and twitching motility (Beatson et al., 2002). Compared with its parent strain, a *P. aeruginosa* *vfr* mutant was severely defective in twitching motility (Beatson et al., 2002). Vfr also regulates the expression of pilin biogenesis genes (Beatson et al., 2002). Fulcher et al. (2010) suggested that the Pil-Chp system influences twitching motility through the cAMP-dependent activity of Vfr. To determine if Vfr plays a role in the effect of mucin, we compared biofilm development and twitching motility produced by PAO1 and its *vfr* isogenic mutant PAO *vfr*. Compared with PAO1, PAO *vfr* produced a significantly reduced biofilm (Fig. 9A). This defect was rescued by a plasmid carrying intact *vfr* (pKF906) but not a cloning vector (Fig. 9A). In the presence of mucin, PAO1 and PAO *vfr*/pKF906 produced a significantly reduced biofilm (Fig. 9A). However, mucin did not eliminate the already reduced biofilm produced by either PAO *vfr* or PAO *vfr*/pUCP19 (Fig.9A).

We then compared the twitching motility of PAO1 and PAO *vfr*. The defect of PAO *vfr* in twitching motility was not as pronounced as the defect in biofilm formation (13 mm in PAO1 vs. 8.6 mm in PAO *vfr*) (Fig. 9B). Mucin enhanced twitching motility in both strains. However, the enhancement in PAO *vfr* was significantly lower than that in PAO1 (Fig. 9B). The mucin-induced enhancement of the twitching motility of PAO *vfr*/pKF906 paralleled that of PAO1 (Fig. 9B). These results suggest that mucin requires functional Vfr to influence PAO1 twitching motility. We observed similar results with the *P. aeruginosa* strain PA14 and its *vfr* mutant. Even at 48 h, mucin significantly reduced biofilm development by PA14 and the complemented mutant (data not shown). Additionally, the mucin-induced enhancement in PA14 *vfr* twitching motility was significantly less than that detected in PA14, a defect that was complemented by pKF906 (data not shown).

If Vfr plays a role in the observed mucin-induced reduction in SABF formation, it may also be required for the mucin-induced development of BLS. To examine this possibility, we introduced the GFP plasmid pMRP9-1 plasmid (Table 1) in PAO1 and PAO *vfr*. We grew

the transformants (PAO1/pMRP9-1 and PAO *vfr*/pMRP9-1) in the mucin medium ASM⁺, which we previously utilized to examine different aspects of the BLS (Haley et al., 2012), and compared the BLS formed by the two strains using the COMSTAT. Compared with the BLS formed by PAO1/pMRP9-1, the total biomass, mean thickness, and total surface areas of BLS formed by PAO *vfr*/pMRP9-1 were significantly decreased (Table S1). This suggests that, in addition to its role in the development of SABF, Vfr is critical for the mucin induced development of BLS.

Discussion

We previously showed that in the artificial sputum medium ASM+, *P. aeruginosa* formed free-floating BLS (Haley et al., 2012). Mucin, a major component of this medium, contributed significantly to the formation of BLS (Haley et al., 2012). In this study, we confirmed that regardless of the medium, mucin facilitates the development of BLS at the expense of the development of a surface-attached biofilm. Mucin accomplishes this effect in part by enhancing *P. aeruginosa* pilin-mediated twitching motility (Figs. 2 and 9). The influence of mucin on twitching motility is not a new finding. Huang et al. (2003) previously showed that mucin as well as bovine serum albumin enhanced *P. aeruginosa* twitching motility. However, our findings showed that beyond the initiation, the excessive mucin-induced twitching prevented *P. aeruginosa* from forming SABF. This phenomenon is not limited to the PAO1 strain, but occurs in other *P. aeruginosa* strains such as PA14, PAK and PA103 (Fig. 6). However, the excessive twitching motility does not seem to interfere with the formation of the BLS, indicating that the mechanism of development of these structures is different from that of SABF (Haley et al., 2012).

Yeung et al. (2012) recently reported that, on swimming agar plates, mucin induced a highly modified *P. aeruginosa* surface motility termed surfing, in which *P. aeruginosa* swims relatively more rapidly across the surface of the agar. The phenomenon requires a functional flagellum but not type IV pili as none of the tested chemosensory mutants were surfing defective (Yeung et al., 2012). We did not observe a difference in swimming motility of *P. aeruginosa* that was inoculated on either regular swimming agar or swimming agar containing mucin (data not shown). The most likely explanation for the variation between the two studies is the difference in the types of media that were used in the swimming agar plates. We utilized a tryptone base for swimming plates (0.3 % agar) and an LB base for twitching motility (1 % agar), media that have been used by other investigators (Deziel et al., 2001), whereas Yeung et al. (2012) used BM2 or MSCFM medium in their swimming plates.

The influence of host factors on biofilm development through the twitching motility was previously proposed by Singh et al. (2002), as they demonstrated that lactoferrin enhances PAO1 twitching motility and compromises biofilm development. What is surprising in our findings, however, is that in the absence of twitching motility, mucin enhances rather than reduces the formation of SABF (Fig. 7). This effect requires the presence of pilin protein. A possible explanation is that mucin interacts with *P. aeruginosa* outer surface structures including pilin. While this scenario is plausible for PA14 *pilH*, which is piliated but twitching defective, it is not likely for PA14 *pilI*, PA14 *pilJ*, and PA14 *pilG*, which

synthesize pilin but are not piliated. At this time, the mechanism for the pilin-dependent increase in SABF by mucin is not known. We have already eliminated a possible role for the flagellum or the flagellum-based swimming motility in this phenomenon. Mucin did not affect the swimming motility of PAO1 (data not shown).

Vfr is required for an efficient twitching motility in *P. aeruginosa* (Beatson et al., 2002). Compared with its parent strain, *P. aeruginosa* *vfr*-deficient mutants displayed a significantly reduced twitching motility (Beatson et al., 2002) (Fig. 9B). Although *vfr* mutants produced surface pilin, the amount of pilin protein was considerably lower than that produced by the parent strain (Beatson et al., 2002). Based on these results, Beatson et al. (2002) concluded that Vfr does not influence *pilA* expression or pilin synthesis. Rather, Vfr may affect the pilin assembly or the function of the assembled pilus (Beatson et al., 2002). However, transcriptional analyses demonstrated that, compared with its parent strain, the expression of several genes of the chemosensory system including *pilG*, *pilI*, *pilJ*, *pilK* and *fimV* was significantly reduced in the *vfr*-deletion mutant (Wolfgang et al., 2003). Kanack et al. (2006) demonstrated specific Vfr binding within the upstream region of *fimS* and *pilM*. Similarly, we showed that in the absence of functional Vfr both PAO1 and PA14 mutants displayed a deficient twitching motility, a defect that was complemented by a *vfr* plasmid. Therefore, based on these results and other previous studies, Vfr may not be directly involved in the mucin effect on twitching motility. Rather, Vfr may influence genes through which mucin enhances twitching motility, including some of the chemosensory systems. However, mucin was capable of enhancing twitching motility even in the absence of functional Vfr (Fig. 9B). This partial enhancement in twitching motility suggests that Vfr affects twitching motility through two mechanisms, one Vfr-dependent and one Vfr-independent. The Vfr-dependent mechanism is clearly represented by the increase in the twitching motility of PAO1 and PA14 vs. PAO *vfr* and PA14 *vfr*. The Vfr-independent mechanism is represented by the mucin-induced increase in the twitching motility produced in the *vfr* mutants (Fig. 9B). While evidence suggests that the Vfr-dependent mechanism involves some of the chemosensory genes, the Vfr-independent mechanism is yet to be defined. Our analysis of PA14 and PAO *vfr* mutants supports the potential two mechanisms.

With respect to twitching motility, mucin's primary target may be the Pil-Chp system. Fulcher et al. (2010) previously suggested that the Pil-Chp system influences the *P. aeruginosa* twitching motility by cAMP-independent and cAMP-dependent mechanisms. In the cAMP-dependent one, the Pil-Chp system modulates the activity of the adenylate cyclase that synthesizes cAMP. Vfr requires cAMP to activate the transcription of several factors including those involved in the synthesis and regulation of TFP (Fulcher et al., 2010). Based on our current results, mucin may affect the Pil-Chp system, which in turn enhances twitching motility through the cAMP-independent and cAMP-dependent mechanisms. The cAMP-dependent mechanism occurs through Vfr (Fig. 9). We clearly showed that only part of the mucin effect on twitching motility occurs through Vfr (Fig. 9). Analysis of *P. aeruginosa* cAMP-deficient mutants is required to confirm this hypothesis.

At this time, we do not know the exact host factors that stimulate *P. aeruginosa* motility. We excluded the possibility that mucin chelates iron and enhances *P. aeruginosa* twitching motility. The presence of mucin in TBSD-DC repressed the expression of the *P. aeruginosa*

iron-regulated gene *pvdS* (Fig. 8B). These results differ from those of previous studies that suggested the presence of an iron-chelating activity within the CF mucus (Fung et al., 2010; Wang et al., 1996). Wang et al (1996) showed that the growth of *P. aeruginosa* in a minimal medium containing 10% CF sputum induced the expression of the pyochelin receptor gene, *fptA*. However, no increase in the expression of other iron-regulated genes including *toxA*, *toxR*, *pvdS*, and *pvdA* was reported (Wang et al., 1996). Fung et al. (2010) also showed that the growth of *P. aeruginosa* in their modified artificial sputum medium induced the expression of iron acquisition genes. Besides mucin, this synthetic medium contained other components (Fung et al., 2010; Sriramulu et al., 2005). In contrast to these studies, our current analysis was focused on mucin only. Mucin significantly enhanced twitching motility even when added to the iron-deficient medium TSB-DC, an effect that was not influenced by exogenously added iron (Fig. 8A). More importantly, the expression of the iron-regulated gene *pvdS* was repressed by mucin or iron alone, or both (Fig. 8B). Similar to previous studies (Fung et al., 2010; Sriramulu et al., 2005), we utilized type-3 porcine mucin. Whether other sources of mucin produce a similar effect on the iron-regulated genes is yet to be determined.

Mucin is a complex macromolecule and one or more of its components may induce the enhancement in *P. aeruginosa* twitching motility. We previously suggested that the binding of *P. aeruginosa* to certain constituents of mucin contributes to the development of BLS (Haley et al., 2012). Whether such binding affects both the twitching motility and biofilm development is yet to be determined. Five of the 22 known human mucins (MUC) play a key role in respiratory tract health and defense. The membrane-tethered MUC1, which is upregulated by TNF α -mediated inflammatory responses that occur in *P. aeruginosa* infection in response to TLR5 signaling, is crucial to both protection during infection and in resolution of the anti-inflammatory response (Kim, 2012). Membrane-tethered MUC1 is crucial for the formation of the inner gel formed immediately above the apical cell surfaces by MUC4 and MUC16, as well as formation of the outer, more fluid gel layer by MUC5AC and MUC5B (Kim, 2012). Additionally, the MUC1 ectodomain that is cleaved by neutrophil elastase during acute infection serves as a decoy receptor for *P. aeruginosa*, keeping the bacteria away from the cell surfaces (Kim, 2012). Certainly, the level of mucin varies within the lungs of CF patients, with decreases of up to 89% (to 0.55 mg or less mucin/mL) during periods of stable disease (no progression) and increases of over 900% (to 45 mg or more mucin/mL) during exacerbations of pulmonary disease (Henke et al., 2007). As a result, the twitching motility would be affected in response to changes in the host, thereby favoring the production of either a surface-attached biofilm at low mucin concentrations or free-floating biofilm-like structures at high mucin concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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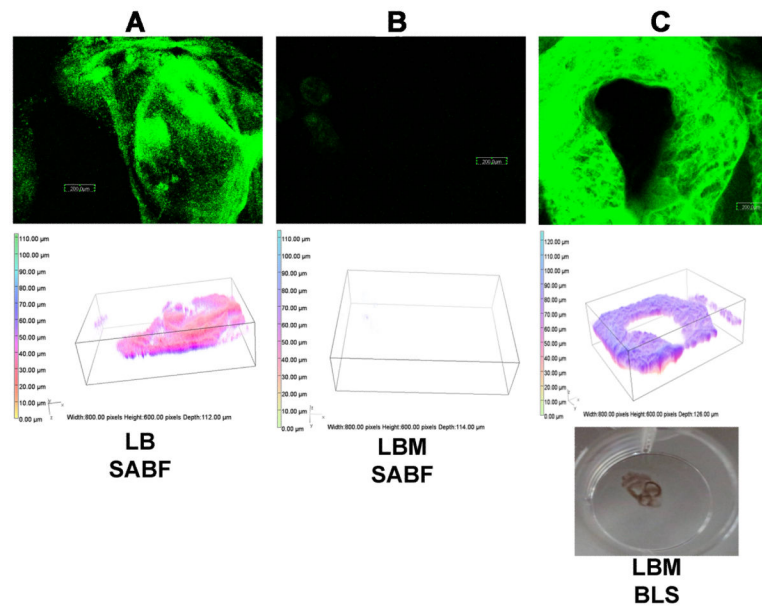


Fig. 1. *P. aeruginosa* PAO1 forms biofilm-like structures (BLS) rather than surface attached biofilm (SABF) in the presence of mucin. PAO1/pMRP9-1, which expresses GFP, was grown aerobically on plastic coverslips in a 24-well microtiter plate in either LB or LBM (5 mg/mL) broth for 24 h at 37 °C under aerobic conditions. CLSM and 3D image analysis of (A) PAO1 SABF in LB broth, (B) PAO1 SABF in LBM broth, (C) PAO1 BLS in LBM broth including photograph of the free-floating BLS within the microtiter plate well (bottom); magnification, 10×; bars, 200.00 nm. Images shown are representative of three independent experiments.

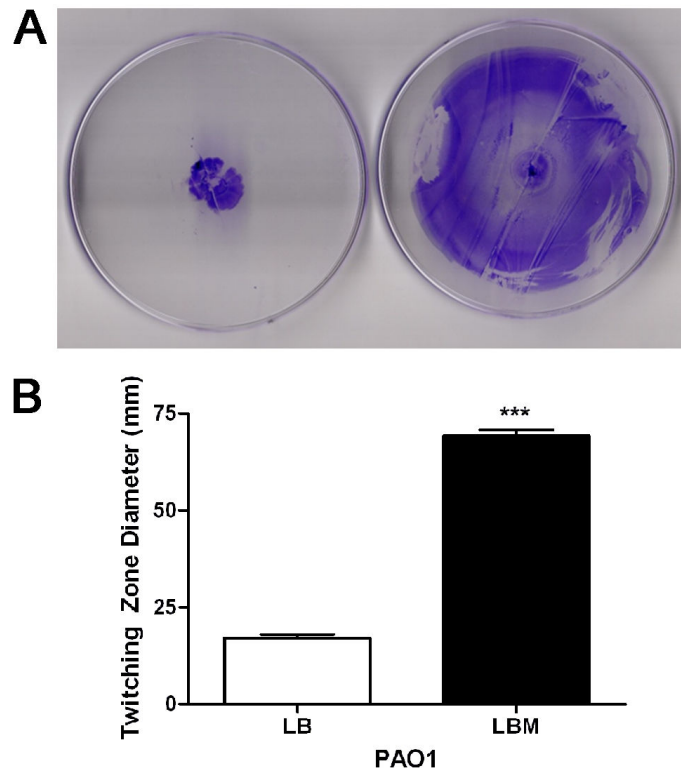


Fig. 2. Mucin enhances PAO1 twitching motility. **(A)** PAO1 was stab-inoculated onto either LB (left) or LBM (right) twitching plates (1 % agar). The plates were incubated at 37 °C for 48 h then at 23°C for an additional 48 h. The agar was removed and the twitching zone revealed by staining with crystal violet. Images shown are representative of three independent experiments. **(B)** Graphic comparison of twitching zone diameters for PAO1 in LB and LBM broths; values represent the means of 3 independent experiments \pm SEM; *** P <0.001.

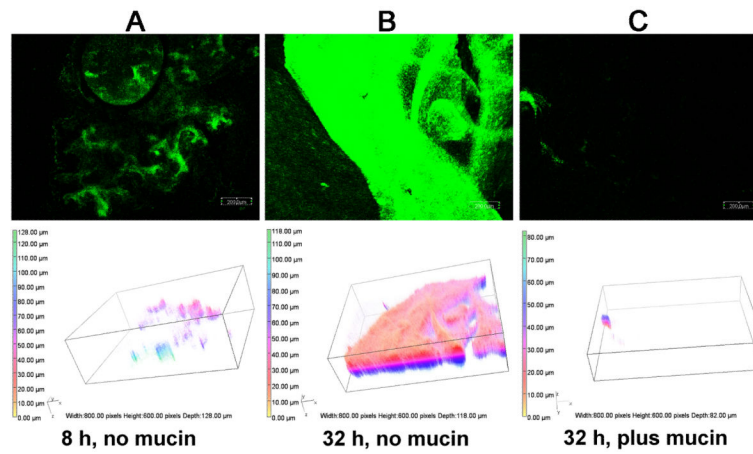


Fig. 3.

Mucin disrupts partially established PAO1 biofilms. PAO1/pMRP9-1 was grown aerobically at 37 °C on plastic coverslips in a 24-well microtiter plate in LB broth for 8 h. Mucin was added (5 mg/mL) to one-third of the samples and the growth was continued for an additional 24 h. CLSM micrographs and 3D image analysis of the SABF that were developed for (A) 8 h without mucin, (B) 32 h without mucin, or (C) 32 h with mucin (24 h post addition); magnification, 10×; bars, 200.00 nm. Images shown are representative of three independent experiments.

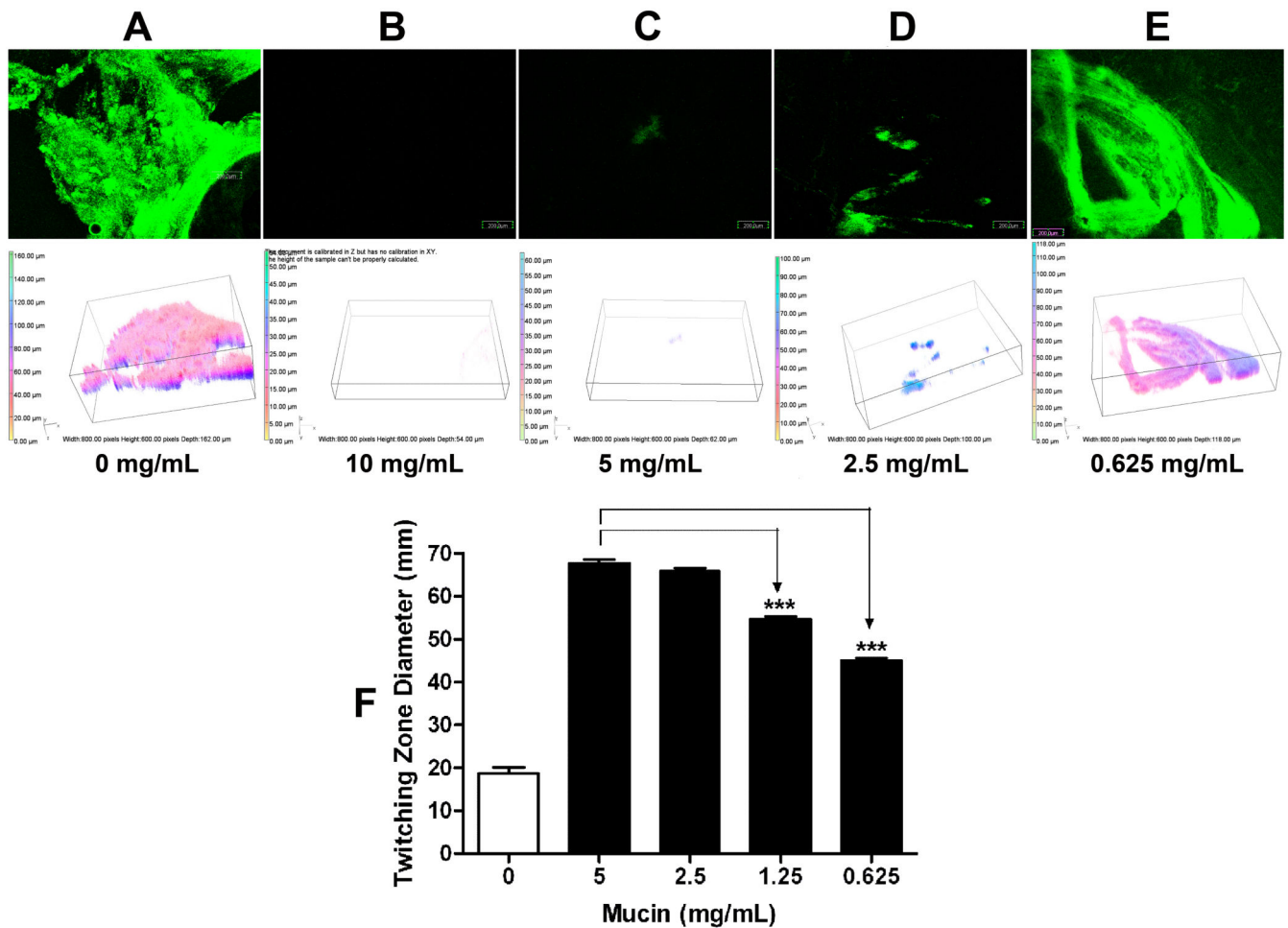


Fig. 4. Mucin inhibition of biofilm formation by PAO1 is concentration dependent. PAO1/pMRP9-1 was grown aerobically on plastic coverslips in a 24-well microtiter plate in either LB or LBM broth containing variable concentrations of mucin for 24 h at 37 °C under aerobic conditions. CLSM micrographs and 3D image analysis of the PAO1 SABF grown (A) without mucin, and with concentrations of mucin indicated: (B) 10 mg/mL, (C) 5 mg/mL, (D) 2.5 mg mucin/mL, and (E) 0.625 mg/mL; magnification, 10×; bars, 200.00 nm. Images shown are representative of three independent experiments. (F) Twitching motility of PAO1 in different concentrations of mucin. PAO1 was stab-inoculated onto either LB or LBM (5 mg mucin/mL) twitching plates (1 % agar) containing variable concentrations of mucin. The plates were incubated at 37 °C for 48 h then at 23°C for an additional 48 h. The agar was removed and the twitching zones revealed by staining with crystal violet. Values represent the means of 3 independent experiments ± SEM. Twitching motility in decreasing concentrations of mucin was compared to that in 5 mg/ml mucin by one-way ANOVA; *** $P < 0.001$.

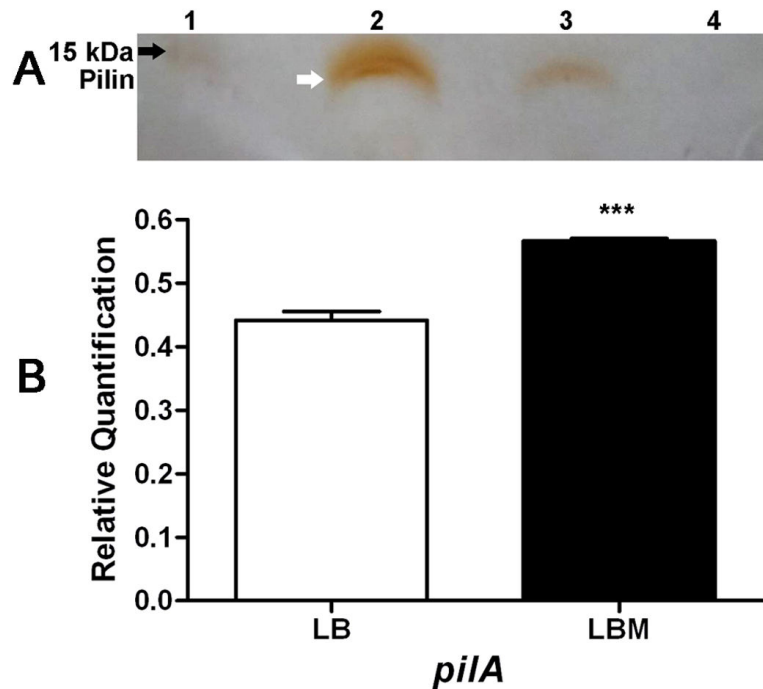


Fig. 5. Mucin enhances the production of pilin and the expression of the *pilA* structural gene. PAO1 was grown on LB and LBM agar for 16 h at 37 °C. **(A)** Pilin was sheared from the surface of PAO1 and PAO *pilA* and pilin proteins were analyzed by 15% SDS-PAGE and silver staining. Lanes: 1) Molecular weight standard, 2) PAO1 from LBM agar, 3) PAO1 from LB agar, 4) PAO *pilA* from LBM agar. **(B)** Mucin enhances *pilA* expression. Overnight cultures of PAO1 were spread on four LB agar plates and four LBM agar plates and incubated at 37 °C for 16 h. Cells were harvested from the surface of the plates with a sterile glass rod, RNA was extracted and purified and used in qRT-PCR to analyze the levels of *pilA* expression. Samples were normalized using 30S ribosomal RNA (*rplS*) as an internal standard. Values represent the means of 3 independent replicates \pm SEM; *** $P < 0.001$.

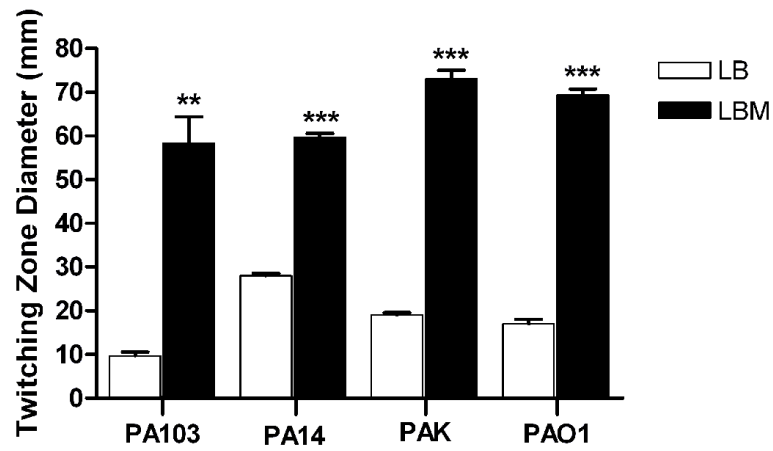


Fig. 6.

Mucin enhances twitching motility of multiple *P. aeruginosa* strains. PA103, PA14, PAK and PAO1 were stab-inoculated onto either LB or LBM twitching agar (1 % agar). The plates were incubated at 37 °C for 48 h then at 23°C for an additional 48 h. The agar was removed and the twitching zones revealed by staining with crystal violet. Values represent the means of 3 independent experiments \pm SEM. Twitching zones of each strain in LBM were compared to those in LB using the *t*-test; *** P <0.001, ** P <0.01.

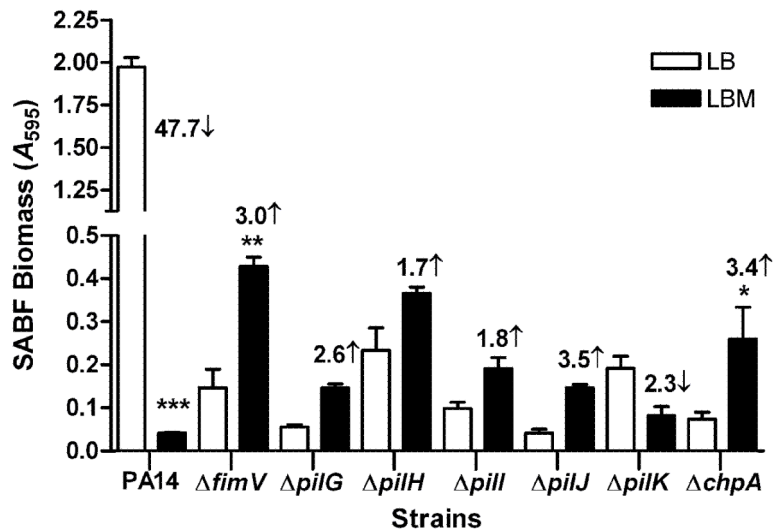


Fig. 7.

In the absence of twitching motility, mucin increases SABF formation by PA14. PA14 and its different PA14 pilin biogenesis mutant strains were grown in LB and LBM broth. SABF formation was determined as described in Fig. 1. In the presence of mucin, the SABF biomass was significantly increased in PA14 *fimV* (3 fold), PA14 *pilG* (2.6 fold), PA14 *pilH* (1.7 fold), PA14 *pili* (1.8 fold), PA14 *pilJ* (3.5 fold), and PA14 *chpA* (3.4 fold). In contrast, mucin significantly decreased the SABF biomass in PA14 *pilK* (2.3 fold) and PA14 (47.7 fold). Values represent the means of 3 independent experiments \pm SEM. SABF of each strain in LBM broth were compared to those in LB broth using the *t*-test; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

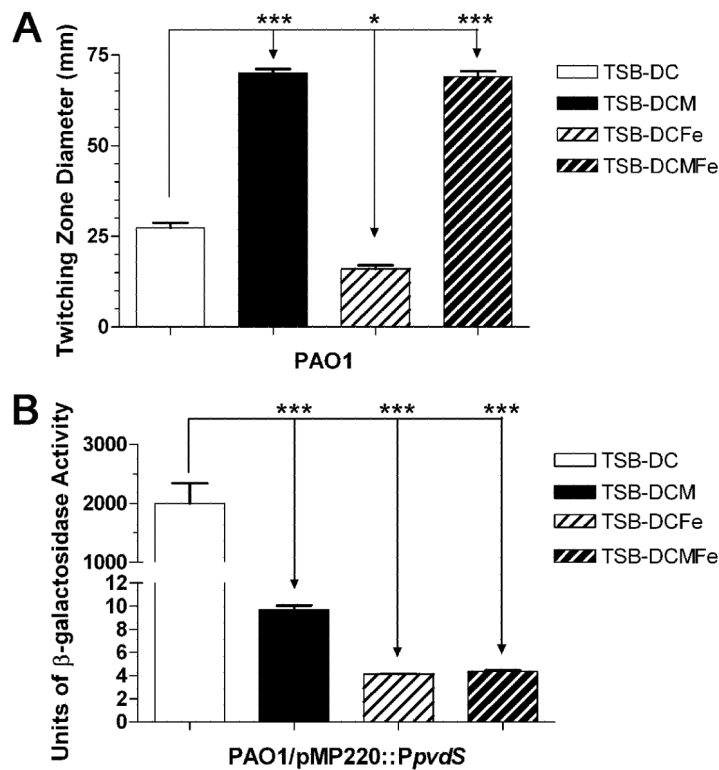


Fig. 8. Mucin does not enhance *P. aeruginosa* twitching motility by chelating iron. **(A)** PAO1 was stab-inoculated onto TSB-DC, TSB-DC with mucin (5 mg/mL), TSB-DC with iron (20 μ g/mL), or TSB-DC with mucin (5 mg/mL) and iron (20 μ g/mL) twitching plates. The plates were incubated at 37 $^{\circ}$ C for 48 h then at 23 $^{\circ}$ C for an additional 48 h. The agar was removed and the twitching zone revealed by staining with crystal violet. **(B)** Either mucin or iron significantly reduced the expression of the iron regulated gene *pvdS*. PAO1 containing a *pvdS-lacZ* fusion plasmid (pMP220::PpvdS) was grown in TSB-DC, TSB-DC with mucin, TSB-DC with iron, or TSB-DC with both mucin and iron under shaking conditions for 6 h at 37 $^{\circ}$ C. Samples were collected and the β -galactosidase activity was determined. Values in A and B represent the means of 3 independent experiments \pm SEM. Values obtained in the presence of mucin, iron, or both were compared to values obtained in TSB-DC alone by *t*-test; *** P <0.001, * P <0.05.

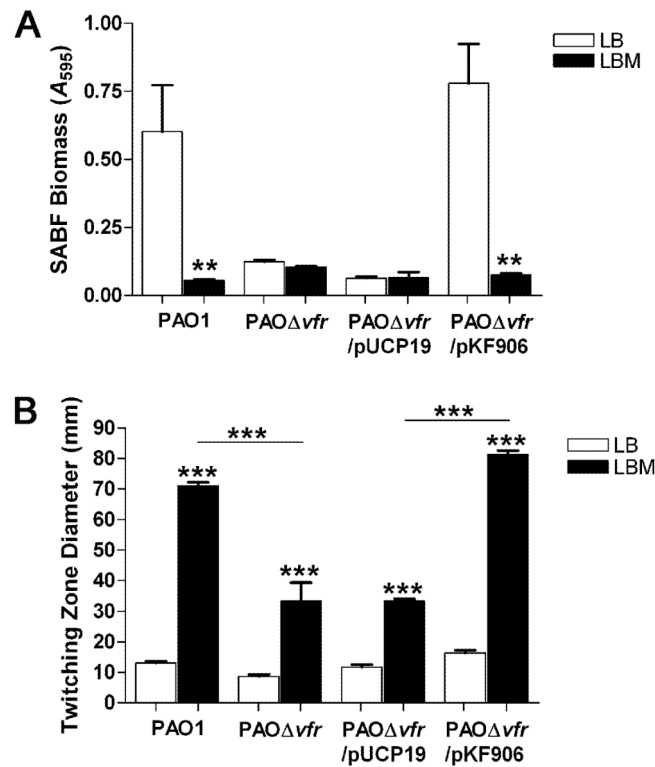


Fig. 9. Mucin enhances twitching motility and reduces biofilm development of PAO1 through the virulence factor regulator, Vfr. We assessed biofilm development and twitching motility of PAO1, PAO Δvfr , PAO $\Delta vfr/pUCP19$, and PAO $\Delta vfr/pKF906$ as described in figures 1 and 2. **(A)** The effects of mucin and Vfr on SABF development. **(B)** The effects of mucin and Vfr on twitching motility. Values in A and B represent the means of 3 independent experiments \pm SEM. Twitching zones of each strain on LBM agar were compared to those on LB agar using the *t*-test; and in B, values for the mutant were compared to the parent (upper bar); *** $P < 0.001$, ** $P < 0.01$.

Table 1
P. aeruginosa strains and plasmid used in this study

	Description	Source
Strains		
PAO1	Prototroph; human isolate	(Holloway et al., 1979)
PA103	Human isolate	(Liu, 1966)
PAK	Prototroph; human isolate	(Strom and Lory, 1986)
PA14	UCBPP-PA14; burn wound isolate	(Rahme et al., 1995)
PAO <i>pilA</i>	<i>pilA</i> inactivated by allelic displacement; tagged with eGFP, in a mini-Tn7 construct; Gm ^r	(Klausen et al., 2003)
PA14 <i>fimV</i> *	PA14 with <i>TnMAR2xT7</i> insertion in <i>fimV</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilG</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/G</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilH</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/H</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilI</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/I</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilJ</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/J</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilK</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/K</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>chpA</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>chpA</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>pilR</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>pi/R</i> ; Gm ^r	(Liberati et al., 2006)
PA14 <i>rpoN</i>	PA14 with <i>TnMAR2xT7</i> insertion in <i>rpoN</i> ; Gm ^r	(Liberati et al., 2006)
PW1762 [†]	<i>chpC-F07::IS/acZ/hah</i> at bp 79 or 507 in the ORF; Tc ^r	(Jacobs et al., 2003)
Plasmids		
pMRP9-1	pUCP18 carrying a gene encoding enhanced green fluorescent protein (GFP); Cb ^r	(Davies et al., 1998)
pUCP19	<i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector; Cb ^r	(Schweizer, 1991)
pKF906	pUCP19 carrying intact <i>vfr</i> ; Cb ^r	(West et al., 1994)
pMP220::P <i>pvdS</i>	pMP220 carrying a <i>pvdS</i> promoter transcriptional <i>lacZ</i> fusion, Tc ^r	(Ambrosi et al., 2002)

* PA14 mutants were purchased from the PA14 Transposon Insertion Mutant Library of non-redundant *Pseudomonas aeruginosa* transposon insertion mutants (<http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi> [accessed 22 January 2014])

[†] PAO1 transposon mutant purchased from the University of Washington Genome Center (<http://www.gs.washington.edu/labs/manoil/libraryindex.htm> [accessed 22 January 2014])

^r resistant; Cb, carbenicillin; Gm, gentamicin; Tc, tetracycline

Table 2
Effect of mucin on *P. aeruginosa* biofilm development

Variable	Images per stack (#) [*]	Total biovolume ($\mu\text{m}^3/\mu\text{m}^2$) [†]	Mean thickness (μm) [‡]	Roughness coefficient [§]	Total surface area $\times 10^7$ (μm^2)	Surface to volume ratio ($\mu\text{m}^2/\mu\text{m}^3$) [¶]
Reduction of SABF						
8 h SABF	10	1.38 \pm 0.40	2.18 \pm 0.49	1.34 \pm 0.12	0.36 \pm 0.08	1.36 \pm 0.13
no mucin [#]	10	7.90 \pm 0.29	8.42 \pm 0.09	0.11 \pm 0.01	0.80 \pm 0.07	0.53 \pm 0.06
+ mucin [#]	10	0.25 \pm 0.06	0.35 \pm 0.09	1.88 \pm 0.03	0.10 \pm 0.02	2.06 \pm 0.25
Mucin titration (mg/mL)						
0	10	5.63 \pm 0.30	6.12 \pm 0.41	0.55 \pm 0.07	0.68 \pm 0.03	0.63 \pm 0.02
10	10	0.02 \pm 0.00	0.00 \pm 0.00	1.20 \pm 0.00	0.01 \pm 0.00	2.89 \pm 0.02
5	10	0.29 \pm 0.06	0.40 \pm 0.13	1.87 \pm 0.03	0.10 \pm 0.18	1.88 \pm 0.08
2.5	10	0.71 \pm 0.07	0.98 \pm 0.09	1.58 \pm 0.09	0.24 \pm 0.40	1.71 \pm 0.21
1.25	10	3.84 \pm 0.13	4.68 \pm 0.39	1.38 \pm 0.05	0.45 \pm 0.08	0.60 \pm 0.11
0.625	10	4.44 \pm 0.57	5.43 \pm 0.52	1.30 \pm 0.06	0.55 \pm 0.10	0.69 \pm 0.18

* PAO1/pMRP9-1 was used in all experiments described in the table. Each experiment was done in duplicate. Two 10-image stacks were obtained from random positions within each SABF. Image stacks were analyzed using the COMSTAT program (Heydorn et al., 2000). Values represent the mean \pm SEM.

[†] Estimates the biomass of the SABF

[‡] Measures spatial size of the SABF

[§] Assessment of the variation in the thickness of the SABF

^{||} Total of the area occupied in each image stack

[¶] Estimates the portion of the biofilm exposed to nutrients; biovolume divided by the surface area of the substratum

[#] Three sets of 2 coverslips each were inoculated with PAO1/pMRP9-1 and incubated for 8 h to allow initiation and partial development of SABF. At 8 h, one set of coverslips was harvested for analysis, mucin (5 mg/mL) was added to one set, no mucin to the other and incubation continued for 24 h.

Table 3
Significance of differences in values presented in Table 2

Variable	Total biovolume ($\mu\text{m}^3/\mu\text{m}^2$)*	Mean thickness (μm)*	Roughness coefficient*	Total surface area $\times 10^7$ (μm^2)*	Surface to volume ratio ($\mu\text{m}^2/\mu\text{m}^3$)*
Reduction of SABF					
No mucin vs. 8 h	Increase [†]	Increase	Decrease	Increase	Decrease
	0.0302	0.0103	0.0045	0.0293	0.0292
Mucin vs. 8 h	Decrease	Decrease	Increase	Decrease	Increase
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Mucin titration (mg/mL)					
10 vs. 0	Decrease	Decrease	Increase	Decrease	Increase
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
5 vs. 0	Decrease	Decrease	Increase	Decrease	Increase
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2.5 vs. 0	Decrease	Decrease	Increase	Decrease	Increase
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
1.25 vs. 0	Decrease	Decrease	Increase	Decrease	No change
	<0.0001	<0.0001	<0.0001	<0.0001	NS [‡]
0.625 vs. 0	Decrease	Decrease	Increase	Decrease	No change
	NS	NS	<0.0001	NS	NS

* See Table 2 for description of parameters

[†] Significant change with *P* value indicated

[‡] NS, no significant difference