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Report

Mucin Biopolymers Prevent Bacterial Aggregation by Retaining Cells in the Free-Swimming State

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Summary

Many species of bacteria form surface-attached communities known as biofilms. Surrounded in secreted polymers, these aggregates are difficult both to prevent and eradicate, posing problems for medicine and industry [1, 2]. Humans play host to hundreds of trillions of microbes that live adjacent to our epithelia, and we are typically able to prevent harmful colonization. Mucus, the hydrogel overlying all wet epithelia in the body, can prevent bacterial contact with the underlying tissue. The digestive tract, for example, is lined by a firmly adherent mucus layer that is typically devoid of bacteria, followed by a second, loosely adherent layer that contains numerous bacteria [3]. Here, we investigate the role of mucus as a principle arena for host-microbe interactions. Using defined in vitro assays, we found that mucin biopolymers, the main functional constituents of mucus, promote the motility of planktonic bacteria and prevent their adhesion to underlying surfaces. The deletion of motility genes, however, allows *Pseudomonas aeruginosa* to overcome the dispersive effects of mucus and form suspended antibiotic-resistant flocs, which mirror the clustered morphology of immotile natural isolates found in the cystic fibrosis lung mucus [4, 5]. Mucus may offer new strategies to target bacterial virulence, such as the design of antibiofilm coatings for implants.

Results and Discussion

Mucins Reduce Surface Adhesion and Biofilm Formation of *P. aeruginosa*

To begin to dissect mucin-bacterial interactions, we developed an in vitro assay that uses defined concentrations of native mucins. As a source of mucins, we purified native porcine gastric mucus to obtain an extract composed predominantly of MUC5AC, which is one of the major gel-forming components of the mucus in the lungs and stomach [6]. The use of natively purified mucins is decisive for the utility of this assay, because commercially available mucins are processed and have lost the ability to form viscoelastic hydrogels, as are generated by the native polymers [7, 8]. The second critical feature for this assay is the presentation of mucins in solution, as they exist in the secreted lung mucus, instead of depositing them onto a surface. This detail is important because the surface deposition of mucins is likely to adsorb functional groups, thereby partially dehydrating and altering the biochemical activity of the polymer.

First, we tested the effect of mucins on the ability of bacteria to colonize an immersed surface. A glass coverslip was suspended in culture medium that contained physiological concentrations of mucins [9]. Using the motile, opportunistic pathogen *Pseudomonas aeruginosa*, we quantified firm attachment by placing exponential-phase cells in contact with the coverslip and imaging, using phase-contrast microscopy. Cells that adhered to the surface and fully arrested (based on overlaying pairs of images separated by 2 s) were considered firmly attached and were counted at 20 min intervals (Figure 1A).

We found that mucins reduced bacterial surface adhesion by 20-fold over a 70 min period (Figure 1B). To test whether this inhibitory effect was specific to the mucins or a generic result of the presence of polymers, we compared the effects of mucins to the effects of solutions of polyethylene glycol (PEG), a polymer often used as an antiadhesive coating [10], and dextran, a branched, high-molecular-weight polysaccharide. In comparison to mucins, PEG and dextran demonstrated only mild reductions in bacterial adhesion at these early time points, indicating that mucins have singular effects that cannot be attributed to nonspecific effects of polysaccharides or soluble polymers alone. At 6 hr, a time at which biofilms have begun to form, approximately 90% of *P. aeruginosa* cells remained planktonic in the presence of mucins, compared with 50%–60% in tryptone broth (TB) alone or TB plus PEG or dextran (Figure 1C).

Mucin Gels Maintain or Augment Bacterial Swimming Motility

It is tempting to speculate that bacteria failed to access the underlying surface because they were trapped within the mucin network. If this is true, we should expect to see a measurable decrease of motility within the mucin hydrogel. First, to test whether motion was hindered in the presence of mucins, we tracked the movements of *P. aeruginosa* cells that carried a deletion in the flagellar hook gene (*flgE*) and were thus deficient in self-propulsion. These cells demonstrated a significant decrease in diffusivity ($p < 0.001$) in mucin environments, from $2.4 \pm 0.2 \times 10^{-9}$ cm^2/s to $1.0 \pm 0.1 \times 10^{-9}$ cm^2/s ($n \geq 96$ cells), reflecting a higher apparent viscosity of mucin-containing gels and suggesting that geometric hindrance was present. However, the wild-type cells remained highly motile in the presence of the mucins (see Movies S1, S2, S3, S4, and S5 available online). The distribution of velocities of swimming

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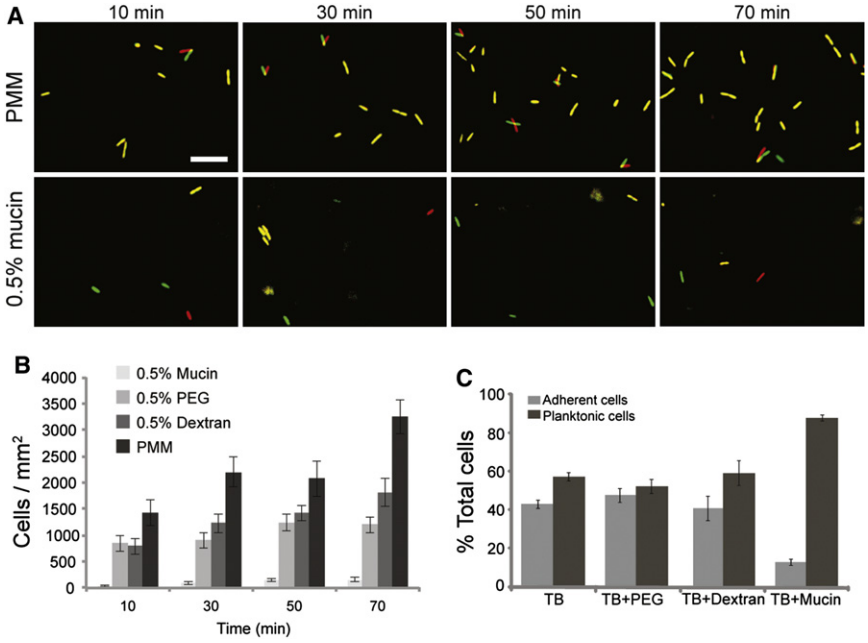

Figure 1. Mucins Block *P. aeruginosa* Attachment to Surfaces

(A) Images of coverglass surfaces at the indicated time points, depicting cell adhesion. Cells in PMM or PMM plus mucins were photographed at 2 s intervals at each time point. Images from these intervals were false colored in red and green, respectively, and overlaid, allowing visualization of active cell motility or Brownian motion versus firm adhesion. Scale bar represents 10 μm.

(B) Number of wild-type cells firmly adherent to coverglass in PMM, or PMM supplemented with PEG, dextran, or mucins, after the indicated incubation periods. Error bars indicate SEM of 8–11 different data points.

(C) PAO1 wild-type bacteria were grown in polypropylene tubes containing TB or TB plus 1% (w/v) PEG, dextran, or mucin. After 6 hr, the relative amount of planktonic versus surface-attached cells was quantified using MTT staining. Error bars represent the SD.

cells in mucins was similar to that in liquid medium, despite the differences in apparent viscosity (Figures 2A and S1A available online). This effect was apparent when we compared cells in *Pseudomonas* minimal medium (PMM) as well as in tryptone broth (TB) with or without mucins. To test whether this effect is specific for *Pseudomonas* or whether it is a more general phenomenon that affects other swimming bacteria, we tracked a different motile bacterium, *Escherichia coli*. Despite a significant decrease in diffusivity ($p < 0.001$) of nonflagellated cells (Δ*fliC*) in mucins, from $2.2 \pm 0.2 \times 10^{-9}$ cm^2/s to $0.7 \pm 0.1 \times 10^{-9}$ cm^2/s ($n \geq 92$ cells), the wild-type cells had significantly increased swimming velocities in mucins compared with medium only (Figures S1B and S1C).

Immotile *P. aeruginosa* Cells Can Form Suspended Flocs in Mucin Gels

If mucins can prevent surface colonization by maintaining cellular motility, we speculated that cells lacking motility may be able to overcome this dispersion effect and succeed in adhesion and biofilm formation in mucin environments. This line of inquiry may have direct physiological relevance, as isolates of *P. aeruginosa* from cystic fibrosis (CF) mucus are often nonmotile [5]. As with the wild-type, mucins detectably reduced surface adhesion of nonmotile cells (Δ*flgE*), which are already poorly adherent (Figure S1D; compare to Figure 1B). To look beyond surface adhesion in the presence of mucins, we observed the bacteria in the volume of the mucin gel after 20 hr of incubation. The wild-type cells remained largely as individual cells or small, suspended colonies (Figures 2B and 2C) of up to 20 μm^2 (this corresponds roughly to clusters of 10–20 cells) distributed throughout the volume of the mucin medium. Increasing mucin concentration did not visibly increase cellular cluster size (Figure 2E). However, when observing the Δ*flgE* mutant, we noticed a striking difference compared to the behavior of wild-type cells. The flagella mutant formed large aggregated flocs of up to 250 μm^2 (Figures 2B and 2C). These differences are not likely due to variations in cellular populations in the mucin medium, because PAO1 displayed similar growth rates in the presence and absence of mucins (Figure S1E). A similar behavior was found for two additional flagella mutants, Δ*flgK*, which lack a hook filament junction protein, and Δ*fliD*, which lack an adhesive protein at the tip of the flagellar filament (Figures 2B, 2C, and S1F), but not for Δ*pilB*, which lack pilus-mediated adhesion and twitching motility (Figures 2B and 2C). The ability of cells to form suspended flocs was inversely correlated with their ability to form surface biofilms in mucin-free environments (Figure 2D). For example, wild-type and Δ*pilB* cells formed substantial surface biofilms but failed to form large suspended flocs in the presence of mucins. Conversely, the various flagellar mutants formed large flocs, but had reduced surface biofilms in the absence of mucins. All mutants tested displayed similar growth rates (Figure S1G). The flocs formed by Δ*flgE* strains increased in maximum size with increasing mucin concentration (Figure 2F).

We hypothesized that loss of flagellar motility (rather than other properties of flagella, such as adhesion) was the dominant contributor to the observed aggregation. To test this, we measured mucin-dependent flocculation by a PA14 strain that carries a fully assembled flagellum but is paralyzed, due to deletions in all four stators in the motor complex (Δ*motAB*Δ*motCD*). This mutant formed substantially larger flocs (up to 60 μm^2) than the wild-type (Figures S1H and S1I), but the structures were smaller than those formed by the Δ*flgK* strain. Again, floc-forming ability in mucins tended to be negatively correlated with surface biofilm formation in medium-only environments (Figure S1J). Both a loss of motility and loss of the flagella itself, therefore, appear to contribute to mucus colonization. Complementing the *flgE* deletion in PAO1 Δ*flgE* restored swimming motility and diminished the capacity of the bacteria to form flocs in mucin, indicating that it is indeed the lack of functional flagella that caused the formation of flocs (Figure S2).

Our data suggest that mucins are highly effective at preventing swimming cells from surface attachment and forming suspended aggregates. Previous work has indicated that fliD is an adhesin for mucin [12], yet it does not appear to be required for the aggregative phenotype (Figure 2C). How then do the flagella mutants achieve aggregate formation? It appears that their lack of motility enables cells to form clonal

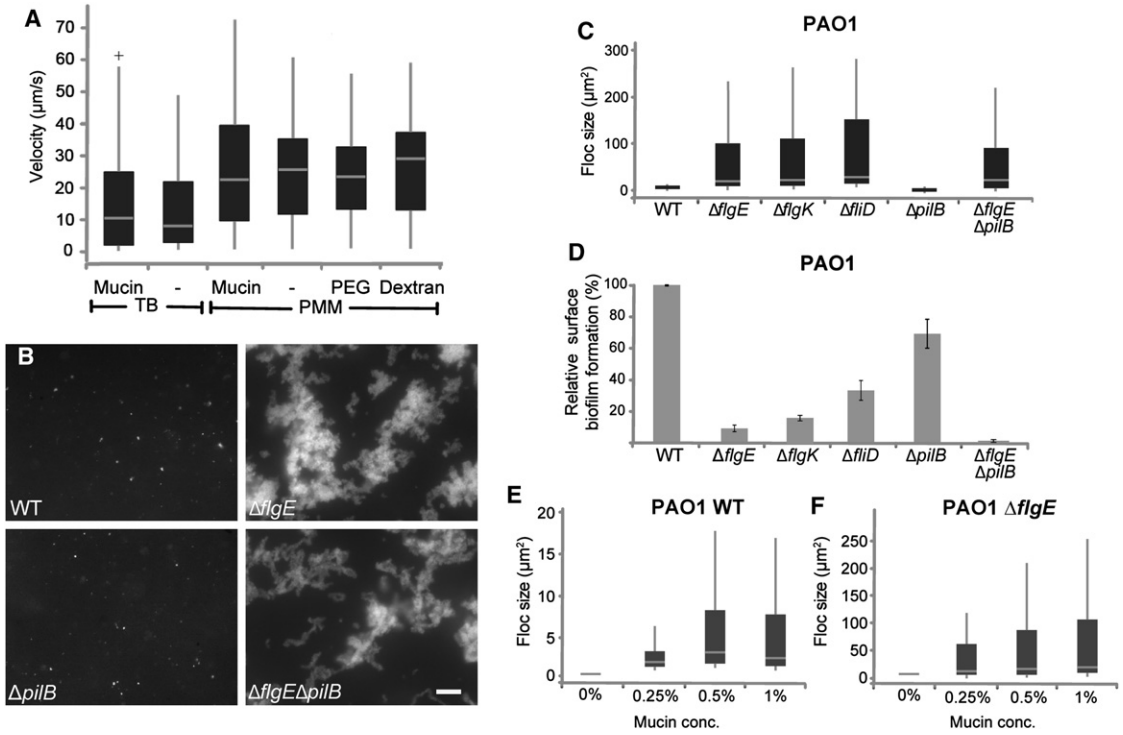

Figure 2. Nonmotile Flagella Mutants, but Not Their Motile Counterparts, Form Flocs in Mucin Environments

(A) Box plots depicting swimming velocities of *P. aeruginosa* in various conditions. Cells were grown in the media indicated, but swimming experiments were in 50% strength media. Velocities were obtained from particle-tracking analyses of 20 s swimming videos obtained at 20 frames per second. See also Movies S1, S2, S3, S4, and S5.

(B) Floc formation of wild-type cells, flagella mutant (Δ*flgE*), pili mutant (Δ*pilB*), and double flagella and pili mutant (Δ*flgE*Δ*pilB*) in PMM with 1% mucins after 20 hr of incubation. Images are of cells in suspension only. Scale bar is 20 μm.

(C, E, and F) Box plots quantifying floc size of wild-type, flagella, pili, matrix, and motility mutants for the strains indicated in μm^2 after 20 hr of growth in 1% mucin (unless otherwise indicated). For details on the quantification method see Experimental Procedures. For all box plots, boxes extend from the 25th to the 75th percentile, the central line is the median, and whiskers extend to the data point nearest to 1.5 times the interquartile range above and below the box. Outliers are plotted as plus signs.

(D) Surface-attached biofilm formation was quantified by crystal violet (CV): liquid cultures of the strains indicated were inoculated in 96-well plates at an OD_{600} of 0.01 and incubated for 7 hr at 37°C. The biofilms that formed were quantified by staining with 0.1% CV as described previously [11]. After staining, each plate was rinsed, and the remaining CV was destained with 33% acetic acid for 15 min, and measured using a plate reader (OD_{595}). Data are presented as percent biofilm formation relative to wild-type. The error bars represent SD. See also Figure S1.

outgrowths of individual cells within the mucus. This was supported by culturing mixtures of fluorescent and nonfluorescent immotile cells in mucin medium. Over the course of 20 hr, small homogeneous patches of 10–20 cells emerged and further expanded (Figure S2). Notably, floc formation did not occur in PEG, dextran, or industrially purified mucins (Figure 3A). It appears that this phenomenon depends on specific features unique to native mucins.

P. aeruginosa Floc Formation Is Dependent on the Production of Psl and Alginate

Flagella loss allows bacteria to effectively colonize mucus in a manner reminiscent of surface-attached biofilms. Just how similar are these two forms of bacterial aggregation? To address this, we tested whether floc formation by nonmotile cells required an extracellular matrix, a hallmark of biofilms. Specifically, we looked at Psl, which plays a structural role in the maturation of surface-attached biofilms [13], and alginate, which appears to play only a minor role in biofilm formation (Figure 3B; [14]) but is overexpressed in colonies adapted to growth in CF lung mucus [15, 16]. Using previously characterized single *algD* and *psl* mutant strains [13, 17], we introduced additional *flgE* mutations to study the importance of the extracellular matrix on the immotile flocs. Complementation of the double mutants with *flgE* was able to restore motility (Figure S2). We found that both polymers, particularly alginate, were important for floc formation (Figures 3C and 3D), because in their absence, floc size was greatly reduced. This phenotype may be relevant to CF pathology, where the formation of *P. aeruginosa* flocs inside the lung mucus is associated with the rise of antibiotic resistance [18]. These data suggest that mucin-based flocs and surface-attached biofilms have the same broad reliance on extracellular matrices, but the mechanistic details differ in important ways. Specifically, flocs rely on alginate and flagella loss in a manner not seen in surface-attached biofilms.

P. aeruginosa Flocs that Emerge in Mucin Gels Are Antibiotic Resistant

Last, we asked whether floc formation can provide bacteria with a selective advantage. Again by analogy with biofilms, we hypothesized that the immotile cellular aggregates that emerge in the presence of mucins also have a higher resistance toward antibiotics compared to motile wild-type cells. We grew wild-type and the nonmotile Δ*flgE* cells in mucin media for 20 hr and then subjected both strains to two clinically

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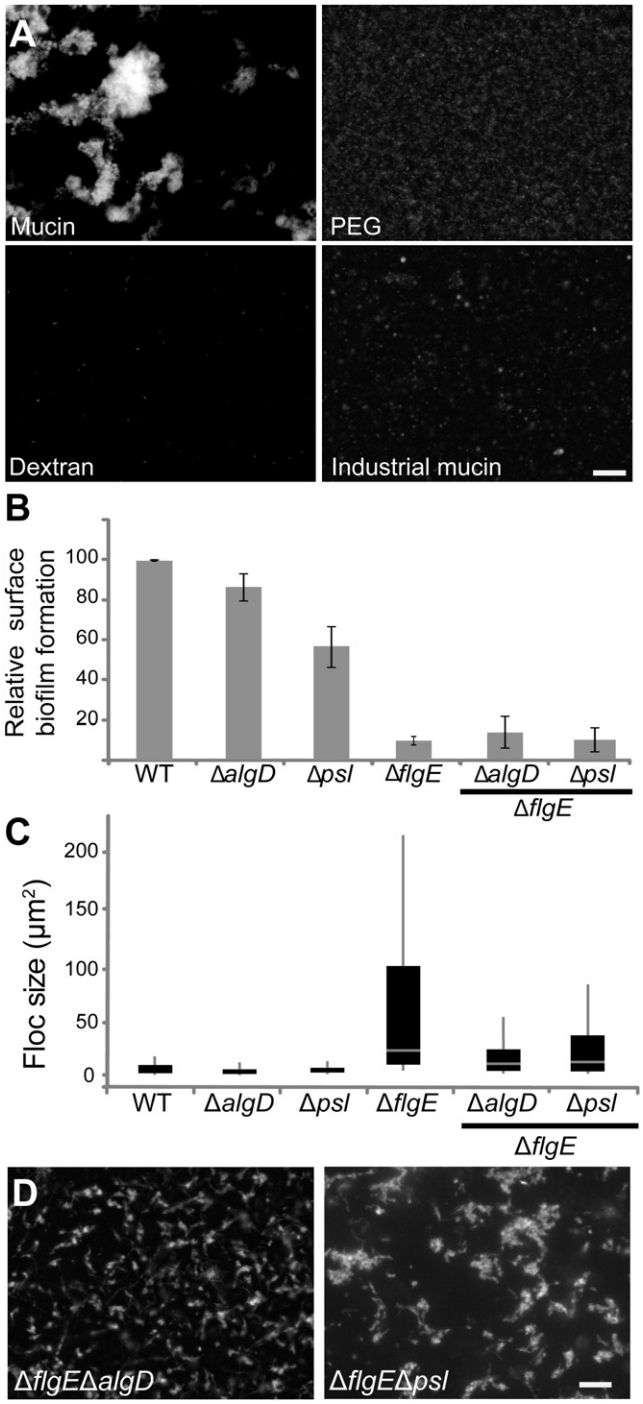

Figure 3. Floc Formation in Mucin Environments Is Exopolysaccharide Dependent

(A) Δ*flgE* strains were grown for 20 hr in PMM containing 1% (w/v) PEG, dextran, or industrially purified mucins (NBS Biologicals). Only in the presence of native mucins is floc formation observed. Scale bar is 20 μm.

(B) Liquid cultures of EPS secretion mutants and motility mutants were quantified by CV, as described in Figure 2. The experiments were performed in triplicate. The error bars represent the SDs.

(C) Box plots of floc size of wild-type cells and the indicated motility and matrix mutants in PMM with 1% mucins after 20 hr of incubation. Box plots are drawn as described in Figure 2. See also Figure S2.

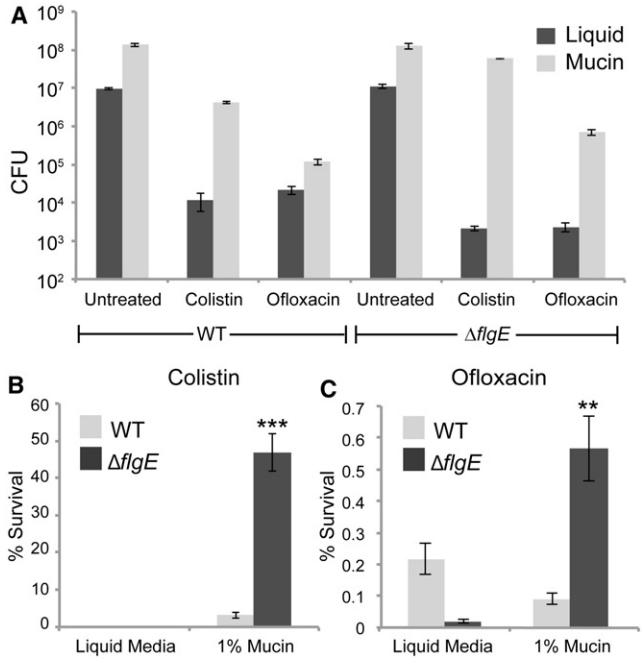

Figure 4. Flocs Grown in Mucin Environments Are Antibiotic Resistant

(A) Wild-type and Δ*flgE* cells were grown in liquid culture or in 1% mucin for 20 hr and then exposed to colistin and ofloxacin. After 3 hr of antibiotic exposure, the cells were plated to determine the number of surviving cells. (B and C) Data from (A) replotted as survival of antibiotic-treated cells (in percentage, normalized to untreated cells). Each trial was repeated at least three times. Error bars represent SEM. **$p < 0.01$; ***$p < 0.001$, comparing survival of Δ*flgE* to wild-type in 1% mucins.

relevant antibiotics that differ in their mode of action (Figure 4). This experiment revealed two points: first, both wild-type and Δ*flgE* bacteria were more resistant to colistin in the presence of mucins, as compared to liquid culture without mucins. This suggests that the mucins themselves have the capacity to reduce the efficacy of colistin, regardless of whether cells are planktonic (wild-type) or form flocs (Δ*flgE*). Second, it appeared that the floc-forming Δ*flgE* cells were more resistant to both antibiotics in the mucin medium than the motile wild-type cells. To test for this possibility, we determined the percent survival of the bacteria in either condition, by normalizing to the cell numbers in the untreated samples in liquid and mucin medium. Inside the mucin medium, the nonmotile flagella mutants were on average 14 times more resistant to colistin (Figure 4B) and approximately 6 times more resistant to ofloxacin (Figure 4C) than wild-type cells, both of which are statistically significant differences. We conclude that the aggregates that emerge upon loss of motility indeed have an increased resistance compared to motile wild-type cells, possibly due to the presence of an altered composition or quantity of extracellular matrix components or due to a protective effect of increased cell density [19].

Conclusions and Outlook

Here, we have found that animals provide a candidate solution to inhibit biofilm formation, namely mucin polymers. Critically, our results demonstrate that mucins can limit bacterial surface attachment and biofilm formation without killing or trapping bacteria, which will help to limit selective pressure for resistance. Indeed, our only evidence for a resistance phenotype

comes in the form of nonmotile cells, which are likely to be strongly limited in other modes of virulence [5, 20]. Our observations of motility and reduced adhesion in mucin media are similar to findings for *Campylobacter jejuni* in mouse intestinal crypts. In a previous study, extracted epithelial scrapings from *C. jejuni*-colonized gnotobiotic mice demonstrated a lack of adhesion and unhindered motility within the crypts [21]. Similar to this, a recent study showed that when supplemented in agar plates, mucins appear to increase motility of *P. aeruginosa* [22]. At first sight, these and our findings contrast with reports on surface-immobilized mucins, which arrest [12, 23] and can cause large aggregate formation of *P. aeruginosa* cells [24]. However, these findings can be reconciled if one considers that the effects of mucins on motility may depend on their native three-dimensional structure and hence biophysical properties, such as viscoelasticity and lubricity, which are preserved in native mucus and presumably inside agar gels but not when adsorbed to a two-dimensional surface [22]. The gel-forming mucin MUC2 has an ordered repeating ring structure [25], and we speculate that also other gel-forming mucins, such as the MUC5AC used in our experiments, display three-dimensional features that affect their interactions with bacteria. Indeed, Berg and Turner have observed that certain structured viscous solutions allow increased velocities of motile bacteria by providing a rigid framework for generating propulsive forces [26]. We anticipate that studying mucins in their native three-dimensional form will reveal valuable novel information about bacterial behavior that cannot be captured by collapsed mucin monolayers.

Experimental Procedures

Mucin Purification

The source for purification of native MUC5AC was pig stomachs, which secrete MUC5AC, homologous to the human glycoprotein [27]. Porcine gastric mucins were purified as described previously, with the omission of the CsCl density gradient centrifugation [28]. Mass spectrometry analysis was used to determine the composition of the mucin preparation as described previously [29]. Briefly, the analysis was performed at the Harvard Microchemistry and Proteomics Analysis Facility by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Thermo LTQ-Orbitrap mass spectrometer. The spectra were analyzed using the algorithm Sequest [30]. The analysis showed that MUC5AC was the predominant mucin present in our purified extract, which also contained MUC2, MUC5B, and MUC6, as well as other proteins including histones, actin, and albumin. In addition, its quality was tested by rheology, as described in Kocevar-Nared et al. and Celli et al. [7, 28], which confirmed that the isolated mucins displayed viscoelastic properties similar to those of native mucus.

Microbial Adhesion Assays

For adhesion experiments, PAO1 wild-type and PAO1 Δ*flgE* were inoculated in LB and grown overnight at 37°C, shaking. Overnight cultures were diluted 1:100 into PMM and grown shaking at 37°C for 4 hr. One milliliter of exponential-phase cells (OD_{600} = 0.4 to 0.45) was centrifuged, and cells were resuspended in 400 μl sterile PMM. These cells were diluted 1:10 in PMM and then further diluted 1:10 into the medium to be tested (PMM only, 0.5% mucin, 0.5% PEG 3350, or 0.5% dextran). Forty microliters of this mixture was pipetted onto glass slides with shallow spherical depressions, covered with a glass coverslip, and inverted. Pairs of images were taken 2 s apart in multiple fields for each sample at 10, 30, 50, and 70 min. Image pairs were compared to differentiate firmly attached cells from moving cells in each frame. Adherent cells were counted for each time point. Pairs of dividing cells were counted as single cells.

Quantification of Biofilm Formation in Mucin Gels

Freshly growing cells at an OD_{600} of 0.01 were inoculated in polypropylene PCR tubes and incubated at 37°C in TB or in TB containing 0.5% (w/v) mucins. After 6 hr, the planktonic cells were removed for quantification, and the adherent cells in the tubes were washed two times with PBS to remove nonadherent cells. Planktonic and adherent cells were stained with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 hr at 37°C and subsequently destained with 20% sodium dodecyl sulfate in 50% dimethylformamide (adjusted to pH = 4.7) overnight at 37°C. The resulting solutions were quantified using a plate reader (OD_{595}).

Particle Tracking

For measurement of cell velocities, bacteria were grown to exponential phase as described above, stained with Syto9 live cell stain by adding Syto9 1:1000 into the culture, and incubated for 10 min at room temperature. The stained cells were diluted 1:10 into a 50% strength solution of growth medium (as indicated in Figures 2A and S1A–S1C) or growth medium supplemented with mucin, dextran, or PEG. These solutions were mixed and dispensed into chambers for visualization. Videos of cells were taken on an inverted fluorescent microscope at 20 frames per second to obtain trajectories (see Supplemental Information for additional details). The trajectories obtained were processed using MATLAB to determine velocities and diffusivities. Diffusivities were based upon mean squared displacement values for a range of lag times. Trajectories were also examined visually to ensure accuracy.

Antibiotic Treatment

To determine the antibiotic resistance of flocs grown in mucin media, we grew cells in PMM with 1% (w/v) mucin. After 20 hr, the number of cells was determined by counting cfu; this number was used as the reference number prior to treatment. The antibiotics ofloxacin and colistin were added to the cultures at final concentrations of 20 μg/ml, and the cultures were grown at 37°C for 3 hr. After treatment, the number of survivors was estimated by measuring the cfu. To avoid aggregates, we bead-bashed each sample for 30 s before diluting and plating. Each experiment was carried out in triplicate. To determine the resistance of cells grown in the absence of mucins, we adjusted an exponential-phase culture to contain the same number of cells as had grown in 1% mucin in 20 hr and challenged with antibiotics as described above.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.10.028.

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