

CHARACTERIZATION OF A MUCOID-LIKE *PSEUDOMONAS* *AERUGINOSA* BIOFILM

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MANUSCRIPT RECEIVED 2 APRIL, 2015; ACCEPTED 29 JUNE, 2015

ABSTRACT

Pseudomonas aeruginosa biofilms are implicated in chronic infections. A key element of *P. aeruginosa* pathogenicity is the formation of a biofilm, a community of bacteria encased in an exopolymeric substance (EPS) that shields the bacteria from the host immune response and antibiotic treatment. A crucial step in biofilm production is a switch in motility from freely swimming, planktonic bacteria to twitching movement and then to attached and sedentary bacteria that develop into a mature pillar-shaped biofilm. A mucoid biofilm produces an excess of alginate and is clinically the most pathogenic and the most resistant to antibiotics. Biofilms from patients exhibit a wide variety of structure, motility, and levels of attachment. *In vitro* biofilms do not exhibit such a wide variety of structure and physiology. The difference between *in vivo* and *in vitro* biofilms has made the translation of *in vitro* studies into *in vivo* treatments difficult. Under different growth conditions in our lab, the *P. aeruginosa* strain PAO1 demonstrates two phenotypes: a non-mucoid and a mucoid-like phenotype. Confocal laser scanning microscopy (CLSM) indicates the mucoid-like phenotype is intermediate in height to the non-mucoid phenotype and biofilms formed in a once-flow-through chamber. Both mucoid-like and non-mucoid phenotypes exhibit a significant increase in twitching between 24 and 72 hours of development. The mucoid-like phenotype had greater attachment at 72 hours compared to non-mucoid phenotype. Therefore, the two phenotypes observed in our lab may represent the effect of environment to stimulate development of two types of biofilms by PAO1.

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KEYWORDS

- Psl
- Pel
- alginate
- type IV pili
- flagella

INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen implicated in chronic infections in cystic fibrosis (CF) (15, 17, 33) and chronic wounds (4, 8, 17). Its virulence

is due, in part, to formation of biofilms which confer resistance to both the innate immune system and antibiotics. Biofilms recovered from the sputum of CF patients

can exhibit a widespread variability in biofilm formation both in structure and virulence (1, 10, 27, 33).

BIOFILM FORMATION

Biofilms are understood to have several steps in their formation (8, 33). There is an initial attachment to a surface followed by microcolony formation. The biofilm may then mature to a pillar-shaped structure which can release planktonic (freely moving) bacteria to colonize new areas. Inside biofilms, cells in different regions exhibit differences in gene expression as well as functional heterogeneity, indicating a complex community of cells within biofilms (8). The time span for the formation of a pillar-shaped biofilm depends on many factors, but has been estimated to take 5–7 days at minimum (33).

The triggers for biofilm formation are complex, but one trigger is the formation of a quorum sensing (QS) signaling cascade. One QS molecule, N-(3-oxo-decanoyl)-L-homoserine lactone (3OC12-HSL) is constitutively released from *P. aeruginosa*. When *P. aeruginosa* has multiplied and reached a large enough population (i.e. a “quorum”), 3OC12-HSL levels reach a threshold and signal extensive changes in gene expression through the LasR–LasI system (8, 9, 28, 33). One of these changes includes the formation of a second QS molecule, N-butyryl-L-homoserine lactone (C4-HSL). C4-HSL acts through the RhIR–RhII system independently and in a coordinated fashion with 3OC12-HSL further influences gene expression. In fact, blocking QS molecule action has been one target of therapeutic interventions, but with limited success (2).

EXOPOLYMERIC SUBSTANCE (EPS)

Biofilm maturation requires the formation of an exopolymeric substance (EPS) which confers antibiotic resistance and protection from host immunity by the biofilm (4, 8, 33). The EPS in a *P. aeruginosa* biofilm is composed of three components: alginate, Psl, and Pel, although other polymers such as certain proteins, lipids, and extracellular DNA also contribute to biofilm structure (12, 24, 25, 36).

Psl, composed of rhamnose, mannose, and glucose, is required for maintenance of any biofilm structure, and for biofilm construction (31, 36). Overproduction of Psl promotes mushroom-shaped biofilms (36). Pel, a glucose-rich component of the EPS, is required for initiation of any biofilm, as well as for the growth of air-liquid interface biofilms (31, 36).

The final EPS component, alginate, is a complex polysaccharide composed of two different sugars, β -D-mannuronate and L-glucuronate, and is one of the most studied and well understood portions of the matrix. Over-expression of alginate causes what has been termed the mucoid biofilm phenotype for its mucus-like appearance (31). Mucoid biofilms have been shown to be more virulent and are often found in chronic infections (11, 23). Mutant strains of *P. aeruginosa* which produce mucoid biofilms can be studied *in vitro*; however, the transformation of wild-type *P. aeruginosa* into a mucoid biofilm is not well understood (7). Because of the homogeneity of *in vitro* biofilms grown in the laboratory setting and the heterogeneity of *in vivo* biofilms as recovered from CF patients, it has been difficult to study these pathogenic mucoid biofilms within the lab.

This heterogeneity of biofilms recovered from CF patients is well documented. They differ structurally as demonstrated

in scanning electron microscopy as pillar-shaped, knobby, or flat (10). The PAO1 within biofilms are also functionally distinct as seen in the QS molecules released, their virulence, the types of motility they exhibit, their ability to attach to a surface, and their response to treatment (1, 27, 33). In recent years several strategies have been employed to characterize *P. aeruginosa* isolates from patients in order to identify the most effective treatment. Virulent strains of *P. aeruginosa* form biofilms, but also have decreased motility and decreased production of virulence factors such as pyocyanin (1, 27). It has been hypothesized that a lack of production of flagella or type IV pili might help *P. aeruginosa* evade the host immune system since both innate and adaptive immune systems respond to epitopes on flagella and type IV pili (1, 27). A lack of motility may also increase the ability of *P. aeruginosa* to attach to a surface and produce a biofilm. These studies also demonstrated a decrease in production of virulence factors such as pyocyanin (1, 27). Virulence factors are common in acute infection and may spur the immune system response. Therefore, eradicating or decreasing the production of virulence factors may decrease stimulation of the host immune system by *P. aeruginosa*, increasing the ability of *P. aeruginosa* to evade the host immune response. Finally, the production of biofilms increases the ability of *P. aeruginosa* to evade the host immune system by creating the exopolysaccharide (EPS) matrix that limits the ability of the host immune system to reach *P. aeruginosa*.

Clinical isolates of *P. aeruginosa* biofilms can be classified by their motility patterns and biofilm structure (1, 10, 27). Mulet *et al.* (2013) demonstrated that high risk clones of *P. aeruginosa* from CF sputum contained greater drug resistance as well

as increased biofilm production, decreased twitching, and decreased production of pyoverdine and pyocyanin, two virulence factors. Therefore, changes in motility from swimming to swarming to twitching are a standard part of the formation of biofilms. These changes allow *P. aeruginosa* to attach to a surface, form a microcolony, and finally, form a biofilm. However, the emerging clinical picture indicates that motility patterns are being used to classify and characterize clinical isolates based on their virulence and ability to develop chronic infections (10, 27). In the clinical setting then, motility may not only serve a role in biofilm formation, but may also serve as an additional marker that may lead to identification of the most effective methods for treatment of chronic *P. aeruginosa* infections.

MOTILITY

P. aeruginosa exhibits swimming, swarming, and twitching types of motility. Twitching is mediated by type IV pili which act as retractable arms that can pull or “slingshot” cells across a surface (5, 19). Twitching is usually seen in more viscous types of media or environments. Swimming through a liquid medium is performed by the use of flagella (18, 36). Swarming movements use both type IV pili and flagella resulting in a movement in between swimming and twitching (14, 36). To test motility, assays are used measuring distance displaced by *P. aeruginosa* on plates of media with different fluidity (5, 10).

As previously stated, the motility of *P. aeruginosa* within a biofilm can be used to identify virulent strains that are more likely to cause chronic infection. However, the correlation between motility and its role in biofilm formation is not entirely clear. Swimming motility may not be required

for biofilm formation, but may be important in allowing *P. aeruginosa* to reach an environment with greater nutrients or to colonize a new area (36). Therefore, although it may not be directly involved in the stage of biofilm formation, swimming motility may play a role in determining sites where *P. aeruginosa* can travel to form a biofilm.

Perhaps the most critical type of motility for *P. aeruginosa* during biofilm formation is the ability to twitch. This is how bacteria move on a solid surface, perhaps as they are attaching to a surface to form a microcolony (10, 36, 37). Psl may be secreted to coat a solid surface as bacteria twitch in a spider web fashion. Other bacteria then attach to this surface to form a microcolony, which is one of the first steps in biofilm formation (36, 37). There is evidence that bacteria favor the use of flagella to swim or type IV pili to twitch; once there is a switch to a different type of movement, it may be difficult for the bacteria to revert to the previous type of motility (35).

Swarming, which uses both flagella and type IV pili, may represent *P. aeruginosa* in transition from swimming to attachment in which twitching will then predominate. Originally, research indicated that swarming is only mediated by flagella (10); however, more recent data indicate the use of both flagella and type IV pili are required (5, 36). Therefore, swarming may be an intermediate form of motility for *P. aeruginosa* between swimming which

requires only flagella and twitching which requires only type IV pili. In fact, data indicate that bacteria can twitch or swim, but rarely do both well (35), indicating that modes of motility vary according to stimuli.

TWO PHENOTYPES OF PAO1 DEVELOP UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

PAO1 is a strain of *P. aeruginosa* commonly used in laboratory experiments. We have been able to grow PAO1 in our lab with two distinct phenotypes, which have named non-mucoid and “mucoid-like” phenotypes. We wanted to investigate the differences in structure, motility, and surface attachment between these two phenotypes. It is our hypothesis that PAO1 in the mucoid-like phenotype produce a biofilm closer to a “mucoid” biofilm with greater twitching motility, greater attachment to a surface, and greater height and pillar-shaped structure, when compared to the non-mucoid phenotype of PAO1.

To investigate these two phenotypes we used confocal scanning laser microscopy, motility assays, and a surface attachment assay. If the mucoid-like phenotype exhibits greater twitching behavior, surface attachment, and more pillar-shaped morphology than the non-mucoid biofilm, these environmental conditions may be replicated to study different phenotypes of PAO1 biofilms.

MATERIALS AND METHODS

GROWTH OF MUCOID-LIKE AND NON-MUCOID CULTURES:

PAO1 (ATTC BAA-47) cultures were grown overnight in 10 ml tryptic soy broth (TSB) (Difco Laboratories, Sparks, MD) in standard 16 ml glass test tubes with a plastic cap (VWR, 89000-482) before being streaked for isolation on tryptic soy agar (TSA) plates (Difco Laboratories, Sparks, MD). Single colonies were used to inoculate TSB under two environmental conditions to produce biofilms with either mucoid-like or non-mucoid phenotypes. Mucoid-like phenotype PAO1 biofilms were grown in 10 ml TSB in standard 16 ml glass test tubes with a plastic cap (VWR, 89000-482) on a shaker incubator at 37°C and 225 RPM. Non-mucoid phenotype PAO1 biofilms were grown in a 250 ml Erlenmeyer flask (VWR, 29136-060) with 100 ml TSB and a foil cap at the same conditions. The shaking was set so that the biofilms did not grow on the surface of the culture or on a solid surface, but throughout the media. The two phenotypes were easily distinguished. The mucoid-like phenotype developed strings of a mucous-like substance that spun down toward the bottom of the test tube. The shaking was necessary to keep the mucoid-like strings suspended in the TSB and maintain oxygenation of the TSB. Without shaking, the mucoid-like strings of biofilm sank to the bottom of the test tube and died (data not shown). The non-mucoid biofilms developed a thick, consistent composition throughout the TSB. Previous studies have implicated nutrient or oxygen deprivation in initiating biofilm formation (13, 16, 32, 34). Samples were grown for 24, 48, or 72-hour time periods.

GROWTH OF BIOFILMS USING ONCE FLOW THROUGH CHAMBERS:

Mature biofilms are most fully developed *in vitro* in a flow cell apparatus with a constant flow of media over the biofilm. As a positive control for biofilm development, a three-chambered flow cell apparatus (IBI Scientific, Peosta, IA) was set up as previously described (6, 24). TSB at room temperature was pumped through the individual chambers at a rate of 2.5 ml/minute. Prior to inoculation of the apparatus chambers, a single colony of PAO1 was inoculated into 10ml TSB overnight at 37°C. The 10 ml sample was diluted until an absorbance of 0.5 at OD600 was reached using a protocol outlined in Deligianni *et al.* (10). Each chamber was inoculated with 0.5 ml of the diluted sample. During the inoculation period, chambers were inverted and flow was stopped for one hour while bacterial cells were given time to attach (6). Each of the three chambers was assigned to a 24, 48, or 72 hour sample. One chamber was inoculated each day for three days. All three chambers were stopped on the same day, 24 hours after the inoculation of the final chamber. Biofilms were first fixed with 100 ml of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 minutes. Flow chambers were then rinsed with 50 ml PBS for approximately 30 minutes before embedding. The embedding gel was a 20% 200:1 acrylamide:bis-acrylamide polyacrylamide gel that was prepared with 20 µl of AP S (10 mg/ml), 8 µl TEMED, and 1 ml of 20% polyacrylamide 200:1 acrylamide:Bis solution. The gel was injected into each flow chamber using a 1ml tuberculin syringe. (Bio-Rad, Hercules, CA) (6, 26). Flow direction was marked before the glass which covered each chamber was carefully removed and the fixed biofilms embedded in the polyacrylamide gel were kept in PBS until stained for microscopy.

STAINING SAMPLES FOR EXAMINATION BY CONFOCAL LIGHT SCANNING MICROSCOPY:

Cultures of mucoid-like or non-mucoid bacterial cells were allowed one hour to attach to the surface of poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated cover slips before being fixed with 4% paraformaldehyde. Biofilms grown in the flow cell apparatus were stained through the polyacrylamide gel. All biofilms were stained with a 1:300 dilution of DAPI (Invitrogen, Grand Island, NY), which was used as a counterstain to label nucleic acids. A FITC conjugated antibody to lectin from *Hippeastrum amaryllis* (HHA) (EY Laboratories, San Mateo, CA, F-8008-1), which binds to 1,3- or 1,6- mannosyl units in polysaccharides of the Psl component of the biofilms (25), was then applied at 0.2 mg/ml. Slides were rinsed, and mounted using an anti-fade reagent (ProLong, Life Technologies, Grand Island, NY). Flow cell samples were too thick to add a coverslip and were maintained under wet conditions for microscopy.

IMAGING OF BIOFILMS USING CONFOCAL LASER SCANNING MICROSCOPY

A Zeiss LSM 700 confocal laser scanning microscope was used to image the previously stained bacterial samples for DAPI label of nucleic acids or FITC-HHA label of the Psl component of PAO1 biofilms. Single images of the biofilms were taken for DAPI or FITC and Image J was used to generate composite images (30). Z-stacks were also taken (data not shown) and used to give an estimate of biofilm height (20). As a negative control coverslips with mucoid-like PAO1 were examined without any stain or FITC-HHA label. These were viewed through the DAPI filter or the FITC filter to check for autofluorescence of *P. aeruginosa*.

MOTILITY ASSAY

Motility assays were conducted as previously described (5, 10). Briefly, swim medium contained 0.982% tryptone, 0.295% agarose, and 0.491% sodium chloride. This is the most fluid medium that allows the easiest movement. Swarm medium contained 0.786% nutrient broth, 0.491% agar, and 0.491% glucose. This is a thicker medium that restricts movement. Twitch plates contained 0.976% tryptone, 0.9763% agarose, and 0.976% sodium chloride. These plates have the highest concentration of agarose and the most restricted movement.

Non-mucoid and mucoid-like samples for the motility assay were prepared as described above and given 72 hours to develop. At 72 hours, an inoculating needle was sterilized in alcohol and flame, inserted into the non-mucoid or mucoid-like sample just beneath the surface of the biofilm, and each motility plate was stab inoculated to the bottom of the plate, according to Deligianni *et al.* (10). The point of inoculation was marked on the bottom of the plate and the plates were incubated at 37°C. Growth of colonies was measured 24, 48, or 72 hours after inoculation; images were taken after 48 hours. The sample size was three to six plates per time point and plate type. Data were analyzed using two comparisons. The purpose of the first analysis is to evaluate changes in motility over the 72 hour period of the motility assay. To do this, values for each type of motility (swimming, swarming, or twitching), were statistically compared to the 24 hour mucoid-like phenotype with an unpaired t-test using GraphPad. The second analysis determines if there is a difference in motility between the two phenotypes at each time point. In this analysis, an unpaired t-test compared mucoid-like to non-mucoid samples at each time point.

SURFACE ATTACHMENT ASSAY

Surface attachment assays were conducted as previously described (21). Briefly, bacterial cultures were grown under either mucoid-like or non-mucoid conditions for 24, 48, or 72 hours and then diluted down to an absorbance of OD600 of 0.5. The cultures were then diluted 1:2 in TSB and 0.5 ml of each diluted

culture was placed in an individual well of a poly-L-lysine coated 24 well plate for 1.5 hours. Culture fluid was removed before the cultures were fixed with 70% methanol for 30 minutes and stained with 0.5% crystal violet for 30 minutes. Excess crystal violet was then washed from the wells using PBS. Digital images of the plates were obtained.

RESULTS

NON-MUCOID AND MUCOID-LIKE PHENOTYPES

An example of non-mucoid and mucoid-like biofilms developed in our lab is shown in Fig. 1. As can be seen in Fig. 1A, the non-mucoid

phenotype grown for 72 hours is concentrated with a consistent composition. The mucoid-like biofilm seen in Fig. 1B has thick, mucous-like strings that emanate from the surface and spread interior into the TSB. If re-



Figure 1: Non-mucoid and mucoid-like *Pseudomonas aeruginosa* biofilm. The non-mucoid biofilm (A) was grown in 100 ml TSB in a 250 ml Erlenmeyer flask and has a consistent composition. The mucoid-like biofilm (B) was grown in 10 ml TSB in a 16 ml test tube and demonstrates strings of mucoid-like biofilm. All samples were incubated at 37°C on a rotary shaker at 225 rpm for 72 hours. The specific differences in environment conditions leading to the formation of two phenotypes was not investigated. However, studies have shown that oxygen deprivation can induce biofilm formation; thus, it is possible the low surface-to-volume ratio in the test tube may lead to favorable conditions for biofilm development.

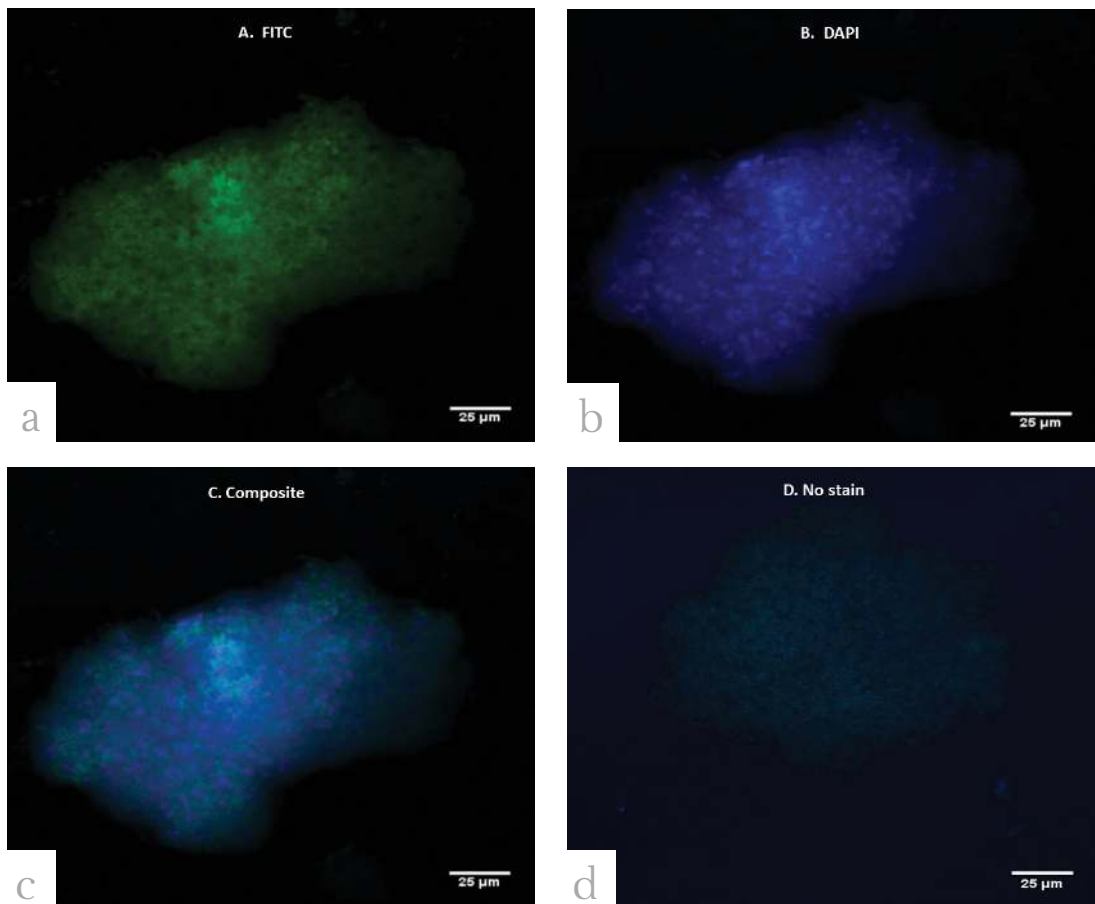


Figure 2. A–D is an example of a 72 hour mucoid-like biofilm stained with DAPI and HHA-FITC shown with the FITC filter (A), the DAPI filter (B) and the composite image (C). D is the corresponding negative control without DAPI or HHA-FITC antibody. Pockets, indicated by dark “holes” within the image, appear to be formed throughout the EPS matrix of the biofilm. At these pockets a higher concentration of nucleic acids are present as seen by the DAPI fluorescence. These “pockets” filled with PA may be the beginning of pillar-shaped biofilms. Scale bars are 25µm.

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moved from the test tubes, these mucous-like strings are easily disrupted and disseminated. However, in the test tube, they are discrete from the surrounding media. The observation of these two phenotypes is the basis of our examination. When cultured for longer than 72 hours, PAO1 in the mucoid-like phenotype died, perhaps from a lack of nutrients.

CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

Fig. 2 A–D demonstrates a composite image of a 72 hour mucoid-like biofilm labeled with FITC-HHA (A), DAPI (B), and the composite image (C). In confocal images of pillar-shaped biofilms, it is common to view the EPS matrix

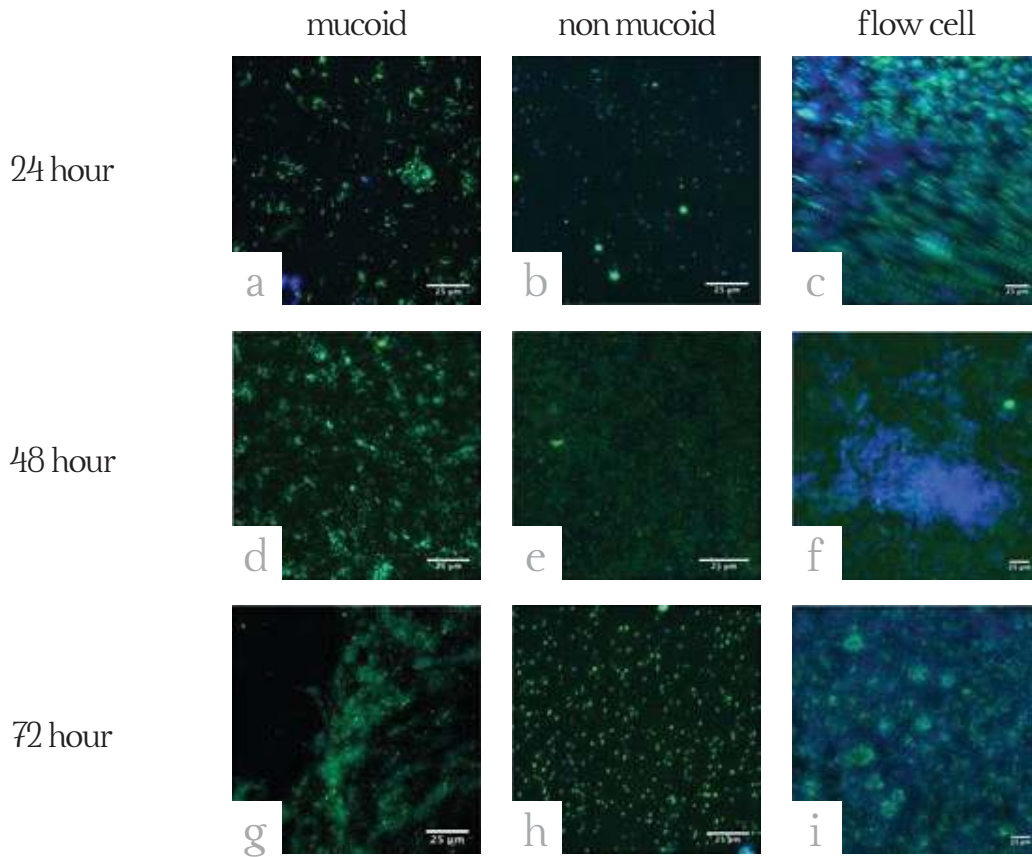


Figure 3. Confocal microscopy of PA biofilms. PA samples were prepared as described in the methods. At 24 hours, all samples demonstrated biofilm growth (A-C). The flow cell samples (C) are slanted in the direction of media flow through the chamber and difficult to obtain focused images. At 48 hours, the mucooid-like and flow cell samples demonstrate greater growth (D, F) compared to the non-mucooid sample (E). The spherical structures in D could be the beginning of pillar-shaped biofilms. In the 72 hour samples (G-I), the mucooid-like sample (G) shows string-like biofilms structures typical of the mucooid-type phenotype compared to the punctate labeling seen in the non-mucooid sample (H). The flow cell sample (I) has circular structures indicative of pillar-shaped biofilms. Calibration bars are 25μm.

surrounding a “hollow-like” center that may contain bacteria (8). Our biofilms contain similar structures. Fig. 2A demonstrates “holes”, black spaces in the biofilm surrounded by FITC-labeled matrix. These “holes” are, however, “filled in” with DAPI label in Fig. 2B, indicating that bacteria are within this structure. The composite image, Fig. 2C demonstrates the

separation of the matrix from the bacteria with the pattern of blue DAPI stain surrounded by the FITC-HHA label for the matrix. Fig. 2D is a negative control of mucooid-like PAO1 biofilm indicating some background label; however, it is much less than the positive label seen in Fig. 2A-C.

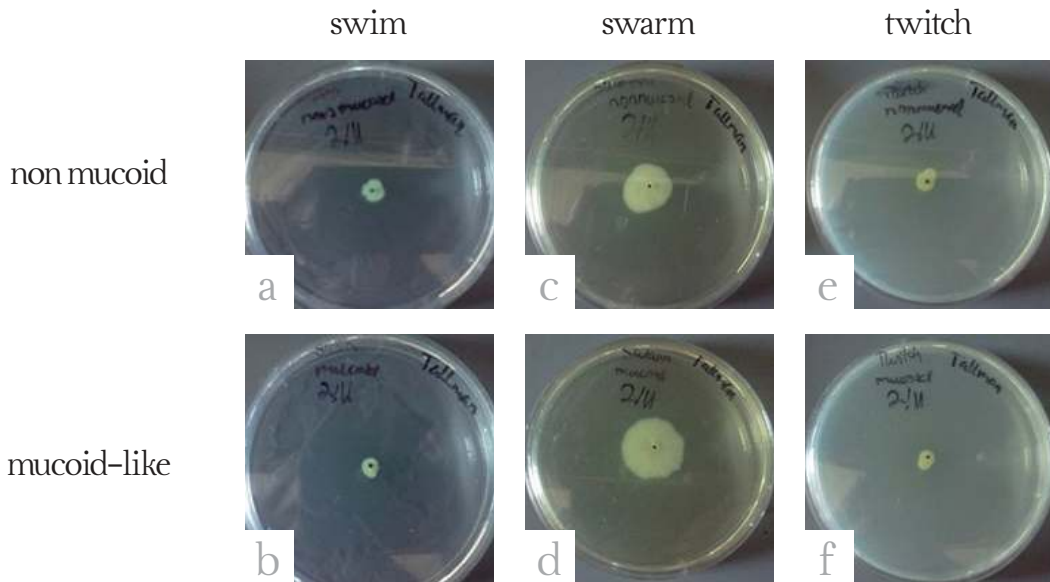


Figure 4. Motility assay. Shown are images of swim (A-B), swarm (C-D), and twitch plates (E-F) for non-mucoid and mucoid phenotypes. All plates were inoculated with 72 hour samples and allowed to develop for 48 hours. Swarming showed the greatest motility of the three types of movement as indicated by the increased diameter. All three types of movement demonstrated similar diameters, and therefore similar amounts of movement, between the non-mucoid and mucoid-like phenotypes.

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In order to compare biofilm development between the non-mucoid and mucoid-like phenotypes generated in this lab, biofilms were also grown in once-through flow cell chambers. These chambers have been shown in the past to provide optimal conditions for biofilm development (3, 29). Fig. 3 compares non-mucoid, mucoid-like, and flow cell biofilms 24, 48, and 72 hours after inoculation of a chamber. Biofilm development was greatest in the mucoid-like and flow cell samples at 72 hours (Fig. 3G and I). There was some labeling in the 72 hour non-mucoid sample (Fig. 3H), but it is much more punctate and does not show the elaborate Psl network seen in the mucoid-like or flow-cell apparatus biofilms.

In Fig. 3I, the 72 hour flow-cell biofilm sample demonstrates circular structures similar to the

pillar shaped biofilms shown in Fig. 2C. The mucoid-like biofilms (Fig. 3D, 3G) demonstrate biofilm structure that has the Psl matrix as observed by the FITC-HHA label, but is not always a discrete or circular structure (Fig. 3G). However, there is much greater development of the Psl matrix than in the non-mucoid biofilm which is a more consistent, punctate FITC-HHA label indicating a much less complex and fully formed biofilm matrix in the non-mucoid sample.

To measure biofilm height, z-stacks of 72 hour samples were used since biofilms at this point are the most developed (data not shown). Non-mucoid phenotype biofilms were smaller and flatter with an average height about 6 μm . Mucoid-like phenotype biofilms were taller with an average height of 6-12 μm . Flow

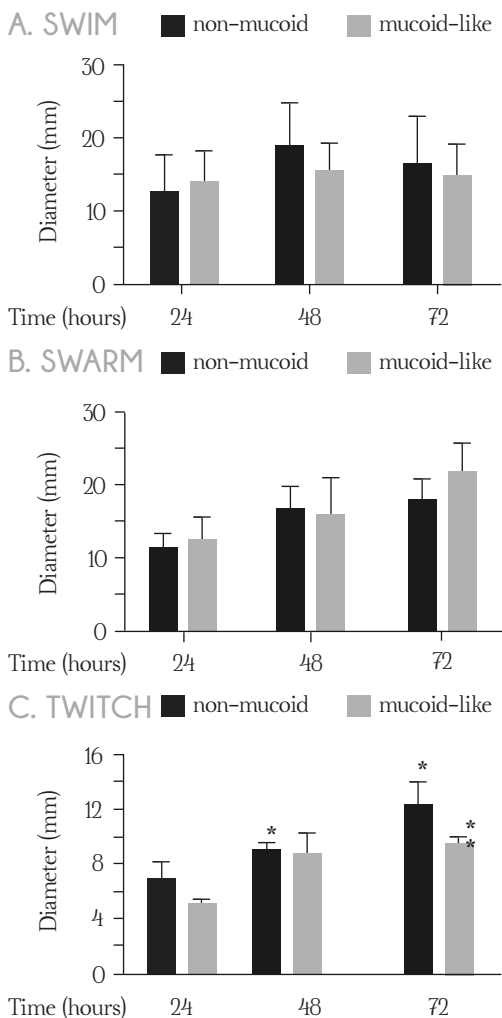


Figure 5. Graphs of motility data. A–C are graphs of the average diameter for each assay. Data were compared to the 24 hour mucoid sample for each motility using an unpaired t-test (GraphPad). Twitch motility demonstrated a significant increase from the 24 hour sample for the 72 hour mucoid, 48 hour non-mucoid, and 72 hour non-mucoid. This indicates that over time, both mucoid and non-mucoid phenotypes exhibited a significant increase in twitching. This indicates that both phenotypes are exhibiting characteristics of biofilm formation; however, the biofilm structure between the two phenotypes remains different as observed by a flatter biofilm structure for non-mucoid biofilms using microscopy. Non-mucoid p values: $p = 0.0019$, 48 hours; $p = 0.0031$, 72 hours. Mucoid-like phenotype p value at 72 hours, $p = 0.0001$.

cell biofilms were the largest biofilms with average heights between 30–40 μm . Biofilms grown in a flow cell chamber were slanted in the direction of the flow of media, making it difficult to focus the image.

MOTILITY ASSAY

Fig. 4 A–F demonstrates images of the motility assays. As can be seen swarming motility demonstrated the greatest diameters (Fig. 4 C, D). Visual inspection alone indicates that motility is very similar between PAO1 in each phenotype.

The average movements of swarm motilities at 72 hours as shown on the column graphs (Fig. 5B) were 18.1 mm (non-mucoid) and 21.8 mm (mucoid-like) as compared to the swim plate values (Fig. 5A) of 16.3 mm (non-mucoid) 14.6 mm (mucoid-like). Swarming is the only motility in which the 72 hour mucoid-like biofilm exhibited greater motility as compared to the non-mucoid sample (Fig. 5B) although there was no statistical difference. It was hypothesized that twitching movement would be greater in the mucoid-like sample (Fig. 5C); however, it was slightly greater in the 72 hour non-mucoid sample (12.2 mm vs. 9.5 mm). Twitching had the only statistically significant data with both the 48 hour non-mucoid and 72 hour non-mucoid being significantly higher in twitching ($p = 0.0019$ for 48 hour; $p = 0.0031$ for 72 hour) than the 24 hour mucoid-like phenotype. The 72 hour mucoid-like phenotype also demonstrated significantly more twitching than the 24 hour mucoid-like sample ($p = 0.0001$). Changes in motility are a part of biofilm formation as *P. aeruginosa* moves from swimming to swarming to twitching, eventually attaching to a surface and forming a microcolony and then a biofilm. Clinical data from CF patient sputum indicate that clinical isolates containing *P. aeruginosa* exhibit widely varying patterns of motility (10, 27). These differences in motility may indicate differences in virulence and ability to develop chronic

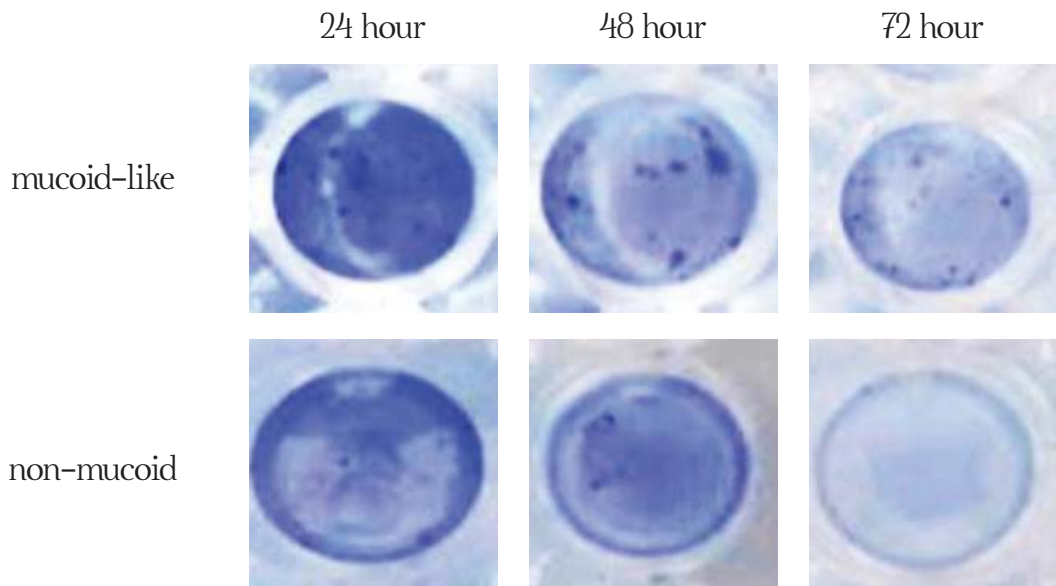


Figure 6. Surface attachment assay. All samples were given 1.5 hours to attach to the well before being fixed and stained with crystal violet. As can be seen, attachment decreased for both mucooid-like and non-mucooid samples between 24 and 72 hours. Biofilm formation is usually associated with an increase in attachment. While the trend in attachment was not as expected, it can be noted that the 72 hour mucooid-like sample exhibited greater punctate staining; these punctate areas of staining could be biofilms that attached. There are clearly qualitative differences in the development of the mucooid-like phenotype as compared to the non-mucooid phenotype.

infections. Motility is now being used as a method to further identify and characterize *P. aeruginosa* in sputum from CF patients (10, 27). These motility patterns may help identify the most effective treatments (27).

SURFACE ATTACHMENT

Attachment decreased as one moved from 24 hour to 72 hour samples (Fig. 6). There was a qualitative difference in that the mucooid-like samples attached with more punctate staining indicating clusters or colonies of PAO1 whereas the non-mucooid phenotype exhibited a more diffuse, even attachment. Each sample was given 1.5 hours to attach so the punctate staining represents clusters of bacteria that existed at the time of sample attachment not colonies formed through cell division after attachment. The

clusters may be similar to the circular structures observed in Fig. 2A–C in which a cluster of PAO1 is surrounded by Psl matrix. Absorbance data from the surface attachment assays did not indicate any significant difference in surface attachment when compared to the 24 hour mucooid-like sample or when comparing mucooid-like and non-mucooid samples at each time point.

DISCUSSION

Biofilm formation is a critical component of bacterial infections in a clinical setting; for example, in chronic infections in CF that increase mortality and morbidity (17). Biofilms are also implicated in chronic sinusitis and chronic wounds as well as other infections (4). It is well documented that mucoid biofilms are more pathogenic (11, 23). Alginate, one of the prime components of a mucoid biofilm elicits different responses from airway epithelium that attenuates the host response (7). It is also well documented that biofilms isolated from clinical patients exhibit widely different morphology and physiology with some forming pillar-shaped biofilms, some forming knobby shaped biofilms, and some with flat biofilms (10, 27). Motility assays are one way in which clinical strains are analyzed to determine trends in pathogenicity. Multiple studies using motility assays have found that pathogenic strains have decreased motility (1, 27).

The classic biofilm structure is that of a pillar-shaped structure containing bacteria and surrounded by an exopolymeric substance (EPS) composed of Psl, Pel, and alginate. It is hypothesized that biofilm formation may be driven by environmental conditions such as nutrient depletion, although the environmental triggers driving biofilm formation are not completely understood (1, 4, 7). A common way in which biofilms are grown within the lab is with rotation in a shaker, similar to the non-mucoid phenotype in this study using an Erlenmeyer flask. This biofilm produces different ratios of quorum-sensing molecules than biofilms formed within patient sputum (33). The environmental conditions that trigger biofilm transformation from a non-mucoid to a mucoid phenotype remain unknown (7).

Our lab was able to grow two distinct pheno-

types of PAO1 biofilms under two environmental conditions that may include oxygen or nutrient deprivation. It was our hypothesis that PAO1 in the mucoid-like phenotype would produce a biofilm closer to a clinical “mucoid” biofilm with greater twitching motility, greater attachment to a surface, and greater height and pillar-shaped structure when compared to the non-mucoid phenotype of PAO1.

Based on our data, both phenotypes exhibit some markers of biofilm development such as increased twitching behavior by PAO1 as well as a clearly defined matrix, indicated by FITC-HHA labeling. However, the mucoid-like phenotype exhibits characteristics closer to the more fully formed, mature pillar-shaped biofilms described in literature (8, 10). The mucoid-like biofilm developing in our lab had evidence of small pillar-shaped structures that were beginning to form (Fig. 2 A-C), but did not reach the height or complexity of biofilms formed in a flow-through chamber (Fig. 3G and 3I).

Biofilms are associated with a decrease in motility, both swimming and swarming (10, 27). Swimming utilizes flagella to propel bacteria to a new location, possibly to obtain a better food source. Twitching utilizes type IV pili to slowly move across a surface and is associated with surface attachment and the beginning stages of biofilm formation. Swarming is one of several intermediate forms of motility that utilizes both flagella and type IV pili as well as a bacterial-secreted rhamnolipid that acts as a surfactant to create a more fluid surface for bacterial movement (5, 22, 35). PAO1 in our mucoid-like phenotype exhibited swimming and swarming at 24, 48, and 72 hour time points (Fig. 4F); however, the only significant differences

in motility when compared to the 24 hour mucoid-like phenotype were in twitching. At both 48 hours and 72 hours the non-mucoid phenotype had significantly greater twitching than the 24 hour mucoid-like phenotype (Fig. 5C, $p = 0.0019$, 48 hours; $p = 0.0031$, 72 hours). At 72 hours, the mucoid-like phenotype had significantly greater twitching than the 24 hour mucoid-like (Fig. 5C, $p = 0.0001$). These data signal an increase in twitching across a 72 hour period and support that both of these phenotypes may be progressing through the early stages of biofilm development.

Surface attachment is also an established and necessary component for biofilm formation (8, 17). This may be facilitated by a switch from swimming motility to swarming and then to twitching as the bacteria become sedentary. In our phenotypes, attachment consistently decreased from 24 to 72 hours in both the mucoid-like and non-mucoid phenotypes. This is counter to the generally accepted view that surface attachment of bacteria increases as biofilms form. However, there were also qualitative differences, especially in the mucoid-like phenotype which exhibited more

punctate staining. These punctate spots could be small biofilms (Fig. 6) which would be consistent with biofilm formation.

In conclusion, our data indicate both phenotypes are in the process of biofilm formation due to FITC-HHA label and increased twitching. However, there are also differences between the phenotypes as shown in CLSM images in which the mucoid-like biofilm has more pillar-shaped biofilms and in the increased level of punctate staining in the surface attachment assay. Not much is known about the environmental factors that drive the formation of the wide variety of biofilms observed in clinical isolates. Nutrient and oxygen deprivation have been implicated in the formation of mucoid-like biofilms (16, 32, 37). The role of specific environmental triggers was not investigated in this study; however, knowing that environments as simple as using a test tube or an Erlenmeyer flask can produce two different phenotypes of biofilms might lead to further identification of the triggers that lead to the development of pathogenic biofilms in patients.

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ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge funding for this project from the following sources: Azusa Pacific University Faculty Research Council, Beta Beta Beta National Honor Society Research Foundation, and the Department of Biology and Chemistry at Azusa Pacific University. We would also like to thank Megan Prosser and Skyla Herod for their valuable feedback in preparing this manuscript.

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REFERENCES

1. Bianconi, I., Milani, A., Cigana, C., Paroni, M., Levesque, R. C., Bertoni, G., & Bragonzi, A. (2011). Positive signature-tagged mutagenesis in *Pseudomonas aeruginosa*: tracking patho-adaptive mutations promoting airways chronic infection. *PLoS Pathogens*, 7(2), e1001270–e1001270.
2. Bjarnsholt, T., & Givskov, M. (2007). Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens. *Philosophical Transactions of the Royal Society B: Biological Sciences* 362(1483), 1213–1222.

3. Bjarnsholt, T., Jensen, P. O., Burmølle, M., Hentzer, M., Haagenen, J. A. J., Hougen, H. P., Givskov, M. (2005). *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology*, 151, 373–383.
4. Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P. O., Madsen, K. G., Phipps, R., Krogfelt, K., Givskov, M. (2008). Why chronic wounds will not heal: a novel hypothesis. *Wound Repair And Regeneration: Official Publication Of The Wound Healing Society [And] The European Tissue Repair Society*, 16(1), 2–10.
5. Burrows, L. L. (2012). *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annual Review of Microbiology*, 66, 493–520.
6. Christensen, B. B., Sternberg, C., Andersen, J. B., Palmer, R. J., Jr., Nielsen, A. T., Givskov, M., & Molin, S. (1999). Molecular tools for study of biofilm physiology. *Methods in Enzymology*, 310, 20–42.
7. Cobb, L. M., Mychaleckyj, J. C., Wozniak, D. J., & López-Boado, Y. S. (2004). *Pseudomonas aeruginosa* flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. *Journal of Immunology*, 173(9), 5659–5670.
8. Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, 284(5418), 1318–1322.
9. Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, 280(5361), 295–298.
10. Deligianni, E., Pattison, S., Berrar, D., Ternan, N. G., Haylock, R. W., Moore, J. E., Dooley, J. S. (2010). *Pseudomonas aeruginosa* cystic fibrosis isolates of similar RAPD genotype exhibit diversity in biofilm forming ability *in vitro*. *BMC Microbiology*, 10, 38.
11. Doggett, R. G., Harrison, G. M., Stillwell, R. N., & Wallis, E. S. (1966). An Atypical *Pseudomonas aeruginosa* Associated with Cystic Fibrosis of Pancreas. *Journal of Pediatrics*, 68(2), 215
12. Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, 8(9), 623–633.
13. Folsom, J. P., Richards, L., Pitts, B., Roe, F., Ehrlich, G. D., Parker, A., Stewart, P. S. (2010). Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. *BMC Microbiology*, 10, 294–294.
14. Fraser, G. M., & Hughes, C. (1999). Swarming motility. *Current Opinion In Microbiology*, 2(6), 630–635.
15. Govan, J. R., & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev*, 60(3), 539–574.
16. Haley, C. L., Colmer-Hamood, J. A., & Hamood, A. N. (2012). Characterization of biofilm-like structures formed by *Pseudomonas aeruginosa* in a synthetic mucus medium. *BMC Microbiology*, 12, 181–181.
17. Hall-Stoodley, L., & Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cellular Microbiology*, 11(7), 1034–1043.
18. Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annual Review of Microbiology*, 57, 249–273.
19. Jin, F., Conrad, J. C., Gibiansky, M. L., & Wong, G. C. L. (2011). Bacteria use type-IV pili to slingshot on surfaces. *Proceedings of the National Academy of Sciences USA*, 108(31), 12617–12622.
20. Kim, H.-S., Lee, S.-H., Byun, Y., & Park, H.-D. (2015). 6-Gingerol reduces *Pseudomonas aeruginosa* biofilm formation and virulence via quorum sensing inhibition. *Scientific Reports*, 5, 8656–8656.
21. Koehler, S. E., & Ladner, R. D. (2004). Small interfering RNA-mediated suppression of dUTPase sensitizes cancer cell lines to thymidylate synthase inhibition. *Molecular Pharmacology*, 66(3), 620–626.
22. Kuchma, S. L., Griffin, E. F., & O'Toole, G. A. (2012). Minor pilins of the type IV pilus system participate in the negative regulation of swarming motility. *Journal of Bacteriology*, 194(19), 5388–5403.
23. Lam, J., Chan, R., Lam, K., & Costerton, J. W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infection and Immunity*, 28(2), 546–556.
24. Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., & Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathogens*, 5(3), e1000354.
25. Ma, L., Lu, H., Sprinkle, A., Parsek, M. R., & Wozniak, D. J. (2007). *Pseudomonas aeruginosa* Psl is a galactose- and mannose-rich exopolysaccharide. *Journal of Bacteriology*, 189(22), 8353–8356.

26. Møller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M., & Molin, S. (1998). *In situ* gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Applied and Environmental Microbiology*, 64(2), 721–732.
27. Mulet, X., Cabot, G., Ocampo-Sosa, A. A., Domínguez, M. A., Zamorano, L., Juan, C., Oliver, A. (2013). Biological markers of *Pseudomonas aeruginosa* epidemic high-risk clones. *Antimicrobial Agents and Chemotherapy*, 57(11), 5527–5535.
28. Pesci, E. C., & Iglewski, B. H. (1997). The chain of command in *Pseudomonas aeruginosa* quorum sensing. *Trends in Microbiology*, 5(4), 132–134.
29. Purevdorj, B., Costerton, J. W., & Stoodley, P. (2002). Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, 68(9), 4457–4464.
30. Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671–675.
31. Schurr, M. J. (2013). Which bacterial biofilm exopolysaccharide is preferred, Psl or alginate? *Journal of Bacteriology*, 195(8), 1623–1626.
32. Sigurdsson, G., Fleming, R. M. T., Heinken, A., & Thiele, I. (2012). A systems biology approach to drug targets in *Pseudomonas aeruginosa* biofilm. *Plos One*, 7(4), e34337–e34337.
33. Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J., & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*, 407(6805), 762–764.
34. Steinberger, R. E., Allen, A. R., Hansa, H. G., & Holden, P. A. (2002). Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa*-unsaturates biofilms. *Microbial Ecology*, 43(4), 416–423.
35. Taylor, T. B., & Buckling, A. (2011). Selection experiments reveal trade-offs between swimming and twitching motilities in *Pseudomonas aeruginosa*. *Evolution; International Journal Of Organic Evolution*, 65(11), 3060–3069.
36. Wang, S., Parsek, M. R., Wozniak, D. J., & Ma, L. Z. (2013). A spider web strategy of type IV pili-mediated migration to build a fibre-like Psl polysaccharide matrix in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol*, 15(8), 2238–2253.
37. Zhao, K., Tseng, B. S., Beckerman, B., Jin, F., Gibiansky, M. L., Harrison, J. J., Wong, G. C. L. (2013). Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature*, 497(7449), 388–391.