Role of flagella, type IV pili, bio-surfactants, and EPS polysaccharides on the formation of pellicles by *Pseudomonas aeruginosa*

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Abstract: Microbial biofilms are viscoelastic materials formed by bacteria, which occur on solid surfaces, at liquid interfaces, or in free solution. Although solid surface biofilms have been widely studied, pellicles, biofilms at liquid interfaces, have had significantly less focus. In this work, interfacial shear rheology and SEM imaging are used to characterize how flagella, type IV pili, bio-surfactants, and EPS polysaccharides affect formation of pellicles formed by *Pseudomonas aeruginosa* at an air/water interface. Pellicles still form with the loss of a single biological attachment mechanism, which is hypothesized to be due to surface tension aided attachment. Changes in surface structure of the pellicles are observed when changing both the function/structure of type IV pili, removing flagella, or stopping expression of biosurfactants. However, these changes do not appear to affect pellicle elasticity in a consistent way. Traits that affect adsorption and growth/spreading appear to affect pellicles in a manner consistent with literature results for solid surface biofilms; small differences are seen in attachment related mechanisms, which may occur due to surface tension.

Key words: Biofilm, P. Aeruginosa, pellicle, interfacial rheology

Introduction

Almost any surface in any environment can be colonized by bacteria. Colonization can lead to the formation of biofilms, microbial communities encased in extracellular polymeric substances (EPS) that create an environment that allows bacteria to survive hostile conditions and disperse cells to colonize new areas.[1-7] The formation of biofilms at solid surfaces has been extensively studied, which has led to some understanding of the role of environment and biological response on their formation;[8-10] the same cannot be said for pellicles, biofilms at liquid interfaces.

Pellicles are found in several important applications/processes. They contribute to lung infections and complications in cystic fibrosis patients,[11-13] help to degrade organic pollutants in soil and water,[14-16] affect the efficacy of microbial enhanced oil recovery,[17-19] and impact the processing of crude oil.[20-22] Therefore, understanding both the development and material properties of pellicles is beneficial for their removal or utilization. Many liquid interfaces typical of the above applications are complex and have additional adsorbed species. Pellicle formation is affected by the hydrodynamic, steric, and physio-chemical interfacial boundary conditions created by such complex interfaces.[23-24] A better understanding of the interaction between bacteria and interfaces is necessary to manipulate pellicle formation.

Although not extensive, the effects of changing interfacial properties on pellicle development have been examined for some conditions. When moving towards an interface, interfacial viscosity affects the rotation direction of adsorbing bacteria for a range of species.[24-26] Adhesion to an interface by *Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida, Salmonella typhimurium, Escherichia coli,* and *Bacillus subtilis* can be impacted by modifying surface tension and charge through addition of interfacially adsorbed components.[27-29] Growth and final pellicle strength of *Escherichia coli* can be affected by addition of surfactant immediately prior to pellicle formation, which prevents formation of cellular aggregates.[30] Addition of surfactant after and during pellicle formation reduces viscoelasticity and delays growth of *Escherichia coli, Pseudomonas fluorescens, Vibrio cholera,* and *Bacillus subtili* pellicles.[30-34] Metal nanoparticles at a liquid interfaces have been found to create stronger pellicles by upregulating cellulose production in *Pseudomonas fluorescens* pellicles.[35] Our own work,[36] has shown that *Escherichia coli* pellicles can be made to form faster by adding negatively charged

microparticles to an interface, which increases bacteria attachment rates. Conversely, interfacially adsorbed silver nanoparticles can be used to prevent the formation of pellicles.

Despite the above, there is a gap in understanding the basic ways in which liquid interfaces affect the biological mechanisms known to control biofilm formation on solid surfaces. It is not known how bacterial motility and attachment mechanisms are altered by liquid interfaces. This work attempts to understand how specific biological components of *Pseudomonas aeruginosa* known to be important in solid surface biofilms affect pellicle formation using interfacial rheology and SEM imaging. The role of motility and EPS components on the formation of *P. aeruginosa* solid surface biofilms are well established,[37-46] which will allow us to note differences between solid surface biofilms and pellicles. Comparing our results to published literature results will highlight differences, which will provide insight into the relevant physics and mechanisms that dictate *P. aeruginosa* pellicle formation on liquid interfaces.

Background

Stages of Biofilm Formation

There are three basic stages of biofilm formation: adsorption, attachment, growth.[47] During adsorption, bacteria move from the bulk to a surface/interface driven by propulsive mechanisms or through diffusion in the case of non-motile bacteria. Once bacteria initiate surface contact, they adhere to the surface during attachment.[47-48] Growth has two separate mechanisms: attached bacteria both produce EPS and actively spread over surfaces. EPS polysaccharides secreted by the bacteria create the soft viscoelastic base of the biofilm by making a material similar to a cross-linked polymer gel. Spreading increases the boundaries of the biofilm. The final microstructure of the biofilm will have colonies of varying shapes on top of underlying film of varying thickness.[1-3]

P. aeruginosa Biofilm Formation at Solid Surfaces

All bacteria that form biofilms go through the same basic stages of formation, but each have unique EPS components, motility traits, and behaviors. Below, the properties of *P. aeruginosa* biofilms on solid surfaces are outlined.

The adsorption stage of formation is controlled by organelles employed by bacteria for bulk transport. *P. aeruginosa* swim by means of flagella at speeds up to 50 μm/s.[39, 49] Mutants without flagella are poor colonizers of solid surfaces, due to diffusion being the only means of bulk transport. However, even without flagella, *P. aeruginosa* biofilms will eventually form , due to diffusion of bacteria from the bulk close to the surface. [37]

Attachment occurs as bacteria contact and adhere to a solid surface. For *P. aeruginosa,* attachment is controlled by two mechanisms: type IV pili and EPS. Type IV pili are external appendages, which extend and retract out of the bacteria surface.[37] The pili aid in overcoming repulsive forces that prevent bacteria from making contact with a solid surface.[38, 40] Mutants lacking pili still colonize surfaces but are less efficient and cannot easily detach from a surface, which affects biofilm microstructure.[37] EPS, besides being secreted to form the biofilm matrix, is present on bacteria surfaces. One particular component of EPS, Psl, is well known to provide adhesion between bacteria and solid surfaces.[41-42] In the absence of pili, a second EPS component, Pel, can also aid in attachment to a solid surface.[41]

After attachment, biofilms spread through surface motility and grow by EPS secretion. Extension and retraction of type IV pili power surface motility parallel to the surface, which is referred to as twitching.[37-40] Twitching propels bacteria across a surface with average speed of about of 1mm/h.[37] A number of studies have shown that bacteria modified to not form or form atypical type IV pili, create biofilms with fewer micro-colonies on top of a thinner underlying layer of biofilm in comparison to wild type bacteria.[37-38, 43]

A second motility that aids in growth is swarming. Swarming is the rapid movement in unison of many side by side bacteria on a surface, and it occurs at a critical concentration of bacteria. This motion is powered by flagella, pili, and naturally secreted biosurfactants.[45] Swarming is characterized by the formation of dendrite shaped structures in the biofilm.[37] Mutants which lack rhlA, a biosurfactant important to swarming, create thicker biofilms than wild type bacteria.[44] However, biofilms formed by the same mutants are easier to detach from a solid surface in comparison to wild type.[42]

The other activity that occurs during growth is the secretion of EPS. The EPS of *P. aeruginosa* biofilm is a mixture of three polysaccharides: alginate, Psl, and Pel. Alginate is a high molecular

weight, acidic polysaccharide composed of non-repeating units of mannuronic acid and guluronic acid.[50] It is not required for biofilm formation on solid surfaces, but *P. aeruginosa* strains with defective alginate form weaker biofilms.[46] Alginate is important to the overall microstructure stability and aids in water retention.[41] Pel is a cationic exopolysaccharide, whose structure has not been determined, but derives its name from its perceived importance in pellicle formation. It serves as a scaffold, and is necessary for the formation of solid surface biofilms.[51] Psl is a repeating pentamer of mannose, rhamnose and glucose.[50] It is also a major component of the structural scaffold of biofilms, and due to its aforementioned role in adhesion is also the initiator of biofilm growth and the development of new micro colonies.[41-42]

Materials and Methods

Bacterial stains and growth conditions

Individual *P. aeruginosa* are approximately 1.5~1.6 μm and 0.8~0.9 μm in length and width.[52] Two primary strains of *P. aeruginosa* were used in this work: PAO1 and PA14. PAO1 is a spontaneous chloramphenicol-resistant mutant of the PAO strain, and is a common reference strain.[53] PA14 lacks Psl producing genes, but still forms biofilms.[54] Several mutant strains of each type were used as outlined in Table 1. PA14 and mutant strains were obtained from the PA14 nonredundant transposon mutant library (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi). PAO1 and mutant strains were obtained from the Seattle P. aeruginosa PAO1 transposon mutant library (http://www.gs.washington.edu/labs/manoil/libraryindex.htm).

All bacteria starting cultures were grown from frozen stock by scraping a small amount of bacteria onto an LB agar plate and incubating overnight at 37 °C. A single colony of bacteria on the agar plate was picked and suspended in a tube of 5 mL of LB broth. The fresh medium was prepared with deionized water and sterilized by autoclaving at 121°C, 15 psi for 45 min. This starting culture was placed into incubator at 37 °C for 20 h, shaking at 150 rpm. After 15 h, the planktonic cell concentration of working culture was measured by optical density at 600 nm; a density of 2.4A was found. Afterwards, 0.75ml of culture with the strain in the stationary phase were diluted to 150 ml using LB broth. This solution was used to grow pellicles for SEM and interfacial rheology measurements. For both tests, pellicles were grown in a custom designed flow cell that kept

temperature at 25 °C and infused LB broth at a rate of 0.006 mL/min while maintaining a constant height of the interface.[36]

Scanning Electron Microscopy (SEM)

The formation of biofilms can be difficult to observe at a microscopic level but is possible with the use of fluorescent dyes, fluorescent mutants, and confocal microscopy.[55-57] Unfortunately, to grow pellicles for imaging in conditions identical to rheology experiments required a flow cell that was not compatible with available confocal instruments. Therefore, pellicles were grown and then surface structure was observed using cryo-SEM. It should be noted, that images of the surface structure provide information on the top layer of the pellicle, but do not illuminate the entire 3D microstructure of the pellicle. Therefore, these surface structure are not a direct indicator of measured elasticity.

Pellicles floating at the air/liquid interface in the flow cell were collected on glass slides and flash frozen using liquid nitrogen. These frozen sample slides were kept in a freeze dryer at −25 °C for 3 days. Before imaging, the slides were sputter-coated with a thin layer of Au/Pd alloy to improve the sample conductivity. Samples were imaged using a Hitachi, Model SE/N 4300 scanning electron microscope. SEM images were taken in one location of each side of the pellicle at 3 magnifications. Representative images are shown in the paper and additional images can be found in supporting information.

Images were taken from both the "water side" and the "air side" of the pellicle. There is a marked similarity in the "water side" images, all of which have a hexagonal cell structure. This occurs due to the relatively slow freezing process, which creates nitrogen ice crystals that force the EPS into the structure observed.[58-59] These "water side" images are not used for any analysis of the pellicles, but can be observed in the supporting information.

On the "air side" images, the freezing process does not affect the number of bacteria on the interface, their density, or their relative positions.[60] In some SEM images, it is noted that bacteria in generally appear to be lying flatter than in others. However, it is difficult to ascertain whether this bacteria orientation (i.e. parallel or perpendicular to the interface) is affected by freezing.

Therefore, porosity and complexity of surface structure shapeare relied on for quantitative analysis.

To analyze porosity and complexity, images were converted to binary using ImageJ's auto threshold. The resulting image had the top layer of bacteria white while "holes," which revealed the interior of the pellicle, were black. These images were then quantified through porosity of "holes", $\phi = A_{black\ pixels}/A_{total}$, and complexity using box counting to find fractal dimension, D_f. The porosity indicates how filled the top layer of the pellicle was and had a standard deviation of ±1% when image processing was repeated with changes in thresholding schemes. The fractal dimension is indicative of space filling and shape complexity, approaching 2 for a filled plane and lower numbers representing more network like shapes. This was found to be accurate to ±0.01 when image processing was repeated with changes in thresholding scheme.

Interfacial rheology

Biofilm storage and loss moduli aid in mechanical protection and attachment, and their values are established to change through the stages of biofilm formation.[47] Furthermore, interfacial viscoelasticity measurements are sensitive to the inception of biofilm formation, and hence molecular-level features of pellicles can be correlated with mechanical properties.[30-31] Therefore, rheology is an excellent tool for the quantitative characterization of biofilm formation.[61] During the adsorption stage, there is no measurable interfacial viscoelasticity because no biofilm has developed. During attachment, bacteria become packed, which creates a small but measurable viscoelasticity. Finally, as biofilms grow, there is an increase in viscoelastic moduli due to changes in EPS composition and microstructure.[62-64] For instance, denser biofilms create larger moduli that are more elastic.[52, 65] Although microstructure varies throughout a biofilm,[1-2, 42] global viscoelastic parameters can be measured and used to characterize average behavior of the developing biofilm. Therefore, rheological testing of biofilms (and/or pellicles) is a means to analyze how changes in biology are affecting formation, while evaluating mechanical strength which is also important to pellicle survival.

It is possible that EPS components secreted by bacteria before transferring to the rheometer could adsorb to the interface and affect the viscoelasticity of the interface. However, it is not believed this is particularly impactful for 2 reasons. One, the working cultures were diluted 200 times before

pellicle growth began, so the quantity of free EPS in solution is small. Secondly, the most likely time this free EPS would impact the measurement is during adsorption before pellicle growth; in this stage, there was no measurable moduli within the sensitivity of the rheometer used. Therefore, the effect if present is too small to detect.

All interfacial rheology measurements in this work were done using a double-wall ring geometry[66] attached to an AR-G2 stress-controlled rheometer (TA Instruments) that has been redesigned to allow for the controlled growth of pellicles as outlined in earlier work.[36] Measured interfacial moduli have units of [Pa m]. Each experiment ran for 72 h, during which the pellicle grew, and surface viscoelastic moduli were measured for 10 min at the end of each hour with an angular frequency of 0.5 rad/s and strain of 1%.

All pellicles exhibited interfacial storage modulus G' and interfacial loss modulus G''. The pellicles were dominated by interfacial elasticity, and the loss moduli followed similar trends to the storage moduli, which was similar to previous work.[30, 36] In the following results, only storage moduli are reported. All loss moduli are included in supporting information.

The time dependent interfacial moduli of pellicles from 2 strains of *P. aeruginosa* are shown in Figure 1. At the beginning of an experiment, the interface is clean, and bacteria have not adsorbed close or attached to the interface. During this phase, moduli are below measurement sensitivity. As more bacteria attach to the interface, a small but growing elasticity is observed. Eventually, the bacteria increase EPS production to form a pellicle; there is a significant increase of the interfacial elasticity. The exact transition from attachment to growth is difficult to pinpoint for *P. aeruginosa* using interfacial rheology. Furthermore, determining spreading vs. EPS growth through this technique alone is impossible. Macroscopically, pellicle formation is seen at some point after moduli are measurable.

Variability in biofilm mechanical properties has been well established. It is impossible a priori to estimate such variability, but it is well over any error in measurement due to sensitivity of the rheometer. In typical interfacial rheology studies of pellicles, no error or statistical analysis of the compared moduli are given.[29-36] In order to gauge variability, three tests were done on each wild type strain (PAO1 & PA14). There was some variability in the timing of various stages during the formation, but due to the time step between data points, variability in timing could not be

accurately determined. The variability in the moduli at any particular time step was evaluated by taking the average and standard deviation of all tests. The standard deviations were converted to standard error and then to percentage error. The average over all moduli was found to be 35%, which is shown using error bars on all figures. For all wild type strains, the data shown is average response from 3 repeated tests. In addition, comparison between all strains and wild type final moduli were quantitatively compared using a P-value calculated using a student one tailed t-test. The null hypothesis was that any two strains final moduli value should be identical, and hence the difference of the final storage modulus value should be 0.

Results

Role of EPS on Pellicle Elasticity and Surface Structure

In order to gauge the role of Psl, the growth of PA14 WT over time was characterized, and compared with the result of PAO1 WT (Figure1). PA14 wild type and PAO1 wild type pellicles exhibit nearly identical trends in elasticity over the entire growth period; although, timing between various stages of formation are clearly different. During the first 2 hours for the PA14 and first 12 for the PAO1, elastic moduli are too small to be detected because too few bacteria have absorbed close to or attached to the interface. From 2 to 12 hours for the PA14 and 12 to 24 for the PAO1, interfacial moduli grew gradually as bacteria absorb and attach to the interface and EPS production begins to occur. PA14 pellicles reach a peak modulus at ~hour 16 and PAO1 pellicles at hour 25, and then moduli for both gradually decreased until hour 72. The growing pellicles can be visually observed forming on the air/water interface, filling the interface and becoming opaquer as they thicken. The PA14 wild type strain pellicle exhibits moduli larger than the PAO1 wild type strain pellicle for the first 48 hours and then the two curves merge. Using the final moduli, the P-value for these two pellicles is 0.4, indicating that the null hypothesis is affirmed, and the two pellicles have nearly identical moduli after 72 hours.

From the SEM results (Figure 2), the PAO1 pellicle appears to have fine “holes” in the dense top layer of bacteria, whereas the PA14 pellicle has much larger “holes” around the edges of a central bacterial mat in the top layer. This is quantitatively characterized by examining the porosity of the top layer which is less than 1% for the PAO1 vs. 24% for the PA14. The fractal dimensions are large given the primarily filled top layer both pellicles create, but the PA14 pellicle does have

a smaller fractal dimension given the more network like structure around the "holes." At higher magnifications, the PAO1 bacteria appear to be lying flatter on the surface, but this is likely an artifact of the freezing process.

The effect of the Pel on pellicle formation was studied by comparing the result of mutant PAO1 Δpel and PAO1 WT pellicles (Figure 1). The general behavior trends observed for PAO1 and PA14 are seen again. The Pel lacking mutant attaches to the interface slightly faster in comparison to the wild type bacteria, and its pellicle was similarly faster in reaching peak modulus. The value of elastic modulus for the Pel lacking mutant pellicle after the peak values appears to be consistently larger than the PAO1 wild type pellicle. These moduli are well above measured variability and the P-value between the mutant PAO1 Δpel pellicle and the wild type pellicle was found to be 0.00001, rejecting our null hypothesis, confirming the moduli are different.

From the SEM results (Figure 2), the Pel mutant pellicle appears to have more "holes" in comparison to the wild type strain, which is confirmed by the larger porosity value in comparison to the wild type pellicle. Furthermore, the structure is slightly more network like given the reduction in the fractal dimension in comparison to the wild type pellicle. In close, the two pellicles again look similar to one another with the bacteria appearing to be relatively flat on the interface for each, which again is likely a result of the freezing process.

Role of Motility on Pellicle Formation

Significantly different behavior is observed when examining the wild type and mutant strains that lack flagella (Figure 3). The mutant without flagella needed much longer time to absorb to the interface in comparison to the wild type. Furthermore, over the 72-hour period, the mutant without flagella exhibit different behavior in the moduli. There is still the initial attachment stage of rapid growth. However, this is followed by a much slower rate of growth from hour 40 on. Furthermore, no peak is ever observed for the pellicle of the mutant without flagella. Instead, the moduli slowly increase up to hour 72. The pellicle formed by mutants without flagella has a higher modulus at the end of the test, which is consistent with the reported P-value of 0.02, which indicates that the null hypothesis is weakly rejected.

From the SEM results (Figure 4), surface structure of the two pellicles are quite different. For both pellicles, dense bacterial mats on the top layer are seen; however, the wild type pellicle has large "holes" around its edge. There are no observable "holes" in the pellicle formed by the flagella lacking mutants. This can be seen in its reported porosity, which is effectively 0. There is no fractal dimension reported for the mutant strain pellicle because they form a filled plane. The fractal dimension for the wild type pellicles indicates greater complexity to the structure of the top layer, which is qualitatively observed at the edges of the "holes." The close-up images, which focus on the dense mats on the top layer, appear essentially similar.

Surface motility was examined by looking at a series of mutants that lack the ability to make substructures which aid in the function/structure of type IV pili including PA14 lacking pilD which aids in rotation of the pili, PA14 lacking pilP which aids in aligning the extended pili at a particular angle, and PA14 lacking pilA which is the major structural unit of the pili (Figure 5).[37] The general trend in behavior for all pellicles in Figure 5 are identical. Delayed attachment is clearly visible for all the mutant strains by the initial flat lines in the data in comparison to the PA14 wild type. The rate of growth after attachment also seems to be altered for all the mutants, but in no consistent way. There is no consistent trend in moduli, with the pellicles of the pilP and pilD mutants slightly less than the wild type pellicle (outside of measured variability) and the pellicle formed from pilA mutants slightly greater after 72 hours. Looking at the P-values, similar results are observed for the pilD and pilP with P-values of 0.2 affirming null hypothesis that the final pellicle moduli are similar. The pilA pellicle has a P-value of 0.03, which is a weak rejection of the null hypothesis.

From the SEM results (Figure 6), the surface structures of the pili mutants' pellicles all appear quite different than the wild type pellicle. Starting with the pilA pellicle, a more network like appearance is observed. Compared to the wild type pellicle, the porosity of the top layer for the pilA pellicle is about 10% greater. The fractal dimensions are also quite different with the pilA mutant pellicle having a smaller value indicating a more complicated/networked shape. In the closeup images, the wild type pellicle top layer is flat, whereas the pilA pellicle appears to be more three dimensional. Both pilD and pilP mutants' pellicles appear similar to the wild type pellicle, with a dense solid mat of bacteria in the top layer. However, these mutants' pellicles do not exhibit the "holes" at the edge of a dense mat that are seen in the wild type pellicle, and instead have smaller "holes" throughout their structure. This can be seen quantitively in porosity, which are

smaller in comparison to the wild type pellicle. The fractal dimensions of the pilP pellicle is larger but within error of the wild type pellicle, but the pilD pellicle is larger and outside of error. In close-up, the bacteria all appear to be flat on the interface for the pilD and pilP like the wild type.

The contribution of genes that regulate pili formation also were studied by comparing the results of PAO1 lacking pilJ, which aids in type IV pili construction, and PAO1 lacking pilS, which regulates pili production (Figure 7). PAO1 wild type and the ΔpilJ mutant pellicles exhibit moduli within the measured variability throughout the entire growth process. However, the P-value for these pellicles is 0.0, which is a weak rejection of the null hypothesis. The pilS mutant shows a faster attachment than the wild type, and the moduli reach a value close to but larger than measured variability of error of the wild type pellicle. The P-value for these pellicles was 0.002, strongly rejecting our null hypothesis and the moduli can be considered different.

From the SEM results (Figure 8), the surface structure for pellicles of all pili formation mutants appear to have no particular difference in comparison to the wild type. Comparing the porosity of all three pellicles, the pilS is slightly larger than the wild type at 5%, but the pilJ is effectively identical. The fractal dimensions of all pellicles are within error of the measurement, indicating the structures are all essentially similar space filling planes. The closeup images all look relatively similar with bacteria flat on the interface in the top layer.

In order to investigate the role of biosurfactants, the results of PA14 ΔrhlA and PA14 ΔrhlB are compared to wild type strains (Figure 9). The mutants which lack the 2 types of rhamnolipid biosurfactants,[41] take more time to initially absorb compared with wild type bacteria. The mutants' pellicles both have larger maximum values of G' than the PA14 wild type pellicle after 72 hours. The respective P-values for the final moduli of PA14 ΔrhlA and PA14 ΔrhlB pellicles are 0.02 and 0.008, which weakly and strongly reject our null hypothesis respectively, indicating that there is some change in the moduli.

From the SEM results (Figure 10), the mutants' pellicles appear to have a somewhat similar structure to the wild type. There are large dense mats on the top layer. However, the porosity of the wild type pellicles is greater at 24% than both ΔrhlA and ΔrhlB pellicles, at 18% and 5% respectively. Based on fractal dimensions, the ΔrhlB mutant's pellicle is more space filling. At higher magnifications, no particular difference can be seen.

Discussion

General Trends

The trend in the elastic moduli of a peak value reached followed by slow decay are non-intuitive, but consistent in all tests done. We have considered 3 possible mechanisms for this behavior. One is mechanical buckling/wrinkling of the film that may weaken its global resistance to deformation. Similar wrinkling has been observed in other pellicle studies.[29, 31-32] However, no loss in mechanical strength was observed in those works. Alternatively, as the pellicle grows rapidly to its peak strength, the amount of nutrient being supplied through the inflow of broth may not be enough to sustain the bacteria in the film. This would eventually inhibit further growth of the pellicle and possibly weaken the existing structure, resulting in the slow decreases of G'. However, in previous work with *E. coli,*[36] this peak and decay behavior is not observed, but rather a plateau value reached in the elastic moduli of the pellicle. If nutrient loss were occurring, it might be suspected that a similar behavior would be found in the *E. coli* systems. The final possibility in the loss of mechanical strength may be due to the constant testing of the growing film. Although small amplitude oscillatory shear was used, which should minimize any strain/stress induced damage to the film, it is possible that the repeated testing weakened the film. However, as mentioned above, similar trends were not seen in *E. coli* systems in previous work, which was tested identically. Given the above conclusions, the reason for this weakening is still not evident, and may perhaps be due to another biological feature/mechanism that has not been considered.

Adsorption

The primary effect of adsorption in this study was done by looking at flagella lacking mutants. As expected, there was a long delay in adsorption to the interface for these mutants due to their reliance on diffusion rather than having propulsive motility. Indeed, motile cells are known to travel up to 1000 times faster than non-motile cells.[67] However, once the bacteria attached on the interface, there was a rapid growth phase and creation of pellicle, indicating that flagella were not necessary for attachment and/or growth. This is consistent with results for solid surface biofilms that were grown under different conditions.[37] Furthermore, the results are consistent with the work of Niepa et al,[68] who studied similar strains and mutants at water-hexadecane interfaces. In their particle tracking and dilatational rheometry, Δflgk mutants of PAO1 had the ability to create an

elastic interface but showed some delay in the dynamics of this formation, which was attributed to the slower speed of the non-motile mutants.

Attachment

Psl is established to play a key role in attachment of solid surface biofilms. Pel has also been noted to aid in attachment to a solid surface.[41] Furthermore , Pel is considered important to the formation of pellicles.[51] The attachment stage is slower for the PAO1 in comparison to the Psl lacking PA14. Attachment is also faster for PAO1 Δpel in comparison to PAO1 WT. Both the EPS missing strains appear to attach more quickly than the wild type and still form pellicles. These results indicate that attachment is occurring even without the two individual EPS components studied in this work, which are known to aid attachment on solid surfaces. Why attachment appears to happen more quickly when either Psl or Pel are missing is not clear and cannot be rigorously evaluated given the experiments done here. One possible hypothesis is that losing either of these EPS components alters the bacteria surface chemistry such that a larger surface tension force exists to trap the bacteria to the air/water interface. However, more specific tests would need to be done to confirm this idea.

Those mutants with some form of pili deficiency related to the structure/function (pilA, pilD, and pilP) of the pili took longer to attach to a surface. It would appear given these results that the existence and/or functionality of surface pili are relevant to biological attachment at liquid interfaces. These results are also consistent with results reported for the oil-water interface,[68] where pellicles of PA01 were still seen to form for ΔpilA mutants, but there was a delay in the formation of the pellicle. Changes to type IV pili structure/function affect attachment but are not essential for attachment.

Results from pilS and pilJ mutants, which regulate formation of pili are less conclusive. The attachment stage of the pilJ mutant is identical to the wild type, whereas the pilS occurs more quickly.

For all strains/mutants in this study, pellicle formation eventually occurs, indicating attachment is resistant to the loss of a single biological mechanism. If attachment occurs when more than one biological mechanism is removed is unclear. These results indicate that attachment may be aided

by surface tension. It is well established,[69] that particles of the same size of the bacteria are permanently trapped by surface tension when at a liquid interfaces. Such strong forces might aid in trapping/attaching bacteria to the interface when a natural biological mechanism is missing. In previous studies of bacterial film formation,[47] attachment was observed to be an individual event that could occur in multiple orientations. Once bacteria were on the interface they would stay on the interface and swim in that 2D plane. This is consistent with the idea that once on interface, bacteria are trapped there by surface tension. These results and hypothesis are also consistent with studies that show pellicle formation is disrupted by the addition of surfactant for a range of bacterial species.[27-29]

Mutants unable to produce biosurfactants also exhibited slower pellicle formation times similar to pili mutants. This appears counterintuitive, since less surfactant would result in greater surface tension, increasing the strength of interfacial attachment. The reason for this discrepancy is currently unclear; pellicle formation of surfactant deficient mutants needs to be further studied to see if the surfactant is affecting more than just the surface tension of the interface.

Motility's role on Elasticity and Surface structure

Given the measurement technique, it is difficult to discern the distinction between growth and spreading. However, it is possible to evaluate overall elasticity which is an indirect measure of the pellicle growth as well as analyze surface structure from SEM data which is an indirect indicator of both spreading and growth.

Typically, on a solid surface, pili play a key role in surface structure formation through spreading, which creates colonies and regulates biofilm size. Whether such motility behaviors extend to the liquid interfaces is not known. Bacteria motility on liquid interfaces has been observed previously with wild type mutants; what biological mechanism cause this motility is not established.[47] In terms of pellicle surface structure, there are some changes observed in pellicles of mutants directly related to the structure/function of pili (pilA, pilD, and pilP). The pilA mutant pellicle was similarly porous to the wild type pellicle but had different fractal dimension. The pilD and pilP pellicles had similar dense bacterial mats on the top layer, but were less porous when compared to the wild type pellicle. Given these observations, it would appear that pili structure/function affect pellicle surface structure and hence possibly spreading and/or growth.

For pilP and pilD mutants, the moduli of the pellicles were similar to the wild type pellicle based on P-value. However, pilA pellicle's modulus was a weak rejection of the null hypothesis. Therefore, it is inconsistent how changes to these structure/function pili mutants affect pellicle elasticity.

The pellicle of pili mutants that relate to regulation/formation (pilS and pilJ) rejected the null hypothesis, indicating there was some difference in final moduli. Why these mutants appear to have an effect in comparison to the structure/function mutants is unclear. Moreover, they have less effect on the pellicle surface structure as evaluated by the top layer porosity and fractal dimension.

Mutants which lacked biosurfactants formed stronger pellicles than wild type. This can be seen based on final G' which were larger than the wild type pellicle moduli (outside of variability shown on the graph through error bars) and further confirmed through rejection of the null hypothesis. It is established that similar mutants create thicker biofilms than wild type bacteria on solid surfaces, which would likely result in stronger films.[44] Compared to the wild type pellicle, the surface structure of these pellicles was somewhat different. Both mutant pellicles were less porous, and the Δrhlb pellicle had denser more plane like structures in comparison to the wild type pellicle. Again, it is established that on solid surfaces similar mutants also affect surface structure, except the changes are observed in biofilm thickness not porosity/complexity.

Flagella lacking mutant's pellicle surface structure did not have any obvious holes in the top layer, which was similar to the biosurfactants mutants' pellicles. Flagella and biosurfactants play a role in swarming motility on solid surfaces; whether such motility also affects the liquid interface is not determined by these tests, but it may be that the similar surface structures of these strains' pellicles could be due to changes to swarming. In comparison to wild type strain, the flagella mutants' pellicle exhibits no peak modulus. Also, the final moduli reject the null hypothesis indicating they are statistically different.

EPS role on Elasticity and Surface structure

From SEM data, the PAO1 pellicle formed dense mats on the top layer with small porosity and a plane like shape. The PAO1 Δpel pellicle had similar behavior, but increased porosity and more complicated structure. The PA14 pellicle also had a dense top layer, but images showed large

"holes" around the edge. Its porosity and fractal dimension where closer to the PAO1 Δpel pellicle than the PAO1 WT pellicle. It would appear that losing either of these 2 EPS components affects pellicle surface structure in quantitively similar ways although why is not clear from these experiments.

In our work, PAO1 Δpel lacking mutant created pellicles at liquid interface that had a larger final modulus than wild type pellicles. Biofilms on solid surfaces form without Pel;[41] our results are consistent with these observations. However, our results are at odds with previous work in which Pel was found to be necessary for pellicle formation in a standing culture.[41, 51] It is unclear why this difference is observed. However, the culture in this study had underlying flow and was not standing.

Psl lacking PA14 mutant pellicles were nearly identical in elasticity when compared to PAO1 pellicles. This indicates that Psl may not be essential in the formation of pellicles, and its functions may be replicated by Pel or Alginate. However, it is not clear how the various EPS components, including alginate (not tested here), work together to form the pellicle.

Comparison to Oil/Water Interfaces

It should be noted, that these results diverge somewhat from previous studies of oil-water interfaces for PA14 and PAO1. In the work of Niepa et al,[68] PA14 and its deflagellated and depilliated mutants were not seen to form elastic like interfaces through dilatational rheometry or particle tracking. However, there are significant differences between this work and that study which may explain these differences. The oil-water interface is significantly different than air-water interface in terms of physio-chemical boundary conditions. For instance, colloids at the oil water interface experience stronger repulsive forces than those at air-water interfaces.[70] Although it is not known by these authors if similar forces are at play, the difference in physio-chemical boundary conditions of the two interfaces may delay biofilm formation. In this work, pellicle formation by PA14 occurred between 12-24 hours; all of the work by Niepa and coworkers ended at ~24 hours of growth. Therefore, if the oil interface did suppress formation, it is possible that the films were simply not given enough time to form in the Niepa work. This hypothesis is supported by the fact that one PA14 mutant, ΔpilC, began to show signs of viscoelasticity at the end of an experiment with pendant drop in the Niepa work. Therefore, we suspect the differences between

the two papers regarding the role of Psl may be due to the changes in attachment time caused by the differences in the oil/water and air/water interface, and that given greater formation times, similar results to ours would be observed at an oil/water interface.

There is additionally another reason that differences in these studies occurred, which is the difference in the media used to grow the pellicles. In particular, this work used LB broth and constantly infused new broth to the bacteria. Whereas, in the Niepa work measurements were performed on salt solution suspensions and the only resource available to bacteria was the hexadecane oil. The work of Niepa and coworkers focused on the differing ability of PAO1 and PA14 to metabolize hexadecane, which is why the difference between the strains was observed in their study. In this work, with a nutrient rich environment, there is no difference in the behavior of the strains which where both able to support themselves on the nutrient rich LB broth.

Conclusion

Pellicle formation of *P. aeruginosa* at simple liquid interfaces displays interesting behavior in attachment. In particular, the ability of the bacteria to attach to the liquid interface while missing individual biological attachment mechanisms including expression of Pel or Psl and changes to the structure/function of pili (as seen through the pilA, pilP and pilD strains) has been observed. Furthermore, faster attachment in the absence of Pel or Psl have also been observed. This is hypothesized to be due to the surface tension affecting the attachment stage, which would not occur for solid surface biofilms.

Due to the measurement techniques we cannot make definitive comments about spreading and growth but can about final surface structure and elasticity. However, these results are inconsistent. In some cases, there were changes to surface structure and elasticity (Pel, pilA, ΔrhlA, ΔrhlB, flagella), in other changes to surface structure but none to elasticity (pilD, pilP), and finally no changes in surface structure were seen with changes in elasticity (Psl, pilJ pilS). It is unclear from these results, how changes to biological mechanisms that affect surface structure, as analyzed in this work, translate to elasticity.

Given the complexity of pellicle formation and the large number of mechanisms involved, it is highly possible that there are other biological mechanisms not studied here that may be affecting

the formation of pellicle that may change conclusions. However, we hypothesize that pellicle formation is aided by surface tension in a way that solid surfaces cannot aid biofilm formation. As we continue to study such systems, physical changes to the interface that modify its properties such as addition of surfactants, particles, or proteins may have pronounced impact on pellicle formation. This study provides a baseline in understanding why these changes may occur, and how to proceed forward in characterizing pellicle systems when interfaces are modified.

Acknowledgements

We wish to acknowledge the NSF CMMI for funding this work (#1635245). The authors would also like to thank Dr. Kendra Rumbaugh of TTU HSC and Dr. Vernita Gordon of UT for all her help in supplying bacteria and educating the authors in bacteria culturing technique.

Supporting Information

Supporting information in the form of the following can be found online: Data and discussion of loss moduli and additional SEM images of bacteria.

Table List

Table 1: Primary strains and associated mutants used to study pellicle interfacial rheology. *Pseudomonas aeruginosa strain PAO1 were obtained from the Seattle P. aeruginosa PAO1 transposon mutant library (http://www.gs.washington.edu/labs/manoil/libraryindex.htm) †Pseudomonas aeruginosa strain PA14 were obtained from the PA14 nonredundant transposon mutant library (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi)

Strain	Gene Knockout	Description
PAO1*	WT	wild type with natural chloramphenicol resistance
	Pel	Pel is a component of the EPS
	pilS	pilS is a sensor kinase that regulates pilA production
	pilJ	pilJ aids in pili assembly results in low pili surface coverage
PA14†	WT	Wild type strain that cannot produce PSL in EPS
	rhIA	rhamnolipidA is a biosurfactant that aids in swarming
	rhIB	rhamnolipidB is a biosurfactant that aids in swarming
	flgK	flagella aid in free solution motility and swarming
	pilA	pilA is a major subunit of pili - results in low pili surface coverage
	pilP	pilP is a component used to align pili
	pilD	pilD is a component of pili motor

Figure List

Figure 1: Storage modulus of PA14 WT (black circles), PAO1 WT (black squares) and PAO1 Δpel (gray squares) pellicles grown over 72 hours. PA14 and PAO1 were measured in 3 independent trials, and average results are shown.

Figure 2: SEM images of air side of the pellicles from Figure 1. These images were taken when pellicles had reached their peak modulus values based on the Figure 1.

Figure 3: Storage modulus of PA14 WT (black circle) and PA14 ΔflgK (white circle) pellicles grown over 72 hours. PA14 was measured in 3 independent trials and PA14 ΔflgK was in 1 independent trial.

Figure 4: SEM images of air side of the pellicles from Figure 3. These images were taken when pellicles had reached their peak modulus values based on the Figure 3. For the PA14 ΔflgK, the modulus at 41 hours were considered the peak value.

Figure 5: Storage modulus of PA14 WT (black circle), PA14 ΔpilD (white circle), PA14 ΔpilP (light gray circle) and PA14 ΔpilA (dark gray circle) pellicles grown over 72 hours. PA14 was measured in 3 independent trials and PA14 ΔpilD, PA14 ΔpilP, and PA14 ΔpilA were in 1 independent trial each.

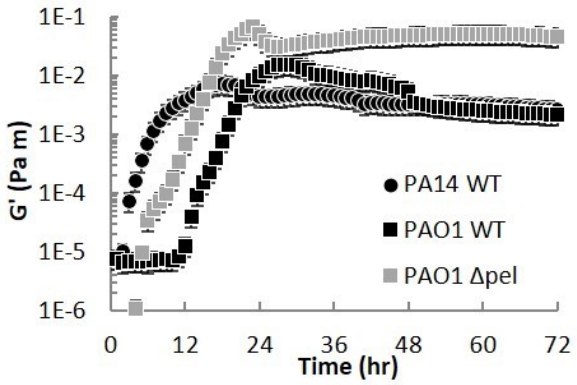
Figure 6: SEM images of air side of the pellicles from Figure 5. These images were taken when pellicles had reached their peak modulus values based on the Figure 5.

Figure 7: Storage modulus of PAO1 WT (black squares), PAO1 ΔpilJ (white squares), and PAO1 ΔpilS (gray squares) pellicles grown over 72 hours. PAO1 was measured in 3 independent trials and PAO1 ΔpilJ and PAO1 ΔpilS Δ were in 1 independent trial each

Figure 8: SEM images of air side of the pellicles from Figure 7. These images were taken when pellicles had reached their peak modulus values based on the Figure 7.

Figure 9: Storage modulus of PA14 WT (black circle), PA14 ΔrhIA (light gray circle), and PA14 ΔrhIB (dark gray circle) pellicles grown over 72 hours. PA14 was measured in 3 independent trials and PA14 ΔrhIA and PA14 ΔrhIB were in 1 independent trial each.

Figure 10: SEM images of air side of the pellicles from Figure 9. These images were taken when pellicles had reached their peak modulus values based on the Figure 9.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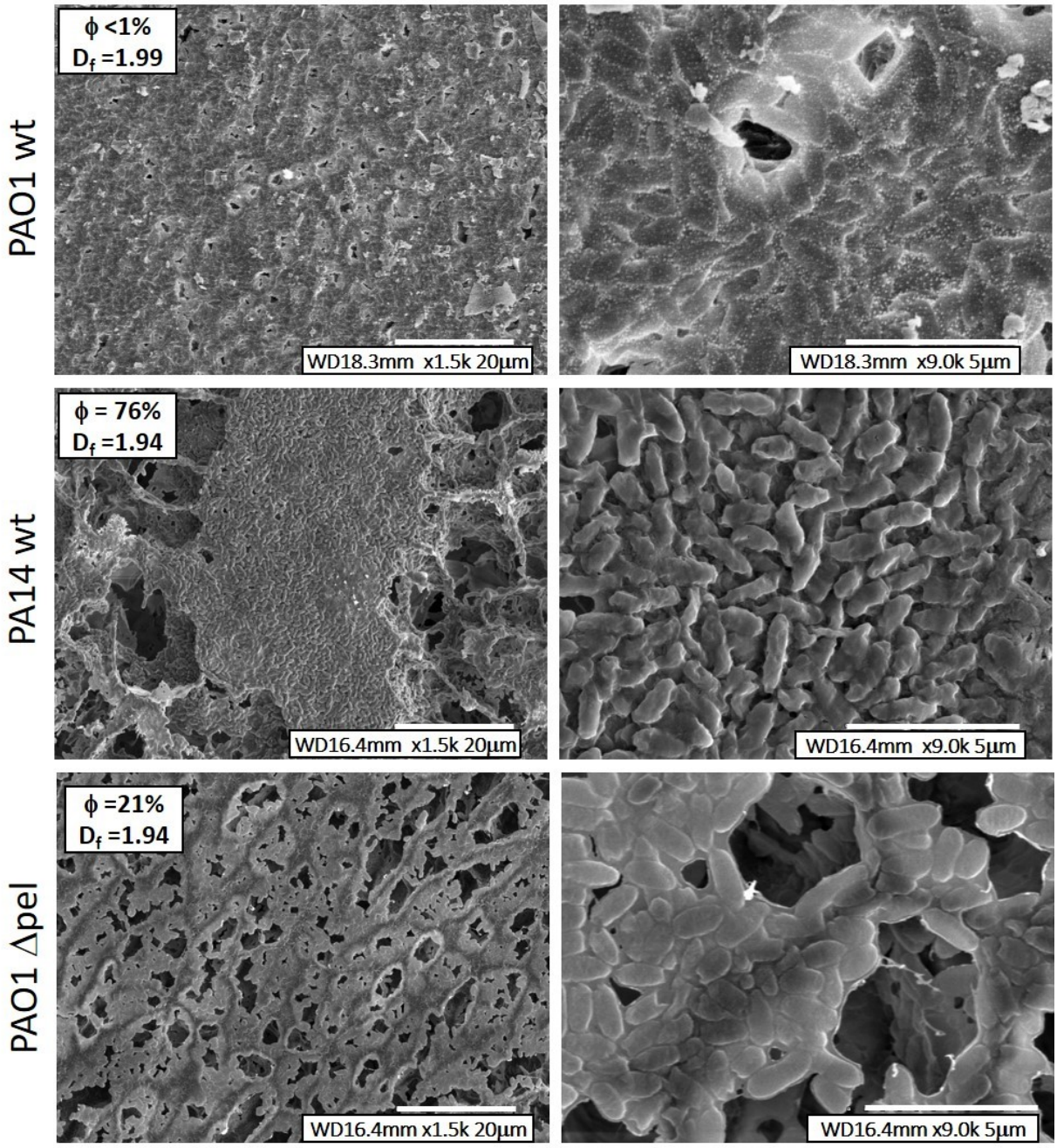
1E-2
1E-3
1E-4
1E-5
1E-6
G' (Pa m)
0
12
24
36
48
60
72
Time (hr)
PA14 WT
PAO1 WT
PAO1 Δpel

Figure 1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WD18.3mm x9.0k 5µm
PA14 wt
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D_f =1.94
WD16.4mm x1.5k 20µm
WD16.4mm x9.0k 5µm
PAO1 Δpel
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D_f =1.94
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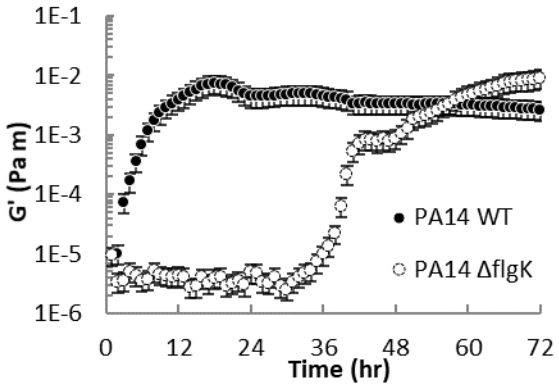
WD16.4mm x9.0k 5µm

Figure 2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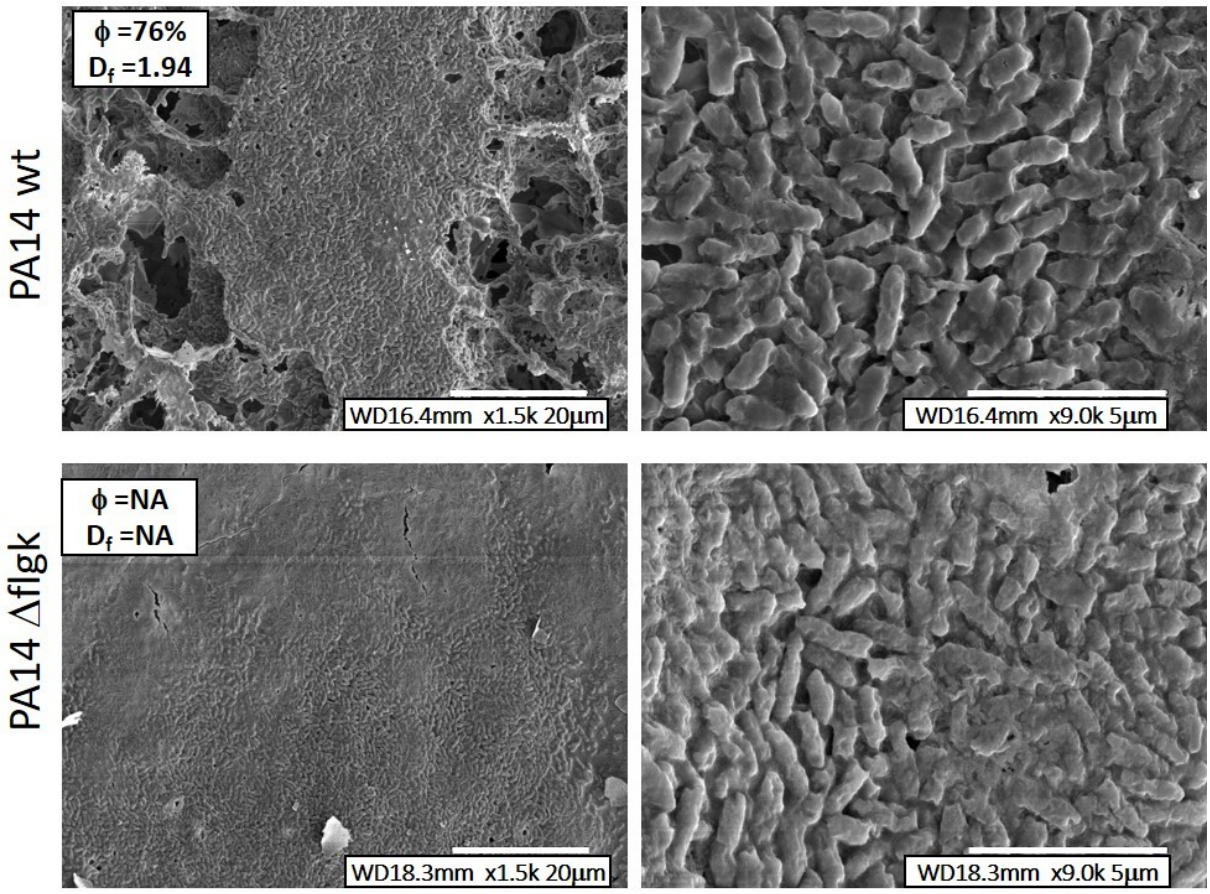
1E-2
1E-3
1E-4
1E-5
1E-6
G' (Pa m)
0
12
24
36
48
60
72
Time (hr)
PA14 WT
PA14 ΔflgK

Figure 3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D_f =NA
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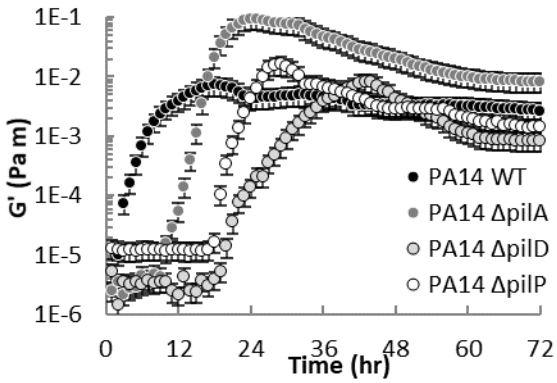
WD18.3mm x9.0k 5µm

Figure 4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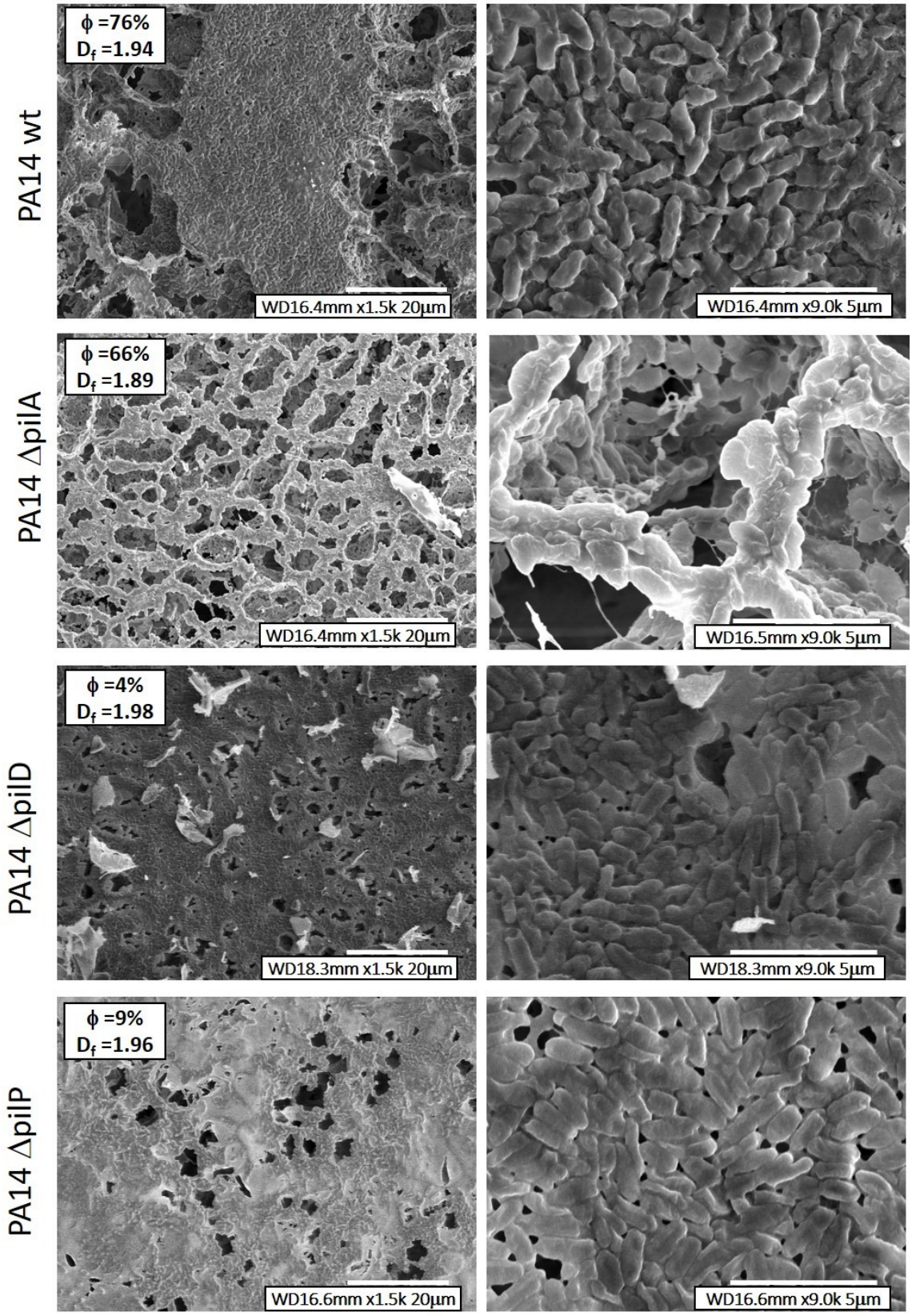
1E-3
1E-4
1E-5
1E-6
G' (Pa m)
0
12
24
36
48
60
72
Time (hr)
PA14 WT
PA14 ΔpilA
PA14 ΔpilD
PA14 ΔpilP

Figure 5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WD16.5mm x9.0k 5µm
PA14 ΔpilD
ϕ =4%
D_f =1.98
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WD18.3mm x9.0k 5µm
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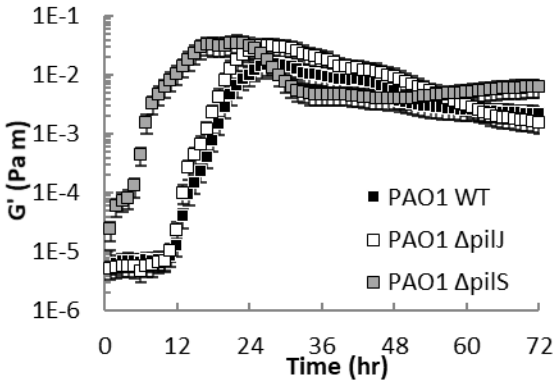
WD16.6mm x9.0k 5µm

Figure 6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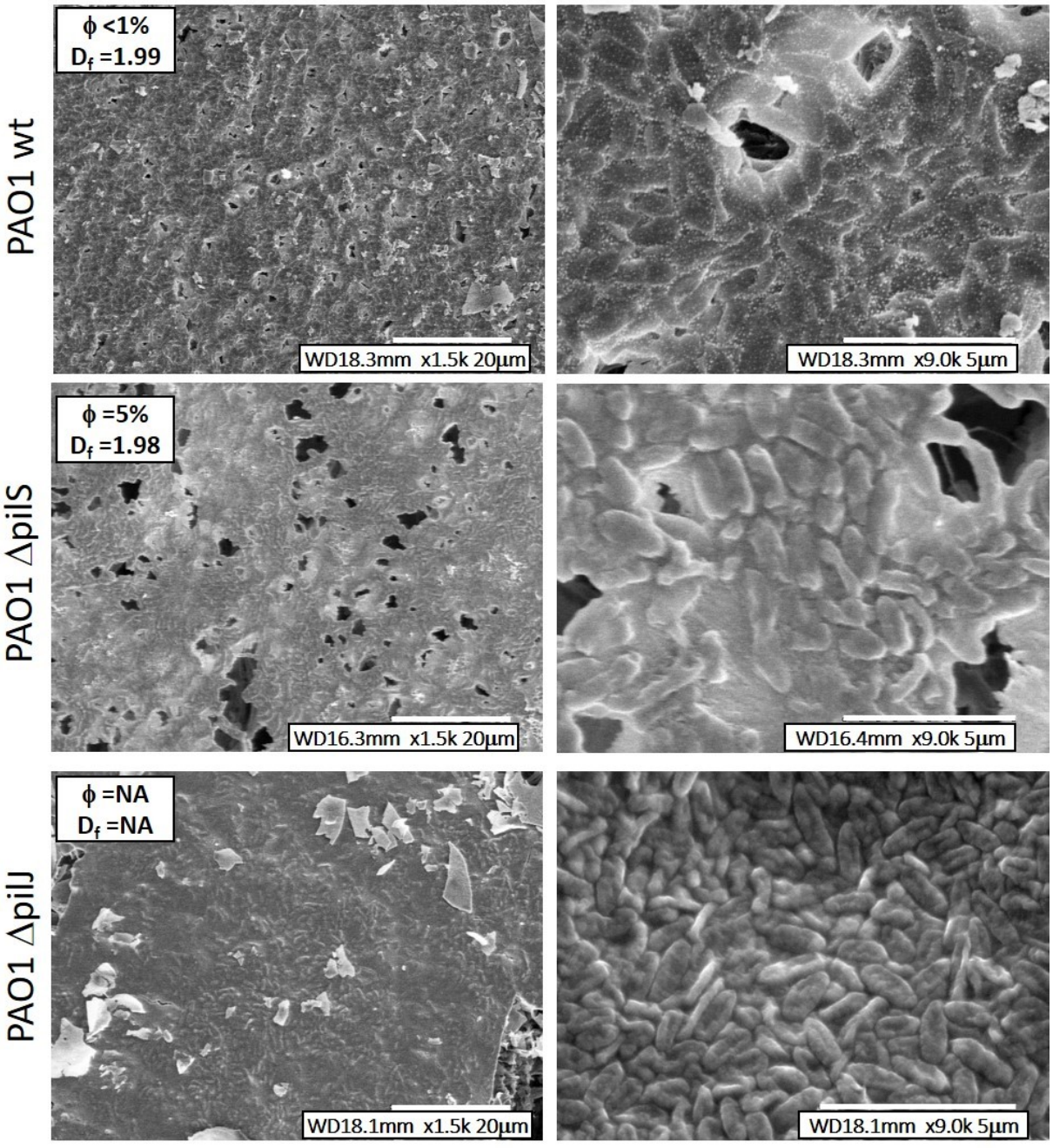
1E-2
1E-3
1E-4
1E-5
1E-6
G' (Pa m)
0
12
24
36
48
60
72
Time (hr)
PAO1 WT
PAO1 ΔpilJ
PAO1 ΔpilS

Figure 7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ϕ <1%
D_f =1.99
WD18.3mm x1.5k 20µm
WD18.3mm x9.0k 5µm
PAO1 ΔpilS
ϕ =5%
D_f =1.98
WD16.3mm x1.5k 20µm
WD16.4mm x9.0k 5µm
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ϕ =NA
D_f =NA
WD18.1mm x1.5k 20µm
WD18.1mm x9.0k 5µm

Figure 8

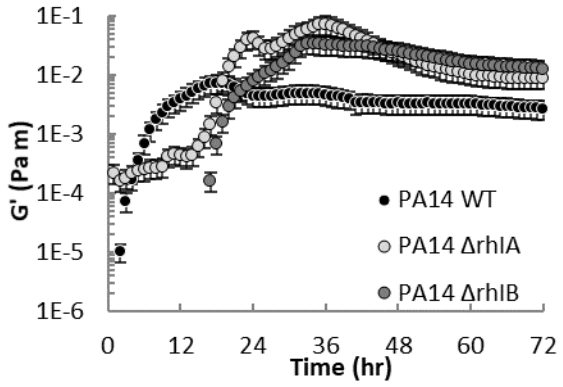
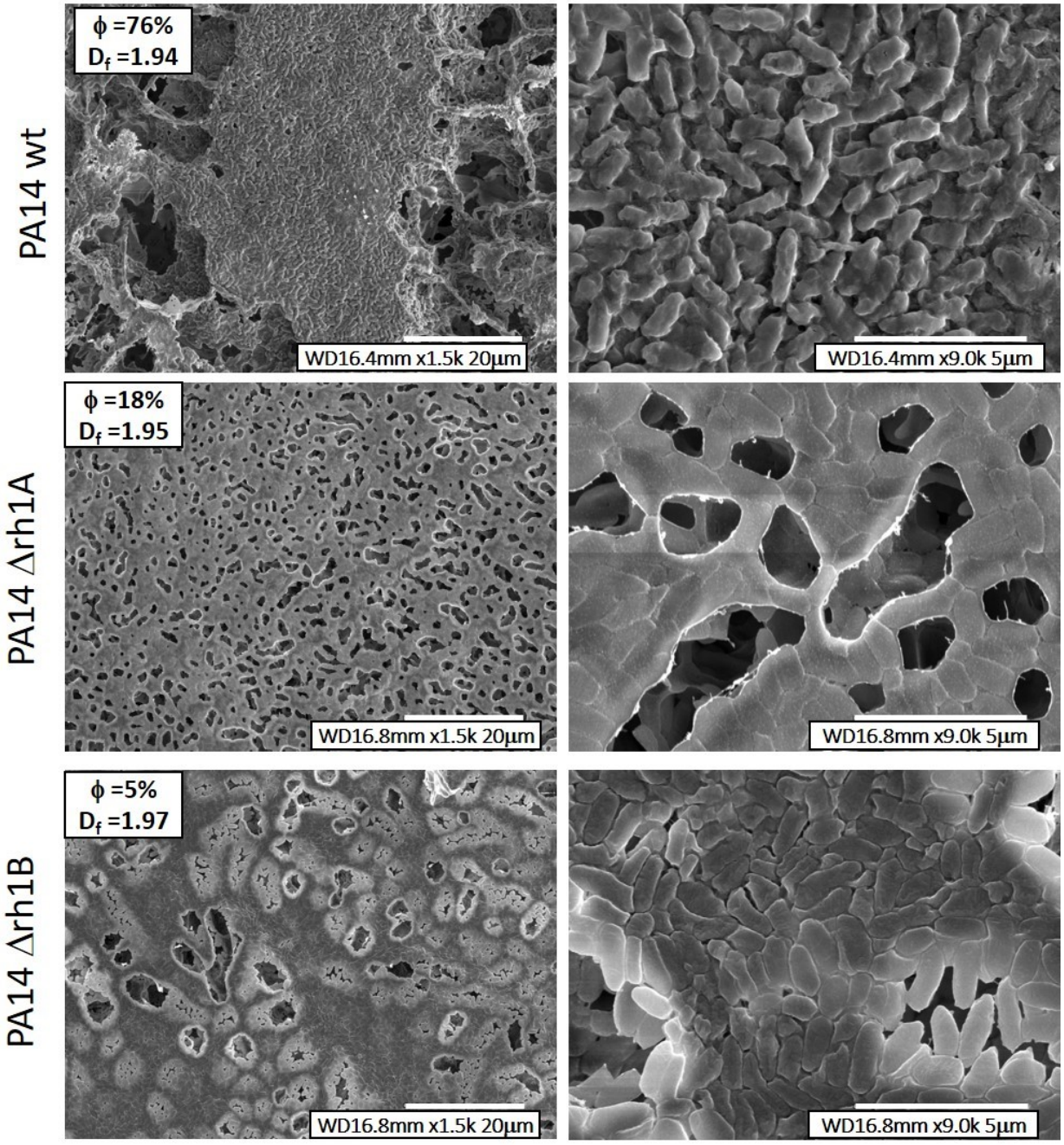

1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
G' (Pa m)
0
12
24
36
48
60
72
Time (hr)
PA14 WT
PA14 ΔrhlA
PA14 ΔrhlB

Figure 9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WD16.4mm x9.0k 5µm
PA14 Δrh1A
ϕ =18%
D_f =1.95
WD16.8mm x1.5k 20µm
WD16.8mm x9.0k 5µm
PA14 Δrh1B
ϕ =5%
D_f =1.97
WD16.8mm x1.5k 20µm
WD16.8mm x9.0k 5µm

Figure 10

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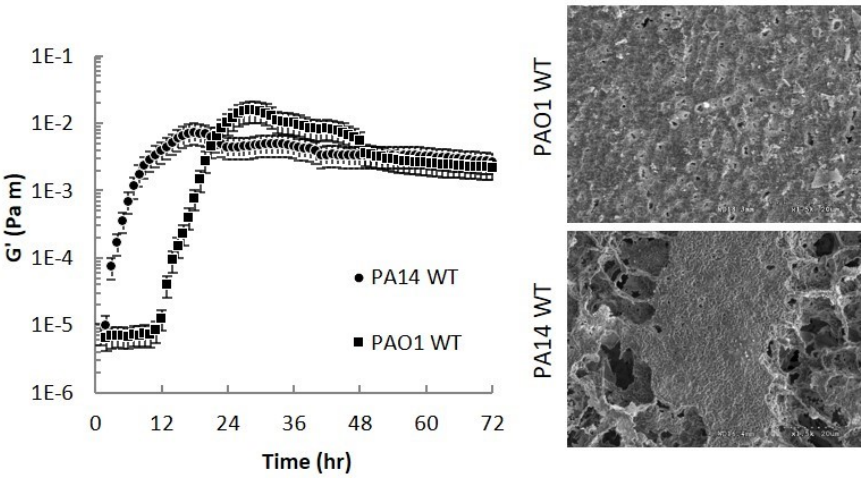
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