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Alginate does not appear to be essential for biofilm production by PAO1 *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **can form biofilms which is of vital importance for a variety of industries. It has been reported that antibiotic resistance is a hallmark of biofilms which is critically significant for the medical industry as the persistence of infectious agents is dangerous. For cystic fibrosis patients, colonization of the lungs with *Pseudomonas* can be damaging. Recently, the antibiotic resistance of biofilms was attributed to alginate, a long chain polymer of uronic acids. Utilizing two strains, each with selective mutations either in *algU*, a transcriptional regulator of the alginate synthesis operon, or *algL*, a lyase that will sever bonds between the monomers of the alginate polymer, biofilm was quantified using a crystal violet assay over 3 days. Since it was not clear if the crystal violet assay will stain the biofilm cells, the extracellular polymeric substance, or both a concurrent growth assay was conducted. The growth patterns between the mutant strains and control were consistent, with the number of biofilm bacteria declining after 1 day of incubation at 37°C. The amount of biofilm increased steadily for the mutant strains but at a slower pace than the wild-type biofilm which declined after one day of considerable expansion. From these results, we speculate that alginate synthesis is not a required attribute in biofilm formation in the PAO1 strain.**

Biofilms are defined as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" (6). These are clinically, industrially, and epidemiologically relevant due their intrinsic ability to resist dispersion and the cytocidal effects of many detergents and disinfectants. The cycle of biofilm formation begins with the adherence of planktonic, free-swimming, bacteria to a surface. The generation of a microcolony or cluster is followed by a size increase of the microcolony, and an eventual inter-connection via an extracellular matrix (25), referred to as the extracellular polymeric substance (EPS) (25). It has been observed that biofilms can increase the resistance to antibiotics and oxidative stress (7, 22, 25). Although resistance classically refers to a unicellular mechanism of evasion of antibiotic effects, the term shall be used to indicate the reduced susceptibility of bacterial cells within the biofilm to inherently toxic conditions. The thick mass of EPS can create an internal nutrient gradient which can isolate cells at the centre and render them metabolically inactive (14). One explanation for biofilm resistance is the fact that many antibiotics only affect metabolically active cells, which would spare the inactive cells contained within the proteinous glycocalyx. Another reported theory is that the antibiotic resistance is due to alginate (7, 14).

Alginate, a virulence factor, is a linear unbranched polymer composed of 1-4' linked saccharides β-D-mannuronic Acid (M) and its C-5 epimer α-L-guluronic Acid (G) (10). Almost all the genes for the biosynthesis of alginate are located on an 18-kb operon under the control of the σ-factor AlgU. Also known as AlgT, AlgU is the anti σ-factor for *mucA*. Mutations in, or inactivation of, *mucA* result in a mucoid phenotype, characterized by shiny colonies when grown on agar; this is due to the presence of a carbohydrate capsule (25). AlgU is a stress-response σ-factor that exhibits functional homology with the *Escherichia coli* σ^E heat shock protein. It acts in conjunction with mucoid proteins MucA and MucB to regulate the transcription of key alginate biosynthesis components, such as the *algK* and *algL* genes (23). Hershberger *et al.* (15) noted that the AlgU protein is autoregulatory in nature, binding its own promoter region. An *algU* knockout would, hypothetically, result in a *P. aeruginosa* strain lacking alginate.

Alginate lyase (AlgL) is encoded by the *P. aeruginosa algL* gene (1). AlgL functions to break up alginate polymers; specifically, alginate lyase cleaves glycosidic bonds between uronate residues in such polymers (4). Previous studies have noted the detrimental effects of administered AlgL on existing biofilms, resulting in depolymerisation of the alginate polymers and a decrease of biofilm integrity, as expected, considering the enzymatic activity of AlgL (7). It is worthy to note, however, that other studies have observed that AlgL plays a substantial role in the formation of the alginate biofilms; experiments have demonstrated that *algL*

knockouts exhibit the loss of the mucoid *P. aeruginosa* phenotype, which is associated with high alginate production (2). The significance of AlgL's role in alginate production is questionable; however, as Bakkevig *et al.* found, *algA* expression restored mucoid phenotypes in *algL* knockouts (2).

Here, the role of alginate in the formation of *P. aeruginosa* biofilms was investigated by contrasting control PAO1 and mutant PAO1 strains Δ*algU*, and Δ*alg*L, which presumably exhibit alterations in alginate production. By quantifying bacterial growth in parallel to biofilm generation across the three strains it was determined that alginate was not essential for biofilm formation.

MATERIALS & METHODS

Bacterial strains and growth conditions. *Pseudomonas aeruginosa* PAO1 was obtained from the University of British Columbia MICB 421 Culture Collection of the Microbiology & Immunology Department. The *ΔalgU* and *ΔalgL* strains, PA01_lux_43_A2 and PA01_lux_26_H7 respectively, were obtained from the *Pseudomonas aeruginosa* PAO1 mini-Tn*5 lux* transposon mutant library (18) maintained by the Hancock Laboratory at The Centre for Microbial Diseases and Immunity Research, UBC. Luria-Burtani (LB) media (1 L; 5 g yeast extract, 10 g tryptone, 5 g NaCl, pH 7.1 ± 0.1) was selected to support growth because it can support the mucoid biofilm phenotype (14). When solid media was needed 15 g of agar was added to 1 L of liquid culture. Overnight cultures were grown at 37ºC in a shaking air incubator while streaked plates for isolation were left on the benchtop and allowed to mature for two days before sampling. All overnight cultures were diluted by a factor of ten before being read at 600 nm in a spectrophotometer (#333182 Spectronic 20+, Spectronic Instruments, Madison, WI).

Biofilm culture. Ninety-six well plates (Falcon #351177, Becton Dickinson & Company, Franklin Lakes, NJ) were seeded with 150 µL of culture diluted to a putative OD_{600} of 0.005 per well at Day -3, -2, -1 and -0.1 (2.5 hrs). Each sample was seeded into 6 replicate wells at each time point. The edges of the plates were filled with LB media, used as a negative control, to avoid edge effects. The 96-well plate was placed in a 37°C incubator overnight. Each incubation was aerobic and not shaken.

Crystal violet assay. The Crystal Violet Assay also known as the Abiotic Solid Surface Assay (SSA) (19) was performed as previously described (5, 12, 14). Briefly, 96-well plates were washed thoroughly with distilled water 3 times. 175 µL of 1% (w/v) crystal violet (#C581-25, Fisher Scientific, Pittsburgh, PA) was added to each well and left to incubate at room temperature to stain any adherent bacteria or negatively charged glycocalyx. After 20 minutes the plates were washed vigorously with water three times after which 200 µL of 100% ethanol was added to the wells and left at room temperature for 15 minutes to release the stain. The ethanol was transferred into a fresh 96-well plate which was subsequently read at 595 nm in a microplate reader (Model #3550, Bio-Rad Laboratories, Hercules, CA). Graph was created and analysed using GraphPad Prism V5.

Growth assay. 13 x 100 mm glass test tubes were inoculated with 1 mL of culture with a putative OD_{600} of 0.005. The tubes were incubated alongside the 96 well plates at the various conditions. At the time of harvest the cultures were emptied of their liquid contents and washed vigorously with water twice. The bottom and sides of the test tubes were scraped with a sterile spatula after which 1 mL of PBS was added to resuspend any scraped cells. The contents of a tube were added to a 2 mL tissue homogenizer tube (narrow ground glass made by Radnoti Glass, Monrovia, CA) to break up any biofilm by using a twist and push motion 12 times. The homogenizer was sterilized with ethanol in between sample processing.

Five hundred microlitres of the mechanical processed samples were added to 500 µL of 40 units/mL alginate lyase (#A1603, Sigma Aldrich, Oakville, ON) and left to incubate at room temperature. After 20 minutes, 100 µL of enzyme treated sample was either diluted in PBS or plated immediately, in the case of the 2.5 hour incubation. The final plated dilutions were 10^{-3}, 10^{-4}, and 10^{-5} for the Day 1 to 3 incubations while the final plated dilutions for the 2.5 hour incubation were 10^{-1}, 10^{-2}, and 10^{-3}. Graph was created and analysed using GraphPad Prism V5.

RESULTS

Bacterial growth curve over a three day culture. In determining the pattern of bacterial growth for all three strains of PAO1, Δ*algU* and Δ*algL* were allowed to grow in glass tubes at 37°C for a period of three days, with harvests performed 3 days, 2 days, 1 day and 0.1 days (~2.5 hours) after bacteria were seeded. Only the bacteria that were stuck in the fixed biofilm were collected and plated, while the free-floating bacteria and suspended biofilm were discarded.

All three strains followed similar growth patterns, shown in Fig.1, with an initial increase in the first 24 hrs, followed by a steady decrease in the latter two time points. In comparing the first 24 hours of each strain, PAO1 had the largest amount of growth (Fig. 1), with a 4000-fold increase in cell quantity measured within the period between the 2.5 hour time-point and the 1 day time-point. This was followed by the Δ*algL* strain, which grew to nearly 500-fold its original cell number, while Δ*algU* exhibited the slowest growth in that period, increasing its cell number by a factor of about 90. It must be noted, however, that, at the 2 hour time-point, the PAO1 had the lowest cell count of the three strains (Fig. 1), suggesting that the PAO1 growth was much slower in the first two hours of incubation, likely due to a prolonged lag phase.

For all three strains, the cell number peaked at Day 1 time-point, as shown in Fig. 1. After day 1, the counts steadily decreased, with the most prevalent drop

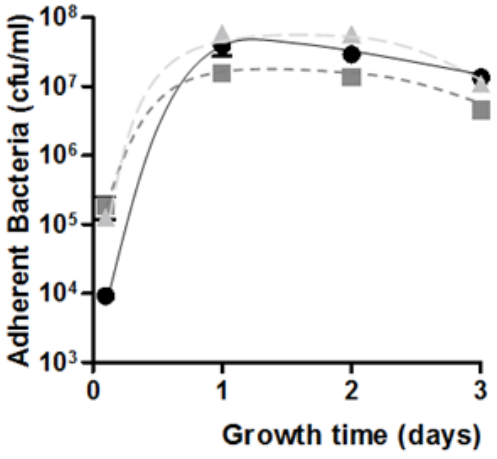

FIG. 1. Counts of PAO1 (●), Δ*algU* (■), and Δ*algL* (▲) in the attached biofilm component. Values represent means ± SEM of two replicates.

existing, unanimously, between Day 2 and Day 3 (Fig. 1). The Day 2 cell counts for PAO1, Δ*algU* and Δ*algL* experienced 27%, 11% and 2% drops, respectively, from their Day 1 counterparts. The Day 3 counts, however, demonstrated more drastic drops when compared to the Day 2 counts: the cultured PAO1, Δ*algU* and Δ*algL* experienced respective drops of 53%, 67% and 81%. As seen, the decrease in cell counts were consistent across all three strains, suggesting the presence of a significant reversal in adhered cell behaviour beyond the first day, from active growth in biofilm to a loss of adherent cells.

Biofilm production over a three day culture. Biofilm production of PAO1, Δ*algU* and Δ*algL*, the three strains of *P. aeruginosa* cultured in 96 well plates over three days was observed. Biofilm that had formed on, and was attached to, the surfaces of the wells was quantified by crystal violet staining.

As depicted in Fig. 2, the quantity of biofilm detected across the time-points showed notable variation across all three strains. The biofilm produced by the PAO1 culture peaked at Day 1, undergoing a steady decrease in the subsequent two days. The biofilm quantity, as quantified by A_{595} readings, dropped 10% between Day 1 and Day 2, and a 14% between Day 2 and Day 3. In contrast, the mutant strains generally showed an increase in biofilm content across the three day culture. The Δ*algU* culture exhibited a fairly notable 28% increase in the A_{595} reading between Day 1 and Day 2, subsequently reaching almost a plateau, with only a slight 5% increase after that. Curiously, the Δ*algL* culture exhibited the opposite pattern of increasing biofilm content, with a minor 0.8% increase in the A_{595} readings between Day 1 and Day 2, followed by a much larger 37% increase between Day 2 and Day 3.

Aside from concurrent increases between the 2.5 hour and the Day 1 time-points, no explicit correlation was observed to exist between the growth of the adherent cells and the measured biofilm content. Examining the individual strain data, the PAO1 biofilm content seemed to match the best with the growth data, with both exhibiting a peak at Day 1 and a steady decrease during the latter two days. The mutant strains, however, both exhibited biofilm content data that did not seem consistent with the growth data. Biofilm content measured in the Δ*algU* and Δ*algL* cultures were observed to increase continuously across the experiment, while the associated growth data showed a peak at Day 1 with a decrease in cell number afterwards. This disparity seems to indicate the lack of a consistently defined correlation between the adhered cell growth and the biofilm content.

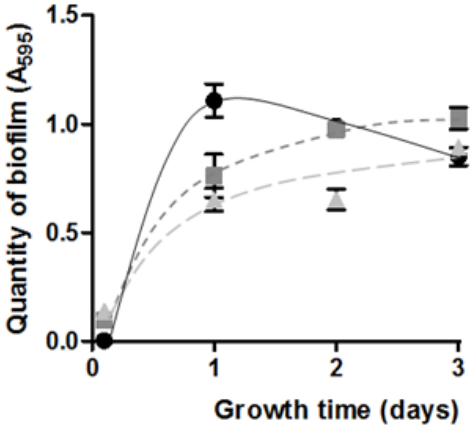

FIG. 2. Production of biofilm by PAO1 (●), Δ*algU* (■), and Δ*algL* (▲). Values represent means ± SEM of six replicates.

DISCUSSION

Although alginate may be important in the structure and persistence of mature biofilms in opportunistic infections (25), its role in the PAO1 developing biofilm is debatable. The results in Fig. 2 suggest that although biofilm levels are lower in the *ΔalgU* mutant at day 1, no observable differences can be seen by days 2 and 3. The data is supported by Wozniak *et al*. (25) who showed that Δ*algD* PAO1 mutants are capable of forming biofilm with similar architecture and antibiotic resistance profiles to wild type PAO1. The *algD* gene product catalyzes the first committed step in alginate synthesis and is not known to be required for any other cellular process (25). The observation contradicted our initial hypothesis, namely that *algU* mutants are unable to produce biofilm. The results found by Cotton *et al*. showed that resistance of PAO1 to gentamicin and ciprofloxacin is due to alginate (7) but the resistance of biofilms to antibiotics is due to its polyanionic profile which can bind cationic antibiotics (16, 21) or to the limited metabolic activity of biofilm cells.

It was observed that the number of adhered cells decreased across the three day culture, and this trend was unanimous across the three strains. The growth profiles (Fig. 1) indicate that the bacteria are either detaching from the biofilm or they are entering a death phase. It is known that bacteria can detach from biofilm to move to a new surface (16, 24); in this case, the bacteria would detach from the biofilm adhered to the glass test tube walls and become free-floating in solution. Since these free-floating bacteria were washed out and discarded, and only the adhered cells were collected, any net migration into the broth would result in decreased cell counts. However when the experiment was continued over day 4, 5, and 6, the wells began to dry up and turn brown indicating that the bacteria were

indeed dying, which was confirmed by the absence of colony forming units on spread plates (data not shown).

The use of crystal violet to assay the quantity of biofilm hinges on its ability to bind negatively charged polymers. It is not clear if crystal violet is binding to colonies of sessile bacteria or to the extracellular matrix. Although the patterns of growth (Fig. 1) and biofilm quantitation (Fig. 2) appear similar for the wild-type strain, it is clear that the biofilm quantities are not directly linked to the pattern of growth. This would suggest that the crystal violet is staining something other than the culturable viable cells in the biofilm. Since DNA, a long chain polymer of negatively charged phosphates, can be stained by crystal violet (9) it is not unreasonable to suppose that the negatively charged exopolysaccharide polymers would be electrostatically bound by the small cation (8).

The lower biofilm levels in Δ*algU* at Day 1 may be supported by a recent finding that Δ*algU* PAO1 mutants exhibited reduced attachment capabilities and impaired stability of biofilm community as compared to wild-type (3). Our results may imply that biofilm formation in AlgU mutants is an initially slow process, but once established, undergoes steady growth. This implication is supported by the observation that AlgD mutants show an initial lag in biofilm development (11). Furthermore, Bazire *et al*. (3) demonstrated that despite having similar thicknesses of biofilm after 2 days of growth the *algU* mutant biofilm was considerably more susceptible to shearing and mechanical breakage, and upon stringent washing less bacteria were retained in the biofilm. Since we did not test for the integrity of the biofilm, no conclusions can be drawn about the structure of the biofilm in the *algU* mutant; however, stringent washing conditions were employed for the growth assay, and bacterial counts were similar between all three strains (Fig. 1). Assuming that the different strains grew to similar levels, it would appear that *algU* only had a minor, if any effect on the retention of bacteria in the biofilm (Fig. 1).

The defects associated with the *algU* mutant are not thought to be due to alginate but rather its role in regulating the production of Psl polysaccharide, another exopolysaccharide component, shown to be integral for the PAO1 biofilm (11). It has been shown to promote cell-cell interaction and assembly of a matrix which holds bacteria attached to the biofilm (19). *AlgU* mutants were shown to have reduced exopolysaccharides as well as lowered levels of *Psl* mRNA expression (3).

The increasing biofilm content apparent in the Δ*algL* culture seems to indicate that the alginate lyase protein does not play an essential role in the formation of new biofilm. We initially speculated that, due to the alginate-depolymerizing action of AlgL, an alginate lyase knockout would result in an increased intracellular level of alginate, and, as such, used the Δ*algL* culture for the purpose of examining the effects of increased alginate on biofilm production, hypothesizing that the increased alginate would correlate with increased biofilm production. The results obtained seem to confirm this hypothesis, as the alginate lyase knockout showed clear increases in the biofilm content over a three-day growth period. The results also appear consistent with data presented by Bakkevig *et al*. (2), who note that alginate lyase is utilized in other *Pseudomonas* species to 'clean up' any excess periplasmic alginate that had failed to undergo extracellular export (2). Instead of eliminating the excess alginate, the PAO1 Δ*algL* bacteria would presumably accumulate alginate in the periplasm which would increase the viscosity and subsequently lower the metabolism of the cell or lead to cell lysis (16). Conversely, the increased periplasmic alginate polymers could undergo eventual exportation. This exportation would invariably lead to an increased availability of alginate in the extracellular matrix, which may incite the increased biofilm synthesis exhibited by the Δ*algL* culture over the three day period.

This supposition, however, is contradicted by the findings of Albrecht *et al.* (1), who have proposed an opposing perspective on the role of alginate lyase in biofilm production, suggesting that AlgL is, in fact, an essential component of the alginate biosynthesis pathway. According to the results presented by Albrecht *et al.*, AlgL is involved in a protein scaffold complex consisting of AlgG, AlgK and AlgX, which, in turn, is involved in the late stages of alginate biosynthesis and thus, an *algL* knockout would result in no biofilm made (1, 16). If this is indeed the case, then the increases in biofilm production seen in the Δ*algL* culture would be deemed alginate-independent. Similarly, it has been hypothesized that the lyase can prime the polymerization complex by contributing small fragments of alginate polymer (26). Therefore, a loss of AlgL would lead to defective alginate polymerization and contribution to the biofilm.

As such, there are three plausible models for the mechanism by which AlgL acts to alter the alginate content in *P. aeruginosa*, and thus, by which to interpret the results obtained from the Δ*algL* biofilm quantification. In one interpretational model, following the assertions made by Yian Wong *et al.* (26) and Albrecht *et al.* (1), the increased biofilm content is interpreted as being completely independent of alginate production, as we assume the *algL* knockout strain would produce no alginate at all. If we follow the inference that a lack of alginate lyase would in fact increase the amount of alginate being exported for further biofilm synthesis, then it is possible to attribute the increased biofilm content over the three day culture with the presumed increased alginate levels in the cells.

Ultimately, it is impossible to differentiate between these two models, and thus, referring to only the Δ*algL* biofilm numbers, the results are inconclusive as to whether alginate plays an essential role in biofilm synthesis or not. When analyzed in conjunction with the erratic Δ*algU* biofilm data, however, one is inclined to reach the assertion that alginate does not play an indispensible role in *P. aeruginosa* biofilm formation.

It is not surprising that it was found that alginate does not play an integral role in biofilms since it has been shown that alginate is not significant component of EPS in *Pseudomonas aeruginosa* (25). In fact the results of that study indicate that the AlgD mutants were more resistant to ciprofloxacin treatment which is in opposition of the findings by Cotton *et al.* (7). The main components of the exopolysaccharides of PAO1 were found to be glucose, rhamnose, and mannose in conjunction with an absence of alginate (25). The remainder of the EPS was likely made of LPS and/or nucleic acids (25). Although there was no alginate present in the supernatant of PAO1 cultures in the study by Wozniak *et al.* (25), PAO1 can produce alginate (14) especially under stress. Leaving the cells to incubate at 37°C with no aeration in LB for up to 3 days may have been sufficient to induce the alginate operon. Whether there was alginate production in the wildtype PAO1 or not it is clear that alginate is not required for the production of biofilm based on the results obtained from the alginate mutants. This conclusion is emboldened by the observation by that PAO1 itself does not produce alginate *in vitro* under favorable conditions (25).

FUTURE DIRECTIONS

The results of our study provided evidence that alginate does not appear to be essential for production of biofilm however the role of alginate in biofilm production by PA01 still remains elusive. Further experiments involving the specific quantification of alginate produced (16, 25) by the different strains should be performed to validate the levels of alginate produced by the mutant strains. Since the Δ*algU* and Δ*algL* PAO1 mutants that were employed in this experiment possess a luciferase reporter, it would be of additional interest to perform a luciferase assay on the temporal expression of these genes, to possibly ascertain expressional changes due to aging of the biofilm as the literature differs in the timing of alginate synthesis (14, 16). Changing the growth conditions from static to dynamic with varying degrees of flow prior and post adherence would allow for the evaluation of the integrity of the varying biofilms (3). There are also more sensitive techniques for the quantification of biofilm that, if used, may eliminate some of the inherent problems of the crystal violet assay; crystal violet staining is not restricted to biofilm, making the final analysis difficult. For example, utilizing ruthenium red (13) or wheat germ agglutinin conjugates (5), in binding specific biofilm components, such as alginate, may provide more definitive results. Additionally, better methods to quantify adherent cells could be employed such as a fluorescent stain, for example acridine orange (19). In order to truly reveal the validity of the crystal violet assay, a constitutively expressing green fluorescent protein strain capable of forming biofilms could be assayed to compare the relative change in fluorescence to the relative change in crystal violet binding.

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