Journal of Experimental Microbiology and Immunology (JEMI) Vol. 15: 71 – 78

Role of Alginate in Gentamicin Antibiotic Susceptibility during the Early Stages of *Pseudomonas aeruginosa* PAO1 Biofilm Establishment

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The primary cause attributed to decreased susceptibility of *Pseudomonas aeruginosa* to antibiotics is its ability to form dense, mucoid microbial communities known as biofilms, of which alginate is an important component. In this experiment we attempted to determine the point at which alginate becomes critical for reducing gentamicin susceptibility of *P. aeruginosa* PAO1 biofilms. We predicted that treatment of *P. aeruginosa* with alginate lyase would increase susceptibility, particularly in mature biofilms when alginate becomes a predominant biofilm component. Gentamicin susceptibility was tested on three strains, wild type PAO1, alginate overexpressing mutant *ΔmucA,* and underexpressing mutant *ΔalgA*. Results showed that although antibiotic susceptibility decreased with increased growth, there was no significant difference between the lyase treated and control samples in any strain. No significant change in susceptibility was observed between wild type PAO1 and *ΔmucA*, but *ΔalgA* exhibited a higher susceptibility within the 6 to 24 hour time frame. Using the wild type strain only, we showed that alginate concentration increased over time, although the alginate lyase treatment did not appear to have any effect. However, the concentration of reducing sugars showed a significantly higher increase in the enzyme treated biofilms, suggesting alginate degradation. Due to these conflicting results, we were unable to determine whether or not the lyase treatment was effective in decreasing the alginate concentration. Further experiments are required to either support or refute our hypothesis.

Pseudomonas aeruginosa is a Gram-negative bacteria commonly associated with opportunistic and nosocomial infections, most notably among the immunocompromised and cystic fibrosis (CF) patients (23, 26). A major challenge in the treatment of *P. aeruginosa* infections has been largely accredited to its ability to aggregate into microbial communities referred to as biofilms which is associated with its broad-range resistance against many commonly utilized antibiotics (20).

Biofilm mode of growth facilitates increased bacterial survival and persistence under unfavorable environmental conditions (8, 11). Biofilms have significant implications in medical settings, primarily due to their resistance to antimicrobial agents and the host immune system, resulting in contamination of medical equipment, chronic infections, and high mortality rates (15, 23, 29). Studies measuring the sensitivity of bacteria in biofilms to various antibiotics in comparison to their planktonic counterparts have indicated a decrease in susceptibility from 10- to 1000-fold when bacteria are in the biofilm mode of growth (14). An essential component of bacterial biofilms is the extrapolymeric substance (EPS) which serves to reinforce its structural integrity and confers increased antimicrobial resistance though multiple mechanisms which remain unclear (19, 23). The EPS is a matrix of polysaccharides, proteins, and nucleic acids, and plays important roles in initial attachment, cell-cell interactions and tolerance (14, 15). The composition of the matrix is dynamic and is directly influenced by environmental conditions, biofilm age, and bacterial strain (15).

Until recently, alginate has been considered the major polysaccharide component of EPS responsible for biofilm structure and recalcitrance, and thus has been the predominant prognostic indicator of *P. aeruginosa* infection in the CF lung (19, 29). Alginate is an acetylated homopolymeric polysaccharide consisting of β-1,4-linked L-guluronic acid and D-mannuronic acid (15). For the past decade, the exact role of alginate in biofilm development has been under investigation (28).

Evidence from several recently conducted studies suggests that alginate is not an essential component of the EPS during biofilm attachment and early formation (19, 23, 29). A more detailed investigation into the growth pattern of *P. aeruginosa* biofilms has indicated that at some point after microcolony formation, there is a phenotypic conversion from a nonmucoid to a mucoid form, characterized by an overproduction of alginate (19, 21, 29, 32). Prior to switching to a mucoid phenotype, alternative polysaccharide-encoding gene clusters, *psl* and *pel*, are necessary factors in the early stages of biofilm attachment and formation in *P. aeruginosa* PAO1 and PA14 strains, respectively (19, 23, 29). Alginate is currently believed to dominate in mature biofilm stages and is believed to be more important within the context of persistent infections and enhanced antimicrobial tolerance rather than in biofilm attachment and initiation (14, 29). In support of this, analyses of the carbohydrate composition of the EPS of nonmucoid PAO1 and PA14 *P. aeruginosa* strains have shown that the predominant carbohydrates present include glucose, mannose, and rhamnose and not mannuronic or guluronic acids, indicating that alginate is not present in the nonmucoid EPS (19, 32). This is furthered by biofilm kinetics studies which have demonstrated that there is no significant difference in biofilm formation rates between wild type PAO1 and PA14 strains compared to *algD* mutant strains, *algD* being an alginate biosynthetic gene responsible for the first step in alginate biosynthesis (32).

Cotton *et al.* showed that treatment of *P. aeruginosa* PAO1 biofilms with alginate lyase resulted in reduced cell survival when exposed to gentamicin (9), although it has yet to be determined at which point in the establishment of a biofilm alginate becomes primarily responsible for antibiotic resistance. In this experiment, we aimed to determine the point in *Pseudomonas aeruginosa* PAO1 biofilm maturation at which alginate becomes the necessary structural component of the EPS for lowered antibiotic susceptibility. We predicted that over time, treatment of *P. aeruginosa* PAO1 with alginate lyase would result in an increased sensitivity to the antibiotic gentamicin, as compared to untreated cells. Our results show an accumulation of alginate over time, but digestion with alginate lyase did not show any increase in gentamicin susceptibility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas aeruginosa* strains wild type PAO1, PAO1-UW *ΔmucA* (locus ID: PA0763) and PAO1-UW *ΔalgA* (locus ID: PA3551) were obtained from the University of Washington *Pseudomonas* mutant library (18). Initial overnight cultures were made by inoculating 250 ml of Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract, 5 g NaCl per l at a final pH 7.0) with the appropriate strain and allowed to grow at 37°C on a shaking platform at 150 rpm. Cultures were grown by inoculating 3 ml of LB media with 250 ul of the corresponding overnight starter culture, and incubated at 37°C without shaking for 0, 1, 3, 6, 24, or 48 hours.

Dry weight determination. Dry weight determination was performed to assess biofilm growth over the experimental time course. For each of the biofilm ages, all three strains were grown in triplicate. Biofilms were detached from the culture tubes by vigorous vortexing, and the contents of the tube were emptied on a pre-weighed Whatman filter paper (9.0 cm, low ash). The tubes were washed several times with distilled water to ensure that all the biofilm was detached and transferred onto the filter paper. Excess fluid was removed via vacuum filtration and filters were further dried overnight at 90°C.

Alginate lyase digestion. At the appropriate biofilm age, 20 units/ml of alginate lyase from *Flavobacterium sp.* (Sigma-Aldrich Cat #A1603; 28000 units/g) were added to the treatment tubes and mixed in by gentle vortexing. Both treatment and control cultures were incubated for 1 hour at 37°C without shaking to allow for optimal digestion.

Kill curve assay. The kill curve assay was performed on all three *P. aeruginosa* strains to assess differences in antibiotic susceptibility between treatment and control of each respective strain as a result of their varying alginate expression levels following alginate lyase digestion. After enzymatic treatment, 1.8 mg/ml of gentamicin sulphate (Sigma-Aldrich Cat #3632) was added to both control and treatment cultures of each strain. Cultures were vigorously vortexed until homogenized. Spread plates (LB media supplemented with 15 g/l of agar) were performed using 50 ul aliquots of each sample over the course of 2 hours to observe their rate of death. The cultures were plated prior to antibiotic addition (0 min) and at 15, 45, 75 and 120 min post-antibiotic treatment. Culture tubes were kept in the 37°C incubator without shaking in between assaying time points. Plates were incubated overnight at 37°C and counted the following day.

Biofilm physical homogenization. Physical disruption of the biofilms was performed to create a homogenous culture appropriate for assaying. Following enzymatic digestion, both control and treatment cultures were vortexed vigorously for 10 seconds and physically disrupted using a tissue homogenizer (Pyrex, 19 x 150 tissue homogenizer, #7725-19) by repeating a push/twist motion ten times. The homogenizer was washed once with 70% ethanol and again with distilled water between samples. Samples were briefly revortexed before being separated into two aliquots; one for the Park & Johnson and another for the carbazole assay.

Park & Johnson assay. This assay was used to detect changes in reducing sugar concentration, in the form of algino-oligosaccharides with reducing sugar terminals, as a result of alginate digestion. The assay was scaled down by half from the original publication by Park and Johnson, and KCN was substituted with NaCN (26). Briefly, 0.5 ml each of carbonate cyanide (5.3 g/l Na_2CO_3 and 0.65 g/l KCN) and ferricyanide (0.5 g/l $K_3[Fe(CN)_6]$) were added to the samples, which were then incubated for 15 minutes in a 100°C water bath. Samples were allowed to cool down at room temperature for 5 minutes, and then reacted with 2.5 ml of ferric iron solution (1.5 g/l $FeNH_4(SO_4)_2$, 1.0 g/l SDS, prepared in 0.05 N H_2SO_4). Color was allowed to develop by incubating the samples at room temperature for 15 minutes prior to reading the absorbance at 690 nm. Values were compared against D-glucose standards ranging from a 0 ug/ml blank to16 ug/ml.

Alginate precipitation and uronic acid-carbazole assay. The carbazole assay was performed to quantify differences in total amount of uncleaved alginate in digested and undigested samples. An initial precipitation step utilizing cetyltrimethylammonium bromide (CTAB) was performed to precipitate and purify uncleaved alginate from the culture tubes. The procedure was carried out as described by de Jong *et al.* and Bitter and Muir (4, 12). Aliquots of 1.5 ml of previously homogenized sample were transferred into clean 16 mm x 125 mm test tubes. An equal volume of 0.9% saline was added to each tube,

vortexed briefly, and then adjusted to pH 4.0-4.5 using 0.1M HCl or 0.1M NaOH as necessary. 30 ul of 1% aqueous CTAB was added to each acidifed sample. Following overnight incubation at 4˚C, the tubes were centrifuged at 10000 rpm for 15 minutes (Beckman J2-HS centrifuge; JA-20 rotor). Pellets were subsequently washed with 1.0 ml of ethanol (saturated with sodium acetate), re-centrifuged, and dried overnight at 42˚C. Dried pellets were stored at room temperature until assayed for uronic acids at which time they were reconstituted in 0.5 ml of 0.1M NaOH. For uronic acid quantification, the entire 0.5 ml volume of each sample was carefully layered onto 3 ml of previously frozen (-80˚C) sulfuric acid and mixed by gentle pipetting up and down three times while on ice. Mixed samples were then shaken at 300 rpm at 4˚C until cooled. The tubes were heated for 10 minutes in a 100˚C water bath and cooled to room temperature. Then 0.1 ml of carbazole solution (0.125% carbazole in 100% ethanol) was added; tubes were vortexed and then reheated in a 100˚C water bath for 15 minutes. Samples were cooled to room temperature before optical density (OD) was read at 530 nm. D-glucuronic acid standards of 1 to 40 ug/ml were prepared by dilution with distilled water. All samples or standards were blanked against sulfuric acid reagent.

Statistical analysis. Data points shown represent the average value of three replicates. Error for spread plates was calculated by taking the square root of the number of colonies per plate. For the kill curve assay, error in the slope was documented both as the correlation between the calculated line and the data (Pearson's correlation coefficient: R2) and the standard error of the slope. The error associated with the ratio of control to treatment kill curve slopes were calculated though standard error division. All other error bars were calculated using 95% confidence intervals.

RESULTS

Assessment of biofilm growth. Biofilm growth was monitored via a dry weight assay to determine if *P. aeruginosa* entered a stage of metabolic quiescence typical of established biofilms over the course of the experiment. This data was further meant to serve as a normalization factor for the remaining assays. Despite being run multiple times, the results were not useful because its high error range led to large fluctuations in the biofilm mass which were not consistent with a normal growth pattern or with our visual observations (data not shown).

Alginate lyase causes biofilm detachment and promotes its physical disruption. Biofilm formation was observed after 3 h of growth, starting with the formation of a thin layer around on the inner walls of the test tube at the liquid-air interface. Following the 1 h digestion period with alginate lyase, biofilms were visibly detached from the tube in the 24 h and 48 h biofilm ages. During homogenization, control cultures at all ages generated a significant amount of foaming, a phenomenon not seen among the treatment tubes, and were physically harder to disrupt.

Wild type, *ΔmucA* and *ΔalgA* PAO1 death rates inversely correlate with increased alginate production. The susceptibility to gentamicin decreased as the biofilm aged for all three strains, represented by the progressive decrease in the steepness of the kill curve (graphs not show). To be able to compare the three strains across time, the absolute value of the slope of each individual kill curve was plotted against the biofilm ages for the control (Figure 1) and alginate lyase treated biofilms (Figure 2). Higher values indicate a greater sensitivity to gentamicin. These figures both illustrate a 2- to 3-fold decrease in gentamicin susceptibility in all three strains between ages 0 to 6 h for the control and enzyme-treated samples. Past the 6 h stage, the *ΔalgA* strain showed a relatively consistent level of susceptibility to the antibiotic treatment. In contrast, the wild type and *ΔmucA* strains continued to decrease in sensitivity to gentamicin, reaching a minimum antibiotic susceptibility at the age of 24 h, before becoming more sensitive again at 48 h. The near identical curves of these two strains indicates that an increase in alginate expression, as compared to the wild type, did not further protect *P. aeruginosa* from gentamicin within the first 48 h of biofilm formation. Loss of alginate as represented by *ΔalgA,* however, did result in an increased susceptibility to gentamicin in the mid-ranged ages of our experimental growth. These results suggest that alginate does play an important role in antibiotic sensitivity during this time period (6-24 h). Finally, the convergence of all three curves at the 48h stage indicates that alginate expression levels ceased to be the main factor in gentamicin resistant in mature biofilms.

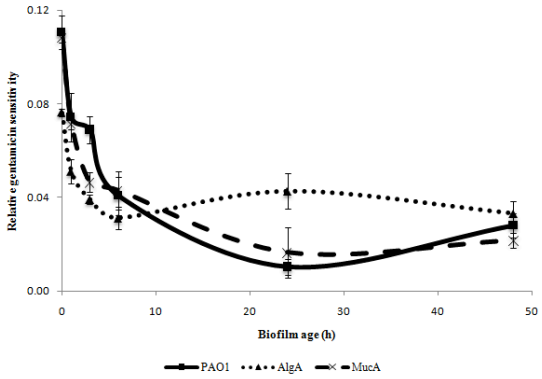

FIG. 1. The relative sensitivity to gentamicin of control cultures of wild type, *ΔalgA,* and *ΔmucA* PAO1 *P. aeruginosa*. The relative sensitivity values plotted represent the absolute value of the semi-log slope of kill curves performed for each biofilm age. Higher values indicate a greater sensitivity to gentamicin. Error bars represent the standard error derived from regression analysis on each individual kill curve.

No significant change in antibiotic susceptibility was observed from lyase treatment for wild type, *ΔmucA* or *ΔalgA* PAO1. Comparison of Figures 1 and 2 indicates there is no significant difference in the overall trend of gentamicin sensitivity between the control and lyase treated samples. For all three strains, the initial susceptibility was high and decreased with

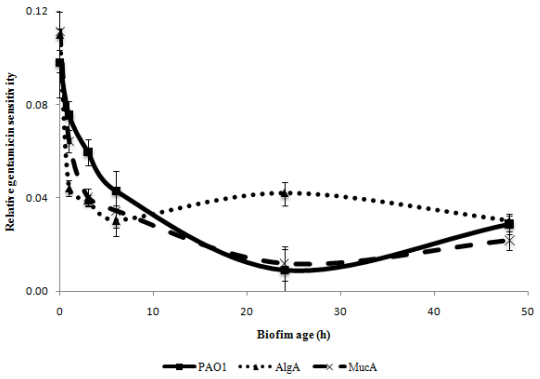

FIG. 2. The relative sensitivity to gentamicin of alginate lyase treated cultures of wild type, *ΔalgA,* and *ΔmucA* PAO1 *P. aeruginosa*. The relative sensitivity values plotted represent the absolute value of the semi-log slope of kill curves performed for each biofilm age. Higher values indicate a greater sensitivity to gentamicin. Error bars represent the standard error derived from regression analysis on each individual kill curve.

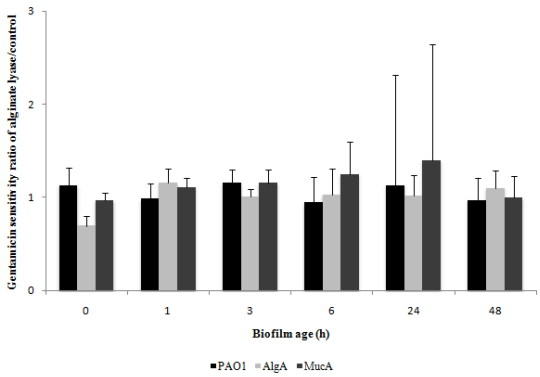

FIG. 3. Gentamicin sensitivity ratio of alginate lyase treatment-to-control for the wild type, *ΔalgA, and ΔmucA* PAO1 *P. aeruginosa.* The consistent variation around 1.0 indicates no difference between the control or treatment values. Error bars represent the values obtained after conventional error division of those associated with the treatment and control kill curve slopes.

time in a similar fashion. Further illustration of this lack of variability is represented in Figure 3, which graphs the ratio of the control to treatment kill curve slope at each assayed age and for each strain. A ratio of 1 indicates that there was no difference in the rate of death between the control and treatment. All ratios include, or are close to, 1. No significant difference was observed between different strains or different ages, indicating that the changes in antibiotic susceptibility over time in each strain cannot be attributed to the lyase treatment.

Uncleaved alginate levels are not altered by alginate lyase digestion. The carbazole assay was used to quantify the levels of alginate in *P. aeruginosa* PAO1 biofilms at each of the six sampled ages. This assay was performed on CTAB precipitates, which allowed isolation of only uncleaved alginate, rather than monomeric uronic acids from the biofilm. Figure 4 shows that the initial cultures (0 h) already had some alginate derived from the starter culture at concentrations of 2.23 and 2.96 ug/ml for the treatment and control cultures, respectively. Alginate levels had slight fluctuations without any significant synthesis during the first 6 hours of incubation, followed by a rapid increase of approximately 3-fold between 6 and 24 h, and by an additional 1.5-fold increase between 24 and 48 h, to final values of around 15 ug/ml. The is no statistical difference in alginate concentrations between treated and control cultures.

Reducing sugars in biofilm increase with alginate lyase digestion. The Park & Johnson assay (27) was carried out to determine the concentration of algino-oligosacchaides with reducing sugar terminals released as a result of alginate lyase digestion. Figure 5 shows the concentration of reducing sugars for each biofilm age in the treatment versus control biofilms. Data for 0 and 1 h are omitted due to errors in procedure. Both treatment and control cultures show a steady, but non-linear increase in the concentration of reducing sugars as the cultures aged. The levels are increasingly higher in the alginate lyase treated cells, with the most noticeable difference at the 48 h age, where there is a difference of 5 ug/ml.

DISCUSSION

Multifactorial antibiotic resistance in *Pseudomonas aeruginosa* has been associated with its ability to form biofilms. To date, the role of alginate, a polysaccharide comprising β-1,4-linked L-guluronic acid and D-mannuronic acid (15), in biofilm formation and antibiotic resistance is still unclear. This investigation was aimed at determining the point during early biofilm establishment at which alginate becomes an essential component of the extrapolymeric substance (EPS) facilitating a decrease in antibiotic sensitivity. We hypothesized that treating biofilms with alginate lyase would sensitize *P. aeruginosa* to the antibiotic gentamicin, and that this effect would be most noticeable at mature stages, when alginate was present at higher levels in the EPS. Our results, however, showed no difference in gentamicin resistance between treated and untreated biofilms.

It has been previously demonstrated that *P. aeruginosa* PAO1 strain biofilms exhibit a significant increase in resistance to numerous antibiotics in

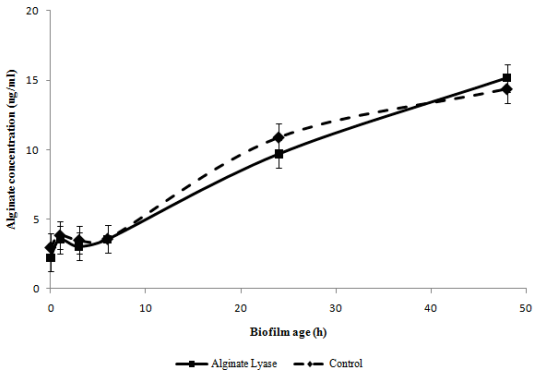

FIG. 4. Alginate concentration (ng/ml) of various biofilm ages of *P. aeruginosa* PAO1.

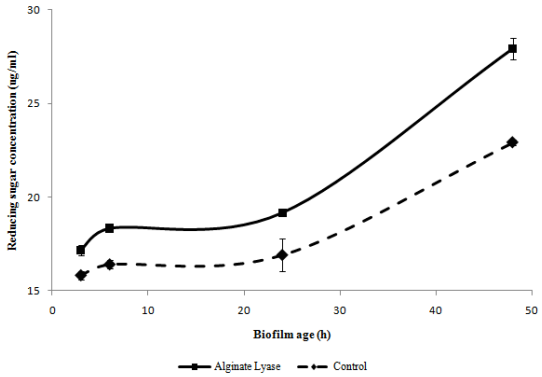

FIG. 5. Effect of alginate lyase digestion on the concentration of reducing sugars in *P. aeruginosa* PAO1 biofilm. Data for 0 and 1 h not shown. Error bars represent 95% confidence intervals. Some error bars were too small to be visible.

comparison to planktonic cells (6). The susceptibility of a 5 day-old biofilm to gentamicin in particular was 128-fold lower than that of the planktonic counterpart (6). Treatment of biofilms with alginate lyase has been shown to enhance the diffusion of various antibiotics, notably gentamicin and tobramycin, though the EPS formed by mucoid *P. aeruginosa* (15, 16). Cotton *et al.* demonstrated this in a procedure involving treatment of *P. aeruginosa* PAO1 biofilms with a combination of alginate lyase and gentamicin. At the early stage of growth (1 day after formation), the percentage of survival of *P. aeruginosa* cells following gentamicin treatment was 9%, and after gentamicin and alginate lyase treatment combined, it was 5%. In mature biofilms (5 days after formation) alginate lyase reduced cell survival from 31% to 0% when administered in combination with gentamicin (9). The results of these studies indicate that not only does alginate act as a barrier to effective antibiotic activity, but also that this phenomena increases as the biofilm matures.

In this experiment, we used two mutant strains to assess whether or not differences in sensitivity to gentamicin changed with different expression levels of alginate. The *algA* gene codes for phosphomannose isomerase-guanosine diphospho-mannose pyro-phosphorylase (PMI-GMP), an enzyme that catalyses two steps in the alginate biosynthetic pathway, converting fructose-6-phosphate to mannose-6-phosphate in the first step, and later mannose-1-phosphate to GDP mannose (7). The strain used in this experiment is a knockout *ΔalgA* and therefore has very low alginate levels in its EPS. The *ΔmucA* mutant, in contrast, overexpresses alginate since MucA is a repressor of AlgU, a factor that promotes transcription of the alginate biosynthetic operon (10). We therefore predicted that alginate lyase treatment would have little to no effect on the *algA* mutant, and a greater effect on *ΔmucA* gentamicin sensitivity compared to the wild type PAO1 strain.

Based on our results, we can correlate an increase in PAO1 alginate production (Figure 4) to a decrease in PAO1 antibiotic susceptibility (Figures 1 and 2). The lack of significant difference in gentamicin susceptibility between alginate lyase-treated and control biofilms suggests, however, that this may not be a causative relationship. This is supported by the observation that the largest decrease in antibiotic susceptibility (0-6h, Figures 1 and 2) did not correspond to the largest increase in alginate concentration (6-48h, Figure 4), indicating that alginate production does not linearly correlate with changers in susceptibility and there are possibly other factors involved. Indeed, various publications have indicated that the EPS is composed of several other polysaccharides which may contribute significantly to biofilm-associated antibiotic resistance (31). These findings are contradictory to the results reported by Cotton *et al.*, which indicated that gentamicin susceptibility increased by 4% in early PAO1 biofilms (24 h) after treatment with alginate lyase (9). No significant difference was observed in antibiotic sensitivity between the strains which overexpress and underexpress alginate at the mature stages, in which alginate is present at its highest concentrations, suggesting that alginate production may not be a vital factor in antibiotic resistance in early biofilm formation.

The validity of our observations is contingent on whether or not alginate lyase was able to perform a significant degree of digestion. Our concern on this point arose mainly from the lack of difference in alginate levels between control and treatment cultures as seen in Figure 4. The results seemed to suggest either

that there was little enzymatic digestion or that we mistakenly assayed for total alginate concentration. The latter explanation, however, is unlikely since previous studies have corroborated with several uronic acid-containing carbohydrates that addition of CTAB to previously digested samples did not precipitate the monomers, confirming the method's specificity for the intact polymeric form (13, 31). An important caveat is that although CTAB is specific for acidic polysaccharides, as compared to other precipitation methods, it has been shown to recover as little as 50% of the total alginate (3). Added to this is the fact that we were unable to normalize our results to biofilm growth since the dry weight assay did not give reproducible results.

The carbazole assay was performed on only the purified CTAB precipitates, and it should have only detected the undigested alginate. We, therefore, expected to see a decrease in alginate levels in the treatment, particularly at the later ages. As shown in Figure 4, however, there were no significant differences in alginate levels between treatment and control samples at any of the sampled ages. Figure 4 illustrated the already well documented progression of alginate synthesis during early *P. aeruginosa* biofilm maturation. Alginate synthesis is a dynamic process involving both polymerization and depolymerisation of the growing chain, presumably as an editing mechanism (24). The fluctuation in alginate levels observed during the first 6 hours could be due to this process. During this age interval, we did not anticipate a noticeable effect of the alginate lyase digestion given its low levels. Importantly, previous research shows that the carbazole assay does not reliably quantify uronic acid levels below 4 ug/ml which encompasses the above mentioned readings. This raises questions about their accuracy (4). The later rapid increase in alginate production was also consistent with the literature.

The added experimental limitations of the CTAB precipitation and the carbazole assay may have prevented accurate detection of small decreases of undigested alginate levels in the treatment cultures. Nevertheless, it is plausible that alginate levels were indeed decreasing in the treatment cultures at levels below our detection limit. This is supported by the Park & Johnson assay results which showed that treated samples had statistically higher levels of reducing sugar levels. Their general increase in both the control and treatment is justified by a greater need for available glucose and rhamnose in order to sustain the increased rate of alginate synthesis. It has been well established that AlgC, a protein in the alginate biosynthetic pathway, recruits and incorporates these two sugars to the growing polysaccharide. Mutant strains lacking this protein are incapable of forming alginate, and also have defective LPS structures without glucose or rhamnose (24). Throughout the duration of the experiment, reducing sugars were higher in the treatment, suggesting the release of monosaccharides as a result of enzymatic digestion. In a paper that analyzed the effect of alginate lyase derived from *Flavobacterium sp.,* the same enzyme in the present protocol, on biofilms, An *et al.* showed a figure establishing a direct correlation between a decrease in alginate polymerization and an increase in reducing sugar levels (2). Although the authors did not specify reducing sugars species, it can be concluded from their discussion that digestion of alginate does lead to an increase in such sugars. Our results from the Park & Johnson assay, however, have replicated An *et al.*'s findings, but seem to contradict our own carbazole assay results (2).

A final consideration that applies both to the efficacy of the enzymatic digestion as well as antibiotic treatment is that the dense and slimy nature of biofilms prevents substances from diffusing as they would under planktonic culture mode. Several authors have shown that biofilm penetration of gentamicin is not completely prevented, but is significantly retarded (17, 30). Part of this effect is due to the highly negative nature of alginate, which can then sequester positively charged antibiotics such as gentamicin (30). It is possible that even when digested by alginate lyase, the monomeric uronic acids could still bind gentamicin, thus conferring some protection to *P. aeruginosa*. Following this same logic, it would be reasonable to assume that enzymatic digestion occurred primarily at the surface of the biofilm. In anticipation of this, cultures were vortexed immediately after adding the enzyme, but it may still have been insufficient for its even distribution. Judging from the physical appearance of the digested biofilms, which tended to sag towards the bottom of the tube and detach from the test tube walls, it a appears that a degree of digestion did take place.

The goal of our investigation was to determine the point in biofilm development at which alginate becomes a critical player in reducing antibiotic susceptibility. Our results showed that neither treatment with alginate lyase nor an increase in alginate accumulation over time within the biofilm changed its susceptibility to gentamicin within the first 48 hours. In contrast, the complete lack of alginate, as represented by the *ΔalgA* mutant, corresponded to higher gentamicin susceptibility between 6 and 24 hours. These conclusions are suspectedly due to the vital inconsistency observed between the carbazole assay, which indicated alginate lyase had no significant effect on the intact alginate concentration, and the Park & Johnson assay, which indicated that the treatment did significantly increase the amount of reducing sugar in the samples. Overall, we believe that the enzymatic

digestion was not thorough enough to provide useful data.

FUTURE DIRECTIONS

The results of our experiment indicated that enzymatic treatment of all *P. aeruginosa* wild type, Δ*mucA* and Δ*algA* PAO1 strains had no observable effect on the susceptibility to gentamicin. Retesting the same hypothesis may be necessary to obtain conclusive results taking into account a number of procedural modifications and limitations discussed in this paper. Further experiments involving varying concentrations of alginate lyase and increased digestion time should be performed to optimize enzyme treatment conditions at all age points. Additionally, homogenization of the culture prior to alginate lyase may facilitate a more complete enzymatic digestion. Alternatively alginate lyase could be added in conjunction with another antimicrobial agent specific for a different EPS component, such as DNase to target extracellular DNA (eDNA) to further facilitate EPS degradation (1, 22). An increase in antibiotic treatment length may be necessary to allow for sufficient permeation across the EPS. The CTAB precipitation could be substituted for another alginate precipitation and purification method, such as nitron precipitation, to increase alginate recovery (up to 99%) on a smaller culture volume (3). A more accurate and specific technique for biofilm growth quantification, such as a crystal violet assay (27) or wheat germ agglutinin conjugates which bind to EPS components (5), may provide more definitive results in terms of overall growth rate and allow for more accurate normalization. Due to the possible continuation of trends in both alginate production and increase in gentamicin susceptibility profiles past 48 hours, it would also be beneficial to extend the culture ages to 72 or 96 hours. Finally, alginate levels should be observed for all three strains to conclusively correlate the effect of the enzymatic activity on antibiotic susceptibility.

ACKNOWLEDGEMENTS

The funding and materials for this project were generously provided by the Department of Microbiology and Immunology at the University of British Columbia. We would like to thank Dr. William Ramey for his guidance on this project, Shaan Gellatly for her advice and guidance with working with *P. aeruginosa* and the kill curve assay, and Lando Robillo and the media room staff for providing equipment.

REFERENCES

1. **Alipour, M., Z. E. Suntres, and A. Omri.** 2009. Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against *Pseudomonas aeruginosa*. J. Antimicrob. Chemoth. **64:**317-325.
2. **An, Q-D., G-L. Zhang, H-T. Wu, Z-C. Zhang, G-S. Zheng, L. Luan, Y. Murata, and X. Li.** 2008. Alginate-deriving oligosaccharide production by alginase from newly isolated *Flavobacterium* sp. LXA and its potential application in protection against pathogens. J. Appl. Microbiol. **106:**161-170.
3. **Azeredo, J., and R. Oliveira.** 1996. A new method for precipitating bacterial exopolysaccharides. Biotechnol. Tech. **10:**341-344.
4. **Bitter, T., and H. M. Muir.** 1962. A modified uronic acid carbazole reaction. Anal. Biochem. **4:**330-334.
5. **Burton, E., N. Yakandawala, K. LoVetri, and M. S. Madhyastha.** 2007. A microplate spectrofluorometric assay for bacterial biofilms. J. Ind. Microbiol. Biot. **34:**1-4.
6. **Chen, A., H. Wu, M. Ni, and D. Tian.** 2009. Comparative analysis of proteins, drug resistance, and biofilm formation in free-swimming bacteria and their surface-attached counterparts. Afr. J. Microbiol. Res. **3:**767-773.
7. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol. Microbiol. **8:**583-590.
8. **Conibear, T. C. R., S. L. Collins, and J. S. Webb.** 2009. Role of mutation in *Pseudomonas aeruginosa* biofilm development. PLoS ONE. **4:**e6289.
9. **Cotton, L. A., R. J. Graham, and R. J. Lee.** 2009. The role of alginate in *P. aeruginosa* PAO1 biofilm structural resistance to gentamicin and ciprofloxacin. J. Exp. Microbiol. Immunol. **13:**58-62.
10. **Damron, F. H., D. Qiu, and H. D. Yu.** 2009. The *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production though AlgW-dependent MucA proteolysis. J. Bacteriol. **191:**2285-2295.
11. **de Carvalho, C. C. C. R.** 2007. Biofilms: recent developments on an old battle. Recent Pat. Biotechnol. **1:**49-57.
12. **de Jong, J. G. N., R. A. Wevers, C. Laarakkers, and B. J. H. M. Poorthuls.** 1989. Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine: a rapid screening procedure for mucopolysaccharidoses. Clin. Chem. **35:**1472-1477.
13. **Di Ferrante, N.** 1956. Turbidmetric measurement of acid mucopoly-saccharides and hyaluronidase activity. J. Biol. Chem. **220:**303-306.
14. **Friedman, L., and R. Kolter.** 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J. Bacteriol. **186:**4457-4465.
15. **Harmsen, M., L. Yang, S. J. Pamp, and T. Tolker-Nielsen.** 2010. An update on *Pseudomonas aeruginosa* biofilm tolerance, and dispersal. FEMS Immunol. Med. Mic. **59:**253-268.
16. **Hatch, R. A., and N. L. Schiller.** 1998. Alginate lyase promotes diffusion of aminoglycosides though the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa.* Antimicrob. Agents Ch. **42:**974-977.
17. **Hoyle, B. D., J. Alcantara, and J. W. Costerton.** 1992. *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. Antimicrob. Agents Ch. **36:**2054-2056.
18. **Jacobs, M.**A., et al. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa.* Proc. Natl. Acad. Sci. USA. **100:** 14339 44.
19. **Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak.** 2004. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J. Bacteriol. **186:**4466-4475.
20. **Lambert, P. A.** 2002. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa.* J. Roy. Soc. Med. **95:**22-26.

21. **Li, X., L. Nielsen, C. Nolan, and L. J. Halverson.** 2010. Transient alginate gene expression by *Pseudomonas putida* biofilm residents under water-limiting conditions reflects adaptation to the local environment. Environ. Microbiol. **12:**1578-1590.
22. **Ma, L., M. Conover, H. Lu, M. R. Parsek, K. Bayles, and D. J. Wozniak.** 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog. 5:e1000354.
23. **Ma, L., H. Lu, A. Sprinkle, M. R. Parsek, and D. J. Wozniak.** 2007. *Pseudomonas aeruginosa* Psl is a galactose- and mannose-rich exopolysaccharide. J. Bacteriol. **189:**8353-8356.
24. **May, T. B., and A. M. Chakrabarty.** 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol. **2:**151-157.
25. **Mulet, X., M. D. Maciá, A. Mena, C. Juan, J. L. Pérez, and A. Oliver.** 2009. Azithomycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of *nfxB* mutants. Antimicrob. Agents Ch. **53:**1552-1560.
26. **Obritsch, M. D., D. N. Fish, R. MacLaren, and R. Jung.** 2005. Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacotherapy. **25:**1353-1364.
27. **Park, J. T., and M. J. Johnson.** 1949. A submicrodetermination of glucose. J. Biol. Chem. **181:**149-151.
28. **Piletska, E. V., G. Stavroulakis, L. D. Larcombe, M. J. Whitcombe, A. Sharma, S. Primrose, G. K. Robinson, and S. A. Piletsky.** 1 March 2011, posting date. Passive control of quorum sensing: prevention of *Pseudomonas aeruginosa* biofilm formation by imprinted polymers. Biomacromolecules.
29. **Ramsey, D. M., and D. J. Wozniak.** 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chonic infections in cystic fibrosis. Mol. Microbiol. **56:**309-322.
30. **Shigeta, M., G. Tanaka, H. Komatsuzawa, M. Sugai, H. Suginaka, and T. Usui.** 1997. Permeation of antimicrobial agents though *Pseudomonas aeruginosa* biofilms: a simple method. Chemotherapy. **43:**340-345.
31. **Song, J-M., J-H. Im, J-H. Kang, and D-J. Kang.** 2009. A simple method for hyaluronic acid quantification in culture broth. Carbohyd. Polym. **78:**633-634.
32. **Wozniak, D. J., T. J. O. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. Proc. Natl. Acad. Sci. USA. **100:**7907-7912.