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The Role of Alginate and Extracellular DNA in Biofilm-Meditated *Pseudomonas aeruginosa* Gentamicin Resistance

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The role of extracellular DNA in gentamicin resistance of *Pseudomonas aeruginosa* biofilms was examined in the presence and absence of alginate, a major component of the bacterial biofilm. A PAO1 *P. aeruginosa* variant with a chromosomal *lux* gene insertion was used to measure bacteria viability during experimentation. Mature biofilms grown in cation-adjusted Mueller-Hinton media were treated with alginate lyase, deoxyribonuclease, or both, and resistance to gentamicin was examined by bioluminescence measured over 2 hours. All enzymatic digestions resulted in increased susceptibility to gentamicin treatment, with both enzymes in conjunction having a maximized effect on antibacterial sensitivity. The findings indicate that extracellular DNA is a partial determinant of *P. aeruginosa* biofilm-mediated resistance to gentamicin. The combination of alginate lyase and deoxyribonuclease treatment of *P. aeruginosa* infections may be beneficial prior to administration of antibiotics and warrants further investigation.

Microbial biofilms are of high clinical significance in a number of infectious diseases. To this end, much research has been done on *Pseudomonas aeruginosa*, the most frequently identified pathogen cultured from the respiratory samples of cystic fibrosis (CF) patients, which is implicated in a majority of premature CF patient deaths (8). The pulmonary symptoms caused by viscous respiratory secretions common in CF are exacerbated by immune inflammation upon *P. aeruginosa* infection of the lungs (3, 27). Chronic *P. aeruginosa* infection of CF patient lungs is characterized by cells within raft-like biofilm matrices(12), which display a greatly decreased susceptibility to antibiotics compared to planktonic cells (4, 9). Thus, chronic infections have proven to be difficult to eradicate by traditional antibiotic means once they have established in the CF lung (12). Therefore studies into biofilm resistance are important for treating this type of infections.

Biofilms are composed of water, proteins, polysaccharides and nucleic acids, all of which originated as secreted polymers, absorbed nutrients, products of cell lysis, or particulate material from the environment (22). Of particular relevance to biofilm-mediated antibiotic resistance are alginate and extracellular DNA (eDNA). Although not essential for the formation of biofilms (15), the negatively-charged sugar polymer alginate is a major component of naturally forming biofilms (7). eDNA is actively secreted from *P. aeruginosa* (14), is necessary for the initial formation of the biofilm (24), and confers increased viscosity (1).

It was originally thought that biofilms increase antibiotic resistance primarily by acting as a physical barrier, preventing or significantly impairing antibiotics from penetrating through the heterogeneous mix of major cellular macromolecules to reach the cells below (13). However, mechanical disruption of mature 5-day-old biofilms demonstrated little effect on susceptibility of the underlying *P. aeruginosa* bacteria to the aminoglycoside antibiotic gentamicin (4, 11). In contrast, treatment of biofilms established over 24 h or 5 days with alginate lyase (AlgL) drastically decreased resistance to gentamicin (2, 5). This suggests that the antibiotic resistance attributed to biofilms is not simply conferred by means of a physical barrier, but by the chemical properties of the biofilms' components. While examining the kinetics of gentamicin susceptibility following AlgL treatment, Diaz *et al* suggested biofilm components other than alginate also contribute to gentamicin resistance in *P. aeruginosa* biofilms (6). The group confirmed that the biofilm's alginate has been degraded by AlgL, but no difference in gentamicin resistance was observed. This implies that other chemical factors such as eDNA might play a role in antibiotic resistance.

While it is not fully characterized, some progress has been made in understanding the role of eDNA in antibiotic resistance and pathogenesis. Firstly, eDNA may have a compensatory structural role in the absence of alginate; mutant *P. aeruginosa* strains that under-express alginate have been found to display increased secretion of eDNA in biofilms (10). Furthermore, eDNA has been shown to play a regulatory role in

induction of antibiotic resistance genes. Mulcahy *et al.* elucidated one protective role of eDNA through its induction of the phoPQ and pmrAB operons following eDNA chelation of Mg^{2+}, causing an addition of aminoarabinose to lipid A (18). This reduced permeability of the outer membrane to the cationic antimicrobial peptides (18), but was not convincingly reproduced for gentamicin. Thus, the authors proposed that eDNA presented a phoPQ-pmrAB independent mechanism of gentamicin resistance (18). Gentamicin is a polycationic aminoglycoside antibiotic; its ionic properties allow it to bind negatively-charged residues such as those found on the outer membrane of Gram-negative bacteria, RNA, and the 16S rRNA of the 30S ribosomal subunit (20). However, the ionic charges necessary to the functioning of gentamicin may also render it less effective in the presence of bacterial biofilms. Negatively-charged species that make up a significant portion of the biofilm, specifically alginate and eDNA, may bind and sequester or inhibit gentamicin (1).

This study aimed to build on the previously established observation that mechanical disruption of the biofilm does not result in a loss of gentamicin resistance (4, 11), and that inherent properties of the biofilm's constituent macromolecules must be the cause for the persisting resistance (6). Antibiotic resistance attributed to *P. aeruginosa* biofilms seems to be due to the chemical properties of the biofilm material. In the case of gentamicin, alginate and eDNA are two biofilm components of significance interest as their negative charge may allow them to interact with and inhibit the efficacy of the cationic aminoglycoside (1). Enzymatic digestion of alginate has been shown to increase gentamicin susceptibility in *P. aeruginosa* (1, 2, 5) but the results of Diaz *et al.* has highlighted the importance of other molecules such as eDNA (6). Chelation by negatively-charged alginate and polyanionic DNA in particular (1), would explain both the inconclusive results found by Diaz *et al.*, and the anomalous data presented by Mulcahy *et al.* (6, 18). Thus, it was hypothesized that enzymatic digestion of the biofilm alginate and eDNA by AlgL and deoxyribonuclease (DNase) would decrease gentamicin resistance more effectively than either treatment alone.

METHODS AND MATERIALS:

Biofilm preparation and growth conditions. An initial overnight culture of the chromosomal *lux P. aeruginosa* PAO1 strain was prepared by inoculating 3 mL of Cation-Adjusted Mueller-Hinton (CAMH) media (21 g/l dehydrated Mueller-Hinton broth at pH 7.4, 25 mg/l magnesium chloride, and 12.5 mg/l calcium chloride) (25) with the bacteria. CAMH was selected for optimal gentamicin functioning (25). After 24 h of growth with aeration in a 37 °C waterbath shaking at 150 rpm, 20 µl aliquots of the culture were distributed into eight test tubes containing 1.8 ml of CAMH. The test tubes were then incubated aerobically in the 37 °C waterbath shaking at 150 rpm for 100 h.

Chemical treatments and incubation times. After the 100 h incubation, the eight test tubes were homogenized using a 7 ml Dounce Tissue Homogenizer with corresponding pestle A for 60 sec with a push/twist motion as previously described by Cotton *et al,* (4). Two 100 µl homogenized samples were used for the Crystal Violet assay for quantification of the biofilm. The rest of the homogenized culture was divided into eight 1.8 ml aliquots. The following treatments were applied to each of 2 aliquots to a final volume of 2 ml: control (CAMH), AlgL (Sigma-Aldrich, Cat. no. A1603) in CAMH (20 U/ml) (4), DNase (Sigma-Aldrich, Cat. no. D0876) in CAMH (100 mg/l) (1), and DNase in combination with AlgL in CAMH (100 mg/l and 20 U/ml, respectively). These treatments were applied for 2 h with aeration in a 37 °C waterbath shaking at 150 rpm. Following the 2 h incubation, each test tube was distributed into four 190 µl aliquots in an opaque 96-well plate (Perkin-Elmer, Cat. no. 6005182). The plate contained 32 filled wells: 3 enzymatic treatments and 1 control (for both gentamicin-treated (Gen^+) (Sigma-Aldrich, Cat. no. G3632) and non-gentamicin-treated (Gen^-) conditions) in duplicate. Duplicate technical replicates were also used following separation of cultures after homogenization. Experiments were repeated 5 times using distinct bacterial cultures.

Antibiotic treatment and luminescence recording. 10 µl of gentamicin in CAMH was added to 2 of the 4 x 190 µl aliquots for each sample in the 96-well plate (final gentamicin concentration 4 µg/ml). In addition, a positive control was made by adding 10 µl of gentamicin to 190 µl of overnight culture. 10 µl of CAMH was added to each of the remaining half of the 4 x 190 µl aliquots as Gen^- controls. Immediately after, the OD_{620} and luminescence of wells were measured with the Tecan Spectra II Microplate Reader. Subsequent luminescence (RLU) readings were taken every 10 min for 2 h, during which the plate was incubated at 37°C and shaken between readings.

Crystal violet assay. The assay was carried out with some modifications to the previously described method (11). The two 100 µl homogenized samples were centrifuged separately and the supernatants were discarded. Next, the pellets were each resuspended in 100 µl of 0.1% crystal violet solution. The solutions were then centrifuged and the supernatants were discarded. The pellets were then resuspended with water, centrifuged, and the supernatants discarded. This washing step was repeated until the supernatants were clear. Lastly, the pellets were each resuspended with 100 µl of 95% ethanol and transferred to a 96-well plate. The absorbance of the ethanol resuspensions was measured at 595 nm with the Epoch Microplate Spectrophotometer.

Luminescence, optical density and plate count standards. To correlate between luminescence and OD_{620}, a series of 2-fold dilutions were prepared of the initial overnight culture and both of the readings were done with the Tecan Spectra II Microplate Reader prior to each 2-h series of RLU readings for biofilm samples. To correlate between OD_{620} and plate counts, a series of 2-fold and 10-fold dilutions were done on the initial overnight culture. The OD_{620} readings were measured using the Spectronic 20 spectrophotometer. 100 µl of the diluted samples were spread-plated onto Luria-Bertani agar. The plates were incubated at 37°C for 24 h and then counted.

Data Analysis. The luminescence readings were all converted to viable cell counts using the standard curve constructed above. The points were base line corrected to time 0 min or 10 min values as indicated in figure legends. Repeated measure two-way ANOVA tests were used to determine the statistical significance of differences in the 120 min time points over (n=5) biological replicates. Statistical analyses including repeated measure two-way ANOVA with

Bonferroni post-test and generation of standard error of mean bars were performed using GraphPad Prism 5.00 (Trial Software).

RESULTS

Enzymatic treatments altered the growth kinetics of biofilms. A common trend observed across all biological replicates was differences in growth patterns between the enzymatically treated biofilms and the control biofilm, regardless of the presence of gentamicin. This was observed in differences in the quantity of metabolically active cells at the beginning of the time course experiments and was indirectly measured by luminescence since it can quantitatively estimate the concentration of metabolically active cells that express the *lux* gene (19). The initial mean concentration of metabolically active cells was increased by enzymatic treatment with AlgL, DNase, and combination of enzymes by 46.7%, 42.6%, and 63.5% respectively (Table 1).

Gentamicin had negative effects on the non-enzymatically treated control biofilm. All three graphs in Fig. 1 showed the same control curves. The Gen^+ and Gen^- controls had similar increasing growth patterns in the first 60 min. However, the growth rate of the Gen^+ control reduced to static at 60 min while the Gen^- control continued to grow at a constant rate. The final difference in cell concentrations between the Gen^+ and the Gen^- controls was 4.0 x 10^5 CFU/ml. Also, as a positive control, overnight planktonic samples without the protective influence of extracellular biofilm components were treated with gentamicin. These samples demonstrated the highest mean initial concentration of 2.03 x CFU/ml 10^7 at 0 min and subsequently decreased in concentration exponentially to a final mean of 1.09 x CFU/ml 10^7 at 120 min.

TABLE 1. Initial luminescence of cells in biofilm after enzymatic treatments immediately following addition of gentamicin.

Enzymatic Treatment	Mean initial luminescence (RLU x 10^3)		* Luminescence increase versus control (%) [1]
	Gen^+	Gen^-	
Control	3.2	3.3	
Alginase	4.8	5.0	47
DNase	4.6	4.6	43
Alginase and DNase	5.3	5.6	63

*1. No statistically significant difference is found between Gen^+ and Gen^- mean initial luminescence values so these are averaged together for percent comparisons to control.

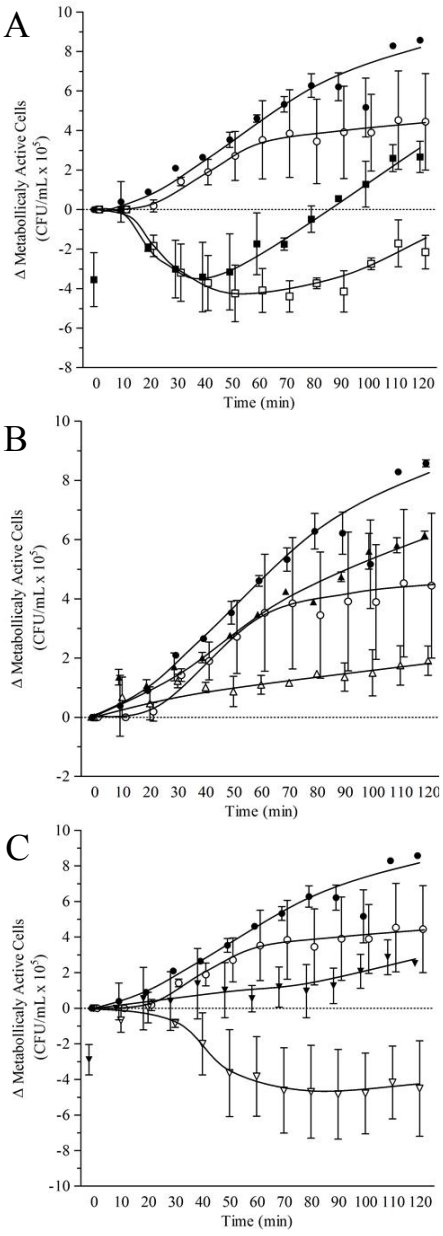

FIG. 1. Susceptibility of *Pseudomonas aeruginosa* to 4 µg/ml gentamicin following enzymatic degradation of mature biofilm. Data are from 1 of 5 biological replicates representative of trends observed across all replicates. (A) AlgL, (B) DNase, and (C) AlgL in combination with DNase are shown versus controls with and without gentamicin. Data points are the mean ± standard error of mean from n=2 replicate wells. Curves are regression lines that describe the growth rate of *P. aeruginosa*. Outliers are defined as one-or-two-point-trends that cannot be explained with biological means, and thus are not plotted onto regression lines. Values are baseline corrected to 0 min values with the exceptions of the Gen^- AlgL sample in Fig. 1A and the Gen^- combined AlgL and DNase sample in Fig. 1C, which are corrected to 10 min values as the initial readings are outliers. ■Gen^- AlgL; □ Gen^+ AlgL; ▲Gen^- DNase; □Gen^+ DNase; ▼Gen^- combined AlgL and DNase; □Gen^+ combined AlgL and DNase; ●Gen^- Control; ○Gen^+ Control.

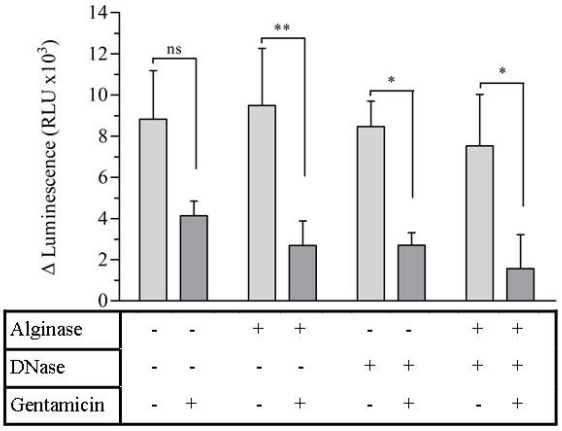

FIG. 2. Mean susceptibility of *Pseudomonas aeruginosa* to 4 µg/ml gentamicin following enzymatic degradation of mature biofilm after 120 min. Error bars represent the standard error of means across 5 biological replicates; ns signifies non-significant difference, (∗) signifies $p<0.05$, (∗∗) signifies $p<0.01$.

TABLE 2. Quantification of mature biofilm growth via crystal violet assay following 100 h incubation prior to enzymatic treatments.

Biological Replicate	Mean Biofilm Production (Abs 595)
1	0.89
2	0.09
3	0.09
4	0.62
5	0.62

AlgL, DNase, or a combination of both enzymes resulted in increased susceptibility to gentamicin. At least two of the following three trends were observed in each treatment, suggesting enzymatic treatments of biofilms increased susceptibility to gentamicin: i) an initial decrease in cell concentration in the presence of gentamicin ii) a static growth rate in the presence of gentamicin or iii) a lower final active cell count after incubation with gentamicin.

Fig. 1A showed that AlgL treatment increased susceptibility to gentamicin as two of the previously stated trends (i and iii) were observed. AlgL treatment initially caused a negative growth rate on both the Gen^- and the Gen^+ samples. The growth rate recovered after 40 min for the Gen^- sample while it took 60 min for the Gen^+ sample to recover. By 70 min, the Gen^- sample climbed back to a 2.0×10^5 CFU/ml deficit, in contrast the Gen^+ sample was still at a 4.0×10^5 CFU/ml deficit. Lastly, the difference in the cell concentrations between the Gen^+ and the Gen^- cultures was 5.0×10^5 CFU/ml at 120 min.

As shown in Fig. 1B, DNase treatment resulted in two of the stated trends (ii and iii) of increased gentamicin susceptibility. Interestingly, the initial decrease in growth rate observed in both of the AlgL samples (Fig. 1A) was not seen in either of the DNase samples. The Gen^- sample had a constant linear increase in growth from the start. In comparison, the Gen^+ cells remained static throughout the time course. Therefore, the final difference in cell concentrations at 120 min was 4.5×10^5 CFU/ml.

In Fig. 1C, the combination of both AlgL and DNase treatments resulted in all three trend characteristics of increased gentamicin susceptibility. The first striking observation was that the decreased cell count exclusive to the AlgL treatment and the static growth rate exclusive to the DNase treatment were both present in the Gen^+ combined AlgL and DNase treatment. Similar to the Gen^+ AlgL treatment in Fig. 1A, the growth rate of the biofilm treated with both enzymes and gentamicin was negative from 20 min to 60 min. In addition, the non-recovering trend seen after 60 min was similar to the Gen^+ DNase treatment in Fig. 1B. In comparison, the Gen^- sample remained relatively constant at 1.0×10^5 CFU/ml until 60 min and then started to grow. The difference in concentration of cells at 120 min was 8.0×10^5 CFU/ml.

This increase in gentamicin susceptibility following AlgL, DNase, or the combined treatment was shown to be reproducible. Comparison of the final differences in metabolically active cells shown in Fig. 2 indicated that the Gen^+ enzymatic treatments resulted in a statistically significant decrease in growth which was not observed in the Gen^+ control. For the AlgL treatment, the decrease in cell count in the presence of gentamicin at 120 min was statistically significant ($p<0.01$) over the five biological replicates. For both the DNase and the combined treatment, the decrease in cell count in the presence of gentamicin at 120 min was also statistically significant ($p<0.05$) over the five biological replicates.

Greater gentamicin susceptibility was observed in the combined treatment than either of the two treatments alone. While both the Gen^+ AlgL and the Gen^+ DNase treatments reduced the cell count to almost two-thirds that of the Gen^+ control after 120 min, the Gen^+ combined treatment reduced the cell count to half

that of the Gen^+ control (Fig. 2). This was evidential in Fig. 1 as well. The difference in cell count between the Gen^+ combined treatment and the Gen^- combined treatment was the greatest of all three enzymatic treatments at 120 min. It exceeded the AlgL treatment by 3.0 x 10^5 CFU/ml and the DNase treatment by 3.5 x 10^5 CFU/ml.

The crystal violet assay was performed to assess and correlate between biofilm growths across different experimental dates but the results were highly variable. It did not suggest reproducibility of similar biofilms between biological replicates as the amount of biofilms between different days had a ten-fold difference (Table 2). The variability in the results of this assay was too large for the data to be used for standardization of bioluminescence to relative amount of biofilm for each biological replicate.

DISCUSSION

The non-enzymatically treated controls reflected the hypothesized result. Namely, it demonstrated greater luminescence in the presence of gentamicin compared to any of the enzymatically treated samples. Gentamicin was found to reduce the cell counts of the control treatment, which corresponded with Charlesworth *et al.*'s suggestion that homogenized biofilm treated with gentamicin showed increased susceptibility to the antibiotic (4). This observation could be explained if physical disruption of the biofilm caused some of the cells to be exposed and prevented protection by the biofilm. We did not treat unhomogenized biofilm with gentamicin as a control to compare the effect of the 4 μg/ml gentamicin used, so this explanation should not be mistaken to be substantiated by this study. The linearized change in growth rate seen across all Gen^+ treatments and controls could be attributed to the bacteriostatic effects of gentamicin mediated through binding to ribosomal subunits and halting protein production (20).

One replicate of this experiment was performed with 2 μg/ml of gentamicin. Since no difference in growth pattern was observed for the treatments or the controls, no further experiments with this concentration were carried out. At 16 μg/ml, all of the treatments and controls showed dramatically decreased luminescence. This observation was expected since gentamicin at higher concentrations should induce bactericidal effects mediated through disruption of cellular membranes by interfering with Mg^{2+} bridges in lipopolysaccharides (20). However, at 4 μg/ml some death of the control sample was observed, but a distinguishable pattern between treatments was evident. Thus, Figures 1 and 2 reflected data collected from samples treated with 4 μg/ml of gentamicin.

The data shown in Fig. 1 was normalized to an initial CFU/ml value of zero in order to account for the different initial metabolic activities in the treatment cultures, and to enable accurate comparisons in the growth patterns of the cultures. Of interest, the enzymatically treated cultures were found to have a higher initial CFU/ml of biologically active cells relative to control cultures. This phenomenon could be due to the release of metabolically inactive cells from the 100-hour-old biofilm, as cells within a single biofilm exist in different physiological conditions and metabolic states (26). Upon chemical disruption of biofilm matrices, these cells could then be exposed to fresh aerobic media and enter a logarithmic growth phase more quickly than cells contained within a biofilm environment, as with the non-treated controls. This explained the high initial cell concentrations of the enzymatically treated samples compared to the controls following the 2-hour incubation period prior to the time course. This observation suggested that gentamicin not only decreased the number of metabolically active cells, but also inhibited the proliferation of planktonic cells that were released from the biofilm.

DNase treatment (Fig. 1B) resulted in very slow, linear growth in Gen^+ cultures. The rapid decrease in biologically active cells seen in AlgL cultures (Fig. 1A) was not reflected in the DNase cultures (Fig. 1B). This could be due to the fact that the proportion of alginate in mature biofilms was more pronounced than that of eDNA (8). Therefore, digestion of eDNA was expected to have a less-pronounced effect on the susceptibility of the bacteria to gentamicin than digestion of alginate. However, there appeared to be some protective function for eDNA in an established biofilm. Growth of *P. aeruginosa* that was treated with DNase was significantly reduced compared to untreated controls under similar gentamicin exposure, suggesting the contribution of eDNA to resistance (Fig. 1B, Fig. 2). DNase was administered to *P. aeruginosa*-infected CF patients to decrease viscosity of mucoid build-ups, both to alleviate pulmonary distress and to increase efficacy of chemotherapies in reaching their pathogen target (12). In our study, however, mechanical disruption and homogenization of biofilms was expected to effectively eliminate eDNA-modulated viscosity and potential inhibition of antibiotic diffusion by the biofilm. Thus, the protective effects of eDNA were likely to be due instead to chemical factors, such as the polyanionic character of the molecules (1). Given the polycationic nature of the antibiotic used, it seemed reasonable that

eDNA might have increased resistance by binding and sequestering or inhibiting the action of the gentamicin.

Unfortunately the results of the crystal violet assay were too variable to be used for the standardization of CFU/ml to the relative amount of biofilm for each of the experimental replicates. This was a newly adapted protocol and there was not enough time to alter the assay to provide more accurate results. However, the precision of measurements increased with each trial of the assay, and it would likely be useful for other researchers investigating biofilms grown in a similar manner to this experiment.

Another limitation of this work was in the derivation of CFU/ml results from RLU readings. This required interpretations to be made from both an OD_{620}-CFU standard and OD_{620}-RLU standards specific to each day of experiments. It should also be noted that the chromosomal location of the *lux* insertion in the *P. aeruginosa* strain used in this study was unknown. This limitation opened the results to the possibility that they were not a representative model of PAO1 behaviour under similar conditions. The insertion could disrupt one of any number of genes which might have significance to this work, including stress response, biofilm production, and cell wall structural genes.

In conclusion, chemical disruption of an established *P. aeruginosa* biofilm with AlgL or DNase (separately or in combination) was found to increase the susceptibility of the culture to the aminoglycoside gentamicin. The non-enzymatically treated control was shown to have the least decrease in the concentration of metabolically active cells in the presence of gentamicin from 0 to 120 min while the combined treatment was shown to have the greatest decrease. AlgL and eDNA were found to play different roles in gentamicin resistance. Digestion of AlgL caused a negative growth rate while digestion of eDNA produced a static growth rate. Simultaneous digestion of both eDNA and alginate resulted in an additive effect – a negative growth rate followed by non-recovery.

FUTURE DIRECTIONS

One major uncertainty associated with the experiment was the location of the random insertion site of the *lux* operon into the chromosome of the *P. aeruginosa* PAO1 strain. The random insertion might have damaged the biofilm-forming abilities or other cellular functions of the strain. Even though the crystal violet assay confirmed biofilm production by the strain, neither the chemical make-up nor volume of the biofilm produced was compared to that of a wild-type PAO1 strain. In order to determine the differences between the biofilms produced by PAO1 and by the mutant strain, the crystal violet assay and the uronic acid assay (17) could be used to measure and compare the amount of exopolysaccharides, like alginate, that constitute the biofilms.

Luminescence generated by the *lux* operon was used to determine changes in active cell concentrations in response to gentamicin in this experiment. However, luminescence does not directly reflect alterations to the biofilm. Future experiments could focus on the direct effect of AlgL and DNase on the biofilm itself. To this end the crystal violet assay performed in this experiment was also only a crude quantification of the biofilm. Specific assays such as the uronic acid assay (17) and the eDNA assay (23) could be applied to determine the difference in the levels of alginate and eDNA before and after the enzymatic treatments. The uronic acid assay works by detecting the amount of chromagen when uronic acids like alginate is heated in the presence of tetrborate (17). The eDNA assay works by detecting the amount of fluorescence released by ethidium bromide when it is added to the electrophoresis gel (23). More direct relationships between the enzymatic treatments and the biofilm composition could be understood in this way.

Finally, the variable and altered kinetics of cellular metabolism following enzymatic treatments were not explained during this experiment. As described above, it was typically observed that enzymatic treatment altered the initial cellular metabolism and had an effect on the time for the culture to establish a constant growth rate. Examination of this trend without the presence of antibiotics would be useful to elucidate the mechanisms and rate of rematuration of biofilms degraded with AlgL or DNase.

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