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Resistance to Tobramycin in *Pseudomonas aeruginosa* PA14 and PA14 Δ*pel*B Co-culture Biofilm through Antibiotic Sequestering

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Biofilms consist of complex communities of bacteria, spatially structured with functionally dependent interactions. The extracellular polymeric substances in biofilms, consisting of polysaccharides such as Pel, have been shown to enhance resistance of these communities towards aminoglycoside antibiotics. The ability of Pel, produced by the wild type, to confer resistance for communities of different wt to *pel*B mutant ratios towards tobramycin through a gradient or threshold mechanism was examined by the co-culturing of *Pseudomonas aeruginosa* PA14 wild type and PA14 Δ*pel*B mutant. The PA14 wild type and PA14 *pel*B mutant were cultured independently and then co-cultured in ratios of 3:1, 1:1, and 1:3. PA14 wild type produced more biofilm than the PA14 *pel*B mutant, with an increasing amount produced as the ratio of PA14 wild-type to PA14 *pel*B mutant increased. The co-culture ratios were not maintained, while the average colony counts were significantly higher for *pel*B mutants compared to PA14 wild type. Further studies with improved protocols to optimize the disruption of biofilms are needed to determine the survivability ratios after exposure of the biofilm to tobramycin.

A biofilm is an aggregate of microorganisms embedded within a matrix of extracellular polymeric substance (EPS) mainly composed of high molecular weight polysaccharide polymers, proteins, and nucleic acids (4). Biofilm formation is well characterized to be based on quorum sensing (QS) (9, 11). Biofilms confer protection from environmental conditions and help maintain persistence (10). Resistance against antibiotics is also conferred through biofilm-associated patterns of gene expression, slower growth rate, and reduced diffusibility of antibiotics within the biofilm (5).

Three extracellular polysaccharides acting as primary structural components in *Pseudomonas* biofilm development are alginate, Psl and Pel. Pel has been shown to enhance resistance to aminoglycoside antibiotics such as tobramycin (4). Pel is a glucose-rich exopolysaccharide essential for the formation of surface associated biofilms and in *Pseudomonas aeruginosa* is synthesized through a metabolic pathway encoded by a seven gene operon, *pel*A-G (7). A multiple losses of function in these seven genes lead to a loss of biofilm formation (4). *pel*B is hypothesized to hold the greatest effect amongst the seven genes in the operon (7).

P. aeruginosa PA14 (wt) is unable to synthesize Psl, and the PA14 *pel*B mutant (*pel*B) is also unable to synthesize functional Pel due to a transposon insertion in the *pel*B operon with an antibiotic resistance marker encoding for a gentamicin efflux pump.

It has been hypothesized that Pel confers resistance to tobramycin by antibiotic sequestering through ionic binding (4). Since the wt and *pel*B only differ in the *pel*B mutation, it was hypothesized there would be no hindrance in the ability for QS to occur and the sequestering of tobramycin would occur through either a gradient or threshold effect. The wt and *pel*B were co-cultured in ratios of 3 wt to 1 *pel*B (3:1), 1 wt to 1 *pel*B (1:1) and 1 wt to 3 *pel*B (1:3). Using the inherent resistance to gentamicin of *pel*B, the changes in the ratios after 1 day of incubation on tobramycin was observed at the determined minimum inhibitory concentration of 8 ug/ml.

MATERIALS AND METHODS

Strains, media and reagents. *P. aeruginosa* PA14 and PA14 Δ*pel*B were provided by R.E.W. Hancock Laboratory (Department of Microbiology and Immunology, University of British Columbia). Overnight cultures were grown in tryptic soy broth (TSB; pH 7.3; EMD Millipore, #1.05459) with aeration at 200 rpm for 21 h at 37°C. For trypic soy agar (TSA), 1.5% of select agar (Invitrogen, #30391-023) was added to TSB before autoclaving. Tobramycin sulfate salt (#T1783), gentamicin sulfate (#G3632), and congo red (#C6767) were obtained from Sigma. Crystal violet was obtained from Fisher Scientific (#C581). Phosphate buffered saline (PBS; pH 7.2) was made by combining 2.71 mM of $Na_2HPO_4 \cdot 7H_2O$ (Fisher Scientific, #S373), 1.54 mM of KH_2PO_4 (EM Science, #PX1565-1), and 0.15 M of NaCl (Fisher Scientific, #BP358).

Drop plating (12). Each plate was divided into six equal quadrants and 10 µl of a sample was added five times to each quadrant. The plates were incubated overnight at 37°C. After

incubation, the number of colony forming units (CFUs) in each quadrant was counted and recorded.

Minimum inhibitory concentration (MIC) determination (13). The overnight cultures of *P. aeruginosa* PA14 and PA14 Δ*pel*B were diluted by a factor of 1/100 using TSB and 100 µl of the diluted overnight cultures were added to a polypropylene 96-well microplate. Tobramycin was added at 0, 3, 4, 5, 6, 8 and 10 µg/ml in 6 replicates. The microplate was incubated at 37°C for 24 h. After incubation, each well was diluted 1/100 using TSB. The diluted cultures were then drop plated on TSA plates in duplicate. The MIC was then determined by examining where the number of viable cells were reduced to zero.

Quantification of biofilm (13). The overnight cultures were diluted to an optical density of 0.12 - 0.14 at 600 nm before putting 100 µl of the cultures into each of the wells on a polystyrene 96-well microplate. Twelve replicates were performed on each of the cultures. The microplate was covered and incubated at 37°C for 24 h. After 24 h, the cultures were removed and the wells washed with dH_2O. After drying, 100 µl of crystal violet (1%) was added to each well and incubated at room temperature for 20 min. The crystal violet was then removed and the plate was washed with dH_2O. Absolute ethanol (110 µl) was added to each well and the microplate was read on a spectrometer at 595 nm.

Effect of tobramycin on the co-culture of *P. aeruginosa* PA14 and PA14 Δ*pel*B (12). Biofilm cultures were obtained by growing *P. aeruginosa* on nitrocellulose membrane filters (diameter, 25mm; pore size, 0.22 µm; EMD Millipore, #GSWP02500) resting on TSA plates. The membranes were sterilized by autoclaving at 121°C / 15 psi for 20 min and each filter was inoculated with 5 µl of an overnight culture. The overnight cultures were grown in 5 ml of TSB then diluted using the same medium to an optical density of 0.12 - 0.14 at 600 nm before making the wt, mutant, 3:1, 1:1, and 1:3 samples. The resulting mixtures were inoculated onto nitrocellulose membranes in duplicate, placed on TSA plates, and incubated at 37°C for 48 h. The membranes were transferred onto fresh TSA plates every 24 h. The filters containing the biofilm were transferred upright onto TSA plates with 8 µg/ml tobramycin (2 filters per tobramycin plate) and incubated at 37°C for 24 h. After incubation, the filters were removed from the tobramycin plates and placed in separate tubes containing 4.5 ml of PBS for 3 min of vortexing. After vortexing, an additional 4.5 ml of PBS were added to the solution and homogenized using an homogenizer for 1 min. Serial dilution was performed and the 10^{-6} and 10^{-7} dilutions were bead plated onto both congo red (40 µg/ml) and gentamicin (12.5 µg/ml) TSA plates in duplicates. The plates were incubated at 37°C for 24 h.

Data analysis. The data obtained in this experiment were statistically analyzed using Microsoft Excel 2007. The same software was used to generate the figures.

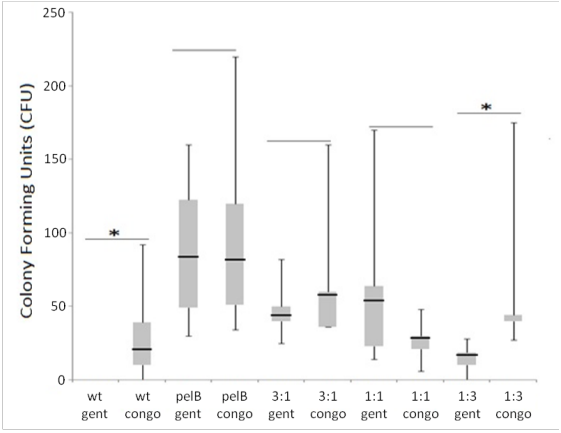

FIG. 1. Box plot of cultured and co-cultured ratios subjected to 24 h of 8 µg/ml tobramycin.* Significant with $P < 0.05$ in a two-tail paired t-test analysis. Co-culture of 3 wt to 1 *pel*B (3:1), 1 wt to 1 *pel*B (1:1), and 1 wt to 3 *pel*B (1:3), replicate plated onto congo red 40 µg/ml (congo) TSA plates as well as onto 12.5 µg/ml gentamicin (gent) TSA plates.

TABLE 1. Table of P values in a unpaired T-test of corrected average CFU counts of culture and co-cultures. significance shown with * for $P < 0.05$.

Comparison	Gentamicin	Congo Red
wt/3:1	0.006*	0.18
wt/1:1	0.079	0.76
wt/1:3	0.004*	0.24
*pel*B/3:1	0.09	0.45
*pel*B/1:1	0.51	0.03*
*pel*B/1:3	0.009*	0.25
3:1/1:1	0.59	0.13
3:1/1:3	0.02*	0.71
1:1/1:3	0.15	0.17
wt/*pel*B	0.004*	0.04*

RESULTS

P. aeruginosa PA14 and PA14 Δ*pel*B were co-cultured in ratios of 3:1, 1:1 and 1:3 at MIC of tobramycin to observe the sequestration of tobramycin by the biofilm to be that of a gradient or a threshold effect. *P. aeruginosa* wt are susceptible to gentamicin (12.5ug/mL) and therefore will grow on congo red TSA plates but not gentamicin TSA plates, whereas the *pel*B mutant will grow on both plates. Therefore, congo red TSA plates and gentamicin plates were used to quantify and obtain the end ratios of wt and *pel*B. If the ratios were maintained throughout the experiment the observed numbers would be four times as much for 3:1 initial ratio, two times as much for 1:1 initial ratio, and 25% more for the 1:3 initial ratio, of colony counts on congo red plates compared to the gentamicin plates. The expected the results is to have no gentamicin plate counts for the all wt control, and equal numbers of plate counts on both congo red and gentamicin plates for the all *pel*B control. While the controls showed the

TABLE 2. Ratios of co-cultures before and after exposure to tobramycin at 8 µg/ml for 24 h. Ratios were calculated as: (colonies on congo red - colonies on TSA) / (colonies on TSA).

Starting Ratio (wt : *pel*B)	End Ratio (wt : *pel*B)
3:1	2:5
1:1	0:1
1:3	3:1

expected results, none of the expected results were observed for the initial co-culture ratios of 3:1, 1:1 and 1:3 (Fig. 1). The results suggested for both 3:1 and 1:1 initial ratios of wt to Δ*pel*B resulted in an end ratio with more Δ*pel*B than wt. This is shown by the non-significant difference between congo red and gentamicin plate counts in Fig.1. There was, however, a significant difference in the 1:3 initial co-culture ratio plate counts, showing a higher amount of counts in the congo red plates than the gentamicin plates (Fig. 1). This suggests that the majority of the bacteria in the 1:3 initial ratio co-cultured colony were wt *P. aeruginosa* PA14.

The MIC of PA14 wt to tobramycin was found to be 8 ug/ml; the MIC of *pel*B to tobramycin was found to be 6 ug/mL (data not shown). The concentration of tobramycin used in the co-culture experiment was 8 ug/ml, and thus was enough to inhibit both wt and *pel*B cells in our various initial ratios. However, as seen in Fig. 1, there was a significantly higher average amount of *pel*B compared to that of wt, suggesting that the *pel*B grew better than the wt under stress from tobramycin.

There were no significant differences in the total bacteria counts after co-culture (congo red plate counts) in Table 1. This suggest that while the initial ratios of wt to *pel*B were 3:1, 1:1 and 1:3, this did not have an overall effect on the survival of the bacteria. The significant differences observed in the counts of 3:1 to 1:3 initial ratios of wt to *pel*B co-cultures in the gentamicin plates (Table 1) suggest a decrease in wt in the initial co-cultures, correlates with a decrease in the survivability of the *pel*B mutant. For the gentamicin counts, there was no significant difference when comparing the 3:1 to 1:1 and the 1:1 to 1:3 co-cultures (Table 1).

Looking at Table 2, none of the ratios from the original co-culturing was maintained throughout the experiment. The co-culturing of 3:1 went from ratio of 3:1 to 2:5 suggesting that *pel*B mutant was more resistant to tobramycin treatment compared to the wt. Similarly, the 1:1 co-culturing went from a ratio of 1:1 to all *pel*B. The 1:3 co-culturing showed a different trend. The ratios went from 1:3 to 3:1 suggesting that *pel*B mutants were reduced in numbers throughout the experiment.

As expected, the wt displayed a higher amount of biofilm compared the *pel*B mutant in the biofilm quantification assay (Fig. 2). The trend, regardless of protection mechanism, is expected to be downward, with 3:1 being the highest and 1:3 being the lowest in biofilm amount. However, after repeating the experiment twice, both sets of data (only one set shown) indicate an increase in the amount of biofilm as the amount of *pel*B in co-culture increases (Fig. 2).

DISCUSSION

The *pel*B mutant showed significantly more growth in the presence of tobramycin when compared to wt (Fig. 1). One explanation of this is the metabolic changes cells exhibit while growing in biofilms due to the difference in gene expression that is associated with biofilms (2, 5). Since the wt cells associated with biofilms can undergo cell growth arrest and *pel*B mutant are unable to interact with biofilms, it was expected that the numbers of *pel*B cells would be higher even before subjecting either to tobramycin (5). If the cells were plated right after the two day incubation on TSA, our data would have altered to include the relative inhibitory effects of tobramycin rather than just the final cell counts (Fig. 1).

A trend of increasing biofilm production as the ratio of wt to *pel*B decreased was also observed (Fig. 2) This result is in agreement with the final ratios (Table 2). Comparing Fig. 2 with Fig. 1, the cultures that produced the greatest amount of biofilm also had the least amount of colonies counted, possibly indicating the method of homogenization was insufficient in fully releasing bacteria from biofilms. While the data showed a possible trend of a lower bacteria count in the congo red plates correlating with an increased amounts of biofilm produced (*pel*B, 3:1, and 1:1 in Fig. 1 and Fig. 2), the averages of these values are not significant enough to support this claim.

There has been evidence suggesting that tissue homogenizers are unsuitable for dislodging microorganisms or producing fully homogenized solutions (1, 3). An approach to increasing the release efficiency is perhaps to treat biofilms with alginase to reduce the rigidity of the biofilm.

It is also important to note that the MIC determination in this study was done through a non-conventional method; therefore our determined MIC of

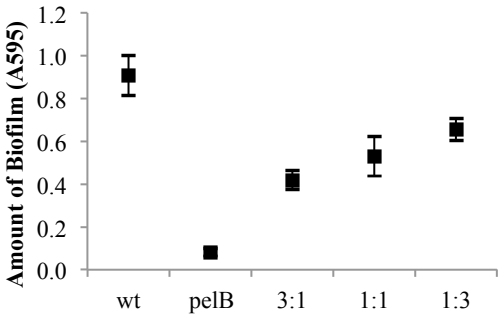

FIG. 2. Quantification of biofilm production by *P. aeruginosa* cultured and co-cultured strains. The error bars were generated by using the two-sided student t-test at 95% confidence level with n = 12.

8 µg/ml of tobramycin may be different than the optimal MIC. This is because most MIC determinations have antibiotic concentration increased in two-fold (6). If our determined MIC of tobramycin was unable to reduce the growth of *pel*B mutants, that could also explain the higher *pel*B CFU compared to the wt (Fig. 1).

Another possible explanation for the difference between wt and *pel*B counts in Fig. 1, is the fact that both wt and *pel*B were grown overnight in TSA. This would have given ample time for some biofilm to form at the air liquid interface and thus activate biofilm-associated genes (5, 8). Since the co-culturing was based on the OD_{600} readings, there may have been some differences between the wt and *pel*B mutant. Due to cell arrest, the wt may be physically different in terms of size compared with the *pel*B mutant leading to different intended initial ratios. To correct for this, a similar plating method with congo red TSA and gentamicin TSA plates prior to co-culturing should be done to ensure the correct ratios.

This experiment gave unexpected results. One of which is the increased growth of *pel*B during the co-culture period after setting the initial ratios of 3:1 and 1:1. If the biofilm confer uniform protection against tobramycin, the expected results would be to have the initial ratios before co-culturing to be maintained. The other result is the progressive increase in the amount of biofilm produced as the ratios of wt to pelB went from 3:1, 1:1, and 1:3. If the *pel*B was unable to influence wt cells in the co-culture, there would have been a gradual decrease in the production of biofilm. The lack of time and controls affected difficulty in establishing the specific mechanism that Pel conferred protection to cells within the biofilm against tobramycin, an aminoglycoside antibiotic. However, the difference observed through the co-cultures also gave insight into the complexity of the regulation of biofilm formation and that genetics are not the sole determinant of growth, as it is not enough to explain our results. There are likely factors associated with cell to cell signaling as well as environmental factors.

FUTURE EXPERIMENTS

Although the greatest amount of biofilm was observed in the co-culture of 3:1 and the least in the 1:3 ratio, the opposite results were shown from the plate count after tobramycin exposure (Table 2). To confirm that the method of homogenization releases sufficient amount of biofilm, using chemicals that break biofilm to a greater extent could be employed, rather than just the vortexing and homogenizing used in this experiment.

Also, more diverse ratios of co-cultures such as 6:1 and 1:6 could be employed to yield trends and significant values to either support or refute the claim that a gradually lower average of bacteria counts correlates with an increase in biofilm formation.

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