Journal of Experimental Microbiology and Immunology Vol. 12:27-33

The Role of Biofilm Structure in the Mechanism of Gentamicin and Ciprofloxacin Antibiotic Resistance in *P. aeruginosa* PAO1 Biofilms

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Biofilms of the opportunistic pathogen *Pseudomonas aeruginosa* are implicated in the pathology of numerous infections. These biofilms are resistant to multiple antibiotics, and several mechanisms have been proposed to explain this phenomenon. This study investigated whether the resistance of *P. aeruginosa* PAO1 biofilms to gentamicin and ciprofloxacin can be explained by the inability of these antibiotics to penetrate the extrapolymeric substances (EPS) layer of the biofilm. Disrupted and undisrupted biofilms were treated with antibiotic or Tryptic Soy Broth (TSB) media for four hours. Disrupted samples were prepared by subjecting biofilms to tissue homogenization prior to antibiotic treatment in order to model complete antibiotic penetration, whereas undisrupted samples were prepared by simply leaving biofilms intact. In addition, qualitative pellet size observations were made of undisrupted, treated biofilms. Results indicated that 1- and 5-day old disrupted biofilms had similar cell concentrations to their respective undisrupted counterparts. We also observed that gentamicin effectively reduced cell concentrations in 1- and 5-day old biofilms, while ciprofloxacin was only effective against 5-day old biofilms. Antibiotics appeared to reduce overall biofilm size. We conclude that resistance of *P. aeruginosa* biofilm cells to gentamicin and ciprofloxacin after four hours of incubation cannot be explained solely by an inability of the antibiotics to penetrate the EPS layer, and that other resistance mechanisms must also be contributing.

In nature, bacteria are often found living as part of biofilms: a collection of immobile cells that adhere to surfaces and live as communities within an excreted matrix known as extrapolymeric substances (EPS) (3). The EPS is a complex of polysaccharides, lipids, nucleic acids and proteins, which is known to form a barrier around the cellular community (13) and hinder the entry of chemical substances (3). Hence, cells living as part of a biofilm have higher tolerances to antibiotics (4).

One common biofilm former is *Pseudomonas aeruginosa*, a gram negative, rod-shaped, aerobic bacterium, whose biofilm formation is implicated in numerous health related problems (8). *P. aeruginosa* is an opportunistic pathogen that is responsible for numerous nosocomial infections including urinary tract infections resulting from biofilm formation on catheters (8), various infections resulting from biofilm formation on medical implant devices (3), infection in patients with serious burns or other skin traumas, and most notably, pulmonary infections in Cystic Fibrosis patients (8). It is also a common cause of systemic infections, and is known to be resistant to a wide range of antibiotics (8). Determining the mechanisms of antibiotic resistance in *P. aeruginosa* biofilms is clearly important for advances in medical treatment of *P. aeruginosa* infections.

The development of *P. aeruginosa* biofilms begins with transient attachment of cells onto a surface (11). After two hours, the cells begin to attach irreversibly to the surface and to one another, which initiates the formation of the biofilm community (11). By three days the first stage of maturation has been reached, characterized by a film thickness of at least 10 μm (11). This is also the first time point at which the EPS layer can be visualized and cellular protein expression is significantly different from planktonic cellular protein expression (11). After approximately six days, biofilms growing in the presence of a continuous flow of nutrients have reached maximum thickness (usually 100 μm), and have fully transitioned from planktonic cells into members of a biofilm community (11).

Two antibiotics that are currently used to treat clinical infections of *P. aeruginosa* are gentamicin and ciprofloxacin. Both of these antibiotics are bacteriocidal and hence result in cell death (6, 7). Gentamicin is an aminoglycoside antibiotic that is able to inhibit protein synthesis by binding to cellular ribosomes (6, 16). Ciprofloxacin, a fluoroquinolone antibiotic, directly inhibits DNA replication by preventing the enzymatic action of DNA gyrase (7, 1).

There are several proposed explanations for the resistance of biofilms to antibiotics. One asserts that the reason biofilms are resistant to antibiotics is due to the inability of the antibiotic to completely penetrate the EPS and enter the biofilm (10, 3, 12). A

second explanation states that resistance is due to the fact that some of the cells within the biofilm are in a metabolically inactive state (3). It may be the case that some cells within a biofilm experience conditions under which nutrients are scarce, and therefore enter this state of reduced metabolic activity, making bacteriocidal antibiotics less effective at killing them (3). Alternatively, changes in gene expression levels, and therefore the upregulation or downregulation of specific gene products, may alter the cells' susceptibility to antibiotics (11). In each case, the surviving persisting cells which are not killed by the antibiotic can later regenerate the biofilm (2).

The purpose of this experiment is to test the theory that the inability of antibiotics to penetrate the structure of the EPS of a biofilm is responsible for the resistance of *P. aeruginosa* PAO1 biofilms to gentamicin and ciprofloxacin antibiotics. To do this, an experimental model was designed which uses "disrupted" and "undisrupted" biofilms. "Disrupted" biofilms were those that were homogenized to simulate complete penetration by an antibiotic, while the "undisrupted" biofilms were simply left intact.

MATERIALS AND METHODS

Strains and chemicals. *P. aeruginosa* PAO1 was obtained from the Microbiology 421 culture collection. Gentamicin sulphate was purchased from Sigma (Cat. no. G-3632). Ciprofloxacin was purchased from Sigma-Aldrich (Cat. no. 17850).

Growth conditions and biofilm formation. In this experiment, 1-day old *P. aeruginosa* biofilms grown without constant nutrient flow were used to represent an immature biofilm, as the EPS layer is still in the beginning stages of growth and is not yet fully formed; and 5-day old biofilms grown without constant nutrient flow were used to represent mature biofilms with a complete, intact EPS layer. To prepare overnight cultures, bacteria were grown in 30 mL Tryptic Soy Broth (TSB) for 25.5 hours at 37˚C with aeration and agitation of 150 rpm in a shaking incubator. TSB (pH 7.4) was prepared using a powdered base purchased from Merck (Cat. no. 5459). Following overnight incubation, 36 biofilms were prepared for each time point by adding aliquots of 30 µl overnight culture to 3.0 ml TSB in 16 x 125 mm test tubes, and continuing incubation at 37˚C without aeration or agitation. Biofilms were allowed to grow for 1 or 5 days.

Disruption of biofilms. Following biofilm growth over the indicated time period (1 or 5 days), a metal spatula was used to scrape the edges of the biofilms to be disrupted off the sides of the tubes. A 7 ml Dounce Tissue Homogenizer and corresponding tight pestle A (Wheaton, Cat. no. 357542) was used for 60 seconds with a push/twist motion to break up the cells of each biofilm and disrupt the biofilm structure without damaging cell viability. The effectiveness of this homogenization and disruption method was verified using phase-contrast microscopy to visualize individually separated cells (data not shown).

Antibiotic addition. 100 µl of 23.2 µg/ml gentamicin solution was added to the appropriate replicates of the disrupted and undisrupted biofilms treated with gentamicin, 100 µl of 17.4 µg/ml ciprofloxacin solution was added to the appropriate replicates of the ciprofloxacin disrupted and undisrupted biofilms, and a mock treatment with 100 µl of TSB medium was added to the appropriate replicates of disrupted and undisrupted control biofilms. Antibiotic was added over the top of both the disrupted and undisrupted biofilms to prevent it from immediately reaching the bottom layers of the undisrupted biofilms. All biofilms were then further incubated at 37˚C without aeration or agitation for four hours.

Estimating biofilm size. The relative sizes of the biofilms before and after experimental treatment were subjectively compared using a centrifugation and pelleting method. The contents of each biofilm to be pelleted was transferred to a 2 ml microcentrifuge tube and centrifuged at 16,000 x g for 10 minutes to allow a pellet to form. The size of each pellet was then visually compared to a control pellet prepared from untreated biofilms at the time of antibiotic addition. These preparations and comparisons were all made by a single observer, and a two-fold or greater difference was observed to be significant. Figure 1 illustrates the magnitude of size reduction taken to be significant.

FIG. 1. Qualitative observational method shows that antibiotic treatment reduces the overall mass of one-day old undisrupted biofilms. From left: control, gentamicin, and ciprofloxacin-treated samples.

Quantifying viable planktonic cells. To determine the number of planktonic cells remaining in the liquid medium after biofilm formation, plate counts were performed. Following the four hour incubation, planktonic bacteria were separated from the biofilms of three untreated replicates by draining the liquid medium from the test tubes through small holes in parafilm stretched across the top of the tube. Some biofilm material was inevitably transferred along with the liquid media, which likely affected the results of this assay. Serial dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} of the planktonic cell-containing supernatants were prepared, and 100 µl of each dilution was spread plated onto Tryptic Soy Agar (TSA) media containing TSB and 15% Select Agar (Invitrogen, Cat. no. 30391-023) in triplicate. The plates were incubated in an inverted position at 37˚C for approximately 18 hours to allow for growth to occur. The results were subtracted from the total viable cells calculated for the control biofilms in order to eliminate planktonic bacteria from the results. It was assumed that treatment with antibiotics killed all planktonic cells in the disrupted and undisrupted antibiotic treated biofilms, as the concentrations of each antibiotic used were well above minimum inhibitory concentrations for planktonic *P. aeruginosa*.

Quantifying viable cells of biofilms. To quantify the number of viable cells present following antibiotic treatment, each undisrupted biofilm was disrupted using the method described above, and both previously disrupted and newly disrupted biofilms were vigorously vortexed. The contents of each biofilm was transferred to a 2 ml microcentrifuge tube and briefly centrifuged at 8160 x g for 6 minutes to pellet cells of the biofilm. The antibiotic-containing supernatant was removed and the pellet resuspended in 1.0 ml TSB medium by pipetting and vortexing. Serial dilutions were prepared, and 100 µl of each dilution was spread plated in triplicate onto TSA media. All plates were incubated in an inverted position at 37˚C for approximately 18 hours to allow for growth to occur.

RESULTS

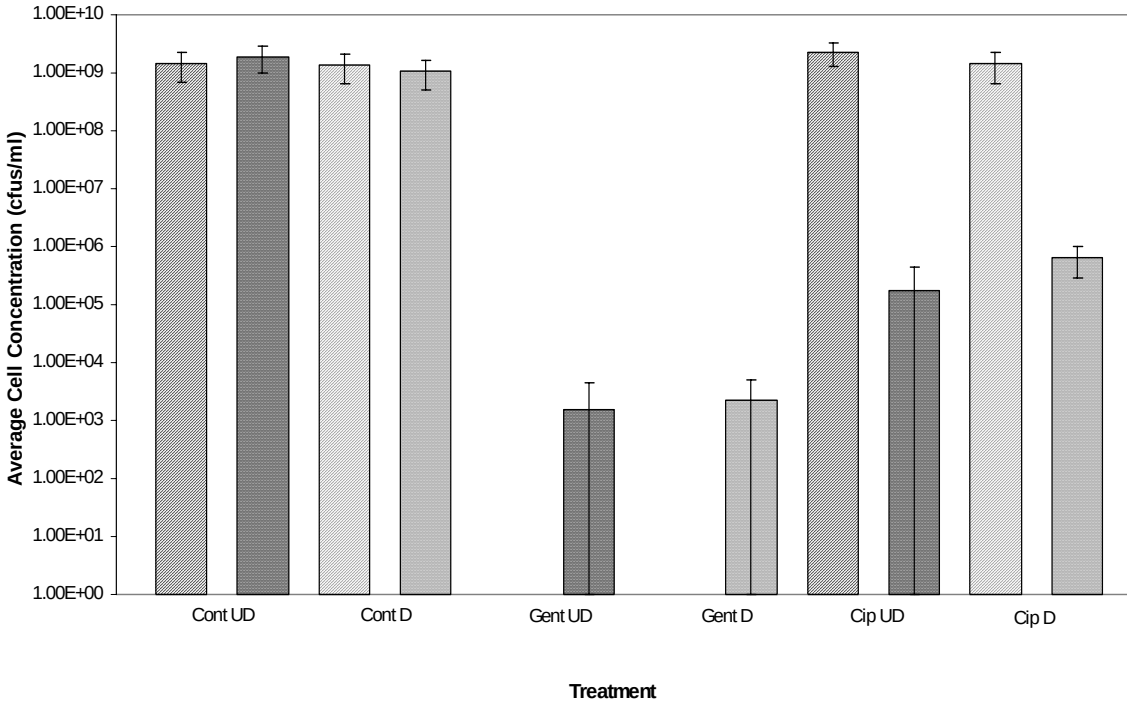

FIG. 2. Comparison of responses of 1-day old immature biofilms (diagonal lines) and 5-day old mature biofilms (shaded) of *P. aeruginosa* PAO1 to gentamicin and ciprofloxacin antibiotics as determined by viable plate counts. Error bars indicate 95% confidence intervals.

Comparison of 1-day old biofilms disrupted prior to treatment with their respective undisrupted counterparts reveals equally effective cell killing. The control undisrupted and control disrupted biofilms were not significantly different from one another, as indicated by the overlapping error bars for these two treatments (Fig. 2). Both gentamicin samples had a value of zero viable cells, and therefore they were also not significantly different from one another. However, this could be due to the fact that the spread plate dilutions were not low enough to capture the true concentrations of these samples. In addition, both the disrupted and undisrupted ciprofloxacin treated biofilms were not significantly different from one another. In general, disruption of 1-day old biofilms prior to treatment did not significantly alter cellular concentrations in comparison with the undisrupted biofilms.

Table 1. Qualitative size of pelleted undisrupted *P.aeruginosa PAO1* biofilms. Relative size is indicated by the number of pluses. Sample not available (X).

	Replicate	Initial control	Final Control	Ciprofloxacin treated	Gentamicin treated
1-day old biofilm	A	++++	++++++	++	+
	B	++++	+++++	++	+
	C	++++	+++++	++	+
5-day old biofilm	A	++++	+++	++	++
	B	++++	X	++	+++

Table 2. Comparative analysis of viable cell counts and pellet size for observing the effects of antibiotics on *P. aeruginosa* PAO1 undisrupted biofilms.

	Observed Change in Ciprofloxacin treated biofilms		Observed Change in Gentamicin treated biofilms	
	Pellet size	Cell Concentration	Pellet size	Cell Concentration
1-day biofilm	Reduction	No change	Reduction	Reduction
5-day biofilm	Reduction	Reduction	Reduction	Reduction

Comparison of 5-day old biofilms that were disrupted prior to treatment with their respective undisrupted counterparts reveals equally effective cell killing. As indicated by the overlapping error bars (Fig. 2.), there was no significant difference in viable cell concentration between the disrupted and undisrupted biofilms that were treated with TSB media as a control. The same results were found for gentamicin treated and ciprofloxacin treated biofilms. Overall, disrupting a 5-day old biofilm prior to treatment does not appear to result in significantly different viable cell concentrations in comparison with a corresponding undisrupted sample.

Gentamicin effectively killed *P. aeruginosa* PAO1 biofilms of varying maturity levels, while ciprofloxacin was effective only against mature biofilms. Figure 2 summarizes the results obtained from the 1 and 5-day time points. 1-day old biofilms treated with ciprofloxacin were not significantly different than their corresponding control. Gentamicin treated samples appear to have been completely eliminated; however, we cannot be certain of complete elimination because of the limited range of dilutions plated. Nonetheless, the data supports the suggestion that viable cell counts following gentamicin antibiotic treatment were significantly lower than control values.

In the 5-day biofilms, the viable cell counts of the undisrupted control sample were significantly different from both the gentamicin undisrupted sample (by a minimum difference of 5 orders of magnitude) and the ciprofloxacin undisrupted sample (by a minimum difference of 3 orders of magnitude). Similarly, the disrupted control biofilms had a significantly higher number of viable cells than the gentamicin and ciprofloxacin disrupted biofilms (a minimum difference of 3 orders of magnitude for both).

In general, as illustrated in Figure 2, incubation of the biofilms with gentamicin caused a significant reduction in the viable cell concentrations of both mature and immature biofilms, regardless of whether the biofilms were disrupted or undisrupted.

Color change observed in antibiotic treated *P. aeruginosa PAO1* biofilms. Prior to pelleting, it was observed that all biofilms which had been incubated with an antibiotic (gentamicin or ciprofloxacin) exhibited dark green coloration in the media beneath the biofilm. This effect was not noted in control cultures treated with TSB media.

Both ciprofloxacin and gentamicin treatment reduced the physical size of *P. aeruginosa* biofilms. The Crystal Violet (CV) assay initially considered for use in quantifying overall biofilm size was inaccurate, as the excess crystal violet stain could not be sufficiently removed from the biofilm cells following addition. Therefore, an alternative, qualitative method of estimating biofilm size by visually comparing pellet size was devised. Table 1 summarizes the results from pelleting experiments. Following treatment, the 1-day control biofilm pellets were, on average, slightly larger than

the initial pellets. This result is expected, as the final control pellets were incubated for an additional four hours while the test samples were being treated with inhibitory antibiotics. The 1-day ciprofloxacin and gentamicin treated biofilm pellets were both greatly diminished in size in comparison with the initial control, which further exemplifies the effectiveness of these antibiotics in inhibiting cells of a one-day immature biofilm. The 5-day media treated control pellet was slightly smaller than the initial control sample, suggesting some biofilm may have been lost during the experimental process. However, the ciprofloxacin and gentamicin treated samples were again substantially smaller than the initial control sample. After normalizing for procedural error by normalizing the results with the corresponding control, both ciprofloxacin and gentamicin treated samples were smaller than the initial pellet size, therefore suggesting that both antibiotics were effective in reducing the size of the 5-day biofilm.

Similar trends were observed for both quantitative cell counts and qualitative pellet size analysis of *P. aeruginosa* biofilms treated with either ciprofloxacin or gentamicin. Similar trends were observed upon comparison of the two methods used in determining the effect of antibiotic addition on undisrupted biofilms (as shown in Table 2). Treatment of 1-day old biofilms with gentamicin reduced both the size of the biofilm pellet and the viable cell concentration, indicating that the antibiotic was able to effectively reduce cell concentrations as well as the overall mass of the biofilm. The same effect was observed in both the 5-day gentamicin and 5-day ciprofloxacin treated samples. However the 1-day ciprofloxacin treated sample was not consistent with this pattern, as the viable cell concentration remained relatively constant while the size of the biofilm pellet was noticeably reduced. This suggests that ciprofloxacin may have been able to reduce the size of the EPS layer, the physical size of the cells, or increase the density of the pelleted cells, thus making the pellet appear smaller while the cellular concentration remained constant.

DISCUSSION

The similarity of the results for treated undisrupted and disrupted biofilms appear to confirm that the intact structure of a biofilm does not play a role in increasing the resistance of *Pseudomonas aeruginosa* PAO1 to aminoglycoside antibiotics such as gentamicin or fluoroquinolone antibiotics such as ciprofloxacin. This is demonstrated in Figure 2, in which no difference is observed between disrupted and undisrupted biofilms in terms of antibiotic resistance. However, this does not necessarily mean that the EPS matrix generated by *P. aeruginosa* is unimportant to antibiotic resistance, as the EPS forming molecules were still present in the disrupted biofilms. Thus, any EPS-associated factors responsible for antibiotic resistance would remain present in the growth medium at the same concentration as the undisrupted, intact biofilms. Comparable experiments performed by Brooun *et al.* also demonstrated that there was no change in antibiotic resistance of *P. aeruginosa* biofilms before and after disruption (2). However, they found that dilution of the disrupted biofilm and likely, though unconfirmed, dilution of EPS associated resistance factors, did increase susceptibility of *P. aeruginosa* to aminoglycoside antibiotics up to a certain dilution, though resistance was still substantially higher than that observed in planktonic cells (2). This implies that although EPS associated resistance factors likely contribute to resistance, other more intrinsic mechanisms such as changes in the gene expression profile of biofilm-associated cells, are important (2).

It is important to note that diffusion of aminoglycosides is known to be somewhat impeded in biofilms (15). This effect may not have been apparent in our experiment because the four hour antibiotic treatment may have allowed sufficient time for complete antibiotic equilibration across the biofilm. This would explain the observed lack of difference in viable cell counts between the disrupted and undisrupted treatments (Fig.2.). However, it has been reported in multiple studies that resuspended biofilm cells still show increased antibiotic resistance over planktonic cells (15, 2). Again, more intrinsic mechanisms are likely contributing to this phenomenon.

Although biofilm associated *P. aeruginosa* show much greater resistance to antibiotics when compared to planktonic cells, there is still a significant amount of killing observed (2). We observed that biofilm treatment with gentamicin and ciprofloxacin after 5 days of growth reduced the number of colony forming units (and thus viable cells in the biofilm) by 5 and 3 orders of magnitude respectively (Fig. 2.). This is not a unique finding; it has been previously reported that antibiotics reduce viable cell counts by 3 to 4 orders of magnitude, at which point the residual viable cell counts plateau, even as antibiotic concentrations increase (2, 5). It is these remaining super resistant persisting cells which persevere and enable the biofilm to survive (2). Furthermore, it has been demonstrated that these persisting cells do not arise by mutation, but are a part of the normal *P. aeruginosa* population (2). Once antibiotics are removed, the cells are able to re-form biofilms with nearly identical susceptibility patterns and minimum

inhibitory antibiotic concentrations to the biofilms from which they originally came (2).

A key to explaining why persisting cells can remain viable after antibiotic treatment is the magnitude of changes in gene expression which occur during biofilm formation. It has been previously reported that a developing *P. aeruginosa* biofilm will undergo five stages of development (11). Each of these stages demonstrate substantial changes in gene expression patterns, with nearly 50% of all *Pseudomonas* proteins being upregulated and only 7% downregulated (11). To better understand the size of this difference, it may be beneficial to note that in terms of gene expression patterns, a biofilm associated *P. aeruginosa* cell is potentially as different from a planktonic cell as planktonic cells are different from another *Pseudomonas* strain (11).

Another important observation to consider is the dark green color which developed most prominently in our antibiotic treated, disrupted biofilms. This color development is believed to be due to the production of pyocyanin, a blue-green phenazine pigment known to promote neutrophil apoptosis, and have toxic effects on competing species of prokaryotes (14). It is thus possible that when *P. aeruginosa* is under stress, such as antibiotic treatment, pyocyanin is released as a non-specific defense mechanism (14). Interestingly, it has been reported that pyocyanin also is a signaling molecule involved in quorum sensing, and thus can initiate modification of gene expression (14). Perhaps pyocyanin release plays a role in the initiation of an adaptive response to stresses such as antibiotic treatment. There is some evidence to support this proposal, as the gene *mexGHI-ompD*, a multidrug efflux pump, has been designated as part of a proposed pyocyanin stimulon (9). This, along with the observation that no difference in viable cell counts was evident between disrupted and undisrupted biofilms, suggests that biofilm structure penetration is not the major mechanism contributing to biofilm antibiotic resistance. Further testing will be required to determine the role of pyocyanin and gene expression profiles in antibiotic resistance.

Another interesting observation is the drastic reduction in size (Table 2) and apparent complete eradication of all bacteria, including persisting cells (Fig. 2.), in 1-day-old biofilms treated with gentamicin. Aminoglycoside antibiotics are known to be highly effective against *P. aeruginosa*, but the organism does possess highly specific defense mechanisms against these antibiotics (2). In fact, *P. aeruginosa* is so well adapted to aminoglycoside antibiotics that Hoffman *et al.* have demonstrated a specific response regulator which allows an increase in cell concentrations within a biofilm when subinhibitory concentrations of aminoglycosides are detected (5). It is therefore the opinion of the authors that this result is more likely due to the plating of overly diluted samples than complete killing of all biofilm and persisting cells.

Variability was another important factor that must be taken into consideration during analysis of our experimental results, as can been seen by the large 95% confidence intervals in Figure 2. An entire 3-day time point was discarded because variability was so high that the resulting plate counts could not be interpreted with any certainty. A major contributing factor was the error generated during enumeration of viable cells in biofilms. In order to plate biofilm cells, it was required that we separate as many of the planktonic cells as possible from the biofilm, resuspend the biofilm, and homogenize it. Variability can be introduced in any of these steps. First, during removal of growth medium containing planktonic cells, it is possible that biofilm could be lost, thus reducing the number of viable cells detected. Conversely, if the entire medium is not removed, planktonic cells could contaminate the biofilm, falsely indicating higher viable cell counts. Second, it is possible that homogenization of the biofilm did not produce a homogeneous mixture, leading to plating of sections of biofilm which contained clusters of cells. This would imply a lower concentration of viable cells than was actually present. The importance of this variability can be seen when we consider that it masked any potential significant differences between the ciprofloxacin and gentamicin treatments of the 5-day-old biofilms, despite a trend towards more killing by gentamicin.

Despite the variability in plate counts, the conclusions drawn from them are in agreement with the sizes of our antibiotic treated and pelleted biofilms. Thus, the results from the two diverse experiments exhibit a common trend, showing that the structure of the biofilm does not appear to be an effective barrier to either ciprofloxacin or gentamicin when treated for four hours. The correlation between the experiments provides credibility to our findings.

Antibiotic resistance in *P. aeruginosa PAO1* biofilms after four hours of treatment with gentamicin and ciprofloxacin can not be solely explained by retarded diffusion through the EPS matrix. Although this non-specific mechanism likely does play a role in antibiotic resistance, many other highly specific mechanisms are also in place within the bacterial cells. Understanding how these key mechanisms function is essential if we are to have any hope of successfully treating persistent *P. aeruginosa* infections.

FUTURE EXPERIMENTS

This experiment demonstrated that the effect of antibiotic treatment on disrupted and undisrupted biofilms was identical following a four hour incubation period. It would be beneficial to do a time-course experiment investigating the effects of antibiotics incubated with disrupted and undisrupted biofilms for varying lengths of time, in order to confirm that the results observed in this experiment are not simply due to the prolonged exposure time, and therefore complete antibiotic equilibration across the EPS. In addition, the experiment should also be further refined by varying the dilutions of disrupted biofilms in order to dilute the EPS and determine whether concentration of EPS-associated factors in the matrix plays a role in antibiotic susceptibility.

Other mechanisms, possibly the overall decrease in metabolic and growth rate of persisting cells or the changes in gene expression patterns which occur in differentiated biofilm-associated cells, must be contributing to antibiotic resistance. In order to determine whether growth rate is a determining factor in antibiotic susceptibility, time-course experiments could be performed which measure both the growth rate of *P. aeruginosa* biofilm-associated cells and their antibiotic resistance. If growth rate does indeed contribute to biofilm resistance to specific antibiotics, an inverse relationship between growth rate and antibiotic resistance is expected, as faster growing cells will be more susceptible to antibiotics.

ACKNOWLEDGEMENTS

This work was supported by funds from the Department of Microbiology and Immunology of the University of British Columbia, and the supervision and direction of Dr. William Ramey. The authors would like to thank Jennifer Sibley for providing the *P. aeruginosa* PAO1 bacterial strain used in this experiment, Lando Robillo for his useful advice and tireless work autoclaving media and supplies, Shaan Gellatly for her helpful suggestions and guidance throughout this project, Karen Smith for her assistance in developing our methods, as well as Dr. Ramey for his support and assistance in the preparation and planning of this experiment.

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