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The Effect of Biofilm Disruption on Growth and Metabolism of *Pseudomonas aeruginosa* PAO1

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Disruption of *Pseudomonas aeruginosa* PAO1 biofilms with alginate lyase has been shown to increase antibiotic susceptibility while physical disruption of the biofilm has not. The current study examined whether cell division and synthesis of RNA, DNA and protein differed between cells that were physically disrupted in the presence or absence of alginate lyase. Incorporation of radioactive uracil, thymine and glycine was used to assess RNA, DNA and protein synthesis, respectively, over a 4 hour time course. Plate counts were used to assess cell number at each time point. Cells treated with alginate lyase were found to increase specific incorporation of uracil, thymine and glycine until 2 hours, after which the levels decreased. This coincided with the time when treated cells began to divide. In contrast, the untreated cells were capable of synthesizing RNA, DNA and protein to high levels, but were unable to divide. The results indicate that, while macromolecule synthesis did not vary between alginate lyase treated and untreated cells, the ability of those cells to divide did. This may account for the differential antibiotic susceptibility of physically disrupted versus alginate lyase treated *P. aeruginosa* PAO1 cells.

Alginate lyase is an enzyme that catalyzes the degradation of alginate (13). Several papers have reported successful treatment of established *P. aeruginosa* biofilms in vitro with a combination of alginate lyase and antibiotic. Alkawash *et al.* observed almost complete elimination of a biofilm maintained in a continuous flow culture when treated with alginate lyase and gentamicin, while biofilms treated only with gentamicin persisted (1). Cotton *et al.* saw similar results in a 4 hour treatment with ciprofloxacin (4). However, the exact mechanism of the alginate lyase-mediated increase in susceptibility has yet to be elucidated. While alginate lyase could have enabled higher antibiotic penetration of the biofilm, leading to increased exposure of cells to antibiotics, other factors may have been involved. Charlesworth *et al.* did not observe an increase in antibiotic susceptibility from physical disruption of biofilms (2).

The ability of *P. aeruginosa* to form a biofilm confers much of its pathology as it decreases its susceptibility to antibiotics and enables partial evasion of the host's immune system (8, 7). The steps of biofilm formation include attachment of planktonic cells to a biotic or abiotic surface, proliferation to form micro-colonies and secretion of a polymer matrix consisting of polysaccharide, protein and DNA (7, 8). The stability and structure of mucoid *P. aeruginosa* biofilms is mainly due to the polysaccharide component, alginate, which is a complex co-polymer of α-L-guluronate and β-D-mannuronate (4, 13). Cells in the biofilm are significantly less sensitive to antibiotic treatment. Indeed, the minimal inhibitory concentration of antibiotics is 100 to 1000 fold higher in biofilm cells compared to planktonic cells (7, 3). This is due to several factors. Firstly, the close proximity of the bacteria to the exopolysaccharide matrix may physically occlude the antibiotic (3, 6, 8). Secondly, bacteria located further away from the biofilm periphery have decreased access to nutrients and oxygen. As a result, these cells experience reduced metabolic activity and increased doubling times which can render antibiotics that target metabolic processes ineffective (6, 7, 3). Thirdly, cells in the biofilm can up-regulate β-lactamases and drug efflux pump expression (8).

Here, we investigated the effects of alginate lyase on the metabolic state of physically homogenized *P. aeruginosa* biofilm cells compared to homogenization alone. A change in metabolism could help explain the observed increase in antibiotic susceptibility in cells treated with alginate lyase but not in physically homogenized cells (4, 2). As both methods of biofilm disruption would likely remove the physical barrier to antibiotic penetration, it is possible that alginate lyase might invoke an additional change in the cells that affects their ability to resist antibiotic treatment. As many antibiotics are effective only against metabolically active cells, we hypothesized that alginate lyase might have increased the metabolic state of *P. aeruginosa* biofilm cells.

MATERIALS AND METHODS

Bacterial strains, media and chemicals. *P. aeruginosa* strain PAO1 was supplied from the MICB 421 Culture Collection in the Department of Microbiology and Immunology Department at the University of British Columbia. All liquid cultures were grown in Tryptic Soy Broth (TSB) and spread plate cultures were plated on Tryptic Soy Agar (TSA), both purchased from Becton, Dickinson and Company (Oakville, ON). Cultures were treated with alginate lyase from *Flavobacterium sp.*, thymine (Sigma Aldrich, Oakville, ON, Cat # A1603, T0376), uracil (Nutritional Biochemicals Incorporation, Cleveland, OH), ^{14}C-glycine (Radiochemical Centre, Amersham, UK), ^{14}C-uracil and ^{14}C-thymine (both New England Nuclear Corporation, Boston, USA).

Biofilm preparation. Biofilms were prepared as described by Cotton *et al.* (4). Briefly, an overnight culture of *P. aeruginosa* was prepared by inoculating 3 ml of TSB with a loop-full of bacteria which was grown with aeration for approximately 24 hours at 37°C on a shaking platform set at 150 rpm. 30 µl of the overnight culture was added to each of five 16 x 125 mm test tubes containing 3 ml of TSB. Biofilms were allowed to grow in the same conditions as stated above for 5 days to characterize mature biofilms (7).

Biofilm isolation and physical homogenization. Biofilms were isolated and physically homogenized as described by Cotton *et al.* (4). Briefly, following biofilm growth for 5 days, each of the 5 cultures was split into two 1.5 ml microfuge tubes and centrifuged for 15 minutes at 18,300 x *g*. Following centrifugation, the biofilm was left suspended in the supernatant while the planktonic cells were pelleted at the bottom of the tube. Biofilms were vacuum filtered using 47 mm, 0.45 micron metrical membrane filters (Gelman, Pensacola, FL) until excess fluid was removed. One filter was used per five microfuge tubes of centrifuged culture. Filtered biofilm from all the tubes were combined and the volume made up to 3 ml with TSB. Biofilms were physically homogenized using a 7 ml Dounce Tissue Homogenizer and corresponding pestle A (Wheaton, Millville, NJ) for 120 seconds using a push/twist motion. Integrity of the individual cells was assessed using phase contrast microscopy.

Preparation of stock solutions. Stock solutions of alginate lyase (60 mg/ml), non-radioactive uracil and thymine (both 2 mg/ml), and ^{14}C-uracil, ^{14}C-thymine, and ^{14}C-glycine (all 10.5 µCi/ml) were prepared in sterile distilled water in 1.5 ml microfuge tubes and mixed vigorously by vortexing, except for the radioactive solutions which were mixed by gentle pipetting. Alginate lyase and radioactive stock solution were prepared fresh while non-radioactive uracil and thymine stock solutions were stored at 4ºC.

Preparation of cultures for incorporation and growth experiment. A master solution containing non-radioactive carrier uracil (6 ug/ml), non-radioactive carrier thymine (4 µl/ml), 1.575 ml of homogenized *P. aeruginosa* biofilm and 11.36 ml of TSB for a final volume of 13 ml was prepared. No carrier glycine was required as TSB contains approximately 40 ug/ml of glycine (5). The culture was then split into two 16 x 125 mm tubes and 224 µl of stock alginate lyase (60 mg/ml) was added to one tube for a final concentration of 20 units/ml while 224 µl of TSB was added to the other. Three 1.5 ml aliquots were taken from each of the alginate lyase treated and untreated cultures and placed in 16 x 125 mm tubes for assessment of radioactive incorporation. ^{14}C-uracil was added to one alginate lyase treated and one untreated tube at a final concentration of 0.017 µCi/ml. Similarly, ^{14}C-thymine and ^{14}C-glycine were added to separate pairs of tubes at a final concentration of 0.092 µCi/ml and 0.112 µCi/ml, respectively. Nine 150 µl aliquots were taken from each of the alginate lyase treated and untreated samples and placed in 1.5 ml microfuge tubes to be used for plate counts. Cultures were separated at the start as there was a concern for biofilm re-formation throughout the time course. This could potentially provide a misrepresentation regarding the number of cells present as conglomerations of cells could be pipetted in the sampling process. All samples were placed in a 37°C water bath with a shaking platform set at 150 RPM. Radioactivity measurements and plate counts were taken at half hour intervals commencing at the 0.0 hour time point and ending at the 4.0 hour time point.

Quantification of biofilms. At each half hour interval one 1.5 ml tube containing 150 µl of alginate lyase treated or untreated sample was removed from the water bath and cultures were homogenized in the microfuge tubes using disposable pestles (Nalge Nunc International, Rochester, NY) for 60 seconds using a push-twist motion. Each homogenized culture was then used to prepare serial dilutions of 10^{-5}, 10^{-6}, and 10^{-7} in TSB. 100 µl of each were spread-plated in duplicate on TSA plates, incubated at 37°C for approximately 17 hours and colonies were counted.

Radioactivity incorporation assays. At each half hour interval radioactive uracil, thymine and glycine incorporation in the alginate lyase treated and untreated cultures were measured by pipetting 50 µl of each culture onto a filter disk (Whatman International Ltd, Springfield Mill, UK, Grade 3) in duplicate. Samples were allowed to absorb and then transferred to a flask of chilled 5% trichloroacetic acid (TCA). In addition samples from the 0.0 hour time point were allowed to air dry to measure total radioactivity and clean filter discs were used to determine background absorption. An additional 5% TCA wash was performed ten minutes after the final disc was added to the TCA and a final 95% ethanol wash was performed ten minutes after the TCA wash. Discs were dried for approximately 18 hours at 100°C and transferred into scintillation vials with 4 ml of ScintiSafe Plus 50% (Fisher Scientific, Ottawa, ON) scintillation fluid. Samples were counted for 2 minutes in a liquid scintillation counter (Beckman Coulter, Kiefeld, Germany, Cat # LS 6000TA).

Statistical analysis. The 95% confidence limits were calculated taking the square root of the total number of colonies or counts (9). Overlapping error bars were interpreted as indicating a non-significant difference between measurements.

RESULTS

Plate count data from physically homogenized biofilms in the presence or absence of alginate lyase indicates increased growth in alginate lyase treated samples. There were significant differences in colony forming units (CFU) between alginate lyase treated and untreated samples (Fig. 1). The alginate lyase treated samples began to increase at 2.0 hours and increased in total number to a maximum of 2.75 x 10^9 CFU/ml. In contrast, the untreated samples did not increase in CFU number over the time course, with the final concentration of 2.16 x 10^8 CFU/ml being only slightly higher than the starting value, 8.10 x 10^7 CFU/ml. The almost 13-fold greater number of CFU in the alginate lyase treated samples indicates that the digestion of alginate may liberate cells from the biofilm more effectively than physical disruption alone, allowing them to begin dividing. In addition to initiating division, the data suggest that the alginate lyase prevented re-establishment of biofilms over the 4 hour period following physical disruption. The plate count results from the 1.5 hour time points appear to be erroneous, as it seems unlikely that 1.64 x 10^8 CFU/ml failed to survive in this interval for the alginate lyase treated samples and 5.90 x 10^7 CFU/ml also failed to survive in the untreated samples, only for them to

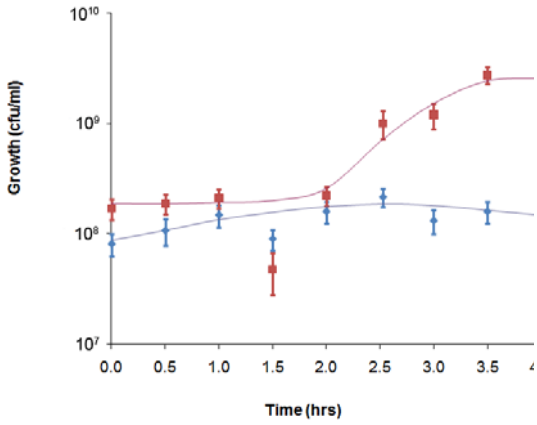

FIG. 1. The growth of alginate lyase treated (square) and untreated (diamond) *P. aeruginosa* PAO1 disrupted biofilms over four hours. Error bars indicate 95% confidence limits.

recover by the 2.0 hour time point. The low values are likely due to an unidentified experimental error.

Radioactive uracil, thymine and glycine incorporation was similar between alginate lyase treated and untreated samples. Incorporation data for radioactive uracil indicates that there were no significant differences in RNA synthesis rates between alginate lyase treated and untreated samples throughout the 4 hour time course. While initial rates of uracil incorporation for both samples were similar, the rate of incorporation in untreated samples looked slightly less compared to treated cells beginning at 1.5 hours post-treatment, though this was not statistically significant. The thymine incorporation patterns were also comparable between alginate lyase treated and untreated samples. In both, the rates were steady until 3.0 hours, at which point they began to increase. Radioactive glycine incorporation appeared to be analogous between the treated and untreated samples. Both samples initially showed a relatively slow rate of incorporation until approximately 3.5 hours, after which the rates increased rapidly. Together, these data indicate that synthesis of RNA, DNA and protein was similar between alginate lyase treated and untreated samples throughout the 4 hour time course (Fig. 2A, 2B, 2C).

Specific incorporation of radioactive uracil, thymine and glycine reveals differential metabolic activity between the alginate lyase treated and untreated samples. Specific incorporation of radioactive uracil indicates significant differences between the RNA synthesis taking place at a cellular level between treated and untreated samples. Initially, the rates mirror each other, but they diverge after 2.0 hours. The untreated samples appear to continuously

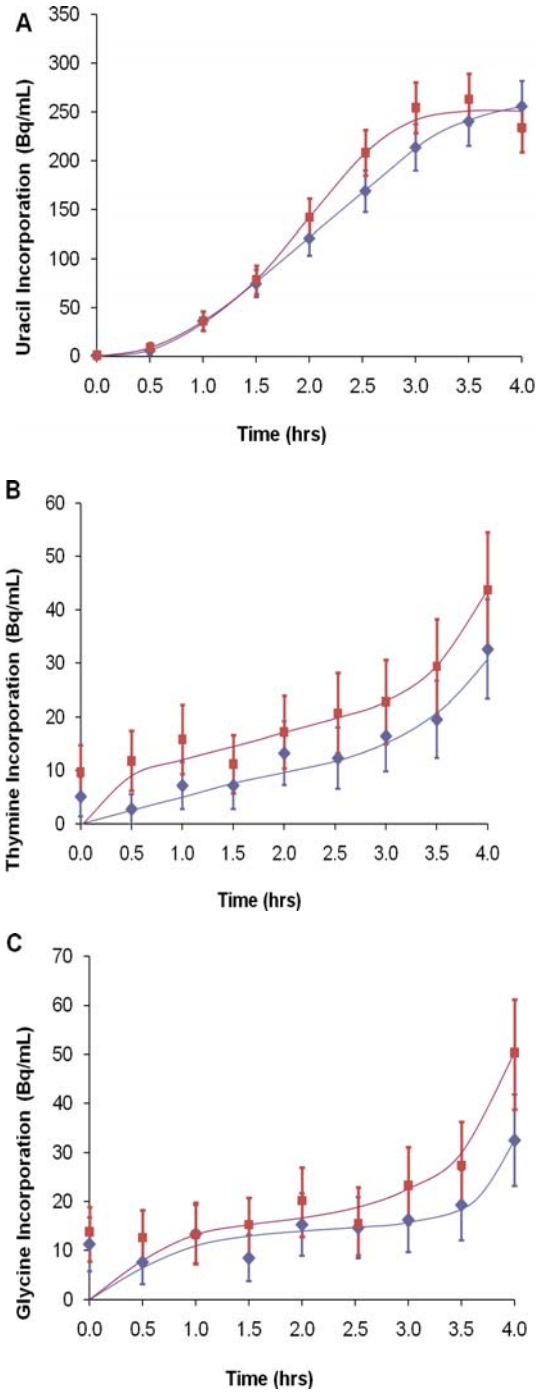

FIG. 2. The incorporation of radioactive (A) uracil, (B) thymine and (C) glycine in alginate lyase treated (square) and untreated (diamond) *P. aeruginosa* PAO1 disrupted biofilms. Error bars indicate 95% confidence limits.

increase the amount of radioactive uracil incorporation throughout the experiment, with 2.13 µBq/CFU present by 4.0 hours. In contrast, the treated disrupted biofilm

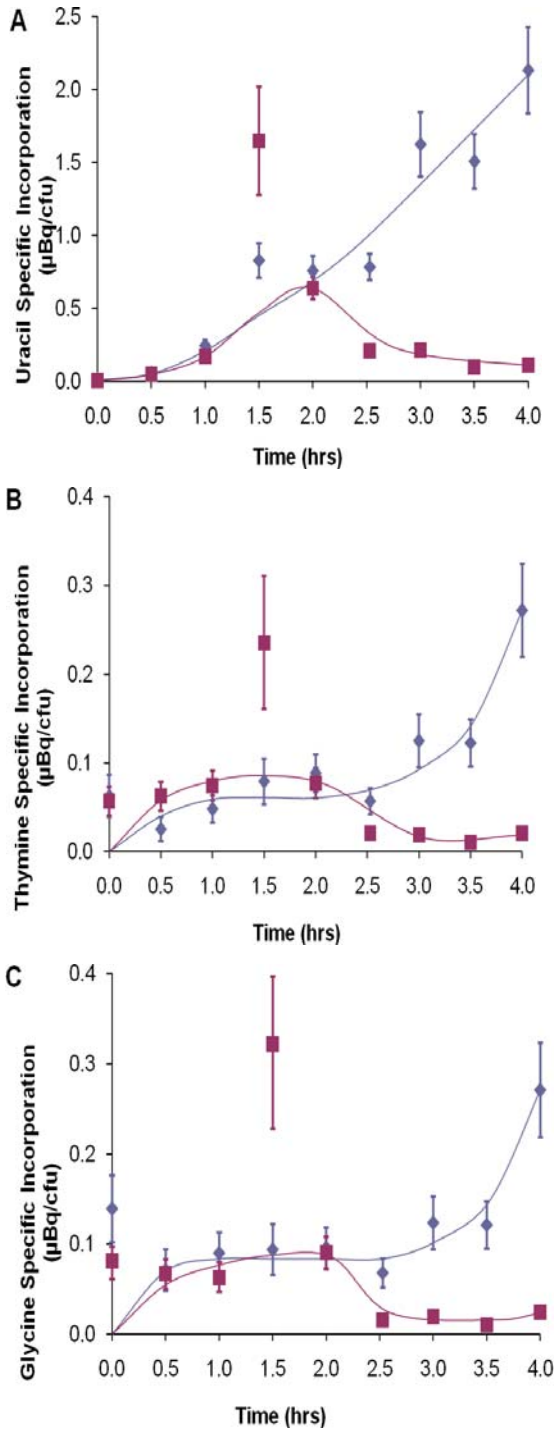

FIG. 3. The specific incorporation of radioactive (A) uracil, (B) thymine and (C) glycine in alginate lyase treated (square) and untreated (diamond) *P. aeruginosa* PAO1 disrupted biofilms. Error bars indicate 95% confidence limits.

appeared to increase the amount of RNA synthesized, to a peak between 1.0 and 2.0 hours, after which the amount of radioactive uracil incorporated decreased (Fig. 3A).

A similar pattern is observed in the specific incorporation of thymine. The untreated samples appear to mirror the incorporation of radioactive thymine in untreated samples from 0.0 to 2.0 hours. Then, the specific incorporation continues increasing from 2.0 to 4.0 hours in untreated samples. In alginate lyase treated cells, a pattern similar to that of uracil specific incorporation is observed, where values increase to a peak between 1.0 and 2.0 hours. After the peak, the amount of radioactive thymine per CFU decreases to approximately 0.17 µBq/CFU from 2.5 to 4.0 hours (Fig. 3B).

The specific incorporation of glycine, as shown in Figure 3C, reveals a similar pattern to that of uracil and thymine. Treated and untreated samples have similar rates of specific incorporation from 0.0 to 2.0 hours. Once again, the patterns diverge after this point. Untreated samples continue increase their specific incorporation to a total of 0.27 µBq/CFU after 4.0 hours. In the treated samples, the incorporation of glycine peaks between 1.0 and 2.0 hours, and subsequently decreases to 0.02 µBq/CFU by 4.0 hours. At the final time point, over 13 times more radioactive glycine was incorporated into untreated than treated samples.

Taken together, the specific incorporation of uracil, thymine and glycine indicate that the alginate lyase treated samples increased the amount of RNA, DNA and protein present until between 1.0 and 2.0 hours, after which they sharply decreased the amount present per CFU. This coincides with the increase in CFUs seen in the plate counts at 2.5 hours and beyond (Fig. 1), indicating that cells in treated samples were preparing to replicate from 0.0 to approximately 2.0 hours. At around 2.0 hours, the cells began dividing, which decreased the amount of RNA, DNA and protein present per CFU. In contrast, the cells in untreated samples appear capable of synthesizing RNA, DNA and protein to much higher levels than those observed in treated samples, but the plate counts show that they were unable to replicate (Fig. 1).

DISCUSSION

The plate counts from biofilms treated with alginate lyase had increased growth compared to untreated samples. There was distinct cell growth observed beginning after 2.0 hours of exposure. This suggests that the activity of alginate lyase liberated the cells from the biofilm and helped create an environment conducive to growth. The untreated cells did not exhibit any evidence of division, as there was no

significant increase in CFU throughout the time course. In mature *P. aeruginosa* biofilms, like our 5 day old cultures, the metabolic activity of cells is heterogenous (10). Oxygen and nutrients, such as glucose, are limited in the core of the biofilm and consequently cells exhibit differences in gene expression and metabolism, most notably in the tricarboxylic acid cycle and amino acid metabolic pathways (10). Release of cells from the biofilm, for instance by treatment with alginate lyase, could induce changes in these cellular processes and others, such as transcription and translation.

The concentration of incorporated radioactive uracil, thymine and glycine in alginate lyase treated versus untreated bacteria were similar throughout the experiment (Fig. 2A, 2B, 2C). However, when one accounts for the number of cells by dividing the concentration of the incorporated isotope by the number of CFU, a different pattern emerges. The untreated samples increased their levels of all three isotopes throughout the 4 hour time course. In contrast, the alginate lyase treated samples mirrored the untreated samples in specific incorporation until 2.0 hours post-treatment, at which point their levels declined and remained low for the remainder of the experiment. The plate count data indicate that alginate lyase treated cells began dividing between the 1.5 and 2.0 hour time points. Together, this indicates that the decrease in specific incorporation for the treated cells after 2.0 hours is due to the division of cells and the resulting decrease in intracellular isotope due to dilution.

The experiment performed by Charlesworth *et al.* found that there was no increase in susceptibility of *P. aeruginosa* PAO1 to ciprofloxacin or gentamicin after physical disruption of 5 day old biofilms (2). The concentrations of ciprofloxacin and gentamicin were 0.56 μg/ml and 0.74 μg/ml, respectively. At these concentrations, both antibiotics act bacteriostatically (12, 11). Cotton *et al*., in a similar experiment, found that disruption of the biofilms with alginate lyase treatment, rather than physical disruption, was able to induce susceptibility to these antibiotics (4). The concentrations of ciprofloxacin and gentamicin used were 0.25 μg/ml and 1.00 μg/ml, respectively. These concentrations ensure that the antibiotics were also acting bacteriostatically (12, 11).

The results of this experiment can help elucidate the reasons behind the difference between the findings of Charlesworth *et al*. and Cotton *et al*. (2, 4). In our experiment, both alginate lyase treated and untreated cells were able to synthesize RNA, DNA and protein. However, only the alginate lyase treated underwent cell division. The untreated samples continued to synthesize the macromolecules throughout the time course, but were not able to divide. This discrepancy can explain the Charlesworth *et al*. and Cotton *et al*. results (2, 4). The effect of bacteriostatic antibiotics will only be detectable in cells which are dividing.

Readers should be cautious in interpreting our results. It is possible that alginate lyase treated samples did not undergo cell division, but only appeared to do so. We cannot be certain that our homogenization step immediately prior to plating had effectively separated clumps of cells. Since alginate lyase breaks up an important biofilm matrix component, it could have further separated clumped cells, thus leading to higher CFU counts. For instance, alginate lyase may have only started to become effective after 2.5 hours of treatment, leading to increased CFU counts towards the end of the time course. It is not possible to discern colonies derived from several clumped cells versus a single cell. While this possibility is unlikely, we do not rule it out.

The findings of our experiment indicate that alginate lyase appeared to be successful in liberating cells from their biofilm. Physically homogenized cells in the presence or absence of alginate lyase continued to produce macromolecules throughout the time course. Therefore, metabolism, as indicated by macromolecular synthesis, did not differ between samples. If the increase in CFUs observed in the alginate lyase treated samples compared to the untreated samples was indeed due to greater division rather than continued release, this may explain why physical disruption of *P. aeruginosa* PAO1 biofilms did not increase antibiotic susceptibility, while disruption with alginate lyase did (2, 4). Future research is needed to elucidate this uncertainty.

FUTURE DIRECTIONS

Future studies could also include other methods of enumerating cell number, which would be critical in determining whether the increase in CFU is due to greater division of cells or continued release from the biofilm. The luminescence of *P. aeruginosa* lux mutants with stable gene expression with and without alginate lyase treatment could be measured and used as an indicator of cell number. Alternatively, treatment of disrupted and undisrupted biofilms with alginate lyase for a period of 20 minutes would allow for the differentiation between release and cell division, as the doubling time of *P. aeruginosa* is greater than the treatment time.

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