Analysis of *Pseudomonas aeruginosa* Biofilms

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Introduction:

Cystic Fibrosis is a genetic disorder caused by at least one of approximately 1,500 possible mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene; it has a prevalence of approximately 1 in 2,500 live births [1]. A defect in this gene leads to the loss of a chlorine ion channel which transports chlorine ions across epithelial cell surfaces. An individual with a malfunctioning channel will have decreased volume of periciliary fluid, or the layer of fluid that is present on the surface epithelial cells, in the respiratory tract. With decreased periciliary fluid volume, the mucociliary escalator has a more difficult time removing inhaled pathogens from the respiratory tract. This process in non-CF patients vs. CF infected patients is shown in figure 1 below. With the mucociliary escalator unable to remove pathogens efficiently, microorganisms such as *Pseudomonas aeruginosa* are able to form colonies in the lungs. This initiates further immune responses by leukocytes and antibodies which scar the lung tissue, impairing its function. Even with antibiotic treatment, the average life span of a patient with cystic fibrosis is 40 years [1].

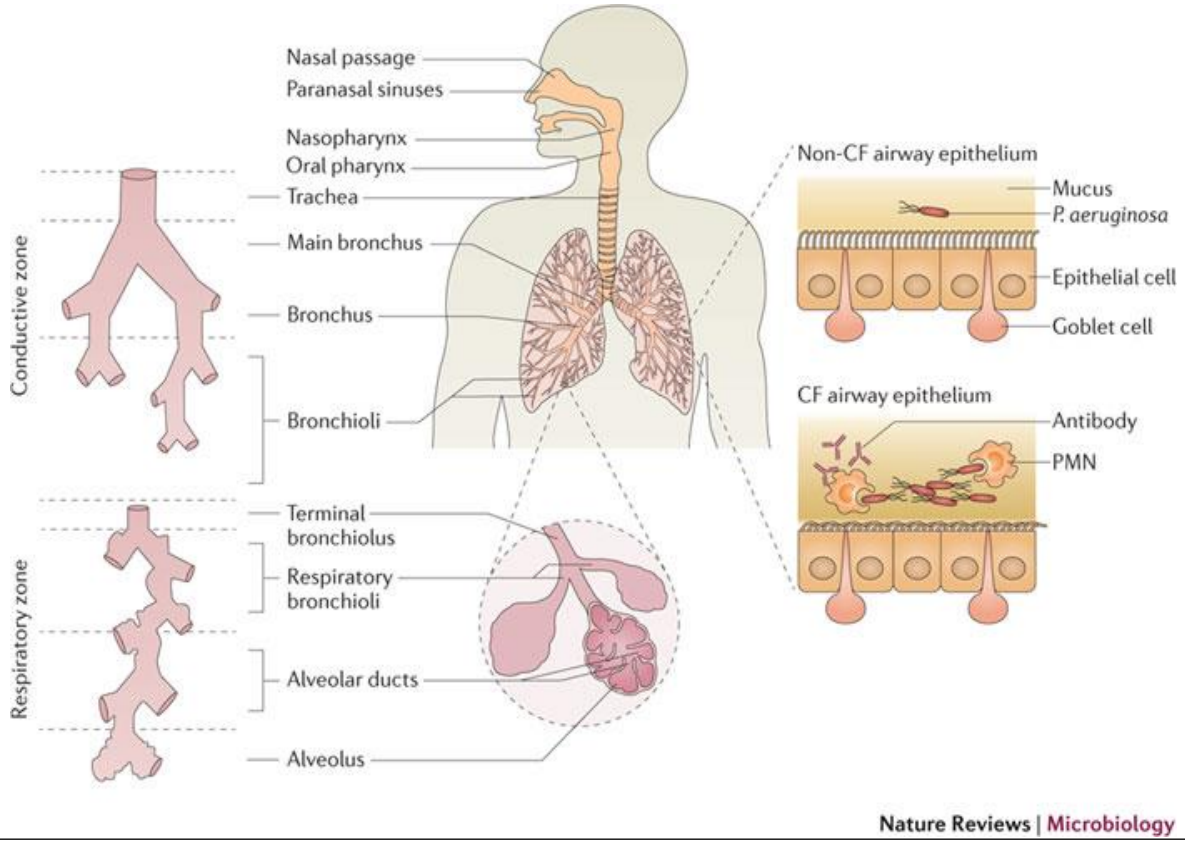

Figure 1: Lungs of Non-CF vs CF patients. In non-CF individuals, particles are trapped in the mucus using the cilia of epithelial cells. In individuals who have CF, cilia cannot function as efficiently, allowing for the formation of bacterial colonies. This leads to a more severe immune response, resulting in scaring of lung tissue which impairs its function [1].

Pseudomonas aeruginosa is the most common microorganism to infect patients with cystic fibrosis, with the bacterium present in the respiratory tract of 60-70% of cystic fibrosis patients [1]. Most infections caused by *P. aeruginosa* persist and turn into chronic infections, leading to respiratory failure and eventually death. One method, by which it is believed that *P. aeruginosa* is able to persist in the lungs is through the formation of biofilms [2]. The mechanism of biofilm formation is shown in figure 2 below. Planktonic cells, or free floating cells, will attach to the epithelial layer of the lungs using their flagella. This will initiate the release of quorum sensing molecules which alter gene expression and downregulate the gene involved in flagella development. Cells will adhere to one another and the surface initiating biofilm formation; biofilm cells will proliferate resulting in continued biofilm growth. Eventually, cells will mature and differentiate. Planktonic cells are released, completing the cycle and leaving inactive persister cells which are resistant to antibiotics [4]. It has been shown that exposure to antibiotics results in the intracellular synthesis of guanosine tetraphosphate (ppGpp) by the ribosomes. After synthesis, ppGpp will suppress translation and degrade the ribosomes [5].

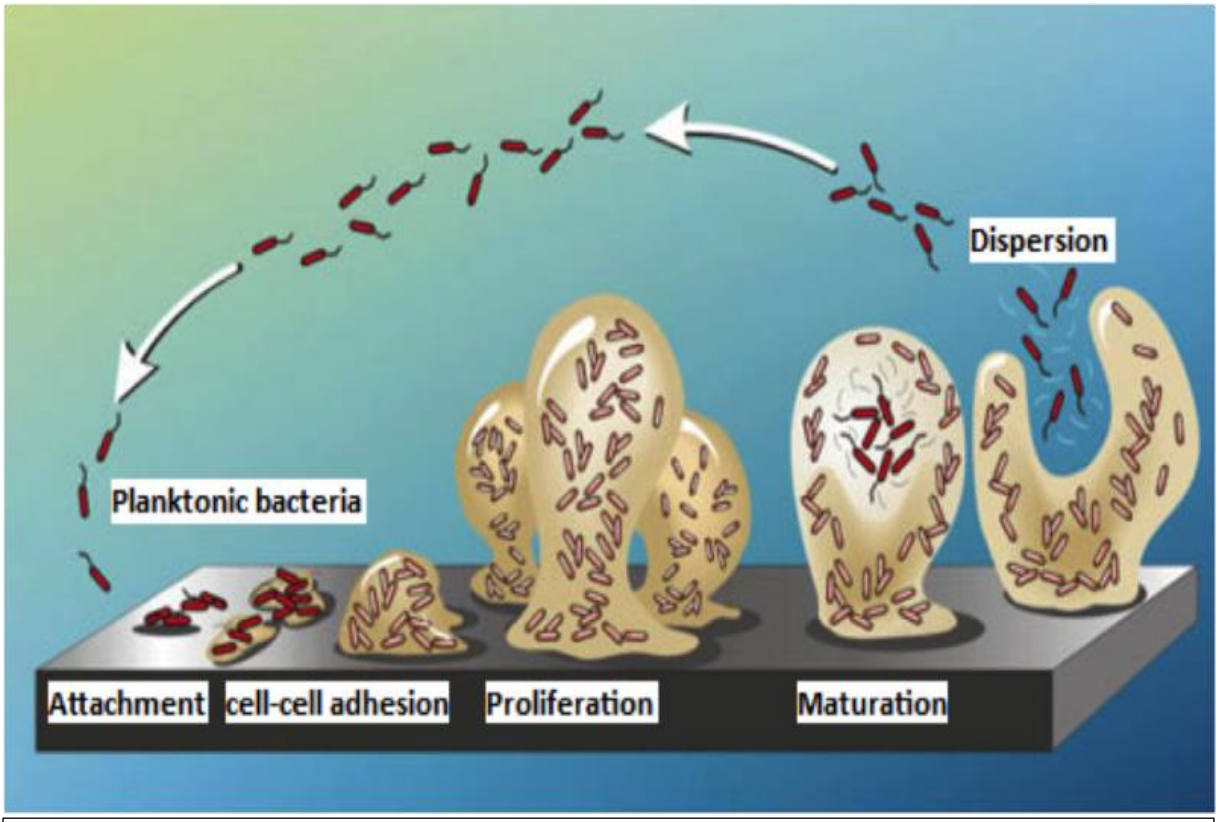

Figure 2: Biofilm formation process. Biofilm formation begins with planktonic cells reversibly binding to a surface. Production of adhesion molecules located on the pili and fimbriae interact with the surface making the process irreversible. Cells proliferate and develop a biofilm mass. The biofilm mass matures and develops a different physiology and metabolism from planktonic cells. Dispersion of cells from the biofilm into the surrounding environment occurs, releasing some planktonic cells. Inactive persister cells which are resistant to antibiotics remain [3].

There are two long term aims to this research project. The first aim is to determine what effect the presence of ppGpp has on *P. aeruginosa* planktonic and biofilm cells. The second aim is to determine what effect antibiotics have in altering the ribosome profiles of *P. aeruginosa* planktonic and biofilm cells. In regard to aim 1, we hypothesize that ppGpp will trigger the stringent response, causing gene expression in the bacterium to change as a result of cellular stress. This process has been shown to occur in E. coli [5], so it is possible it would occur in other bacterial species as well. It is our belief that the change in gene expression caused by ppGpp results in *P. aeruginosa* biofilms being resistant to antibiotics.

Experimental Procedure Background:

Because of the sensitivity of the ribosomes the methods protocol for this experiment has been adapted and amended many times. When the project was first stated in the Wilson lab in early 2015, *Escherichia coli* was the organism being studied. But because *E. coli* does not grow biofilms as readily as other organisms, such as *P. aeruginosa*, focus was shifted to study *Pseudomonas* when I joined the project in the fall of 2015.

At the beginning of the project we employed a multi-step lysis procedure involving both chemical and mechanical lysis. The mechanical lysis procedure involved using a mortar and pestle to physically grind up the biofilm and planktonic sample into a white powder after it had been frozen with liquid nitrogen. Our initial fractionation results were not good and we have since moved away from using a mechanical lysis and now only use a chemical lysis procedure that has given better fractionation results. The protocol outlined below uses only a chemical lysis procedure.

Additionally, we initially grew both planktonic cells and biofilm cells in the same beaker and then extracted them from that one beaker. This meant that both biofilm and planktonic cells had to be grown for the same period of time, rate, and temperature. Retrieval of the cells proved taxing at times as biofilm is especially viscous, making it difficult to remove liquid planktonic solution without accidently taking up some biofilm with it. Now, biofilm cells are grown using a peristaltic pump for three days as opposed to overnight. This allows for more mature biofilms to form. The planktonic cells are grown in a similar manner as before, however they are grown in a shaking incubator at a rate (220 rpm) that inhibits the growth of biofilms. This makes the planktonic cell retrieval process much easier and more efficient than before. Additionally, ice is now added to the planktonic cell solution to keep it cold during the lysis procedure and prevent the denaturation of the ribosomes.

The fractionation process is largely the same as before but has been slightly modified. Early on in the experiment, 5 drops of liquid was collected in 30 individual microcentrifuge tubes as the gradient was pumped through the fractionator and spectrophotometer. Now, all the liquid is collected in one large test tube until a peak is detected by the spectrophotometer. Once a peak is detected, we wait for 23 drops to flow through the apparatus until we switch to a new test tube. This is because after the sample is read by the spectrophotometer, it must flow through 23 drops of tubing before it exits the apparatus. We then collect the drops corresponding to a specific peak in a new test tube until another peak is recorded and we repeat the process. This makes it

possible for us to analyze a specific peak corresponding to a specific subunit of the ribosome via gel analysis. The changes that we have made to the protocol have led to more promising results.

Results:

Growth and Lysis of the Bacterial Biofilms and Planktonic Cells:

P. aeruginosa PA01, a known biofilm forming strain whose genome has been sequenced [6], was used for the project. Two other mutants of the PA01 strain were also used for the project. The strains used were ΔRelA, which has the ΔRelA gene removed (involved in the production of ppGpp) and ΔRelAΔSpoT, which has both the ΔRelA gene and ΔSpoT gene (encodes an enzyme that hydrolyzes ppGpp) removed [7].

Planktonic cells were grown in liquid Luria broth (LB) that was shook (220 rpm) overnight in a 37℃ shaker. The speed of the shaker prevented biofilms from forming in the planktonic cell flask. The process for growing planktonic cells is as follows: 3 separate 250 mL flasks were filled with 50 mL of LB broth. Next, the 3 planktonic strands used (PA01, ΔRelA, and ΔRelAΔSpoT) in this experiment were inoculated into their specific flask and labeled. The flasks were then placed in a 37℃ shaker set to 220 rpm and left overnight to grow. After growing the samples overnight, the planktonic samples were then subcultured using 1:100 dilutions in 200 mL of LB media and grown for four hours. After four hours, the OD was tested. An OD of approximately 0.600 is ideal. Once an OD of 0.600 was reached, ice was added to the beaker containing planktonic cells. We then poured the sample into a 200 mL centrifuge tube and spun it at 4,200 rpm for 10 minutes. We discarded the remaining LB media, resuspended the cell pellets using 300 µL of lysis buffer (described below) and transferred the solution into 3 labeled microcentrifuge tubes in preparation for the lysis process. The planktonic cells were lysed in the process described below.

Biofilm cells are grown in liquid Luria broth (LB) using a flow incubation system. LB broth is sent through a peristaltic pump at a flow rate of 200 µL/min for three days. After three days Biofilms are removed using a pipet and placed into a 15 mL test tube. The test tube was spun at 3,000 rpm for 5 minutes in a 4 ℃ centrifuge. This left a biofilm pellet at the bottom of the test tube and a planktonic supernatant layer above it that was removed using a pipet. To the best of our ability, excess liquid containing planktonic cells was removed leaving only the biofilm. The isolated biofilm mass (about 100 µL) was resuspended using 100 µL of lysis buffer then transferred into a microcentrifuge tube in preparation for the lysis process.

The lysis process for both planktonic and biofilm cells is the same. First, the lysis buffer must be prepared. 10 mL of lysis buffer was prepared using 100 µL of 1M $MgCl_2$, 100 µL of 1M Tris chloride (pH= 7.5), 0.1g of lysozyme, and 9.8 ml of water. In their separate microcentrifuge tubes, the biofilm sample had 100 µL of lysis buffer while the planktonic samples had 300 µL of lysis buffer added to them. The lysis buffer is used to transfer the cell samples to a microcentrifuge tube. The tubes were then flash frozen using liquid nitrogen and thawed in room temperature water. Next, 10 µL of a 10% solution of Sodium deoxycholate was added to the biofilm sample while 30 µL was added to the planktonic cell sample. The tubes were then

vortexed, flash frozen in liquid nitrogen, and thawed in room temperature water. Lastly, the tubes were flash frozen with liquid nitrogen and stored in the -80 ℃ freezer.

Creation of Sucrose Gradient and Fractionation:

The method for analyzing the ribosomes was adapted from a similar study previously conducted in Dr. Wilson's lab on *Escherichia coli* persister cells [8].

The creation of the 10%-40% sucrose gradient is as follows: 5 ml of 40% sucrose was placed into a large 14 mL test tube. Next, 5 mL of 10% sucrose was placed on top of the 40% gradient in the same test tube. To prevent spillage, parafilm was placed on top of the gradient and the test tube was gently placed on its side for 4 hours to allow the gradient to form. After 4 hours, the test tube was slowly turned back upright and allowed to cool overnight in the 4 ℃ fridge. This process of creating the sucrose gradient was repeated 4 times for the 4 samples.

After allowing the sucrose gradient to cool overnight, the samples were ready to be loaded onto the gradient. PA01 planktonic, PA01 biofilm, ΔRelA planktonic, and ΔRelAΔSpoT planktonic cell extract was loaded on top of four separate (3 planktonic samples and 1 biofilm sample) sucrose gradients (composed of 10%-40% sucrose). The loading process is conducted in the following manner: firstly, the samples were removed from the -80 ℃ freezer and allowed to thaw in room temperature water. While the samples were thawing, the sucrose gradients were removed from the 4 ℃ fridge and their concentrations were checked. Once the samples had thawed, about 200 µL of biofilm sample and 100 µL of planktonic sample was added to their respective sucrose gradients. Once the samples had been loaded and covered with parafilm, the gradients were ready for ribosomal separation via high speed centrifugation. The sucrose gradients were placed into a high speed centrifuge (25,000 rpm) for 6 hours at 4℃. This process separates the ribosomes into their different subunits.

After high speed centrifugation, the samples were ready to be fractionated using a fractionator. The spectrophotometer generates the graphs that are seen below. To begin the process the sucrose gradient was placed into the fractionation apparatus by piercing the bottom of the tube with a needle. The fractionation pump was set to run at a speed of 1.05 mL per minute. The fractionator was turned on and the machine began to push the sucrose through the fractionator and generate a graph using the spectrophotometer. As the samples pumped through and exited the apparatus they were collected into a large test tube until a peak was read. Once a peak was read, another test tube was used to collect all the sample that is associated with that specific peak. It was determined that because the location of the spectrophotometer reader and where the sample is pumped out are not the same, there is a slight delay of approximately 23 drops between the time the peak shows up and the time the sample that is pumped out actually exits the apparatus. Thus, once a peak showed up on the graph, 23 more drops were allowed to pump through the apparatus before a new test tube was used to collect all the drops related to a specific peak. This process was repeated with every new peak that showed up on the graph until all of the sucrose gradient had been pumped through the apparatus. Peaks corresponding to ribosome subunits (70S, 50S, and 30S) were identified using RNA absorbance at 254 nm. These graphics

are indicated in the results section below. The test tubes containing the samples corresponding to specific peaks were labeled and then stored in the 4°C fridge for future use.

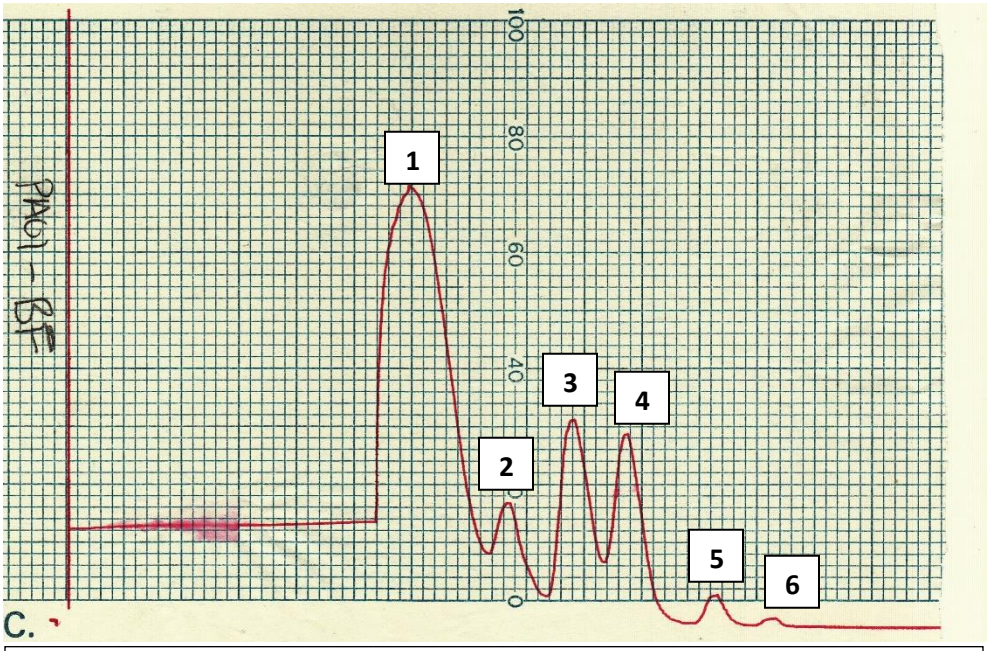

Figure 3: Ribosome fractionation of PAO1 wild type biofilm. Peaks 1-6 represent tRNA, 30S subunit, 50S subunit, 70S ribosome, disomes and trisomes, respectively.

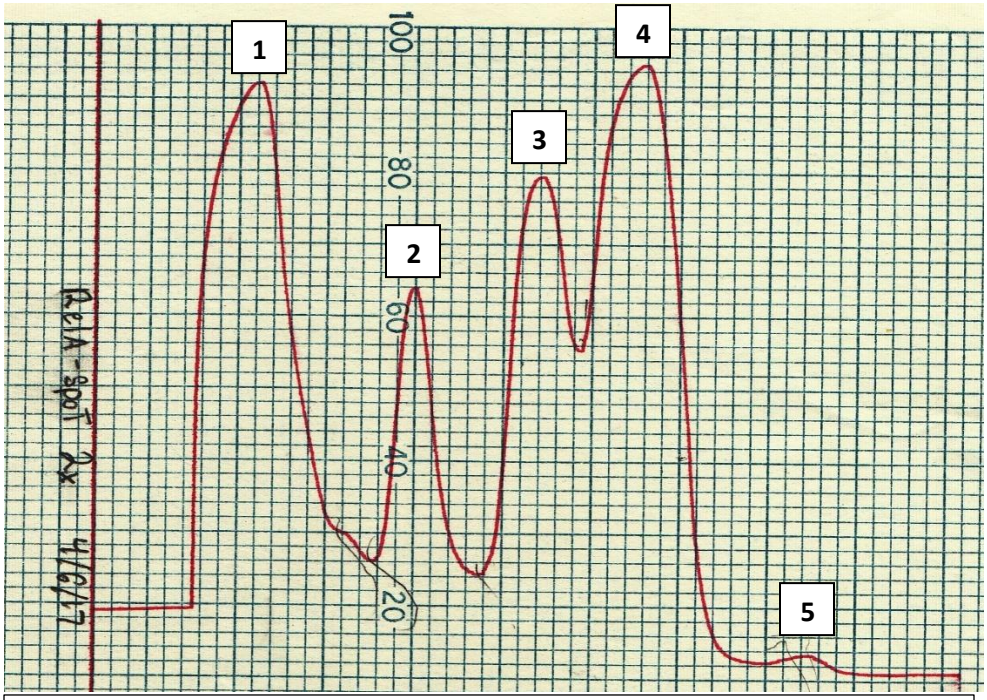

Figure 4: Ribosome fractionation of ΔRelASpoT planktonic cells. Peaks 1-5 represent tRNA, 30S subunit, 50S subunit, 70S ribosome, and disomes, respectively.

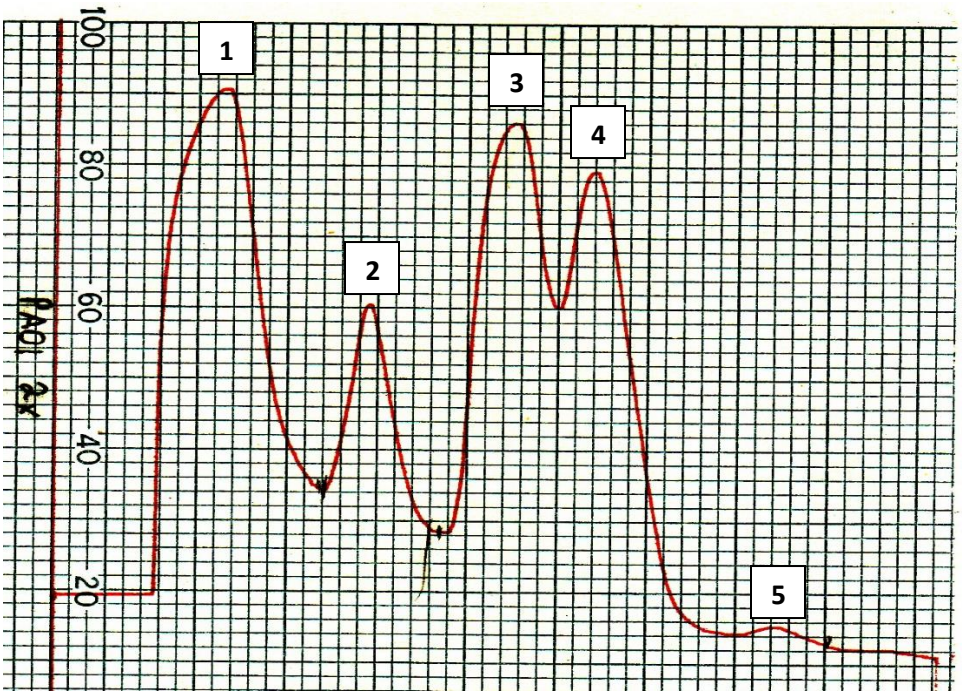

Figure 5: Ribosome fractionation of PAO1 wild type planktonic cells. Peaks 1-5 represent tRNA, 30S subunit, 50S subunit, 70S ribosome, and disomes, respectively.

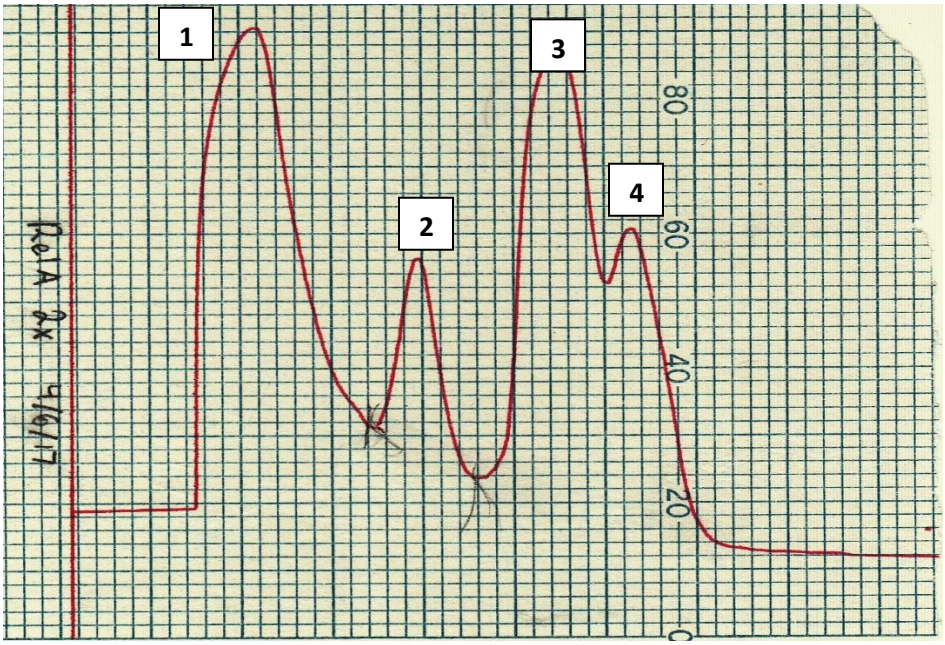

Figure 6: Ribosome fractionation of ΔRelA planktonic cells. Peaks 1-4 represent tRNA, 30S subunit, 50S subunit, and 70S ribosome, respectively.

As indicated in figures 3-6, the samples were successfully separated into their different subunits at the very least. This is demonstrated by the presence of different peaks for tRNA, 30S subunit, 50S subunit, and 70S ribosome. Now that the sample has been successfully separated, a greater understanding of the protein profile present in each fractionated sample can be attained via mass spectrometry. We expect that there are differences in the ribosome profiles between planktonic and biofilm cells.

Agarose Gel Electrophoresis:

A 10 lane agarose gel was run using planktonic samples recovered from the fractionation. These samples were the PA01 wild type (30S, 50S, and 70S fractions), the ΔRelA strain (30S, 50S, and 70S fractions), and the ΔRelASpoT strain (30S, 50S, 70S fractions, and a disome fraction). 5 µL of each sample was added with 1 µL of 6X dye to their respective lanes. The gel was run at a voltage of 108V. After the gel finished running, it was stained using a 1 to 10,000 solution of Ethidium bromide (15 µL EtBr to 150 ml of water) and placed on a rocker for 30 minutes.

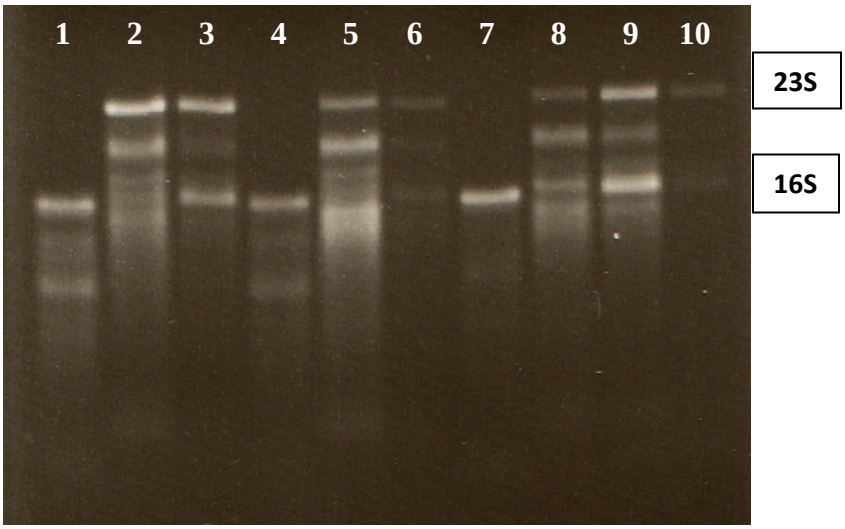

Figure 7: Agarose gel of planktonic cell samples. Lanes 1-3 represent PA01 30S, 50S, and 70S fractions. Lanes 4-6 represent ΔRelA 30S, 50S, and 70S fractions. Lanes 7-9 represent ΔRelASpoT 30S, 50S, and 70S fractions. Lane 10 represents ΔRelASpoT disome fraction. The 23S and 16S rRNA bands are indicated to the right of the graph.

The separation of the fractions was furthered confirmed on the agarose gel. Because the 23S rRNA subunit is only present in the 50S large subunit it would be expected that only the lanes that have the large subunit would also have the 23S subunit. The 23S subunit is present in lanes 2, 3, 5, 6 (faint), 8(faint), 9, and 10(faint). These lanes represent the 50S and 70S ribosomes as expected. The 16S rRNA subunit is present only in the 30S small subunit; thus, it would be expected that only the lanes that have the small subunit would have a substantial amount (indicated by a bright band) of the 16S rRNA subunit. This 16S subunit is present in lanes 1, 3, 4, 6 (very faint), 7, 9, and 10 (very faint). These lanes correspond to the 30S and 70S ribosomes as expected. Interestingly, lane 6 (which corresponds to the 70S ribosome of ΔRelA strain) shows up very faintly on the gel. This result is line with the fractionation result (see figure 6), in which the ΔRelA strain 70S ribosome has a small peak. These results confirm that ribosome fraction isolation has taken place.

Discussion:

As expected the peaks corresponding to the different ribosome fractions of the four samples are not exactly the same. All four samples have a large tRNA peak present which could indicate that biofilm formation, specifically the production of ppGpp, does not greatly effect tRNA.

The second peak on the graph, which corresponds to the 30S subunit of the ribosome varies between the biofilm and planktonic cells. Figures 5 and 6 (corresponding to PAO1 and ΔRelA planktonic cells, respectively) have 30S subunit peaks that are nearly identical. Figure 4 (the ΔRelA-SpoT planktonic cell sample) appears to have a slightly larger 30S subunit peak but it doesn't appear to be significantly larger. Figure 3 (the PA01 wild type biofilm) has a 2nd peak that is much smaller than all the planktonic cell 30S subunit peaks. This could potentially represent that ppGpp is disrupting translation at the 30S subunit in biofilm cells. Future analysis via protein mass spectrometry will give us insight into what specific proteins are present in the 30S subunit and whether they are present at abnormally high or low levels.

As indicated in figures 5 and 6, the third peak (which corresponds to the 50S subunit) is similar in size in the PAO1 and ΔRelA planktonic cells. The ΔRelA-SpoT planktonic cell sample (figure 4) has a slightly smaller 3rd peak size than the other planktonic cell samples but it may not be significant. The PA01 biofilm (figure 3) has a smaller 3rd peak size in relation to the planktonic cells. However, in relation to its 2nd peak, the 3rd peak looks to be proportionally the same as the PA01 and ΔRelA planktonic cells. In other words, the size of peak 3 in relation to peak 2 appears to be proportionally the same in the PA01 biofilm, PA01 planktonic and ΔRelA planktonic. That being said, the smaller 50S subunit could represent decreased translational activity in the biofilm cells. Further quantification of the peaks is needed to determine if the difference between biofilm peak and planktonic cell peak is due to translational inhibition or simply because less biofilm sample is loaded onto the fractionator than planktonic sample.

The 4th peak present represents the 70S ribosomes and varies greatly from one sample to the next. The ΔRelASpoT Planktonic had the largest 70S peak followed by PAO1 Planktonic, PA01 biofilm and ΔRelA Planktonic. The larger ΔRelASpoT peak is expected as the double mutant would produce very little ppGpp, thus the 70S ribosome would not be degraded and a large peak would still be expected. The ΔRelA result is interesting. One would expect that the mutant would produce less ppGpp and thus have a larger 70S ribosome peak; however this in not the case in this experiment.

Future Directions:

Using protein mass spectrometry, we will be able to compare the proteomes of biofilms and planktonic cells of *P. aeruginosa*. Analysis via protein mass spectrometry is achieved by cleaving the proteins present in the ribosome fractions with trypsin, a protease; this is referred to as bottom-up mass spectrometry. The fractions are then sent through the mass spectrometry machine which will separate the proteins based on mass to charge ratio and put out a large data set of the proteins that are present in a specific ribosome fraction. Proteins that have the greatest mass and smallest charge are deflected the least and will hit a detection plate at the end of the device which records what ion with a specific mass to charge ratio hit the plate. The more ions

present of a specific mass to charge ratio, the larger the peak that is produced. Based on this information, the mass spectrometry machine will generate a large set of proteins that are present in a specific ribosomal fraction. While mass spectrometry will generate a large data set of proteins, focus will be directed to proteins that control global translation. This can be achieved through the use of a proteomics database of *P. aeruginosa*. In regard to biofilms, global translation proteins are significant because it is believed that biofilms that are exposed to antibiotics result in the intracellular synthesis of guanosine tetraphosphate (ppGpp) by the ribosomes [5]; ppGpp will then interact with translation proteins such as Prokaryotic initiation factor-2 (IF2) which controls the entry of tRNA onto the ribosome. Binding of translational proteins, such as IF2, could cause translation to be inhibited. Proteins that methylate or phosphorylate any ribosomal component should also be investigated as they could represent a potential mechanism that biofilm cells use to disrupt translation.

The effect that antibiotics have in altering the ribosome profiles of *P. aeruginosa* planktonic and biofilm cells (aim 2) will be studied in the future. Tobramycin is an antibiotic that targets the ribosomes of bacteria and is commonly used in cystic fibrosis patients [1]. Tobramycin has been shown to induce the formation of biofilms in *P. aeruginosa* cells [9], making it clinically relevant to study. Planktonic and biofilm cells will be harvested in the same manner that was described above. Cells will then be treated with a lethal concentration of tobramycin for planktonic cells and a sub-lethal concentration for biofilms. The cells will then be lysed in the same manner as previously described and their RNA components that were collected via ribosome fractionation will be analyzed by mass spectrometry. Further analysis will be conducted using SDS-PAGE gels which allows analysis of monosomes and polysomes into their ribosomal RNA subunits (5S, 16S, and 23S).

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