

## Comparative Ribosome Analysis of *Pseudomonas aeruginosa* Planktonic and Biofilm Cells

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogen commonly found in the lungs of cystic fibrosis patients. *P. aeruginosa* is notable for its ability to form biofilms within the lungs. Biofilms exhibit incredible antibiotic tolerance. Tolerance differs from resistance: while resistant cells may actively grow in the presence of antibiotics, tolerant cells slow their metabolic rates, preventing most antibiotics from taking effect. The tolerance of *P. aeruginosa* biofilms is the cause of recalcitrant infections in cystic fibrosis infections. In this project, we sought to characterize the ribosomes of *P. aeruginosa* planktonic and biofilm cells in samples which either have or have not been exposed to tobramycin. Tobramycin is an aminoglycoside commonly used in *P. aeruginosa* infections. Recent studies have shown environmental stressors including antibiotic exposure cause ribosome degradation, remodeling, and dimerization in *E. coli*, leading to translational inhibition and in turn, antibiotic tolerance. We hypothesized similar events occur in tolerant cells of *P. aeruginosa*. We isolated ribosomes of planktonic and biofilm cells through sucrose gradient centrifugation. Following this, we qualified the relative distributions of ribosome fractions and analyzed the protein makeup of these ribosomes by mass spectrophotometry. We expect to observe a difference between the protein makeup of ribosomes from biofilm cells and those from planktonic cells. We expect similar results between samples treated with/without tobramycin. The results of this project may mediate future research on how to target cells exhibiting antibiotic tolerance.

**Keywords:** Antibiotic Tolerance, Biofilm, Tobramycin, Ribosome Dimerization, Translational Inhibition

### Introduction

Cystic Fibrosis is a severe and often fatal disease resulting from a mutation to both alleles encoding the protein cystic fibrosis transmembrane conductance regulator (CFTR), affecting 1 in 2,500 live births (O'Sullivan and Freedman 2009, Folkesson et al. 2012). CFTR is responsible for conducting chloride ions across epithelial membranes (Anderson et al. 1991). Disturbance of this process can result in mucus buildup within the lungs, creating a prime location for infection by opportunistic pathogens (O'Sullivan and Freedman 2009).

*Pseudomonas aeruginosa* is the most common pathogen causing chronic lung infections in patients with CF (Govan and Deretic 1996). Like other pathogenic

bacteria, *P. aeruginosa* has become difficult to treat with contemporary antibiotics due to various adaptations enabling the bacterium to survive hostile conditions through antibiotic resistance or tolerance (Folkesson et al. 2012). Of particular interest is *P. aeruginosa*'s ability to form biofilms within the lungs of CF patients (Hoffman et al. 2005, Bjarnsholt et al. 2009).

Biofilms are populations of bacterial cells embedded in an extracellular matrix of polysaccharides and proteins known as extracellular polymeric substance (EPS) (Hall-Stoodley et al. 2004). Cells from biofilms are phenotypically different from planktonic (free-floating) cells (Hall-Stoodley et al. 2004). *P. aeruginosa* biofilm cells exhibit higher tolerance than planktonic *P. aeruginosa* cells (Drenkard

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2003, Bjarnsholt et al. 2009). Researchers have shown this tolerance is not attributed to the EPS. Rather, it is the result of phenotypic differences between the cells of the biofilm and those of the planktonic population (Walters et al. 2003).

Cells of biofilms are thought to exhibit translational inhibition, giving rise to antibiotic tolerance (Hall-Stoodley et al. 2004). Tolerance is thought to arise because of translational inhibition since most antibiotics require disruption of a particular cellular synthesis process to take effect (Hall-Stoodley et al. 2004). Consequently, dormant cells often remain viable in a population following antibiotic exposure. Guanosine tetraphosphate (ppGpp) is a molecule responsible for the stringent response and translational inhibition in various bacteria (including *P. aeruginosa*) as a response to certain environmental stresses including antibiotic exposure (Potrykus and Cashel 2008). This inhibition of translation allows for *P. aeruginosa* biofilm cells to remain alive and able to proliferate within the lungs of a CF patient, often resulting in chronic infection.

The aim of our study was to characterize the ribosomes of *P. aeruginosa* following exposure to relevant antibiotics. Cho et al. (2015) discovered that most ribosomes of *E. coli* persister cells are degraded to their 50S and 30S subunit forms when exposed to ampicillin, contributing to the translation inhibition based antibiotic tolerance mentioned above. The remaining functional ribosomes of the *E. coli* persisters were remodeled, giving rise to the “persister proteome” (Cho et al. 2015). These persister cells were able to survive not by genetic means, but due to a phenotypic shift resulting in translational suppression.

There is a sufficient lack in understanding regarding ribosomal modification in *P. aeruginosa* biofilm cells to mediate research upon the subject. However, a greater understanding of ribosomal modification could lead to future research focused on how to treat tolerant bacteria. We hypothesized that *P. aeruginosa* biofilm cells may also remodel their ribosomes similar to *E. coli* persister cells that have been exposed to bactericidal antibiotics (Cho et al. 2015).

Tobramycin is a commonly used antibiotic for patients with CF (Ramsey et al. 1993, Ratjen et al. 2001). Tobramycin targets the ribosomes of bacteria, causing them to create erroneous proteins (Fosso et al. 2015). Because of its clinical relevance and ribosomal targeting, we used tobramycin in our treatment groups. Comparison of the protein and rRNA makeup of ribosomes derived from *P. aeruginosa* biofilm cells versus those from planktonic cells of the same strain following exposure to tobramycin will give insight on the stringent response effects ribosome profiles.

Cho et al. (2015) found that seven key proteins (S3, S5, S10, S11, S14, S21, and L25) were missing from the ribosomes of *E. coli* persister cells following treatment with ampicillin while two others (L10 and L12) were found in higher amounts than normal. We hypothesized to see similar results in the ribosomes from *P. aeruginosa* biofilm ribosomes. Along with ribosomal remodeling, we also expected to see ribosomal degradation (into inactive subunits) as seen by Cho et al. (2015).

## Methods

### Cell Growth and Lysis

Three strains of *P. aeruginosa* were chosen for experimentation. Strain PA01 served as a wild-type. Strain  $\Delta relA$  was used as a mutant type lacking a gene responsible for the synthesis of ppGpp. Strain  $\Delta relAspoT$  was used as a second mutant strain lacking both the RelA and SpoT proteins, both of which are located on the same operon with roles in the synthesis of ppGpp. The mutant strains lack the ability to enter the stringent response pathway because of their inability to synthesize ppGpp. Therefore, these strains should not exhibit translational inhibition nor ribosome degradation.

Planktonic cells were grown in standard LB media in an overnight culture, then subcultured into fresh media at a 1/100 ratio the next morning. Subcultures were grown to OD550 = 0.6. Overnight cultures were then quickly chilled on ice and centrifuged (4200 rpm, 10 minutes, 4 °C). Following centrifugation, cells were

chemically lysed with a lysis buffer (Tris-HCl, pH 7.8, MgCl<sub>2</sub>, lysozyme) and flash frozen in liquid nitrogen. Cells were then thawed and treated with a 10% (w/v) deoxycholate solution and introduced to liquid nitrogen once more. Cells were then ready to be stored long term at -80 °C. Cells treated with tobramycin followed the same protocol with the exception of the addition of the antibiotic (0.5 µg/ml) at OD550 = 0.2.

Biofilm cells were grown in a flow incubator apparatus designed with plastic tubing and a peristaltic pump flowing at a rate of 200 µL/minute with standard LB media. Samples were collected after three days of growth and centrifuged (4200 rpm, 10 minutes, 4 °C). Biofilm matrices were discarded and the cell precipitate was lysed as described above. Samples treated with tobramycin followed the same protocol with the exception of the addition of the antibiotic (10 µL/ml) to the starting LB media.

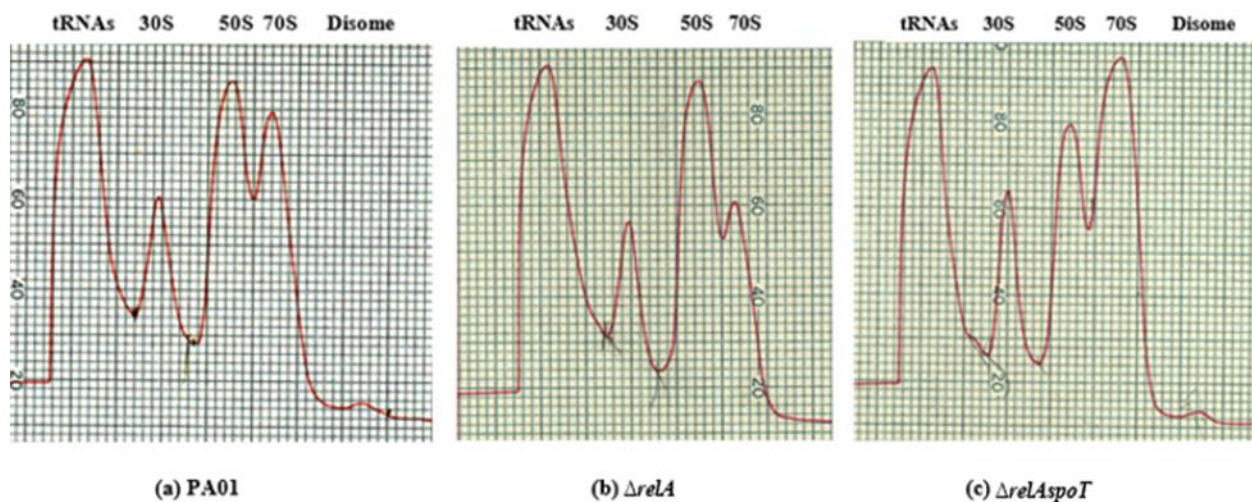


Figure 1 - Planktonic ribosome fractions were achieved through sucrose gradient centrifugation. Each peak represents relative absorbance at 254 nm. Both PA01 and  $\Delta relAspoT$  show potential for the presentation of disomes. Disomes are two ribosomes found on a single strand of mRNA which indicate high levels of translation.

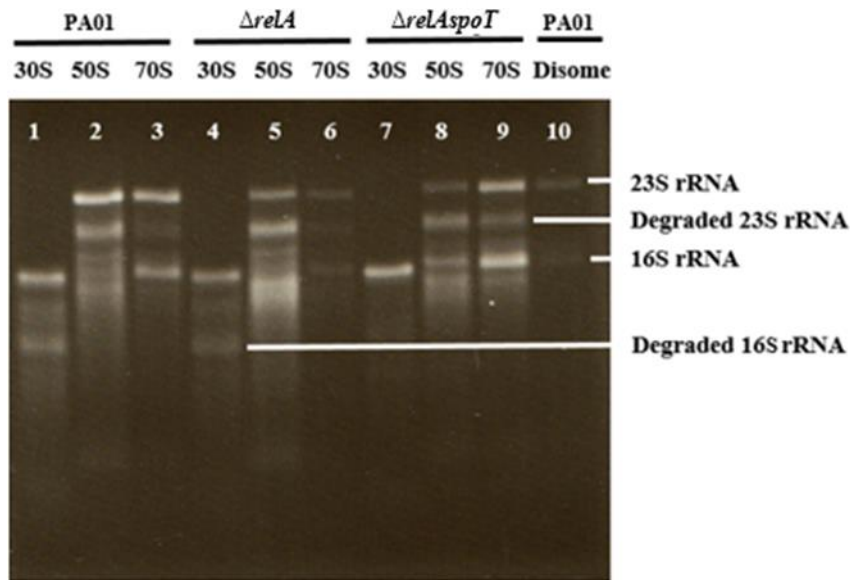


Figure 2 - Planktonic ribosome fractions were run on a native agarose gel to confirm separation. Each subunit contains rRNA: the large 50S subunit contains 23S rRNA while the 30S subunit contains 16S rRNA. Fractions containing the 70S full ribosome display both 23S and 16S rRNA. A notable characteristic of this gel is the presence of site-specification degradation of the rRNA. This could be a hint as to how *P. aeruginosa* degrades its ribosomes.

### Ribosome Fractionation

Samples lysed as above were thawed and treated with 1  $\mu$ L of RNase inhibitor (1 Unit/ $\mu$ L) and 1  $\mu$ L of DNase I (1 Unit/ $\mu$ L). These were then centrifuged (14,000 rpm, 10 minutes, 4  $^{\circ}$ C). The resulting supernatant was collected for further analysis. Sample absorbance values were checked by a photospectrometer at the wavelength of 260 and 280 nanometers. Concentrations were calculated using Beer's Law. Samples were then loaded onto 10 – 40% sucrose gradients and centrifuged (25,000 rpm, 6 hours, 4  $^{\circ}$ C, vacuum). Following centrifugation, samples were fractionated and detected by a real-time photospectrometer at 254 nm. A chart recorded relative absorbance values in real-time. Ribosome fractions were collected in separate tubes as the sample left the photospectrometer. 23 drops were counted

when a peak was observed on the chart to compensate for the lag time between detection and collection. From these fractions, SDS-PAGE, UREA-PAGE, and mass spectrometry would be conducted.

### Native Agarose Gels, SDS-PAGE, and UREA-PAGE

Qualitative protein and rRNA content of the fractionated samples was conducted by SDS and UREA-PAGE, respectively. SDS-PAGE gels (10% SDS, 6% polyacrylamide) were stained with Coomassie Brilliant Blue. UREA-PAGE gels (7M urea) were stained with ethidium bromide. UREA-PAGE gels were observed under ultraviolet light. Agarose gels (1%) were stained with ethidium bromide and observed under ultraviolet light.

### Mass Spectrometry

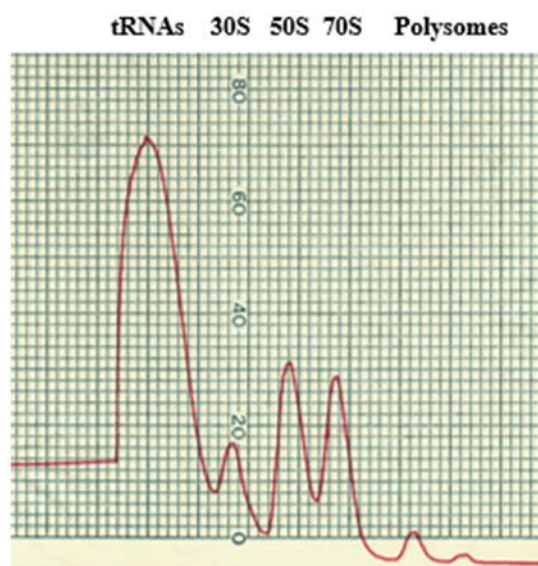
Successfully fractionated samples were treated with trypsin to digest protein components into peptides which were analyzed by mass spectrometry (LC-MS/MS hybrid LTQ-OrbitrapXL). Identified proteins in each sample were quantified and compared to those of *E. coli* control strain MG1655.

## **Progress to Date**

### Ribosome Fractionation

Through methods refined by Cho et al. (2015), we have successfully isolated ribosome fractions from PA01,  $\Delta relA$ , and  $\Delta relAspoT$  of the planktonic phenotype by

sucrose gradient centrifugation (Figure 1). Isolation of ribosome subunits from the planktonic samples was confirmed by native agarose gel electrophoresis (Figure 2). These samples were analyzed by mass spectrometry (data in preparation). Along with the three planktonic samples, we successfully isolated ribosome fractions from PA01 biofilm cells (Figure 3) which was analyzed by mass spectrometry (data in preparation).



*Figure 3 - Fractionation of a PA01 biofilm sample demonstrates a much lower concentration of ribosome material than planktonic samples, indicating translational inhibition. We hypothesize the disome peak may actually be evidence of ribosome dimers. Ribosome dimers are formed from two regular ribosomes, but exist in a state of preserved hibernation.*

Comparing the ribosome fractions of the planktonic samples against that of the PA01 biofilm sample shows a decrease in amount of ribosome material in the biofilm relative to the planktonic samples. This may be attributed to the slowing of translation in the biofilm culture. Less ribosome material

indicates a slowed metabolic rate in the cells of the biofilm. We expect to observe the same patterns in the other two biofilm samples.

Future mass spectrometry data will allow us to compare the ribosome profiles of the planktonic samples to those of the biofilm samples. We expect to see ribosome remodeling as found in *E. coli* by Cho et al. (2015).

#### Native Agarose Gel Electrophoresis

Isolation of ribosome fractions of the planktonic phenotype for PA01,  $\Delta relA$ , and  $\Delta relA spoT$  was confirmed through native agarose gel electrophoresis (Figure 2). The small 30S subunit contains a strand of 16S rRNA while the large 50S subunit contains a strand of 23S rRNA. The presence of rRNA in the ribosome subunits allows us to observe them on an agarose gel when stained with ethidium bromide.

When observed on the gel, the subunits appeared to show site-specific degradation of the rRNA (Figure 2). This could give insight on how the ribosomes of *P. aeruginosa* are disassembled. This disassembly process could be a vital component in translational inhibition of *P. aeruginosa* biofilm cells.

## **Discussion and Future Research**

### Ribosome Fractionation

Ribosomes of non-replicating cells are able to dimerize with ribosome modulation factor (RMF) (Yoshida et al. 2002). This dimerization allows for ribosomes to remain intact, but causes them to deactivate, resulting in inhibited translation. We presume the ribosomes of *P. aeruginosa* biofilm cells exhibit ribosome dimerization similarly to non-replicating cells in order to suppress translation. In our

PA01 biofilm analysis, we have hypothesized the disome peak may actually be a composite of disomes and ribosome dimers (Figure 3). We anticipate results from mass spectrometry to indicate the presence of RMF in our biofilm samples.

Along with dimerization, we anticipate to observe degradation of biofilm ribosomes into their inactive subunits and the proteomic remodeling of active ribosomes as found in *E. coli* by Cho et al. (2015). Our fractionation of PA01 biofilm ribosomes indicates evidence for degradation into inactive subunits (Figure 3). Mass spectrometry will allow us to compare the protein makeup of ribosomes from planktonic cells to those of biofilm cells. If our hypothesis is supported, we will observe proteomic differences between the profile of *P. aeruginosa* biofilm cells and planktonic cells. Specifically, we expect to see several key ribosome proteins to be missing from the biofilm samples.

The native agarose gel of the planktonic samples indicates site-specific degradation of rRNA. This degradation may be unique to *P. aeruginosa* and could yield hints on how its ribosomes are disassembled. We expect to witness more of this degradation in samples treated with tobramycin and biofilms.

### Effect of Tobramycin

The next step of the project is to identify the effects of tobramycin on the ribosomes of planktonic and biofilm cells of *P. aeruginosa*. Because of tobramycin's ability to stall bacterial ribosomes, we expect to see a build-up of polysomes in replicating cells (Fosso et al. 2015). However, in non-replicating or metabolically slowed cells such as those found in biofilms, we expect to observe

ribosomal modifications that result in translational inhibition including dimerization, degradation, and remodeling.

The results of this branch of the project will give insight into the mechanism *P. aeruginosa* takes on in order to inhibit translation. In the future, we hope to sequence strands of mRNA which are being transcribed by biofilm cells treated with tobramycin in order to determine what ribosome associated proteins result in the tolerance of *P. aeruginosa* biofilm cells.

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