CHARACTERIZATION OF THE STABILITY OF *PSEUDOMONAS AERUGINOSA*

RIBOSOMAL PROTEINS UNDER STRESS CONDITIONS

by

Sila Yanardag

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2017
DEDICATION

I would like to dedicate this work to women who stand up for justice and who never hesitate to stand up for each other.
ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Franklin for being a great mentor and for always supporting me. Thank you for giving me the opportunity to work for you. I also would like to thank to every member of Franklin Lab that I have worked with. Knowing you and learning from you is a privilege.

My dear family, thank you for believing in me and supporting me all the ways possible. Without your endless love and support, I could not be who I am today. Canım annem, canım babam; iyi ki varsiniz. Sizin sonsuz sevginiz ve desteginiz olmasaydı ben bugunku ben olamazdim.
TABLE OF CONTENTS

1. INTRODUCTION ...........................................................................................................1
   Overview of Planktonic and Biofilm Bacteria .................................................................1
   General Information on *Pseudomonas aeruginosa* .........................................................5
   *Pseudomonas aeruginosa* Infections ...............................................................................5
      Pulmonary Tissue Infections of Cystic Fibrosis Patients ........................................5
   *Pseudomonas aeruginosa* Infections of Chronic and Acute Wounds .......................7
   Review of HPF and RMF Proteins ..................................................................................8
      Review of HPF and RMF proteins in 
      *Escherichia coli* and other bacteria ..........................................................................8
      Role of HPF and RMF in *Pseudomonas aeruginosa* ..............................................11

2. GOALS OF THIS STUDY ............................................................................................14
   Goals of This Study .......................................................................................................14

3. METHODS ....................................................................................................................17
   Plasmid Construction .....................................................................................................17
   Protein Purification ........................................................................................................19
      HPF ........................................................................................................................19
      S13 .........................................................................................................................22
      L5 ...........................................................................................................................23
   Antibody Generation ..............................................................................................23
   Antibody Purification Approach ............................................................................23
   Western Blot ..........................................................................................................23
   Detection Limit Assay ...........................................................................................24
   Planktonic Growth Curve Experiment ................................................................25
   Colony Biofilm Experiment ...................................................................................25
   Planktonic Starvation Experiment ..........................................................................26
   Determination of Cell Viability .............................................................................26

4. RESULTS ......................................................................................................................27
   HPF Protein Is Expressed during Early-Exponential Phase and Stable for 24 Hours under Nutrient-Rich Growth Conditions ........................................................................................................27
TABLE OF CONTENTS — CONTINUED

HPF is Required for the Maintenance of P. aeruginosa PAO1 Cell Viability under Nutrient-Limited Conditions .................................................................30
Hpf is Required to Repress Protein Synthesis under Nutrient Limitation and during the Late Stationary Phase in Nutrient Rich Media ..................................................31
Large Subunit Ribosomal Protein L5 Is Stable and Not Degraded after the Prolonged Starvation of PAO1Δhpf .................................................................32
L5 Degrades at Later Time-Points of Colony Biofilm Growth of Δhpf Mutant Cells ..........................................................................................33

5. DISCUSSION ........................................................................................................37
Discussion ..................................................................................................................37

6. FUTURE DIRECTIONS .......................................................................................39
Future Directions ..................................................................................................39

REFERENCES CITED ..............................................................................................41

APPENDICES ..........................................................................................................49

APPENDIX A: Protocols .......................................................................................50
APPENDIX B: Minimum Inhibitory Concentrations ........................................57
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Primers used to PCR amplify HPF, L5, and S13 genes for plasmid construction</td>
<td>17</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Predicted structures of Pseudomonas RMF(A) and HPF(B) proteins</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Hypothesized activity of HPF on ribosome dimerization of P. aeruginosa PAO1</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Expression and degradation of ribosomal proteins during nutrient rich growth</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>CFU counts and total protein content during starvation</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Stability of large and small ribosomal subunits during the nutrient limited growth</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>Abundances of small and large ribosomal subunit proteins during colony biofilm growth on nutrient rich media</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>CFU counts and total protein content during starvation. (A)CFU counts of starved wild type and Δhpf cells</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Stability of large and small ribosomal subunit proteins during the nutrient limitation</td>
<td>33</td>
</tr>
<tr>
<td>9</td>
<td>Abundances of small and large ribosomal subunit proteins during colony biofilm growth on nutrient rich media</td>
<td>34</td>
</tr>
</tbody>
</table>
CHAPTER ONE — INTRODUCTION

Overview of Planktonic and Biofilm Bacteria

Bacteria are capable of surviving and proliferating in a wide variety of conditions, including extreme environments like hydrothermal vents, supraglacial sulfur springs, and even clouds (Reysenbach & Cady, 2001), (Grasby et al., 2003). Also in the human body, there are a tremendous number of bacterial cells, which are approximately ten fold more than the number of cells that make up our bodies. They can be found on the skin surface, but the vast majority of the non-human organisms in our body reside in the gastrointestinal tract and are mostly beneficial (Savage, 2003). On the other hand, many bacterial species are pathogenic and they are the causes of infections in humans, other mammals, and plants (Johnny W. Peterson, 1996; Monack, Mueller, & Falkow, 2004). In this broad range of environmental conditions, bacteria can live planktonically or can form biofilm (Reysenbach & Cady, 2001), (Takai et al., 2008), (Ollivier, Caumette, Garcia, & Mah, 1994). When the conditions favor biofilm formation, freely swimming bacteria experience a phenotypic switch (J et al., n.d.; Romeo, 2008). As a result of this phenotypic switch, motility is lost, and an extracellular matrix is produced. Biofilm-forming bacteria differ from their planktonic equals (Høiby et al., 2011) regarding self-produced extracellular structures such as nucleic acids, proteins, and polysaccharides that they use to attach to surfaces and to one another (Costerton, Geesey, & Cheng, 1978), (Stewart & Franklin, 2008). Biofilms cannot simply be defined as organisms stuck to surfaces (L Hall-Stoodley, Costerton, & Stoodley, 2004). They are rather complex, dynamic, structurally protective, and they give bacteria an immense ability to colonize in new, even hostile, environments (Stewart & Franklin,
Living within a matrix provides biofilm forming bacteria several advantages over their planktonic counterparts. Biofilm bacteria are more resistant to environmental stress as well as having enhanced cell to cell communication. Cell-to-cell communication, in other words, quorum sensing, is a process in which a chemical or a product synthesized by one cell triggers the expression or repression of some genes in other cells. Findings of Greenberg and his colleagues in 1998 suggested that quorum sensing is a critical component of biofilm formation and development as well as the virulence of *Pseudomonas aeruginosa*.

As bacteria can survive in a variety of conditions, colonization and biofilm formation in medical devices are not unthinkable for these organisms. Urinary catheters, joint prostheses, artificial heart valves, central venous catheters, and contact lenses are some of the examples where bacteria form biofilm and cause significant health problems due to elimination difficulties.

Traditionally, planktonic bacteria become tolerant to antibiotics by inactivating them, modifying their target, or excluding the antimicrobial agent. However, in biofilms, the contribution of any of these mechanisms to antibiotic tolerance is minor. Antibiotic resistance mechanisms of biofilms are widely studied but...
remain an ambiguous subject of microbiology. One highly discussed and studied theory is dormancy (Nathalie Q Balaban, Gerdes, Lewis, & McKinney, 2013). This argument is based on the knowledge that when biofilm cells which are known to be tolerant or resistant to antibiotic treatment are dispersed in liquid culture, they recover antibiotic susceptibility (Stewart et al., 2015). This suggests that biofilm cells enter a reversible physiological phase where they protect themselves from the effects of antibiotics (Stewart et al., 2015). However, biofilms are physically heterogeneous and contain different physiological states of the cells forming them. (De Beer, Stoodley, Roe, & Lewandowski, 1994), (Xu, Stewart, Xia, Mcfeters, & Huang, 1998). This difference in physiological states of the cells might be due to the microenvironments in biofilm (Anand et al., 2015; Stewart & Franklin, 2008). Diffusion rate, age of the biofilm, temperature etc. are factors affecting the amount of nutrients and dissolved oxygen available throughout the biofilm, which in turn play a crucial role in the formation of these microenvironments (De Beer et al., 1994), (Xu et al., 1998), (Stewart & Franklin, 2008). The presence of physical complexity in these dense and hydrated structures is the driving force for the formation of heterogeneity within the biofilm (Stewart & Franklin, 2008), (Williamson et al., 2012). As inferred from the locational mRNA abundances in P. aeruginosa PAO1 biofilms, the nutrient interface of the biofilm, which is oxygen depleted also shows slow metabolic activity compared to the top parts of a colony biofilm (Williamson et al., 2012), (Pérez-Osorio, Williamson, & Franklin, 2010). Cells associated with slow metabolic activity might be in the dormant phase (Pérez-Osorio et al., 2010), (Nathalie Q Balaban, Merrin, Chait, Kowalik, & Leibler, 2004). Dormant phase cells are known to be tolerant to
antibiotics and other stress factors and repopulate the biofilms when the conditions are favorable for active growth again (Akiyama et al., 2017).

Joseph Bigger (Bigger, 1994) described the term persister for the first time in 1944 in his paper where he used penicillin on \textit{Staphylococcus spp}. Bigger (Bigger, 1944) found that while the majority of the cells die following treatment, some could survive and he named those cells who survived as “persisters”. Lewis describes persister cells as being dormant, non-dividing cells which are not simply antibiotic-resistant mutants (Lewis, 2007). However, not all persister cells are non-dividing cells. There are observations suggesting that some types of persister cells use certain mechanisms actively, such as efflux pumps, to export antibacterial agents from the cell or toxin proteins to enable those cells with high toxin to persist through longer growth arrest (N Q Balaban, 2011; Nathalie Q Balaban et al., 2013). Another theory on persisters concerns the idea of slow-growth. By general definition, induction of slow growth is the mechanism for the formation of persister cell sub-populations (Nathalie Q Balaban et al., 2013). Williamson et.al. showed that cells at the bottom of the thick \textit{P.aeruginosa} biofilm are slow growing, and have low metabolic activity with reduced sensitivity to the antibiotics Ciprofloxacin and Tobramycin(Williamson et al., 2012), which can be classified as stress factors for the bacteria. When the bacteria have little to no metabolic activity, antibiotics are still able to bind to their target, but are unable to interrupt the function of their target, thus leaving the cells intact or functional (Lewis, 2007).
General Information on *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a gram-negative, rod-shaped, pathogenic and opportunistic bacterium associated with several animal and plant infections (Hardalo & Edberg, 1997). Genome size of *P. aeruginosa* strains varies among strains and ranges from 5.5 to 7.5 Mbp with ~60% Guanine-Cytosine content. *P. aeruginosa* is known for its ability to survive in inhospitable environments such as jet fuels, soap, chlorhexidine solutions as well as plant and animal tissues, soil and water bodies (Botzenhart & Döring, 1993). *P. aeruginosa* also colonizes at the roots of *Arabidopsis thaliana* and sweet basil and causes mortality of the plants seven days post inoculum (Walker et al., 2004). This ubiquitous organism is usually found in water bodies polluted by human activities and readily colonizes immunocompromised people such as patients suffering from cystic fibrosis and AIDS or people with autoimmune diseases (Hardalo & Edberg, 1997; Lyczak, Cannon, & Pier, 2002). The ability of *P. aeruginosa* to survive in a wide spectrum of environments and cause severe infections make it an important candidate for scientific studies.

*Pseudomonas aeruginosa* Infections

Pulmonary Tissue Infections of Cystic Fibrosis Patients

Cystic Fibrosis (CF) is a genetic disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein and it is one of the most common reasons of death by autosomal diseases (J. C. Davies, 2007; Veit et al., 2016; Wang, Wrennall, Cai, Li, & Sheppard, 2014). After Sickle Cell Disease (SCD), CF is the second
most common life-shortening, inherited disorder in the US, where approximately 1,000 people are diagnosed with CF annually (Grosse Scott D., 2004). One in 3500 people in the USA and one in 2500 people in EU suffer from this life-quality-diminishing disorder (Farrell, 2008; Pettit & Fellner, 2014). Patients with CF have high levels of sodium chloride in the airway-surface liquid in their lungs. Davies et al. hypothesized that high levels of sodium chloride would inactivate native antimicrobial peptides in the surface, thereby making the patients more susceptible to infections (J. C. Davies, 2007). Pulmonary tissue infections in Cystic Fibrosis patients with P. aeruginosa is proven to be the main transgressive of declined lung functions and death (Lyczak et al., 2002). Once the CF lung is infected with P. aeruginosa, the bacteria settle and are hard to eliminate with current clinical treatment methods (Jeukens et al., 2014). In-vivo models characterizing the P. aeruginosa infections show that the bacteria are cleared from the tissue after 7 days of treatment, but that they are able to re-colonize 28 days after the clearing with a reduced fitness but improved colonization ability (Weiss, 2016). Re-colonization of the bacteria suggests that there is heterogeneity of the initial population with some antibiotic tolerant cells (Lewis, 2007; Williamson et al., 2012). In-vitro studies completed to characterize the heterogeneous nature of P. aeruginosa biofilms show that the antibiotic tolerant sub-populations are the variants of the initial population and resume to the normal, susceptible lifestyle when the antibiotic is cleared from the environment (Lewis, 2007, 2010). Thus, development of new treatment strategies that would target and eliminate the antibiotic tolerant sub-populations of P. aeruginosa biofilms colonized in the lung tissues of CF patients is primarily important to improve the life quality of the patients.
Sites of chronic wound infections are the home for bacteria that are good at forming biofilms and adopting the biofilm living conditions (Donlan et al., 2002; Parsek, 2003; Stewart & Costerton, 2001). Chronic wound environments are dynamic places where pathogenic activity of the biofilm forming bacteria overcome the host defense system throughout time (Phalak, Chen, Carlson, & Henson, 2016). Over $16M of federal funds were given for chronic wound infection research in 2012. Among the non-NIH based granting agencies, the biggest contributor, with 72% of the total grants given out, is the Department of Defense (Davidson, Martins-green, Sen, & Tomic-, 2015). Biofilms formed at the wound area are composed of several organisms, (Rashid et al., 2000; Serralta et al., 2017) which are predominantly *Staphylacoccus sp.*, *Acinetobacter sp.*, *Escherichia sp* and *Pseudomonas sp*. (Dowd et al., 2008; James et al., 2007). These bacteria live in a consortium depending on their oxygen requirements. Results of the two-bacterium-biofilm study by Phalak et al. show that *P. aeruginosa* is found in the oxygenic regions of the biofilm while the facultative anaerobe *S. aureus* is predominantly present in the anaerobic region when these two organisms are grown together and form a biofilm (Phalak et al., 2016). A study conducted on 77 patients with chronic wounds showed that 35% of bacteria at the infection site are *Pseudomonas spp* (James et al., 2007). Another study where 50 consecutive patients admitted to the hospital with the complaints of chronic wounds are included showed that 52.2% of the bacteria harbored in wound environment are *Pseudomonas aeruginosa* (Gjødsbøl et al., 2006). As this opportunistic pathogen
experiences a phenotypic switch and becomes dormant when it faces an external stressor like an antibiotic, it is hard to eliminate it from the wound environment. Identification of the ways this bacterium enters dormancy and resuscitates when the ambient is favorable is essential and important in order to develop new therapeutic agents that can eliminate the entire population, regardless of the physiological state of the bacterium.

Review of HPF and RMF Proteins

Review of HPF and RMF Proteins in *Escherichia coli* and other Bacteria

During the exponential growth phase, bacteria actively transcribe the genes required for the cellular activity. The quantity and type of the genes transcribed differ depending on the growth condition and the requirements of the organism (Alberts B, Johnson A, Lewis J, 2002). Upon entry into the stationary phase, bacteria slow down protein synthesis (Wada, Yamazaki, Fujita, & Ishihama, 1990),(Polikanov, Blaha, & Steitz, 2012). For example, in *Escherichia coli*, during the log phase, various proteins are actively synthesized, but under starvation conditions, ribosomal biosynthesis is repressed, thus protein synthesis is also repressed (Yoshida, Ueta, Maki, Sakai, & Wada, 2009). In *E.coli*, 70S active ribosomes are inactivated by forming 100S (Fig. 2A) dimers, which reduces metabolic activity and prevents ribosomal degradation following the transition from the exponential phase to the stationary phase (Wada et al., 1990). Formation of the 100S ribosome leads to ribosome hibernation, which in turn helps cell survival (Polikanov et al., 2012). The dimerization process takes place by the involvement of several proteins in *E.coli*. These proteins are Ribosome Modulation Factor (RMF), Hibernation Promoting
Factor (HPF) and YfiA (Polikanov et al., 2012; Ueta et al., 2013; Ueta, Wada, & Wada, 2010; Yoshida, Yamamoto, Uchiumi, & Wada, 2004). The crystal structures of RMF, HPF and YfiA were reported by Polikanov et.al in May, 2012. Polikanov et.al. stated that RMF is a small protein which binds next to the 3’ end of 16S ribosomal RNA (rRNA) at the anti Shine-Dalgarno sequence (anti-SD) in the mRNA exit channel of the 70S ribosome (Polikanov et al., 2012). RMF prevents initiation of protein synthesis by preventing interaction between the Anti-SD and the SD of mRNA, which is a required step for translation initiation (Polikanov et al., 2012; Shine & Dalgarno, 1974; Steitz & Jakes, 1975). Binding of RMF to both of the 70S subunits of ribosomes is required, although the RMF proteins in each subunit do not interact, as they are 100 Å apart from each other (Polikanov et al., 2012) On the other hand, it is known that binding of YfiA prevents RMF binding, as its C-terminus tail blocks the RMF binding site. YfiA bound ribosomes are called inactivated 70S ribosomes, but these ribosomes cannot further form 100S inactive dimers due to the blockage of the binding site for HPF by YfiA (Polikanov et al., 2012).

Different bacteria have orthologs of the HPF protein. In E.coli, there are two paralogs of HPF available; HPF and YfiA. HPF is a 95- amino acid protein which has 40% sequence similarity and high structural homology with another stationary phase protein of E.coli, YfiA, which has an additional short amino acid sequence at the C-terminus and inhibits formation of 100S ribosomes(Ueta et al., 2005). Long-HPF, which is only found in Gram positive bacteria, differs from HPF by an addition of a long C-terminus tail (Ueta et al., 2008). Similar to RMF, HPF plays a crucial role in ribosome dimerization. Upon the binding of RMF, two 70S ribosomes form a 90S complex, which
is further stabilized by the binding of HPF (Ueta et al., 2005). YfiA has a slightly different function in ribosome dimerization. Binding of YfiA prevents the formation of 100S mature ribosome and releases already bound RMF from the 90S ribosomes, whereas HPF further stabilizes 90S ribosomes (Ueta et al., 2005). This is due to the long C-terminus tail of YfiA, which blocks RMF binding (or which destabilizes already bound RMF and brings 70S ribosomes back) (Polikanov et al., 2012).

In *Staphylococcus aureus* (*S. aureus*), only one of the two proteins that take part in ribosome dimerization is found; HPF. The HPF homolog in *S. aureus* is called saHPF and saHPF’s molecular weight is twice as much as the short-HPF. saHPF functions in a similar way to HPF. In *S. aureus*, it is also found that during the exponential phase, saHPF preferentially binds to a 70S ribosome rather than a 100S ribosome (80% of the time). Ueta et al. hypothesize that preferential binding of HPF is due to the fact that the re-activation of the ribosome is easier from 70S-saHPF complex than from 100S ribosomes (Ueta et al., 2010).

Similar to *S. aureus*, *Bacillus subtilis* has a long-HPF homolog, but doesn’t have a RMF homolog in its genome. Akanuma et al. (Akanuma et al., 2016) found that HPF protein is required to maintain ribosome stability during the prolonged stationary phase. They also concluded that lack of HPF protein results in a prolonged lag phase when the cells are regrown, due to an interruption in transcription initiation. Results concluded on the role of HPF on the regrowth of *B. subtilis* correlate with that of *L. monocytogenes* (Akanuma et al., 2016; Kline, Mckay, Tang, & Portnoy, 2015). The HPF homolog of
*L. monocytogenes* participates in similar biological and genetic functions as in *E. coli* and *S. aureus*.

**Role of HPF and RMF in *Pseudomonas aeruginosa***

RMF and HPF orthologs are present in *Pseudomonas aeruginosa*, but their functions are not fully characterized yet. Studies performed by Williamson et al. (Williamson et al., 2012) on *P. aeruginosa* PAO1 strain showed that mRNA transcripts of these two proteins are highly abundant in the slow growing antibiotic-tolerant subpopulations of PAO1 biofilms. For further analysis, these two genes were deleted from the genome of *P. aeruginosa* (Williamson et al., 2012). When mutant and wild type strains were subjected to nutrient-deprived environments, the *rmf*-deleted strain (∆*rmf*) did not show a different phenotype than the wild type strain, whereas the *hpf*-deleted strain (∆*hpf*) had loss in recovery from starvation (Akiyama et al., 2017). Similarly, when colony morphologies of these three strains were compared after they were starved in nutrient-limited media and transferred to Tryptic Soy Agar (TSA) plates, it was observed that ∆*hpf* strain had heterogeneous colony size, while wild type and ∆*rmf* were homogeneous in colony morphology (Akiyama et al., 2017). Apart from the phenotypical and morphological evidence of reduced fitness of ∆*hpf* strain, ribosomal RNA content of this mutant was also different than the wild type and ∆*rmf* strain. Deletion of the *hpf* gene led to the degradation of 23S rRNA by the fifth day of starvation. According to the experiments where cellular 16S and 23S rRNA content were measured and compared, stationary phase wild type PAO1 cells had a 23S/16S ratio of 1.6. This ratio was maintained in wild type PAO1 and ∆*rmf* cells, but not in ∆*hpf* mutant cells during the course of starvation. Complementary to
the community level fitness characteristics of wild type, hpf, and rmf deletion mutants, loss of rRNA was also observed at the single cell level for the Δhpf strain, where a small number of the cells still maintained their ribosomes. Fluorescence in-situ Hybridization (FISH) analysis showed that wild type cells were able to protect their rRNA under the nutrient limitation, but from day 1 through day 4 of starvation, loss of rRNA was clearly observed in Δhpf strain (Akiyama et al., 2017).

During all phases of the starvation assay, Williamson et al. (Williamson et al., 2012) observed the recovery of phenotype (colony morphology and CFU counts), fitness and rRNA levels when the hpf deletion mutant was complemented with a plasmid containing a copy of the hpf gene. Moreover, a double deletion mutant (PAO1ΔhpfΔrmf) showed a similar phenotype to the hpf single deletion mutant. Colony morphology, fitness and rRNA levels of the PAO1ΔhpfΔrmf mutant was recovered when complemented with a plasmid containing the hpf gene, but not with the rmf gene.

Based on the observations about the role of HPF in the recovery of P. aeruginosa from starvation, in this study, I chose to characterize HPF at the protein level. For this research, I purified HPF and two ribosomal proteins for the production of polyclonal antibodies, so that I could characterize the stability of these proteins during starvation of P. aeruginosa and during growth of P. aeruginosa in liquid culture and as biofilms.

Figure 1 shows the predicted structures of RMF and HPF of P. aeruginosa. RMF consists of 3 alpha-helices (Fig. 1A), while HPF has 2 alpha-helices and 4 beta-sheets (Fig. 1B).
Figure 1: Predicted structures of Pseudomonas aeruginosa (A)RMF and (B)HPF proteins. Structures are predicted via the Bioinformatics Tool Kit, HHPRED. The most similar proteins to the query proteins with e-values less than $10^{-5}$ and SS score more than 90 were selected.
CHAPTER TWO — GOALS OF THIS STUDY

Goals of This Study

In this study, I aimed to standardize western blot methods for probing large and small ribosomal subunits of *Pseudomonas aeruginosa* grown under different environmental conditions, and to characterize the stability of ribosomal proteins to bring light to the heterogeneous composition of the population, which is hypothesized as one mechanism for antibiotic tolerance. Long-term studies done with *P.aeruginosa* PAO1 showed that mRNA transcripts of two proteins, RMF and HPF, are highly abundant at the biofilm-nutrient interface of the thick *P.aeruginosa* biofilms. Also, it was previously shown by Perez et al. and Williamson et al. (Pérez-Osorio et al., 2010; Williamson et al., 2012) that the cells located at the oxygen limited interphase of the biofilm were metabolically inactive or slow-growing. Akiyama et al. (Akiyama et al., 2017) and Williamson et al. (Williamson et al., 2012) found that HPF is a critical protein for the maintenance of 23S rRNA and overall ribosomal RNA stability after prolonged stress exposure (Akiyama et al., 2017; Williamson et al., 2012). In light of this information, Akiyama et al. (Akiyama et al., 2017) showed that in the absence of the HPF protein, *P.aeruginosa* cannot protect its ribosome integrity (Fig. 2B) and cannot resuscitate from dormancy after the environmental stressors are gone. Perez et al. (Pérez-Osorio et al., 2010) showed that *P.aeruginosa* biofilms are heterogeneous in physiology, and it is posited that persister cells of the biofilm are located at the bottom of the biofilm, unaffected by the antibiotic exposure and therefore can repopulate the biofilm (Williamson et al., 2012). Localization
of ribosomal subunits and determination of the abundance of ribosomes within the heterogeneous biofilms will provide valuable insights on the mechanisms of persister cell formation, dormancy, and resuscitation from dormancy. In order to do so, I have isolated two ribosomal proteins, L5 and S13, and HPF. In this study, I generated polyclonal antibodies against those three proteins. I used the antibodies to determine the abundance of these proteins during the normal course of growth of the wild type and Δhpf mutant strains. Growth analysis in nutrient rich media gave us an understanding of the stability of 70S ribosomes when the bacterium was growing without any stress. Later, the wild type and Δhpf strain were grown in a carbon and nitrogen-limited environment for seven days to examine the response of the cells to the starvation stress regarding ribosomal stability. Finally, I tested the hypothesis that cells located at the bottom of the biofilm are abundant in the HPF protein, and therefore contain more inactive ribosomes compared to the cells located at the top of biofilm.
Figure 2: Hypothesized activity of HPF on ribosome dimerization of P. aeruginosa PAO1. (A) Ribosomal integrity of WT cells are protected by forming 100S dimer with the binding of HPF when the cells are exposed to stress and able to become active again when the stress conditions are removed. (B) HPF-lacking cells lose some of their 50S ribosomal subunits when the cells experience stress as they are cannot form 100S dimers and translational activity of the cell is reduced due to the loss in total ribosome content.
Plasmid Construction

Target DNA sequences were PCR amplified by Taq polymerase. Amplified DNAs were gel extracted and cloned into the pCR2.1 vector (Thermo Fisher) according to the manufacturer’s instructions. Competent \textit{E.coli} top10 cells containing pCR2.1 with the gene of interest were grown overnight on nutrient rich Tryptic Soy Agar (TSA) plates. Single colonies containing the gene of interest were identified according to their β-galactosidase activity and grown overnight in TSB. After the plasmid extraction, target genes were cut from pCR2.1 by using specific restriction enzymes (XhoI and NdeI, NEB) and ligated into the pET28a vector. The pET28a vector with gene of interest (Fig. 3) was first transformed into \textit{E.coli} top10 cells, and then to BL21DE3 cells. Cells were stored at -80°C until needed.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPF Forward</td>
<td>CGC ATA TGC AAG TCA ACA TCA GTG GCC ATC</td>
</tr>
<tr>
<td>HPF Reverse</td>
<td>AGC TCG AGT CAG CGG GCG CCT ACG CCT TGC</td>
</tr>
<tr>
<td>L5 Forward</td>
<td>GGC ATA TGG CAC GAT TGA AAG AAA TTT ATC</td>
</tr>
<tr>
<td>L5 Reverse</td>
<td>CGC TCG AGC TCG CGG TTC TTC ATG CTC TC</td>
</tr>
<tr>
<td>S13 Forward</td>
<td>GGC ATA TGG CCC GTA TTG CAG GCG TCA AC</td>
</tr>
<tr>
<td>S13 Reverse</td>
<td>GGC TCG AGC AGC AGG TTT TGC CAT GAC TAG</td>
</tr>
</tbody>
</table>

Table 1: Primers used to PCR amplify HPF, L5, and S13 genes for plasmid construction.
Figure 3 Continued

Figure 3: Plasmid maps of (A) HPF, (B) L5, and (C) S13 constructs. Genes were PCR amplified from *Pseudomonas aeruginosa* PAO1 with the given primer couples in Table 1 and gel extracted. After the genes were cloned into pCR2.1 vector, XhoI and NdeI restriction enzymes were used to cut the genes from the vector. Restriction enzyme digested genes were inserted into pET28a vector to construct C-terminus Histidine tagged proteins.

**Protein Purification**

**HPF**

*E. coli* BL21DE3 strain was grown overnight from frozen stock in 3mL TSB with 30ug/mL Km at 37°C in a roller incubator. Overnight cultures were transferred to fresh ZYP-5052 media (1:1000) containing 30ug/mL Km and incubated for 16 hours at 37°C at 200 rpm. Cells were pelleted at 5,000rpm for 30 min at 4°C.
Lysis and Protein Purification: Cell pellets were first lysed via three freeze and thaw cycles. The cell pellets were re-suspended in a 0.85% saline solution and sonicated for 20 cycles of 30 seconds on and 30 seconds off. After the sonication, lysates were centrifuged at 15,000 rpm, at 4°C for 60 min to remove the cellular debris. Proteins were purified by cobalt affinity chromatography, where the protein extracts were applied to a 500uL Cobalt resin (Thermo Fisher, HisPur™ Cobalt Resin, catalog number: 89864) trapped between two polypropylene disks (Pierce disposable columns, catalog number: 29920). The cobalt resin was washed with 10mM imidazole buffer (10mM imidazole, 300mM NaCl, 20mM Sodium phosphate) until the absorbance at 280nm was zero. Cobalt affinity purified proteins were eluted with 4 aliquots of 500uL of 150mM imidazole buffer (150mM imidazole, 300mM NaCl, 20mM Sodium phosphate) (Fig. 4A).
Figure 4: 6xHis-tagged purified proteins on SDS-PAGE. Purifications were performed as explained previously. *E. coli* BL21DE3 containing pET28a with fused (A) *hpf*, (B) L5 and (C) S13 were lysed and used as positive control. (-) control is the same host cell and with empty vector. (FT: Flow Through, E: Elute, Numbers indicate the order of elutes or flow through.)

**Quantification:** Protein quantification was performed with the ProStain Protein Quantification Kit (Active Motif). The Manufacturer’s instructions were followed.

**SDS-PAGE and Western Blot Analysis:** To assess the quality of the protein expression, cell lysis, and protein purification cell pellets (before and after the lysis), flow through and
elutes were separated by SDS-PAGE electrophoresis and also transferred to nitrocellulose membranes by electrophoresis. 6x-His Conjugated primary rabbit antibody (Pierce) and goat-anti-rabbit IgG, peroxidase conjugated secondary antibody (Pierce) were used in immunoblots. Immunoblots were developed and visualized with chemiluminescent visualization method with enhanced luminol.

S13

Growth: *E.coli* BL21DE3 containing pET28a-6xHis-S13 was grown overnight from frozen stock in 3mL TSB with 30ug/mL Km at 37C in roller incubator. The bacteria were transferred to fresh TSB media (1:100) containing 30ug/mL Km and grown for 2 hours without Isopropyl β-D-1-thiogalactopyranoside (IPTG), and 10 hours with 1mM IPTG at 37C at 200 rpm. Cells were pelleted at 5,000rpm for 30 min at 4C.

Lysis and Purification: Cell lysis and protein purification was performed same as HPF (Fig. 4B).
L5

Growth: Cells were grown the same as HPF.

Lysis and protein purification: For cell lysis and purification of the L5 protein, QIAexpressionist handbook was used as reference. “Protocol 10. Preparation of cleared *E. coli* lysates under denaturing conditions” was followed (ref. infor like number page etc). Cells were lysed and proteins were purified under denaturing conditions (Fig. 4C).

Antibody Generation

To generate anti-rabbit polyclonal antibodies against the proteins of interest initially, purified proteins were transferred to 1x PBS buffer at pH 7. 1.5 ug total protein is sent to Lampire Biologicals for three separate immunization of the 0.5 ug protein for each immunization.

Antibody Purification Approach

HPF, L5 and S13 antibodies were purified using Thermofisher Scientific Nab Spin column kit (Cat#). For purification, the protocol provided by the manufacturer was followed. From 400 uL of crude serum, ~3.5 mg/mL HPF antibody, ~2.51mg/mL L5 antibody, and ~5.34mg/mL S13 antibody was purified.

Western Blot

1 ug total protein was loaded in each well to determine the relative abundance of the protein of interest over the course of experiment. For growth curve experiment samples were taken every two hours, for starvation experiments sampling was performed every two days. Proteins were transferred from 16% SDS-PAGE gels to 0.2 micrometer nitrocellulose
membrane in wet transfer conditions in transfer buffer (25mM Tris-HCl, 192mM glycine, 20% methanol at pH 7.6) for 1 hour at 180mAmp/gel or 350mAmp/2 gels.

Figure 5: 6xHis-tagged proteins and *Pseudomonas aeruginosa* PAO1 cell lysates probed with specifically raised (A) anti-L5, (B) anti-HPF and (C) anti-S13 polyclonal antibodies.

Detection Limit Assay

To test the detection limits of the antibodies generated against HPF, L5, and S13 proteins, two strategies were followed: 1) The minimum amount of colony forming units (CFUs) required for detections and 2) the minimum amount of protein required for detection. For this purpose, wild type *P. aeruginosa* PAO1 was grown for 18 hours at 37°C, 200 rpm, and 200 uL of cells were pelleted down with a benchtop centrifuge (name model etc) at room temperature, 13,000 RPM for 1 min. Cell pellets were then resuspended in 100uL of 0.85% NaCl solution and 100uL of protein loading buffer. 10 fold dilutions of cell resuspensions were loaded into wells for western blot analysis. Cell numbers of 18-hour
grown wild type *P. aeruginosa* was determined by drop plate count method to be $3 \times 10^{11}$ CFU/mL. Proteins were transferred to nitrocellulose membranes as explained previously. Membranes were blocked in 3% skim milk in tris-buffered saline (50mM Tris-Cl, 150mM NaCl ph7.5) (TBS) with 0.1% tween 20 (TBST) for 1 hour, incubated overnight with 1:10000 diluted respective primary antibodies and 3 hours with 1:10000 diluted secondary antibodies. Membranes were washed with TBST 3-5 times for 10 minutes between each incubation. Membranes were developed using chemiluminescent detection method and visualized on x-ray films.

**Planktonic Growth Curve Experiment**

Overnight cultures grown in TSB were transferred to fresh 25mL TSB to a final OD of ~0.04. Cells were incubated at 37°C, 200 rpm, for 36 hours. Starting from hour 4, 1mL of cell suspension is removed every two hours, spun down, and cell pellets were stored in a -80°C freezer for future use.

**Colony Biofilm Experiment**

13mm filters were UV sterilized and placed on TSA plates. Overnight grown wild type *P. aeruginosa, Δhpf*, and Δ*rmf* strains were diluted to OD 0.05. 20uL of diluted cultures were placed onto filters. Biofilms were grown at 37°C and every 24 hours one biofilm was taken and resuspended in 10mL 0.85% saline with vigorous vortexing until all the cells were detached from the filter. The proper dilutions of cell suspensions were plated on TSA for CFU counts. 1mL of 1x cell suspension was centrifuged and kept at -20°C for western blot analysis.
Planktonic Starvation Experiment

Strains were cultured overnight in TSB with 1mM IPTG and 150ug/mL Carbenicillin when appropriate. 120uL of each overnight culture was used to inoculate 3mLs TSB with 1mM IPTG and 150ug/mL Carbenicillin when necessary. Cultures were grown for 7 hours or until an OD<sub>600</sub> ~7 was reached. Depending on the OD of the cultures, enough volume of cells was pelleted and washed twice with 1mL PBS to make the final OD of a 1mL suspension ~6.6. Cell pellets were resuspended in 1mL PBS and added to 25mL pre-warmed PBS in 125mL baffled flasks with appropriate concentrations of IPTG and Carbenicillin.

Determination of Cell Viability

Viability was determined as CFU/mL. Cells were serially diluted in 0.85% NaCl solution, and 100uL of appropriate dilutions were plated on TSA plates as 10 drops each containing 10uL. Colonies were counted after 18 hours of growth.
CHAPTER FOUR — RESULTS

HPF Protein Is Expressed during Early-Exponential Phase and Stable for 24 Hours under Nutrient-Rich Growth Conditions

To outline HPF expression and stability during planktonic growth, *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1Δhpf were grown in nutrient-rich media for 48 h. Starting from 4 h after the initial sub-culturing, samples were collected every 2 hours and stored at -80°C to be used for western blot analysis. After assessing the total protein concentration in the cells, 1 µg of total protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. 3 identically-prepared membranes were probed with anti-L5, anti-S13 and anti-HPF primary antibodies to assay the relative abundances of the two ribosomal proteins and one ribosome binding protein, HPF. Growth curve experiments showed that the amount of total protein increased from 4 hours until 12 hours, remained steady until 24 hours growth and dropped thereafter (Fig. 6A). Western blot analysis showed that the expression of the HPF protein starts around 4 hours after the initial inoculation. The relative abundance of HPF increased during the first 12 hours, and dropped after 24 hours of planktonic growth in nutrient-rich media. I observed that HPF was below the limit of detection by 48 hours of growth (Fig. 6C). Western blot analysis also showed that abundance of L5 was diminished when observed after 24 hours of growth in the hpf-deleted strain. However in WT it was observed that L5 was stable after 24 hours of growth, but degraded at 48 hours. On the other hand, in both WT and Δhpf, S13 remained stable for 24 hours, but was completely degraded by 48 hours of growth (Fig. 6D-E).
Figure 6

A

**Total Protein - Nutrient Rich Growth**

B

**Nutrient Rich Growth-CFU Counts**
Figure 6: Total protein content, CFU counts, and expression and degradation of ribosomal proteins during nutrient rich growth. Cells were grown in nutrient-rich media starting from OD 0.04 and harvested every 2 hours for western blot and total protein analysis. Primary antibodies used to probe the specific proteins are indicated in boxes. (A) Hpf deleted cells experienced protein loss earlier than WT strain. (B) CFU counts of WT and Δhpf cells differ by 100 fold at 48 hours of growth in nutrient rich media. (C) HPF is expressed during the early exponential phase and degradation starts after 12 hours. By the time of 48 hours of growth, HPF is below the limit of detection. (D) L5 is expressed during the early exponential phase and the protein is stably kept within the cells for 24 hours in WT. Degradation of L5 took place earlier at Δhpf strain compared to wild type. (E) Similar to L5, S13 is expressed at an earlier time point and was stable for the first 24 hours of growth, both in the wild type and Δhpf strain. Values shown in the graphs are the mean of three biological replicates. Error bars represent the standard deviation of the three biological replicates.
HPF is Required for the Maintenance of *P. aeruginosa* PAO1 Cell Viability under Nutrient-Limited Conditions

Similar to the results found by Akiyama et al. (Akiyama et al., 2017) I found that HPF protein is required to maintain cell viability after prolonged nutrient deprivation. To test this, the mutant strain where the hpf gene was deleted from the genome of *P. aeruginosa* was grown in nutrient-rich media until stationary phase and then transferred to nutrient-limited PBS. Over the course of 7 days, wild type PAO1 maintained cell viability while ∆hpf strain had reduced viability on day 5 and day 7 (Fig. 7A). However, when the total protein content of the two strains was examined, I observed that the amount of protein for both strains was decreased, regardless of the presence of HPF (Fig. 7B).

Figure 7
Figure 7 Continued

Figure 7: CFU counts and total protein content during starvation. (A) CFU counts of starved wild type and Δhpf cells. WT and Δhpf cells were grown in PBS for 7 days. Some of the Δhpf cells die by day 5 while WT cells can survive through day 7. (B) Total protein content of cells collected on indicated days. Under the starvation conditions, WT and Δhpf cells maintain similar quantities of total protein. Values shown in the graphs are the mean of three biological replicates. Error bars represent the standard deviation of the three biological replicates.

HPF is Required to Repress Protein Synthesis under Nutrient Limitation and during the Late Stationary Phase in Nutrient Rich Media

It is known that in *E. coli*, HPF participates in the inactivation process of active ribosomes (Polikanov et al., 2012). Similarly, in *B. subtilis*, sucrose density gradient experiments show that absence of HPF negatively affects the formation of 100S ribosome dimers (Akanuma et al., 2016). Akiyama et al. showed that HPF is required for ribosome preservation after prolonged nutrient starvation. In this work, when the total protein
concentrations of WT and Δhpf strains were compared during the later time points of starvation (day 5 and 7) (Fig. 6D-E) and nutrient rich growth (day 2) (Fig. 7A-B), it was observed that, although the CFU counts of WT are around 100 fold higher than the Δhpf mutant, total protein concentrations of the two strains are comparable. It is suggested by Yoshida et al. (Yoshida et al., 2009) that in *E.coli* when the cells are starved, protein synthesis is repressed due to the repressed ribosomal biosynthesis. Similar to the previous findings and hypothesized activity of HPF protein, this finding may suggest that HPF protein limits protein synthesis by inactivating active 70S ribosomes. Although WT has ~100 fold more CFUs, another reason why WT and Δhpf mutant have comparable total protein content might be due to the viable but non-culturable Δhpf mutant cells.

Akiyama et al. (Akiyama et al., 2017) reported that deletion of the hpf gene from the genome of *P.aeruginosa* PAO1 caused degradation of 23S ribosomal RNA following the three days of carbon and nitrogen starvation, as well as reduction in CFU counts while the wild type *P. aeruginosa* could protect their 23S rRNA during starvation and the CFUs following starvation. Here I showed that, although CFU counts do correlate with their findings after prolonged starvation in nutrient-deprived media, these conditions do not lead to the degradation of the large subunit ribosomal protein, L5, of the hpf-deleted strain. Results of western blots conducted on both wild type and hpf-deleted strains suggest that the L5 protein is abundant in both of the strains in similar quantities. On the other hand, signal intensity of the S13 protein is always higher than the L5 proteins, although the same
amount of total protein was loaded in each well and the SDS-PAGE and western blot analysis were done in parallel.

Figure 8: Stability of large and small ribosomal subunit proteins during the nutrient limitation. (A) Large subunit protein L5 is comparable during the experiment between WT and Δhpf cells and degradation isn’t the case under nutrient limited growth conditions. (B) Small subunit protein S13 is also comparable each day during the experiment between WT and Δhpf cells.

L5 Degrades at Later Time-Points of Colony Biofilm Growth of Δhpf Mutant Cells

When bacteria switch from planktonic growth to biofilm growth, they undergo several physiological changes to adapt to biofilm conditions. Apart from the secretion of extracellular material to form the matrix, they also need to adapt to nutrient and oxygen limitations (Costerton et al., 1978; Stewart & Franklin, 2008). One strategy that bacteria follow to survive in a biofilm environment is spatial gene expression. When GFP-expressing *P. aeruginosa* cells were grown in biofilms for 48 hours, the cells located at the
biofilm-air interphase actively expressed fluorescence, while cells at the biofilm-nutrient interphase do not (Williamson et al., 2012). The difference in spatial fluorescence suggests that the cells located at the bottom of the biofilm are either not in an active growth state or transcription is not actively taking place. In light of previous planktonic experiments, the stability of the ribosomal proteins during the biofilm growth was tested both with wild type and Δhpf mutant cells. Western blot analysis of the community level ribosomal stability showed that during the later time points of the biofilm growth in nutrient rich environments, lower levels of L5 ribosomal proteins is available in hpf-deleted cells while L5 remains in unchanged quantities in WT cells. However, I observed that S13 is uniform and in similar abundance in WT and hpf-deleted cells at the later time points of the biofilm growth.

Figure 9
Figure 9: Abundances of small and large ribosomal subunit proteins during colony biofilm growth on nutrient rich media. When the cells are grown for 3 days on a 0.2micron filter on nutrient agar, (A) L5 protein significantly degrades on day 3 of hpf-deleted cells while WT cells are able to protect the protein from degradation. (B) S13 protein appears to be in similar quantities in a given time point of WT and hpf-deleted cells and not degraded after 3 days of biofilm growth. (C) CFU counts on indicated days of WT and Δhpf cells during the colony biofilm growth shows no significant difference.
(D) Total protein content of the two cell types are also comparable and does not show a significant difference at any time of the growth. Values shown in the graphs are the mean of three biological replicates. Error bars represent the standard deviation of the three biological replicates.
Discussion

I found that, when *P. aeruginosa* PAO1 was grown in a nutrient-rich environment for 48 hours, the large subunit protein L5 was still stable after the 24 hours of growth whereas in PAO1Δhpf strain, the large subunit protein L5 was already degraded when the cells reached the same time point (Fig. 3D). In this work, the degradation of L5 in the wild type was observed after 48 hours of growth (Fig. 3E). Akanuma et al. (Akanuma et al., 2016) found that in *B. subtilis*, in the absence of HPF protein, 23S rRNA degrades during the late stationary phase, but until now we did not have any knowledge about the fate of ribosomal proteins. In this study, I found that the small and large subunit ribosomal proteins are more stable in WT compared to the Δhpf strain. However, both proteins are found to be degraded by the end of 48 hours of growth in nutrient-rich media.

The HPF protein is hypothesized to be carrying a vital function to stability of ribosomes when the cells are under stress (Akiyama et al., 2017; Williamson et al., 2012). Akiyama et al. (Akiyama et al., 2017) demonstrated that when *P. aeruginosa* cells are exposed to nutrient limitation, after 4 days HPF-lacking cells lose their 23S rRNA, whereas wild type cells have high levels of 23S rRNA even after starvation. Because rRNAs are rapidly degraded when not associated with ribosomes (Papers & Deutscher, 2003), I expected to see degradation in ribosomal proteins, potentially with a delay, as they are solely part of the ribosomes. However, regardless of the HPF availability, detection of these two ribosomal proteins via western blot after prolonged nutrient deprivation indicates that
these proteins remain within the cell without being degraded. Proteins are thermodynamically stable and are expensive for the cell to make and bring to their stable and active state. I believe the non-correlating results of the rRNA data and the protein data are rooted in the differences of structural stability of these two macromolecules, especially under nutrient limitation.

In colony biofilm experiments, cells located at the bottom part of the biofilms have access to nutrient while the cells located at the top portion of the biofilm have limited nutrient but adequate oxygen (Stewart & Franklin, 2008). In this study, western blot analysis was used to measure ribosomal protein abundances at the community level. Thus, presence or absence of ribosomal proteins tell us the average appearance of ribosomal content throughout the biofilm (Fig 6A-B). Similar to the growth curve analysis (Fig 3D-E), here we see the degradation of ribosomal proteins during the later time points of biofilm growth. Protein degradation was more pronounced in L5 compared to S13. One reason why proteins are prone to degradation when they are grown in nutrient rich media, but not in nutrient limited media, could be the overall energy availability for the cells. As there are enough nutrients to use in case cells need to make more copies of ribosomal proteins, it may not be a necessity for them to protect those proteins from degradation.
CHAPTER SIX — FUTURE DIRECTIONS

Future Directions

With the rapid development of technology and an exponentially increasing human population, translocation of people became very easy and sometimes a necessity which led to increased interstate and intercontinental human interaction. This elevated human interaction possibly causes high rates of passages of pathogens from one host to another. Considering the arms race between the pathogen and the host, it is evident that pathogens are good at overcoming the challenges they face. Winning this battle is not easy but not impossible, either. One of the key steps to be taken is the understanding and the characterization of the persister nature of *P. aeruginosa* in biofilms. Developing new techniques is necessary for this purpose. We know that biofilms are heterogeneous environments and the cells residing in a biofilm are different than their planktonic, freely-swimming mates. In addition to this, biofilm-forming cells differentially regulate certain mechanisms depending on the environment they live in (due to the availability of oxygen and the stressors they are exposed to). Identification of the end results of these physical differences and the pathways in which these outcomes play crucial roles should be the priority, as it could bring light to the bigger picture. In this study, I investigated the stability of the ribosomal proteins during the normal course of planktonic growth, 7 days of planktonic starvation, and 3 days of colony biofilm growth. I used polyclonal antibodies specifically generated for L5, S13, and HPF. Results of the western blot analysis are community-level, and not quantitative per cell. To better understand the physiological
adaptation of *P. aeruginosa*, single cell level experiments where the abundance of the proteins can be detected is necessary. One possible approach is the detection of ribosomal proteins and HPF within cells with immunogold labeling and transmission electron microscopy. For that purpose, cells under starving conditions or cells taken from the different sections of biofilms can be probed with specific primary antibodies and then visualized with nano-gold labeled goat anti-rabbit secondary antibodies. Another possible analysis that can be useful to strengthen the hypothesis of ribosomal degradation is the comparison of the availability of L5 and S13 proteins in the presence and absence of HPF with FACS when the cells are grown with different stressors. Apart from these, other types of cellular stressors could also be tested to make a better characterization of ribosomal stability under the stress. In this study, I tested the effect of starvation stress on the stability of the ribosomal proteins. In addition to or coupled with starvation stress, antibiotic, oxidative or iron limitation stress could expand our understanding about the stress tolerance of this bacterium.
REFERENCES CITED


http://doi.org/10.1104/pp.103.027888.such

http://doi.org/10.1016/j.biocel.2014.04.001

http://doi.org/10.1002/1873-3468.12454


http://doi.org/10.1128/JB.00022-12


http://doi.org/10.1111/j.1365-2443.2008.01272.x

APPENDICES
General Protein Purification Method by Sila Yanardag

- Grow *E.coli* BL21DE3 with the plasmid containing gene of interest overnight in nutrient rich media. Include antibiotic to prevent plasmid curing (30ug/mL Km for pET28a)

- Next day transfer the culture to fresh media
  - If you are using TSB (or general nutrient rich growth media) to express the protein; 1:100, if using **ZYP-5052**; 1:1000
  - Do not forget to add antibiotic (30ug/mL Km for pET28a)
  - If you are using TSB (or general nutrient rich growth media), add 1mM IPTG after two hours of the initial inoculation

- Grow the cells for 16 hours at 37C shaking at 200rpm
  - This changes depending on the protein. To find out the best condition for your protein, grow them in small cultures with varying media, temperature and time and take samples every few hours, run the cell pellet through SDS-PAGE and find out which condition and time give the higher protein yield
  - For **HPF**- ZYP media, 37C, 200rpm, 16-18 hours
  - For **L5**- ZYP media, 37C, 200rpm, 16-18 hours
  - For **S13**- TSB, 37C, 2 hours without IPTG, 10 hours with IPTG

- Centrifuge the cells at 5000rpm for 30min at 4C (I usually grow 4x400mL cell culture, so I used 1L centrifuge tubes with 800mL cell culture in them)

- Carefully discard supernatant and freeze the cell pellets

- Keep the cell pellets in -80C until next time you need it or freeze and thaw 3 times to lyse the cells if you are planning to do the purification right away

- Resuspend the cell pellets in 25mL 0.85% saline and sonicate in ice
  - 30sec on 30sec off sonication on ice for 20 cycles (the more the better)
  - Then apply 1sec on 3sec off sonication on ice for 5-10 min if you think more lysis is necessary (cell suspension will be less viscous and get darker color when lysed with sonication)
  - For **L5**- L5 was purified in denatured condition, I followed the protocol 10 of “The QiaExpressionist”. I resuspended the cell pellets in lysis buffer (**Buffer B**) at 5mL per gram wet weight. Stir cells for 15-60 min at room temp, avoid foaming (cell suspension will look lighter in color and sticky and viscous when they are lysed). *See below for the purification of L5.*

- Remove the cellular debris by centrifuging them at 15,000rpm at 4C for 1hr
  - Keep the supernatant on ice all the time after this step

- Prepare purification column
  - Fill the column (cap on) with **10mM imidazole buffer**, insert polypropylene disk and push it all the way down with the help of a Pasteur Pipette, add Cobalt resin (300-400uL is usually enough for 1.6 L cell
culture. If you use more than that you will likely get unspecific binding to the column. Using too little resin will result in loss of protein) and wait till it settles down (you will observe the pink color on the disk), add the second disk to trap the resin between two disks.

- Equilibrate the column with 10mM imidazole buffer (or buffer B if purifying L5) by running 5 times 5mL buffer through the column
- After the equilibration, run the supernatant through the column
  - Flow rate should be 1mL/min (should take about 5 minute if the column is fully loaded. If it goes faster, collect the supernatant and re-run it)
- Wash the column with 10mM imidazole buffer (buffer B for L5)
- Collect 1mL sample (Flow through) closer to the end of each wash and keep it for SDS-PAGE analysis.
- Measure OD 280 after every 5 washes
  - OD 280 will be zero after 13-15 wash. Once you make sure OD280 is zero, start eluting your protein
- Put the cap on and load 400uL of 150mM imidazole buffer, let it sit for 5 minutes and collect the elute. Repeat this for a total of 4 elutes.

**L5 purification steps:** Prepare purification column by replacing 10mM imidazole buffer with Buffer C. Equilibrate the column with buffer C. Run the supernatant through the column. Wash the column 4 times with buffer C and then 4 times with Buffer D. When the OD280 reaches zero, add 500uL of buffer E, let it sit for 5 minutes and collect the elutes. Repeat this for a total of 4 elutes.

**10mM imidazole Buffer pH 7.4:**
- 10 mM imidazole
- 300 mM NaCl
- 20 mM Sodium phosphate

**150mM imidazole Buffer pH 7.4**
- 150 mM imidazole
- 300 mM NaCl
- 20 mM Sodium phosphate

**Buffer B:**
- 100 mM NaH2PO4
- 10 mM Tris·Cl
- 8 M urea
- Adjust pH to 8.0 using NaOH
Buffer C:
- 100 mM NaH2PO4
- 10 mM Tris·Cl
- 8 M urea
- Adjust pH to 6.3 using HCl

Buffer D:
- 100 mM NaH2PO4
- 10 mM Tris·Cl
- 8 M urea
- Adjust pH to 5.9 using HCl

Buffer E:
- 100 mM NaH2PO4
- 10 mM Tris·Cl
- 8 M urea
- Adjust pH to 405 using HCl

Preperation of 16% and 4% SDS-PAGE Separating and Stacking Gels

<table>
<thead>
<tr>
<th>12% Separating Gel*</th>
<th>Reagents</th>
<th>4% Stocking Gel*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6 mL</td>
<td>DI Water</td>
<td>1.55 mL</td>
</tr>
<tr>
<td>2 mL</td>
<td>Separating gel buffer</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Stocking gel buffer</td>
<td>625 uL</td>
</tr>
<tr>
<td>160 uL</td>
<td>10% SDS</td>
<td>25 uL</td>
</tr>
<tr>
<td>160 uL</td>
<td>10% APS</td>
<td>25 uL</td>
</tr>
<tr>
<td>16 uL</td>
<td>TEMED</td>
<td>2.5 uL</td>
</tr>
<tr>
<td>3.2 mL</td>
<td>40% Polyacrylamide</td>
<td>250 uL</td>
</tr>
</tbody>
</table>

*Makes one 1.5-mm gel. To make more, simply multiply all the reagents by the order you need.

12% separating gel
- Mix the reagents in the given order
- Pour 6-8 mL (~5 cm from the bottom) immediately
  - 16% gel polymerizes very quickly, you should pour it before it starts getting thicker
  - Avoid air bubbles
- Gently pour DI water on top of separating gel (this will ensure a flat surface)
- Wait 10-20 minute or until the gel polymerizes
- Pour off the water before preparing stocking gel

4% stocking gel
- Mix the reagents in the given order
- Pour on top of separating gel all the way to the top of the glass (~2 mL)
- Carefully place the comb. Make sure there is no air bubbles left
- Wait until the gel polymerizes (30min-1hr).

Separating gel buffer (1.5M Tris-Cl)
36.3g Tris-Cl /200mL DI water, pH8.8

Stacking gel buffer (0.5M Tris-Cl)
3g Tris-Cl /50mL DI water, pH6.8

10% SDS (Sodium Dodecyl Sulfate)
10g SDS/100mL DI water

10% APS (Ammonium Persulfate)
0.5g APS/ 5 mL DI water

Transferring Protein from Gel to Membrane (Semi-wet transfer)
- Cut 8.3cmx5.5cm 12 pieces of filter paper and submerge in transfer buffer (tank buffer+15% MetOH)
- Cut same size of nitrocellulose membrane and submerge in transfer buffer
- Carefully place one filter paper on trans-blot avoiding air bubbles
- Repeat this step 5 more times (in total 6 filter papers) and place the nitrocellulose membrane as the 7th layer
- On top of nitrocellulose membrane, place SDS-PAGE and 6 more filter papers
- Close the lid and run 20min at constant 20V

Transferring Protein from Gel to Membrane (Wet transfer)
- Cut 8.3cmx5.5cm 6 pieces of filter paper and submerge in transfer buffer (tank buffer without SDS+15% MetOH)
- Cut same size of nitrocellulose membrane and submerge in transfer buffer
- Submerge the sponges in transfer buffer
- Place the clear side of the cassette on the dish
- Place one sponge on the disk and three filter paper on top of the sponge
- Place the membrane on filter papers
- On top of nitrocellulose membrane, place SDS-PAGE and 3 more filter papers
- Place the sponge on top
Close the cassette and place it to the tank
Add transfer buffer without SDS to the tank and run for 1 hour at 0.18 mAmp. Keep the tank cold while running

**Detecting 6xHis-tag proteins with 1° and 2° Ab**
- Block the membrane in blocking buffer (TBST+3% BSA) minimum 3 hours
- Drain blocking buffer and add 1° Antibody (1:1000) in 10 mL blocking buffer
- Incubate in room temperature with gentle agitation for 3 hours
- Drain 1° antibody
- Wash the membrane with 10mL blocking buffer (3x, 5min)
- Add 2° antibody (1:10000) in 10 mL blocking buffer and incubate 3 hours

**Developing Membrane (colorimetric method)**
- Wash membrane with TBS 3x 5 min
- Pour 20mL TBS to a falcon tube and add 12 uL 30% hydrogen peroxide
- Pour 4 mL ice-cold MetOH to another falcon tube and add 12 mg 4CN (4-Chloro-1-Naphtol)
- Mix these two solutions and use immediately

**TBS (10x) pH 7.6**
- 24.23 g Tris-HCl
- 80.06 g NaCl
- Mix in 800mL sterile water
- Top up to 1L

**TBST**
- 100mL TBS 10x+900mL Sterile water +1mL Tween 20

**Antibodies**
- 1° antibody: 6x-His Epitope tag Antibody or anti-HPF, anti-S13, anti-L5 polyclonal antibodies
  - Host animal: rabbit
- 2° antibody: Goat anti-rabbit, horseradish peroxidase conjugated

**Developing Membrane**

**Colorimetric Method**
- Wash membrane with TBS 3x 5 min
• Pour 20mL TBS to a falcon tube and add 12 uL 30% hydrogen peroxide
• Pour 4 mL ice-cold MetOH to another falcon tube and add 12 mg 4CN (4-Chloro-1-Naphtol)
• Mix these two solutions and use immediately
  o You will see purple-black color bands on membrane, this is your protein!

Chemiluminescent Method

• Wash membrane with TBS 3x 5 min
• Mix Solution A and Solution B right before pouring off

Solution A (keep in dark)
  o 5mL 500mM Tris at pH 8.5
  o 1.25mM Luminol
  o 0.225mM Coumeric acid
Solution B
  o 500mM Tris at pH 8.5
  o 4uL 30% Hydrogen peroxide

Mix solution A and B, pour on the membrane and take picture with chemiluminescent imaging device.
APPENDIX B

MINIMUM INHIBITORY CONCENTRATIONS
Appendix-A
Minimum Inhibitory Concentrations
MIC of several antibiotics on WT PAO1 and Δhpf, Δrmf, ΔhpfΔrmf, ΔrpoH::rpoH and rpoH transposon mutants were measured. Optical densities of the overnight grown cultures of the mentioned strains were measured for 18-20 hours every 15 minutes after they are transferred to fresh media with starting OD of 0.05.

Minimum Inhibitory Concentration of Tobramycin on PAO1 and Mutants
PAO1ΔHPF

PAO1ΔRMFΔHPF
Minimum Inhibitory Concentration of Ciprofloxacin on PAO1 and Mutants

PAO1

PAO1ΔRMF
Minimum Inhibitory Concentration of Kanamycin on PAO1 and ΔrpoH::rpoH Mutant with Varying Arabinose Concentrations

PAO1-Km-0% Arabinose

PAO1ΔrpoH::rpoH-Km-0% Arabinose
Minimum Inhibitory Concentration of Gentamycin on PAO1 and ΔrpoH::rpoH Mutant with Varying Arabinose Concentrations
Minimum Inhibitory Concentration of Ciprofloxacin on PAO1 and ΔrpoH::rpoH Mutant with Varying Arabinose Concentrations
Minimum Inhibitory Concentration of Tobramycin on PAO1 and ΔrpoH::rpoH Mutant with Varying Arabinose Concentrations
### PAO1ΔrpoH::rpoH-Tb-1% Arabinose

![Graph showing growth curves for PAO1ΔrpoH::rpoH-Tb-1% Arabinose](image)

### Table: Antibiotic/Arabinose Concentration Effects

<table>
<thead>
<tr>
<th>Antibiotic/Arabinose Concentration</th>
<th>PAO1</th>
<th>ΔrpoH::rpoH</th>
<th>PAO1</th>
<th>ΔrpoH::rpoH</th>
<th>PAO1</th>
<th>ΔrpoH::rpoH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km</td>
<td>200</td>
<td>50</td>
<td>200</td>
<td>50</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>Gm</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Tb</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cip</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>