THE ROLE OF RIBOSOME HIBERNATION FACTOR AND THE STRINGENT RESPONSE IN THE SURVIVAL OF *PSEUDOMONAS AERUGINOSA* DURING DORMANCY

by

Sokuntheary Theng

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2019

DEDICATION

All of this work is dedicated to all people who live with a little hope and want to give up but never give up, and people who give hope and chance. I did not have many hopes that this work would happen; but it happened now because I gave a try and my advisor gave me a chance. This work was a slow progress to other people but this was my best speed for this challenging work in my life. A little hope and not giving up make things happen.

ACKNOWLEDGEMENTS

I would first of all like to express my deepest appreciation to my wonderful advisor, Dr. Michael Franklin, who provided me a chance to study microbiology at Montana State University. I am grateful for his good advices, kindness, guidance, generous support, encouragement, valuable comments and suggestions, which made me not give up on my Master's research. My thesis research could not have been carried out successfully without a great guidance and support from Franklin lab. I deeply thank to all Franklin lab members who assisted me during the two years of my Master's research. I am very grateful to Dr. Ben Lei and Dr. Jovanka Voyich for being my great committee and advising me, which realized out this thesis successfully. Grateful acknowledgement and sincere appreciation are extended to all professors and colleagues at Microbiology and Immunology Department.

A special thank goes to the Harpswell Foundation for providing me a chance to experience American education in the U.S before starting my Master program. I deeply thank all my friends in Georgia, my wonderful American woman Catherine, uncle Fred, Mak Om, and the Kemp family for grateful love and care and letting me be a part of their family. I am appreciated my Harpswell sister, Bong Ngim, who never fail to answer my phone and listen all my stressful stories. I thank to friends in Bozeman who took me away from the lab during some weekends. Finally, I would like to express the most gratitude to my parents, family, and friends at my home country for their love, understanding, encouragement, support, and affection.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF FIGURES CONTINUED

ABSTRACT

Pseudomonas aeruginosa survives in a dormant state in low nutrient environments and is able to resuscitate when favorable conditions are available. In response to stressful environmental conditions including antibiotic stress, osmotic stress, and starvation, *P. aeruginosa* undergoes the (p)ppGpp-mediated stringent response to induce a variety of genes for entry into the dormant state. One critical mechanism for *P. aeruginosa* dormancy involves in inactivating translation machinery by converting active ribosomes into inactive 70S and 100S ribosome monomers and dimers. Hibernation promoting factor (HPF~11.6kDa) is a ribosome-associated protein that stabilizes inactive ribosomes. Here, I investigated the relationship between HPF and the stringent response in survival of *P. aeruginosa* during dormancy. I also investigated role of HPF preserving ribosomes in dormant cells by quantifying the abundance of two ribosomal proteins (L5 of the large ribosomal subunit and S13 of the small ribosomal subunit), during *P. aeruginosa* starvation. For quantitative analysis during nutrient-limited conditions, I used immunoblotting and image analysis to quantify L5 and S13 abundances in the wild-type strain, PAO1, and in a *relA*/*spoT* double mutant strain, which is incapable of producing (p)ppGpp. The results show that the *relA/spoT* mutant loses HPF proteins after four days of starvation. To explore the role of HPF in preserving ribosomal proteins, I quantified L5 and S13 in wild-type PAO1 and in the Δ*hpf* deletion mutant and in the *relA/spoT* mutant. Immunoblots showed that both L5 and S13 rapidly decrease by day 2 of starvation in the *Δhpf* mutant strain, but that these proteins are maintained throughout eight days of starvation in the wild-type strain. Notably, L5 and S13 are maintained in the *ΔrelA/ΔspoT* strain throughout starvation. Lastly, I determined if the amount of cellular HPF required for *P. aeruginosa* ribosome maintenance. Growth in minimal medium (MOPS medium) affects the amount of HPF produced, based on the carbon source. Therefore, I tested ribosome maintenance during starvation of cells first cultured in MOPS-fructose or MOPS-glucose. The results indicate that HPF production during growth in MOPS-fructose is higher than in MOPS-glucose. However, the increased amount of HPF did not affect the amount of L5 and S13 during starvation. Therefore, the amount of HPF is not critical for *P. aeruginosa* to maintain its ribosomes during starvation. These results demonstrate HPF is essential for maintenance of ribosomal proteins during starvation of *P. aeruginosa*, and that the ribosomal proteins are likely degraded in the absence of HPF. *P. aeruginosa* needs a minimum amount of HPF to preserve ribosomes during nutrient-limited condition.

CHAPTER ONE

INTRODUCTION

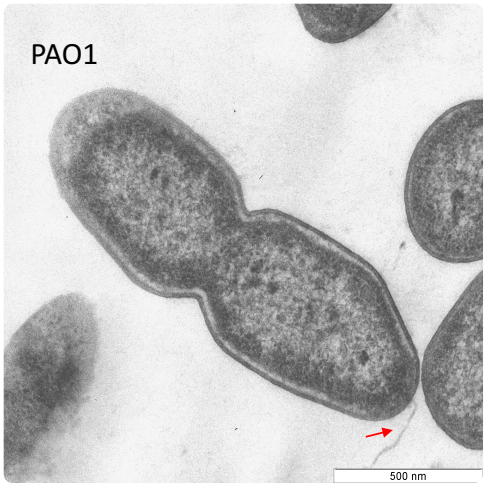
Pseudomonas aeruginosa Strain PAO1

Pseudomonas aeruginosa, is one species of more than 140 species of the genus *Pseudomonas*. *P. aeruginosa* plays a role as a human pathogen due to its ability to produce many virulence factors (Iglewski, 1996). It can be isolated from water, soil, plants, and animal tissues (Hadola & Edberg, 1997). *P. aeruginosa* is able to survive in inhospitable environments such as jet fuels, soap, and chlorhexidine solutions (Botzenhart & Döring, 1993). This bacterium is a major source of bacteremia in urinary-tract infection and burn units of the hospital (Friedrich & Cunha, 2016). Another common infection which is increasingly associated by *P. aeruginosa* is chronic respiratory infections (Yum et al., 2014).

P. aeruginosa is a gram-negative, rod-shaped, and facultative aerobe measuring 0.5 to 0.8, μm by 1.5 to 3.0 μm (Iglewski, 1996). *P. aeruginosa* often produces a yellow-green, fluorescent pigment "pyoverdine" which plays an important role in iron uptake through the bacterial membrane (Orlandi et al., 2015; Lau et al., 2004) and a blue non-fluorescent pigment "pyocyanin" which is the secondary metabolite that contributes and modulates the expression of virulence genes for pathogenicity (Lau et al., 2004) and has antibiotic activity that can kill wide variety of microbes competing against *P. aeruginosa* (Baron & Rowe, 1981). *P. aeruginosa* contains only one single polar flagellum for motility as shown in the Transmission Electron Microscope (TEM) image of *P. aeruginosa* (PAO1) in Figure 1A.

The genome size of *P. aeruginosa* strains ranged between 5.5 and 7.5 Mbp, with 67% Guanine-Cytosine content (Hector & Jonhson, 1990). *P. aeruginosa* strain PA01 has a complete genome sequence available in 2000 (Stover et al., 2000), with the genome size of ~6.3Mbp and 5570 predicted open reading frames (ORFs) (Fig. 1B). The exponential doubling time for PAO1 is 1-1.5 hours in minimal media and 25-35 minutes in a rich broth such as LB (LaBauve & Wargo, 2012). This strain is significant for scientific studies because of its ability to survive in a wide spectrum of environments, roles as a pathogen, and antibiotic resistances.

A



B

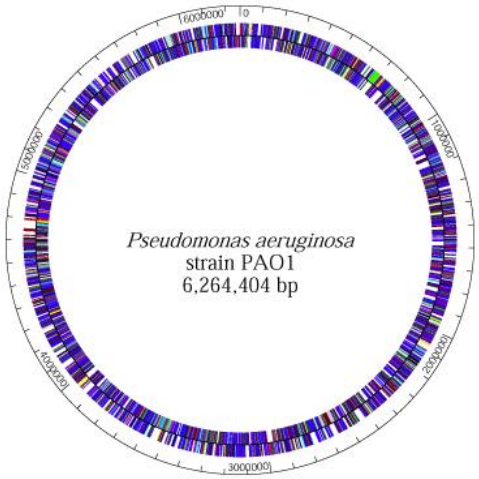


Figure 1: *P. aeruginosa* PAO1 features. (A) Transmission Electron Microscope (TEM) image of *P. aeruginosa* (PAO1): PAO1 overnight culture was grown in the fresh LB media until the culture reached mid-log phase (approximately six hours). PAO1 mid-log phase cells were prepared for TEM processing. This image shows the rod-shape of a single cell was dividing into two cells. At the bottom of the cell where the red arrow points, was the single polar flagellum for cell motility. (B) Circular representation of the *P. aeruginosa* PAO1 genome from the Pseudomonas Genome Database (http://www.pseudomonas.com): The outer circle indicates the chromosomal location in base pairs. The 5570 predicted open reading frames (ORFs) are represented by colored boxes regarding their functional categories and direction of transcription, the outer band is the plus strand; and the inner band is the minus strand

Pseudomonas aeruginosa Infections

Cystic Fibrosis

Cystic fibrosis (CF) is a genetic disease resulting in excess mucus of the lung, which traps bacteria and causes infections (Yu, 2018). According to Cystic Fibrosis Foundation, there are more than 70,000 people worldwide who suffer from CF and more than 30,000 in the US. About 1,000 new cases of CF are diagnosed each year. In America, CF is the second most common life-shortening disease, after sickle cell disease (Grosse, 2004). People with CF have certain symptoms: chronic cough, high salty sweat, poor weight gain, poor growth, very salty-tasting skin, shortness of breath, and pneumonia (James & Lynn, 2013). CF often happens with children. With the modern clinical care, the life expectancy of CF patients has increased to 18 years old or older, according to the data collected by Cystic Fibrosis Foundation. However, there is no cure for CF yet (Elborn, 2004).

A person born with cystic fibrosis, has two copies of CF allele located on the long arm of chromosome 7 (Ratjen & Tullis, 2012). The mutation is in the gene called CFTR (cystic fibrosis transmembrane regulator). The dysfunctional allele leads to the inability to move chloride to the epithelial cell surface (Veit et al., 2016). ΔF508 is the most common CFTR mutation, causes protein misfolding of CFTR that reduces the level of chloride secretion (Riordan, 2008). Without the help of chloride (Cl^-) ion to attract water to cell surface, it causes the thick and sticky mucus along the airway which traps bacteria, resulting in chronic bacterial infections (Livraghi-Butrico et al.,2013; Veit et al., 2016). *P. aeruginosa* is the main pathogen infecting the airway in CF lung (Bragonzi et al., 2004). In the CF lung, the high density of *P. aeruginosa* (10^7–10^9 cfu/mL) can form biofilms

which are hard to treat with antibiotics (Smiley & Hassett, 2005; Turner et al., 2015).

Chronic Wounds

A healthy person is able to process wound healing by 4 main phases: coagulation, inflammation, cell proliferation and repair of the matrix, and epithelialization and remodeling of the scar tissue (Schultz et al., 2003). However, chronic wounds are defined as the wounds that fail to heal within the expected time frame of about three months (Werdin, Tenenhaus, & Rennekampff, 2008). Chronic wounds commonly occur in people with diabetic foot ulcers, venous leg ulcers, and pressure ulcers (Frykberg & Banks, 2015). Six million people in the US and 20 million people in the developed world suffer from chronic wound (Frykberg & Banks, 2015); and 28% of patients with chronic wounds die within a 2-year span, according to the Global Wound Care. The global advanced wound care market is expected to reach $20 billion by 2025 from $10 billion in 2018 (Advanced Wound Care Market, 2018).

P. aeruginosa is known as an opportunistic pathogen that is associated with two different infections, lung infection and chronic wound infection. In a bacterial profiling study, Gjødsbol et al. found that 52.2% of chronic venous leg ulcers were infected by *P. aeruginosa* (Gjødsbol et al., 2006). This opportunistic bacterium is widely known to cause biofilm-based chronic infections in their hosts (Fazli et al., 2009). *P. aeruginosa* has the ability to form biofilm which are organized the communities of bacteria having a matrix of extracellular polymeric substances (EPS) in order to hold microbial cells together to a surface (Costerton et al., 1995). Franklin et al. (Franklin et al., 2011) described that three polysaccharides (alginate, Pel, and Psl) produced by *P. aeruginosa* for the stability of the

biofilm structure. Alginate is a linear unbranched polymer composed of D-mannuronic acid and L-guluronic acid, contributing to the structural stability, protecting biofilms, and retaining of water and nutrients. Pel and Psl polysaccharides play roles as a primary structure scaffold for biofilm development and are involved at the early stages of biofilm formation. The Pel is mainly a glucose-rich matrix material, with still unclarified composition, while Psl comprises a repeating pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose (Ryder, Byrd, & Wozniak, 2007). *P. aeruginosa* has different characteristics from *P. aeruginosa* planktonic cells, such as increased resistance to activities of host immune system and more tolerance to antimicrobial therapy (Jesaitis et al., 2003; Parker et al., 2010). Therefore, detailed knowledge about *P. aeruginosa* characteristics is significant in order to have effective wound treatment and management.

Multiple Mechanisms of Antibiotic Resistance in *P. aeruginosa*

P. aeruginosa is known to be resistant to different classes of antibiotics (Pena et al., 2013), which has become a critical and deadly issue. Currently, there are 8 categories of antibiotics that are used to treat *P. aeruginosa* infections including aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B) (Bassetti, Vena, Croxatto, Righi, & Guery, 2018). In 2016, European Centre for Disease Prevention and Control reported that 33.9% of *P. aeruginosa* were resistant to at least one of the antimicrobial groups such as piperacillin ±

tazobactam, fluoroquinolones, ceftazidime, aminoglycosides, and carbapenems (European Centre for Disease Prevention and Control, 2015). This multi-drug resistance *P. aeruginosa* contains a large genome (~6.3Mbp) that has a high possibility to develop a large number of factors associated with antibiotic resistance involving almost all classes of antibiotics.

Dever & Dermody illustrated three mechanisms of bacterial resistance to antibiotics: 1) bacteria are able to produce enzymes to degrade, inactivate, or modify antibacterial drugs; 2) bacteria are able to alter their proteins that are targeted by antibiotics; and 3) bacteria are able to decrease membrane permeability to antibiotics (Dever & Dermody, 1991). *P. aeruginosa* performed all of these known resistance mechanisms.

β-lactams

Four beta-lactam antibiotics (penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems) share a common core beta-lactam ring that binds to essential penicillin-binding proteins (PBPs) which inhibit the terminal steps of peptidoglycan (murein) cross-linking and cell wall synthesis (Zeng & Lin, 2013). However, wild-type strains of *P. aeruginosa* encode a wide-spectrum class C β-lactamase by bla_{AmpC} gene on the chromosome (Berrazeg et al., 2015). β-lactamase production hydrolyzes the beta-lactam ring, which is an effective strategy for β-lactam resistance (Jacoby & Munoz-Price, 2005). In the case of gram-negative bacteria like *P. aeruginosa*, beta-lactamases *ampC* (originally named *ampA*) is expressed at low level (Zeng & Lin, 2013). However, Berrazeg and his group reported that mutations in the promoter region of *ampC* induced overexpression of beta-lactamases (Berrazeg et al., 2015).

Epidemiologically, *P. aeruginosa* is able to carry mobile genetic elements such as plasmids or integrons which encode enzymes such as carbapenemases, associated with other β-lactam resistance mechanisms (Bassetti, Vena, Croxatto, Righi, & Guery, 2018). Hence, β-lactamases inhibit β-lactam antibiotic activity.

Aminoglycosides

P. aeruginosa carries mobile genetic elements that encoded aminoglycosides modifying enzymes (AME) (Bassetti, Vena, Croxatto, Righi, & Guery, 2018). Those aminoglycoside enzymes include, aminoglycoside phosphoryltransferase [APH], aminoglycoside acetyltransferase [AAC], and aminoglycoside nucleotidyltransferase [ANT] or aminoglycoside adenytransferase (Poole, 2005). AME inactivate aminoglycosides (e.g. tobramycin, gentamicin, and amikacin) by covalent binding of acetyl, phosphate or adenyl groups to amino and hydroxyl on the antibiotic molecules. These modifications reduce the affinity of aminoglycosides to target the 30S ribosomal subunit, hence aminoglycosides cannot block ribosome activity (Llano, Azucena, Kotra, Mobashery, & Chow, 2002). *P. aeruginosa* is resistant to kanamycin, neomycin, and streptomycin by the activity of [APH] phosphorylation; tobramycin, netilmicin, and amikacin by [AAC] acetylation; and streptomycin and gentamicin by [ANT] adenylation of aminoglycosides (Poole, 2005).

Membrane Impermeability and Efflux

Decreased permeability of the bacterial membrane is a cause of antimicrobial resistance. In 1986, Nikaido and Hancock demonstrated that *P. aeruginosa* has low outer membrane permeability (Nikaido & Hancock, 1986), therefore it is hard for antibiotics to

penetrate inside the cells. Nikaido and Yoshimura, measured the rates cephacetrile and cephaloridine hydrolysis in the periplasm. They found that these two compounds had low permeability in the outer membrane, about 100-fold lower than *Escherichia coli* K-12 (Yoshimura & Nikaido, 1982).

Nikaido and Hancock reported low outer membrane permeability but a large exclusion to uptake compounds from the environment. They also determined that *P. aeruginosa* is able to uptake compounds of around 3000 molecular weight, compared to exclusion limit of *E. coli* which is around 500 molecular weight (Nikaido & Hancock, 1986). However, *P. aeruginosa* is able to reduce the number and differentially express of porins (Hancock & Brinkman, 2002). Porin OprD (also called protein D2) of *P. aeruginosa* displays a role in uptake of basic amino acids and antibiotic molecules (Trias & Nikaido, 1990). *P. aeruginosa* modifies OprD structure and reduces *oprD* expression (Li et al., 2012). Li et al., illustrated that OprD was down regulated in the presence of metals (zinc) and small bioactive molecules, and when efflux pump regulator was activated. In this way, *P. aeruginosa* has the ability to lower the antibiotic concentration inside the bacterial cells. Even though, *P. aeruginosa* might still uptakes some of those toxic compounds, *P. aeruginosa* has membrane associated efflux system to remove those compounds (Westbrock-Wadman et al., 1999). The efflux system of *P. aeruginosa* is capable of pumping a wide variety of antibiotics out of the cells, including β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim, as well as various dyes and detergents (Poole, 2001). All of these mechanisms of wild-type strain *P. aeruginosa* PAO1 make *P. aeruginosa* classified as a

multidrug resistant bacterium.

Dormancy

One method that bacteria use to survive harsh conditions is dormancy. An estimated 60% of the global microbial biomass survives in the forms of dormant state (Mueller, 2018). Dormancy refers to the ability of microorganisms to enter a reversible state of low metabolic activity once they are faced with unfavorable environmental conditions (Lennon & Jones, 2011). Dormant bacterial cells are the non-growing bacteria including persister cells (highly antibiotic resistant cells), starved cells in stationary phase, or the viable-but-nonculturable cells (Bergkessel & Newman, 2016). Dormant cells are able to regrow once they are reintroduced into the environment with abundant nutrients (Lewis, 2007). When bacteria enter a dormant state, they notably use less energy. Dormant cells slow down their metabolic activity and rely on their energy stores (Lennon & Jones, 2011). Hence, when cells use dormancy strategy for survival, the cells reduce cell size, degrade macromolecules, and reprogram the cellular resources (Bergkessel & Newman, 2016). Once dormant cells are transferred to high nutrient or any favorable environment, cells are able to sense those nutrients and energies and emerge from dormant state. In the Actinobacteria such as mycobacteria and streptomycetes, Resuscitation-Promoting Factor (RPF) is the protein for nutrient sensing and a signaling molecule at pico-molar concentrations (Gupta & Srivastava, 2012) that dormant cells use to promote growth and metabolic reactivation (Keep, Robertson, Cohen-Gonsaud, & Henderson, 2006).

Lennon & Jones reported that there are three stages of dormancy: initiation, resting, and resuscitation (Lennon & Jones, 2011). Initiation is when active cells make a transition

into dormant stage. In response to unfavorable conditions, bacteria detect the changes of abiotic factor including carbon sources, temperature, osmotic pressure, light, and pH through membrane bound histidine kinase sensor. This detection promotes changes in gene expression and protein synthesis that trigger dormancy. Starvation or resource limitation is one condition that initiates dormancy. During the resting stage, dormant bacteria display a wide range of phenotypes such as reducing cell size or spore formation. The concentrations of nucleic acids, lipids, fatty acids, and proteins are decreased; and there is an increase of storage compounds (glycogen, polyphosphates and polyhydroxyalkanoates) that are significant for meeting energy requirement in order to maintain and survive in the dormant state because dormancy is not an energy cost-free state. Microorganisms use maintenance energy for supporting non-growth functions including, motility, turnover of macromolecules, regulation of osmotic pressure and intracellular pH. Bacterial endospores do not require maintenance energy. Endospores are only known for specific gram-positive, including *Bacillus spp* and *Clostridium spp*. Since there may be no DNA repair during dormancy, genomic chromosomes can be degraded via hydrolysis and oxidation. This damage of genomes lead failure in resuscitation from dormancy. Additionally, if cells cannot maintain their internal resource reserves before resuscitation, dormancy result in death. Hence, the third stage of dormancy is the success of resuscitation and the ability to reproduce. In the case of endospores, when unfavorable conditions change to favorable conditions, spores undergo germination in response to increased availability of resources. In the case of gram-negative bacteria, cells can be a viable but not-culturable (VBNC) stage (Byrd, Xu, & Colwell1991). In mycobacteria, the dormant bacteria induce resuscitation-

promoting factor (RPF), which is a 17 kDa protein that renovates the cell envelope of dormant cells to process cell regrowth and division (Lennon & Jones, 2011).

P. aeruginosa-Dormancy

P. aeruginosa PAO1 is able to survive in water for over 145 days (almost 5 months), significantly longer than *Escherichia coli* and *Staphylococcus aureus* (Lewenza et al., 2018). Lewenza and his group reported that *P. aeruginosa* dormancy involves regulations of many genes which support the long-term survival and antibiotic tolerance (Lewenza et al., 2018). Growth arrest of *P. aeruginosa* can be induced by depletion of carbon and nitrogen sources (David et al., 2017). That study also reported that the switch from active growth to growth arrest in nutrient-starved conditions involves global gene regulators including guanosine tetra- and penta-phosphate (p)ppGpp.

Guanosine Tetra- and Penta- Phosphate (p)ppGpp

During dormancy by starvation or resources limitation, the intracellular concentration of amino acids, fatty acids or other carbon compound is reduced which make cells undergo stringent response (Aertsen & Michiels, 2004). The stringent response is the stress response of bacteria cells in reaction to nutrient limitation including amino acid starvation, fatty acid limitation, and iron limitation (Battesti & Bouveret, 2006; Haseltine & Block, 1973). This response is signaled by guanosine tetra- and penta-phosphate (p)ppGpp (Potrykus & Cashel, 2008; Cashel, 1996). Guanosine 5′-triphosphate-3′diphosphate or Guanosine pentaphosphate (pppGpp) is GTP with pyrophosphate attached at 3’OH. Guanosine 5′-diphosphate-3′diphosphate or Guanosine tetraphosphate (ppGpp) is a GDP with pyrophosphate attached at 3’OH. (p)ppGpp mediates other aspects

of cellular activity such as the activity of the alternative σ-factor, RpoS, which results in expression of a subset of genes that are expressed during stationary phase (Lennon & Jones, 2011). In addition, during stress condition, (p)ppGpp production also affects DNA replication and translation (Bruhn-Olszewska et al., 2018).

During nutrient stress, uncharged tRNA (or deacylated tRNA) bound at the A site of ribosome causes translation elongation to stall and trigger (p)ppGpp productions (Winther, Roghanian, & Gerdes, 2018), through the activity of the enzymes RelA and SpoT. RelA and SpoT are homologues. These two homologous enzymes are involved in (p)ppGpp synthesis by transfer of two phosphates from ATP to 3'OH of GTP/GDP and release of AMP. RelA activates (p)ppGpp synthesis during stress of amino acid starvation, and SpoT activates (p)ppGpp synthesis during other nutrient starvation such as fatty acid starvation (Potrykus & Cashel, 2008). In vitro, the uncharged tRNAs cause RelA to undergo conformational changes (Wendrich et., 2012). RelA acts as (p)ppGpp synthetase to convert GDP/GTP and ATP into ppGpp/pppGpp by adding pyrophosphate (PPi) from ATP on the 3'OH of the ribose in GDP/GTP (which is (p)ppGpp) and releasing AMP.

Ribosome During Dormancy

In a bacterial cell under nutrient-rich condition, ribosomes are the most abundant cellular machinery, containing 40 to 50% of cell weight (Ridgway et al., 2008) and ~55% of the total proteins are ribosomal proteins (Scott et al., 2010). When cells enter the dormant stage through nutrient starvation, the majority of ribosomes are not necessary because cells do not waste energy for unnecessary translation. In starved *Escherichia coli*, more than 80% of ribosomes are degraded (Scott, Gunderson, Mateescu, Zhang, & Hwa, 2010), and

~50% of ribosomes are degraded in *Vibrio spp.* during prolonged carbon starvation (Ridgway et al., 2008).

In *Escherichia coli* in response to stringent control, dormant cells produce phosphate (Pi) due to (p)ppGpp catalyzed reaction. High number of polymer of inorganic phosphate (Pi) can form polyphosphate (polyP). Kuroda et al. illustrated that polyP form a complex with adenosine 5′-triphosphate–dependent protease Lon [Lon protease, is a family of proteases that are induced by stress and degrade polypeptides to yield small peptide fragments that are 5 to 10 amino acids long] in order to degrade most of ribosomal proteins under stress conditions (Kuroda et al., 2001). In 1999, Kuroda's study reported that during nutrient deprivation, cells rely on the generation of polyP in order to degrade ribosomal proteins (Kuroda et al., 1999). This study also showed that a mutant strain of *E. coli* lacking polyphosphate kinase (PPK) was not able to induce proteolysis, and expressed enzyme for amino acid biosynthesis to maintain surviving under carbon starvation (Kuroda et al., 1999). This indicates that proteolysis or degradation of some ribosomal proteins is important for dormant cells during nutrient starvation.

HPF, RMF Ribosome Associated Proteins

Under unfavorable conditions, microorganisms must invest resources into resting structures and the important machinery that are needed for transitioning into and out of a dormant state. In order to achieve a dormancy state, bacterial cells protect some of their ribosomes from degradation. In *E. coli*, the starved cells inactivate 70S ribosomes and form inactive 100S dimers (Polikanov, Blaha, & Steitz, 2012). The cryo-EM study showed that in vivo, a 100S ribosome is formed by two 70S ribosomes linked together via their 30S

small subunits (Kato et al., 2010). In *E. coli*, 100S dimerization occurs in two steps from 70S to 90S, then finally 100S. A crystal structure study of *Thermus thermophiles* revealed that Ribosome Modulation Factor (RMF; 55 amino acids) binds next to the 3′ end of the 16S rRNA at the anti–Shine-Dalgarno region of small ribosome. Thus, the initiation of translation is inhibited by RMF that prevents interaction between Anti-Shine Dalgarno and Shine Dalgarno side of mRNA. The binding of RMF on a ribosome induces a conformational change form 90S ribosome dimers, in which the small subunit of two 70S ribosome came together. Based on Polikanov's model, Hibernation Promoting Factor (HPF; 95 amino acid) is bound to tRNA binding site and stabilizes the 100S dimer (Polikanov, Blaha, & Steitz, 2012). A paralog of HPF, YfiA (113 amino acids) is known to inactivate the 70S ribosome, but inhibits the formation of the 100S dimer. YfiA shares 40% sequence homology with HPF. Formation of 70S monomers or 100S dimers by RMF, HPF, or YfiA leads to ribosome hibernation. HPF is known to be required for *E. coli* (Polikanov et al., 2012), *B. subtilis* (Akanuma et al., 2016), *S. aureus* (Khusainov et al., 2017), *Vibrio*, and *P. aeruginosa* (Akiyama et al. 2017) to form inactive 100S ribosome dimers after prolonged nutrient starvation.

Song and Wood (Song & Wood, 2019) proposed a model of ribosomal dimerization for bacterial persister cells. The persister state occurs under antibiotic stress, osmotic stress, and nutrient limitation in the stationary phase. ppGpp productions by RelA/SpoT induces RMF and HPF associated proteins. Song and Wood proposed that ribosome dimerization is driven by the ribosome modulation factor (RMF) and hibernation promoting factor (HPF); and that ribosomes reactivate translation by HflX (Fig. 2). HflX is the protein that

dissociate 100S hybridized ribosomes back to 70S ribosome, which allows persister cell to resuscitate when nutrients are available.

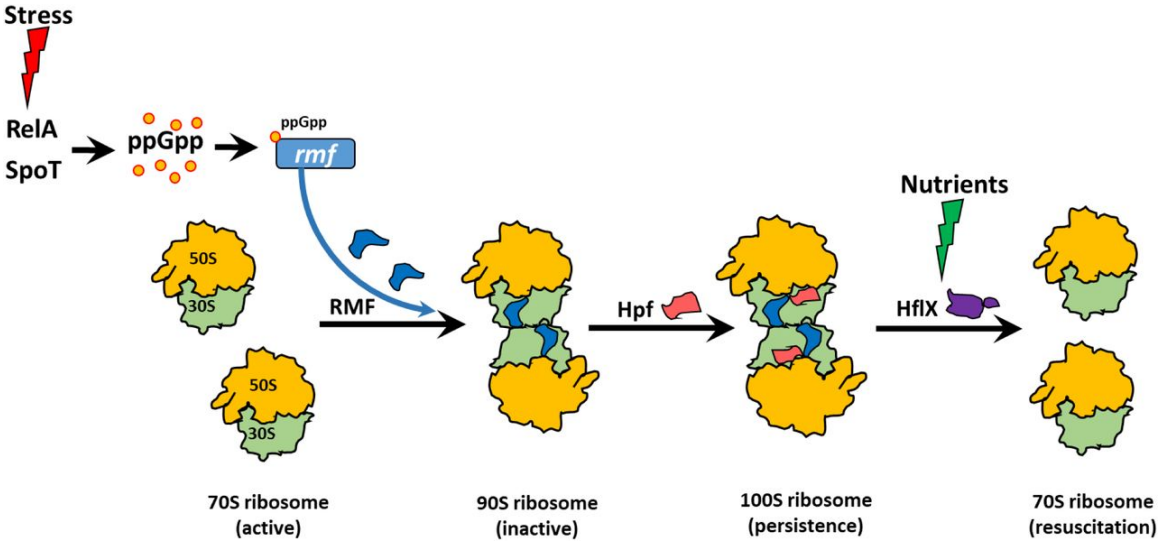


Figure 2: Schematic of the 100S ribosome dimerization model draw by Song and Wood (Song & Wood, 2019). "Myriad stresses (e.g., nutrient limitation, osmotic, acid) induce the stringent response which results in ppGpp formation by RelA/SpoT. ppGpp induces production of both RMF and HPF which converts active 70S ribosomes into inactive 90s and 100s ribosomes, respectively. Upon addition of nutrients and removal of the stress, HflX dissociates 100S ribosomes into active 70S ribosomes and growth resumes." (Song & Wood, 2019).

A structural study of HPF indicated that hibernation promoting factor (SaHPF; ~190 amino acids) promotes formation of 100S ribosomes in *Staphylococcus aureus* (Khusainov et al., 2017). A long variant SaHPF contains a N-terminal domain (NTD, 1 – 95 amino acids) and a C-terminal domain (CTD, 130-190 amino acid), connected by a linker of 35 amino acids. This study reported that 100S dimerization of *S. aureus* does not have a 90S ribosomal stage because *S. aureus* does not encode RMF. In order for *S. aureus* to form a 100S inactivated ribosome, SaHPF molecules bind to a 70S ribosome (Fig. 3). SaHPF-NTD binds to 30S small subunit ribosome which could block translation, and

SaHPF-CTD promotes ribosome dimers. The C-terminus domain of HPF interact with each other and bring two ribosomes to come together to form 100S ribosome dimer.

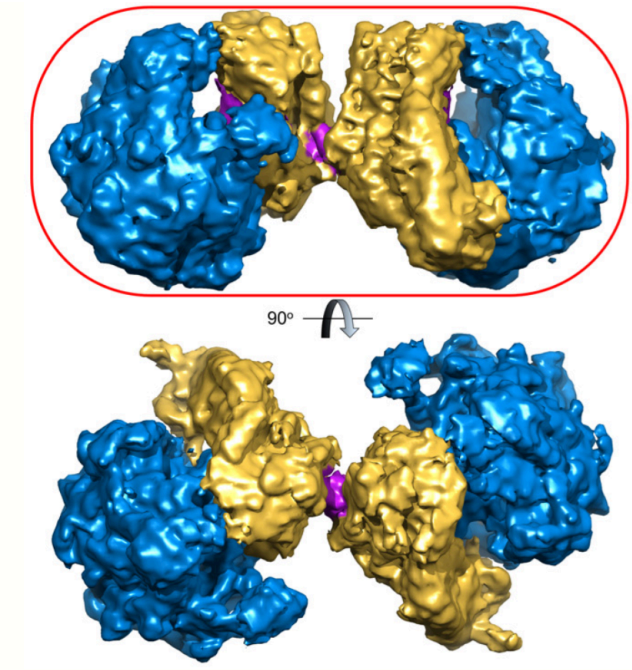


Figure 3: The cryo-EM of *Staphylococcus aureus* ribosome dimers by Khusainov et al (Khusainov et al., 2017). The image shows the structure of the 100S dimer at 11 Å resolution. Yellow represents 30S small subunit, blue is 50S large subunit ribosome, and HPF is colored pink.

A study of Williamson et al. showed that *P. aeruginosa* PAO1 biofilms contain mRNA transcripts of these two proteins (HPF and RMF) in the slow-growing antibiotic-tolerant subpopulations (Williamson et al., 2012). Akiyama et al. demonstrated that only one of these two proteins HPF, but not RMF of *P. aeruginosa* that is involved in ribosome preservation during starvation and resuscitation from dormancy (Akiyama et al., 2017).

L5 and S13 Ribosomal Proteins

Ribosome consists of two subunits, the 30S small subunit contains the 16S rRNA

and 21 ribosomal proteins (labeled S1-S21, in order of their molecular weight). The 50S large subunit contains the 23S rRNA, the 5S rRNA and 31 ribosomal proteins (labeled L1-L31). The two subunits come together to form a 70S ribosome which is the machine for translation. L5 and S13 are located on the 30S and 50S subunit respectively as shown in Figure 4 (Frank et al., 2007). Crystallographic studies of bacterial ribosomes exhibited that L5 plays an important role in the conjunction between 23S rRNA and 5S rRNA (Schuwirth et al., 2005; Selmer et al., 2006; Yusupov et al.,2001). Korepanov et al. demonstrated that *E. coli* failed to assemble the 50S large subunit in the absence of L5, therefore L5 is essential for ribosome assembly in bacterial cells (Korepanov et al., 2012). *E. coli* translation, r-protein L5 on the 50S large subunit and r-protein S13 on the 30S small subunit interact to build bridge B1b that contacts the tRNA molecule at P-site of ribosome in order to synthase polypeptide chains (Ning et al., 2014; Frank et al., 2007).

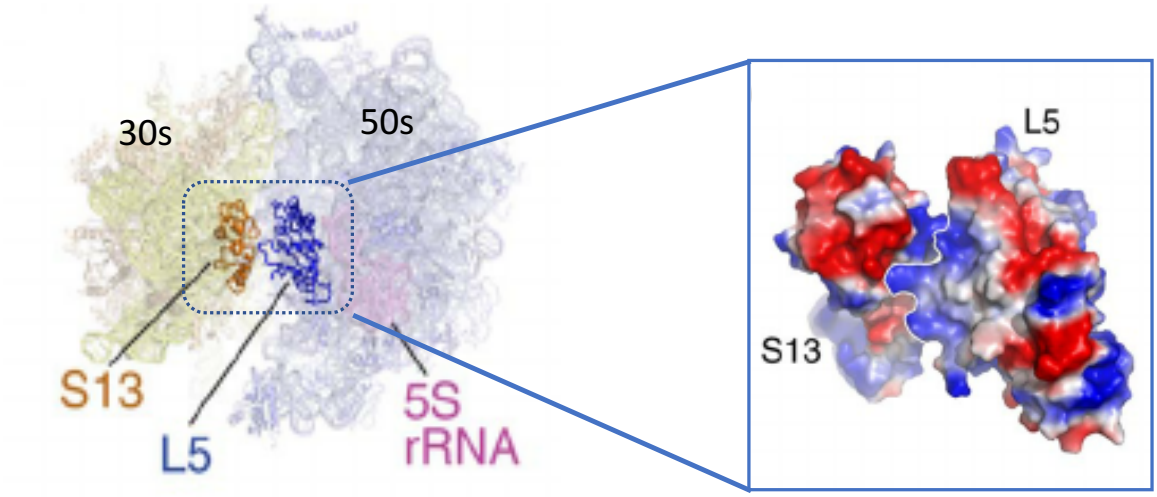


Figure 4: The location of L5 and S13 on ribosome by Frank et al (Frank et al., 2007). The positions of L5 protein is on 50S large subunit ribosome, and S13 is located on the surface of 30S small subunit.

Glycolysis Pathways in *P. aeruginosa*

Metabolism of carbohydrates, including fructose and glucose allows cells to generate pyruvate to acetyle-coA for entering the citric acid cycle (or Krebs cycle) in order to generate energy (White, Drummond, & Fuqua, 2012). Most bacteria perform glycolysis which is the process of converting glucose into pyruvate and generating small amounts of ATP and NADH. In central metabolism important precursor metabolites: six-carbon compounds of glucose-6P and fructose-6P and three-carbon compounds of glycerone-P, glyceraldehyde-3P, glycerate-3P, phosphoenolpyruvate, and final product "pyruvate" are produced. In glycolysis, most of the bacteria generate Fructose-1,6-biphosphate from Fructose-6-phosphate via Embden-Meyerhoff-Parnas (EMP or glycolysis pathway). However, in *P. aeruginosa*, Fructose-6P is not oxidized to Fructose-1,6-biphosphate because PA01 lacks the gene that encodes phospofructokinase (Temple, Sage, Schweizer, & Phibbs,1998). Instead, for growth on glucose, *P. aeruginosa* runs the Entner Doudoroff (ED) pathway to produce Phosphoglyceraldehyde or pyruvate. Glucose and gluconate are oxidized to 6-P-gluconate, then oxidize 6-P-gluconate via the ED pathway, where 6-P-gluconate is dehydrated and then cleaved into glyceraldehyde-3P and pyruvate, which activates in the presence of cations Fe^{2+}, Mn^{2+}, and Mg^{2+}. The ED pathway helps in generation of more NADPH. The enzyme 6-phosphogluconolactonase is an essential enzyme of this pathway activity which is induced in PAO1 by growth on mannitol and repressed by growth on succinate. The ED pathway yields one ATP, one NADH, and one NADPH for each glucose molecule.

Fructose Utilization in *P. aeruginosa*

Fructose is brought into the cell and converted to fructose 1-phosphate by fructose-phosphotransferase (Durham & Phibbs, 1982). During growth on fructose, intracellular fructose 1-phosphate is converted to fructose-1,6-bisphophate by fructose 1-phosphate kinase. Fructose-1,6-bisphophate is converted to fructose-6-phosphate; then to glucose-6-phosphate (by phosphoglucoisomerase) in the ED pathway. A secondary route of Fructose-1,6-bisphophate can be via lower EMP pathway. However, Pibbs (Pibbs, 1988) confirmed that EMP pathway was a minor contribution in metabolizing fructose.

CHAPTER TWO

SIGNIFICANCE AND RESEARCH GOALS

Pseudomonas aeruginosa maintains a sufficient quantity of ribosomes during nutrient stress and dormancy to allow resuscitation under favorable conditions. Since dormancy is an important factor involved in antibiotic resistance, understanding the dormant stage of *P. aeruginosa* is crucial to human health. Many antibiotics commonly used to target CF pulmonary infections and chronic wound infections target the ribosome and translation. Therefore, the goals of these studies were to characterize the response of ribosomes to nutrient limitation conditions, and the roles of the *P. aeruginosa* hibernation promoting factor and the stringent response factors in ribosome maintenance during starvation.

In response to nutrient-limited conditions, *P*. *aeruginiosa* undergoes the (p)ppGpp-mediated stringent response to induce expression of genes that are essential for survival during nutrient limitation (Khakimova et al.,2013). Akiyama et al. (Akiyama et al., 2017) found that in *P. aeruginosa* Hibernation Promoting Factor (HPF) but not Ribosomal Modulation Factor (RMF) is involved in ribosome preservation during starvation and resuscitation from dormancy. They showed that HPF is essential for preservation of the 23S rRNA component of ribosomes when cells are starved. In this study, I characterized the role of HPF in preserving ribosomal proteins during *P. aeruginosa* starvation. I hypothesized besides preserving ribosomal RNA, HPF plays another role in preserving L5 and S13 ribosomal proteins during nutrient limitation. In addition, I investigated the

relationship between RelA/SpoT and HPF on ribosome preservation during starvation. For these studies, I quantified the synthesis and degradation of HPF and of a small subunit ribosomal protein (S13) and a large subunit ribosomal protein (L5) during growth and starvation of wild-type strain PAO1 and its mutant derivatives Δ*hpf* and Δ*relA*/Δ*spoT*. Proteins were quantified using immunoblots and image analysis of immunoblots. In *P. aeruginosa*, HPF is expressed at different rates in minimal medium, when fructose or glucose is used as a sole carbon source. Therefore, I used this phenomenon to quantify the amount of HPF required for preservation of ribosomal proteins during starvation of *P. aeruginosa*.

CHAPTER THREE

MATERIALS AND METHODS

Protein Purification

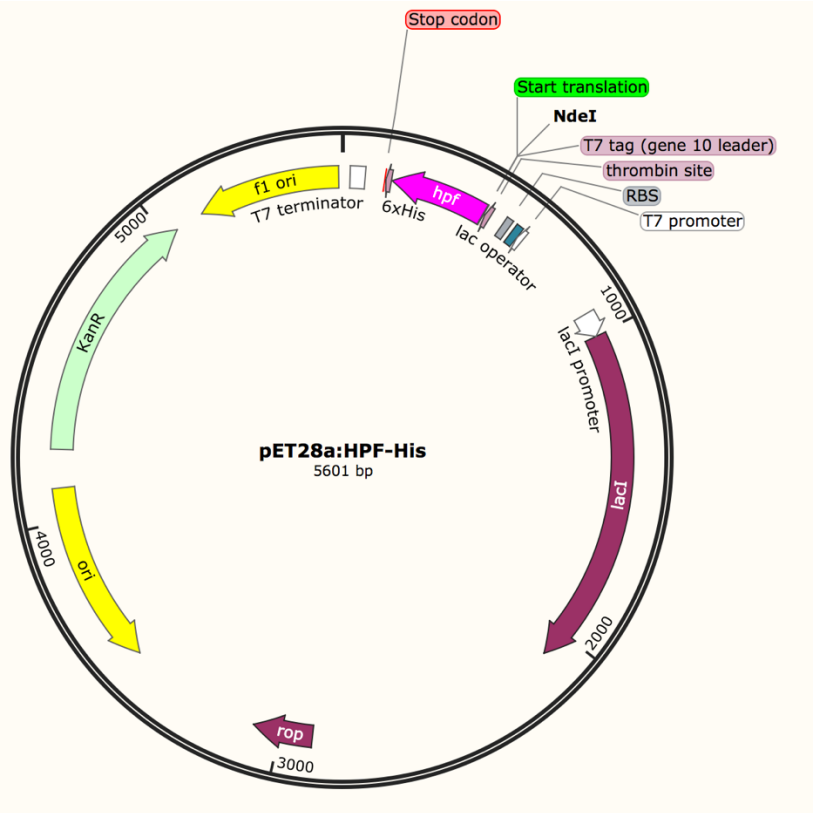
Strains

HPF, L5, and S13 proteins were purified from three strains: *E. coli* BL21 (DE3)-pET28a:HPF-His, *E. coli* BL21 (DE3)-pET28a:L5-His, and *E . coli* BL21 (DE3)-pET28a:S13-His.

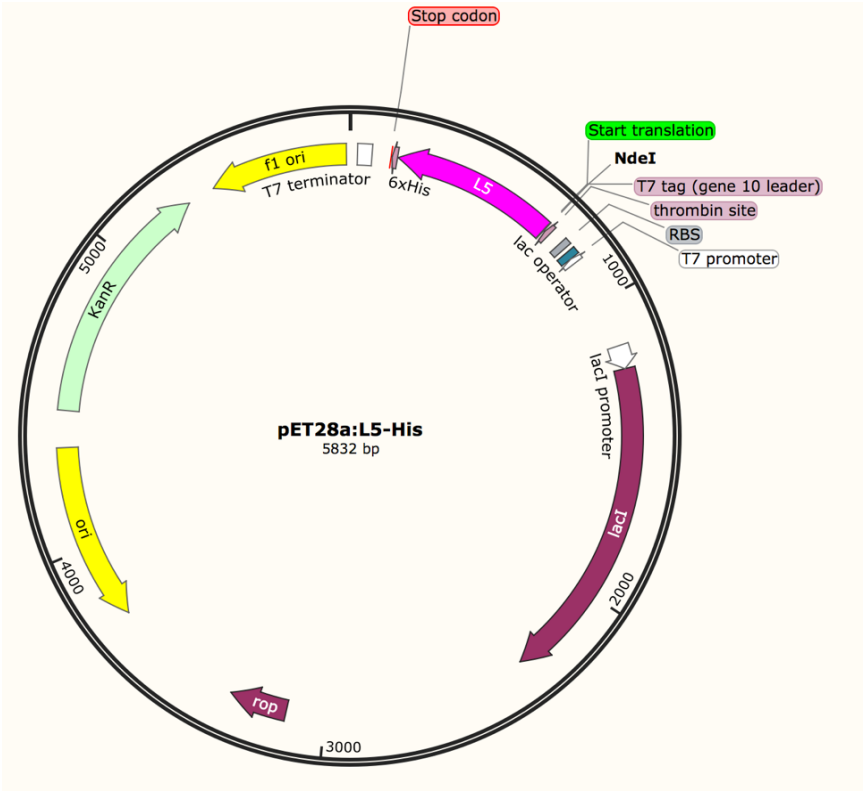
Plasmid Constructs: The plasmids were constructed by Sila Yanardag as I describe here. Three plasmids were contracted by amplified *hpf*, L5, and S13 sequences from *P. aeruginosa* PAO1 genomic DNA, cloned into pET28a vector, and transformed plasmids into *E. coli* BL21 (DE3). Primers used for PCR amplification of *hpf*: forward CGC ATA TGC AAG TCA ACA TCA GTG GCC ATC and reverse AGC TCG AGT CAG CGG GCG CCT ACG CCT TGC5'; L5: forward GGC ATA TGG CAC GAT TGA AAG AAA TTT ATC and reverse CGC TCG AGC TCG CGG TTC TTC ATG CTC TC; and S13: forward GGC ATA TGG CCC GTA TTG CAG GCG TCA AC and reverse GGC TCG AGC AGC AGG TTT TGC CAT GAC TAG. After PCR amplification, each target genes were inserted into pET28a vector at the N-terminus of 6x-poly-histidines using restriction enzymes XhoI and NdeI. Plasmids were constructed successfully, having C terminus 6x-His tag in frame with the target genes (*hpf*, L5, and S13). Lastly, transformations of all three individual plasmids into competent *E. coli* BL21(DE3) cells

were performed. 50µl of *E. coli* BL21(DE3) competent cells were taken from -80°C and immediately thawed on ice for ten minutes. 1µl from ~800ng/µl plasmids were transferred into competent cells and mixed by stirring using pipette tip. The competent cells and plasmids were incubated on ice for 30 minutes, then heated shock for 30 seconds in order for *E. coli* to uptake the plasmids. 250µl of SOC media were added to competent cells and incubated on roller at 37°C for 15 minutes. The culture was spread on LA - 30µg/ml kanamycin plate and incubated at 37°C overnight to obtain colonies. The isolated colonies were picked up for inoculum in 3ml of LB-30µg/ml kanamycin at 37°C for overnight. Finally, frozen stocks were made (0.5ml of overnight culture and 0.5ml of 10% skim milk), then stored at -80°C for inoculating culture in the future.

A



B


Stop codon
Start translation
NdeI
T7 tag (gene 10 leader)
thrombin site
RBS
T7 promoter
f1 ori
T7 terminator
6xHis
L5
lac operator
1000
lacI promoter
lacI
2000
pET28a:L5-His
5832 bp
5000
KanR
ori
4000
rop
3000

C

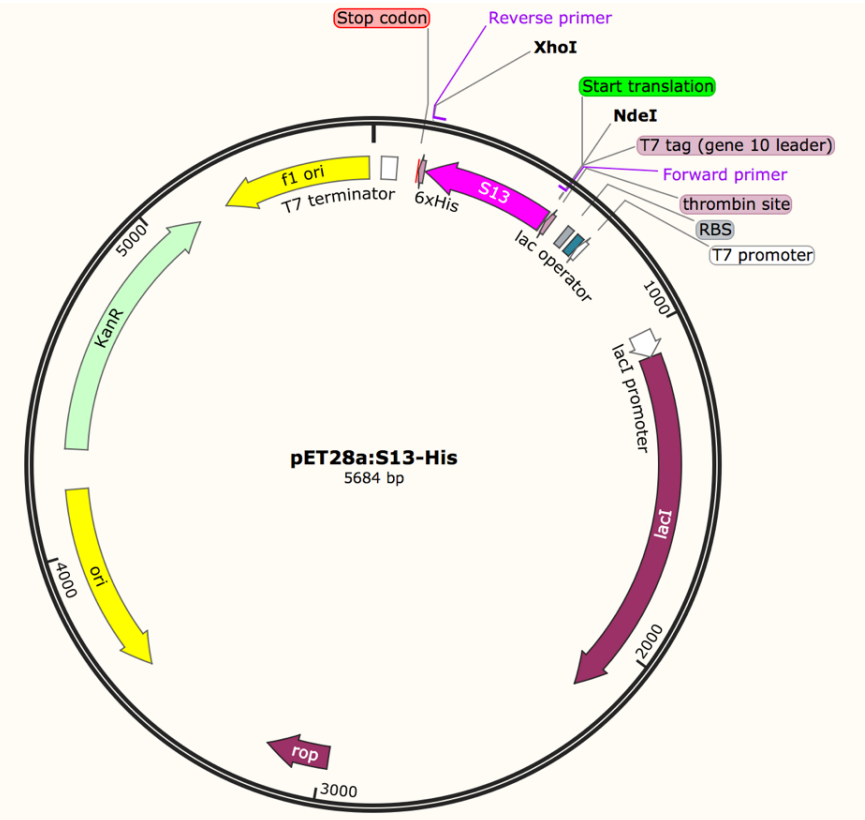

Stop codon
Reverse primer
XhoI
Start translation
NdeI
T7 tag (gene 10 leader)
Forward primer
thrombin site
RBS
T7 promoter
f1 ori
T7 terminator
6xHis
S13
lac operator
1000
lacI promoter
lacI
2000
pET28a:S13-His
5684 bp
5000
KanR
ori
4000
rop
3000

Figure 5: Plasmid maps of (A) HPF, (B) L5, and (C) S13 clones. *hpf*, L5, S13 were inserted on pET28a vector at the restriction sites (NdeI and XhoI). The pink arrow represents the target proteins (HPF, L5, and S13) which have 6xHis tag fussed at the C-terminal in frame with target proteins. The target genes express by T7 promoter and transcriptions are induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG). This pET28a plasmid carries kanamycin resistance.

Protein Expression

To have enough cell pallets of target protein productions for protein extraction, the cultures were inoculated in 15ml LB Broth-Miller medium containing 30μg/ml kanamycin and grown the cultures overnight at 37°C with shaking at 200 rpm. An aliquot 10ml of overnight cultures was used to inoculate in one liter of LB supplemented 30μg/ml kanamycin and incubated at 37°C with shaking at 200 rpm. After eight hours of growth, cells were induced expression by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1mM. The cultures were grown overnight. The next following day, cell pellets were harvested by centrifugation at 5000rpm for 15 minutes, at 4°C, and were stored at -20°C for protein purification.

Protein Purification Using Cobalt Column

Cell pellets were re-suspended in 10ml of lysis buffer (100mM Tris-HCl pH~8, 150mM sodium chloride, 5mM imidazole) and sonicated using Branson Digital Sonifier (Version Number Rev.0612) for six minutes on ice. After sonication, cell lysates were centrifuged at 10,000 xg, at 4°C for 30 minutes to remove the cellular debris. The supernatant (soluble protein) was collected and saved on ice. Proteins were purified by cobalt affinity chromatography. 1 ml of HisPur Cobalt Resin (Thermo Fisher, HisPurTM Cobalt Resin, catalog number: 89864) was added to the column and allowed storage buffer

to drain from the resin by gravity flow at room temperature. The column was equilibrated by washing with 10ml of washing buffer (50mM Sodium phosphate, 300mM sodium chloride, 10mM imidazole; pH 7.4). The supernatant (cleared lysate protein) was run onto the column (re-applied the flow-through three times). The flow-through was collected in a tube. The cobalt resin was washed with wash Buffer in the volume of 50ml and collected the flow-through. Cobalt affinity purified proteins were eluted His-tagged proteins from the resin with Elution Buffer (50mM Sodium phosphate, 300mM sodium chloride, 300mM imidazole; pH 7.4). Ten fractions of purified proteins were collected in separated tubes (2ml for each fraction). The protein fractions were stored at -20°C for running coomassie gels to observe the purity of target proteins.

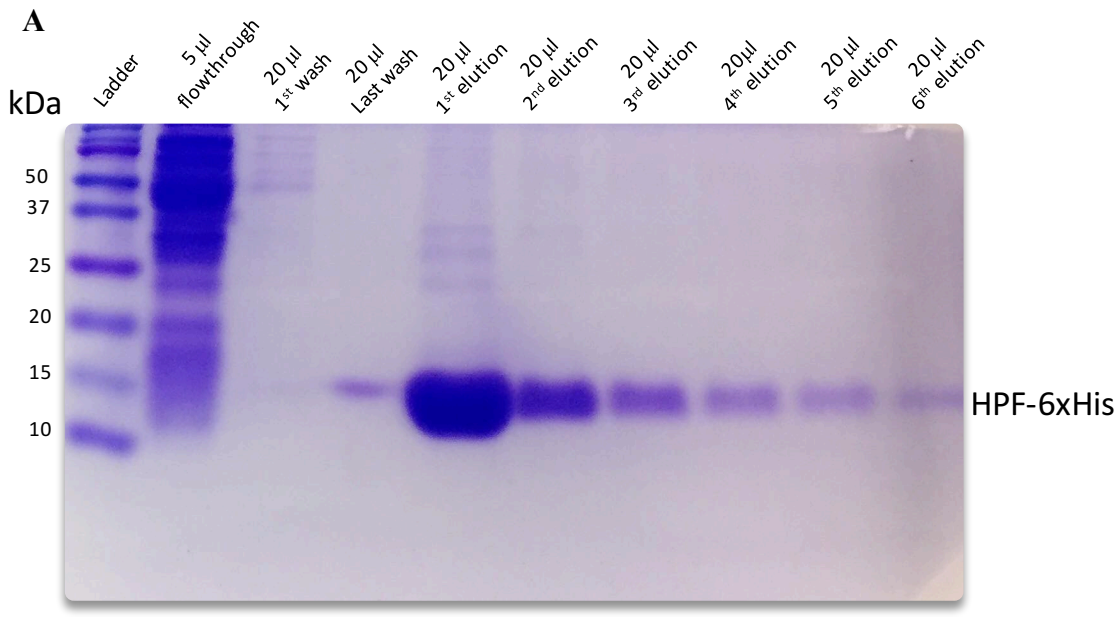
Coomassie Staining SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis)

Reagents	Separating/running gel 12% acrylamide concentration (bottom layer~60%)	Stacking gel 4% acrylamide concentration (top layer~40%)
DI water	5.3ml	6.3ml
Running buffer	4ml	-
Stacking buffer	-	2.5
40% acrylamide	6.4ml	1ml
10% SDS	160µl	100µl
10% APS	160µl	100µl
TEMED	16µl	10µl

Table 1: Reagents for preparing SDS-PAGE gel for two 1.5-mm gels.

The reagents in the given table 1 were mixed and firstly pour 7ml into the two glass plates for separating gel at the bottom, and then 3ml for stacking gel pour were poured on top of the separating gel. The gels were pre-run in 1X Protein Tank Buffer to warm up the

gels for three hours at constant current I=30mA, and electrophoresed the samples at the constant current I= 30mA for two hours. Lastly, the gels were removed off from the glasses for staining with coomassie blue on the rocker for one hour and destained with destain buffer (5% Methanol and 7% Acetic Acid) overnight at room temperature on the rocker. After destaining, the second elution of HPF and S13 (Fig. 3A & 3C) shown to be cleaned enough for sending off to inject into the rabbit in order to generate antibodies. Figure 3B shows that L5 was not perfectly cleaned. There was another band on top of L5 band for all elution.



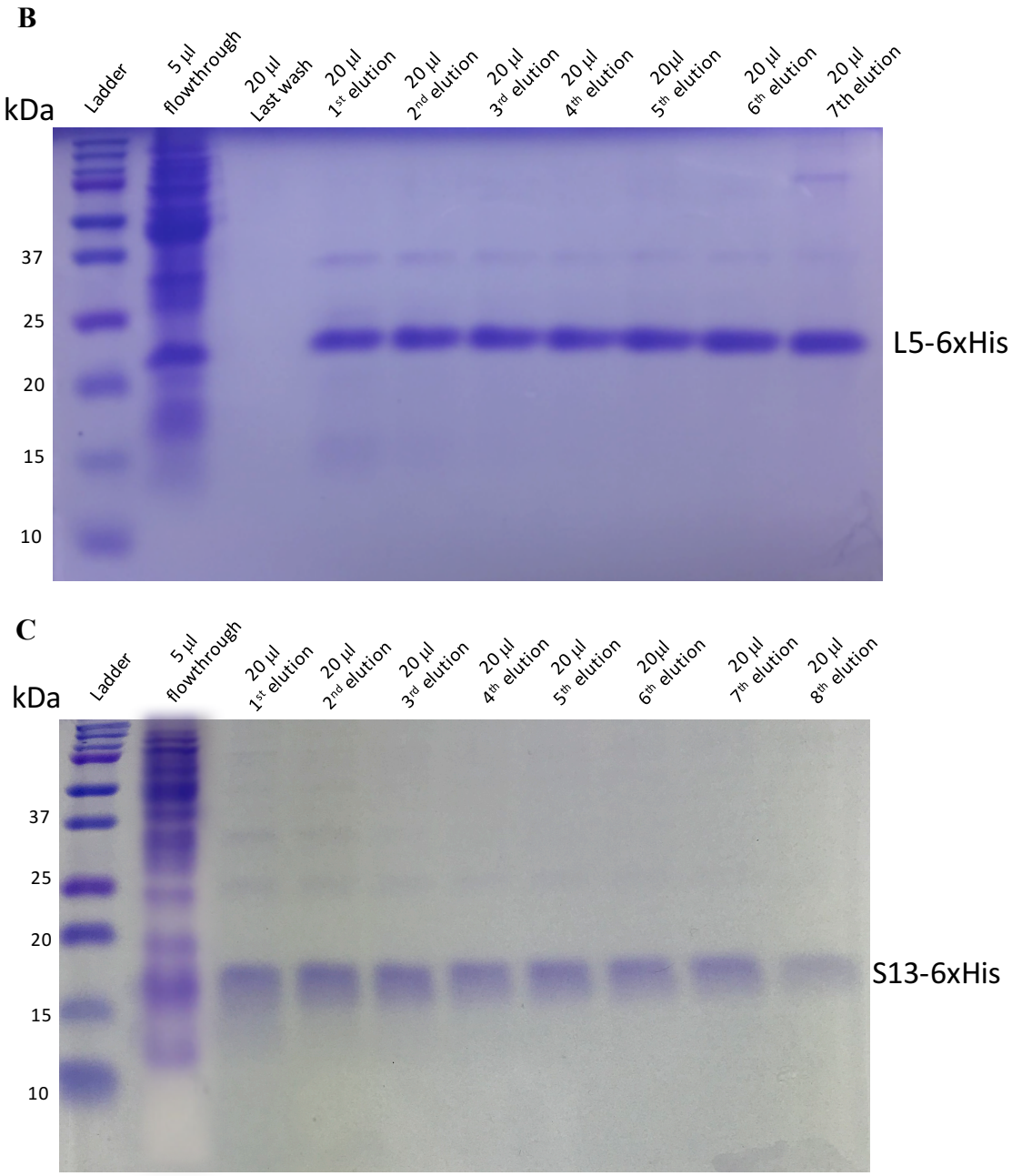


Figure 6: Purified (A) HPF, (B) L5, and (C) S13 proteins on coomassie-blue SDS-PAGE.

BCA (Bicinchoninic Acid) Assay

BCA assay was performed to quantify the protein in each elution sample. Pierce™

BCA Protein Assay Kit (Cat#23225) and bovine serum albumin (BSA) as a standard were used for assaying the protein concentration. 200µl of working reagents from the kit was added to 25µl of each elution samples from protein purification, in the 96well-plates, and incubated the plates at 37°C for 30 minutes. After incubation, the color turning in a purple color formation by bicinchoninic acid. The plates were read the absorbent at OD_{562} by the plate reader.

Elutions	HPF (µg/ml)	L5 (µg/ml)	S13 (µg/ml)
1	593.75	469.57	198.44
2	113.75	339.45	149.44
3	87.92	330.23	144.58
4	43.75	339.45	147.59
5	47.92	344.57	162.52
6	29.58	330.23	162.53
7	29.5	395.8	145.7
8	28.69	350.56	165.02
9	25.85	378.9	140.76
10	20.7	340.81	141.9

Table 2: The proteins concentrations of each elution of HPF, L5, and S13 from protein purification.

Immunoblotting

The SDS-PAGE for two 1.5-mm gels was prepared as described in Table 1. After two hours of electrophorese, proteins were separated and transferred from the gels to Nitrocellulose membrane at 350mA for an hour per two gels. The membrane was gently washed with Tris buffered saline (TBS) on the rocking platform for 30 minutes and blocked with 3% skim in TBS for an hour. After membrane blocking, membrane was incubated in polyclonal

primary antibody raised from rabbit (anti-HPF and anti-L5 1:2000; anti-S13 1:5000 dilution) in 3% skim milk in TBST (Tris buffered saline 0.1% Tween) for overnight. Then, the membrane was washed with TBST four times (rocking for 15 minutes for each time). The membrane was incubated in polyclonal rabbit- conjugated Horseradish Peroxidase secondary antibody (1:10000 dilution) in 3% Bovine Serum Albumin in TBST for three hours. Finally, the membrane was washed with TBST four times (rocking for 15mn for each times). Last step, the membrane was developed with caumaric acid and luminol and hydrogen peroxide for chemiluminescent in the dark room.

Antibody Generation and Purification

To generate anti-rabbit polyclonal HPF, L5, and S13 antibodies, purified HPF, L5, and S13 were dialyzed into PBS buffer (pH~7) and concentrated to 1mg/ml for sending to Lampire Bilological Laboratories. After antibodies were raised in rabbits for five weeks by Lampire lab, HPF, L5, and S13 termination serums were sent back to the lab and purified using Nab^{TM} Protein A Plus Spin Columns, 0.2 mL from Thermo Fisher Scientific (Cat# 89952). The column contains 0.2ml of immobilized protein A resin, which purifies IgG from the serum. Purification followed the protocol provided by the manufacturer in the kid. After purification, antibodies were tested on the western blot analysis as shown in Figure 7. HPF antibody were tested on three strains (PAO1 wild-type, *Δhpf* with copy of plasmid of *hpf*, and *Δhpf*). The molecular weight of HPF protein is ~11.6 kDa. The blot using an anti-HPF antibogy (Fig. 7A) displayed that PAO1 showed a band ~11.6 kDa between 10 and 15 kDa of the ladder, and the positive control that encoded *hpf* on the

plasmid showed a band ~11.6 kDa with higher density compared to PAO1. The negative control is *Δhpf*, which showed no band between 10 to 25 kDa. These results indicate that HPF antibody worked. However, there was a cross-reactive band, run above 25 kDa that appeared to all three strains. PAO1, PAO1:L5-His, and PAO1:L5-YFP were used to test L5 (~20.1 kDa) antibody (Fig. 7B). L5 proteins of these three strains run in a little bit lower than ~20 kDa. But, the blot showed two more positive bands of L5-His ~21.1 kDa and L5-YFP ~48.2 kDa. Thus, L5 antibody worked. Anti-S13 antibody were tested with PAO1, *Δhpf*, and PAO1:S13-YFP. All strains expressed S13 which is ~13kDa (Fig. 7C). PAO1:S13-YFP showed another band of S13-YFP around ~41.1KDa. S13 antibody worked perfectly. In conclusion, anti-HPF, anti-L5, and anti-S13 antibodies worked.

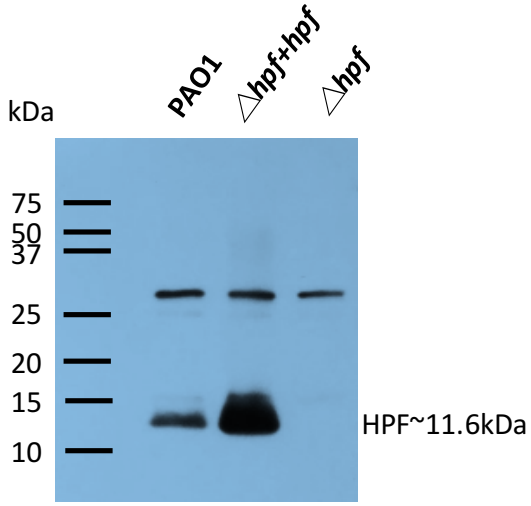
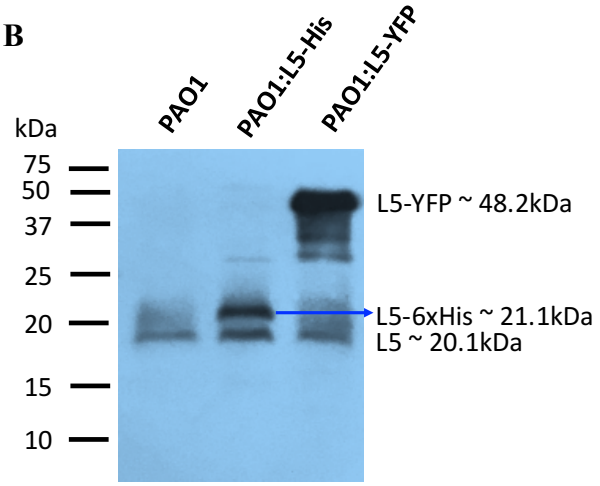
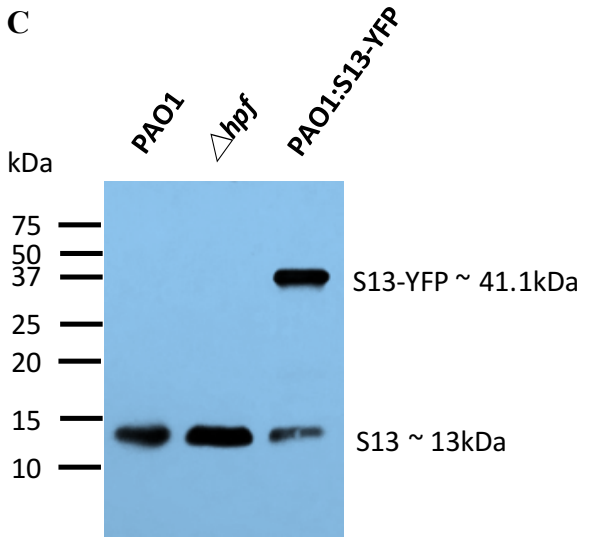


Figure 7: SDS-PAGE analysis of purified HPF, L5, and S13 antibodies.

(A) Purified HPF antibody. The blotting shows HPF antibody worked. HPF protein known to expressed in wild-type P. aeruginosa PAO1, runs in ~11.6kDa; and positive control (*Δhpf+hpf*) complemented strain expressed HPF more than PAO1. The last lane negative control (*Δhpf*), HPF antibody did not react with HPF deletion. The above 25kDa bands are the cross-reactive bands which also happened to PAO1 and complement strains.



(B) Purified L5 antibody. 3 positive control PAO1, PAO1:L5-His, and PAO1:L5-YFP are all expressed L5 ~20kDa; but the band might run a little bit faster than ladder due to the positive a lot of charge of this protein. PAO1: L5-his expressed one band L5-Hid above L5. PAO1:L5-YFP shows a bright band of L5-YFP ~48kDa. In conclusion, L5 antibody worked.



(C) Purified S13 antibody. PAO1 and *Δhpf* both express expressed S13 which is ~13kDa. PAO1:S13-YFP also expressed S13 which is ~13KDa and S13-YFP band around ~41.1KDa. Hence, S13 antibody worked perfectly.

TSB Planktonic Growth Curve Experiment

There were four strains for TSB growth experiment: *P. aeruginosa* PAO1 wild type (WT), complement (*Δhpf*+*hpf*), deletion of the *hpf* gene (*Δhpf*), and deletion of relA/spoT gene (*ΔrelA/ΔspoT*). Frozen stock of the four strains were inoculated for overnight cultures in 3ml Tryptic Soy Broth (TSB) on the rolling incubator 37^{o}C. If plasmid was present, culture was added 3µl of 150mg/mL carbenicillin into TSB overnight to keep the plasmid.

The overnight culture was transferred to 25ml TSB to an OD of ~0.01 at the total volume 26ml. The bacteria grew at 37^{o}C with shacking at 200 rpm. Samples were collected every two hours which started from 0h, 2h, 4h, 6h, 8h, 10h, 12h, 24h, 48h for cell viability drop plate and protein assay.

TSB Planktonic Starvation Experiment

There were four strains for TSB starvation experiment: *P. aeruginosa* PAO1 wild-type (WT), complement (*Δhpf*+*hpf*), deletion of the *hpf* gene (*Δhpf*), and deletion of relA/spoT gene (*ΔrelA/ΔspoT*). 3ml Tryptic Soy Broth (TSB) was used to inoculate frozen stocks and grown overnight on the rolling incubator 37^{o}C. The presence of plasmid in *Δhpf*+*hpf* strain was required adding 150μg/mL carbenicillin into TSB overnight to keep the plasmid. 120μl of overnight culture was added to 4ml TSB with 1mM IPTG. The cultures were incubated on the rolling incubator 37^{o}C until OD_{600} ~ 7. The cells were pelleted and washed with PBS at 10 krpm for three minutes twice, then transferred to fresh 60ml warm Phosphate Buffered Saline (PBS) of OD_{600} 6.6, and incubated at 37^{o}C with shacking at 200 rpm. Samples were collected every day which starts from day zero to day eight for cell viability drop plate and day 0, 2, 4, 6, 8 for protein sampling.

MOPS (Fructose-Glucose) Planktonic Starvation Experiment

There were there strains for MOPS starvation experiment: *P. aeruginosa* PAO1 wild type (WT), complement (*Δhpf*+*hpf*), and deletion of the *hpf* gene (*Δhpf*). MOPS is the abbreviation of 3-Morpholinopropane-1-sulfonic acid, 3-(N-Morpholino) propanesulfonic

acid, and 3-Morpholinopropanesulfonic acid. MOPS media contained 80 mM fructose/glucose and 20 mM nitrogen (pH~7.2). Form the frozen stock of all three strains, the overnight cultures were inoculated in 3ml Lennox Broth (LB) on the rolling incubator at 37^{o}C. For *Δhpf*+*hpf* strain, 150μg/mL carbenicillin was supplemented into LB overnight to keep the plasmid. 1 ml of cells was spun at 10k rpm for one minute at room temperature. Cells pellets were washed two times with 1 ml MOPS-H_2O- K_2HPO_4 pH 7.2. Cells pellets were re-suspended in 1 ml MOPS-H_2O-K_2HPO_4 pH 7.2 and transferred to fresh 100ml MOPS-fructose or MOPS-glucose of OD_{600}~0.05, grew at 37^{o}C with 200 rpm shaking until cutlture reached to OD_{600}~0.3 and OD_{600}~1.0. Cultures were spun at 8xg for five minutes at room temperature, and washed with PBS twice at 10krpm for three minutes. Washed cells were re-suspended in PBS and transferred to fresh 60ml warm Phosphate Buffered Saline (PBS) of OD 6.6, and incubated at 37^{o}C with shaking at 200 rpm. Samples were collected every day which starts from day zero to day eight for cell viability drop plate and day 0, 2, 4, 6, 8 for protein sampling.

Determination of Cell Viability

Using nonsterile U-bottom 96 well-plate, 180μl of 0.85% NaCl was pipetted to each well and added 20μl of samples to the top wells of the dilution series. 10-fold serial dilutions were performed (10^{-1} – 10^{-8} for TSB growth curve and 10^{-1} – 10^{-6} for the starvation). TSA was used for drop-plate. TSA plates were incubated at 37^{o}C overnight for enumeration.

Sampling for Protein Assay

The samples were harvested by pipette 6ml of culture in micro-centrifuge tubes, spun at 9k rpm for three minutes at 4^{o}C, and removed the supernatant avoiding disturbing the pellet and freezed immediately at -80^{o}C for protein assay. Once all the sampling was finished, sets of cell pellet were in lyse 100µl RIPA lysis buffer (pH 7.6) and boiled at 85^{o}C for 5 minutes. After letting the lysate cooled down, 1µl of Benzonase Nuclease enzyme was added and incubated at room temperature for 5 minutes.

CHAPTER THREE

RESULTS

HPF and RelA/SpoT Are Essential for *P. aeruginosa* Viability During Nutrient Limitation

To test the hypothesis that HPF and RelA/SpoT are important for *P. aeruginosa* viability during nutrient starvation, and to confirm previous results of Akiyama et al. (2017), I first test the viability of four strains of *P. aeruginosa* during starvation: PAO1 wild-type (WT), PAO with *hpf* deletion (*Δhpf*), the deletion strain with a plasmid copy of *hpf* (*Δhpf*+*hpf*), and PAO with deletions of *relA* and *spoT* (*ΔrelA/ΔspoT*). I tested viability when the bacteria were cultured under two conditions: rich nutrient tryptic soy broth medium (TSB) and phosphate buffered saline (PBS) starvation medium. Using TSB, all four strains grew similarly and gave growth curves that represented the number of viable cells in a population over 48 hours (Fig. 8A). All strains had similar growth with four phases including lag, exponential (log), stationary, and death. At the first hour, cells were in lag phase, which is the initiation of cells in nutrient rich medium to start synthesizing proteins necessary for replication. No division occurs at this stage, but the cell size might increase (Rolfe et al., 2012). After two hours of lag phase, I observed that bacterial cells entered the exponential or log phase, in which cells were dividing by binary fission and doubling in numbers of cells after each generation time. Once cell growth reached around twelve hours and the environment was less favorable, the competition for nutrient increased. At this stationary phase, bacteria became less metabolically active (Kolter,

Siegele, & Tormo, 1993). After 48 hours, cell viability declined due to less availability of the nutrients, and the build-up of toxic waste products. Some cells died and some entered a dormant stage (Kolter, Siegele, & Tormo, 1993). As a number of cells died and lysed, the nutrient components inside the cells became substrates for the surviving bacteria.

The results indicated that neither HPF nor RelA/SpoT are not necessary for *P. aeruginosa* to grow under rich nutrient conditions. However, under nutrient starvation, *Δhpf* and *ΔrelA/ΔspoT* mutants started losing viability, as indicated by the number of colony forming unit (CFU) after two days of starvation. PAO1 wild-type and the *Δhpf+hpf* complemented strain maintained viability pretty well over eight days of starvation (Fig. 8B). This gave us the conclusion that *P. aeruginosa* needs HPF or RelA/SpoT to maintain its cell viability when cells are being starved. This result also supports results of Akiyama et al. (Akiyama et al, 2017), which showed that HPF is required for PAO1 to maintain its viability over the starvation.

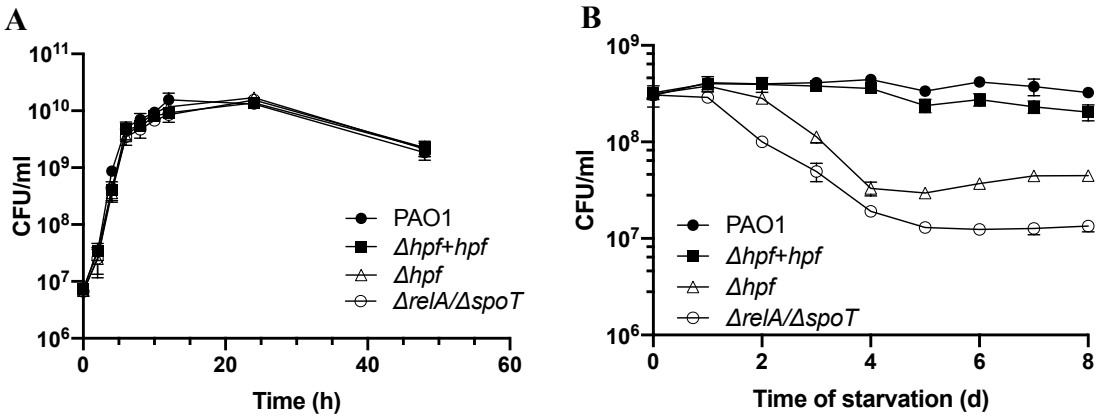


Figure 8: (A) Colony forming unit (CFU) of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* over 48h of growing in TSB. Overnight cultures of PAO1 and mutants were inoculated at OD_{600}~0.01 and incubated at 37^{o}C with 200 rpm shaking. At each time point, 20µl was removed and plated on TSA for cell viability count. (B): Colony forming unit (CFU) of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over eight days of starvation in PBS. Overnight of all four strains were washed two times with PBS and inoculated into PBS at

OD_{600}~6.6 and incubated at 37°C with 200 rpm shaking. Each day, 20µl was removed for cell viability count on TSA plates. Values shown in the graphs are the mean of three biological replicates. Error bars represent the standard deviation of the three biological replicates.

Determination of Total Protein Amount During Nutrient-Rich Condition

In addition to viable counts, I quantified the total cellular protein amount over the course of cell growth of the four strains. For protein analysis, the samples from every time point were stored at -80°C for protein quantification and western blot analysis. The cell pellets were lysed in RIPA buffer and boiled at 85°C for five minutes. The BCA (Bicinchoninic Acid) assay was used to quantify protein amounts and to normalize protein concentrations. During growth in TSB, the total protein of all four strains started from ~30µg/ml and increased over the log phase for 12 hours (Fig. 9). Many studies reported that during log phase, metabolic activity is high in DNA, RNA, proteins, and other substances that are necessary for growth and division (Wang & Levin, 2009). During stationary phase, 12h to 24h, the total protein amounts were stable at ~1.5mg/ml protein concentration. The total protein amount maintained at the same level, as cells did not divide and translation less active (Wada, Yamazaki, Fujita, & Ishihama, 1990; Polikanov, Blaha, & Steitz, 2012). In the next 48 hours, the cells lost total proteins, similar to the CFU growth curve. At 48h, some cells were dead and lysed, so it is possible that total proteins were lost in the supernatant. Moreover, some cells that were able to sense the signal of nutrient limitation, would degrade unnecessary proteins by proteases (Bergkessel & Newman, 2016) and keep energy from synthesis of proteins to maintain storage compounds for

surviving. These arguments supported the results that the total proteins at late stationary phase are lower than early stationary phase.

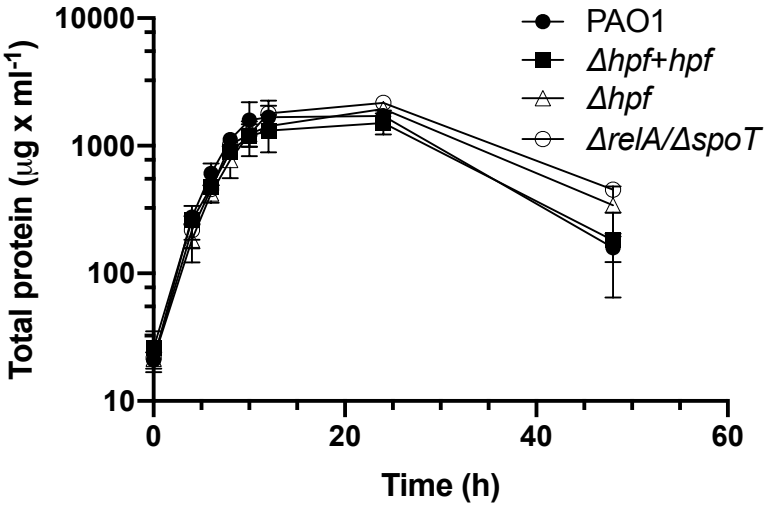


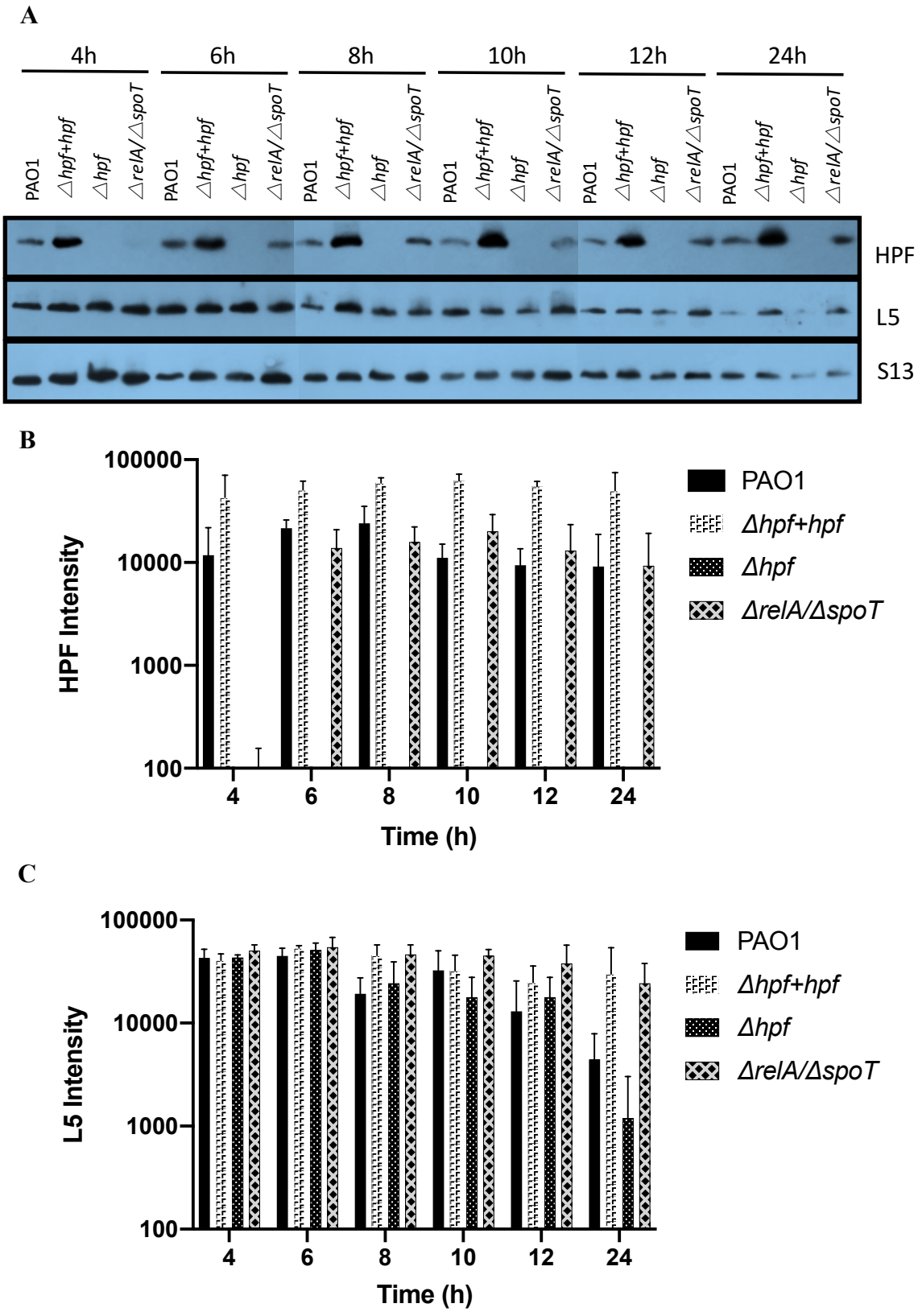
Figure 9: Total protein content of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over 48h of growing in TSB. The cell pellets were lysed in RIPA buffer for performing BCA assay to quantify protein concentrations at every time point. At time 0h, the initial total proteins of all strains are ~30μg/ml of OD_{600}~0.01 which correspond to ~10^7CFU/ml. The total proteins are increasing until ~1.5mg/ml over 12 hours and stable over stationary phase during nutrient-rich conditions. At 48 hours, the total protein amount declined to <0.5mg/ml. Values shown in the graphs are the mean of three biological replicates with standard deviation (SD, n=3).

Determination of HPF, L5, and S13 Abundances During Nutrient-Rich Condition of Wild-type, *Δhpf,* and *ΔrelA/ΔspoT* Mutant Cells at the Population Level

To assay the abundances of HPF, L5 and S13 proteins over the course of growth in the four strains, immunoblots using polyclonal antibodies against HPF, L5, and S13 were performed. All the samples were normalized so that 15μg of total protein was loaded onto each land of the SDS-PAGE gels. Since the total protein of the early time points (0h and 2h) was not enough for loading, I did not perform immunoblotting at the early time points. Figure 10A shows the representative images of immunoblots using the anti-HPF, anti-L5,

and anti-S13 antibodies. Three biological replicates were performed and each immunoblot is presented in appendix A. The first row showed the effect of growth in rich medium on HPF abundance. For wild-type PAO1, HPF is produced from log phase at 4h to stationary phase at 24h (Fig. 10A). The *ΔrelA/ΔspoT* mutant expresses HPF, but the initial amount of HPF was formed two hours later than wild-type. The negative control *Δhpf* strain did not show any HPF production (Fig. 10A), while the *Δhpf*+*hpf* complemented strain had higher HPF production than the wild-type strain. The amount of HPF was quantified by densitometry using the Image Studio program. Figure 10B shows the mean value of protein intensity for the three biological replicates. The intensity of each band was measured using Image Studio program. The results illustrate that the *Δhpf*+*hpf* strain had the highest HPF production among all strains over 24h of growth in TSB. The HPF abundance in the wild-type strain PAO1 was greatest at eight hours, then stable over the stationary stage until 24 hours. The negative control strain *Δhpf* showed <100 units of intensity and was assumed the number of background signal.

Figure 10A (row L5 and S13) shows the abundance of the L5 and S13 proteins over the course of growth in TSB for the four strains. Since TSB is the rich medium, during the log phase from 4h to 12h all four strains produced L5 and S13 as the ribosomal proteins for cells to build ribosomes for active translation, in the nutrient-rich environment. After one day of growing on TSB, all four strains still maintained L5 and S13, except for the *Δhpf* strain, which seemed to loss L5. The image analysis of protein intensity for the three biological replicates show the abundance of L5 and S13 of over 10000 intensity units in all strains from four to twelve hours (Fig. 10C&D).


A
4h
6h
8h
10h
12h
24h
PAO1
△hpf+hpf
△hpf
△relA/△spoT
HPF
L5
S13
B
HPF Intensity
100000
10000
1000
100
4
6
8
10
12
24
Time (h)
PAO1
Δhpf+hpf
Δhpf
ΔrelA/ΔspoT
C
L5 Intensity
100000
10000
1000
100
4
6
8
10
12
24
Time (h)
PAO1
Δhpf+hpf
Δhpf
ΔrelA/ΔspoT

D

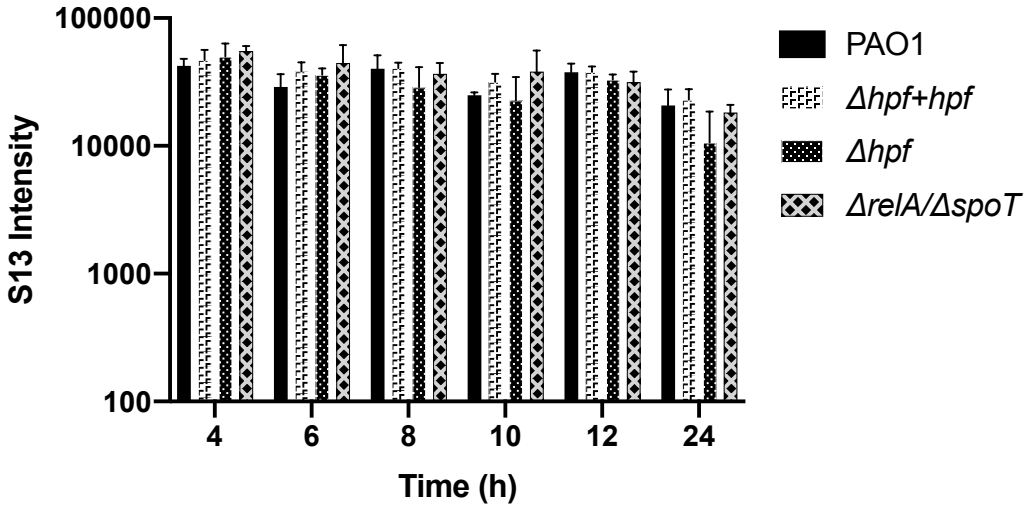


Figure 10: HPF, L5, and S13 immunoblotting of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strain over 24 hours of growth in TSB. (A) Immunoblot analysis: first to third row represent anti-HPF, anti-L5, and anti-S13 blots of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strain from 4 to 24 hours. 15µg proteins were loaded on SDS-PAGE. (B, C, D) represent the relative abundance of HPF, L5, and S13. Relative abundance or intensity levels of each bands are measured using the Image Studio program. The mean intensity for the three replicates are plotted as bar graphs with standard deviation (SD, n=3). *Δhpf* had no detectable blots, so the relative expression value represents less than 100 intensity units (<100 units of intensity properly the background signal number).

Determination of Total Protein Amount During Nutrient-Limitation

I next determined the total protein concentration of the four strains during starvation. The BCA was used to determine total cellular protein amounts during starvation in PBS. Total protein of all four strains started from ~30µg/ml at day zero (Fig. 11). Over eight days of starvation, PAO1, *Δhpf+hpf*, and *Δhpf* strains maintained total protein concentrations of approximately 30µg/ml. Although PAO1 and the complemented strains have approximately eightfold greater CFUs during starvation than the Δ*hpf* mutant, the amount of total protein remained fairly constant. A possible reason why these three strains have comparable total protein content might be due to viable but non-culturable Δ*hpf*

mutant cells. Interestingly, only the *ΔrelA/ΔspoT* decreased total protein from day 2 (Fig. 11). Figure 8B shows that the *ΔrelA/ΔspoT* strain lose cell viability of tenfold comparing to PAO1 and the complemented strain after four days of starvation. This may have been due to cell lyses of the *ΔrelA/ΔspoT* cells, but not the *Δhpf* cells.

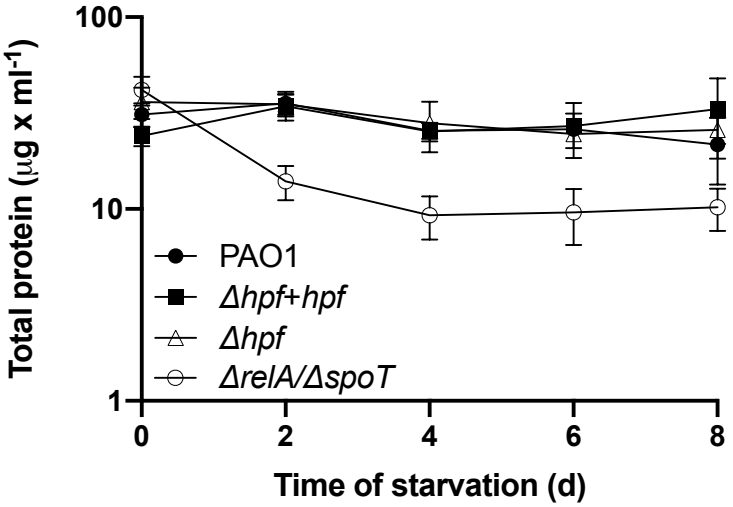


Figure 11: Total protein content of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains during starvation in PBS. All strains were grown in TSB medium until stationary phase and then transferred to nutrient-limited PBS for starvation. Over the course of eight days, wild type PAO1, *Δhpf+hpf*, and *Δhpf* strains maintained ~30μg/ml concentration of cellular proteins, while *ΔrelA/ΔspoT* strain had reduced total proteins content after 2 days of starving in PBS. Each strain represents the mean value with standard deviation of the three biological replicates.

P. aeruginosa Loses the Ability to Maintain HPF Stability in the Absence of RelA/SpoT During Starvation

The cell viability of *Δhpf* and *ΔrelA/ΔspoT* strains are approximately eightfold to tenfold lower than wild-type and than the complemented strain after starving in PBS for four days. To make sure that *Δhpf* and *ΔrelA/ΔspoT* strains have the same protein abundance loaded for immunoblot analyses, an SDS-PAGE coomassie staining gel was

performed to compare total protein intensity at day four and six of starvation. The coomassie stain gel indicated that all four strains appeared to have the same amount of protein, which was normalized to 15 μg proteins total protein per lane (Appendix C). Hence, there was no issue of not having enough total proteins from *Δhpf* and *ΔrelA/ΔspoT* strains for immunoblot analysis.

The abundances of HPF during starvation in the wild-type and *ΔrelA/ΔspoT* mutant at the population level was revealed by immunoblots. Figure 12A shows the HPF protein abundance for PAO1, *Δhpf*+*hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over eight days of PBS starvation. The result indicated that wild-type and the complemented mutant strains accumulated HPF. At the initiation of starvation day zero, I observed that the HPF complemented strain had HPF production approximately threefold higher than wild-type (Fig. 12B). From day two to six of starvation, HPF content in *Δhpf*+*hpf* strain declined to a similar level as wild-type (WT). Interestingly, *ΔrelA/ΔspoT* knockout seemed to lose HPF proteins from day two of starvation and completely disappeared by day six after starving in PBS (Fig. 12B). To verify *ΔrelA/ΔspoT* strain lost the HPF protein, I did the longer exposure time on western blots. The longer exposure time still showed there was no detection of HPF on *ΔrelA/ΔspoT* strain after two days of starvation (Fig. 12A).

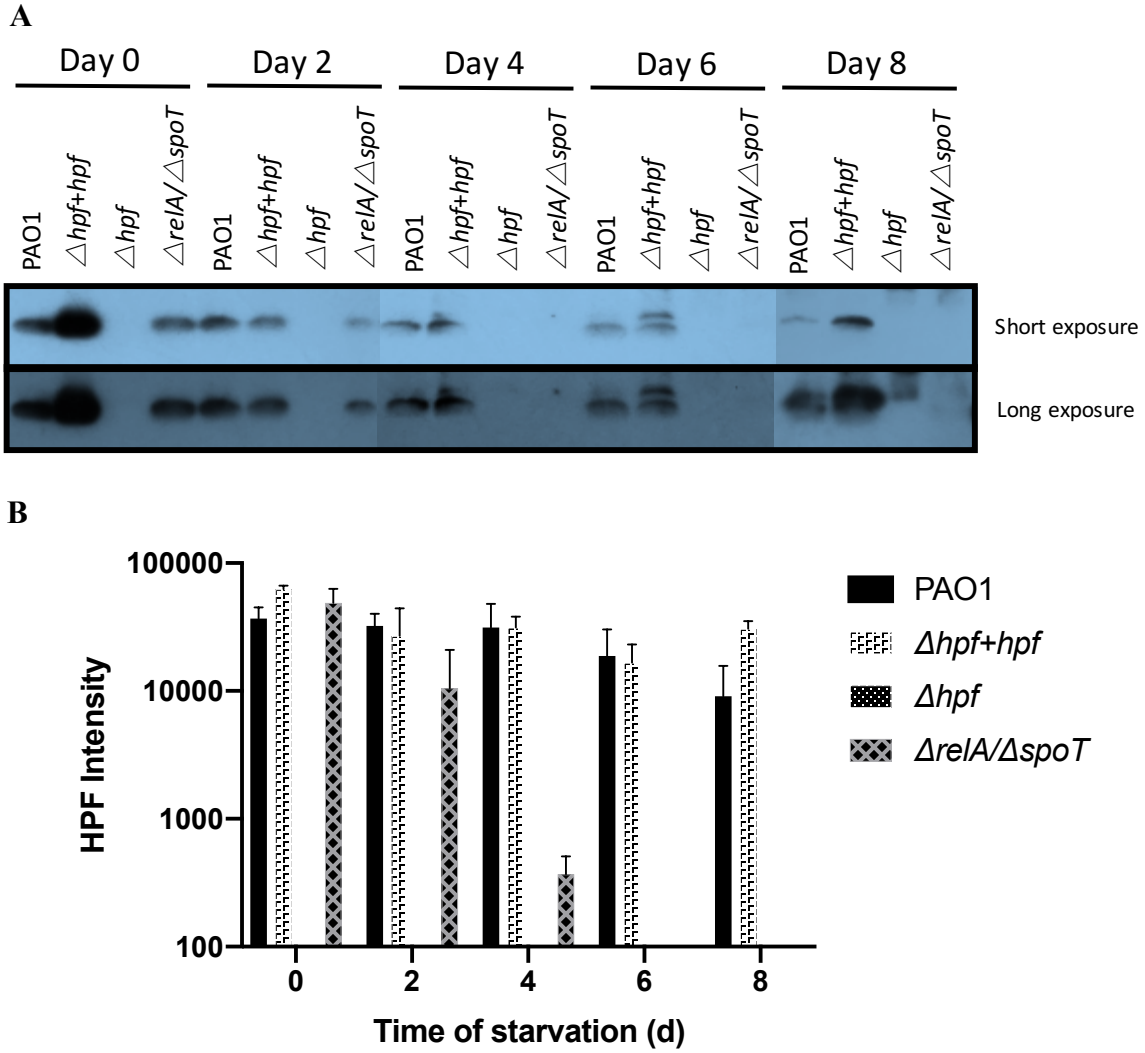


Figure 12: Immunoblot analysis of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over eight days of PBS starvation using anti-HPF antibodies. All strains were grown in TSB medium until stationary phase and then transferred to nutrient-limited PBS for starvation. 15µg proteins were loaded on SDS-PAGE. (A) An image of anti-HPF western blot analysis of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over eight days of PBS starvation. The top row is short exposure time and bottom is long exposure time. (B) represents the mean value with standard deviation of relative expression of short exposure HPF bands from three replicates (SD, n=3). Over eight days, PAO1 and complemented strains maintain HPF intensity over 10000 units. *Δhpf* strains is used as the negative control for HPF antibody, hence there is no detection of HPF on this graph. At the initiation of starvation (day zero), *ΔrelA/ΔspoT* strain produces HPF at a similar level as wild-type PAO1, and depleting after two days of starving. By day six of starvation, HPF in *ΔrelA/ΔspoT* strain is completely disappeared.

HPF Deletion Mutant, But Not RelA/SpoT Deletion Mutant, Loses the Ability to Maintain Ribosomal Proteins (L5 and S13) During nutrient stress

To test this hypothesis, I determined the stability of ribosomal proteins (L5 and S13) during starvation in the of *Δhpf* and *ΔrelA/ΔspoT* strains. All strains were grown in nutrient-rich media until stationary phase and then transferred to nutrient-limited PBS for starvation. 15µg cellular proteins were loaded on SDS-PAGE and immunoblots were performed using anti-L5 and anti-S13 polyclonal antibodies. At the initiation of starvation, PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains contained the similar amount of L5 and S13 proteins as shown by the immunoblotting image (Fig. 13A). The mean value of relative signal intensity was determined for three biological replicates (Fig. 13B & C). Figure 13 indicates that *Δhpf* strain rapidly decreased both L5 and S13 from day two until day eight, while the other three strains maintained both L5 and S13 over eight days of starvation. The presence of HPF in the wild-type strain functions to form 100S inactive ribosomes. Thus, the starved cells do not use energy for translation machinery during PBS incubation. Instead, starved cells save energy to maintain cell viability under stress condition. PAO1 wild-type and complemented strains have functional L5 and S13 on 100S ribosome would not get degraded.

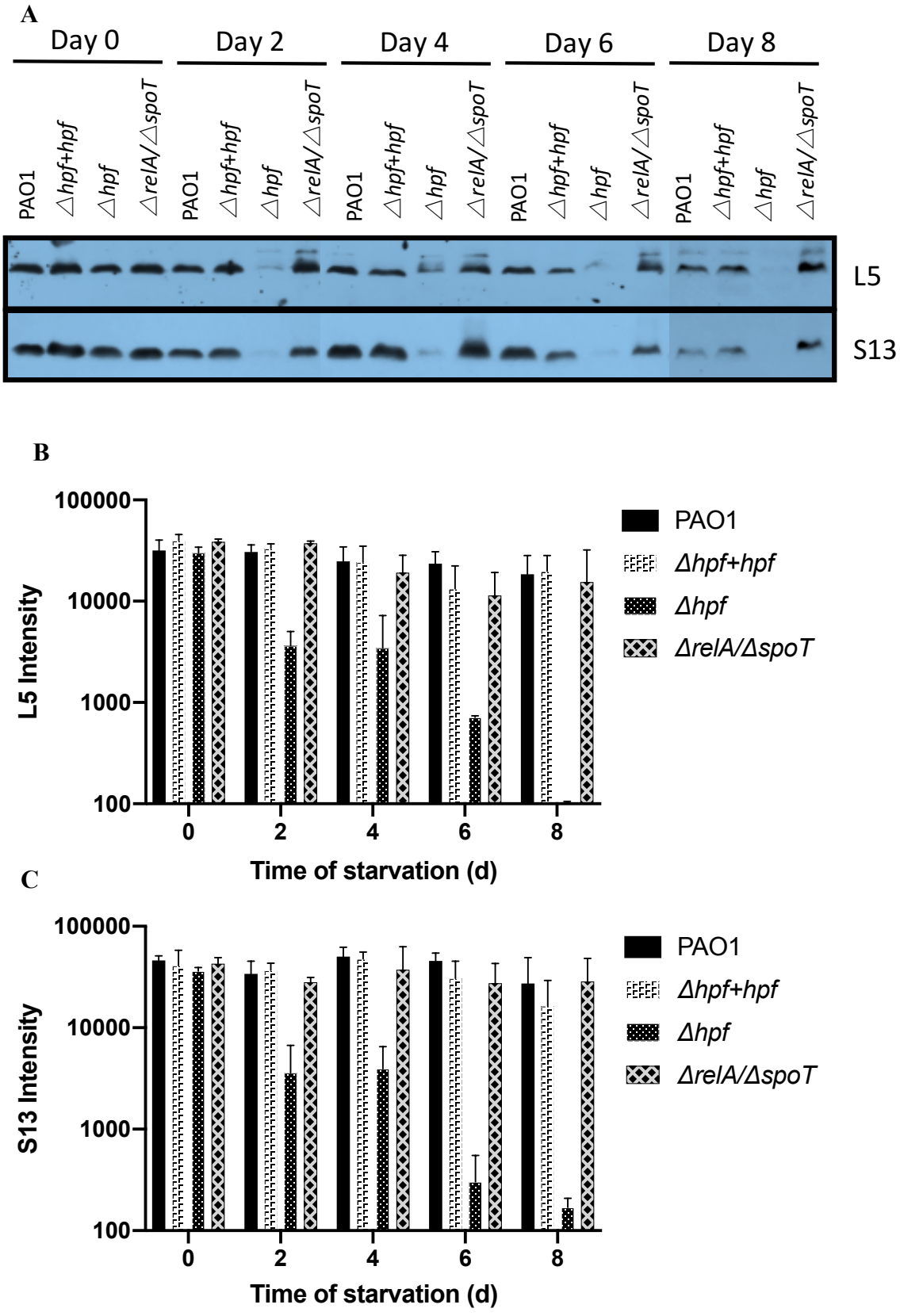

A
Day 0
Day 2
Day 4
Day 6
Day 8
PAO1
△hpf+hpf
△hpf
△relA/△spoT
L5
S13
B
L5 Intensity
100000
10000
1000
100
0
2
4
6
8
Time of starvation (d)
PAO1
Δhpf+hpf
Δhpf
ΔrelA/ΔspoT
C
S13 Intensity
100000
10000
1000
100
0
2
4
6
8
Time of starvation (d)
PAO1
Δhpf+hpf
Δhpf
ΔrelA/ΔspoT

Figure 13: Immunoblot analysis of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over eight days of PBS starvation using anti-L5 and S13 antibodies. All strains were grown in nutrient-rich media until stationary phase and then transferred to nutrient-limited PBS for starvation. Protein samples were normalized and 15µg proteins were loaded onto SDS-PAGE. (A) The represented image from three replicates of anti-L5 and S13 western blot analysis of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* over eight days of PBS starvation [first row is anti-L5 and second row is anti-S13] (each replicate is attached in Appendix B). (B) and (C) represent the mean value of relative expression of L5 and S13 bands from three replicates with standard deviation (SD, n=3). All strains initially contain the similar amount of L5 and S13 on day zero. After two days of starving in PBS, *Δhpf* rapidly decreasing both L5 and S13 until day eight, while the other three strains maintain both L5 and S13 over eight days of starvation.

Carbon Source Effects on *hpf* Expression in Minimal Medium

Growth and *hpf* Expression in Minimal MOPS Medium

Akiyama et al (Akiyama et al., 2018) characterized the regulation of *hpf* expression using hpf-yellow fluorescent protein reporter constructs, to test expression of *hpf* from the native *hpf* promoter P_{hpf}. Using the reporter strain, Akiyama and his group noticed differential expression of hpf if the cells are cultured in minimal medium with glucose or fructose as sole carbon sources. Therefore, I performed a preliminary expression experiment using PAO containing the P_{hpf}-*hpf-yfp* reporter construct on two different conditions to identify conditions where hpf would be expressed at low levels and where hpf would be expressed at high levels. An overnight culture of PAO1 P_{hpf}-*hpf-yfp* was washed and transferred to MOPS medium with different carbon sources (fructose and glucose). Both sugars are monosaccharides and have the same molecular formula $C_6H_{12}O_6$, but glucose makes a six-membered ring structure, and fructose makes a five-membered ring. PAO1 P_{hpf}-*hpf-yfp* grew differently in MOPS-fructose and MOPS-glucose, with the MOPS-fructose having a 12 hours increased lag time (Appendix D). For glucose, this sugar

can be brought directly into the cell by active transport system (Durham & Phibbs, 1982). *P. aeruginosa* converts glucose to glucose-6-phosphate by glucokinase and directly enters the Entner-Doudoroff (ED) Pathway to product pyruvate. During PAO1 growth on fructose, fructose is brought into the cell in the form of fructose 1-phosphate by PEP-dependent phosphotransferase system. The intracellular fructose 1-phosphate is converted to fructose-1,6-bisphophate, then fructose-6-phosphate; and then glucose-6-phosphate, which is ready for the ED pathway. Based on the fluorescence data for the PAO1 P_{hpf}-*hpf-yfp* strain, at OD_{600} ~0.3 (early log phase) MOPS-fructose-grown cells produce HPF-YFP at a similar level like MOPS-glucose-grown cells and at OD_{600}~1.0 (log phase) MOPS-fructose-grown cells produce HPF-YFP higher than MOPS-glucose-grown cells (Appendix D).

HPF Productions From MOPS-fructose/MOPS-glucose

Three strains (PAO1 wild type, *Δhpf* complementing with *hpf*, and deletion of the *hpf* gene) were cultured in rich media overnight. Cell pellets were washed twice in MOPS buffer and then cultured in MOPS-fructose or MOPS-glucose medium at 37^{o}C with 200 rpm shaking until the OD_{600} reached to ~0.3 and ~1.0. Cells were washed two times in PBS and incubated in PBS starvation medium for eight days. I tested the viability when cells were starved under the same PBS starvation condition after the growth in MOPS-fructose and MOPS-glucose at both OD~0.3 and ~1.0. Figure 14A demonstrates the survival cells, as indicated by number of CFUs, of wild-type and complemented strains during PBS starvation from MOPS-fructose growth at both OD was greater than from MOPS-glucose growth. Interestingly, the *Δhpf* strain from MOPS-fructose growth at both OD lost viability

to the similar level as PAO1 and *Δhpf+hpf* strains from MOPS-glucose growth, during starvation. During PBS incubation, the *Δhpf* strains grew from MOPS-glucose at both OD lost the most CFUs compared to other strains. The total protein was investigated. The results showed that total protein of starved bacteria after growing in MOPS-fructose from both OD was a little bit higher than in MOPS-glucose (Fig. 14B). To observe HPF production during PBS starvation after growing in MOPS-fructose and MOPS-glucose at OD~0.3 and OD~1.0, samples were normalized to 15µg of total proteins for loading onto SDS-PAGE gels for immunoblot analysis. The result illustrates that HPF production of wild-type and complemented strain from MOPS-fructose growth at both OD were greater than from MOPS-glucose, under the same nutrient limitation (Fig. 14C). Figure 14C (OD~0.3), the bottom row shows the longer exposure time of blot, which indicates that HPF was below detection in all strains during day two to eight of starvation. The bottom row of OD~1.0 of figure 14C shows the longer exposure time of immunoblot, which HPF production of wild-type and complemented strains from MOPS-fructose growth of was detectable in a low single between two to eight days of starvation, compared to PAO1 and *Δhpf+hpf* strains from MOPS-fructose growth, under the same nutrient limitation. This result suggested that OD~0.3 or OD~1.0 of MOPS cultured cells did not effects HPF production during starvation. However, different sole carbon sources (fructose and glucose) of MOPS minimal medium seem to effect HPF, in which under the same nutrient limitation, wild-type and complemented strain from MOPS-fructose growth produced HPF greater than from MOPS-glucose.

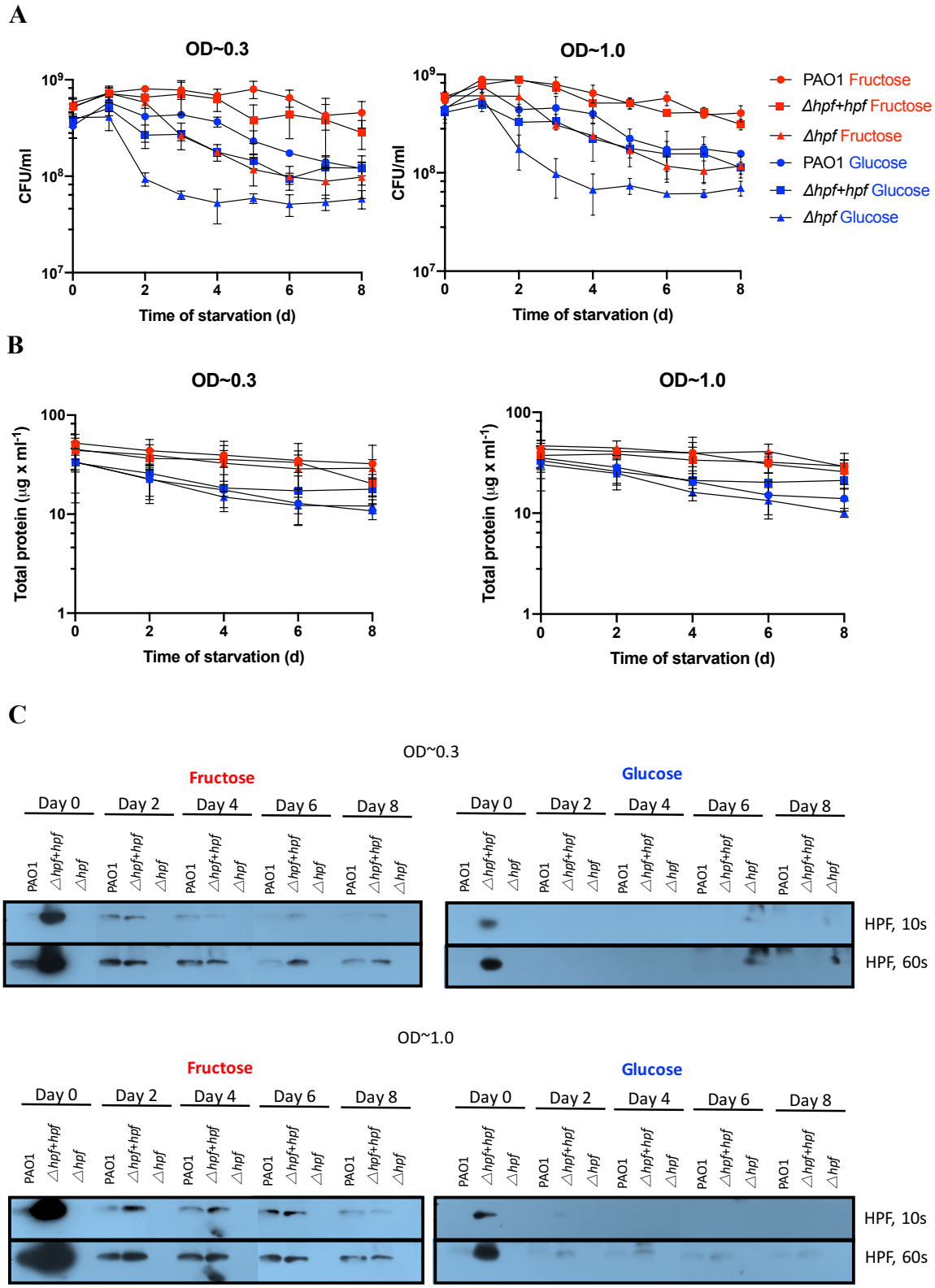
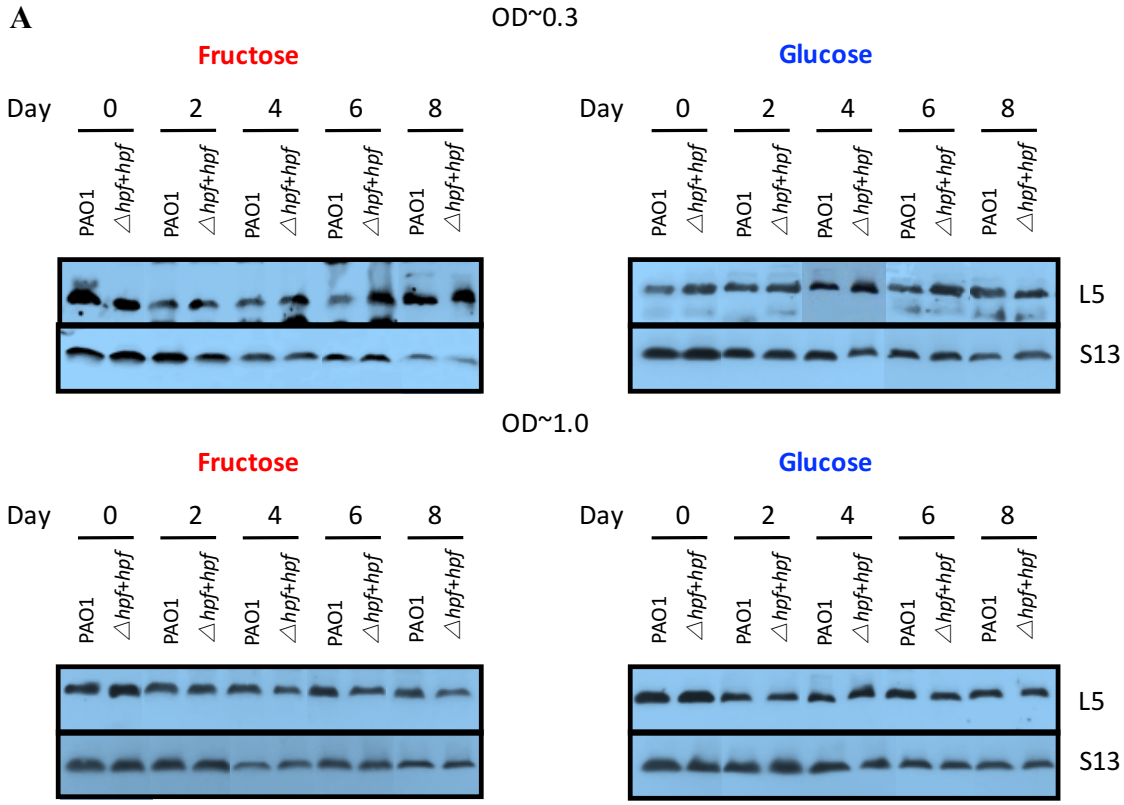

A
OD~0.3
OD~1.0
CFU/ml
Time of starvation (d)
PAO1 Fructose
Δhpf+hpf Fructose
Δhpf Fructose
PAO1 Glucose
Δhpf+hpf Glucose
Δhpf Glucose
B
OD~0.3
OD~1.0
Total protein (μg x ml-1)
Time of starvation (d)
C
OD~0.3
Fructose
Glucose
Day 0
Day 2
Day 4
Day 6
Day 8
PAO1
△hpf+hpf
△hpf
HPF, 10s
HPF, 60s
OD~1.0
Fructose
Glucose
HPF, 10s
HPF, 60s

Figure 14: HPF production during PBS starvation from MOPS-fructose and MOPS-glucose. (A) CFU assays of survival PAO1 wild type, complementing *Δhpf* with *hpf*, and deletion of the *hpf* gene strains, following up to eight days of starvation after growth in MOPS-fructose/MOPS-glucose at OD~0.3 and OD~1.0. Cells were grown in MOPS-fructose or MOPS-glucose until OD_{600} reached to ~0.3 and ~1.0, where hpf-yfp expression in MOPS-fructose and MOPS-glucose was similar at early log phase OD_{600}~0.3 and at OD_{600}~1.0, hpf-yfp expression in MOPS-fructose is greater than in MOPS-glucose. Then, cells were incubated in PBS. CFU count was performed by drop plating following starvation. The circle, square, and triangle symbols indicate PAO1 wild-type, complementing *Δhpf* with *hpf*, and *hpf* strains, respectively. Red color represents strains grew from MOPS-fructose and blue is the strains grew in MOPS-glucose, before starvation. Error bars show the standard deviation of the mean of the three biological replicates per strain, except *Δhpf*+*hpf* from MOPS-fructose at OD~0.3 that was in duplicate. (B) Total protein content of PAO1, *Δhpf*+*hpf*, and *Δhpf* strains under starvation after growing in MOPS-fructose or MOPS-glucose medium at OD~0.3 and 1.0. The total protein shows all strains grew from MOPS-Fructose as colored red contain more protein than grew from MOPS-Glucose as colored blue. (C) Immunoblot of PAO1, *Δhpf*+*hpf*, and *Δhpf* strains, over the course of PBS starvation, growth from MOPS-fructose vs MOPS-glucose and OD~0.3 vs OD~1.0, using an anti-HPF antibody. Immunoblots of OD~0.3 and OD~1.0 show during PBS starvation, wild-type and complemented strains grew from MOPS-fructose expressed more HPF than from MOPS-glucose growth. Within a short exposure time ten seconds, HPF in MOPS-fructose are detectable, while in MOPS-glucose are undetectable except *hpf* complemented strain on day zero. However, HPF signals during starvation from MOPS-glucose at OD~1.0 are detectable within a longer exposure time (60 seconds), while OD~0.3 are undetectable.

Determine the Stability of Ribosomal Proteins (L5 and S13) of *P. aeruginosa* PAO1 During PBS Starvation After MOPS-fructose/MOPS-glucose Growth

MOPS-fructose/MOPS-glucose was a suitable condition to control level of HPF production in *P. aeruginosa*, which allowed me to test L5 and S13 stabilities in the presence of low HPF and high HPF that were originally encoded by chromosome in wild type and by plasmid in complemented strain. The results of immunoblot using anti-L5 and anti-S13 antibodies demonstrate the abundance of L5 and S13 proteins during starvation were similar from both conditions of MOPS-fructose and MOPS-glucose growth at both OD~0.3 and OD~1.0 before transfer to the same nutrient limitation (Fig. 15A). The *Δhpf* strain was

tested along with wild-type and complemented strain under the same condition. The *Δhpf* strain from MOPS-glucose growth lost L5 and S13 abundances after four days of starvation (Appendix E & F). The mean value of relative signal intensity was determined for three biological replicates of OD~1.0 and for a duplicate of OD~0.3. Figure 15B shows over the course of eight days in PBS, PAO1 and complemented strains grew from MOPS-fructose and MOPS-glucose at both OD (0.3 and 1.0) showed an overlap intensity number of L5 and S13 abundances. This result implies that during nutrient-limited conditions, L5 and S13 ribosomal proteins were stable at a similar level in either a tiny amount of HPF or a high HPF production.



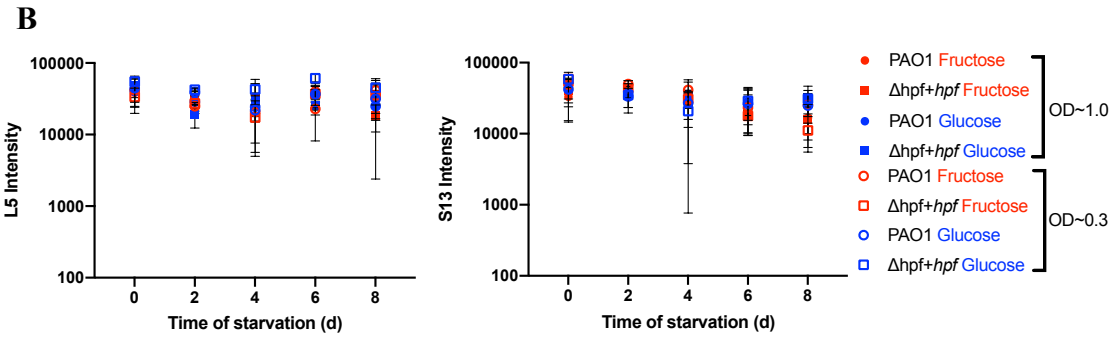


Figure 15: Ribosomal protein (L5 and S13) stability during PAO1 starvation from MOPS-fructose/MOPS-glucose. (A) Immunoblot of PAO1 and complementing *Δhpf* with *hpf* using anti-L5 and S13 antibodies, over eight days of PBS starvation from MOPS-fructose/MOPS-glucose. Immunoblots shows that the amount of L5 and S13 are the similar in both MOPS-fructose and MOPS-glucose. (B) The mean intensity of L5 and S13 proteins from immunoblots of three biological replicates of OD~1.0 (SD, n=3) and a duplicate of OD~0.3 (SD, n=3). The circle and square symbols represent PAO1 and complementing *Δhpf* with *hpf* grew. The red and blue are colored for strains grew in MOPS-fructose and in MOPS-glucose, respectively. Solid symbols and cleared symbols respectively represent strains grew from MOPS medium at OD~1.0 and at OD~0.3.

CHAPTER FIVE

DISCUSSION

Bacteria were among the first life forms to exist on earth and a life that is capable of surviving in a wide variety of extreme environments, including hot springs, ocean bottoms, and clouds (Reysenbach & Cady, 2001). Most pathogenic bacteria have the ability to rest and survive with low nutrient abundance; and reproduce once nutrient become available again (Mueller, 2018). When bacterial cells do not have enough nutrients and energy for their intracellular metabolisms, cells enter the dormant stage (Lennon & Jones, 2011). During dormancy, cells reduce their cell size, degrade unnecessary molecules, and keep the storage molecules for cell viabilities (Bergkessel & Newman, 2016). *P. aeruginosa* carries most of the bacterial characteristics as described above (Lewenza et al., 2018). This opportunistic pathogen is capable of becoming dormant during starvation and resuscitating once nutrients are available again (Akiyama et al, 2017). In response to this nutrient stress or any other stress including antibiotics or osmotic, *P. aeruginosa* undergoes the stringent response induced by RelA/SpoT (Nguyen et al., 2011). A ribosome-associated protein, HPF represses protein translation by converting active 70S ribosomes to inactive 100S ribosomes during stationary phase (Polikanov, Blaha, & Steitz, 2012).

Here, I observed the relationship between HPF and RelA/SpoT in survival *P. aeruginosa* during dormancy. Under nutrient-rich environments, *P. aeruginosa* wild-type, *Δhpf* and *ΔrelA/ΔspoT* strains showed a similar growth curve with four phases including

lag, exponential, stationary, and death (Fig. 8A). However, when the strains were transferred starvation condition in PBS, the *Δhpf* and *ΔrelA/ΔspoT* mutant strains appeared to lose the ability to recover on rich nutrient media, while the wild-type and *hpf* complemented strains maintained cell viabilities during starvation (Fig. 8B). Therefore, both HPF or RelA/SpoT are essential for *P. aeruginosa* to maintain cell viability when nutrients are limited. Akiyama et al (Akiyama et al., 2017) also proved that HPF is required for PAO1 to maintain its viability during starvation. However, Ribosomal Modulation Factor (RMF) was not required for cell viability during starvation (Akiyama et al., 2017). In this study, I demonstrated by using immunoblots that, *ΔrelA/ΔspoT* double knockout mutant strain did not produce HPF during lag and early log phases (Fig. 10) and that HPF was degraded during late stationary phase at 48 hours (Appendix G). Moreover, I observed that the *ΔrelA/ΔspoT* mutant lost HPF proteins after four days of starvation in PBS. This result may explain why the phenotype of *ΔrelA/ΔspoT* strain lost its cells viability faster than *Δhpf* strain during starvation. The *ΔrelA/ΔspoT* strain not only lacked (p)ppGpp production but also lacked sufficient HPF during starvation. Since *ΔrelA/ΔspoT* strain lost two critical functions at the same time, it is possible that these functions lead to faster cell lysing comparing to *Δhpf* strain. Song and Wood also just proposed that (g)ppGpp productions mediate HPF and RMF activity under stringent response (Song & Wood, 2019). Hence, *ΔrelA/ΔspoT* strain lost two critical factors (ppGpp and HPF) that are the keys for *P. aeruginosa* to survive during nutrient stress.

In this study, I characterized the stability of two ribosomal proteins during *P. aeruginosa* starvation in PBS for wild-type strain PAO1 and for the *Δhpf*, *ΔrelA/ΔspoT*,

mutant strains. L5 is one protein of 50S large ribosomal subunit, and S13 is one protein of 30S small ribosomal subunit (Frank et al., 2007). During the stationary phase of 24 hours growth on TSB, lacking HPF led to lose of L5 and S13 (Fig. 10A). Interestingly, during eight days of nutrient starvation, *Δhpf* strain quickly lost both L5 and S13 from day 2, while PAO1 and *ΔrelA/ΔspoT* strains maintained both L5 and S13 proteins throughout the course of starvation (Fig. 13). The abundance of HPF in PAO1 promotes 100S dimerization of ribosome (Song & Wood, 2019), which inhibits translation during unavailable nutrient conditions (Polikanov, Blaha, & Steitz, 2012). Thus, I can conclude that HPF helps stabilizing L5 and S13 ribosomal proteins in order to protect the inactive ribosomes under nutrient depletion and during stationary phase. Without translation, cells save energy for resting during the dormant stage (Lennon & Jones, 2011), and preserve inactive ribosomes that allow regrowth once cells are transferred to favorable conditions (Lewis, 2007). Akiyama et al. (Akiyama et al., 2017) demonstrated that deletion of the *hpf* gene from the genome of *P. aeruginosa* PAO1 caused degradation of 23S ribosomal RNA following the four days of carbon and nitrogen starvation. The wild type *P. aeruginosa* could strain maintained the 23S rRNA during starvation. In this study, I found that lacking *hpf* leads to degradation of L5 on 50S large subunit ribosome and S13 small subunit ribosomal protein after prolonged starvation in nutrient-deprived media. Degradation of ribosomes is initiated by specific cleavage of ribosomal RNAs by ribonucleases (Maruyama et Mizuno, 1970; Zundel, Basturea, & Deutscher, 2009), and then ribosomal proteins started to degrade by formation complex of Lon protease and polyphosphate chains (Kuroda et al., 1999).

Together of 23S rRNA, L5 and S13 ribosomal protein degradations might cause ribosome not to function or ribosome degradation in *P. aeruginosa* under limited nutrient conditions.

During starvation, L5 and S13 are not degraded in the wild-type and the complemented strain. Interestingly, the *ΔrelA/ΔspoT* strain has the stability of L5 and S13 following eight days incubating in PBS, like wild-type (Fig. 13), even though this mutant completely lost HPF by day four (Fig. 12A). A possible reason is that deletion of *relA* and *spoT* genes from PAO1 chromosome resulted in incapacity of generating (p)ppGpp, and (p)ppGpp may be required to induce Lon protease activity to degrade L5 and S13. The catalyzed (p)ppGpp reaction generate inorganic phosphate (Pi). Polyphosphate that is a chain of inorganic phosphates is needed to activate a complex of Lon protease, a specific protease to degrade non-functional ribosomal proteins and only degrade ribosomal proteins that not access to ribosome in nutrient-deprived media (Kuroda et al., 1999). Hence, lacking (p)ppGpp during starvation, *P. aeruginosa* may lose the ability to degrade L5 and S13 ribosomal proteins due to inactivated Lon protease. Another possibility is that under nutrient-deprived conditions*, ΔrelA/ΔspoT P. aeruginosa* still preserve ribosomes, but have others mechanism lead to cell death such as DNA damage or membrane damage.

One primary mechanism for protecting ribosomes during *P. aeruginosa* starvation is HPF converting 70S active to 100S inactive ribosomes. However, we do not know how much HPF concentration is needed to preserve these ribosomes. Thus, in the last part of this study, I discussed this question. A suitable condition to control the level of HPF proteins in *P. aeruginosa* is growing on MOPS-fructose and MOPS-glucose. These two different sources of sole carbon, but not different OD (OD_{600}~1.0 and OD_{600}~0.3), appeared

to show low and high HPF production of *P. aeruginosa*. *P. aeruginosa* PAO1 first cultured in MOPS-fructose and then starved in PBS showed a good number of HPF production, while PAO1 first cultured in MOPS-fructose and then incubated in PBS exhibited a very low amount of HPF (Fig. 14C). However, the results revealed that both conditions did not show the different ability to stabilize both L5 and S13 (Fig. 15). This result suggests that the amount of HPF high or low does not matter for *P. aeruginosa* PAO1 during PBS starvation. Hence, the result proposes a conclusion that *P. aeruginosa* just needs a minimum amount of HPF or the presence of HPF to preserve 100S ribosome during PBS starvation and resuscitate from dormancy when conditions become favorable.

CHAPTER SIX

CONCLUSION

P. aeruginosa is an opportunistic bacterium that is associated with cystic fibrosis lung infections and chronic wound infections. The biofilm of *P. aeruginosa* is highly resistant to antibiotics. When *P. aeruginosa* is faced with unfavorable environmental conditions such as antibiotic stress or nutrient stress, cells enter the dormant stage. Two of the factors involved in the dormancy of *P. aeruginosa* are stringent response and ribosome hibernation. The stringent response is a stress response to nutrient limitation in which induced by (p)ppGpp productions of RelA/SpoT. (p)ppGpp signals to shutdown cellular activities and minimize energy uses during starvation. Ribosome hibernation is the dimerization of active 70S ribosome to inactive 100S ribosome, in which bacteria are not able to synthesize proteins and preserve ribosome during dormancy. HPF plays a role in preserving ribosomal rRNA during *P. aeruginosa* starvation. My main goals of this study were to understand the role of HPF in preserving ribosomal proteins (L5 and S13) and investigated the relationship between RelA/SpoT and HPF on ribosomal proteins during nutrient limitation. I found that *P. aeruginosa* is able to survives in a dormant state in low nutrient environments and is able to resuscitate from dormancy when favorable conditions are available. This opportunistic pathogen requires HPF and RelA/SpoT for viability during nutrient stress. In the absence of RelA/SpoT during starvation, *P. aeruginosa* loses the ability to maintain HPF stability. Additionally, I discovered that HPF is essential for the maintenance of ribosomal proteins during starvation of *P. aeruginosa*, and that the

ribosomal proteins are degraded in the absence of HPF. *P. aeruginosa* seems to need a minimum amount of HPF to preserve ribosomes; therefore, cells are able to survive over the nutrient stress condition. In conclusion, I proposed a schematic of *P. aeruginosa* during nutrient starvation as shown in figure 16.

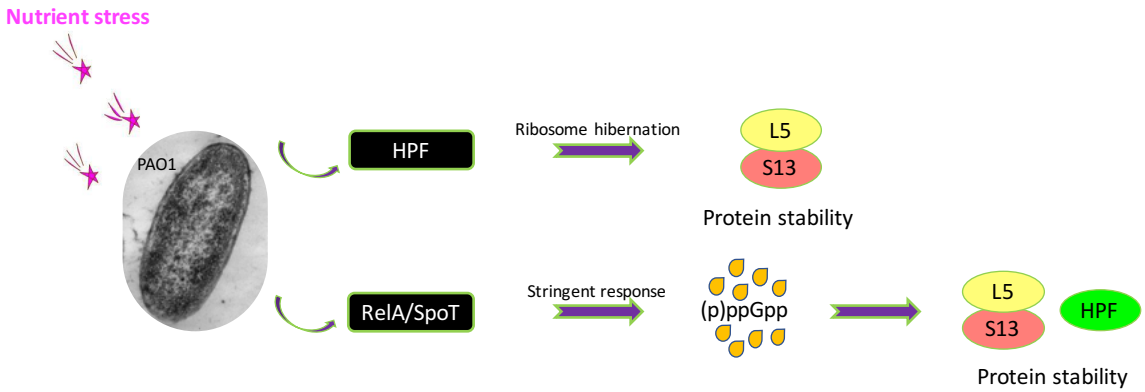


Figure 16: The proposed schematic of *P. aeruginosa* starvation. When *P. aeruginosa* PAO1 is stressed under nutrient limitation, cells undergo stringent response or ribosome hibernation for entering the dormant stage. During starvation, HPF of *P. aeruginosa* maintains L5 and S13 ribosomal protein stabilities in the form of ribosome hibernation. In response to nutrient stress, *P. aeruginosa* undergoes stringent response in which inducing RelA/SpoT in order to induce (p)ppGpp. The presence of RelA/SpoT allows *P. aeruginosa* to maintain L5 and S13 ribosomal proteins and HPF ribosome-associated protein stabilities.

Future Directions

This study demonstrated that HPF deletion of *P. aeruginosa* starvation lost the ability to maintain L5 and S13 ribosomal proteins from TSB pre-culture. One future study from this discovery is to test if the loss of L5 ribosomal protein from 50S large subunit of the ribosome and S13 ribosomal protein from 30S small subunit of the ribosome leads to instability of the rest of ribosomal proteins which result in ribosomal degradation. One method to test this question is ribosome isolation using the sucrose gradient method. This

method will allow us to observe the forms of the ribosome during cell starvation. The sucrose gradient of *P. aeruginosa Δhpf* starvation will also provide the information of 100S ribosome dimer in comparison to *P. aeruginosa* PAO1. If the 70S or 100S ribosomes from *Δhpf P. aeruginosa* exist, the purified ribosomes can be denatured and run on SDS-PAGE coomassie gel which allows identifying other ribosomal proteins and immunoblot of L5 and S13.

However, HPF deletion of *P. aeruginosa* starvation seems to lose the ability to maintain only L5 ribosomal proteins from MOPS-fructose pre-culture. Akiyama et al. (Akiyama et al., 2017) illustrated that HPF deletion of *P. aeruginosa* caused degradation of 23S ribosomal RNA under carbon and nitrogen starvation. These suggest that *P. aeruginosa* might lose L5 ribosomal protein and 23S ribosomal RNA of the large subunit of ribosome faster than ribosomal protein and 16S rRNA of the small subunit. Therefore, the bioanalyzer of HPF deletion strain from the MOPS-fructose pre-culture of this starvation condition will provide information on ribosomal RNA degradation in addition to ribosomal proteins.

From the MOPS pre-culture experiment to control HPF production before PBS starvation, the result indicated that *P. aeruginosa* needs a minimum amount of HPF to preserve ribosomes. Another way to quantify the amount of HPF at the single-cell level is immunogold labeling of anti-HPF antibody will allow the count of HPF molecules at the single cell level and convert to population level with the number of CFU count. This experiment will provide an additional evidence on which MOPS culture conditions control

the different amounts of HPF production. Thus, we can know the minimum amount of HPF that *P. aeruginosa* is required to preserve its ribosomal proteins.

Lastly, *P. aeruginosa* has another factor that is involved in dormancy is the stringent response. In response to nutrient stress, *P. aeruginosa* induces RelA/SpoT in order to produce (p)ppGpp to shutdown cellular activities for survival during dormancy. This study showed that *P. aeruginosa* starvation loses the ability to maintain HPF stability in the absence of RelA/SpoT, however, cells still maintain L5 and S13 ribosomal proteins over the course of eight days of PBS starvation. As in the discussion session, I proposed that *ΔrelA/ΔspoT P. aeruginosa* might decrease the polyphosphate concentration due to no (p)ppGpp reactions. Thus, Lon proteases, that play a specific role to degrade only free ribosomal proteins, are not able to be activated by polyphosphate chains. The future experiment to test this hypothesis is the determination of polyphosphate concentration in *ΔrelA/ΔspoT* strain comparing to wild-type *P. aeruginosa* starvation. In vitro experiment, testing if the L5 and S13 proteins are degraded by Lon protease with or without the presence of polyphosphate. These might answer the question of why L5 and S13 ribosomal proteins are stable during *ΔrelA/ΔspoT P. aeruginosa* starvation, even though cells lost HPF ribosome-associated proteins by four days of starvation.

REFERENCES CITED

Advanced Wound Care Market. (2018). Retrieved from https://www.marketsandmarkets.com/Market-Reports/advanced-wound-care-market88705076.html?gclid=CjwKCAjw4NrpBRBsEiwAUcLcDOI5EHw3P7bKWE2G_xyK8sk658foo8QzuskOs2WrCeuHjcuORR3CihoCOQ8QAvD_BwE

Aertsen, A., & Michiels, C. (2004). Stress and how bacteria cope with death and survival. *Critical Reviews In Microbiology, 30*(4), 263-273.

Akiyama, Tatsuya, Williamson, Kerry S., & Franklin, Michael J. (2018). *Expression and Regulation of the Pseudomonas Aeruginosa Hibernation Promoting Factor.*

Akiyama, T., Williamson, K., Schaefer, R., Pratt, S., Chang, C., & Franklin, M. (2017). Resuscitation of from dormancy requires hibernation promoting factor (PA4463) for ribosome preservation. *Proceedings of the National Academy of Sciences of the United States of America, 114*(12), 3204-3209

Baron, S.S. and J.J. Rowe, 1981. Antibiotic action of pyocyanin. Antimicrobial Agents and Chemotherapy, 20: 814-820.

Battesti, A., & Bouveret, E. (2006). Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. *Molecular Microbiology, 62*(4), 1048-1063.

Bassetti, M., Vena, A., Croxatto, A., Righi, E., & Guery, B. (2018). How to manage *Pseudomonas aeruginosa* infections. *Drugs in context*, *7*, 212527. doi:10.7573/dic.212527

Bergkessel, M., Basta, D., & Newman, D. (2016). The physiology of growth arrest: Uniting molecular and environmental microbiology. *Nature Reviews Microbiology, 14*(9), 549-562.

Berrazeg, M., Jeannot, K., Enguéné, V. Y., Broutin, I., Loeffert, S., Fournier, D., & Plésiat, P. (2015). Mutations in β-Lactamase AmpC Increase Resistance of *Pseudomonas aeruginosa* Isolates to Antipseudomonal Cephalosporins. *Antimicrobial Agents and Chemotherapy,59*(10), 6248-6255. doi:10.1128/aac.00825-15

Botzenhart, K., & Döring, G. (1993). Ecology and epidemiology of *Pseudomonas aeruginosa*. *Pseudomonas Aeruginosa* as an Opportunistic .... Retrieved from http://link.springer.com/chapter/10.1007/978-1-4615-3036-7_1

Bragonzi, Copreni, De Bentzmann, Ulrich, & Conese. (2004). Airway epithelial cell–pathogen interactions. Journal of Cystic Fibrosis, 3, 197-201.

Bruhn-Olszewska, Molodtsov, Sobala, Dylewski, Murakami, Cashel, & Potrykus. (2018).

Byrd, J., Xu, H., & Colwell, R. (1991). VIABLE BUT NONCULTURABLE BACTERIA IN DRINKING-WATER. *Applied And Environmental Microbiology, 57*(3), 875-878.

Structure-function comparisons of (p)ppApp vs (p)ppGpp for Escherichia coli RNA polymerase binding sites and for rrnB P1 promoter regulatory responses in vitro. *BBA - Gene Regulatory Mechanisms, 1861*(8), 731-742.

Costerton, J., Lewandowski, Z., Caldwell, D., Korber, D., & Lappinscott, H. (1995). MICROBIAL BIOFILMS. *Annual Review Of Microbiology, 49*, 711-745.

David W. Basta, Megan Bergkessel, Dianne K. Newman, & Marvin Whiteley. (2017). Identification of Fitness Determinants during Energy-Limited Growth Arrest in *Pseudomonas aeruginosa*. *MBio, 8*(6), E01170-17.

Dever LA, Dermody TS. (1991) Mechanisms of Bacterial Resistance to Antibiotics. Arch Intern Med. 1991;151(5):886–895. doi:10.1001/archinte.1991.00400050040010

Durham, D. R., & Phibbs, P. V., Jr. (1982). Fractionation and characterization of the phosphoenolpyruvate: fructose 1-phosphotransferase system from *Pseudomonas aeruginosa*. *Journal of Bacteriology, 149,* 534–541.

Elborn JS. 2004. Difficult bacteria, antibiotic resistance and transmissibility in cystic fibrosis. Thorax 59:914–915.

European Centre for Disease Prevention and Control. Surveillance Report: Antimicrobial resistance surveillance in Europe 2015. [Last accessed: 22 May 2018]. Available at:https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/antimicrobial-resistance-europe-2015.pdf.

Fazli, M., Bjarnsholt, T., Kirketerp-Moller, K., Jorgensen, B., Andersen, A., Krogfelt, K., . . . Tolker-Nielsen, T. (2009). Nonrandom Distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Chronic Wounds. *Journal Of Clinical Microbiology, 47*(12), 4084-4089.

Frank, J, Gao, Hx, Sengupta, J, Gao, N, & Taylor, Dj. (2007). The process of mRNA-tRNA translocation. *Proceedings Of The National Academy Of Sciences Of The United States Of Ame, 104*(50), 19671-19678.

Franklin, M., Nivens, D., Weadge, J., & Howell, P. (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Frontiers In Microbiology, 2*, 167.

Friedrich, M., & Cunha, B. A. (2016, December 5). *Pseudomonas aeruginosa* Infections. Retrieved October 2, 2017, from https://emedicine.medscape.com/article/226748-overview

Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. Advances in wound care, 4(9), 560–582. doi:10.1089/wound.2015.0635

Gjødsbol, K., J. J. Christiensen, T. Karlsmark, B. Jørgensen, B. M. Klein, and K. A. Krogfelt. (2006). Multiple bacterial species reside in chronic wounds: a longitudinal study. Int. Wound J. 3:225-231.

Gupta, R. K., & Srivastava, R. (2012). Resuscitation promoting factors: a family of microbial proteins in survival and resuscitation of dormant mycobacteria. *Indian journal of microbiology*, *52*(2), 114–121. doi:10.1007/s12088-011-0202-6

Hancock, R., & Brinkman, F. (2002). Function of Pseudomonas porins in uptake and efflux. *Annual Review Of Microbiology, 56*(1), 17-38

Hardalo, C., & Edberg, S. C. (1997). *Pseudomonas aeruginosa*: Assessment of Risk from Drinking Water. Critical Reviews in Microbiology, 23(1), 47-75. doi:10.3109/10408419709115130

Haseltine, W. A., & Block, R. (1973). Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proceedings of the National Academy of Sciences of the United States of America*, *70*(5), 1564–1568. doi:10.1073/pnas.70.5.1564

Hector, J., & Johnson, A. (1990). Determination of genome size of *Pseudomonas aeruginosa* by PFGE: Analysis of restriction fragments. *Nucleic Acids Research, 18*(11), 3171-3174.

Iglewski, H. Barbara. (1996). *Pseudomonas*. In *Medical Microbiology* (4th edition). Galveston, Texas: Samuel Baron

Irving, S. E., & Corrigan, R. M. (2018). Triggering the stringent response: Signals responsible for activating (p)ppGpp synthesis in bacteria. *Microbiology,164*(3), 268-276. doi:10.1099/mic.0.000621

Jacoby G. A., Munoz-Price L. S. (2005). The new beta-lactamases. *N. Engl. J. Med.* 352380–391

James C. Cunningham, & Lynn M. Taussig. (2013). *An Introduction to Cystic Fibrosis for Patients and Their Families* (6th edition). Cystic Fibrosis Foundation

Jesaitis, A., Franklin, M., Berglund, D., Sasaki, M., Lord, C., Bleazard, J., . . . Lewandowski, Z. (2003). Compromised host defense on Pseudomonas aeruginosa biofilms: Characterization of neutrophil and biofilm interactions. *Journal Of Immunology, 171*(8), 4329-4339.

Kato, Yoshida, Miyata, Maki, Wada, & Namba. (2010). Structure of the 100S Ribosome in the Hibernation Stage Revealed by Electron Cryomicroscopy. *Structure, 18*(6), 719-724.

Keep, N., Ward, J., Robertson, G., Cohen-Gonsaud, M., & Henderson, B. (2006). Bacterial resuscitation factors: Revival of viable but non-culturable bacteria. *Cellular And Molecular Life Sciences, 63*(22), 2555-2559.

Khakimova, M., Ahlgren, H., Harrison, J., English, A., Nguyen, M., & Dao Nguyen, H. (2013). The Stringent Response Controls Catalases in *Pseudomonas aeruginosa* and Is Required for Hydrogen Peroxide and Antibiotic Tolerance. *Journal Of Bacteriology, 195*(9), 2011-2020.

Khusainov, I., Vicens, Q., Ayupov, R., Usachev, K., Myasnikov, A., Simonetti, A., . . . Hashem, Y. (2017). Structures and dynamics of hibernating ribosomes from *Staphylococcus aureus* mediated by intermolecular interactions of HPF. *Embo Journal, 36*(14), 2073-2087.

Korepanov, A., Korobeinikova, A., Shestakov, S., Garber, M., & Gongadze, G. (2012). Protein L5 is crucial for in vivo assembly of the bacterial 50S ribosomal subunit central protuberance. *Nucleic Acids Research, 40*(18), 9153-9159.

Kolter, R., Siegele, D., & Tormo, A. (1993). THE STATIONARY-PHASE OF THE BACTERIAL LIFE-CYCLE. *Annual Review Of Microbiology, 47*, 855-874.

Kuroda, A., Tanaka, S., Ikeda, T., Kato, J., Takiguchi, N., & Ohtake, H. (1999). Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in Escherichia coli. *Proceedings Of The National Academy Of Sciences Of The United States Of Ame, 96*(25), 14264-14269.

Kuroda, Nomura, Ohtomo, Kato, Ikeda, Takiguchi, . . . Kornberg. (2001). Role of inorganic polyphosphate in promoting ribosomal, protein degradation by the lon protease in E-coli. *Science, 293*(5530), 705-708.

LaBauve, A. E., & Wargo, M. J. (2012, May). Growth and Laboratory Maintenance of *Pseudomonas aeruginosa*. Retrieved September 26, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4296558/

Lau, Hassett, Ran, & Kong. (2004). The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends in Molecular Medicine,10*(12), 599-606.

Lennon, J., & Jones, S. (2011). Microbial seed banks: The ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology,9*(2), 119-130.

Lewenza, S., Abboud, J., Poon, K., Kobryn, M., Humplik, I., Bell, J. R., … Reckseidler-Zenteno, S. (2018). Pseudomonas aeruginosa displays a dormancy phenotype during long-term survival in water. *PloS one*, *13*(9), e0198384. doi:10.1371/journal.pone.0198384

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology, 5*(1), 48-56.

Li, H., Luo, Y. F., Williams, B. J., Blackwell, T. S., & Xie, C. M. (2012). Structure and function of OprD protein in *Pseudomonas aeruginosa*: from antibiotic resistance to novel therapies. *International journal of medical microbiology : IJMM*, *302*(2), 63–68. doi:10.1016/j.ijmm.2011.10.001

Livraghi-Butrico, A., Kelly, E., Wilkinson, K., Rogers, T., Gilmore, R., Harkema, J., . . . Grubb, B. (2013). Loss of Cftr function exacerbates the phenotype of Na hyperabsorption in murine airways. *American Journal Of Physiology-Lung Cellular And Molecular Physiology, 304*(7), L469-L480.

Llano-Sotelo, Azucena, Kotra, Mobashery, & Chow. (2002). Aminoglycosides Modified by Resistance Enzymes Display Diminished Binding to the Bacterial Ribosomal Aminoacyl-tRNA Site. *Chemistry & Biology, 9*(4), 455-463.

Maruyama, Hiromi, Mizuno, Den&Amp;Apos, & Ichi. (1970). Ribosome degradation and the degradation products in starved *Escherichia coli*: I. Comparison of the degradation rate and of the nucleotide pool between *Escherichia coli* B and Q-13 strains in phosphate deficiency. *BBA Section Nucleic Acids And Protein Synthesis, 199*(1), 159-165.

Martins, D., McKay, G., Sampathkumar, G., Khakimova, M., English, A. M., & Nguyen, D. (2018). Superoxide dismutase activity confers (p)ppGpp-mediated antibiotic tolerance to stationary-phase *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(39), 9797–9802. doi:10.1073/pnas.1804525115

Mueller, E. (2018, November 16). The Microorganism Survival Toolkit: Dormancy. Retrieved from https://blogs.iu.edu/sciu/2018/11/20/microorganism-dormancy/

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., … Singh, P. K. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science (New York, N.Y.)*, *334*(6058), 982–986. doi:10.1126/science.1211037

Nikaido H, Hancock REW. 1986. Outer membrane permeability of *Pseudomonas aeruginosa*. In *The Bacteria: A Treatise on Structure and Function*, ed. JR Sokatch, pp. 145–93. London: Academic

Ning, Wei, Fei, Jingyi, & Gonzalez, Ruben L., Jr. (2014). The ribosome uses cooperative conformational changes to maximize and regulate the efficiency of translation. *Proceedings of the National Academy of Sciences of the United States, 111*(33), 12073-12078.

Orlandi, V., Bolognese, F., Chiodaroli, L., Tolker-Nielsen, T., & Barbieri, P. (2015). Pigments influence the tolerance of *Pseudomonas aeruginosa* PAO1 to photodynamically induced oxidative stress. *Microbiology-Sgm, 161*(12), 2298-2309.

Parker Albert, Ehrlich Garth D, Roe Frank, Pitts Betsey, Richards Lee, Folsom James P, . . . Stewart Philip S. (2010). Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. *BMC Microbiology, 10*(1), 294.

Pena, C., Suarez, C., Ocampo-Sosa, A., Murillas, J., Almirante, B., Pomar, V., . . . Martinez-Martinez, L. (2013). Effect of Adequate Single-Drug vs Combination Antimicrobial Therapy on Mortality in *Pseudomonas aeruginosa* Bloodstream Infections: A Post Hoc Analysis of a Prospective Cohort. Clinical Infectious Diseases, 57(2), 208-216.

Phibbs, P. V., Jr. (1988). Genetic analysis of carbohydrate metabolism in *Pseudomonas*, in: Microbial Metabolism and the Carbon Cycle, (S. R. Hagedorn, R. S. Hanson, and D. A. Kunz, eds.), Harwood Academic Publishers, New York, pp. 412-436.

Polikanov, Y. S., Blaha, G. M., & Steitz, T. a. (2012). How Hibernation Factors RMF, HPF, and YfiA Turn Off Protein Synthesis. *Science*, *336*(6083), 915–918. http://doi.org/10.1126/science.1218538

Poole K. (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms, *J Mol Microbiol Biotechnol* , 2001, vol. 3 (pg. 255-64)

Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents And Chemotherapy, 49*(2), 479-487.

Potrykus, K., & Cashel, M. (2008). (p)ppGpp: Still Magical? *Annual Review Of*

Microbiology, 62(1), 35-51.

Ratjen, F., & Tullis, E. (2012). Cystic Fibrosis. *Clinical Respiratory Medicine,*568-579. doi:10.1016/b978-1-4557-0792-8.00044-1

Reysenbach, A. L., & Cady, S. L. (2001). Microbiology of ancient and modern hydrothermal systems. *Trends in Microbiology*, *9*(2), 79–86. http://doi.org/10.1016/S0966-842X(00)01921-1

Riordan JR. 2008. CFTR function and prospects for therapy. Annu Rev Biochem 77:701–726.

Ridgway, Broderick, Lopez-Campistrous, Ru'aini, Winter, Hamilton, . . . Ellison. (2008). Coarse-Grained Molecular Simulation of Diffusion and Reaction Kinetics in a Crowded Virtual Cytoplasm. *Biophysical Journal,94*(10), 3748-3759.

Rolfe, M., Rice, C., Lucchini, S., Pin, C., Thompson, A., Cameron, A., . . . Hinton, J. (2012). Lag Phase Is a Distinct Growth Phase That Prepares Bacteria for Exponential Growth and Involves Transient Metal Accumulation. *Journal Of Bacteriology, 194*(3), 686-701.

Ryder, Byrd, & Wozniak. (2007). Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Current Opinion in Microbiology,10*(6), 644-648.

Schultz, G., Sibbald, R., Falanga, V., Ayello, E., Dowsett, C., Harding, K., . . . Vanscheidt, W. (2003). Wound bed preparation: A systematic approach to wound management. *Wound Repair And Regeneration,11*(2), S1-S28.

Schuwirth, B., Borovinskaya, M., Hau, C., Zhang, W., Vila-Sanjurjo, A., Holton, J., & Cate, J. (2005). Structures of the bacterial ribosome at 3.5 angstrom resolution. *Science, 310*(5749), 827-834.

Scott, M., Gunderson, C., Mateescu, E., Zhang, Z., & Hwa, T. (2010). Interdependence of Cell Growth and Gene Expression: Origins and Consequences. *Science, 330*(6007), 1099-1102.

Selmer, M., Dunham, C., Murphy, F., Weixlbaumer, A., Petry, S., Kelley, A., . . . Ramakrishnan, V. (2006). Structure of the 70S ribosome complexed with mRNA and tRNA. *Science, 313*(5795), 1935-1942.

Smiley, A., & Hassett, D. (2005). *Pseudomonas aeruginosa* Biofilm Infections in Cystic Fibrosis. *Biofilms, Infection, and Antimicrobial Therapy,*155-169. doi:10.1201/9781420028232.ch8

Song, S., & Wood, T. K. (2019). PpGpp Ribosome Dimerization Model for Bacterial Persister Formation and Resuscitation. doi:10.1101/663658

Stover, C., Pham, X., Erwin, A., & Mizoguchi, S. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature, 406*(6799), 959-964.

Temple L.M., Sage A.E., Schweizer H.P., Phibbs P.V. (1998). Carbohydrate Catabolism in *Pseudomonas aeruginosa*. In: Montie T.C. (eds) Pseudomonas. Biotechnology Handbooks, vol 10. Springer, Boston, MA

Trias, J., & Nikaido, H. (1990). Outer-membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer-membrane of *Pseudomonas-aeruginosa*. *Antimicrobial Agents And Chemotherapy, 34*(1), 52-57.

Turner, K., Wessel, A., Palmer, G., Murray, J., & Whiteley, M. (2015). Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. *Proceedings Of The National Academy Of Sciences Of The United States Of Ame, 112*(13), 4110-4115.

Veit, G., Avramescu, R. G., Chiang, A. N., Houck, S. A., Cai, Z., Peters, K. W., ... Lukacs, G. L. (2016). From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Molecular Biology of the Cell, 27*(3), 424–433. http://doi.org/10.1091/mbc.E14-04-0935

Wada, a, Yamazaki, Y., Fujita, N., & Ishihama, a. (1990). Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proceedings of the National Academy of Sciences of the United States of America, 87*(7), 2657–61. http://doi.org/10.1073/pnas.87.7.2657

Wang, J. D., & Levin, P. A. (2009). Metabolism, cell growth and the bacterial cell cycle. *Nature reviews. Microbiology*, *7*(11), 822–827. doi:10.1038/nrmicro2202

Wendrich, Blaha, Wilson, Marahiel, & Nierhaus. (2002). Dissection of the Mechanism for the Stringent Factor RelA. *Molecular Cell, 10*(4), 779-788.

Werdin, Tenenhaus, & Rennekampff. (2008). Chronic wound care. The Lancet, 372(9653), 1860-1862.

Westbrock-Wadman, S., Sherman, D. R., Hickey, M. J., Coulter, S. N., Zhu, Y. Q., Warrener, P., . . . Stover, C. K. (1999). Characterization of a *Pseudomonas aeruginosa* Efflux Pump Contributing to Aminoglycoside Impermeability.

Antimicrobial Agents and Chemotherapy,43(12), 2975-2983. doi:10.1128/aac.43.12.2975

White, D., Drummond, J., & Fuqua, C. (2012). *The physiology and biochemistry of prokaryotes* (4th ed.). New York: Oxford University Press.

Williamson, K. S., Richards, L. a., Perez-Osorio, A. C., Pitts, B., McInnerney, K., Stewart, P. S., & Franklin, M. J. (2012). Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic- tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *Journal of Bacteriology, 194*(8), 2062–2073. http://doi.org/10.1128/JB.00022-12

Winther, Roghanian, & Gerdes. (2018). Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site. *Molecular Cell, 70*(1), 95-105.e4.

Yoshimura, F., & Nikaido, H. (1982). Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. Journal of bacteriology, 152(2), 636–642.

Yu, E. (2018, March 20). Cystic Fibrosis. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK493206/

Yum, H., Park, I., Shin, B., & Choi, S. (2014). Recurrent *Pseudomonas aeruginosa* Infection in Chronic Lung Diseases: Relapse or Reinfection? *Tuberculosis and Respiratory Diseases, 77*(4), 172-177.

Yusupov, M., Yusupova, G., Baucom, A., Lieberman, K., Earnest, T., Cate, J., & Noller, H. (2001). Crystal structure of the ribosome at 5.5 angstrom resolution. *Science, 292*(5518), 883-896.

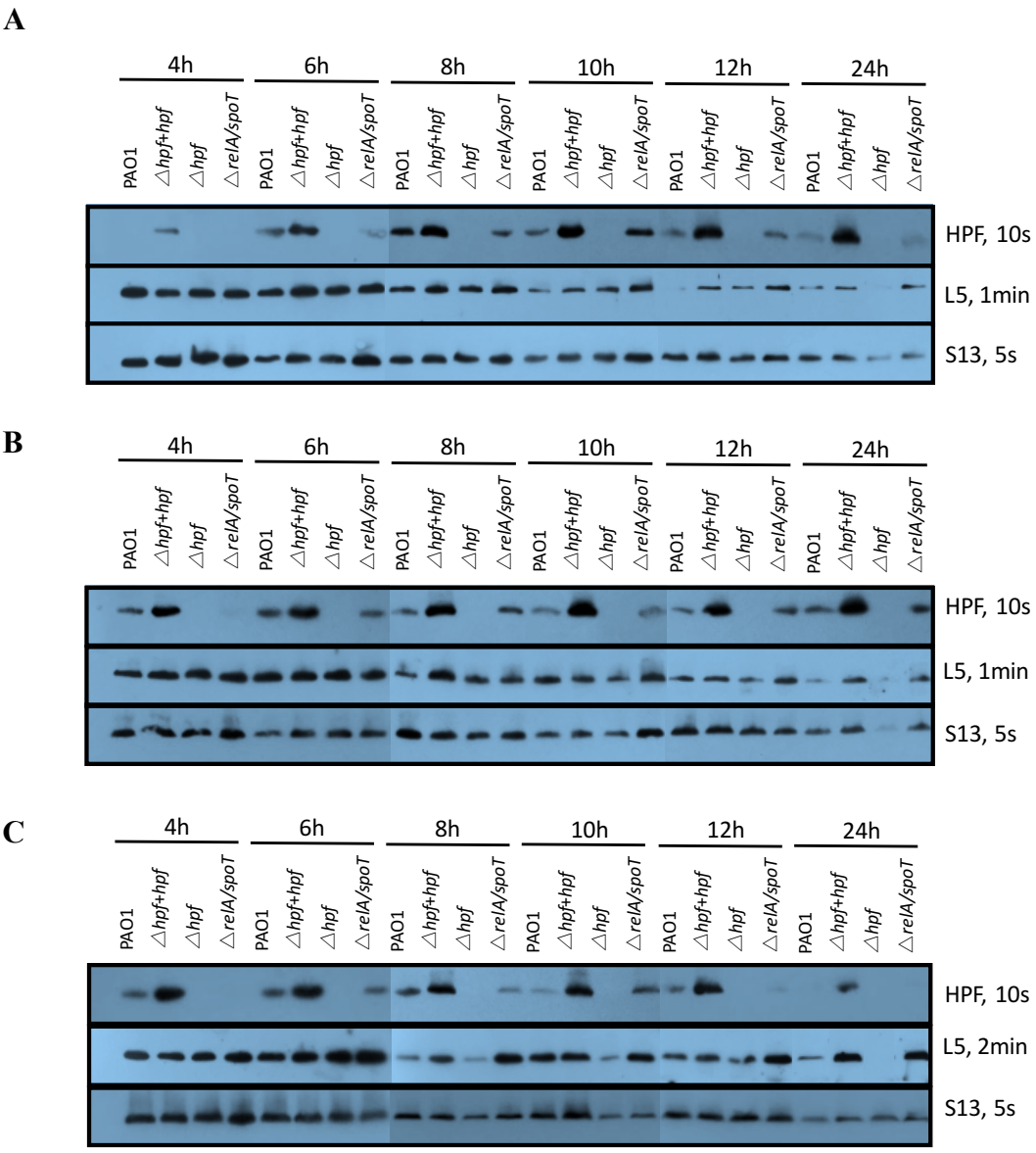
Zeng, X., & Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Frontiers in microbiology*, *4*, 128. doi:10.3389/fmicb.2013.00128

Zundel, M. A., Basturea, G. N., & Deutscher, M. P. (2009). Initiation of ribosome degradation during starvation in Escherichia coli. *RNA (New York, N.Y.)*, *15*(5), 977–983. doi:10.1261/rna.1381309

APPENDICES

APPENDIX A

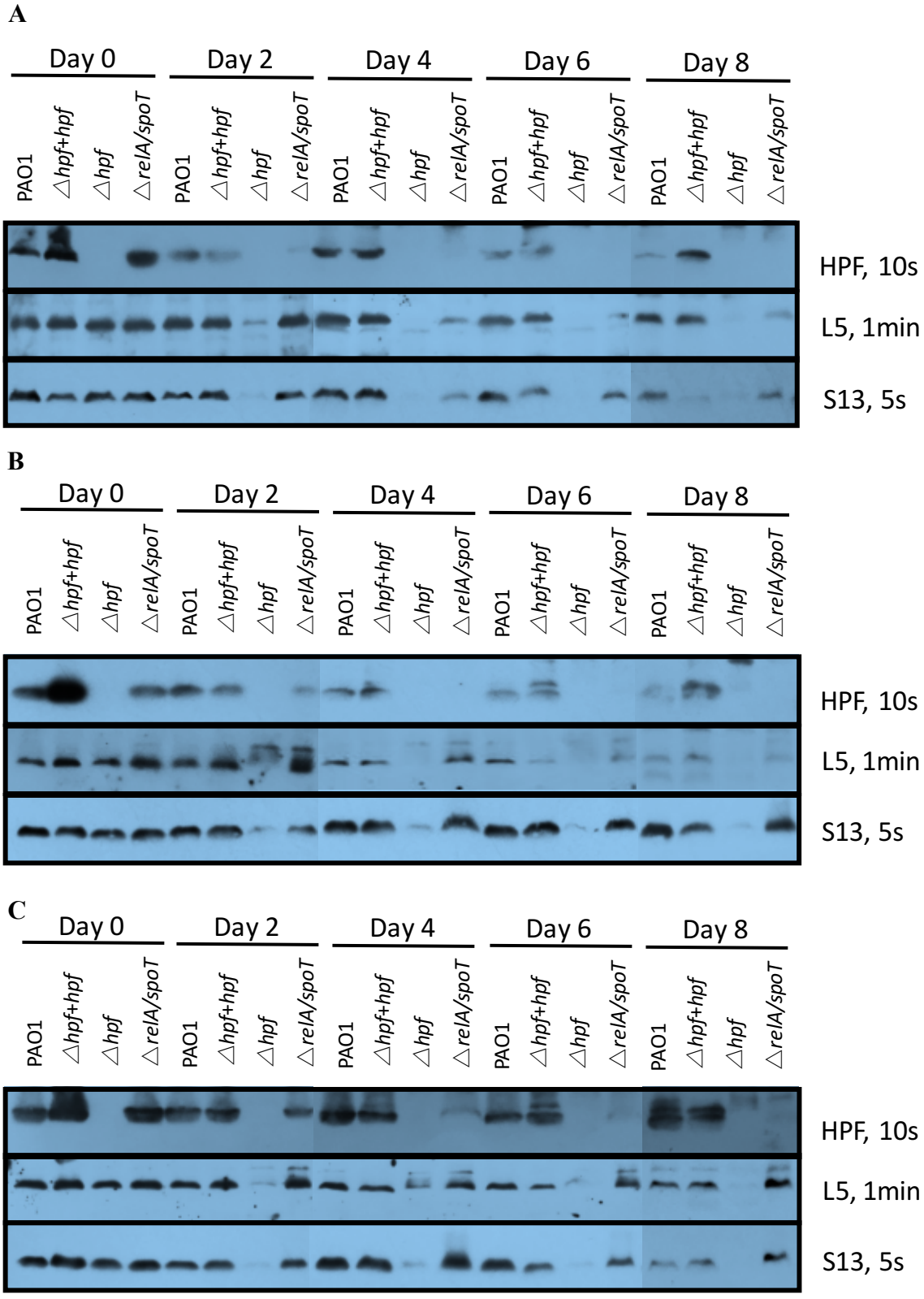
HPF, L5, AND S13 IMMUNOBLOTTING OF PAO1, *Δhpf+hpf*, *Δhpf*, AND *ΔrelA/ΔspoT* STRAINS OVER 24 HOURS OF GROWING IN TSB



HPF, L5, and S13 immunoblotting of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over 24 hours of growing in TSB. Immunoblot analysis: first to third row represent anti-HPF, L5, and S13 blots of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains from 4 to 24 hours. 15µg proteins were loaded on SDS-PAGE. (A), (B), and (C) represent replicate one, two, and three, respectively.

APPENDIX B

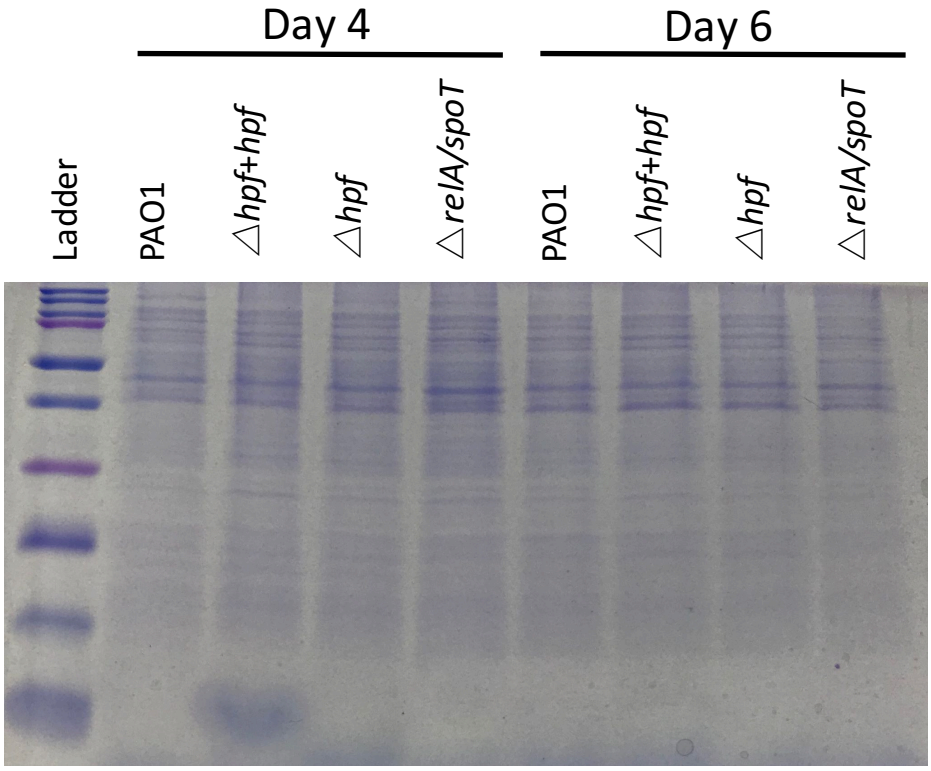
HPF, L5, AND S13 IMMUNOBLOTTING OF PAO1, *Δhpf+hpf*, *Δhpf*, AND *ΔrelA/ΔspoT* STRAIN OVER EIGHT DAYS OF STARVATION


A
Day 0
Day 2
Day 4
Day 6
Day 8
PAO1
△hpf+hpf
△hpf
△relA/spoT
HPF, 10s
L5, 1min
S13, 5s
B
Day 0
Day 2
Day 4
Day 6
Day 8
PAO1
△hpf+hpf
△hpf
△relA/spoT
HPF, 10s
L5, 1min
S13, 5s
C
Day 0
Day 2
Day 4
Day 6
Day 8
PAO1
△hpf+hpf
△hpf
△relA/spoT
HPF, 10s
L5, 1min
S13, 5s

HPF, L5, and S13 immunoblotting of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strain over eight days of starvation. Immunoblot analysis: first to third row represent anti-HPF, L5, and S13 blots of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* following eight days of starvation. 15µg proteins were loaded on SDS-PAGE. (A), (B), and (C) represent replicate one, two, and three, respectively.

APPENDIX C

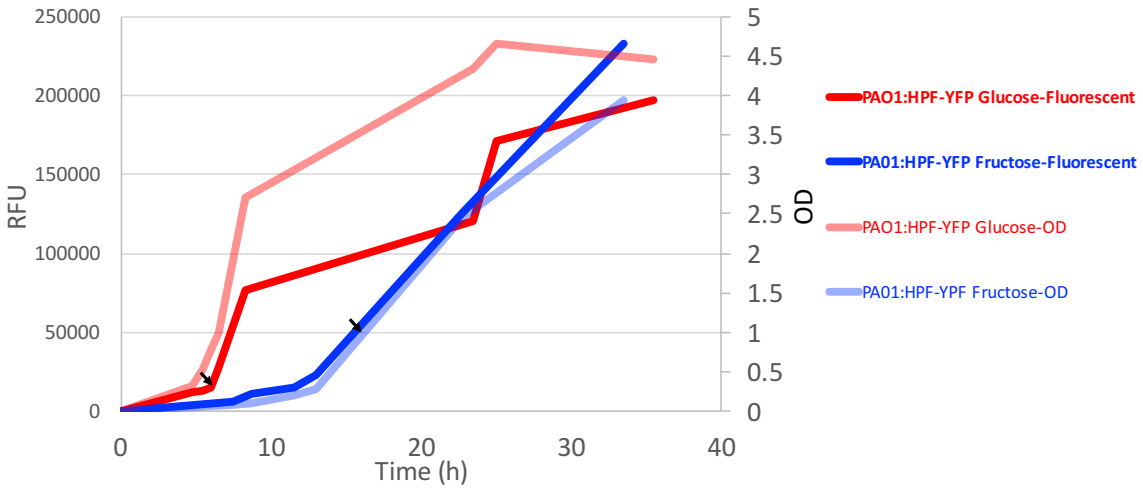
COOMASSIE-BLUE SDS-PAGE GEL OF PAO1, *Δhpf+hpf*, *Δhpf*, AND *ΔrelA/ΔspoT* ON DAY 4 AND DAY 6



PAGE-SDS Coomassie-Blue of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains on day four and Six. 15µg proteins of day four and six samples were loaded on SDS-PAGE. The gel was stained with coomassie blue on the rocker for an hour and destained with destain buffer overnight at room temperature on the rocker. The coomassie gel revealed that each lane contained a similar amount of loaded protein.

APPENDIX D

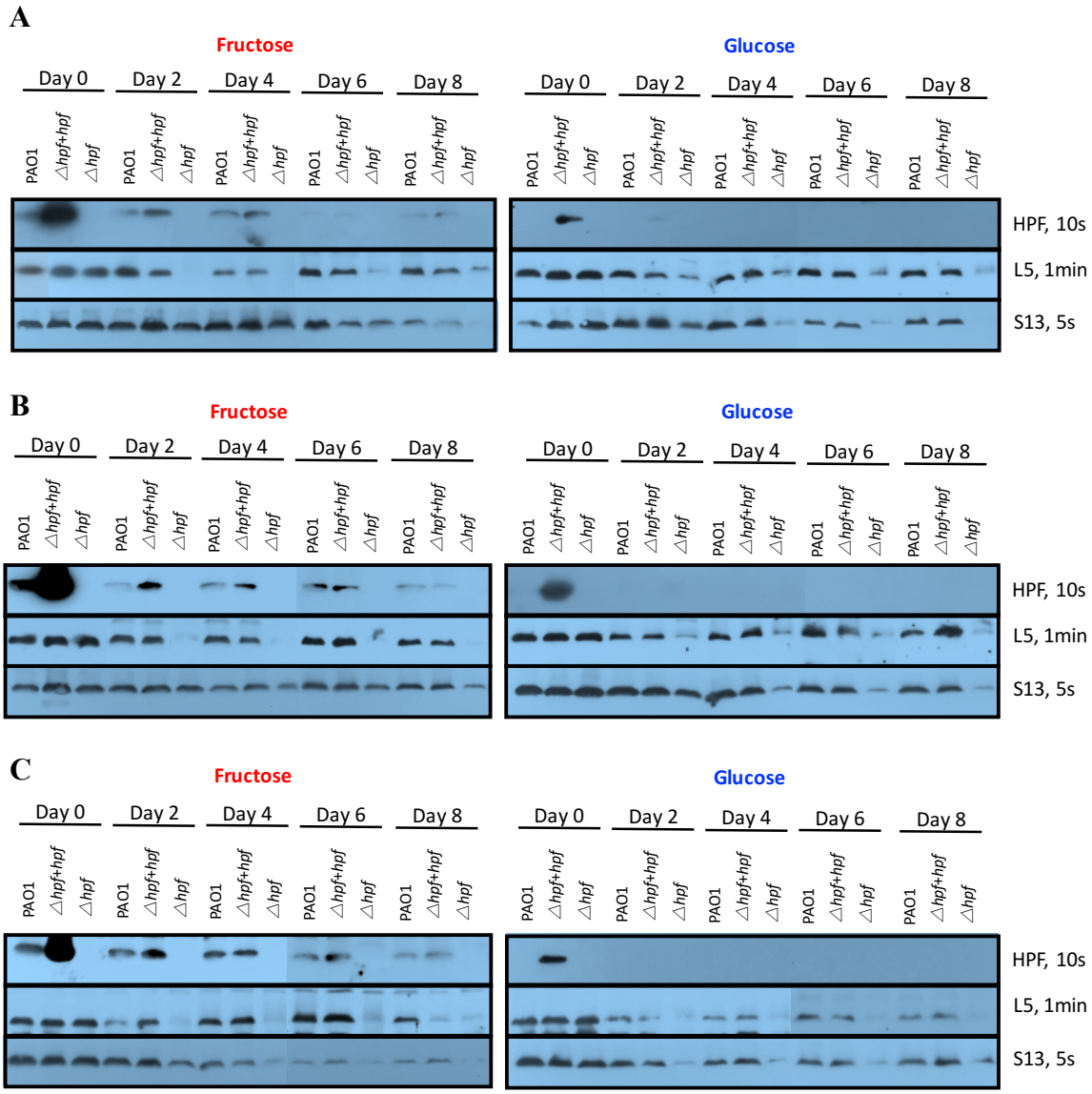
GROWTH AND HPF-YFP EXPRESSION IN MINIMAL MOPS MEDIUM



OD and fluorescent plate reader experiment on PAO1 P_{hpf}-*hpf-yfp* strain growing on two different conditions of minimal medium to test the fluorescence. The overnight culture of PAO1 P_{hpf}-*hpf-yfp* was washed and transferred to MOPS-fructose and MOPS-glucose. PAO1 P_{hpf}-*hpf-yfp* grew in MOPS-fructose with the MOPS-fructose having a 12 hours increased lag time. The OD and fluorescent Y axis show that at OD_{600}~1.0, MOPS-fructose growth cells produce HPF-YFP higher than MOPS-glucose-growth, where the black arrows pointed.

APPENDIX E

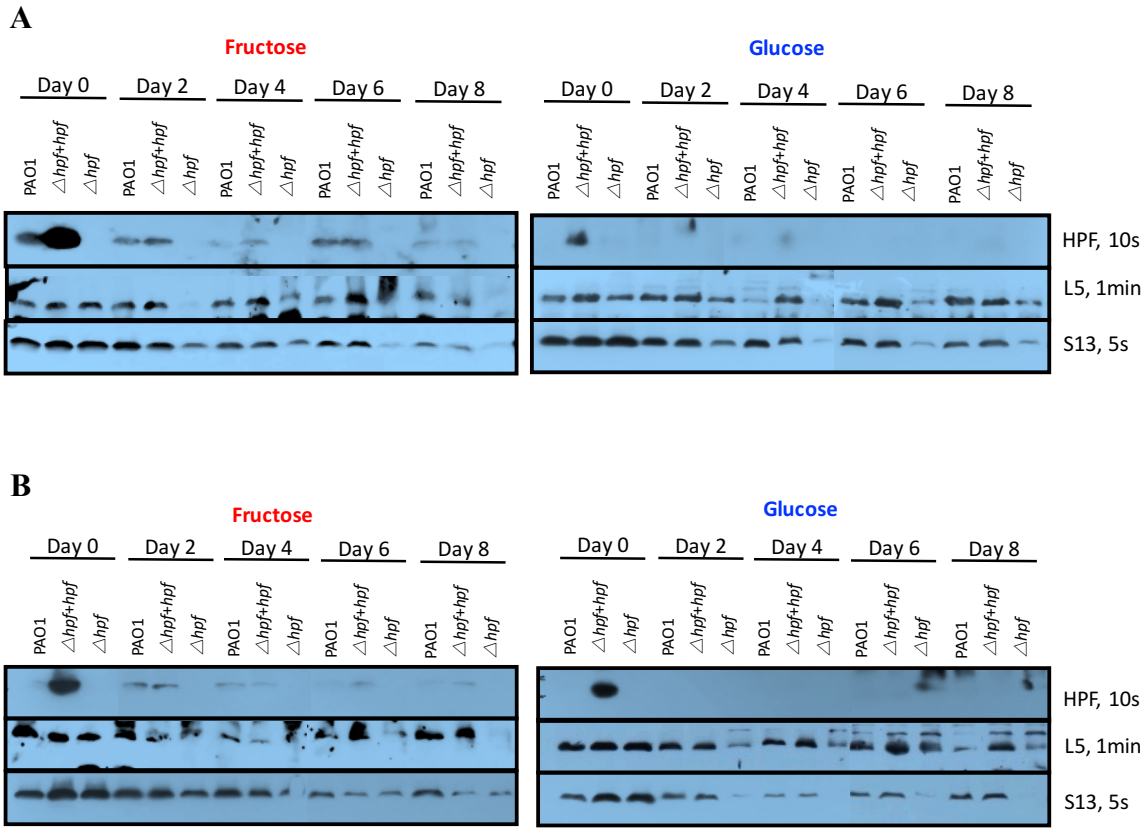
HPF, L5, AND S13 IMMUNOBLOTTING OF PAO1, *Δhpf+hpf*, AND *Δhpf*, STRAINS GREW FROM MOPS-OD~1.0 OVER EIGHT DAYS OF STARVATION



HPF, L5, and S13 immunoblotting of PAO1, *Δhpf+hpf*, and *Δhpf* strains grew on MOPS-fructose and MOPS-glucose OD~1.0 over eight days of starvation. Immunoblot analysis: first to third row represent anti-HPF, L5, and S13 blots of PAO1, *Δhpf+hpf*, and *Δhpf* following eight days of starvation. 15µg proteins were loaded on SDS-PAGE. (A), (B), and (C) represent replicate one, two, and three, respectively.

APPENDIX F

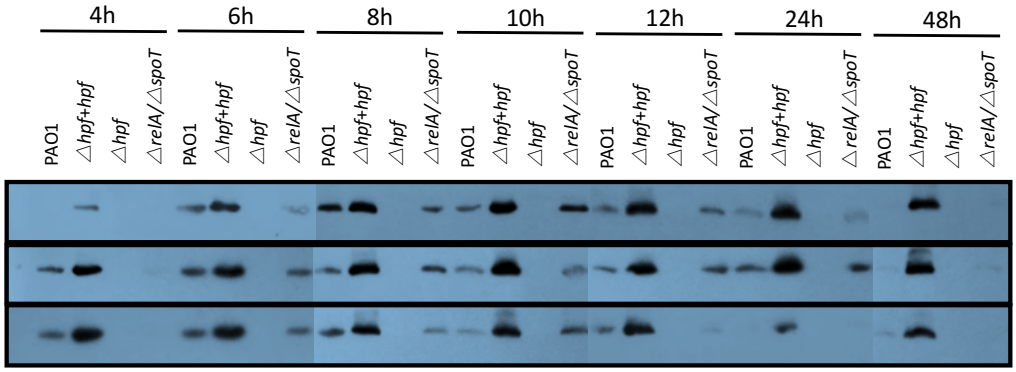
HPF, L5, AND S13 IMMUNOBLOTTING OF PAO1, *Δhpf+hpf*, AND *Δhpf* STRAINS, GREW FROM MOPS-OD~0.3 OVER EIGHT DAYS OF STARVATION



HPF, L5, and S13 immunoblotting of PAO1, *Δhpf+hpf*, and *Δhpf* strains grew on MOPS-fructose and MOPS-glucose OD~0.3 over eight days of starvation. Western blot analysis: first to third row represent anti-HPF, L5, and S13 blots of PAO1, *Δhpf+hpf*, and *Δhpf* following eight days of starvation. 15µg proteins were loaded on SDS-PAGE. (A) and (B) represent replicate one and two, respectively.

APPENDIX G

HPF IMMUNOBLOTTING OF PAO1, *Δhpf+hpf*, *Δhpf*, AND *ΔrelA/ΔspoT* STRAINS OVER 48 HOURS OF GROWING IN TSB



HPF immunoblotting of PAO1, *Δhpf+hpf*, *Δhpf*, and *ΔrelA/ΔspoT* strains over 48 hours of growing in TSB. Immunoblot analysis: first to third row represent replicate one, two, and three, respectively.