Characterization of the global regulatory network of *trans*-translation in *Pseudomonas aeruginosa*

By

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Abstract

Recycling stalled ribosomes is a bioenergetically important process for survival and propagation of bacteria. The primary ribosome rescuing mechanism in bacteria is *trans*-translation which is catalyzed by transfer messenger RNA (tmRNA), a hybrid RNA that contains a tRNA-like domain (TLD) and a mRNA-like domain (MLD). In addition to tmRNA, some bacteria have developed alternative ribosome recycling mechanisms (Arf for alternative ribosome recycling function) of which ArfA is the best characterized. Previous data from the Suh laboratory have shown that tmRNA is required for optimal stress response and production of several virulence factors in the opportunistic human pathogen *Pseudomonas aeruginosa*. To further elucidate the role of *trans*-translation on physiology of *P. aeruginosa*, I performed phenotype analyses to identify global effect of tmRNA. My data indicate that *trans*-translation affects the persister-cell formation, biofilm formation, and metabolism of certain fatty acids, L-valine, and N-acetyl-D-Mannosamine in *P. aeruginosa*. In addition, I identified a putative *arfA* homologue and discovered that *P. aeruginosa* requires at least one of the two ribosome recycling mechanisms, *trans*-translation and ArfA, to maintain viability. Previous data from the Suh laboratory also suggested existence of a regulatory circuit between the general stress response regulator, RpoS, and tmRNA. I expanded on these initial findings and discovered a complex regulatory circuit between tmRNA and several other global regulators including Las and Rhl mediated cell-cell communication systems, in addition to RpoS. According to my data, optimal transcription of *lasR, rhlR,* and *rpoS* require tmRNA and, in turn, each of the

three global regulators are required for optimal transcription of *ssrA*, the structural gene that encodes for tmRNA. Interestingly, this complex regulatory circuit is all at the level of transcription even though *trans*-translation is involved in elongation of the translation machinery. I solved this mystery by discovering that translation of the *relA* mRNA which encodes for (p)ppGpp synthethase requires *trans*-translation. The alarmone, (p)ppGpp, has been demonstrated to be important for regulation of cell-cell communication as well as general stress response. Thus, my data suggest that tmRNA regulation of expression of *las, rhl,* and *rpoS* is likely to be indirect through its role in translation of *relA* mRNA. To complete the regulatory circuit, I discovered that expression of *ssrA* is likely to be modulated by (p)ppGpp. Thus, my data suggest that complex phenotype of a *trans*-translation defective mutant (Δ*ssrA*) is likely due to the pleiotropic effect regulated by (p)ppGpp level in the cell. Finally, I performed a transposon mutagenesis to isolate other putative regulators of *ssrA* expression in *P. aeruginosa*. From insertion mutants with altered *ssrA*::*lacZ* expression, I identified seven genetic loci that are involved in adaptive resistance to antimicrobials, glycine metabolism, RNA processing, and potential glycerol and phosphate metabolisms. These results indicate that regulation of *trans*-translation is complex in *P. aeruginosa*. In summary, my data presented in this dissertation demonstrate the importance of *trans*-translation in the physiology and virulence of *P. aeruginosa*.

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Chapter 1

Literature Review

Introduction

Bacteria are found in almost every environmental niche including air, water, soil, microbiota in animals, and infections in both plant and animals. This ubiquity is attributed to their ability to rapidly divide during optimal growth and to mount efficient responses to various stresses (1). During growth, approximately 50% of the bacterial cellular energy is devoted to protein synthesis of which 20%-40% is devoted to synthesis of ribosome and translational machinery (2). Bacteria, including human pathogens, continuously encounter environmental alterations in nutrients, temperature, osmolarity, oxidative stress, host immunity, and toxic compounds such as antibiotics (3). To survive different niches, bacterial cells must quickly respond to challenges by producing new proteins (2). Protein synthesis is highly regulated at initiation, elongation, termination, and post-translation. During elongation of translation, ribosomes can become stalled as a result of truncation of 3'-end of mRNA (2). Given that bacterial mRNAs are translated by polysomes and so much energy is devoted to synthesis of translation machinery, stalled ribosomes represent a significant energy drain for the cell and affect its viability. Thus, rescuing stalled ribosomes is essential. In order to overcome the devastating effect of stalled ribosomes, bacteria have evolved multiple mechanisms to rescue ribosomes: *trans*-translation, ArfA,

and ArfB (2). Of the three, *trans*-translation is the primary ribosome rescue mechanism and it is found in all bacteria that have been characterized to date.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that can cause severe infections in airways, urinary tracts, the blood stream, and several organs in people with compromised immune systems (4). This bacterium possesses a large genome of approximately 6.3 million nucleotides that contains a large number of genes involved in regulation of gene expression, nutrient acquisition and metabolism, countering various environmental stress (5). Due to its genetic makeup, *P. aeruginosa* is able to metabolize a variety of nutrients and is found almost ubiquitously in nature. Therefore, *P. aeruginosa* is a great model for studying bacterial physiology and pathogenesis.

Previous data from our laboratory demonstrated that mutants defective for *trans*-translation are less adaptive to heat shock and osmotic stress (Suh and Wu, unpublished data). In addition, tmRNA mediated *trans*-translation was required for maximum production of several virulence factors, including elastase, LasA protease, and pyoverdine (Suh and Tucker, unpublished data). Our laboratory has also identified a putative regulatory circuit between RpoS, the mediator of the general stress response, and tmRNA. These data suggested an important role of ribosome rescue on *P. aeruginosa* physiology. In this study, I demonstrate the existence of a complex regulatory network of global regulators of gene expression involving ribosome rescue that governs various aspects of *P. aeruginosa* physiology and pathogenesis.

Pseudomonas aeruginosa

P. aeruginosa is a gram-negative and facultative anaerobic bacterium that is ubiquitous in both nature and nosocomial environments. This bacterium is one of the most important opportunistic pathogens and causes both acute and chronic infections in

immunocompromised individuals (4). The ability of *P. aeruginosa* to survive in various environments, from food-limited soil and water to medical facilities to hostile settings within a host, is due to its metabolic versatility and rapid response to environmental challenges (4). Approximately 10% of its 6.3 million nucleotide genome (Fig. 1.1; adopted from http://v2.pseudomonas.com/index.jsp) is devoted to regulatory genes that are involved in mediating important physiological functions including utilization of a wide variety of nutrients, stress response, and pathogenesis (4, 6). Furthermore, the intrinsic resistance of *P. aeruginosa* to antibiotics makes it a dangerous "superbug" that can cause high-mortality infections for which there are limited efficient therapeutic strategies (7).

The pathogenicity of *P. aeruginosa* is due to the production of many virulence factors, its innate resistance to antimicrobials, and special strategies that are used to form different infections (4, 7). For strains that can cause acute infections, many virulence determinants including flagella, fimbriae, and type III secretion system (T3SS) with the ability to cause inflammatory reactions and damage to the host tissues, are used as strong weapons in their pathogenesis (4). Flagella provide *P. aeruginosa* with the swimming motility and act as adhesins during the development of acute infections (4). Type IV fimbriae play an important role for *P. aeruginosa* in performing twitching motility and biofilm formation (8). T3SS is a key apparatus for *P. aeruginosa* to translocate toxins directly into host cells from bacterial cells through pores formed in the host cell membrane (9). The injected toxins that have been identified in *P. aeruginosa* include ExoY, ExoS, ExoT, and ExoU (9). ExoS that inhibits host translation elongation factor 2 and ExoU that acts as phospholipase are the two major factors that can result in serious toxicity to host cells (9, 10). In addition, multiple proteases secreted by *P. aeruginosa* lead to its increased resistance to immune responses and damage to the host tissues during infections (4). For instance, elastase (LasB) can degrade host surfactant proteins A and D that have opsonizing

activity, allowing *P. aeruginosa* to be less susceptive to phagocytosis (11). Pyocyanin, a redox-active phenazine compound that gives rise to the blue-green color of *P. aeruginosa* cultures, is a member of OxyR regulon that is active against reactive oxygen species generated from the host as well as generating ROS as a part of *P. aeruginosa*'s defense against other organisms (12). Pyoverdine is a siderophore required for iron acquisition for *P. aeruginosa* during infections (4). Production of pyoverdine is correlated to increased production of several virulence other factors including as exotoxin A that inactivates the host translation elongation factor-2 and endoprotease (13). Lipopolysaccharide (LPS) is another important virulence factor that stimulates inflammatory reactions and directly interacts with antimicrobial compounds (14). Lipid A, with endotoxin activity, is a crucial component of LPS that anchors the LPS to the outer membrane, and antimicrobial-induced modification of this component can lead to adaptive resistance to the corresponding antimicrobial in *P. aeruginosa* (15). In contrast to acute isolates, *P. aeruginosa* isolated from chronic infections have lost most of the virulence factors present in acute infections. For example, chronic-infection isolates show decreased production of flagella and fimbriae (16). In addition, these strains are more inclined to form biofilms or over-produce alginate, consistent with the different growth conditions found in chronic infections (10, 17). Thick biofilms, formed during chronic infections of Cystic Fibrosis (CF) patients, are believed to be responsible for enhanced resistance to antibiotic treatments (4). Cells within a biofilm undergo altered gene expression patterns in which several antibiotic resistance systems and stress response mechanisms such as RpoS are positively regulated (4), while some virulence factors used in acute infections are repressed (18). Additionally, *P. aeruginosa* cells with swarming motility have been shown to have a special lifestyle that is more virulent and resistant to various antibiotics (7).

In *P. aeruginosa*, several global regulators of gene expression, including cell-cell communication systems, Vfr, GacA, and RpoS confer advantages for stress tolerance and pathogenesis (4, 7, 19). *P. aeruginosa* possesses multiple cell-cell communication systems that are regulated in a hierarchical manner (20). On the top of the cell-cell communication systems is the LasRI system which positively controls both the RhlRI and PQS systems (20). Inducer signals for Las and Rhl are acyl homoserine lactones (AHLs) 3-oxo-C12 HSL and C4 HSL, respectively, and PQS regulation involves a *Pseudomonas* quinolone signal (20). Interplay between Las, Rhl, and PQS cell-cell communication systems fine tune regulation of stress response, biofilm formation, and pathogenesis of *P. aeruginosa* (20, 21). Vfr is a regulator of exotoxin A, type IV fimbriae, and *lasR*, in *P. aeruginosa* (22). Vfr is a CRP homologue that binds cAMP to interact with promoters to control gene expression (22). However, Vfr has been demonstrated to possesses affinity to cyclic-di-GMP, which may alter Vfr's interaction with target promoters (22-24). RpoS is the global regulator of the general stress response that confers enhanced survivability of cells to stresses including scarcity of food, osmolarity and temperature changes, and oxidizing agents (25). In *P. aeruginosa*, RpoS is required for resistance against heat shock, osmotic stress, H_2O_2 stress, and production of virulence factors such as alginate and Type IV fimbriae (3). In addition to proteins, a bacterial secondary messenger (p)ppGpp plays a crucial part in *P. aeruginosa* physiology by mediating stringent response that is required for persistence and expression of other global regulators including LasR, RhlR, and RpoS (20, 26, 27). RelA and SpoT are the major enzymes that modulate the (p)ppGpp metabolism in bacteria (26, 28). Based on the information available from literature, a regulatory network of these global regulators can be assembled for *P. aeruginosa* (19).

Resistance of *P. aeruginosa* to a wide variety of drugs is due to its intrinsic resistance, acquired resistance, and adaptive resistance (7). There are multiple layers to

the intrinsic resistance for *P. aeruginosa*. The bacterium has a special outer membrane with low permeability for antibiotics (29). Additionally, *P. aeruginosa* possesses effective efflux pumps, such as MexAB-OprM and MexXY-OprM, that prevent antibiotics from reaching their active sites (30). *P. aeruginosa* can also acquire resistance through horizontal gene transfer and mutations (7). It has been noted that cells growing within biofilms undergo both a higher rate of mutations and horizontal gene transfer (7, 31). In *P. aeruginosa*, some antimicrobial agents (*i.e.* polymyxin B) can trigger the two-component regulatory systems (TCRs) ParRS and CprRS which in turn activate expression of *arnBCADTEF* operon, resulting in a modification of the lipid A group of LPS and impairing the ability of LPS to take up the corresponding antimicrobial agents (15). Additionally, low-Mg^{2+} conditions have been demonstrated to induce two TCRs, PhoPQ and PmrAB, that control alteration of LPS in the same manner as ParRS and CprRS (32). Furthermore, other factors, including overproduction of alginate, confer the ability of *P. aeruginosa* to be resistant to various antibiotics (7). *P. aeruginosa* in biofilms has also been shown to become tolerant to drugs by inducing the stringent response against oxidative stress caused by antimicrobials (27). These multiple mechanisms contribute to the inefficiency of antibiotic therapies for treating *P. aeruginosa* infections, especially for chronic infections. Thus, development of more effective therapeutic strategies against *P. aeruginosa* is imperative.

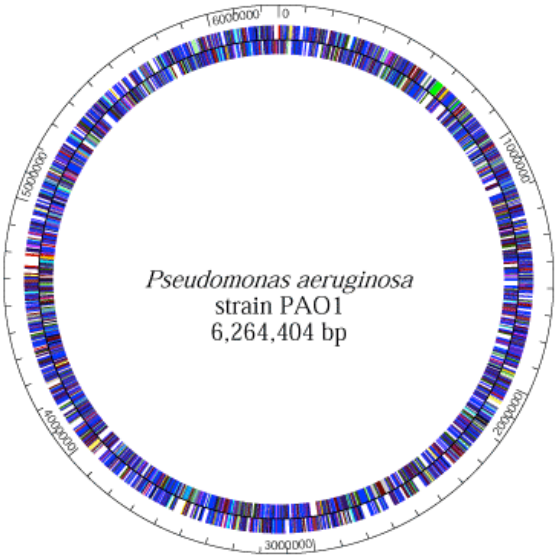

Figure 1.1. Genome of *P. aeruginosa* strain PAO1 (adopted from http://www.pseudomonas.com/index.jsp**).**

tmRNA and *trans*-translation

Coupling of transcription and translation enhances the ability of bacteria to respond to environmental stresses by minimizing the time required to produce new proteins (33). Unfortunately, bacterial mRNAs lack the 3'-poly A tail that protect the eukaryotic mRNA. Therefore, bacterial mRNA can be damaged by early termination of transcription, physical or chemical damage, and exonuclease activity (33). Translation of truncated mRNAs without a stop codon result in stalled ribosomes that cannot be released because there is no nonsense codon in the A site that can be recognized by ribosome release factors (33). Amino acid starvation, presence of rare codons, or weak stop codons also lead to stalled ribosomes followed by an A-site cleavage of the mRNA (2). Together with the mRNA and peptidyl-tRNA, the stalled ribosome forms a nonstop translation complex (2, 33, 34). Studies in *E. coli* showed that a single ribosome on an average stalls in approximately 5 nonstop translation complexes during one cell cycle (35). Thus, without proper rescuing mechanisms, ribosome pool in the cell would rapidly dwindle and protein synthesis would cease in bacteria. The tmRNA mediated *trans*-translation is a unique bacterial system through which bacteria can rescue the stalled ribosome for recycling degrade the damaged mRNA and incomplete peptide (2). *trans*-translation is the main ribosome-rescue system that has been found in all bacteria and its catalytic core of tmRNA-SmpB is highly conserved (2). Due to the importance of ribosome recycling, tmRNA and SmpB are required for viability for bacteria that rely predominantly on *trans*-translation including *Neisseria gonorrhoeae*, *Shigella flexneri*, and *Haemophilus influenzae* (33).

The structure of tmRNA has been studied extensively at biochemical level (Fig. 1.2; (36)). tmRNA is a unique hybrid RNA and contains both a tRNA-like domain (TLD) with the acceptor arm and TΨC arm mimicking tRNAs and an mRNA-like domain (MLD) that contains a reading frame for translation portion of *trans*-translation (33, 36). The lack of

the anticodon stem loop of tmRNA is mitigated by interaction with its cognate partner SmpB (34, 36). The TLD of tmRNA is charged only with alanine because its CCA-tailed acceptor arm includes a G:U wobble base pair that is specific to alanyl-tRNA synthetase (37). In addition, tmRNA has 11 conserved basepair regions (P1-P4 and P6-P12), 8 of which are involved in making 4 pseudoknots (P3-P4 for Ψ1, P6-P7 for Ψ2, P8-P9 for Ψ3, and P10-P11 for Ψ4; (36)). Pseudoknots are believed to contribute to the stiffness and resistance to nucleases of RNAs (38). Additionally, functions of tmRNA pseudoknots include interaction with the ribosomal protein S1 and maturation of tmRNA (39). The MLD of tmRNA is an open reading frame (ORF) that starts with a resume codon (GCA for *E. coli* and GCC for *P. aeruginosa*) and ends with a functional stop codon. The MLD encodes specifically for a peptide sequence tag (ANDENYALAA in *E. coli* and ANDDNYALAA in *P. aeruginosa*; (36)) for recognition and proteolysis by a number of cellular proteases. During *trans*-translation, SmpB binding to tmRNA is essential for interaction between the tmRNA and the ribosomal decoding center and mRNA channel (40, 41).

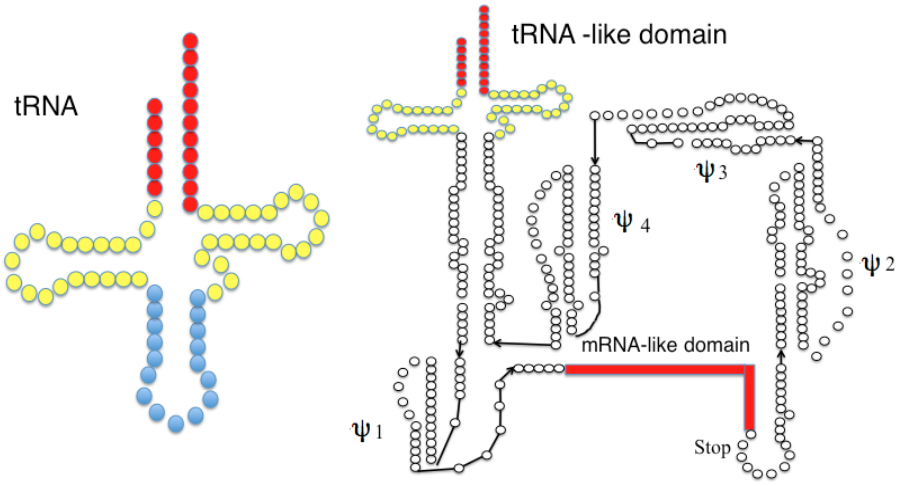

Figure 1.2. Structure of tRNA and *E. coli* tmRNA (Adopted from Wu's dissertation).

Occurrence of mistakes during transcription and translation can result in nonstop translation complexes that are substrates for *trans*-translation (34). Since translation initiation in bacteria does not require an intact 3' end of the mRNA transcript like it is in eukaryotes, bacteria lack mechanisms to ensure the mRNA is fully formed during translation (42). Therefore, truncated mRNAs due to physical and chemical damage and degradation by RNases occur with high frequency in bacterial cells (33, 43). Additionally, transcripts may be prematurely released before transcription of the stop codon (33). Furthermore, translation of an ORF with frameshift or translation that reads through a stop codon can also cause nonstop translation complexes (33). Ribosome stalling that occurs during translation elongation or termination can also be caused by various events, including amino acid starvation, presence of rare codons or weak stop codons, and release factor limitation (2, 33). However, only prolonged ribosome stalling leads to A-site cleavage and therefore a target of *trans*-translation (2, 44). The empty A-site within a ribosome is likely a strong signal for tmRNA-SmpB complex to differentiate between stalled ribosomes from those functioning normally (2).

A model of *trans*-translation is described in Figure 1.3 (33). During *trans*-translation, SmpB-bound tmRNA is aminoacylated with alanine by alanyl-tRNA synthetase, and then the alanyl-tmRNA-SmpB complex is bound by EF-Tu:GTP to form a ternary complex in a manner similar to functional amino acid-charged tRNAs. The $tmRNA^{ala}$-SmpB-EF-Tu:GTP ternary complex recognizes a stalled ribosome and enters the A site. The nascent polypeptide is then transferred to the alanine of $tmRNA^{ala}$, followed by translocation of peptidyl-tmRNA-SmpB from the A site to the P site. During translocation, the stalled ribosome switches from the original mRNA to the MLD of tmRNA with the help of the 30S head of the ribosome to resume translation (33). The original mRNA is released and degraded. Finally, translation termination occurs at the stop codon of the 3'-

end of the MLD of tmRNA and results in rescuing of ribosomes. Because the ORF of MLD encodes for a peptide tag recognized by proteases, the truncated nascent peptide is degraded (34, 45, 46).

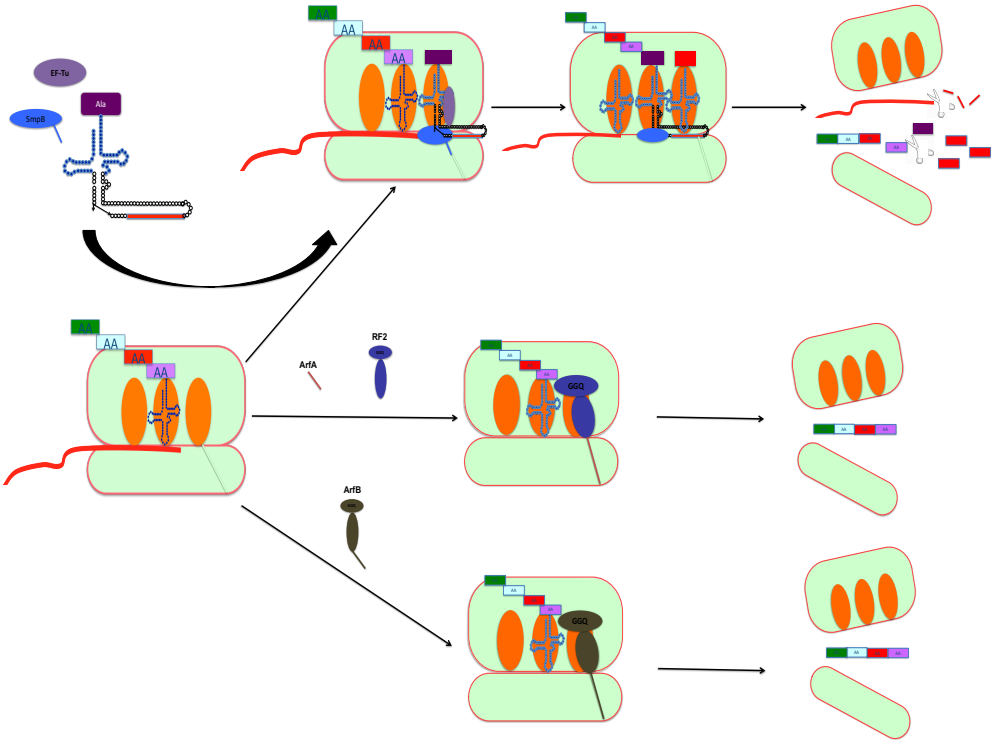

Figure 1.3. Schematic of three ribosome-rescue pathways: *trans*-translation, ArfA, and ArfB (Adopted from (33)).

Role of *trans*-translation in bacterial physiology

The tmRNA mediated *trans*-translation has been demonstrated to be important for regulation of gene expression and physiology including stress response, pathogenesis and cell cycle control in some bacteria (33). The best characterized example of *trans*-translation regulating gene expression is demonstrated in expression of the *E. coli lac* operon (47). The *lac* operon is repressed by the tetramer of LacI that binds to operators O_1 and O_3 of the operon to prevent its expression. Because O_3 is part of the *lacI* gene, LacI binding to O_3 results in a truncated *lacI* mRNA that forms nonstop complex for *trans*-translation for degradation (47). Thus, regulation by *trans*-translation controls the concentration of LacI in the cell and governs catabolism of lactose as carbon source (47). Several other roles of *trans*-translation in physiology have also been demonstrated in several bacteria. As explained above, *trans*-translation is essential for viability of bacteria that only possess this as the sole mechanism of ribosome rescue including *N. gonorrhoeae*, *S. flexneri*, and *H. influenzae* (48-50). In *E. coli*, mutants lacking *ssrA* or *smpB* show defects in response to heat shock stress (51), amino acid starvation (52), and antibiotic treatments (53). Phenotype of *E. coli* mutants deleted for *ssrA* include defects in stress response during stationary phase, indicating that RpoS-mediated general stress response is affected (54). In *P. aeruginosa* Δ*ssrA* mutant, *rpoS* expression is decreased by approximately 50% (Suh and Wu, unpublished data). tmRNA has also been demonstrated to be important for virulence of some pathogens including *Salmonella enterica*, *Streptococcus pneumoniae*, *Yesinia pseudotuberculosis*, and *Yesinia pestis* (33, 50, 55, 56). In *Y. pseudotuberculosis*, translation of VirF, a transcriptional regulator that controls production and secretion of the Yops virulence factors, is dependent on tmRNA (55). tmRNA has also been demonstrated to be required for progression of cell cycle in *Caulobacter crescentus* (57). *C. crescentus ssrA* mutants perform delayed initiation of DNA replication and in the wildtype, the level

of *ssrA* expression is changed throughout the cell cycle (57). My colleagues Wu and Tucker have initially characterized the role of *trans*-translation in physiology of *P. aeruginosa*, and their studies suggest that *P. aeruginosa ssrA* mutants are defective in heat shock and osmotic stress response (Fig. 1.4) and optimal production of several virulence factors (Table 1.1), including LasA protease and pyocyanin. In addition, our previous data suggest a regulatory circuit between tmRNA and RpoS (Fig. 1.5).

Table 1.1. Effect of *trans*-translation on production of several exoproducts in *P. aeruginosa*[a]

	Relative Activity	
Exoproduct	**The wild type**	**The Δ*ssrA* mutant**
Elastase	100	62
LasA protease	100	59
Casein degrading protease	100	86
Pyoverdine	100	77
Pyocyanin	100	156
Rhamnolipid	100	124

[a]Elastase activity was assessed as the rate of hydrolysis of elastin Congo Red (58). LasA activity was measured as the ability to hydrolyze *Staphylococcus aureus* (59). Azocasein proteolysis assay was performed to detect multiple casein degrading proteases including elastase and alkaline protease (3). Pyocyanin was extracted from the supernatant of overnight cell cultures using $CHCl_3$ and the amount of pyocyanin was measured as OD_{695}. The level of pyoverdine was determined as the fluorescence of pyoverdine by excitation at OD_{400} (60). The level of rhamnolipid collected from the supernatant of overnight cell cultures was measured as OD_{625} by conducting anthrone assay (61). All of these assays were performed with the wild type and Δ*ssrA* mutant. Relative activity was calculated as follows: (production of the wild type or Δ*ssrA* mutant/production of the wild type) × 100.

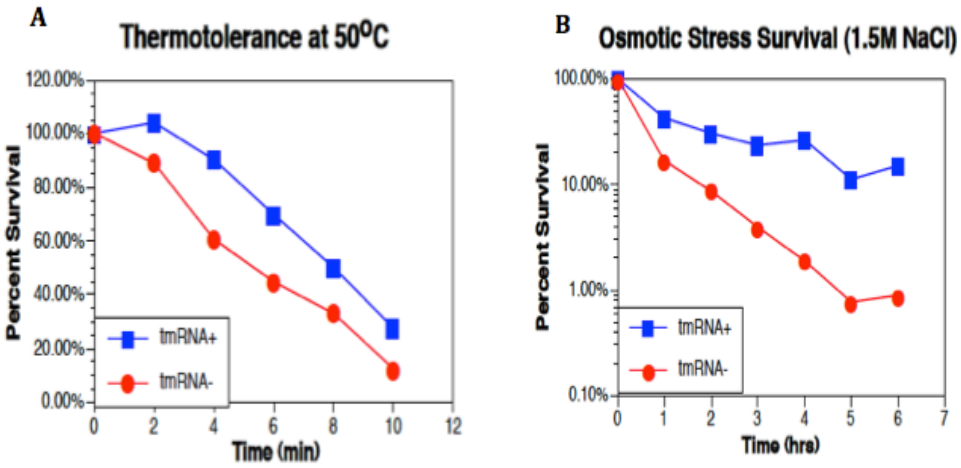

Figure 1.4. Effect of *trans*-translation on thermal and osmotic stress response in *P. aeruginosa*. To assess sensitivity of *P. aeruginosa* strains to thermal stress (A), overnight-grown cells were washed and transferred to prewarmed test tubes. Aliquots removed from cultures exposed to 50°C for 0, 2, 4, 6, 8, and 10 minutes were plated on LB plates for CFU counting to determine the number of viable cells. Viability at each time point is shown as percent survival that means percentage of the number of CFU at time 0. To test sensitivity of *P. aeruginosa* strains to hyperosmolarity (B), overnight-grown cells were washed, resuspended in either NCE or NCE supplemented with 1.5M NaCl, and incubated at 37°C with aeration. Aliquots removed from cultures at 0, 1, 2, 3, 4, 5, and 6h were plated on LB plates for CFU counting. Viability is shown as percent survival. Time 0 stands for the point right after cell resuspension in 1.5M NaCl. Both assays were performed with the wild type and *ssrA* mutant.

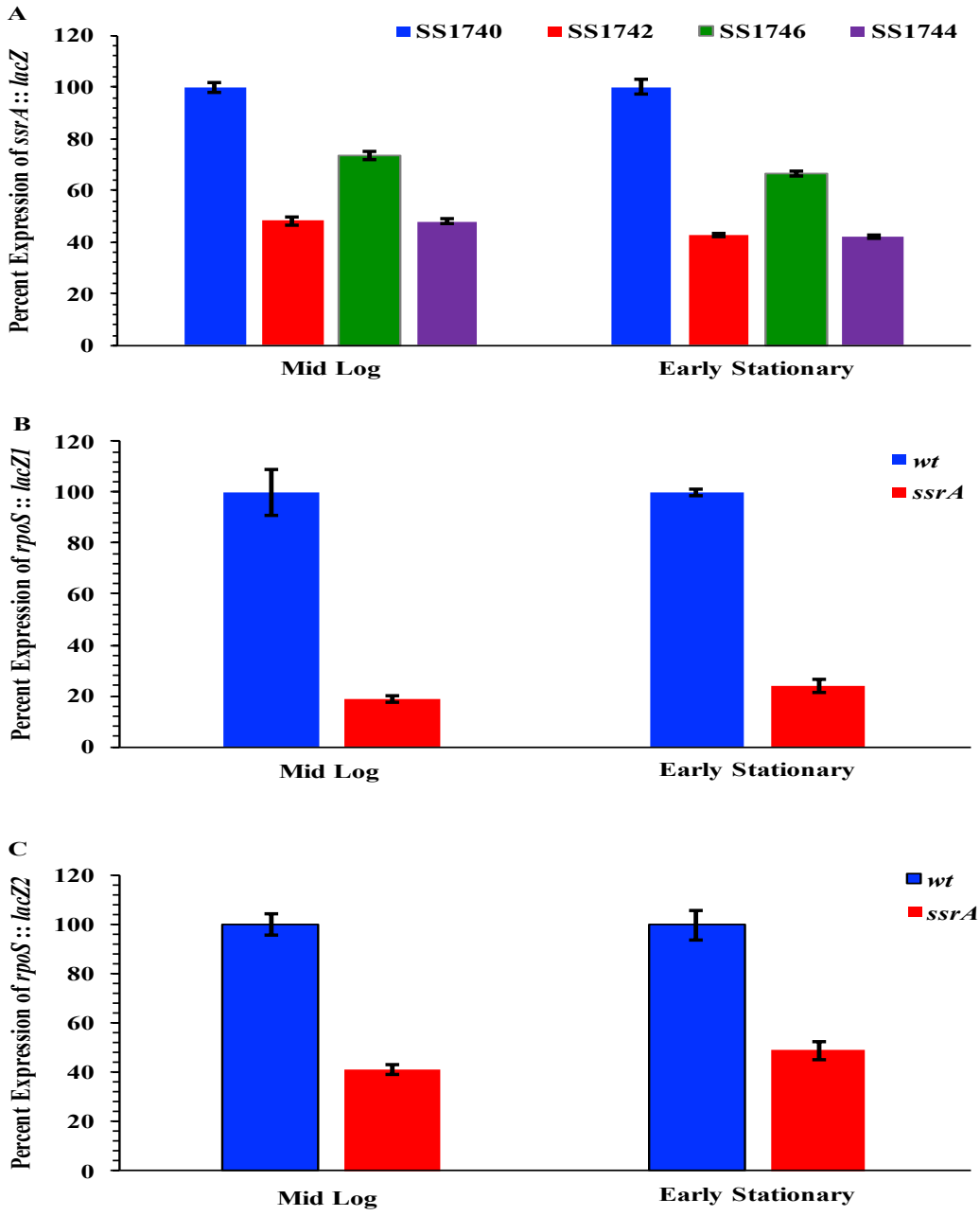

Figure 1.5. The regulatory circuit between *trans*-translation and RpoS in *P. aeruginosa*. (A) RpoS is required for the full expression of *ssrA*. SS1740: the wild type; SS1742: the *rpoS* mutant; SS1746: the complemented *rpoS* mutant; SS1744: the *rpoS* mutant with negative vector control. (B) tmRNA positively regulates *rpoS* expression at transcriptional level. (C) tmRNA positively regulates *rpoS* expression at translational level. Detailed methods used for construction of different gene::*lacZ* fustions are shown in the Materials and Methods of Chapter 3. Bacteria were grown at 37ºC with aeration in LB

supplemented with carbenicillin (100 μg/ml) to mid-log phase (OD_{600} of ~0.8-1.0) and early stationary phase (OD_{600} of ~2.0). The complemented strain (SS1746) and negative vector control (SS1744) were additionally supplemented with tetracycline (50 μg/ml) to prevent the loss of plasmid. β-galactosidase assay was performed according to Miller (62) in duplicate.

Alternative ribosome-rescue factors (Arf)

Based on the importance of ribosome rescue for bioenergetics, it is not surprising that tmRNA mediated *trans*-translation is essential for viability for some bacteria including *N. gonorrhoeae*, *S. flexneri*, and *H. influenzae* (48-50). However, in other bacteria, including *E. coli* and *Bacillus subtilis,* the phenotype of a Δ*ssrA* mutant is subtle, suggesting the existence of a backup system to rescue the stalled ribosomes (33). In support of this hypothesis, a dedicated redundant system, designated as ArfA, has been identified in *E. coli* (63). ArfA is active only in the absence of *trans*-translation and, consistent with the importance of ribosome rescue, *E. coli* must have either tmRNA mediated *trans*-translation or ArfA medidated ribosome recycling mechanism to maintain viability (63). Interestingly, production of functional ArfA in *E. coli* is controlled by *trans*-translation (64). Under "normal conditions," the *arfA* mRNA is cleaved by RNase III into a nonstop complex that requires *trans*-translation for elongation and subsequent degradation of the truncated protein (64). ArfA recycles ribosomes from nonstop translation complexes by recruiting the release factor 2 (RF2) that hydrolyzes the peptide bond of the peptidyl-tRNA on the stalled ribosomes (Fig. 1.3; (65)). *arfA* has been found in many other proteobacteria, and the available data suggest that *arfA* mRNA is regulated similarly as it is in *E. coli* (66). Interestingly, although the *arfA* gene is found in many bacteria including *N. gonorrhoeae* and *S. flexnieri* (66), it does not appear to be enough to maintain the viability of *ssrA* mutants in some organisms. Mild phenotype of the *P. aeruginosa ssrA* mutants suggests the existence of a potential backup system. A gene that encodes for putative *arfA* is mentioned in Schaub et al. (66) and I describe my efforts to identify a *P. aeruginosa arfA* homologue in Chapter 2. ArfB is another alternative found in *E. coli* with the ribosome-recycling activity (2). Interestingly, ArfB appears to be weakly functional and is unable to sustain the *E. coli* viability in the absence of tmRNA and ArfA (2, 63). In contrast, *C.*

crescentus uses ArfB rather than ArfA as the major backup system for *trans*-translation (2). ArfB recycles ribosomes using its own peptidyl-tRNA hydrolase activity (Fig. 1.3 (2)). It has been suggested that bacteria must possesses one or more of these functional ribosome-rescue systems to be viable (33).

Biofilm formation

During infections, *P. aeruginosa* can form biofilms that are bacterial communities surrounded by a matrix consisting of extracellular polysaccharides, lipids, nucleic acids, and proteins (67). The matrix causes low level penetration of antibiotics and host immune cells to the enclosed bacteria and develops channels for circulation of nutrients and liquids (68, 69). Biofilm formation initiates with irreversible attachment of bacteria to the surface that is mainly mediated by type IV fimbria and flagella, in which cell-cell communication systems Las and Rhl play important roles in regulation (4, 70). Subsequently, the encased bacteria divide to form microcolonies and produce polysaccharides which are basis of the matrix (4). To date, three major kinds of polysaccharides have been characterized for *P. aeruginosa* biofilm formation, and they are alginate, Pel, and Psl, expression of which is tightly regulated by different systems including AlgT, RpoN, and c-di-GMP (71). Biofilms are further maturated with channels and afterwards planktonic cells detach from the community and disperse for another round of biofilm formation (4). Multiple systems, including GacAS and c-di-GMP, are responsible for the transition of *P. aeruginosa* from planktonic to biofilm state. Interestingly, during infections, certain nutrients can stimulate biofilm formation. For instance, glycerol metabolism promotes biofilm formation through activation of Pel synthesis (72). In addition, certain amino acids, such as arginine and valine, trigger biofilm formation through the c-di-GMP system (73). Since bacterial cells within biofilms experience stress including nutrition limitation and oxygen deprivation, stress

response systems (p)ppGpp and RpoS also play important parts in *P. aeruginosa* biofilms (27, 74). Once established, biofilms are highly resistant to antibiotics and immune cells, which results in difficulties for elimination of *P. aeruginosa* infections and thus mortality (4). It has been described that persister cells formed in biofilms cause the inability to totally wipe the pathogen and the reoccurrence of infections (75).

Persister cells

In a bacterial population, a small percentage of cells with a fate of dormancy are known as persister cells and highly tolerant to antimicrobials (75). Different from most of the antibiotic-resistant strains, persister cells are genetically identical to the non-tolerant cells (75). Persister cells nearly shut down cellular activities including DNA replication, biosynthesis of cell membrane and cell wall, and protein synthesis, which inhibits the interaction of various antibiotics to their targets and thus the multi-drug tolerance (27). The proportion of persister cells in a bacterial culture increases along the growth phase and peaks from the beginning of stationary phase (76). *In vitro*, both the stationary phase and biofilm cultures contain a larger percentage of persister cells than the log phase cultures (77), indicating that regulation of persister cell formation is dependent on growth rate and some other environmental cues. It is believed that toxin-antitoxin modules primarily mediate persister cell formation in bacteria (78). Type II toxins can cause large amounts of mRNA cleavages to arrest bacterial cell growth (78). Maisonneuve and colleagues modelled that (p)ppGpp promotes the accumulation of inorganic polyphosphates that direct the Lon protease to antitoxins for degradation, which results in the upregulation of toxins (79). In *P. aeruginosa*, (p)ppGpp is required for persister cell formation during stationary phase and oxidative stress, as well as within biofilms (27, 80).

(p)ppGpp and stringent response

In bacteria, secondary messengers, (p)ppGpp, cAMP, and c-di-GMP, are extensively used as cellular signals for rapid reprogramming of the genetic expression pattern to mount an effective response to environmental alterations (81). In most bacteria, formation of (p)ppGpp from GDP or GTP can be catalyzed by RelA or SpoT, two enzymes with highly conserved domains (28). RelA is the major enzyme for (p)ppGpp synthesis, while SpoT is important for modulating (p)ppGpp level via degradation as well as synthesis (28). RelA-mediated (p)ppGpp production is induced by amino acid starvation or heat shock while SpoT is stimulated by carbon, iron, or fatty acid starvation (28, 82). Cellular (p)ppGpp accumulation promotes the stringent response by which expression of rRNA genes is downregulated to reduce protein synthesis and, thus, the growth rate (28, 82). In contrast, (p)ppGpp activates the general stress response and oxidative stress response in *P. aeruginosa* (80).

(p)ppGpp regulation of transcription

The most common mechanism of transcriptional regulation by (p)ppGpp is the destabilization of the transcription open complex of RNA polymerase and the promoter (83). This activity requires the participation of DksA (83). (p)ppGpp also mediates transcription indirectly by binding to RpoD to inhibit its binding to the core RNAP and thereby making the core enzyme more available for alternative sigma factors (84). Finally, (p)ppGpp indirectly induces *rpoS* transcription through elevating polyphosphate levels in *E. coli* (85).

Role of (p)ppGpp in *P. aeruginosa* physiology and pathogenesis

Through its effect on formation of RNAP open complex and inhibition of RpoD, (p)ppGpp has been demonstrated to be important for various aspects of cellular physiology. In *P. aeruginosa*, (p)ppGpp controls expression of two cell-cell communication systems, LasR and RhlR, and accumulation of (p)ppGpp leads to advanced production of Las and Rhl-controlled virulence factors (86). In contrast, the *P. aeruginosa relA spoT* double mutant that synthesizes no (p)ppGpp shows dysregulated biofilm formation and sensitivity to antibiotics as a result of 4-hydroxy-2-alkayquinolines (HAQs) caused promotion of oxidative stress (27). Consistent with results for HAQs, (p)ppGpp represses biosynthesis of pseudomonas quinolone signal (PQS) (87). In addition to amino acid starvation, low magnesium concentration positively regulates *relA* expression (88) in *P. aeruginosa*, which indicates that (p)ppGpp is required during infections in which magnesium concentration is limited. Finally, through its effect on the availability of core RNAP, (p)ppGpp regulates processes controlled by alternative sigma factors including production of pyocyanin and alginate, synthesis of pili, and metabolism of amino acids in *P. aeruginosa* (84).

Summary and Importance

Available data suggest an important role of ribosome rescue mechanisms in bacteria. The effect of *trans*-translation *P. aeruginosa* physiology has been initially characterized in our laboratory. The preliminary data suggested that *trans*-translation generally affect stress response and production of virulence factors in *P. aeruginosa* through mediating expression of regulators such as RpoS. Because stress response and pathogenesis is co-regulated by a fine regulatory network in which cell-cell communication systems and (p)ppGpp play crucial roles (65), elucidation of the relationship between *trans*-translation

with other global regulators of gene expression including LasR, RhlR, and (p)ppGpp is likely to shed a light on understanding the importance of *trans*-translation in *P. aeruginosa*.

In this study, the overall goal is to further characterize *trans*-translation and other potential ribosome rescue systems in *P. aeruginosa*. To accomplish this goal, I continued characterizing *trans*-translation by determining its effect on formation of persister cells, biofilm formation, and metabolism of nutrients. I also identified the *P. aeruginosa* ArfA system. In addition, I uncovered the existence of a complex regulatory circuit between *trans*-translation and various global regulators of gene expression. Finally, I isolated putative regulators of *ssrA* expression using non-predictable approaches in *P. aeruginosa*.

Given that *P. aeruginosa* is highly resistant to a wide spectrum of antibiotics and can be hypermutable to persist, development of novel approaches is imperative via better understanding its physiology. Characterization of the physiological roles of ribosome rescue in *P. aeruginosa* is likely to further the knowledge of mechanisms important for pathogenesis of this pathogen, providing valuable insights in finding new therapeutic strategies in the future.

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Chapter 2

Characterization of *trans*-translation and ArfA in *Pseudomonas aeruginosa*

Abstract

Bacteria possess one or more ribosome-rescue systems to cope with frequently stalled ribosomes on mRNAs during translation. *Trans*-translation, catalyzed by tmRNA, is the main bacterial ribosome-recycle mechanism. In addition to *trans*-translation, some bacteria possess alternative ribosome rescue systems designated as Arf for alternative ribosome rescue function. In *E. coli*, ArfA is the major alternative function for ribosome recycling in the absence of *trans*-translation. Our previous data suggested that tmRNA is required for optimal osmotic and heat shock stress response and affects production of several exoproducts in *P. aeruginosa*. To further characterize the importance of ribosome rescue mechanisms in *P. aeruginosa*, I discovered and performed initial characterization of a putative *P. aeruginosa arfA* (*PA3990*). I discovered that I was unable to construct a Δ*ssrA* Δ*PA3990* double mutant implying that *PA3990* encodes for ArfA. By itself, the Δ*PA3990* mutant showed no demonstrable phenotype except when *ssrA* was not fully expressed. In continuing the characterization of *trans*-translation defective phenotype, I discovered that the Δ*ssrA* mutant formed 40% less persister-cells than the wildtype strain PAO1. For biofilm formation, *trans*-translation had a slight effect on initiation of biofilms and formed 40% more biofilms that did the parent strain as determined via microtiter plate assay. Finally, the Δ*ssrA* mutant was affected for metabolism of certain nutrients including

propionic acid, capric acid, L-valine, and N-Acetyl-D-Mannosamine as determined by the Biolog's phenotypic array. My results suggest a role of *trans*-translation in various aspects of *P. aeruginosa* physiology.

Introduction

Bacteria sense environmental stimuli and respond by reprograming gene expression patterns. The coupled transcription and translation in cytoplasm aids in minimizing the response time (1). Thus, unlike eukaryotes, bacteria can start translation before the mRNA transcript is completely synthesized (2). One consequence of this phenomenon is the stalling of ribosomes on mRNAs lacking a stop codon (1), which could result from ribonucleolytic damages, incomplete transcription, frameshifting, or ribosomal A-site mRNA cleavage from prolonged ribosome pausing during translation elongation (1, 3, 4). In bacteria, stalling of ribosome occurs frequently and can severely impair protein synthesis and affect cell viability if a mechanism for ribosome rescue is not available (1, 5). *Trans*-translation has been found and characterized as the major ribosome-rescue mechanism in all bacteria (for reviews, see reference 1, 3, and 4). During *trans*-translation, tmRNA, a unique small hybrid RNA molecule containing both a tRNA-like domain (TLD) and a mRNA-like domain (MLD) complete with an open reading frame (ORF) and encoded by the *ssrA* gene, is charged with alanine to its TLD and binds to the small protein SmpB to form a complex (6, 7). The charged tmRNA:SmpB protein complex binds to Ef-Tu:GTP and enters the empty A-site of the stalled ribosome and mimics an aminoacylated tRNA to allow transfer of the trapped nascent polypeptide to the alanine charged to tmRNA (1). Following transpeptidation and translocation, the stalled ribosome resumes translation of the ORF on MLD which encodes a tag peptide recognized by cellular proteases (1). Because the ORF on MLD contains a stop codon, the ribosome is released from the RNA and participate in translation of other mRNA and the tagged truncated proteins are degraded to recycle amino acids (1). In addition to the tmRNA-mediated *trans*-translation, ArfA and ArfB have been identified as alternative functional ribosome-rescue mechanisms in some bacteria (1, 8). In *Escherichia coli*, when *trans*-translation is absent or insufficient, ArfA

recognizes the stalled ribosomes and recruits release factor RF2 to hydrolyze the peptidyl-tRNA bond to initiate the release of ribosome (9). ArfB is a RF2 homologue that possesses the ability to hydrolyze the peptidyl-tRNA bond in the stalled ribosomes to participate in ribosome recycling (4). However, ArfB is insufficient to recycle enough stalled ribosome to confer viability in *E. coli* lacking the tmRNA and ArfA (8). Recent studies demonstrate that bacteria must possess at least one functional ribosome-rescue system maintain viability (1).

Trans-translation plays various role in the physiology of bacteria (1). In *E. coli*, *trans*-translation regulates expression of *lacI*, *arfA*, and *rpoS* (at low temperature) and is required for heat shock and stringent stress response (10-14). *Bacillus subtilis ssrA* mutants show defective growths at high and low temperatures (15). In *Caulobacter crescentus*, *trans*-translation is required for correct timing of DNA replication (16). *Yersinia pseudotuberculosis ssrA* mutants are sensitive to antibiotics and oxidative stress. Additionally, they produce less Yops and are less virulent (3, 17).

Pseudomonas aeruginosa is a gram-negative bacterium notorious for its ability to use a variety of nutrients and to resists various environmental stresses. These characteristics allows the bacterium to survive in various environmental niches (18-20). *P. aeruginosa* is also an important opportunistic human pathogen that can cause both acute and chronic infections in immunosuppressed individuals (19, 21, 22). During the establishment of infections, *P. aeruginosa* encounters various stresses, including severe nutritional limitation, alteration in temperature and osmolarity, and reactive oxygen and nitrogen species. However, *P. aeruginosa* is able to survive, colonize, and persist by adapting to the environment (23, 24). Limitation of certain nutrients can trigger stress response and production of virulence factors. For example, amino acid, iron, carbon, or fatty acid starvation leads to accumulation of the alarmone, (p)ppGpp, leading to a variety of cellular

responses including induction of stringent response, oxidative stress response, and general stress response (25-28). The fact that induction of many of these stress responses also result in the production of virulence factors indicate that stress response and production of virulence factors are tightly connected in *P. aeruginosa* (24, 29).

Homologues of *ssrA*, the structural gene encoding tmRNA, and *arfA* have been identified in *P. aeruginosa* genome and sequence analyses suggest structural similarities to the *E. coli* components (4, 30, 31). To elucidate the physiological role of *trans*-translation in *P. aeruginosa*, a Δ*ssrA* mutant was constructed and initial phenotype was assessed. The fact that *P. aeruginosa* Δ*ssrA* mutant is viable indicate that *trans*-translation is not essential for viability of *P. aeruginosa* as it is for *Neisseria gonorrhoeae* and *Shigella flexneri* (4, 32). Our intial characterization of a *P. aeruginosa* Δ*ssrA* mutant demonstrated that *trans*-translation is required for optimal resistance against osmotic and heat shock stress and for optimal production of several virulence factors (Wu, Tucker, and Suh, unpublished results). In addition, a potential regulatory circuit of tmRNA and RpoS, regulator of the general stress response, has been uncovered in *P. aeruginosa*. In this study, I bulit upon our previous data to further characterize the role of ribosome rescue in *P. aeruginosa* physiology.

Materials and Methods

Bacterial, plasmids, media and chemicals

All bacteria and plasmids used for this study are listed in Table 2.1. Unless otherwise indicated, bacteria were grown in L broth (LB; (33); Fisher Scientific, Pittsburgh, PA) or in LB supplemented with appropriate antibiotics at 37°C with aeration. Solid media were prepared using L-agar (BD Biosciences). Pseudomonas isolation agar (PIA; BD Biosciences) was used for tri-parental mating to counterselect *P. aeruginosa* against *E. coli*

as previously described (24). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at following concentrations: ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 100 μg/ml for *P. aeruginosa;* gentamicin, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin, 50 μg/ml for *E. coli*; tetracycline, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*.

DNA manipulations, transformations, and conjugations

For cloning DNA*, E. coli* strain DH10B was routinely used as the host strain. Unless otherwise indicated, DNA was introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (24). Plasmids were purified with QIAprep Spin Miniprep columns (Qiagen, Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (NEB; Beverly, MA). Either *Pfu* polymerase from Stratagene (La Jolla, CA), *Phusion* polymerase (NEB), or *Taq* polymerase (NEB) was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Construction of *P. aeruginosa* mutants

To delete the *ssrA* gene in *P. aeruginosa*, the suicide plasmid pAT1 carrying the Δ*ssrA* allele generated by via spliced overlap extension (SOE) PCR from PAO1 genome (34) was constructed. Oligonucleotide primers used for PCR were SSO-261/F1: 5'-CAT CAT GAG CTC GGC CCC GGC CAT CGC TAA CC-3'; SSO-262/R1: 5'-TCT CTT *TGC ATT GGA TCC AAA ACC TTG ACA* GGT CGA CGA CCA G-3'; SSO-263/F2: 5'-*TGT CAA GGT TTT GGA TCC AAT GCA* AAG AGA TAA GCC CCT G-3'; and SSO-264/R2:

5’-CAT AAG CTT GTC GCG AGA GGT GAT TTT CG-3’. The unique *Sac*I site built into F1 and the unique *Hind*III site built into R2 are highlighted with underline. *Pfu* thermostable DNA polymerase was used to minimize incorporation errors during the amplification. In the first round of PCR, DNA fragments that carry approximately 1000bp upstream of *ssrA* and approximately 1005bp downstream of *ssrA* were PCR amplified from PAO1 genome using primer pairs F1-R1 and F2-R2, respectively. These two fragments overlap with each other by 24 bp (italicized sequences of R1 and F2). For the SOE PCR, two fragments from the first round were used as the templates with F1 and R2 primers to generate the Δ*ssrA* allele.

The Δ*ssrA* allele was then cloned as a *Sac*I-*Hind*III fragment into *Sac*I/*Hin*dIII digested pEX100Tlink (35) to yield pAT1. pEX100Tlink is a suicidal plasmid vector in *P. aeruginosa* and carries the *bla* gene and a counterselectable marker *sacB* gene (Fig. 2.1; (35)). pAT1 was introduced into PAO1 by triparental mating and Cb^r cells with pAT1 integrated into the genome via homologous recombination were isolated. To isolate the deletion mutants that had undergone allelic exchange of the wildtype *ssrA* with Δ*ssrA*, the Cb^r resistant cells were plated on LB agar containing 5% sucrose as previously described (35). Deletion was verified via PCR analysis using internal primers for *ssrA*.

To construct the Δ*arfA* mutant, the suicide plasmid pBL001, carrying the Δ*arfA* allele, was constructed. The Δ*arfA* allele was generated in the same manner as described above for *ssrA*. The oligonucleotide primers used for generation of the Δ*arfA* allele were SSO558/F1: 5’-CAT GGT ACC ACG CCG CGC AGT TCA TCC AG-3’; SSO-559/R1: 5’- *GGA CCA GCG GCT GTT GGC ATA GGT CCT CGA* AGG TGA TCG GGC TGG CGC TC-3’; SSO-560/F2: 5’- *TCG AGG ACC TAT GCC AAC AGC CGC TGG TCC* ATC CTC CCG CAA CGC CCT CC-3’; SSO-561/R2: 5’- CAT AAG CTT TCG GGG TTA CGC ATT GCT CTC CTC-3’. The unique *Kpn*I site built into F1 and the unique *Hin*dIII

site built into R2 are highlighted with underline. The upstream (F1-R1) and downstream (F2-R2) fragments overlap with each other by 30 bp (italicized sequences of R1 and F2). The Δ*arfA* allele was cloned as a *Kpn*I-*Hind*III fragment into pEX100Tlink (35) to yield pBL001. The plasmid pBL001 was conjugated into PAO1 via triparental mating and Δ*arfA* mutant was isolated as described above for Δ*ssrA* mutant.

To construct *P. aeruginosa* with a regulatable *arfA*, a promoter-less *arfA* fragment was PCR amplified using the primers SSO-855 (21 nucleotides upstream from the initiation codon of *arfA*)/F: 5'-CAT GAA TTC TCA CCT TCG AGG ACC TAT GCC AT-3' and SSO-561/R. The unique *EcoR*I site designed within SSO-855 was highlighted with underline. This promoter-less *arfA* fragment was cloned as a *EcoR*I-*Hind*III fragment into pSS213, a suicide plasmid in *P. aeruginosa* with gentamicin resistance (Gm^r) and a regulatable promoter $P_{T7(A1/04/03)}$ (36), to yield pBL052. Subsequently, the m*oriT* cassette (36) was cloned in pBL052 as a *Hind*III fragment to generate mobilizable pBL093. pBL093 was introduced into the *arfA* mutant (SS3178) and *P. aeruginosa* with pBL093 integrated into the genome via homologous recombination at the downstream of *arfA* were isolated as Gm^r colonies to yield SS3017. The Δ*ssrA* allele was then introduced to SS3017 in the presence of IPTG to express *arfA* to construct SS3033 which is a Δ*ssrA* Δ*arfA* double mutant but is viable due to expression of *arfA* from integrated pBL093.

To construct *P. aeruginosa* with a regulatable *ssrA*, a promoter-less *ssrA* fragment was PCR amplified using the primers SSO-1000 (-8 from 5'- of *ssrA*)/F: 5'-CAT GAG CTC AAG GTT TTG GGG CCG ATT AGG-3' and SSO-907/R: 5'-CAT AAG CTT AGT GCG GGT CGG CTG CTC TTC C-3'. The unique *Sac*I site built into SSO-1000 and the unique *Hin*dIII site built into SSO-907 are highlighted with underline. This promoter-less *ssrA* fragment was cloned as a *Sac*I-*Hind*III fragment into pSS213, to construct pBL186. Subsequently, the m*oriT* cassette was cloned into pBL186 as a *Hind*III fragment to generate

pBL187. This mobilizable plasmid was introduced to *P. aeruginosa* via conjugation and strains with integrated plasmid due to homologous recombination at the downstream of *ssrA* were isolated as Gm^r colonies. The *arfA* gene was then deleted in SS3698 in the manner described above (Fig. 2.1) in the presence of IPTG to have *ssrA* expressed to maintain cell viability, to yield SS3703.

Growth curve assays

Growth curve assays were performed with bacterial cells growing in appropriate medium in a 24-well microtiter plate for 24 hours with shaking in a BioTek Synergy HT or BioTek Cytation 3 plate reader (Winooski, VT). To assess growth, overnight culture of cells was diluted 1:200 into appropriate medium and the cells were grown with shaking at the specified conditions. Cell growth was monitored as OD_{600}.

Persister cell formation assay

Cells grown overnight were diluted 1:100 in fresh LB and incubated at 37°C with aeration to an OD_{600} of 2.0-2.5 (early stationary phase). 0.7 ml of the culture was added to 0.7 ml saline and cells were harvested by centrifugation. Culture supernatant was discarded and cell pellet was resuspended in 2 ml of fresh LB supplemented with 10 µg/ml ofloxacin. An aliquot of 100 µl was removed immediately from this resuspension as the starting sample. The cells were incubated at 37°C without aeration for 5 hours, harvested and washed twice with fresh LB, and resuspended in 2 ml LB, and the number of viable cells was determined.

Microtiter plate biofilm formation assay

The ability of cells to form biofilms was determined as previously described (37). Briefly, overnight-grown cells were diluted 1:100 in fresh LB and added to 96-well PVC microtiter dish and incubated at 37°C without aeration for 24 hours. After incubation, biofilms formed in the microtiter dish were stained by crystal violet (CV), dissolved with 30% acetic acid, transferred to a fresh plate and OD_{550} determined (37).

Phenotype Microarrays

The PM1 and PM2 microplates from the BIOLOG's phenotypic array collection (Biolog, Hayward, CA) were used to determine the relative preference of carbon source utilization as per manufacturer's instruction. Briefly, cells were grown overnight, harvested and resuspended in 1x IF-0 to an OD_{600} of approximately 0.035, and this resuspension was further diluted 1:10 in 1x IF-0+dye. Aliquots were added to the PM1 and PM2 plates and incubated at 37°C for 24, 36, and 48 hours. At each time point, utilization of chemicals as the sole carbon source was determined as a function of reduction of the redox dye (tetrazolium; OD_{590}-OD_{750}) as per instructions from the manufacturer. To test utilization of various chemicals as a sole nitrogen source, PM3 (BIOLOG) was used. Cells were grown overnight grown on R_2A plates (38), resuspended in 1x IF-0 to an OD_{600} of approximately 0.035, and further diluted 10-fold in 1x IF-0+dye supplemented with 20 mM sodium succinate/2 μM ferric citrate as carbon source. Cells were added to the PM3 plate and incubated at 37℃ for 24, 36, and 48 hours. At each time point, utilization of chemicals as the sole nitrogen source was determined as a function of reduction of the redox dye (tetrazolium; OD_{590}-OD_{750}) as per instructions from the manufacturer.

Table 2.1. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant characteristics[a]	Source or reference
P. aeruginosa		
PAO1	Prototroph	(39)
SS24	PAO1 Δ*rpoS101*::*aacC1*	(24)
SS1372	PAO1 Δ*ssrA*	This study
SS2975	PAO1 Δ*relA* Δ*spoT*	This study
SS3017	PAO1 Δ*arfA* / pBL093	This study
SS3033	PAO1 Δ*ssrA* Δ*arfA* / pBL093	This study
SS3178	PAO1 Δ*arfA*	This study
SS3698	PAO1 Δ*ssrA* / pBL187	
SS3703	PAO1 Δ*ssrA* Δ*arfA* / pBL187	This study
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ*80dlacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK l-rpsL nupG*	Invitrogen
HB101	F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20 xyl-5 mtl-1 recA13*	Lab Collection
Plasmids		
pAT1	Δ*ssrA* in pEX100T-link (Ap^r/Cb^r)	This study
pBL001	Δ*arfA* in pEX100T-link (Ap^r/Cb^r)	This study
pBL052	$P_{T7(A1/04/03)}$::*arfA* in pSS213 (Gm^r)	This study
pBL093	m*oriT* in pBL052 (Gm^r)	This study
pBL186	$P_{T7(A1/04/03)}$::*ssrA* in pSS213 (Gm^r)	This study
pBL187	m*oriT* in pBL186 (Gm^r)	This study
pEX100T-link	*sacB*, *oriT* (Ap^r/Cb^r)	(35)
pRK2013	Tra1 (RK2), ColE1 (Km^r)	(40)
pSS213	$P_{T7(A1/04/03)}$ (Gm^r)	(36)

[a]Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cb^r , carbenicillin resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant.

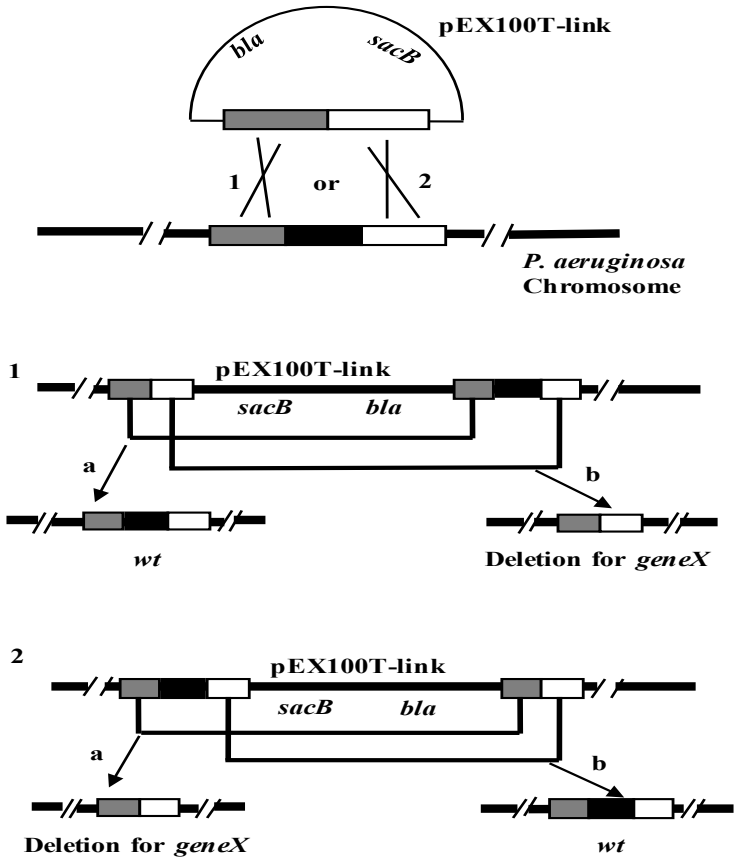

Figure 2.1. Schematic of clean deletion construction in *P. aeruginosa*. Paths 1 and 2 show integration of a suicide plasmid carrying the deletion allele of interest into the *P. aeruginosa* chromosome via homologous recombination of the upstream sequences or the downstream sequences of the gene of interest. Subsequent pressure to lose the *sacB* gene containing plasmid in the presence of sucrose forces the second round of homologous recombination and allelic exchange shown as paths a and b.

Results

Viability of *P. aeruginosa* requires a ribosome rescue system mediated by tmRNA or ArfA

To study ribosome rescue in *P. aeruginosa* physiology, clean deletion mutants of *ssrA* and *arfA* were constructed in the strain PAO1 (Figure 2.1). The *P. aeruginosa ssrA* mutant defective for *trans*-translation was mildly affected in osmotic and heat shock stress, and optimal virulence factor productions (Wu, Tucker, and Suh, unpublished data). In addition, our data suggested the existence of a regulatory circuit between *trans*-translation and RpoS, the mediator of general stress response. In many organisms, a mutation in *ssrA* results in mild phenotype because of existence of a backup system for ribosome rescue (1). In *Escherichia coli*, ArfA has been determined to be the major backup system for ribosome rescue and the bacterium requires at least one of these two systems for viability (8). Given the mild phenotype of *P. aeruginosa* Δ*ssrA* mutant, I surmised the existence of a backup systems analogous to ArfA and found an open reading frame encoded by PA3990 that shared 61% similarity at the predicted amino acid level with *E. coli* ArfA. I constructed a Δ*PA3990* allele and constructed *P. aeruginosa* deletion mutants to characterize the role of its gene product on *P. aeruginosa* physiology.

The *P. aeruginosa* Δ*PA3990* mutant demonstrated no obvious growth defect (Figure 2.2). In addition, I observed no obvious defect when the Δ*PA3990* mutant was subjected to stress tests including osmotic stress (data not shown). In contrast, I was unable to isolate a Δ*ssrA* Δ*PA3990* double mutant. My inability to isolate a Δ*ssrA* Δ*PA3990* double mutant was reminiscent of the inability of Chadani et al. (8) to isolate a double Δ*ssrA* Δ*arfA* double mutant in *E. coli*, suggesting that *PA3990* encoded for a *P. aeruginosa* ArfA homologue. Thus, I propose to designate *PA3990* as the *P. aeruginosa arfA*.

To further demonstrate that *PA3990* encodes for ArfA, I designed approaches to control the expression of either *PA3990* or *ssrA*. I cloned a promoterless *PA3990* or *ssrA* under the control of a regulatory promoter $P_{T7(A1/04/03)}$ in the plasmid vector pSS213 (36) and modulated the expression with IPTG. As shown in Figure 2.3, the Δ*ssrA* Δ*PA3990* double mutant was viable when Δ*PA3990* was complemented *in trans* by chromosomally integrated pBL093 which carries the gene under control of the $P_{T7(A1/04/03)}$ promoter. At 500 μM and 1000 μM concentration of IPTG, the growth of *P. aeruginosa* resembled that of a Δ*ssrA* mutant. When induced with 25 μM IPTG, the growth was partial and the growth was negligible until later due to some leaking from $P_{T7(A1/04/03)}$ when uninduced.

I also performed the complementary experiment of complementing the Δ*ssrA in trans* in the Δ*ssrA* Δ*PA3990* double mutant by expressing *ssrA* from the chromosomally integrated pBL186. As shown in Figure 2.4, the cells were nonviable unless Δ*ssrA* was complemented by the addition of IPTG to induce expression from pBL186. Growth of cells was partial at 100 μM of IPTG while 500 μM and 1000 μM of IPTG restored the growth resembled that of a Δ*arfA* mutant albeit with a slightly longer lag time presumably due to the suboptimal *ssrA* expression from the exogenous promoter $P_{T7(A1/04/03)}$.

My data demonstrate that *PA3990* is an *arfA* homologue that encodes for the major backup ribosome rescue mechanism in *P. aeruginosa*. In addition, the data demonstrate the requirement of at least one of the two ribosome rescue mechanisms, tmRNA-mediated *trans*-translation or ArfA, for viability of *P. aeruginosa*.

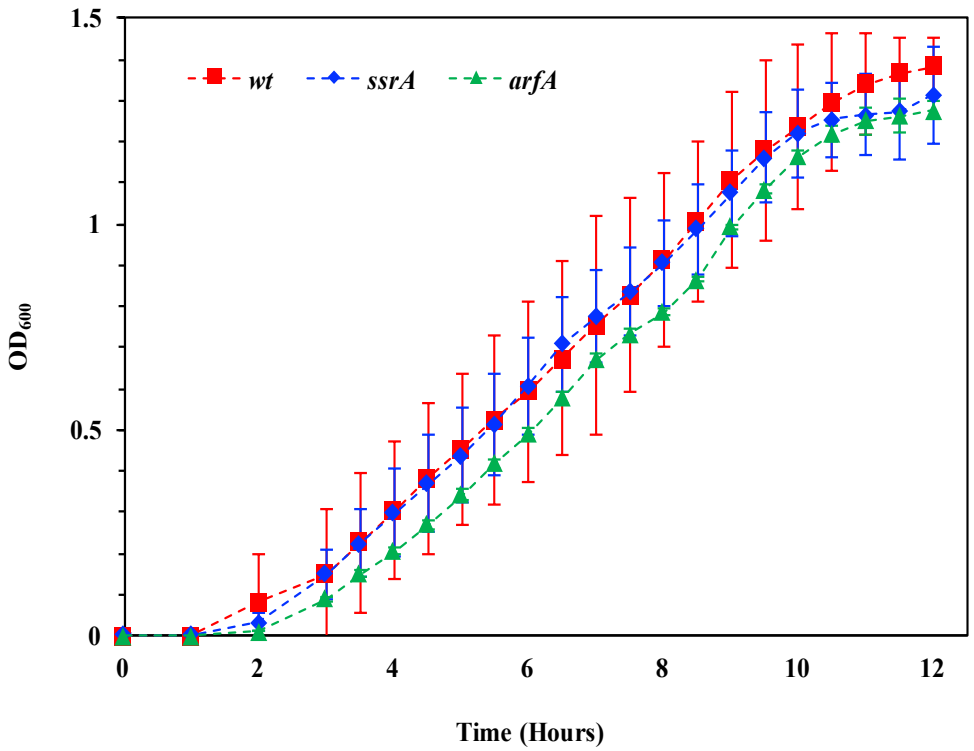

Figure 2.2. Comparison of growths of PAO1, Δ*ssrA*, and Δ*arfA* in LB. Overnight cultures of the wild type PAO1, Δ*ssrA* mutant, and Δ*arfA* mutant were diluted 1:200 in fresh LB, grown at 37°C with aeration in a 24-well microtiter plate in duplicate wells, and growth monitored as OD_{600}. Error bars indicate standard error. Results shown are representative of three independent experiments.

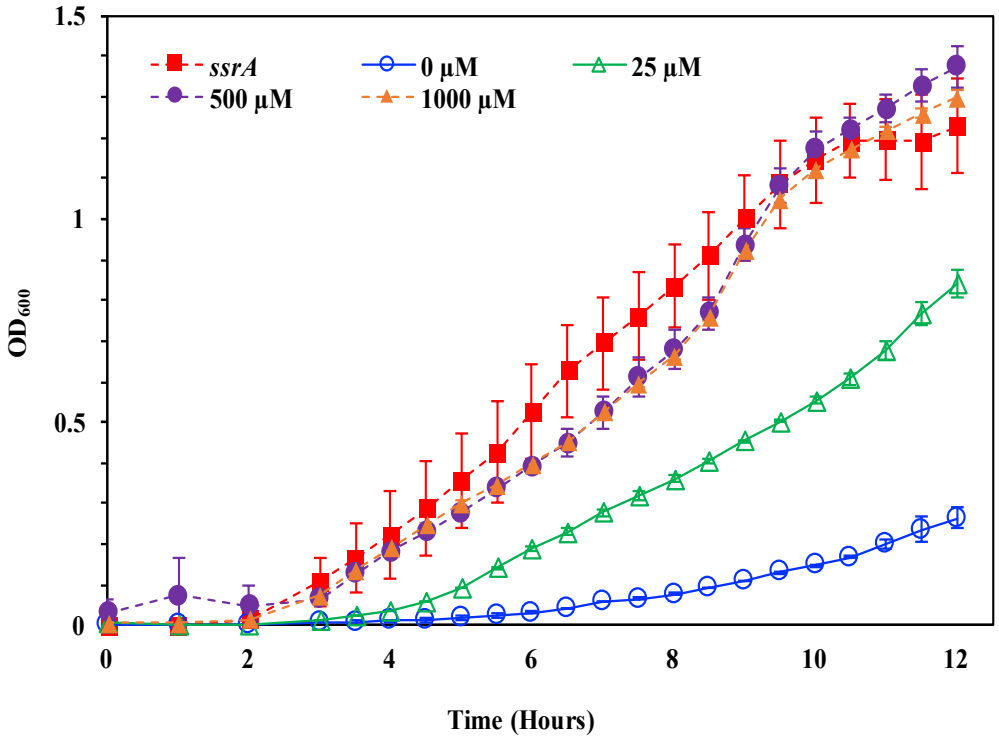

Figure 2.3. Modulation of *PA3990* expression and restoration of viability. SS3033, a Δ*ssrA* Δ*PA3990* mutant with $P_{T7\ (A1/04/03)}$–regulated *arfA* expression from pBL093, was grown overnight at 37°C with aeration in LB supplemented with 20 µM IPTG. The overnight grown cells were washed three times with fresh LB, diluted 1:200 in LB supplemented with 0, 25, 500, or 1000 µM IPTG, grown at 37°C with aeration in a 24-well microtiter plate in duplicate wells, and growth monitored as OD_{600}. Error bars indicate standard error. Results shown are representative of three independent experiments.

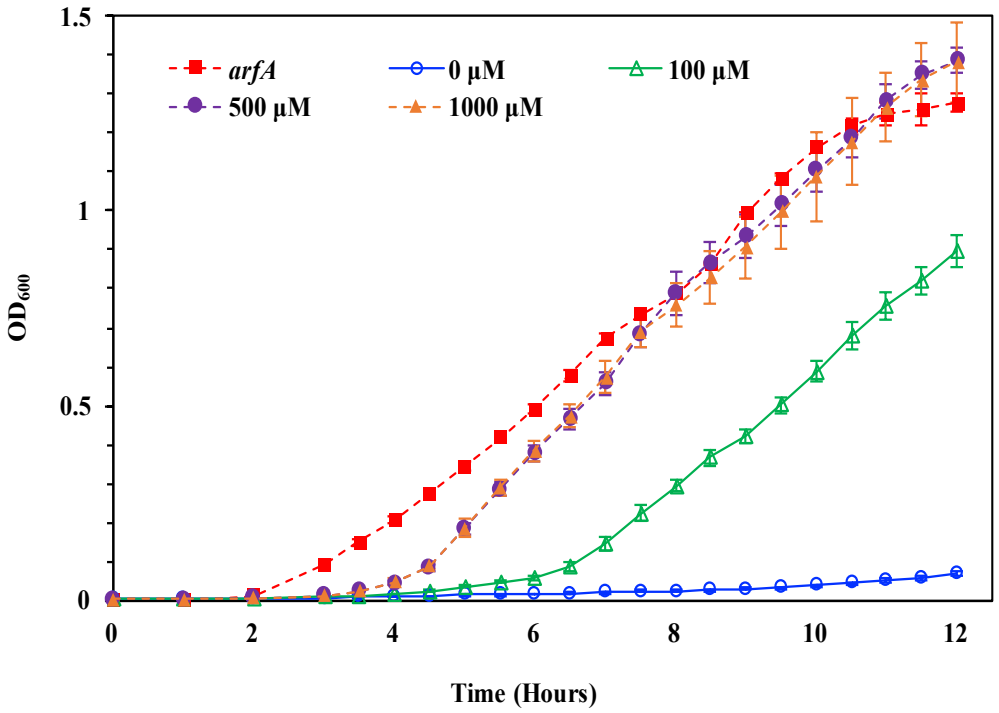

Figure 2.4. Modulation of *ssrA* expression and restoration of viability. SS3703, a Δ*arfA* mutant with $P_{T7\ (A1/04/03)}$–regulated *ssrA* expression, was grown overnight at 37°C with aeration in LB supplemented with 250 µM IPTG. The overnight grown cells were washed three times with fresh LB, diluted 1:200 in LB supplemented with 0, 25, 500, or 1000 µM IPTG, grown at 37°C with aeration in a 24-well microtiter plate in duplicate wells, and growth monitored as OD_{600}. Error bars indicate standard error. Results shown are representative of three independent experiments.

Trans-translation defective mutants form less persister cells in *P. aeruginosa*

Persister cells are a subpopulation of dormant bacteria in a population that are highly resistant to antibiotics (41). Persister cell formation is believed to be regulated primarily by the bacterial toxin-antitoxin modules (42, 43) that are in turn regulated by the cellular alarmone, (p)ppGpp (27, 44). To assess whether *trans*-translation plays a role in persister cell formation in *P. aeruginosa*, I exposed the wildtype PAO1, Δ*ssrA* mutant, (p)ppGpp negative Δ*relA* Δ*spoT* mutant, and the general stress response defective *rpoS* mutant to ofloxacin. The data are shown in Figure 2.5. As expected, the Δ*relA* Δ*spoT* double mutant was severely affected for persister cell formation and formed approximately 90% less persister cells. In contrast, as demonstrated for *E. coli* (45), the *rpoS* mutant formed at least two-fold more persister cells. The Δ*ssrA* mutant formed approximately 40% less persister cells than did the wildtype PAO1. My data show that *trans*-translation plays a role in formation of persister cells in *P. aeruginosa*.

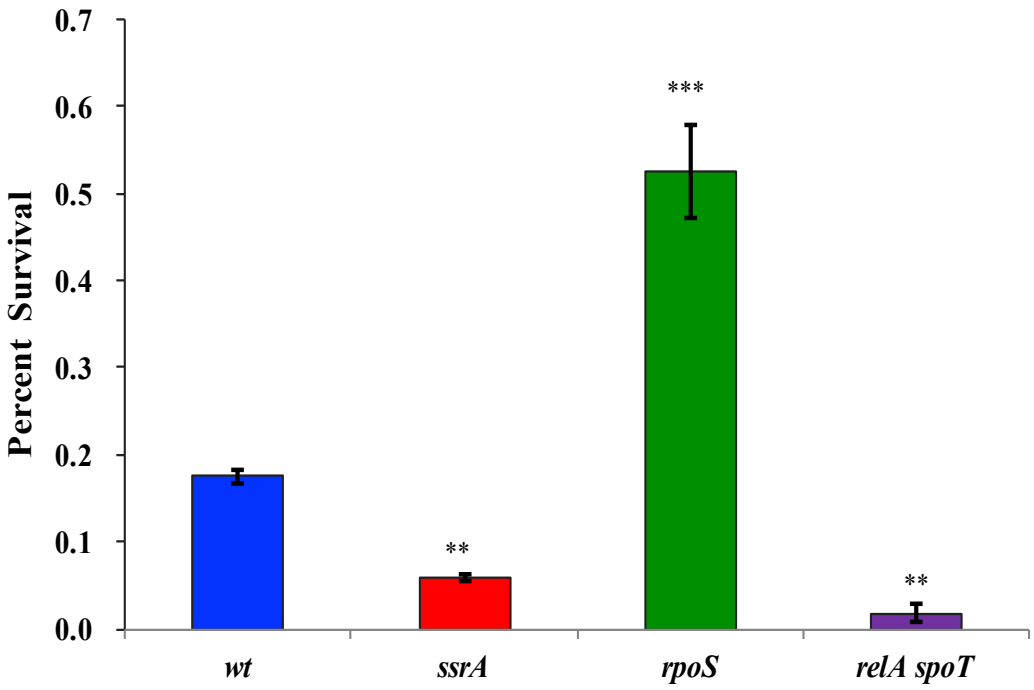

Figure 2.5. Effect of *trans*-translation on persister-cell formation in *P. aeruginosa*. *P. aeruginosa* strains were grown in LB at 37°C with aeration to an OD_{600} of 2.0-2.5 (early stationary phase). Cells were collected and challenged with 10 µg/ml ofloxacin for 5 hours. After five hours, the number of viable cells were determined and expressed as a percentage of the number of viable cells prior ofloxacin exposure. All data are means ± standard deviations (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus persister-cell formation by the wild-type strain, PAO1. Results shown are representative of three independent experiments.

Trans-translation affects biofilm initiation in *P. aeruginosa*

During establishment of infections, *P. aeruginosa* experiences a variety of stress as a result of nutrient deprivation, exposure to oxidative stress, and presence of antibiotics in the environment (19). To survive the harsh environment, *P. aeruginosa* forms biofilms that are highly resistant to various stresses (19). Biofilm formation is a complex process that is cooperatively regulated by many factors including cell-cell communication, GacA, RpoS, and cyclic-di-GMP in *P. aeruginosa* (19, 46, 47). To determine the potential effect of tmRNA mediated *trans*-translation on biofilm formation, I performed microtiter dish biofilm formation assays (37) with the wild-type and the Δ*ssrA* mutant. As shown in Figure 2.6, the Δ*ssrA* mutant formed approximately 40% more biofilm than the parent strain. In contrast, the *P. aeruginosa rpoS* mutant produced approximately 150% more biofilm than the wild type, which is consistent with the published data (46). Thus, my data indicate that *trans*-translation plays a role in biofilm formation in *P. aeruginosa*.

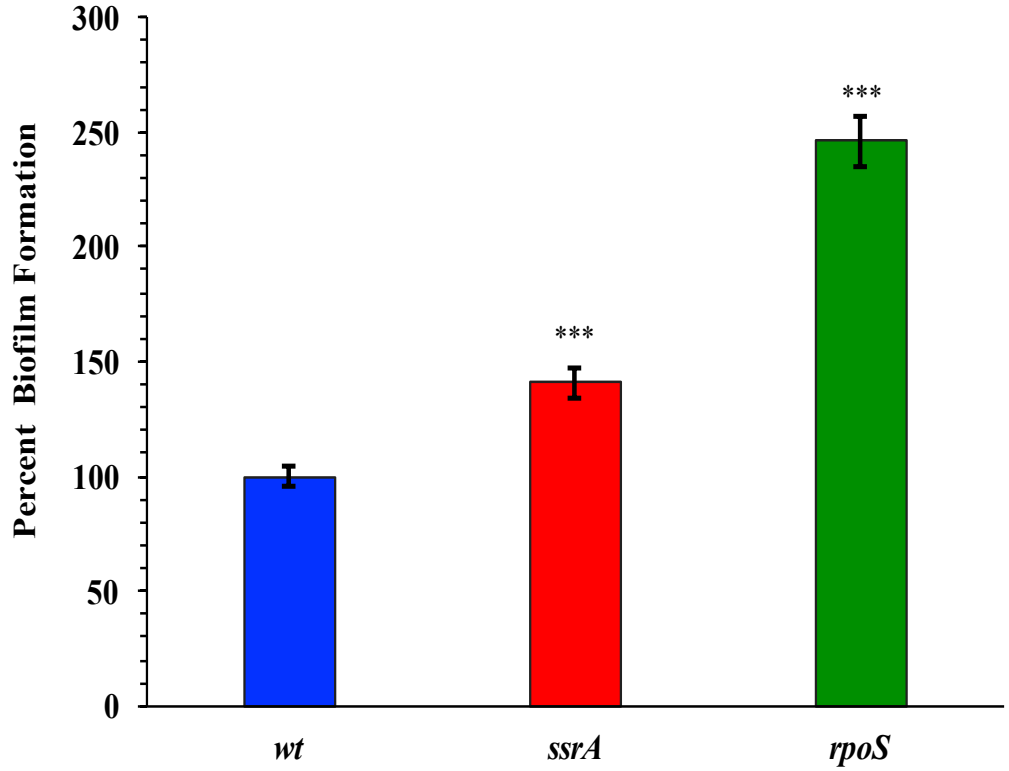

Figure 2.6. Effect of *trans*-translation on biofilm formation in *P. aeruginosa*. Overnight cultures were diluted and incubated in a PVC microtiter plate. For each strain, three independent sampling was performed with each sampling done in sextuplicate to yield a n=18 per microtiter plate. Attached cells in the PVC plate wells were quantitatively measured as OD_{550} according to O'Toole (37). One hundred percent biofilm formation corresponds to the mean OD_{550} reading for the wild type. All data are means ± standard deviations (n=18). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the wild-type strain, PAO1. Results shown are representative of at least three independently performed experiments.

Effect of *trans*-translation on carbon and nitrogen metabolism in *P. aeruginosa*

As shown in Figure 2.2, the Δ*ssrA* mutant show no demonstrable growth defect when grown in a rich medium such as LB. To understand the role of *trans*-translation in nutrient utilization better, I used the Biolog's phenotype array to assess the relative ability of *P. aeruginosa* PAO1 and its Δ*ssrA* mutant to grow on a variety of carbon and nitrogen sources. For carbon source, the PM1 and PM2 plates were used. These plates test for the ability of a bacterium to grow using a variety of carbohydrates and organic acids as a sole carbon source (38). Of the 190 carbon sources tested, *P. aeruginosa* grew on 69 with the best growth on 53 as previously described (48). As shown on Figure 2.7A, the most obvious difference in carbon source utilization between the wildtype strain PAO1 and its Δ*ssrA* mutant was seen for propionic acid, capric acid, and L-valine. The Δ*ssrA* mutant grew approximately 65% better on propionic acid than the parent at 24h but not at 36h or 48h post inoculation (data not shown). In contrast, the *ssrA* mutant grew 100% better on capric acid than the parent by 48 post inoculation. Growth on capric acid at earlier time points were too poor to be measured accurately. In contrast, the Δ*ssrA* mutant grew approximately 60% worse than the parent strain on L-valine by 48h post inoculation. As with capric acid, growth on L-valine at earlier time points were too poor to be measured accurately on our microplate reader. To determine the nitrogen source utilization, I used the PM3 plate. Of 95 nitrogen sources varying from ammonia to amino acids, *P. aeruginosa* grew on 80 with the best growth on 76 as a sole nitrogen source (48). As shown in Figure 2.7B, the only difference I observed between the wildtype and the Δ*ssrA* mutant was for utilization of N-acetyl-D-mannosamine as a sole nitrogen source at 36h post inoculation. The Δ*ssrA* mutant grew twice as well as the parent strain by this point. This growth difference disappeared by 48h post inoculation.

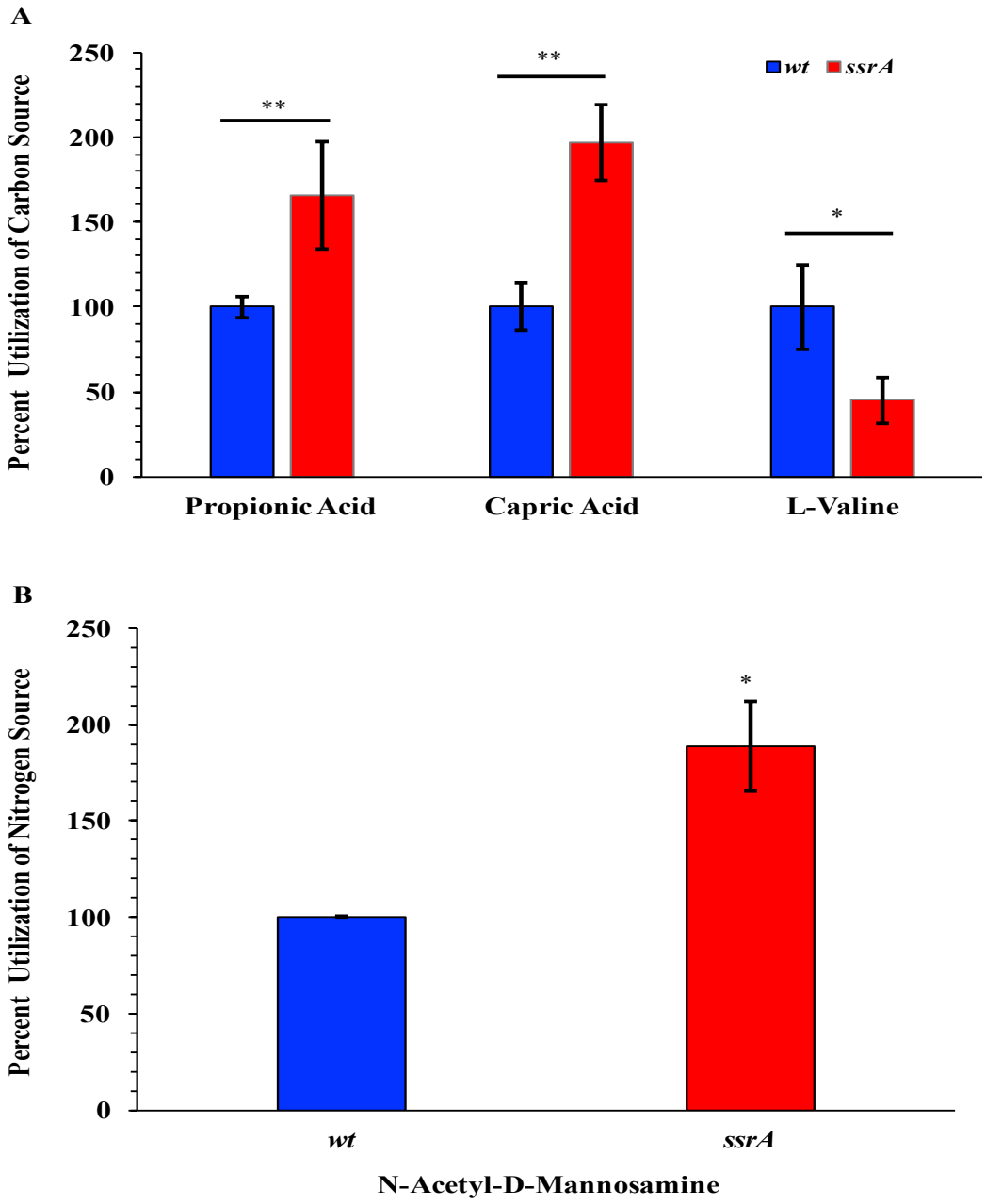

Figure 2.7. Metabolic differences between PAO1 and PAO1 Δ*ssrA* mutant on Biolog's carbon and nitrogen phenotype microarrays. Growth of cells was measured as OD_{590}-OD_{750} as per manufacturer's instructions. (A) Carbon source utilization (PM1 and PM2 plates). Percent utilization of a carbon source is shown as a percentage of the parent strain's growth. For propionic acid, data shown here were collected at 24h post inoculation. For capric acid and L-valine, two poor carbon sources for *P. aeruginosa* (57), data were

collected from 48h (OD_{590}-OD_{750}>0.1). (B) Nitrogen source utilization (PM3 plate). Percent utilization of nitrogen source is determined in the same manner as described above for carbon source. Shown are data for N-acetyl-D-mannosamine utilization collected at 36h post inoculation (OD_{590}-OD_{750}>0.1). All data shown are means ± standard deviations (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the parent strain, PAO1. Results shown are representative of three independent experiments with each experiment performed with three plates.

Discussion

During growth, approximately 50% of the bacterial cellular energy is devoted to protein synthesis and 20%-40% of this 50% of the total energy is devoted to synthesis of ribosome and translational machinery (4). Bacteria, including human pathogens, continuously encounter environmental alterations in nutrients, temperature, osmolarity, oxidative stress, host immunity, and toxic compounds such as antibiotics (24). To survive different niches, bacterial cells must quickly respond to challenges by producing new proteins (4). As a result of catalyzing coupled transcription and translation, bacteria frequently suffer from stalled ribosomes at nonstop complexes (1). These complexes form from incomplete transcription, damage to mRNA, and amino acid or tRNA deprivation (1). Studies in *E. coli* suggest that a single ribosome, on an average, stalls in approximately 5 nonstop translation complexes during one cell cycle (5). Given the energy dedicated to protein synthesis, it is imperative for bacteria have evolved mechanisms to rescue stalled ribosomes at these nonstop complexes.

The tmRNA-mediated *trans*-translation is the primary mechanism by which bacteria rescue stalled ribosomes (1). In addition to *trans*-translation, some organisms have evolved backup systems as represented by ArfA of *E. coli* (9). Our previous characterization of a Δ*ssrA* mutant demonstrated that although the *trans*-translation mutant showed several phenotypes, their overall effect on *P. aeruginosa* physiology were mild (Wu, Tucker, and Suh, unpublished data). This suggested the existence of an ArfA-like backup system in *P. aeruginosa*. As demonstrated in Figure 2.3, I discovered a genetic locus, *PA3990*, that encodes for a ArfA homologue in *P. aeruginosa*. Based on my analyses shown in Figures 2.3 and 2.4, *P. aeruginosa* requires either tmRNA-mediated *trans*-translation or ArfA-mediated ribosome recycling for viability. Although I was unable to construct a viable Δ*ssrA* Δ*arfA* double mutant, I constructed strains in which I could

modulate expression of either *ssrA* or *arfA* to maintain the viability. These strains, SS3033 and SS3703, will be useful for elucidating the relative importance of the two ribosome rescue mechanisms in *P. aeruginosa*.

P. aeruginosa is an important nosocomial opportunistic bacterial pathogen (19, 21, 22). This bacterium is naturally resistant to many antibiotics and it is difficult to eradicate once it establishes infection by forming biofilms which further enhances the antibiotic resistance (49, 50). Biofilm formation is a complex process that is regulated by numerous factors including cell-cell communication systems, GacA, RpoS, and Cyclic-di-GMP in *P. aeruginosa* (19, 46, 47). Interestingly, different nutrients can trigger *P. aeruginosa* biofilm formation during chronic infections. For example, metabolism of glycerol, one of the most common energy sources in the airways of CF patients, has been demonstrated to upregulate biofilm formation by *P. aeruginosa* (51). Additionally, certain amino acids, including arginine and valine, stimulate biofilm formation through promoting cyclic-di-GMP accumulation in *P. aeruginosa* (52). Via mechanism that is not yet clear, *trans*-translation affects biofilm formation in *P. aeruginosa* as demonstrated in Figure 2.6. However, our previous data, which demonstrated that *rpoS* expression is decreased by 50% (Wu, Tucker, and Suh, unpublished data) in the Δ*ssrA* mutant, suggest that the increased biofilm formation of a Δ*ssrA* mutant may be indirect through RpoS.

A major reason for our inability to completely eradicate a bacterial pathogen from infection is the formation of dormant persister cells (41). Persister cell formation appeared to start with the regulation by (p)ppGpp which predominantly responds to amino acid starvation to mount the stringent stress response (27). It was believed that accumulation of polyphosphate as a part of the stringent response was partly responsible for accumulation of toxins due to degradation of antitoxins (44). However, a recent evidence questions this model by demonstrating that polyphosphate accumulation and toxin-antitoxin

accumulation are independent of each other (53). Thus, the linkage between (p)ppGpp and persister cell formation is unclear at present for *E. coli*. However, my data shown in Figure 2.5 and in Chapter 3 demonstrate that in *P. aeruginosa*, *trans*-translation is involved in formation of persister cells and (p)ppGpp is likely to be involved.

Interestingly, there appeared to be very little metabolic differences between the parent strain PAO1 and the Δ*ssrA* mutant when tested on Biolog's phenotype microarrays for carbon and nitrogen sources. I discovered that out of 190 carbon sources and 95 nitrogen sources tested, only three carbon sources and one nitrogen source differentially affected growth of the parent strain PAO1 and the Δ*ssrA* mutant. The Δ*ssrA* mutant grew better on propionic acid and capric acid but grew more poorly on L-valine than the parent strain. Propionic acid is activated through conversion to Propionyl-CoA, an intermediate in the metabolism of odd-chain fatty acids or branched-chain amino acids including leucine, isoleucine, and valine (54). Propionyl-CoA can enter TCA cycle via conversion to succinyl-CoA or be used as the basis for growing long-chain fatty acids (54). Capric acid is a ten-carbon fatty acid and is catalyzed by the fatty acid degradation (FAD) system, including fatty-acid transport and β-oxidation (54). In *P. aeruginosa*, capric acid can be also used as a precursor for cell-cell communication signals or rhamnolipids (19, 54, 55). Unfortunately, based purely on my data, it is not clear exactly how *trans*-translation inhibits either the propionate or capric acid utilization as a sole carbon source. Further experiments are required to decipher the metabolic mechanisms. In contrast to propionate and capric acid, my data showed that utilization of L-valine was decreased in the Δ*ssrA* mutant by approximately 50% (Fig. 2.7A). Interestingly, only the utilization of valine but not leucine or isoleucine was affected in the Δ*ssrA* mutant. In proteobacteria, degradation of valine is catalyzed by the branched-chain amino acid aminotransferase and branched-chain acyl-CoA dehydrogenase, which are also used for degradation of leucine and isoleucine (54).

However, since only valine utilization is affected, it is likely to be further downstream in the metabolic pathway specific only for valine that *trans*-translation plays a role. Recently, utilization of valine as the sole carbon source was shown to be regulated by RpoN and PA2449 in *P. aeruginosa*, likely through the pathway of 3-hydroxyisobutyrate dehydrogenase and methylmalonate-semialdehyde dehydrogenase (56). Thus, *trans*-translation may affect one of these two enzymes. For nitrogen source, only N-acetyl-D-mannosamine gave a measurable growth difference between the parent and the Δ*ssrA* mutant. In *P. aeruginosa*, N-acetyl-D-mannosamine can be used as a precursor for LPS biosynthesis (57). However, more experiments are required to validate these initial findings of the Biolog's phenotype microarray data.

The data presented in this chapter suggest that *trans*-translation is involved in a myriad of physiological responses in *P. aeruginosa* including stress response, biofilm formation and persister cell formation, and that recycling of ribosomes is essential for viability of the bacterium.

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Chapter 3

Genetic evidence for a complex regulatory circuit between *trans*-translation and global regulators of gene expression in *Pseudomonas aeruginosa*

Abstract

A unique bacterial hybrid RNA, tmRNA, catalyzes *trans*-translation which rescues stalled ribosomes during translation. Previous data suggested a regulatory circuit between RpoS and tmRNA that linked the *trans*-translation with the general stress response in *Pseudomonas aeruginosa*. To further characterize the global role of *trans*-translation in *P. aeruginosa*, I determined the relationship between tmRNA and several global regulators of gene expression. I discovered that full *ssrA* gene expression required the two acylhomoserine lactone mediated cell-cell communication systems Las and Rhl. In either a *lasR* or a *rhlR* mutant, the *ssrA* gene expression was decreased by approximately 50-60%. In a *vfr* mutant, the *ssrA* gene expression was decreased by approximately 60%. Because of a regulatory circuit for tmRNA and RpoS, I addressed whether a similar circuit existed between tmRNA, LasR, RhlR, and Vfr. In a Δ*ssrA* mutant, the *lasR* and the *rhlR* gene expressions were decreased by 60% and 70%, respectively, indicative of a regulatory circuit. However, the expression of *vfr* was not affected by the Δ*ssrA* mutation. Interestingly, the tmRNA effect on expression of these genes was at the transcriptional level rather than at the translational level. To elucidate the mechanism(s) by which tmRNA exerts its effect on the transcription of these global regulators, I addressed the potential

effect of tmRNA on the production of (p)ppGpp, an alarmone involved in general stress response, cell-cell communication systems, and virulence factor productions. I determined that *trans*-translation is required for translation but not transcription of *relA*, the structural gene that encodes for (p)ppGpp synthetase. In the Δ*ssrA* mutant, expression of the *relA*::*lacZ* translational fusion but not the transcriptional fusion was decreased by 50%. In contrast, tmRNA had no effect on either the transcription or the translation of *spoT*. I discovered that relationship between tmRNA and RelA is a circuit and that (p)ppGpp level affects *ssrA* expression. In a Δ*relA* mutant, *ssrA* expression was decreased by almost 80%. Interestingly, in a Δ*relA* Δ*spoT* double mutant in which there is no (p)ppGpp, the *ssrA* gene expression was only decreased by 20% suggesting that the cellular level, instead of complete absence of (p)ppGpp, is important for *ssrA* expression. Finally, I discovered that *ssrA* expression is further regulated by another alternative sigma factor, RpoN. In a *rpoN* or a *ptsN* mutant, the *ssrA* gene expression was decreased by 70% and 50%, respectively. My data demonstrate that *ssrA* is a highly regulated gene that is induced by at least six global regulators: LasR, RhlR, Vfr, RpoS, RpoN, and (p)ppGpp. In turn, tmRNA is required for full expression of *rpoS, lasR, rhlR,* and *relA*. It is likely the tmRNA affects expression of these four genes indirectly through *trans*-translation of the *relA* mRNA. The complexity of regulatory mechanisms involved in regulation of *ssrA* reinforces the importance of *trans*-translation and ribosome rescue in *P. aeruginosa*.

Introduction

Trans-translation, catalyzed by a ribonucleoprotein complex, tmRNA-SmpB, is a bacterial mechanism to recycle stalled ribosomes on mRNA. tmRNA contains both a tRNA-like domain and a mRNA-like domain which allows it to act as both tRNA and mRNA on stalled ribosomes (1). *trans*-translation affects various functions in different bacteria including translation of LacI (2) and stringent response in *Escherichia coli* (3), regulation of Yops in *Yersinia pseudotuberculosis* (4), and DNA replication timing in *Caulobacter crescentus* (5). Although *trans*-translation is essential for some bacteria including *Neisseria gonorrhoeae* (6), other bacteria possess at least one alternative function for rescuing stalled ribosomes when tmRNA is not available (7). In *E. coli,* one such function, designated as ArfA, has been identified and characterized as a redundant mechanism for *trans*-translation (8).

Pseudomonas aeruginosa is a gram-negative bacterium that is found almost ubiquitously in nature. The bacterium possesses a large genome of over 6.2 million nucleotides and devotes ten percent of its genome to encode for regulators of gene expression (9). Due to the complexity of regulatory mechanisms, *P. aeruginosa* has become a model bacterium for elucidating complex pathways that govern various aspects of cellular physiology including formation of biofilms, emergence of persister cells, mechanisms of stress response, and virulence factor productions. In *P. aeruginosa*, many of these physiological processes are cooperatively regulated by a complex network consisting of several global regulatory systems, including (p)ppGpp, cell-cell communication systems, GacA, Vfr, RpoS, and cyclic-di-GMP (10, 11). Crosstalk between these global regulatory systems allows *P. aeruginosa* to respond to a variety of stresses and facilitates establishment of infections (10). For example, amino acid starvation stimulates RelA-mediated synthesis and accumulation of (p)ppGpp which induces cell-cell

communication systems Las and Rhl to produce many virulence factors (12, 13). In addition, RpoS, the major regulator of general stress response, controls expression of genes that are also co-regulated by the cell-cell communication regulons in *P. aeruginosa* (14). Furthermore, Vfr, a cAMP receptor protein homologue of *P. aeruginosa*, directly induces expression of virulence factor genes such as *toxA* and indirectly controls other virulence factor productions by inducing *lasR* expression (10, 15). Finally, RpoN, an alternative sigma factor typically associated with nitrogen metabolism, is required for expression of several cell-cell communication controlled gene products including pyocyanin and hydrogen cyanide (16). Thus, many important physiological pathways are regulated by a network of global regulators in *P. aeruginosa* (10).

We previously characterized that *trans*-translation is required for optimal virulence factor production and response to osmotic and thermal stress in *P. aeruginosa* (Wu, Tucker, and Suh, unpublished data). In addition, we discovered a regulatory circuit between RpoS and tmRNA to establish a regulatory link between the general stress response and ribosome rescue. In this study, I elucidated a complex network of global regulators and *trans*-translation that affects various aspects of *P. aeruginosa* physiology.

Materials and Methods

Bacterial strains, plasmids, media, and chemicals

All bacteria and plasmids used for this study are listed in Table 3.1. Unless otherwise indicated, bacteria were grown in L broth (LB; (17); Fisher Scientific, Pittsburgh, PA) or in LB supplemented with appropriate antibiotics at 37°C with aeration. Solid media were prepared by L-agar (BD Biosciences). Pseudomonas isolation agar (PIA; BD Biosciences) was used for tri-parental mating to counterselect *P. aeruginosa* against *E. coli* as previously described (18). Antibiotics were purchased from Sigma-Aldrich (St. Louis,

MO) and used at following concentrations: ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 100 μg/ml for *P. aeruginosa;* gentamicin, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin, 50 μg/ml for *E. coli*; tetracycline, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*.

DNA manipulations, transformations, and conjugations

For cloning DNA*, E. coli* strain DH10B was routinely used as the host strain. Unless otherwise indicated, DNA was introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (18). Plasmids were purified with QIAprep Spin Miniprep columns (Qiagen, Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (NEB; Beverly, MA). Either *Phusion* polymerase (NEB) or *Taq* polymerase (NEB) was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Construction of *P. aeruginosa* mutants

To delete the genes of interest, a clean deletion allele for the gene was constructed via SOE PCR (19), cloned into pEX100T-link (20), introduced into *P. aeruginosa* via triparental mating (18), selected for integration of plasmid by selecting for Cb^r colonies, and forced allelic exchange via negative selection by growing the cells in the presence of 5% sucrose as previously described (20). The unique restriction sites built into the primers are denoted by underlines and the overlapping sequences for SOE PCR are denoted with italics. *Phusion* thermostable DNA polymerase was used to minimize incorporation errors during the amplification. To delete *relA*, oligonucleotide primers used were Δ*relA* SSO-

935/F1: 5'-CAT GGT ACC ATT GCC CGT GCC CTG CCT GCT CTT-3'; SSO-936/R1: 5'-*TTC CGC CTC GCC CTT GCC TAC CCT* TTA CCA C-3'; SSO-937/F2: 5'-*AGG GTA GGC AAG GGC GAG GCG GAA* ACA GG-3'; and SSO-938/R2: 5'-CAT AAG CTT CCA TCG GCG CCC AGG TGA A-3'. To delete *spoT*, ooligonucleotide primers used were SSO930/F1: 5'- CAT GGA TCC GCG AAG CCG TCA GCG AAA TG-3'; SSO-931/R1: 5'-*CTT CTT GAT GAC TTC ACC CCC TGC* CCG TA-3'; SSO-932/F2: 5'-*GCA GGG GGT GAA GTC ATC AAG AAG* CTG CG-3'; SSO-933/R2: 5'-CAT TCT AGA GCG GCT GCG GCG AGG AAA ATC AC-3'. To delete *vfr*, oligonucleotide primers used were SSO957/F1: 5'-CAT GGA TCC GAC CGC GGC CCT TGA CCA CGA A-3'; SSO-958/R1: 5'-*GGG TGC TGT TCA GCC CGA GTC CCG* AAA GAA TAA A-3'; SSO-959/F2: 5'-*CGG GAC TCG GGC TGA ACA GCA CCC* ATG AAA AAG-3'; SSO-960/R2: 5'-CAT AAG CTT CCC GCA AGG CCG AGG AGG TC-3'. For each SOE PCR, F1 and R2 primer sets were used. Deletion of each gene was verified via PCR using internal primers for the gene. A schematic of clean deletion mutant construction is shown in Figure 2.1 in Chapter 2

Construction of *P. aeruginosa* strains with the chromosomal *gene*::*lacZ* fusion

To study regulation of genes of interest, *lacZ* fusions were constructed and integrated into the chromosome via homologous recombination. Our strategy is outlined in Figure 3.1. Briefly, a DNA fragment carrying the promoter and a portion of the gene of interest was amplified by PCR and cloned into pSS223 or pSS231 to generate a transcriptional (*lacZ1*) or a translational (*lacZ2*) fusion, respectively (21). The fusion carrying suicide plasmid was introduced to *P. aeruginosa* via triparental mating and integration of the fusion and the plasmid via homologous recombination in the genome was selected as Cb^r cells as previously described (21). The integration of the fusion in the

chromosome was verified via PCR. For construction of translational fusions, the PCR amplified DNA fragment was cloned in frame to *lacZ2*. All DNA amplification was performed with the *Phusion* thermostable DNA polymerase to minimize errors during the amplification. The unique restriction sites built into the oligonucleotide primers are underlined. To generate *relA*::*lacZ* fusions, the DNA region from -873 to +332 of *relA* was amplified with SSO-980/F: 5'- CAT GAA TTC ATT GCC CGT GCC CTG CCT GCT CTT-3' and SSO-981/R: 5'-CAT GGA TCC ACC GGC CCG AAA TGC TTG TTG A-3'. The *spoT*::*lacZ1* transcriptional fusion (-590 to +1) was generated using the primers SSO-970/F: 5'-CAT GAA TTC GCG AAG CCG TCA GCG AAA TG-3' and SSO-934/R 5'-TTC ACC CCC TGC CCG TA-3'. The *spoT*::*lacZ2* translational fusion (-590 to +176) was generated with the primers SSO-970/F and and SSO-986/R: 5'-CAT GGA TCC ATG TCG GCG AGG ATG TTG GAG A-3'.

Complementation of the Δ*ssrA* mutation in *P. aeruginosa*

In *P. aeruginosa*, Δ*ssrA* mutation cannot be complemented *in trans* on a multicopy plasmid (Tucker and Suh, unpublished data). Thus, the only way to complement the Δ*ssrA* mutation is to use a single copy from an integrated plasmid. I constructed a suicide plasmid pBL185, which harbors the wild-type *ssrA* operon (*PA0827-ssrA*) with its native promoter. Briefly, the DNA fragment carrying *PA0827-ssrA* was PCR amplified as a *Sac*I-*Xba*I fragment and cloned in pBL150, a pSU38 (21, 22) derivative that carries Gm^r in lieu of Km^r and m*oriT* for mobilization. The PCR reaction was performed with *Phusion* DNA polymerase using the primers SSO-618/F: 5'-CAT GAG CTC CCT CCA GCT TGC CGG CGA AAT ACT-3' and SSO-642/R: 5'-CAT TCT AGA CAT AGC AAG GCG AGC CGG CAA CAG GAA-3'. The unique *Sac*I and *Xba*I sites are underlined. pBL185 was

introduced into Δ*ssrA* mutants via triparental mating and Gm^r cells with pBL185 integrated into the genome via homologous recombination were isolated.

Complementation of *lasR, rhlR,* and *vfr* mutants

To complement the *lasR*::*aacC1* mutation, the plasmid pBL032 harboring *lasR* was constructed. To generate pBL032, a non-polar Tet^r cassette (21) was cloned as a *Sac*I fragment into pSS163, a plasmid carrying *lasR* (Suh, unpublished data). pBL032 was introduced into the *lasR* mutant by transformation and Tet^r cells carrying pBL032 were isolated. The Tet^r cassette was cloned in pUCP19 to yield pBL034, which was used as a negative vector control.

To complement the *rhlR*::*tet* mutation, the suicide plasmid pBL167, which harbors the wild-type *rhlRI* operon was constructed. Briefly, the *rhlRI* DNA was PCR amplified as a blunt-*Hin*dIII fragment and cloned in pBL044, a pSU38 (22) derivative that is Gm^r , to yield pBL158. The oligonucleotide primers used for PCR were SSO-972/F: 5'-CAT GAA TTC CGA CAA TGC CGA ACG GCT GGT-3' and SSO-153/R: 5'-CAT AAG CTT AGT GCG CGA AAC GGC TGA CG-3'. Then, m*oriT* was cloned as a *Hin*dIII fragment in pBL158, to yield pBL167. The plasmid pBL167 was introduced into the *rhlR* mutant through triparental mating and Gm^r cells with pBL167 integrated into the genome via homologous recombination were isolated.

To complement the *PA4464*::ISphoA/hah mutant, the plasmid pBL068, which harbors the wild-type *PA4464* was constructed. The *PA4464* DNA was PCR amplified and cloned as an *Eco*RI-*Hin*dIII fragment in pSS213 (21), to yield pBL065. Primers used were SSO-912/F: 5'-CAT GAA TTC CGA ATA AGG TAC TAA GCC CTC AGC C-3' and SSO-913/R: 5'-CAT AAG CTT AGG ATG TGC CGG AAG GTC TGG TG-3'. The

unique *EcoR*I and *Hind*III sites are underlined. Subsequently, mSF was cloned as a *Hin*dIII fragment in pBL065, to generate pBL068 which was introduced into the *PA4464* mutant.

β-galactosidase assay

β-galactosidase assays were performed as described by Miller (23). Absorbance were measured with Shimadzu UV1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Table 3.1. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant characteristics[a]	Source or reference
P. aeruginosa		
PAO1	Prototroph	(24)
SS306	PAO1 *lasR501*::*aacC1*	Lab Collection
SS775	PAO1 $P_{lasR::lacZ1}$	Lab Collection
SS789	PAO1 $P_{rhlR::lacZ1}$	Lab Collection
SS1372	PAO1 Δ*ssrA*	Lab Collection
SS1740	PAO1 $P_{ssrA::lacZ1}$	Lab Collection
SS2798	PAO1 *rpoN*::ISphoA/hah	Dr. Silo-Suh[b]
SS2799	PAO1 *PA4464*::ISphoA/hah	Dr. Silo-Suh
SS2949	PAO1 Δ*relA*	This study
SS2963	PAO1 Δ*relA* $P_{ssrA::lacZ}$	This study
SS2964	PAO1 Δ*vfr*	This study
SS2966	PAO1 Δ*vfr* $P_{ssrA::lacZ}$	This study
SS2975	PAO1 Δ*relA* Δ*spoT*	This study
SS3015	PAO1 Δ*relA* Δ*spoT* $P_{ssrA::lacZ}$	This study
SS3031	PAO1 *rhlR*::*tet*	This study
SS3051	PAO1 *rhlR*::*tet* $P_{ssrA::lacZ}$	This study
SS3058	PAO1 Δ*ssrA* $P_{rhlR::lacZ1}$	This study
SS3070	PAO1 *PA4464*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3076	PAO1 *rpoN*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3080	PAO1 Δ*ssrA* $P_{rhlR::lacZ1}$ / pBL185	This study

SS3262	PAO1 $P_{spoT::lacZ1}$	This study
SS3264	PAO1 Δ*ssrA* $P_{spoT::lacZ1}$	This study
SS3282	PAO1 Δ*ssrA* $P_{lasR::lacZ1}$	This study
SS3285	PAO1 *PA4464*::Tn*5* $P_{ssrA::lacZ}$ / pBL005	This study
SS3287	PAO1 *PA4464*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL068	This study
SS3307	PAO1 Δ*ssrA* $P_{lasR::lacZ1}$ / pBL185	This study
SS3497	PAO1 *rhlR*::*tet* $P_{ssrA::lacZ}$ / pBL167	This study
SS3500	PAO1 Δ*relA* $P_{rhlR::lacZ1}$	This study
SS3502	PAO1 Δ*relA* $P_{lasR::lacZ1}$	This study
SS3506	PAO1 Δ*relA* Δ*spoT* $P_{lasR::lacZ1}$	This study
SS3508	PAO1 Δ*relA* Δ*spoT* $P_{rhlR::lacZ1}$	This study
SS3627	PAO1 $P_{relA::lacZ2}$	This study
SS3629	PAO1 Δ*ssrA* $P_{relA::lacZ2}$	This study
SS3643	PAO1 $P_{spoT::lacZ2}$	This study
SS3645	PAO1 Δ*ssrA* $P_{spoT::lacZ2}$	This study
SS3691	PAO1 Δ*ssrA* $P_{relA::lacZ2}$ / pBL185	This study
SS3732	PAO1 *lasR501*::*aacC1* $P_{ssrA::lacZ}$	This study
SS3749	PAO1 *lasR501*::*aacC1* $P_{ssrA::lacZ}$ / pBL034	This study
SS3751	PAO1 *lasR501*::*aacC1* $P_{ssrA::lacZ}$ / pBL032	This study
SS3802	PAO1 $P_{relA::lacZ1}$	This study
SS3806	PAO1 Δ*ssrA* $P_{relA::lacZ1}$	This study
E. coli		
DH10B	F^- *mcrA Δ*(*mrr-hsdRMS-mcrBC*) ϕ*80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ*(*ara, leu*)*7697 galU galK l-rpsL nupG*	Invitrogen
HB101	F^- *Δ*(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1 Δ*(*mcrC-mrr*) *rpsL20 xyl-5 mtl-1 recA13*	Lab Collection
DH5αF’	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Lab Collection

Plasmids		
pAT3	$P_{ssrA::lacZ1}$ in pSS223 (Ap^r/Cb^r)	Lab Collection
pBL005	mSF in pSS213 (Gm^r)	This study
pBL032	*tet* in pSS163 (Ap^r/Cb^r and Tet^r)	This study
pBL034	*tet* in pUCP19 (Ap^r/Cb^r and Tet^r)	This study
pBL044	*aacC1* in pSU38 (replaced Km^r with Gm^r)	This study
pBL065	*rpoN* operon in pSS213 (Gm^r)	This study
pBL068	mSF in pBL065 (Gm^r)	This study
pBL084	Δ*spoT* in pEX100T-link (Ap^r/Cb^r)	This study
pBL085	Δ*relA* in pEX100T-link (Ap^r/Cb^r)	This study
pBL090	Δ*vfr* in pEX100T-link (Ap^r/Cb^r)	This study
pBL127	$P_{spoT::lacZ1}$ in pSS223 (Ap^r/Cb^r)	This study
pBL135	$P_{relA :: lacZ2}$ in pSS231 (Ap^r/Cb^r)	This study
pBL139	$P_{spoT::lacZ2}$ in pSS231 (Ap^r/Cb^r)	This study
pBL150	m*oriT* in pBL044 (Gm^r)	This study
pBL158	*rhlRI* in pBL044 (Gm^r)	This study
pBL167	m*oriT* in pBL158 (Gm^r)	This study
pBL185	*PA0827-ssrA* in pBL150 (Gm^r)	This study
pBL188	$P_{relA::lacZ1}$ in pSS223 (Ap^r/Cb^r)	This study
pEX100T-link	*sacB*, *oriT* (Ap^r/Cb^r)	(20)
pRK2013	Tra1 (RK2), ColE1 (Km^r)	(25)
pSS163	*lasR* in pUCP19 (Ap^r/Cb^r)	Lab Collection
pSS213	$P_{T7(A1/04/03)}$ (Gm^r)	(21)
pSS223	*trp'-lacZ1* (Ap^r/Cb^r)	(21)
pSS231	*lacZ2* (Ap^r/Cb^r)	(21)
pSS233	$P_{lasR::lacZ1}$ in pSS223 (Ap^r/Cb^r)	Lab Collection
pSS234	$P_{rhlR::lacZ1}$ in pSS223 (Ap^r/Cb^r)	Lab Collection

[a] Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cb^r , carbenicillin resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant. [b] Silo-Suh laboratory collection originally purchased from *P. aeruginosa* stock center (UW-Seattle, Washington)

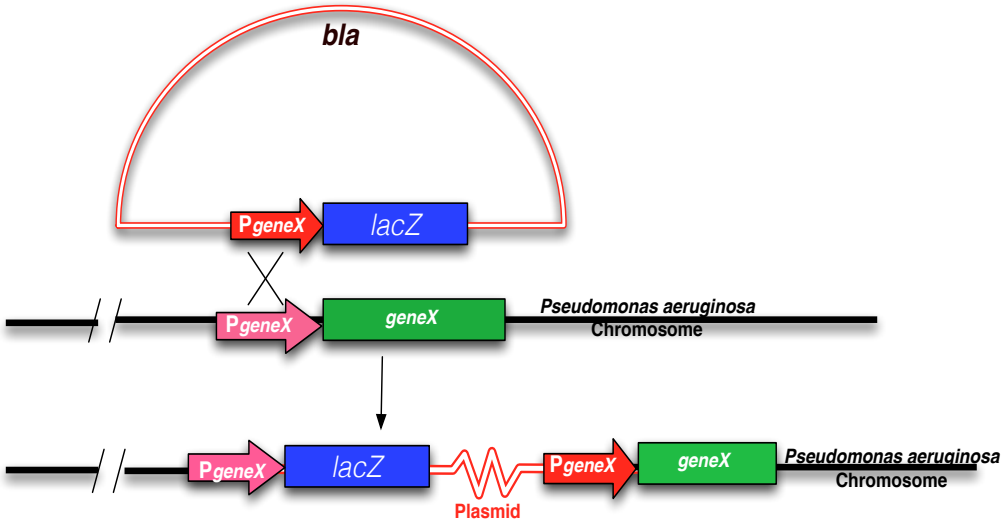

Figure 3.1. Schematic of chromosomal *gene*::*lacZ* construction in *P. aeruginosa* (24).

Results

LasR, RhlR, and Vfr are required for full expression of *ssrA* in *P. aeruginosa*

Our previously data demonstrated that RpoS is required for full expression of *ssrA* (Figure 1.5). In *P. aeruginosa*, the Las and the Rhl cell-cell communication systems participate in control of *rpoS* expression (11). In turn, the Las system is regulated by Vfr (15). I addressed whether Vfr and the two acylhomoserine lactone mediated cell-cell communication systems, Las and Rhl, affect *ssrA* gene expression. As shown in Figure 3.2., I discovered that *ssrA* expression was decreased by almost 80%, 60%, and 50% in the *vfr*, *rhlR,* and *lasR* mutant, respectively. Figure 3.3. show genetic complementation of the *lasR* and *rhlR* mutations. Complementation of the *rhlR* mutant was chromosomal, for the unsuccessful restoration of *ssrA*::*lacZ* expression when the wild type *rhlR* gene was introduced on a multicopy plasmid (data not shown). Thus, no plasmid vector controls were used during the β-galactosidase assay for the *rhlR* mutant complementation (Figure 3.3). My results demonstrate that cell-cell communication systems positively regulate *trans*-translation in *P. aeruginosa*.

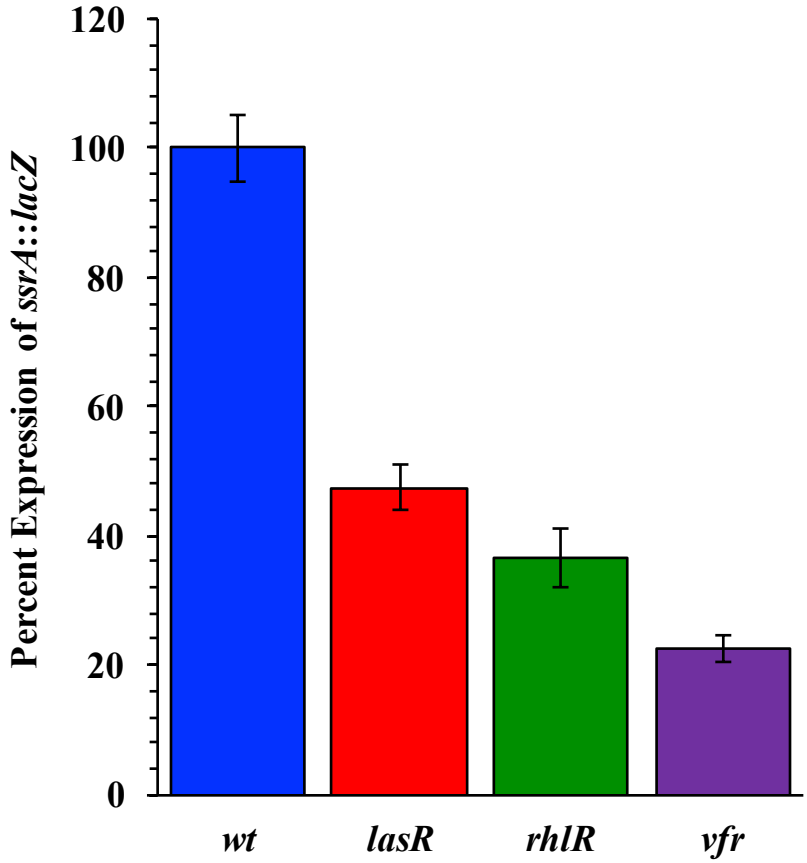

Figure 3.2. Effects of LasR, RhlR, and Vfr on *ssrA* expression in *P. aeruginosa*. Bacterial strains were grown overnight in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 6 independent experiments. The error bars show standard error.

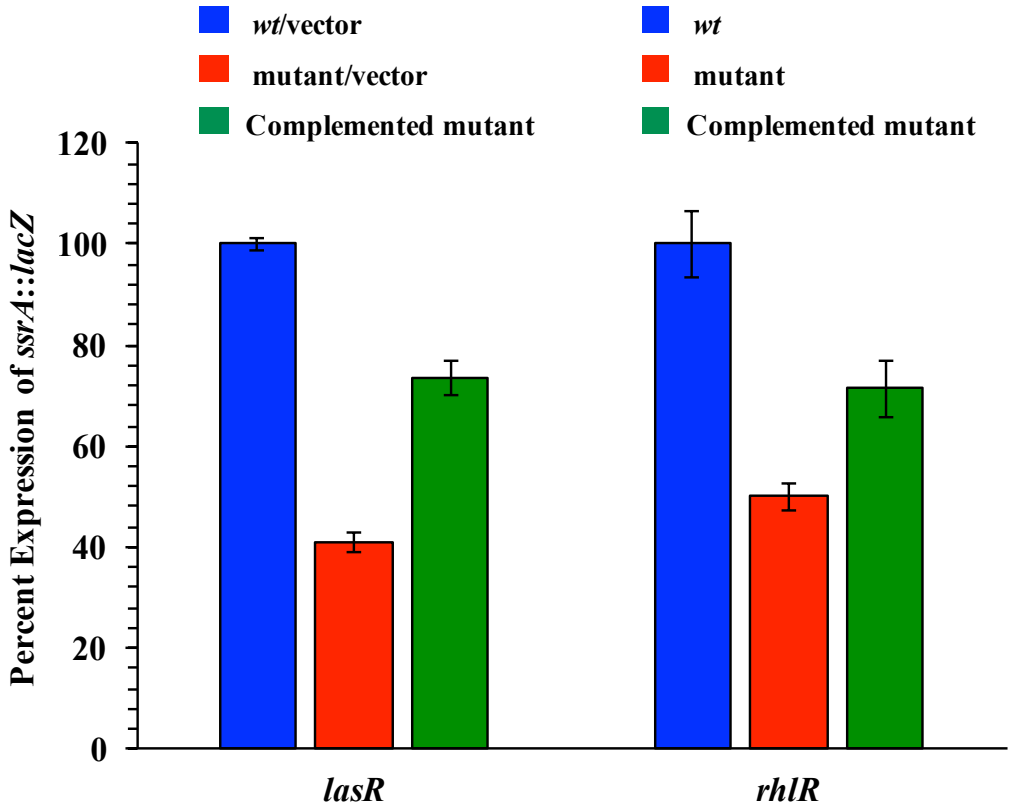

Figure 3.3. Complementation of the *lasR* and *rhlR* mutant on *ssrA* expression. For complementation of the *lasR* mutant, bacterial strains were grown overnight at 37ºC with aeration in LB supplemented with carbenicillin (100 µg/ml) and tetracycline (50 µg/ml). For *rhlR* complementation, the *wt* and mutant were grown overnight at 37ºC with aeration in LB supplemented with carbenicillin (100 µg/ml), and the complemented strains with integrated pBL167 were additionally supplemented with gentamicin (50 µg/ml) to prevent the loss of plasmid β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 2 independent experiments.

Trans-translation is required for full expression of *lasR* and *rhlR*

I addressed whether *trans*-translation formed a regulatory circuit with Vfr, Las, and Rhl as it does with RpoS. As shown in Figure 3.4, expression of both *lasR* and *rhlR* were decreased by 70% and 60%, respectively, in the Δ*ssrA* mutant. The expression of *lasR* was completely complemented with a chromosomally integrated copy of the *PA0827* operon containing *ssrA*. However, I only achieved partial complementation of *rhlR*. To address whether this partial complementation is due to the requirement of LasR for full *rhlR* expression (11), I cloned and expressed *lasR* from an exogenous promoter P_{lac} to liberate it from the tmRNA effect. As shown in Figure 3.5, I was able to achieve full expression of *rhlR* by complementing the Δ*ssrA* mutation while providing LasR *in trans* from an exogenous promoter.

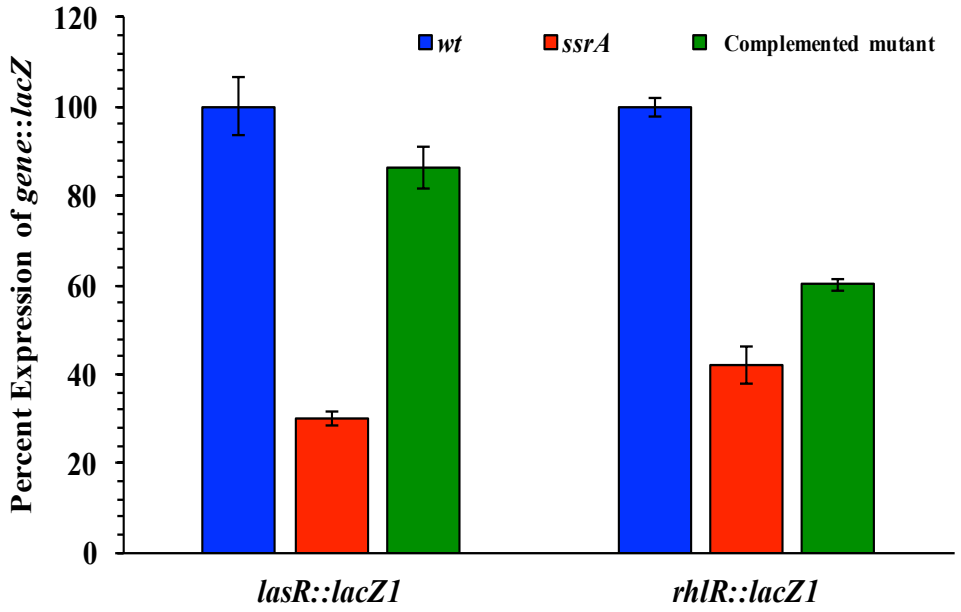

Figure 3.4. *lasR* and *rhlR* expression requires tmRNA. Bacteria were grown at 37ºC with aeration in LB supplemented with carbenicillin (100 µg/ml). The complemented strains with integrated pBL185 were additionally supplemented with gentamicin (50 µg/ml) to prevent the loss of plasmid. β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 3 independent experiments.

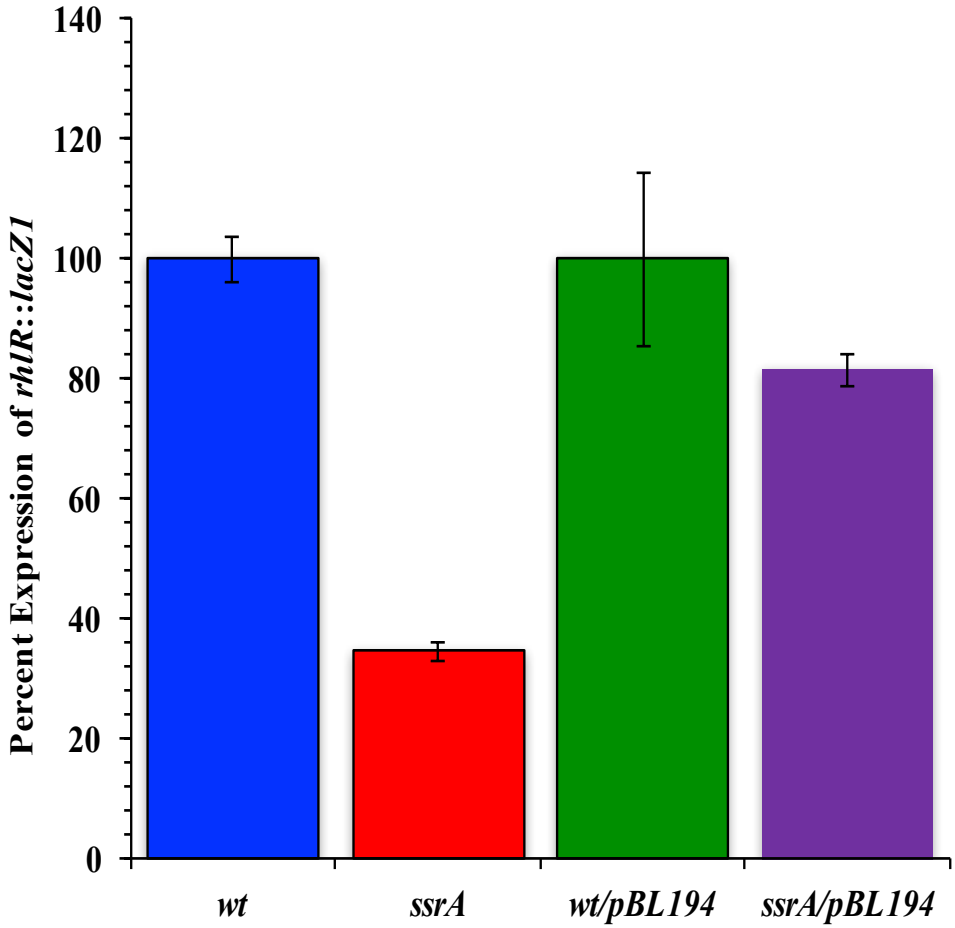

Figure 3.5. Restoration of *rhlR* expression in the *ssrA* mutant. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. Strains with pBL194 carrying *lasR* were additionally supplemented with gentamicin (50 µg/ml). β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 2 independent experiments.

Trans-translation regulates *relA* translation

In bacteria, the alarmone, (p)ppGpp, induces the stringent response when they experience amino acid limitation (26). Similar to *E. coli*, RelA and SpoT are the two major factors controlling cellular (p)ppGpp level in *P. aeruginosa* (27). RelA-mediated (p)ppGpp production is induced by amino acid starvation and heat shock (13, 28). However, SpoT-mediated accumulation of (p)ppGpp is stimulated by carbon, iron, or fatty acid starvation (26). (p)ppGpp has been demonstrated to activate and modulate cell-cell communication and virulence factor productions in *P. aeruginosa* (12, 13). Based on my data demonstrating a decrease in transcription of *lasR* and *rhlR* in a Δ*ssrA* mutant, I addressed whether the tmRNA effect on *lasR* and *rhlR* expression was through (p)ppGpp accumulation. To address the effect of *trans*-translation, I constructed *relA*::*lacZ* and *spoT*::*lacZ* transcription and translation fusions and determined their expressions in the Δ*ssrA* mutant. The results are shown in Figures 3.6 and 3.7. I determined that neither *relA* nor *spoT* expression was affected by the Δ*ssrA* mutation. In contrast, the translation of *relA* but not *spoT* was affected by the Δ*ssrA* mutation. In the Δ*ssrA* mutant, translation of *relA* was decreased by 50%.

My data suggest that the transcriptional effect of tmRNA on *rpoS, lasR,* and *rhlR* is likely to be due to its effect on the translation of *relA* and thus on the accumulation of (p)ppGpp. To address this hypothesis, I determined the expression of *lasR* and *rhlR* in *P. aeruginosa* affected in (p)ppGpp accumulation. As shown in Figure 3.8, expression of *lasR* and *rhlR* were severely reduced in both Δ*relA* and Δ*relA* Δ*spoT* mutants in support of the published data (13). The *lasR* gene expression was decreased by 80% and 95%, and the *rhlR* gene expression was decreased by 45% and 85%, respectively, in the Δ*relA* and Δ*relA* Δ*spoT* mutants.

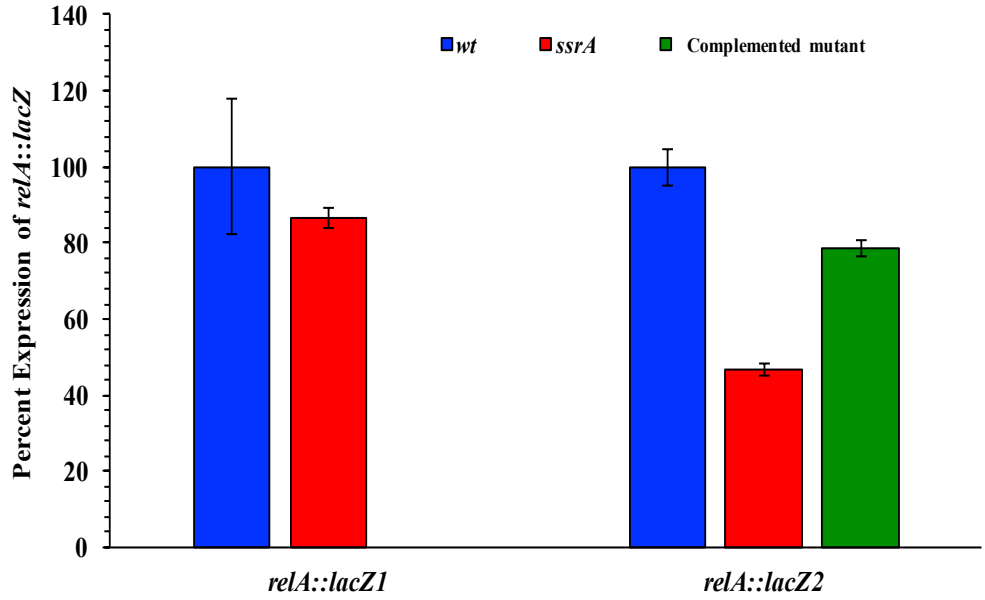

Figure 3.6. Effect of *trans*-translation on *relA* expression. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. The *ssrA* complemented strain was grown in LB supplemented with carbenicillin (100 µg/ml) and gentamicin (50 µg/ml). β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of at least 2 independent experiments.

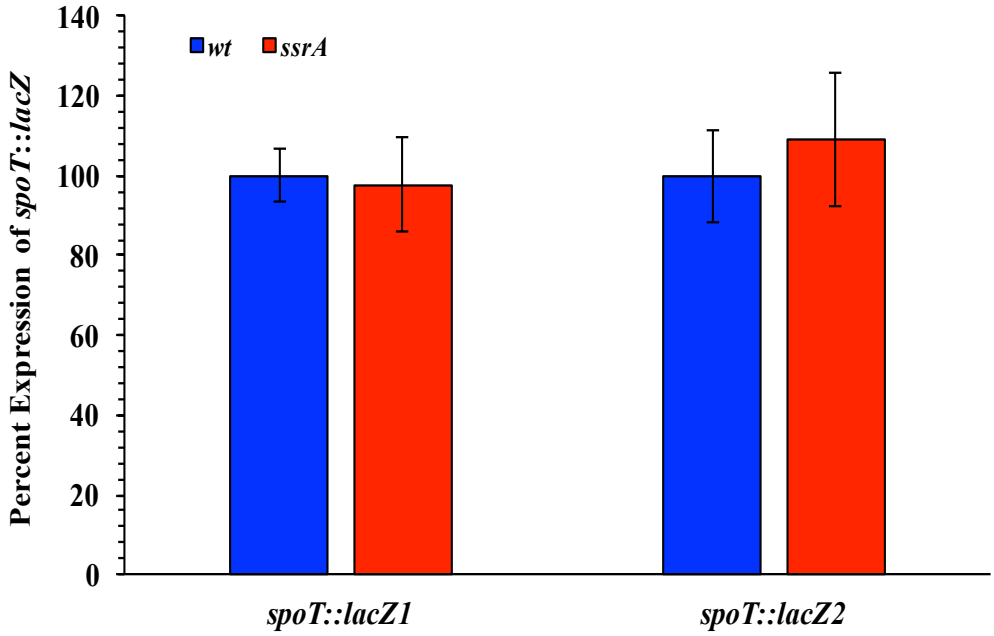

Figure 3.7. Effect of *trans*-translation on *spoT* expression. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 2 independent experiments.

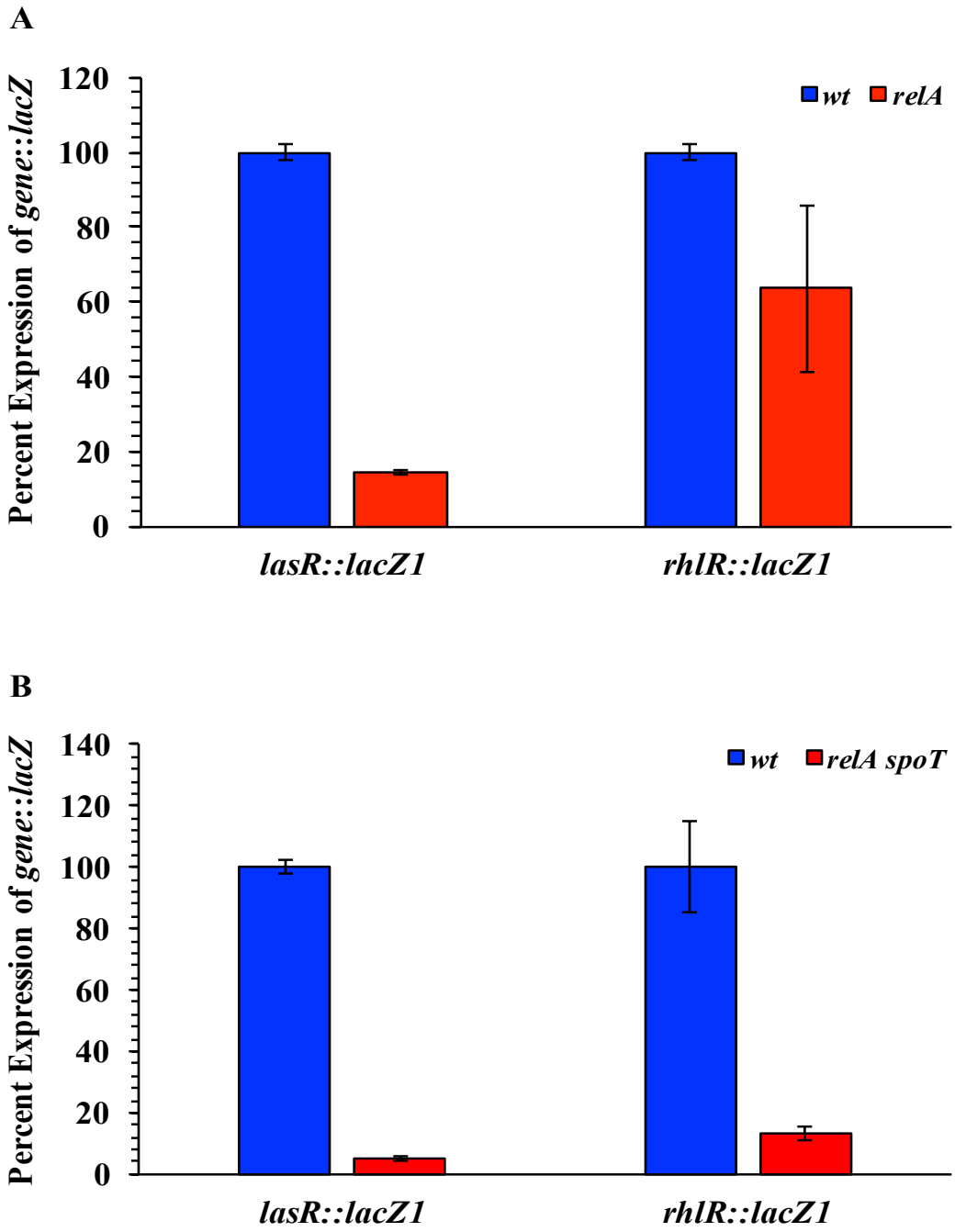

Figure 3.8. Effect of (p)ppGpp on *lasR* and *rhlR* expression. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 2 independent experiments. A: Δ*relA*; B: Δ*relA* Δ*spoT*.

ssrA is a part of the (p)ppGpp regulon

To determine whether *ssrA* expression is regulated by (p)ppGpp, *ssrA*::*lacZ* was introduced into the genomes of the Δ*relA* and Δ*relA ΔspoT* mutant. As shown in Figure 3.9, *ssrA* expression was decreased by 80% in the Δ*relA* mutant. In contrast, *ssrA* expression was decreased only by 20% in the Δ*relA* Δ*spoT* mutant. These results suggest that the level of (p)ppGpp rather than its complete absence is important for regulating *ssrA* expression in *P. aeruginosa*.

ssrA is a part of the RpoN regulon

In *P. aeruginosa*, RpoN controls a variety of genes including those involved in stress response, production of fimbriae, flagella, pyocyanin and alginate production, and amino acid metabolism (31-34). PtsN is an accessory protein that mediates RpoN activity in *P. aeruginosa* (35). Given a wide range of stress response regulated by RpoN, I addressed whether RpoN regulates *trans*-translation in *P. aeruginosa* by determining *ssrA* expression in a *rpoN* and a *ptsN* (*PA4464*) mutant. As shown in Figure 3.10, the *ssrA* gene expression was decreased by 80% and 60% in the *rpoN* and the *ptsN* mutant, respectively. These data suggest that *trans*-translation is a part of the RpoN regulon in *P. aeruginosa*.

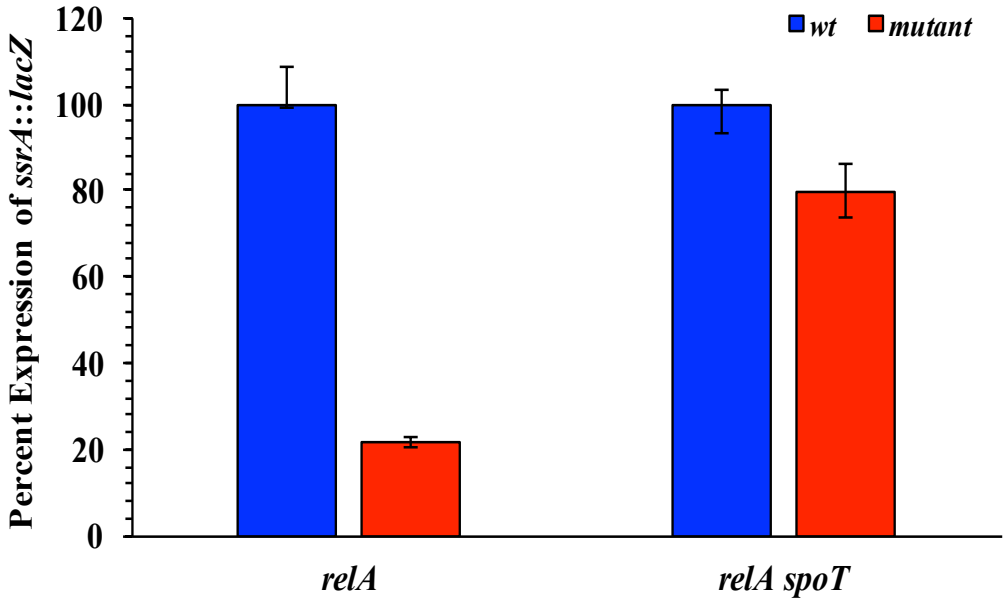

Figure 3.9. Effect of (p)ppGpp on *ssrA* expression in *P. aeruginosa*. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. β-galactosidase assay was performed according to Miller (23) in duplicate. Shown is a representative of 2 independent experiments.

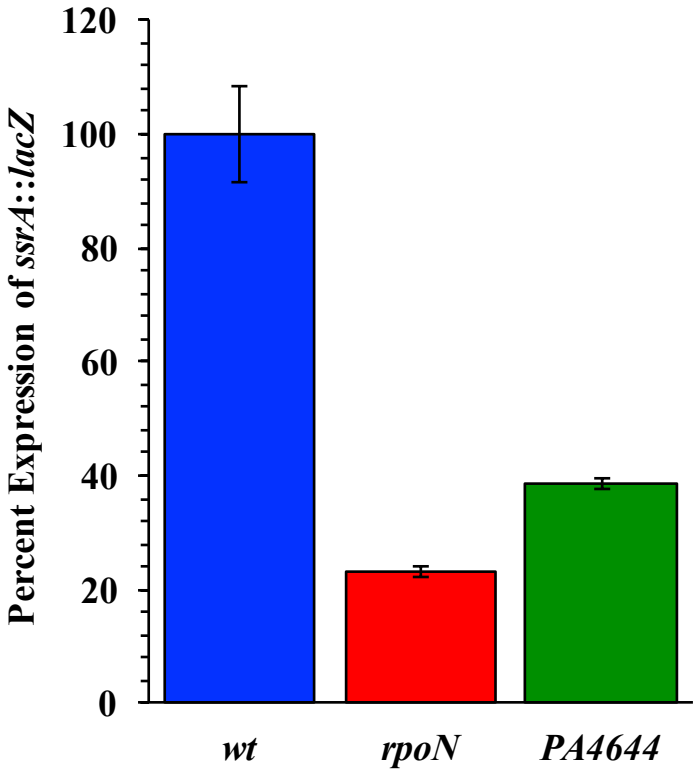

Figure 3.10. Effect of RpoN and PA4464 on *ssrA* expression in *P. aeruginosa*. Bacterial strains were grown in LB supplemented with carbenicillin (100 µg/ml) at 37ºC with aeration. Shown is a representative of 2 independent experiments.

Discussion

The results presented in this chapter demonstrate that *trans*-translation is an integral part of a complex regulatory network in *P. aeruginosa* and is involved in various stress response and virulence factor productions. My data clearly demonstrate that a large number of global regulators participate in *ssrA* expression to control *trans*-translation. These regulators include RpoS, LasR, RhlR, Vfr, RpoN, and (p)ppGpp. At present, it is unclear how each of these regulators controls *ssrA* expression. There is very little available data regarding the regulatory region of *ssrA*. In *P. aeruginosa*, *ssrA* is the second gene of the putative *PA0827* operon. However, although this two genes are considered to make up an operon, no experimental evidence is available to support this assertion. Because the *ssrA* promoter has not yet been mapped, all of my regulatory studies were performed with *lacZ* fusions constructed with a DNA fragment that contained the putative promoter region of *PA0827-ssrA*. Sequence analysis of the entire *PA0827* operon region from the putative promoter region of *PA0827* to the beginning of *ssrA* failed to identify obvious regulator binding sites including Lux box for LasR and RhlR, CRP-binding site for Vfr, or a likely RpoS promoter. Given that (p)ppGpp level affects *ssrA* expression, it is possible that a decreased *ssrA* expression in these regulatory mutants is simply due to the action of (p)ppGpp weakening the RNA polymerase from forming an open complex (26). Interestingly, complete absence of (p)ppGpp only decreased *ssrA* expression by 20%. This suggests that improper concentration of (p)ppGpp is more detrimental to the cell than the complete absence. Additionally, given the highly interconnected nature of the regulatory networks that control expression and activity of these global regulators in *P. aeruginosa* (11), it is possible that many of the effects on *ssrA* expression are indirect.

The complexity of the regulatory networks in *P. aeruginosa* got even more complex with my discovery that *trans*-translation was required for full transcription of several global

regulator genes including *rpoS*, *lasR*, and *rhlR*. Given that *trans*-translation does not affect RNA polymerase directly, my data implied that a master regulator of gene expression was regulated by *trans*-translation. In *P. aeruginosa*, (p)ppGpp has been demonstrated to govern activation of cell-cell communication systems as well as stress response as a result of nutrient deprivation (11-13). (p)ppGpp level in the cell is modulated by two enzymes RelA and SpoT (26). Thus, I surmised that either RelA or SpoT may be the target of *trans*-translation. As demonstrated by my data on Figures 3.6 and 3.7, translation of *relA* but not *spoT* requires tmRNA. Thus, it is likely that the transcriptional phenotype of *rpoS*, *lasR*, and *rhlR* in the Δ*ssrA* mutant is caused by the alteration in (p)ppGpp level in the Δ*ssrA* mutant. What unclear is exactly how *trans*-translation affects *relA* translation and (p)ppGpp level in *P. aeruginosa*. One possibility is that via the tagging and proteolysis (1), *trans*-translation negatively regulates synthesis of certain proteins, which are inhibitors of *relA* translation. Alternatively, in the *ssrA* mutant, excess ribosomes stalling on *relA* mRNA is detrimental for optimal synthesis of (p)ppGpp. It is also possible that partially synthesized RelA peptide may interfere with the activity of fully formed RelA. There are other possibilities including a potential relationship between *trans*-translation and toxin-antitoxin activities in *P. aeruginosa*. A potential link between toxin-antitoxin and stringent response has been established in *E. coli* (33, 34). However, the exact mechanism has not yet been elucidated. My data suggest a potential role of *trans*-translation in this relationship in *P. aeruginosa*. Finally, it is possible that a similar mechanism that is seen in *E. coli* on the effect of *trans*-translation on *rpoS* mRNA translation maybe responsible for the *relA* phenotype I observed in *P. aeruginosa*. In *E. coli*, RpoS translation is impaired in the *ssrA* mutant at a lower temperature due to excessive ribosome stalling between the 477 and 750 nucleotide of the ORF (35). Lack of *trans*-translation results in accumulation of stalled ribosomes and a decrease in the translation machinery. The cumulative effect is an overall

decrease in the elongation of translation on the *rpoS* mRNA and lower concentration of functional RpoS (35). A similar effect maybe occurring on the *relA* mRNA in *P. aeruginosa*.

The genetic data presented in this chapter suggest a complex regulatory between *trans*-translation and other global regulators in *P. aeruginosa*. It will be important to verify my genetic data via biochemical and molecular analysis of this regulatory circuit.

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Chapter 4

Isolation of putative regulator mutants of *ssrA* in *P. aeruginosa*

Abstract

In bacteria, the *ssrA* gene encodes for a small hybrid RNA, tmRNA, that catalyzes the primary ribosome rescue mechanism of *trans*-translation. As described in previous chapters, ribosome rescue is an essential function in bacteria that affects various aspects of cellular physiology. In Chapter 2, I presented that *ssrA* gene expression is regulated by a number of global regulators in *P. aeruginosa*. In this chapter, I present my effort to identify other potential regulators of *ssrA* expression. In contrast to Chapter 2, in this study, I took a non-predictable approach by using the Tn*5*-B30 transposon mutagenesis to isolate insertion mutants in which the *ssrA*::*lacZ* expression was affected. After screening approximately 15,000 mutants, I identified seven genetic loci that affected the *ssrA*::*lacZ* expression in *P. aeruginosa* PAO1. Interestingly, I did not isolate any transposon insertions in any of the global regulators I discussed in Chapter 2. The genetic loci I identified in this chapter indicate that *ssrA* expression is regulated by multiple factors involved in adaptive resistance to antimicrobials, iron acquisition, potential RNA processing, as well as glycine, glycerol and phosphate metabolisms.

Introduction

As described in previous chapters, *trans*-translation is the primary ribosome rescue mechanism in bacteria and its physiological functions have been characterized in several organisms (1).

The research in our laboratory has demonstrated that *trans*-translation plays a role in stress response and virulence factor productions in *Pseudomonas aeruginosa*, an opportunistic human pathogen that causes both acute and chronic infections (2). As described in Chapter 2, I further discovered that *trans*-translation affects persister cell formation, biofilm formation, and utilization of certain nutrients. In Chapter 3, I described the complex network of regulatory factors that affect expression of *ssrA*, the structural gene encoding tmRNA, and how several of these global regulators are, in turn, regulated by tmRNA-mediated *trans*-translation. Although tmRNA has been characterized in great detail at the biochemical and structural level, very little research has been conducted to identify the genetic factors that regulate *trans*-translation activity. The data presented in Chapters 1 and 3 are some of the only data available on factors that govern *trans*-translation at the genetic level. In an effort to further understand the genetic regulation of *trans*-translation, I took a non-predictable approach and conducted a transposon mutagenesis on *P. aeruginosa* carrying a *ssrA*::*lacZ* fusion.

Materials and Methods

Bacterial strains, plasmids, media and chemicals

All bacteria and plasmids used for this study are listed in Table 4.1. Unless otherwise indicated, bacteria were grown in L broth LB ((3); Fisher Scientific, Pittsburgh, PA) or in LB supplemented with appropriate antibiotics at 30°C with aeration. Solid media were prepared using L-agar (BD Biosciences). Pseudomonas isolation agar (PIA; BD

Biosciences) was used for tri-parental mating to counterselect *P. aeruginosa* against *Escherichia coli* as previously described (4). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at following concentrations: ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 100 μg/ml for *P. aeruginosa;* gentamicin, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin, 50 μg/ml for *E. coli*; tetracycline, 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*.

DNA manipulations, transformations, and conjugations

For cloning DNA*, E. coli* strain DH10B was routinely used as the host strain. Unless otherwise indicated, DNA was introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (4). Plasmids were purified with QIAprep Spin Miniprep columns (Qiagen, Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (NEB; Beverly, MA). Either *Phusion* polymerase (NEB) or *Taq* polymerase (NEB) was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

β-galactosidase assay

To determine expression of the target genes, β-galactosidase assays were performed as described by Miller (5). Absorbance measurements were conducted using Shimadzu UV1601 spectrophotometer (Shimadzu, Kyoto, Japan), for tube assays, or BioTek Cytation 3 Microtiter plate reader (Winooski, VT), for 96-well microtiter plate assays.

Transposon mutagenesis

The transposon mutagenesis was conducted using a mini transposon Tn*5*-B30 (6). Tn*5*-B30, which is carried in SS1479, was introduced into SS1740, a PAO1 strain with the chromosomal *ssrA*::*lacZ* fusion, through triparental mating as previously described (7). Tetracycline resistant (Tc^r) colonies with Tn*5*-B30 insertion were sprayed with 1mg/ml the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Insertion mutants with different coloration than the wild type, indicating altered *ssrA*::*lacZ* expression, were picked and patched on PIA plates supplemented with 100 μg/ml carbenicillin (Cb) and 80 μg/ml X-gal for confirmation. Insertion mutants consistently showing altered phenotype on X-gal plates were isolated for β-galactosidase assay in 96-well microtitrer plates (Figure 4.1B) to quantitate the *ssrA*::*lacZ* expression. Cells used for β-galactosidase assay were grown overnight in LB supplemented with 100μg/ml Cb.

Tube β-gal Assay

Insertion mutants that showed altered *ssrA*::*lacZ* expression in 96-well microtiter plate β-galactosidase assay were subjected to more accurate test tubes assays. For tube assays, overnight grown cells were diluted 1:100 in fresh LB supplemented with 100 μg/ml Cb and incubated for 16 hours at 30°C with aeration. Insertion mutants with consistently altered *ssrA*::*lacZ* expression in tube assays were isolated as putative regulator mutants of *ssrA* expression.

Mapping Tn*5*-B30 insertion sites

Tn*5*-B30 insertion sites in the putative regulator mutants were mapped by arbitrary PCR as previously described (8, 9). Arbitrary PCR products were sequenced (Auburn

University Sequence Center) and the affected genes were identified using the BLAST against the genome of PAO1 (http://www.pseudomonas.com/).

Complementation of the putative regulator mutants

Genes of interest were PCR amplified without the native promoter and cloned into pSS213 under control of the $P_{T7(A1/04/03)}$ (7) to construct the complementing plasmids. The mSF was then cloned into these plasmids as a *Hin*dIII fragment to convert them into *P. aeruginosa* replicons (7). The complementing plasmids were introduced into the cognate transposon insertion mutants via conjugation and Gm^R colonies carrying the plasmids were tested for complementation. SS1740 and the insertion mutants carrying the plasmid vector pBL005 were used as controls. Oligonucleotide primers used for constructing complementing plasmids are listed in Table 4.1.

Table 4.1. PCR primers used in this study

Primer	Sequences (5' to 3')	Reference
SSO-130 (Tn5 Ext)	GAA CGT TAC CAT GTT AGG AGG TC	(8)
SSO-131 (Tn5 Int)	CGG GAA AGG TTC CGT TCA GGA CGC	(8)
SSO-133 (Arb2)	GGC CAC GCG TCG ACT AGT AC	(8)
SSO-240 (Arb6)	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC	(8)
SSO-836	CAT GGA TCC CGC CGA GCC CCG CCA AGA CC	This study
SSO-874	CAT GAA TTC CCC ATC CGT CAT GCG AAC AGA TAC C	This study
SSO-818	CAT GGA TCC TGA ACG GGA ACC TCG GCG GAA CTA CG	This study
SSO-899	CAT GAA TTC GCG CGA CTT CAA GGA GAG GGC	This study

SSO-816	CAT GGA TCC ATC GGA CGA AAT CGG CAG TAT GAA C	This study
SSO-900	CAT GAA TTC GGA CTA GAC CAA TAA GGA AGC TGT CG	This study

Table 4.2. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant characteristics[a]	Source or reference
P. aeruginosa		
SS1740	PAO1 $P_{ssrA::lacZ}$	Lab Collection
SS1078	PAO1 *phoP*::*aacC1*	Lab Collection
SS2838	PAO1 *PA0951*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL061	This study
SS2840	PAO1 *PA3249*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL062	This study
SS2842	PAO1 $P_{ssrA::lacZ}$ / pBL005	This study
SS2844	PAO1 *PA0951*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL005	This study
SS2846	PAO1 *PA3249*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL005	This study
SS3830	PAO1 *phoP*::*aacC1* $P_{ssrA::lacZ}$	This study
SS3831	PAO1 *PA4781*::*aacC1*	This study
SS3832	PAO1 *PA4781*::*aacC1* $P_{ssrA::lacZ}$	This study
SS3833	PAO1 *PA3078*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL051	This study
SS3834	PAO1 *PA3078*::Tn*5*-B30 $P_{ssrA::lacZ}$ / pBL005	This study
SS3835 (*prt12*)	PAO1 *PA0951*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3836 (*prt13*)	PAO1 *PA0951*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3837 (*prt14*)	PAO1 *PA4667*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3838 (*prt15*)	PAO1 *PA2445* (*gcvP2*)::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3839 (*prt16*)	PAO1 *PA2445* (*gcvP2*)::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3840 (*prt17*)	PAO1 *PA2445* (*gcvP2*)::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3841 (*prt18*)	PAO1 *PA3249*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3842 (*prt19*)	PAO1 *PA3249*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3843 (*prt20*)	PAO1 *PA1490-PA1489*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3844 (*prt21*)	PAO1 *PA1490-PA1489*::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3845 (*prt22*)	PAO1 *PA3408* (*hasR*)::Tn*5*-B30 $P_{ssrA::lacZ}$	This study
SS3846 (*prt23*)	PAO1 *PA3078* (*cprS*)::Tn*5*-B30 $P_{ssrA::lacZ}$	This study

E. coli		
DH10B	F$^-$ *mcrA Δ*(*mrr-hsdRMS-mcrBC*) ϕ*80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ*(*ara, leu*)*7697 galU galK l-rpsL nupG*	Invitrogen
HB101	F$^-$ *Δ*(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1 Δ*(*mcrC-mrr*) *rpsL20 xyl-5 mtl-1 recA13*	Lab Collection
SS1479[b]	S17-1 pro res$^-$ m$^+$ / *RP4*-Tc::Mu-*km*::TN*7* / $(Gm^R)_{102}$(Zk2219) (SMC154 = C_{70}) pSUP::Tn*5*-B30 (Tc)/pSUP::TN*5*-B30	(6)
Plasmids		
pBL005	mSF in pSS213 (Gm^r)	This study
pBL042	*PA3077* operon in pSS213 (Gm^r)	This study
pBL051	mSF in pBL042 (Gm^r)	This study
pBL058	*PA0951* in pSS213 (Gm^r)	This study
pBL059	*PA3249* in pSS213 (Gm^r)	This study
pBL061	mSF in pBL058 (Gm^r)	This study
pBL062	mSF in pBL059 (Gm^r)	This study
pRK2013	Tra1 (RK2), ColE1 (Km^r)	(10)

[a]Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cb^r , carbenicillin resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant. [b]Suh laboratory designation for the strain published by Simon and colleagues (6).

Results

Isolation of transposon insertion mutants with altered *ssrA*::*lacZ* expression in *P. aeruginosa*

To identify putative regulators of *ssrA* gene expression, I conducted a transposon mutagenesis using Tn*5*-B30 (6) (Figure 4.1). Approximately 15,000 Tn5-B30 insertion mutants were initially screened on PIA plates containing the chromogenic substrate X-gal to identify the mutants with altered *ssrA*::*lacZ* expression. 590 insertion mutants that consistently showed altered level of blue color were further analyzed by β-galactosidase assays in 96-well microtiter plates. From this analysis, 53 insertion mutants showed alteration in *ssrA*::*lacZ* expression by more than 40%. These 53 mutants were further analyzed by tube β-galactosidase assays (an average of six independent experiments with duplicates at each time) and 12 insertion mutants that consistently showed altered *ssrA*::*lacZ* expression were isolated. These mutants were designated as *prt* for putative regulator of tmRNA. All of the 12 *prt*s showed a decrease in *ssrA*::*lacZ* expression by approximately 50% as shown in Figure 4.2.

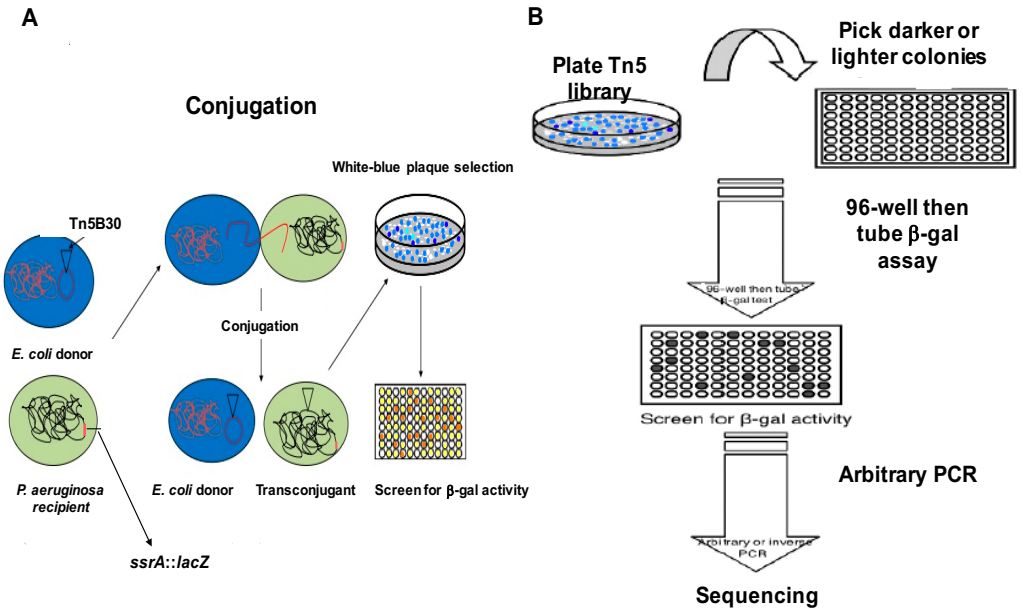

Figure 4.1. Schematic of transposon mutagenesis. (A) Tn*5*-B30 was introduced into SS1740 carrying the *ssrA*::*lacZ* fusion via conjugation. (B) Putative insertion mutants with altered *ssrA*::*lacZ* expression on plates were isolated and screened via microtiter plate β-galactosidase assays.

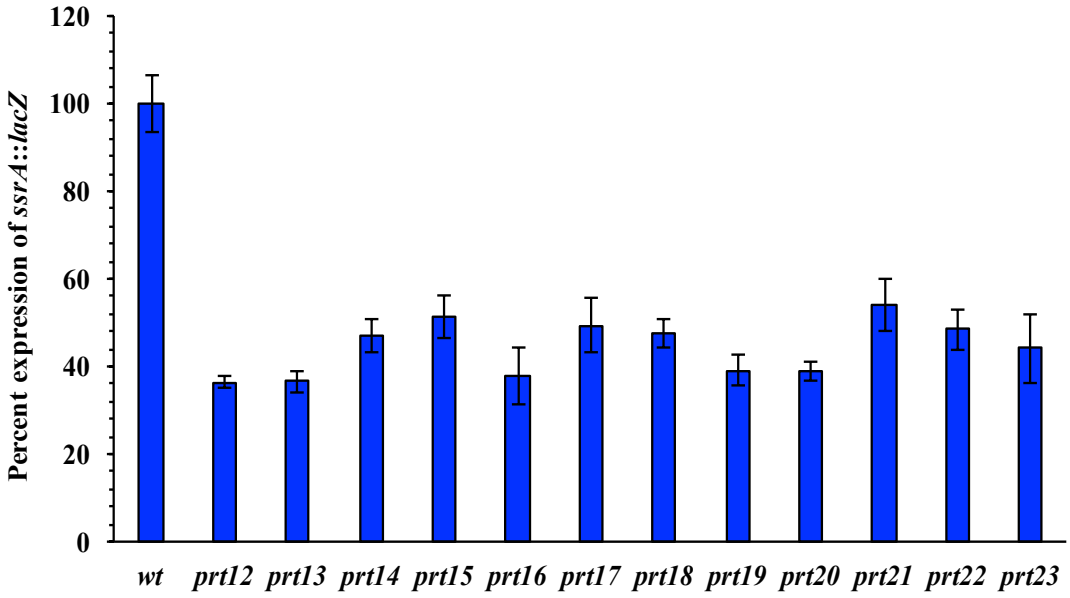

Figure 4.2. *prt*s with altered *ssrA*::*lacZ* expression. Bacterial strains were grown overnight in LB supplemented with carbenicillin (100 μg/ml) at 30ºC with aeration. β-galactosidase assay was performed according to Miller (5) in duplicate. One hundred percent corresponds to *ssrA*::*lacZ* expression in the wild type, SS1740. All data shown are means ± standard deviations (n=4). Results shown are representative of six independent experiments.

Identification of putative regulators of *ssrA*

I used the arbitrary PCR to map the Tn*5*-B30 insertion sites in the isolated *prt* mutants (Figure 4.3) as previously described (8). The arbitrary PCR products were sequenced and compared to the PAO1 genomic sequences in the Pseudomonas database (http://www.pseudomonas.com/) to identify the affected genetic loci. From this analysis, I identified seven genetic loci with Tn*5*-B30 insertions (Table 3).

The *PA3077-3078* operon encodes for CprRS, an adaptive antimicrobial resistant system in *P. aerugisnoa* (11). To date, four systems of adaptive antimicrobial resistance including PhoPQ, PmrAB, ParRS, and CprRS, have been characterized in *P. aeruginosa* (11-14). To determine whether the *ssrA* expression is affected by other mechanisms involved in adaptive antimicrobial resistance, I tested the *ssrA*::*lacZ* expression in the *P. aeruginosa phoP* mutant. As expected, *ssrA*::*lacZ* expression was decreased by more than 50% in the *phoP* mutant than in the parent (Fig. 4.4). One of the common factors among the four adaptive antimicrobial systems is the c-di-GMP-related genetic regulation. *PA4781* encodes for a major phosphodiesterase involved in hydrolysis of c-di-GMP (15). Therefore, I addressed whether the *ssrA*::*lacZ* expression is affected by the c-di-GMP level. My data show that *ssrA* expression was dramatically decreased in the *PA4781* mutant implying that *ssrA* is regulated by the c-di-GMP level in *P. aeruginosa*.

Tn*5*-B30 insertions in *prt20* and *prt21* were mapped to the intergenic space between *PA1490* and *PA1489*, products of which are homologous to GlpR and GlpD, respectively (16). GlpR is the regulator of *glpD* that encodes for glycerol-3-phosphate dehydrogenase (17). PA2445 (GcvP2) is a part of the glycine cleavage system (18). The *PA4667* operon encodes for products that is involved in Flp pilus assembly, outer membrane biogenesis, tRNA-Gln production, and phosphate metabolisms (19-23). Of the three putative transcriptional regulators identified in my analysis, PA3408 (HasR)

functions in iron acquisition (24), PA0951 may affect RNA processing (25), and PA3249-PA3248 affects N-acetylation of ribosomal proteins (16, 26). I have successfully complemented the transposon insertion mutations in *prt12* (*PA0951*), *prt18* (*PA3249*), and *prt23* (*cprS*) (Figure 4.5).

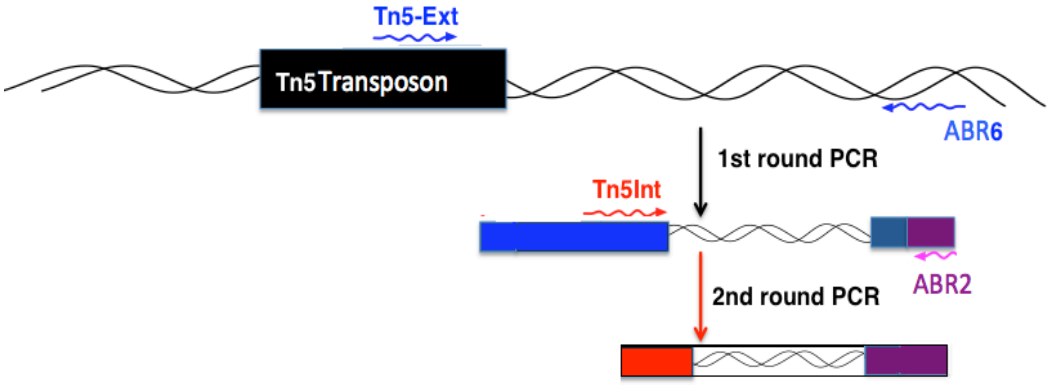

Figure 4.3. Schematic of arbitrary PCR mediated identification of Tn*5*-B30 insertion sites (adopted from (9)).

Table 4.3: List of *prt* mutants

***prt* mutant**	**gene/operon**	**gene product function**
prt12, 13	*PA0951*	Ribonuclease BN family enzyme (25)
prt14	*PA4667* (the first gene of an operon consisting of *PA4667, PA4668, PA4669, PA4669.1* and *PA 4670*)	*PA4667*: Flp pilus assembly protein TadD, contains TPR repeats *PA4668*: Outer membrane lipoprotein involved in outer membrane biogenesis *PA4669*: 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate synthase *PA4669.1*: tRNA-Gln *PA4670:* Phosphoribosylpyrophosphate synthetase (19, 21, 23)
prt15, 16, 17	*PA2445* (*gcvP2,* the second gene of the operon consisting of *PA2446* and *PA2445*)	Glycine cleavage system (18)
prt18, 19	*PA3249* (the first gene of the *PA3249* operon)	*PA3249:* Hypothetical transcriptional regulator *PA3248:* Acetyltransferases, including N-acetylases of ribosomal proteins (16, 26)
prt20, 21	Space between *PA1490* and *PA1489*	*PA1490:* Transcriptional regulator (16) *PA1489*: Putative glycerol-3-phosphate dehydrogenase complex (16) *PA1488*: oxidoreductase activity (16)

		PA1487: Glycerol lipid metabolism (16)
prt22	*PA3408* (*hasR*; the insertion might affect *PA3407*)	*hasR*: outer membrane receptor proteins, mostly Fe transport *PA3407* (*hasAp*): heme acquisition protein HasAp (24)
prt23	*PA3078* (*cprS,* the second gene of the operon consisting of *PA3077* and *PA3078*)	*PA3078*: Signal transduction histidine kinase *PA3077* (*cprR*): Response regulator, positive regulation of phospholipid biosynthetic process CprRS is a two-component system regulating the adaptive resistance of *P. aeruginosa* (11)

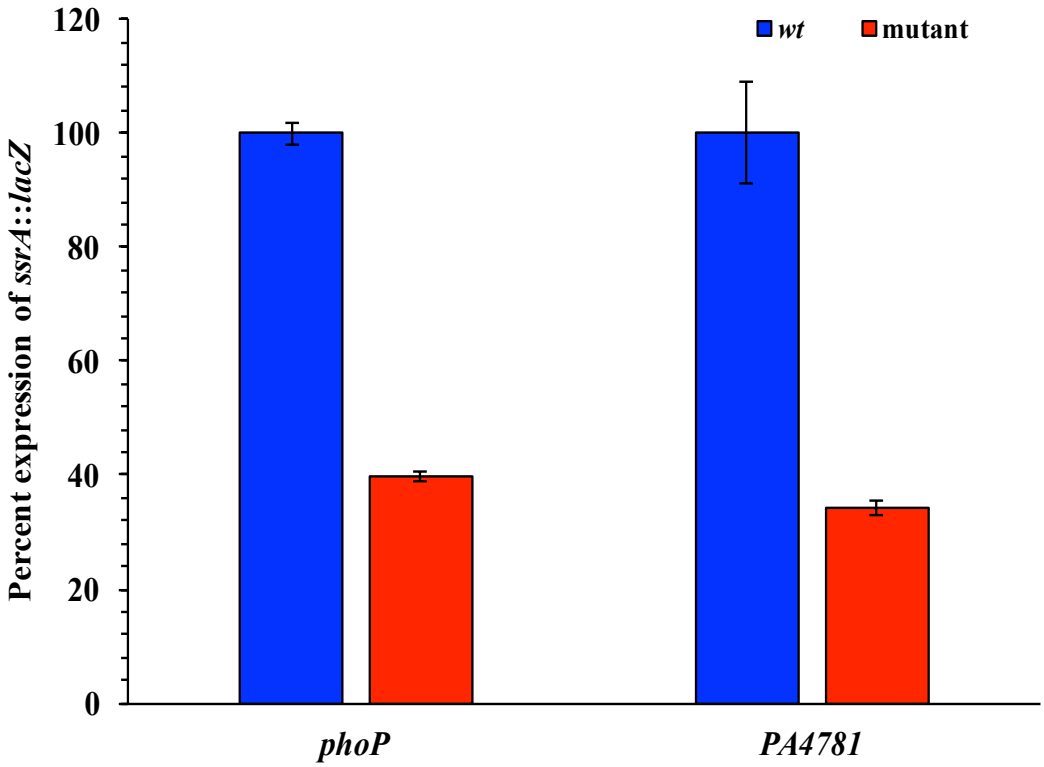

Figure 4.4. Effects of PhoP and PA4781 on *ssrA* expression in *P. aeruginosa*. Bacterial strains were grown overnight in LB supplemented with carbenicillin (100 µg/ml) at 30ºC with aeration. β-galactosidase assay was performed according to Miller (5) in duplicate. One hundred percent corresponds to the *ssrA*::*lacZ* expression in SS1740. All data shown are means ± standard deviations (n=4). Results shown are representative of 6 independent experiments.

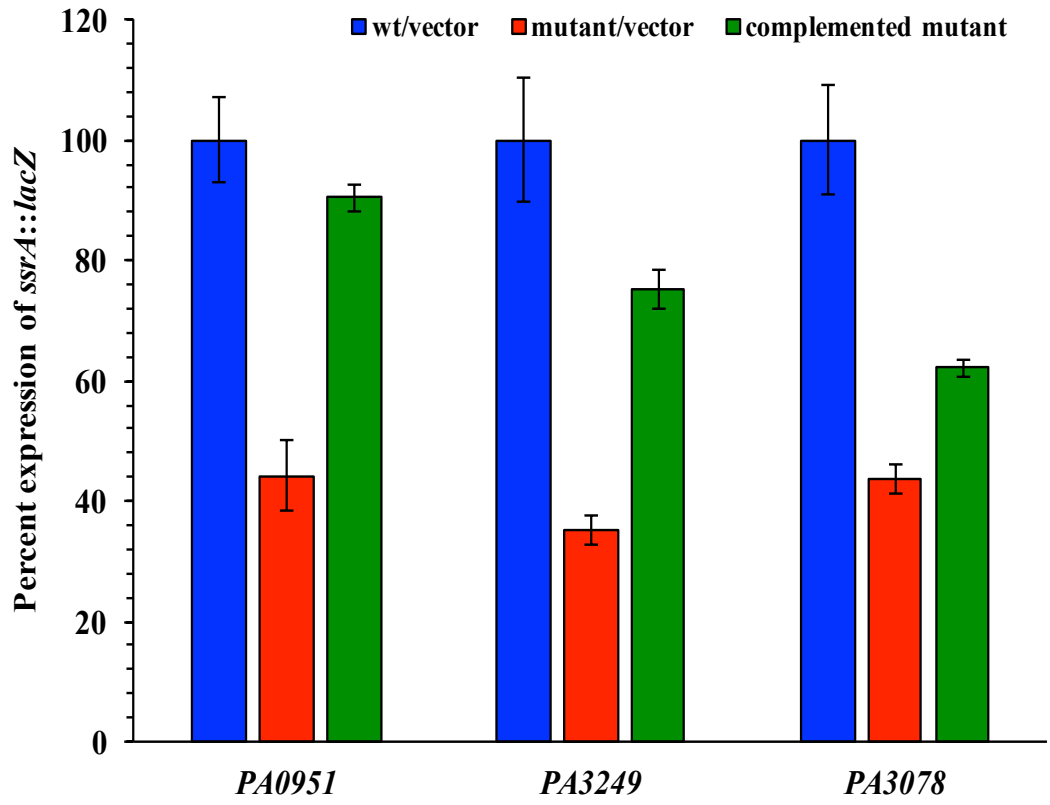

Figure 4.5. Complementation of the *prt12*, *prt18*, and *prt23* mutants. Bacterial strains of were grown overnight in LB supplemented with carbenicillin (100 µg/ml) at 30ºC with aeration. β-galactosidase assay was performed according to Miller (5) in duplicate. All data shown are means ± standard deviations (n=4). Results shown are representative of 3 independent experiments.

Discussion

The tmRNA-catalyzed *trans*-translation is the primary ribosome rescue mechanism in bacteria (1). In addition to the well-known biochemical function, *trans*-translation has effects on physiology in aspects of stress response, pathogenesis, and cell-cycle control in some bacteria (1). As discussed in Chapter 2, *trans*-translation affects osmotic and thermal stress response and production of several virulence factors in *P. aeruginosa* (Tucker and Suh, unpublished data). In addition, we found a complex regulatory circuit between tmRNA and various global regulators of gene expression as described in Chapter 3. All of the data shown in Chapter 3 on regulation were acquired via predicted approach of targeted analysis of potential regulators based on the available phenotypic data. To address whether there are other regulators that affect *ssrA* expression, I took a nonpredictive approach of random transposon insertion mutagenesis and I identified seven genetic loci that affected *ssrA* expression. Interestingly, none of the regulators identified in Chapter 3 were identified in transposon mutagenesis.

One of the most interesting finding was the potential effect of cyclic-di-GMP on *ssrA* expression. This was discovered indirectly by isolating a transposon insertion in *cprS* which led me to address whether other adaptive antimicrobial regulators were involved in *ssrA* expression. As shown in Figure 4.4, I demonstrated that another adaptive antimicrobial regulator, PhoP, is required for *ssrA* expression. In *P. aeruginosa*, PhoP responds to magnesium concentration and is required for swarming motility to cross the host epithelium and for adapting to stresses within biofilms (12, 27). Furthermore, I discovered that PA4781, a major phosphodiesterase for c-di-GMP degradation, affects *ssrA* expression (Fig. 4.4), indicating a role of the secondary messenger c-di-GMP in *trans*-translation in *P. aeruginosa*. *P. aeruginosa* possesses over 40 genes that encode for

products involved in the synthesis and degradation of c-di-GMP (28). It is unclear why I failed to isolate insertion mutations in any of these genes.

I discovered that GcvP2 positively affects *ssrA* expression in *P. aeruginosa* (Fig. 4.3; Table 4.3). GcvP2 is a member of the glycine cleavage system is highly related to pyocyanin biosynthesis in *P. aeruginosa* (18). The insertion mutations in *gcvP2* in *prt15 – prt17* may result in higher cellular level of glycine, a poor energy source that may signal production of enhanced pyocyanin production as a stress response in *P. aeruginosa* (18). In agreement with this hypothesis, our previous data showed that tmRNA negatively affects pyocyanin synthesis (Tucker and Suh, unpublished data).

I also discovered that insertion mutation in *PA4667* leads to decreased *ssrA* expression in *prt14* (Fig. 4.3; Table 4.3). The *PA4667* operon contains five genes, *PA4667*, *PA4668*, *PA4669*, *PA4669.1*, and *PA4670*. PA4667 is homologous to proteins required for Flp pilus assembly (19, 21, 23). PA4668 (LolB) is a factor for lipoprotein localization to outer membrane (19, 21, 23). PA4669 and PA4670 are isopentenyl monophosphate kinase and ribose-phosphate pyrophosphokinase in synthesis pathways of isoprenoids and nucleotides, respectively (20, 22). *PA4669.1* encodes for tRNA-Gln. Unfortunately, because Tn*5*-B30 insertion caused transcriptional polarity, it is unclear at present which of the affected genes in the operon affect *ssrA* expression. In order to address this issue, non-polar deletion in each gene of the operon should be constructed and *ssrA* expression measured.

PA1489 and PA1490 share sequence homology to GlpD and GlpR, respectively. Thus, the data suggest a potential relationship between glycerol metabolism and *trans*-transalation (17). However, further experimentation will be necessary to establish this potential relationship as PA1489 has not yet been demonstrated to possess glycerol-3-phosphate dehydrogenase activity.

Insertion in *hasR* in *prt22* suggests a potential relationship between *trans*-translation and iron metabolism in *P. aeruginosa*. *PA3249* (*prt18* and *prt19*) encodes for a transcriptional regulator that controls production of PA3248, an enzyme enzyme with acetyletransferase (GNAT) domain. This enzyme may facilitate N-acetylation of ribosomal proteins (26) to affect translation. PA0951 (*prt12* and *prt13*) is a homologue of the exonuclease RNase BN (25), which may be involved in the maturation of tmRNA. Alternatively, PA0951 may enhance degradation of mRNA transcripts. Thus, a mutation in *PA0951* may cause less truncated mRNAs and stalled ribosomes, which in turn leads to decrease in *trans*-translation.

The data presented in this chapter suggest that regulation of *trans*-translation may be even more complex than addressed in Chapter 3. Interestingly, none of the global regulators identified in Chapter 3 were found in this study. This is likely due to a potential problem with my transposon mutagenesis. For reasons that are unclear, it appears that my transposon mutagenesis was incomplete and did not cover the whole genome. This could have been due to the emergence of fast growing mutants which led to accumulation of siblings. However, as incomplete as my transposon mutagenesis may have been, it still yielded mutants that affect *ssrA* expression. Together with the data from Chapter 3, the data presented in this study suggest that *trans*-translation is a highly regulated process befitting an essential function.

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Chapter 5

Conclusions and Future Directions

Conclusions

Pseudomonas aeruginosa is an opportunistic pathogen that can cause both acute and chronic infections in immunosuppressed individuals (1). *P. aeruginosa* is highly resistant to a broad spectrum of antimicrobials through various mechanisms (1, 2). Thus, in order to combat this pathogen more effectively, it is imperative to better understand its physiology.

tmRNA-mediated *trans*-translation is the primary bacterial ribosome rescuing system, and its physiological role has been described in some bacteria (3). In this dissertation, I conducted a comprehensive genetic study to demonstrate the various physiological mechanisms that are affected by *trans*-translation in *P. aeruginosa*. I demonstrated that ribosome rescue is an essential function in *P. aeruginosa* and that backup to *trans*-translation is mediated by ArfA homologue. I also demonstrated that *trans*-translation is an integral component of global gene expression in *P. aeruginosa* through its action on translation of the *relA* mRNA which encodes for the enzyme for biosynthesis of (p)ppGpp. This is the first study to clearly demonstrate the myriad of functions that are affected by ribosome rescue in *P. aeruginosa,* and some of my results could be extrapolated to other bacteria. Based on my data, tmRNA and ArfA should be considered as potential targets for developing anti-*P. aeruginosa* drugs. However, as clearly demonstrated in

Chapter 2, both need to be simultaneously targeted as the bacterium requires only one of the two functions to maintain viability. My data presented in this dissertation lay the foundation for future studies for elucidating the molecular mechanisms of the *trans*-translation effect in *P. aeruginosa* physiology and adaptability.

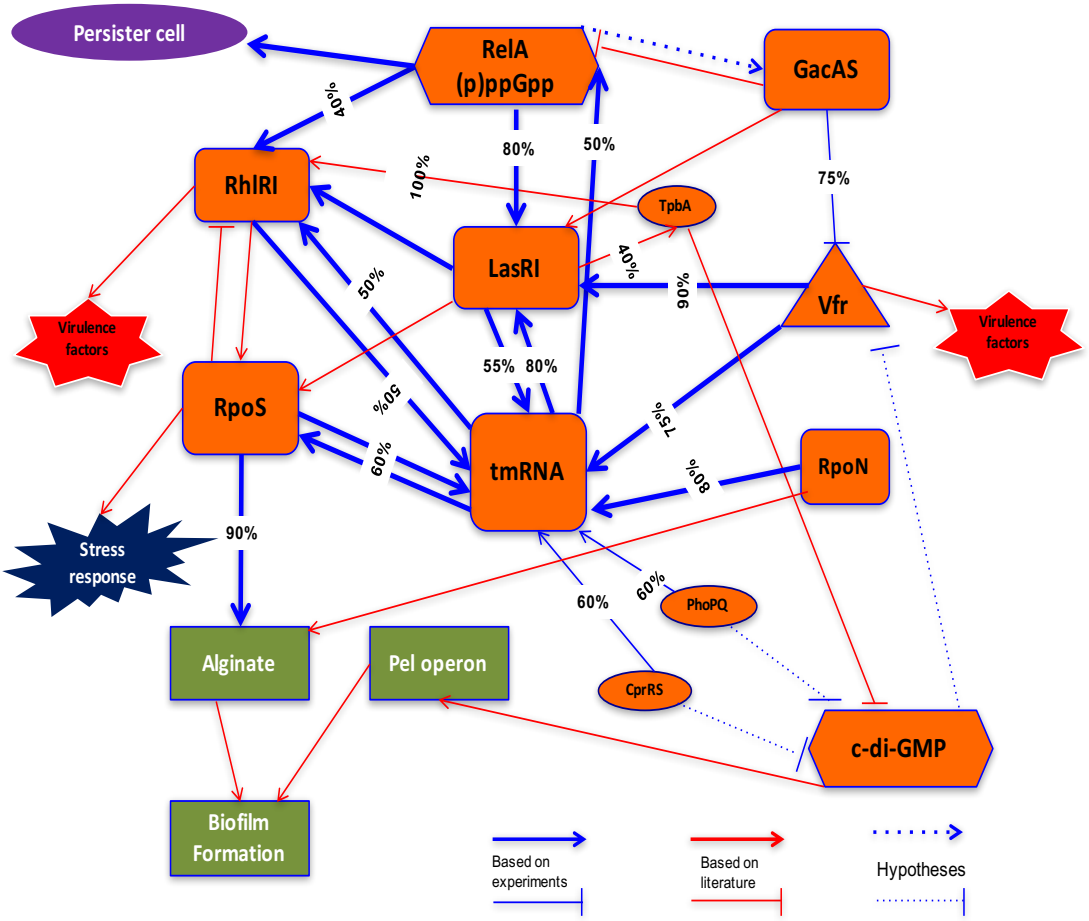

Figure 5.1. Schematic of complex regulatory circuit between *trans*-translation and global regulators of *P. aeruginosa* physiology.

Future Directions

My data presented in this dissertation raised several major questions regarding the *trans*-translation effect on expression of global regulators and certain physiological processes and the regulatory hierarchy of *ssrA* expression in *P. aeruginosa* (Figure 5.1). Discussed below are some of the near future experiments that should be conducted to clarify the complex regulatory circuit of global regulators that control *P. aeruginosa* physiology and pathogenesis.

Elucidation of the *trans*-translation effect on expression of global regulators in *P. aeruginosa*

My data in Chapter 3 suggest that *trans*-translation affects expression of *rpoS*, *lasR*, and *rhlR* at transcription level through RelA-mediated (p)ppGpp accumulation. Thus, intracellular level of (p)ppGpp should be determined in the wildtype and the Δ*ssrA* mutant using the established TLC procedure (4). In addition, to validate whether tmRNA positively regulates expression of *rpoS*, *lasR*, and *rhlR* through promotion of (p)ppGpp level, we should determine whether complementation of (p)ppGpp level can restore the expression of these genes in the Δ*ssrA* mutant using methods, such as overexpressing the *spoT* gene, that can promote (p)ppGpp level in the *ssrA* mutant back to the wild-type level. If the outcoming of these experiments are different from our expectation, we can hypothesize that the *trans*-translation effect on the expression of these global regulators is RelA-independent or RelA-dependent but (p)ppGpp independent, further elucidation of which could be a totally novel interesting topic. Accomplishment of this part might provide us evidence of the molecular basis for the *trans*-translation regulation of gene expression in *P. aeruginosa*.

Elucidation of the *trans*-translation effect on persister cell and biofilm formation in *P. aeruginosa*

Persister cell formation and biofilm formation are important processes that confer *P. aeruginosa* high resistance to antimicrobials. In this dissertation, I found that *trans*-translation positively affects persister cell formation and yet slightly represses biofilm initiation in *P. aeruginosa*. Results from the same assays showed that RpoS negatively affects both persister cell formation and biofilm initiation, which is conflict with the model of tmRNA regulation of *rpoS* expression as described above. Directions shown below may be helpful to resolve this issue.

The mechanism by which *trans*-translation affects persister cell formation should be elucidated first. Persister cell formation is believed to be regulated primarily by the bacterial toxin-antitoxin modules (5, 6). Maisonneuve et al. has described a model in which (p)ppGpp tops the hierarchy of the *E. coli* persistence pathway by accumulating inorganic polyphosphate that directs Lon to degrade the antitoxins, resulting in upregulated activity of toxins (7). As described in Chapter 2, both *trans*-translation and (p)ppGpp positively regulates persister cell formation and the (p)ppGpp level appears to play a more direct role, which is consistent with the *trans*-translation effect on *relA* expression as shown in Chapter 3. Therefore, further understanding whether *trans*-translation affects toxin/antitoxin (TA) systems through mediating the (p)ppGpp level is noteworthy. *P. aeruginosa* possesses several type II TA loci, *higBA*, *relBE*, and *parDE,* and HigA and RelE are known regulated by the Lon protease (8, 9). In the future, we can elucidate the effect of tmRNA on expression of *higBA*, *relBE*, and *parDE*.

It is known that in *E. coli*, mutants for *rpoS* pre-experience different stresses due to the loss of the general stress response regulator RpoS, which allows the *rpoS* mutants to be more fit for antibiotic challenges and thus to form more persister cells than the wild type

(10). It is possible that stresses experienced by the *rpoS* mutants trigger the persistence mechanism through inducing some important factors like (p)ppGpp. Thus, we can hypothesize that in the *ssrA* mutant, although *rpoS* expression is decreased, due to the reduced (p)ppGpp level, induction of persister formation is still inhibited. To elucidate this, we can determine persister cell formation in the *P. aeruginosa rpoS* mutants lacking either *trans*-translation or (p)ppGpp and compared it to the wild type and *rpoS* mutant.

On the other hand, the mechanism of the *trans*-translation effect on biofilm initiation should also be elucidated. Based on data in Chapter 2, I can predict that *trans*-translation affects biofilm initiation during the microtiter dish assay at least partially through its effect on RpoS. Given that *trans*-translation is likely to regulate *rpoS* expression via mediating (p)ppGpp accumulation in *P. aeruginosa*, in the future, whether *trans*-translation affects biofilm initiation through (p)ppGpp should be uncovered as well. However, the microtiter dish method is obviously not enough to assay biofilm formation of *P. aeruginosa* strains accurately. For instance, thicker biofilms detected using this method does not necessarily mean better developed biofilm structures, which may lead to the misunderstanding of the effect of certain mechanisms on biofilm formation. Thus, the use of combination biofilm formation assays, like the combination of the microtiter dish and flow chamber method (11), should be taken into consideration to better determine biofilm formation in different *P. aeruginosa* strains.

Accomplishment of this part will provide us insights in better understanding the regulatory mechanisms by which *trans*-translation affect persister cell and biofilm formation in *P. aeruginosa*, which may shed a light on finding new therapeutic strategies that can reduce the antibiotic tolerance and chance of relapsing of the *P. aeruginosa* infections.

Elucidation of the regulatory hierarchy of *ssrA* expression in *P. aeruginosa*

Regulators of *ssrA* expression in *P. aeruginosa* have been isolated in Chapter 3 and 4, and yet the mechanisms are highly murky (Figure 5.1). To further characterize the regulation of *ssrA* expression in *P. aeruginosa*, the exact transcription start site (s) of *ssrA* gene should be identified first, despite of the predicted promoter for the *PA0827-ssrA* operon. Primer extension can be used for mapping the transcription start site (s). Once the accurate promoter region of *ssrA* is identified, we can further determine whether certain known transcriptional regulators including Vfr, RpoS, and RpoN regulate *ssrA* expression through the direct binding to the promoter region of this gene. In addition, whether the secondary messengers (p)ppGpp and c-di-GMP control *ssrA* expression through affecting the binding of Vfr, RpoS, and RpoN to their target promoters should also be determined, to better elucidate the regulatory hierarchy of *ssrA* expression in *P. aeruginosa*. As regulators of *ssrA* expression, especially transcriptional regulators, are not known studied in any bacteria, it is a high priority for our lab to further the knowledge of *trans*-translation via elucidating the genetic regulation of the *ssrA* gene. Accomplishment of this part may also allow us to detail the regulatory circuits between *trans*-translation and certain global regulators, contributing to elucidation of the global regulatory network of *trans*-translation in *P. aeruginosa*.

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Appendix

Effect of RpoS on carbon and nitrogen metabolism in *P. aeruginosa*

Materials and Methods

Phenotype Microarrays

The PM1 and PM2 microplates from the BIOLOG's phenotypic array collection (Biolog, Hayward, CA) were used to determine the relative preference of carbon source utilization as per manufacturer's instruction. Briefly, cells were grown overnight, harvested and resuspended in 1x IF-0 to an OD_{600} of approximately 0.035, and this resuspension was further diluted 1:10 in 1x IF-0+dye. Aliquots were added to the PM1 and PM2 plates and incubated at 37°C for 24, 36, and 48 hours. At each time point, utilization of chemicals as the sole carbon source was determined as a function of reduction of the redox dye (tetrazolium; OD_{590}-OD_{750}) as per instructions from the manufacturer. To test utilization of various chemicals as a sole nitrogen source, PM3 (BIOLOG) was used. Cells were grown overnight grown on R_2A plates (38), resuspended in 1x IF-0 to an OD_{600} of approximately 0.035, and further diluted 10-fold in 1x IF-0+dye supplemented with 20 mM sodium succinate/2 μM ferric citrate as carbon source. Cells were added to the PM3 plate and incubated at 37℃ for 24, 36, and 48 hours. At each time point, utilization of chemicals as the sole nitrogen source was determined as a function of reduction of the redox dye (tetrazolium; OD_{590}-OD_{750}) as per instructions from the manufacturer.

Results

Effect of RpoS on carbon source utilization

Results are shown in Table A.1-3.

Table A.1. Summary of carbon source utilization at 24h

Carbon Source (24h)	[a]Mean Percent change ± SD
	rpoS (mutant)
Acetic Acid	40.29 ± 5.61
Propionic Acid	44.95 ± 5.69
L-Lactic Acid	60.7 ± 4.33
Pyruvic Acid	103.3 ± 11.48
Methyl Pyruvate	213.3 ± 43.13
Butyric Acid	117.1 ± 21.42
Succinic Acid	81.97 ± 8.26
Mono-Methyl Succinate	123.9 ± 11.33
Bromo Succinate	72.3 ± 24.87
Fumaric Acid	75.01 ± 23.41
Citric Acid	17.5 ± 0.87
Capric Acid	[b]NA
Sorbic Acid	NA
Glycerol	67.2 ± 22.29
Tween 20	-1.69 ± 0.28
D,L-α-Glycerol-Phosphate	429.4 ± 220.31
Pectin	53.5 ± 9.61
Laminarin	65.6 ± 6.13
L-Valine	NA
L-Glutamic Acid	41.4 ± 6.58
N-Acetyl-L-Glutamic Acid	[c]-∞
L-Pyroglutamic Acid	27.6 ± 1.58
L-Arginine	-59.9 ± 9.23

L-Alanine	68.9 ± 6.00
D-Alanine	241.96 ± 83.69
L-Phenylalanine	910.9 ± 646.20
Glycine	713.3 ± 255.86
L-Serine	NA
L-Alaninamide	NA
Inosine	105.9 ± 8.36
Adenosine	NA
Uridine	NA

Table A.2. Summary of carbon source utilization at 36h

Carbon Source (36h)	Mean Percent change ± SD
	rpoS
Acetic Acid	26.6 ± 4.91
Propionic Acid	4.5 ± 0.38
L-Lactic Acid	47.1 ± 2.54
Pyruvic Acid	59.6 ± 8.44
Methyl Pyruvate	196.4 ± 30.90
Butyric Acid	66.6 ± 11.36
Succinic Acid	79.2 ± 7.03
Mono-Methyl Succinate	34.5 ± 4.99
Bromo Succinate	92.9 ± 38.90
Fumaric Acid	43.2 ± 5.33
Citric Acid	12.9 ± 0.88
Capric Acid	-34.5 ± 5.10
Sorbic Acid	27.2 ± 3.08
Glycerol	78.7 ± 10.19
Tween 20	31.8 ± 4.60
D,L-α-Glycerol-Phosphate	832.8 ± 562.20
Pectin	48.1 ± 4.39
Laminarin	58.1 ± 9.40
L-Valine	-4.7 ± 0.28

L-Glutamic Acid	54.1 ± 7.89
N-Acetyl-L-Glutamic Acid	-∞
L-Pyroglutamic Acid	56.1 ± 3.37
L-Arginine	-59.6 ± 5.26
L-Alanine	15.6 ± 1.22
D-Alanine	181.8 ± 74.18
L-Phenylalanine	553.8 ± 230.02
Glycine	365.9 ± 117.05
L-Serine	278.3 ± 93.19
L-Alaninamide	472.4 ± 117.24
Inosine	18.7 ± 3.15
Adenosine	1409.3 ± 1067.19
Uridine	NA

Table A.3. Summary of carbon source utilization at 48h

Carbon Source (48h)	Mean Percent change ± SD
	rpoS
Acetic Acid	20.3 ± 3.10
Propionic Acid	-2.9 ± 0.23
L-Lactic Acid	30.8 ± 1.64
Pyruvic Acid	77.0 ± 12.89
Methyl Pyruvate	131.4 ± 30.69
Butyric Acid	83.5 ± 20.35
Succinic Acid	58.0 ± 2.80
Mono-Methyl Succinate	27.5 ± 2.99
Bromo Succinate	78.9 ± 5.58
Fumaric Acid	33.7 ± 4.82
Citric Acid	-0.7 ± 0.03
Capric Acid	-49.8 ± 11.06
Sorbic Acid	14.1 ± 1.95
Glycerol	35.2 ± 0.79
Tween 20	22.4 ± 2.68

D,L-α-Glycerol-Phosphate	574.0 ± 231.41
Pectin	43.5 ± 6.73
Laminarin	25.2 ± 1.67
L-Valine	-13.6 ± 0.64
L-Glutamic Acid	37.5 ± 5.08
N-Acetyl-L-Glutamic Acid	-∞
L-Pyroglutamic Acid	82.9 ± 9.97
L-Arginine	-59.1 ± 4.38
L-Alanine	10.0 ± 0.81
D-Alanine	122.1 ± 39.04
L-Phenylalanine	335.3 ± 126.22
Glycine	499.6 ± 184.10
L-Serine	193.8 ± 59.55
L-Alaninamide	472.4 ± 117.24
Inosine	18.7 ± 3.15
Adenosine	1254.4 ± 628.31
Uridine	915.2 ± 708.41

[a]Percent change means change in percent utilization in the *rpoS* mutant after compared to the wild type. Percent utilization of a certain carbon source is shown as a percentage of value of OD_{590} minus OD_{750} shown by the wild type; [b]NA means readings are too small to be usable; *c* -∞ means carbon source is not used at all.

Effect of RpoS on nitrogen source utilization

Results are shown in Figure A.1-A.12.

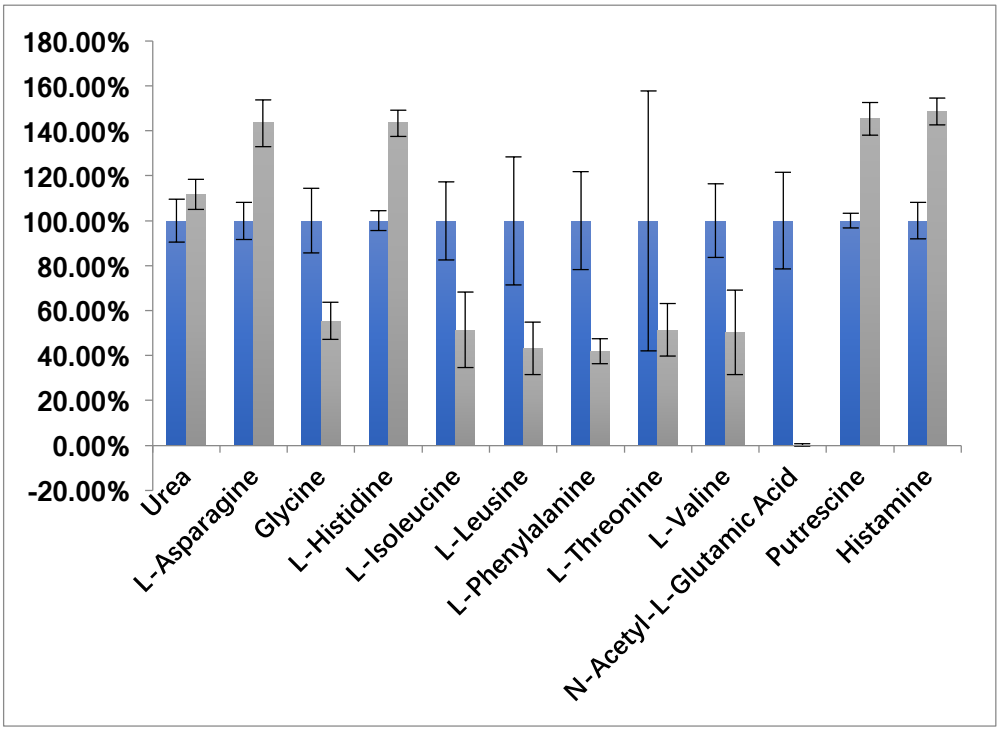

Figure A.1. Utilization of nitrogen sources (24h, part 1). Blue bar: wild type; grey bar: the *rpoS* mutant.

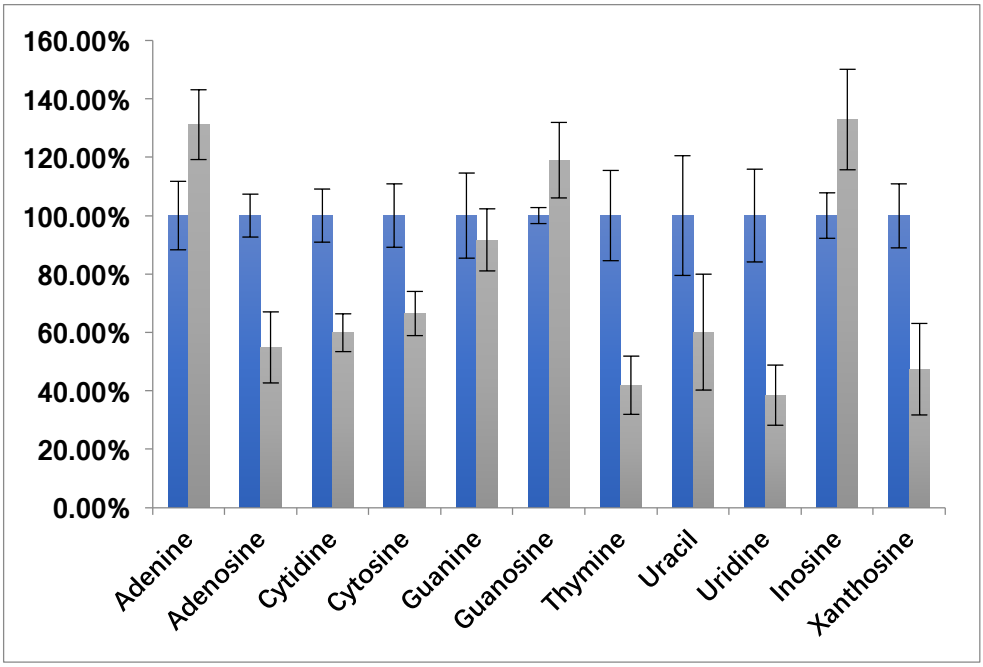

Figure A.2. Utilization of nitrogen sources (24h, part 2). Blue bar: wild type; grey bar: the *rpoS* mutant.

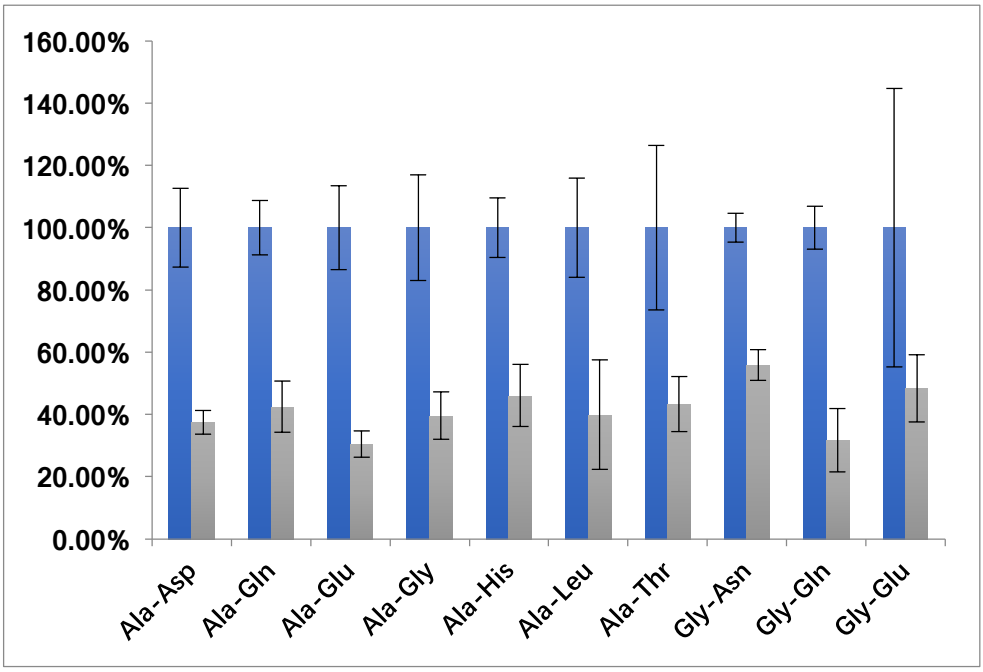

Figure A.3. Utilization of nitrogen sources (24h, part 3). Blue bar: wild type; grey bar: the *rpoS* mutant.

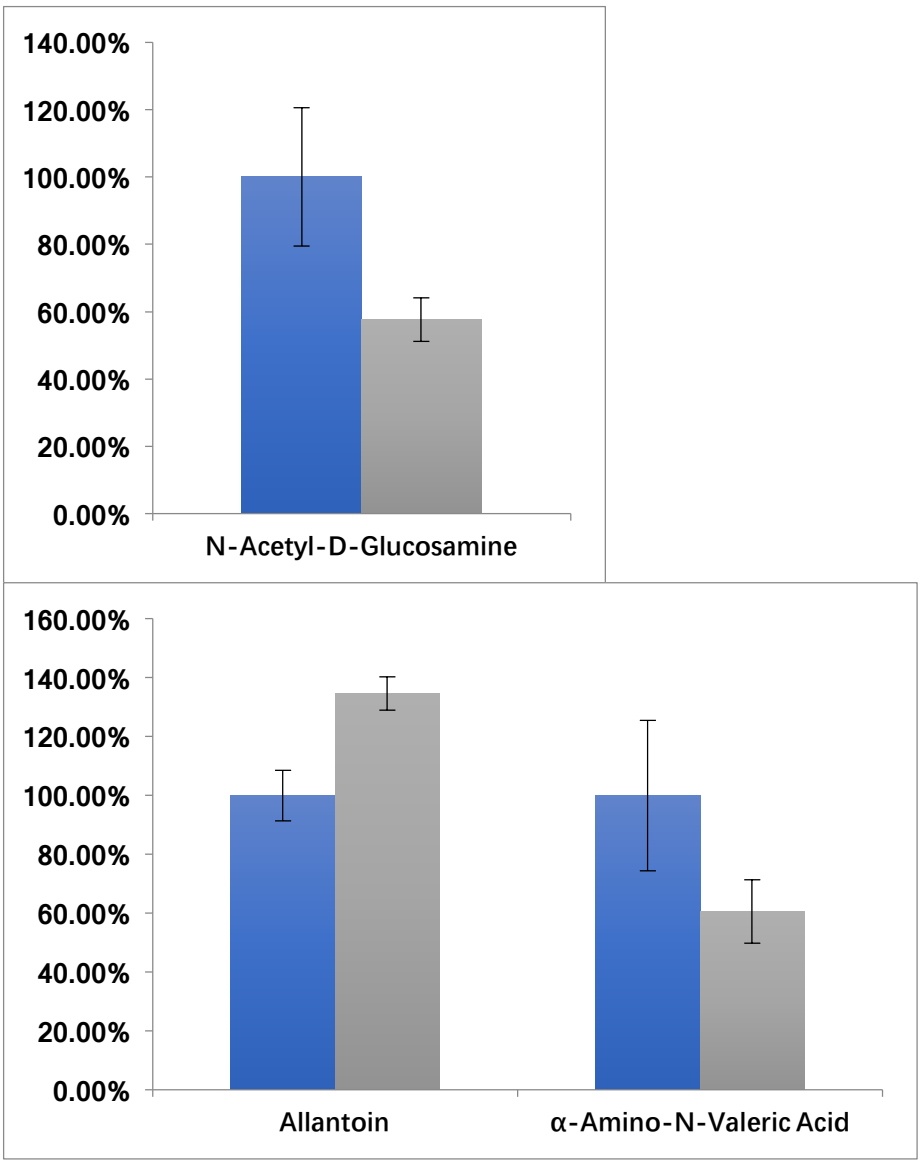

Figure A.4. Utilization of nitrogen sources (24h, part 4). Blue bar: wild type; grey bar: the *rpoS* mutant.

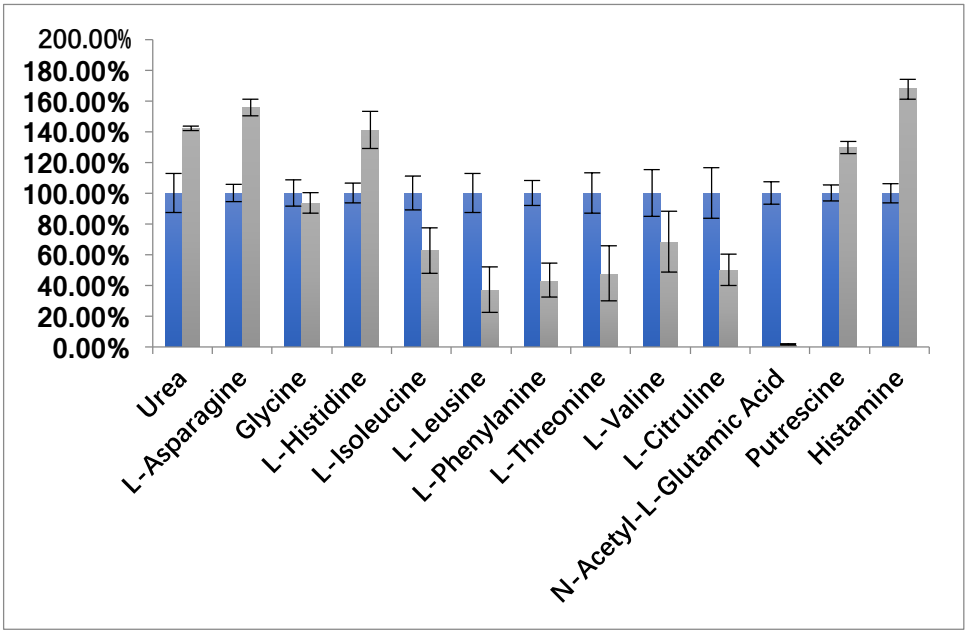

Figure A.5. Utilization of nitrogen sources (36h, part 1). Blue bar: wild type; grey bar: the *rpoS* mutant.

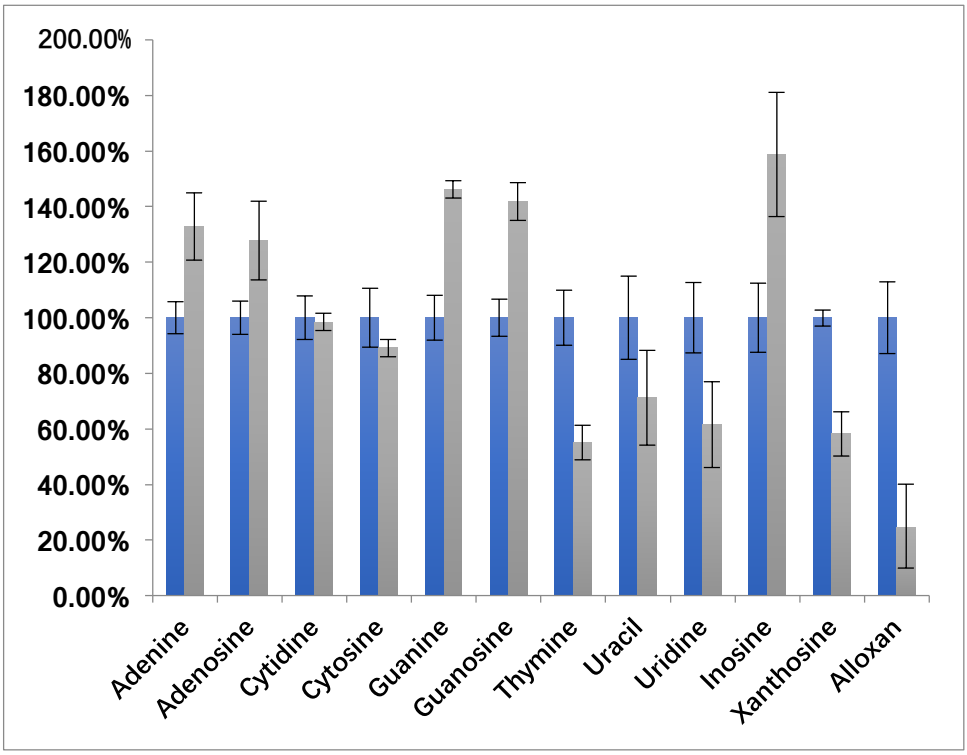

Figure A.6. Utilization of nitrogen sources (36h, part 2). Blue bar: wild type; grey bar: the *rpoS* mutant.

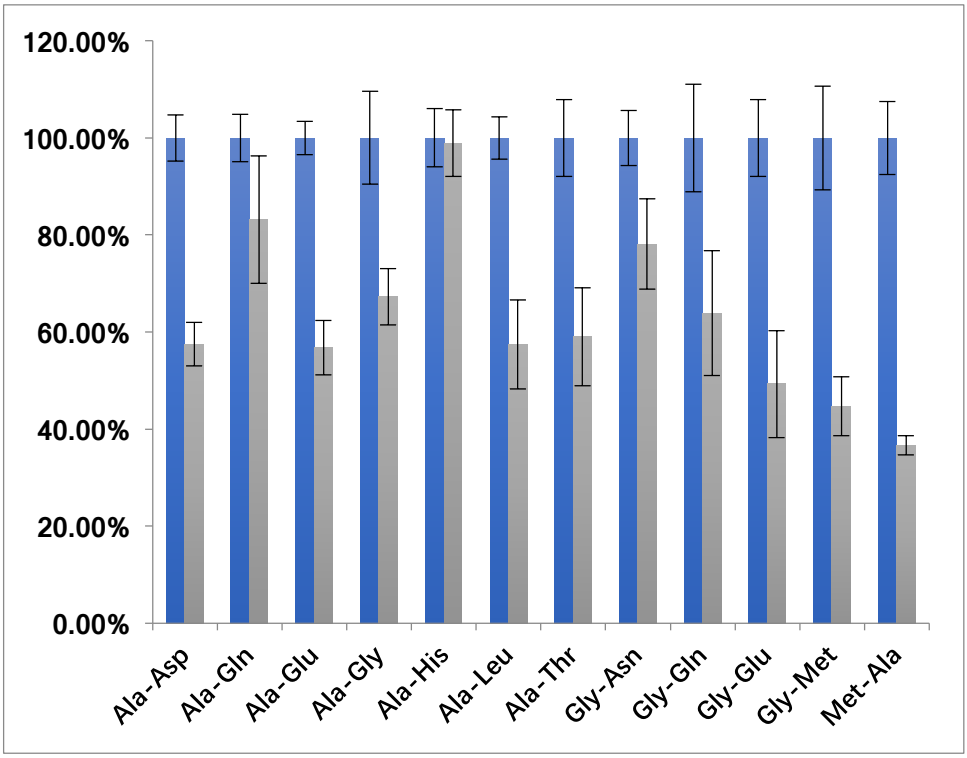

Figure A.7. Utilization of nitrogen sources (36h, part 3). Blue bar: wild type; grey bar: the *rpoS* mutant.

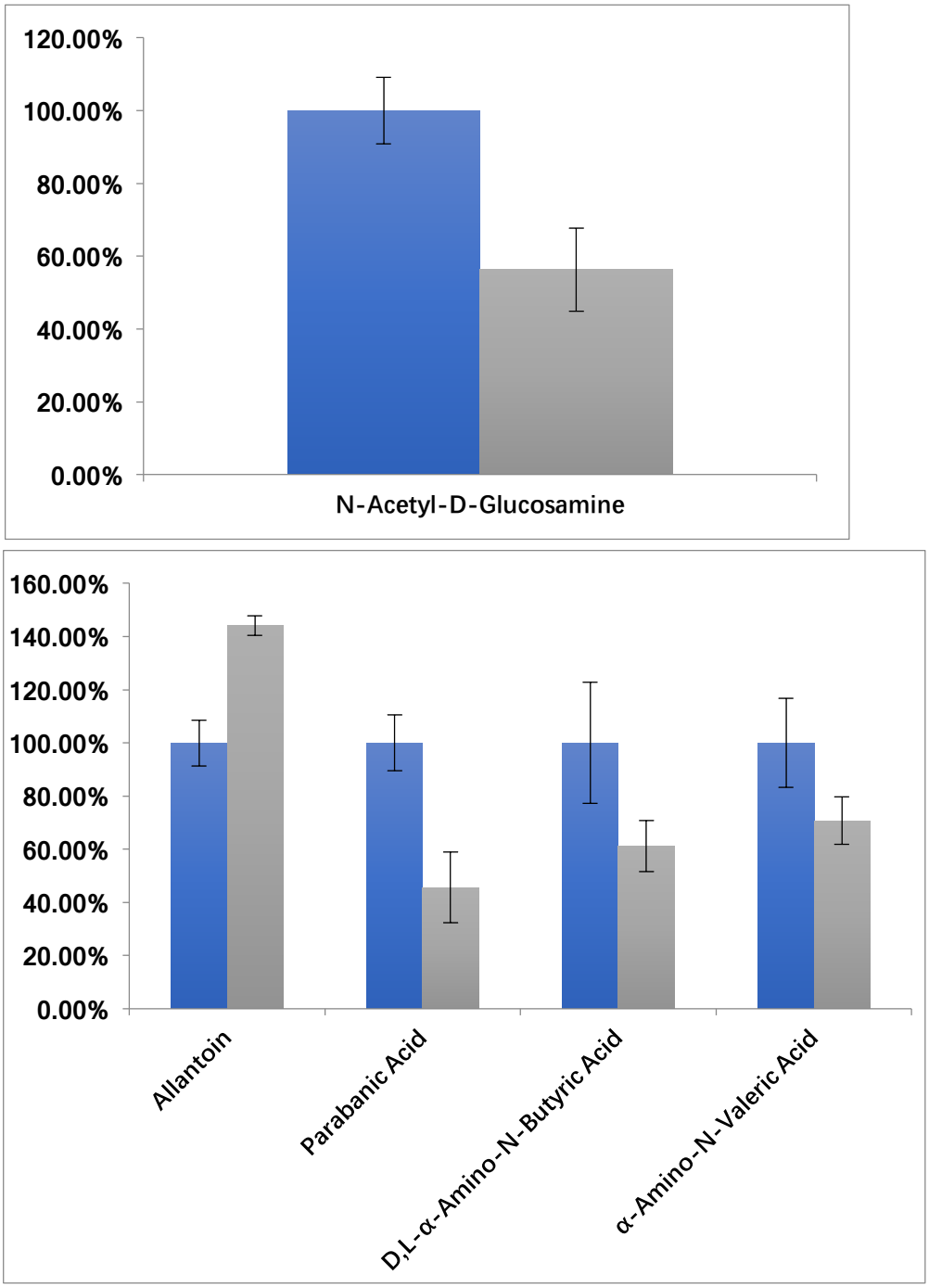

Figure A.8. Utilization of nitrogen sources (36h, part 4). Blue bar: wild type; grey bar: the *rpoS* mutant.

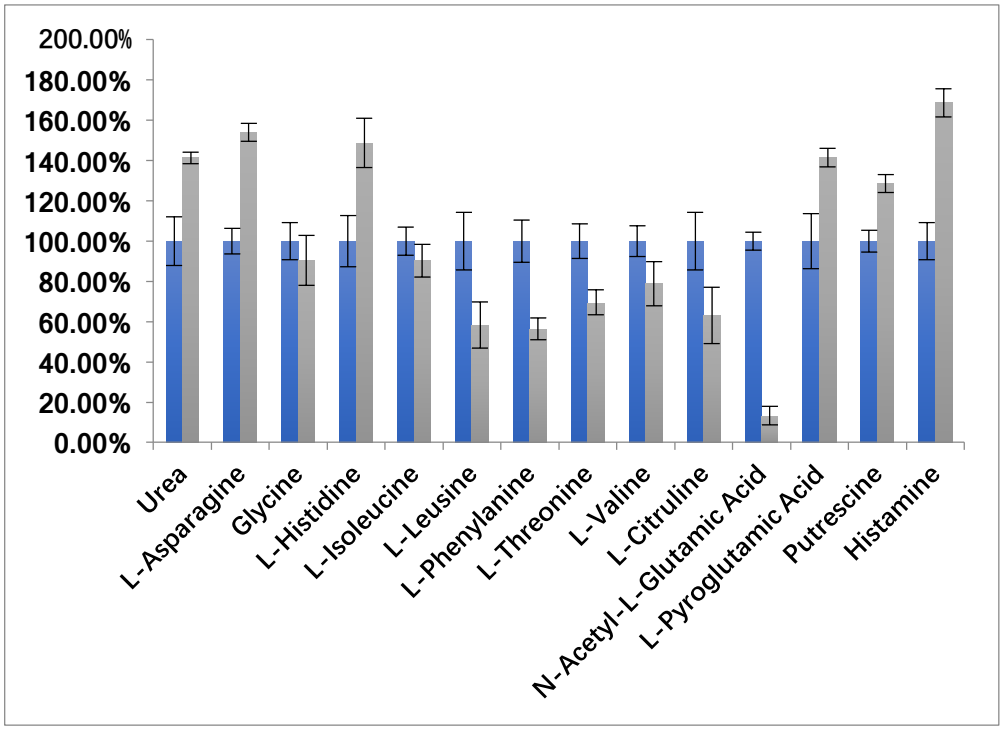

Figure A.9. Utilization of nitrogen sources (48h, part 1). Blue bar: wild type; grey bar: the *rpoS* mutant.

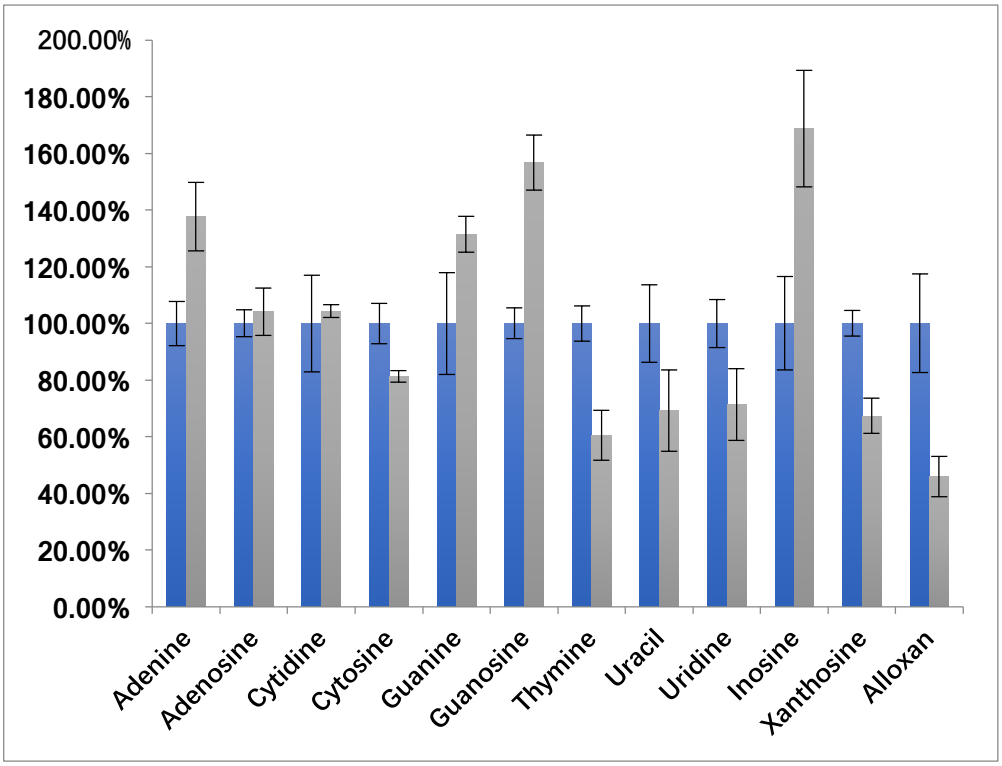

Figure A.10. Utilization of nitrogen sources (48h, part 2). Blue bar: wild type; grey bar: the *rpoS* mutant.

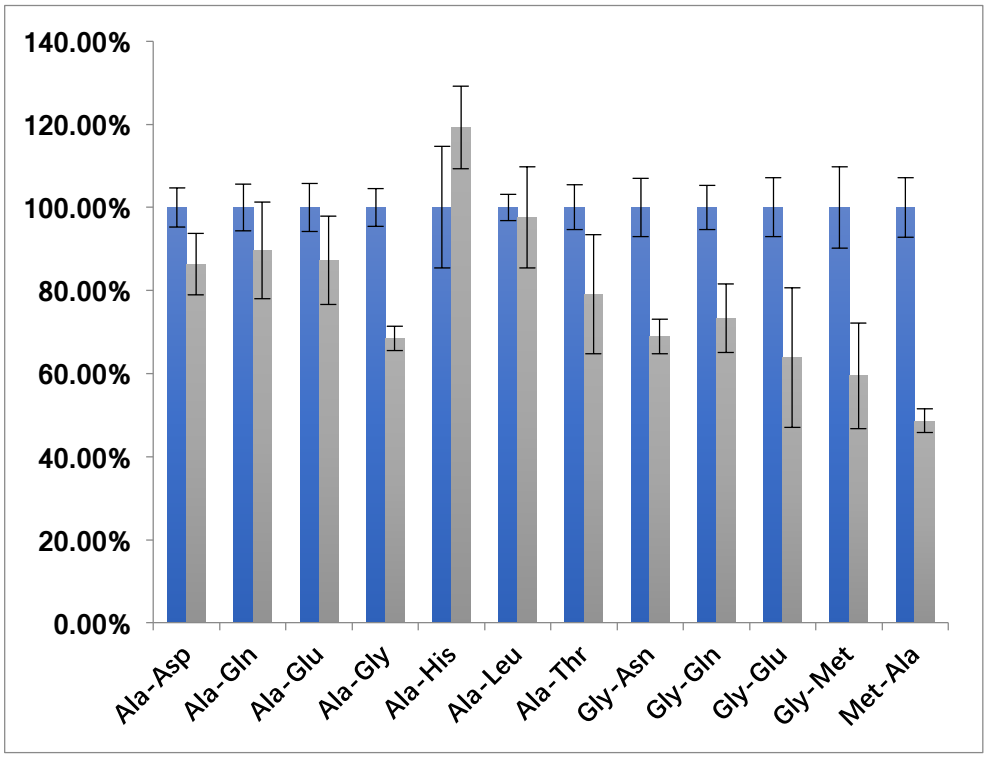

Figure A.11. Utilization of nitrogen sources (48h, part 3). Blue bar: wild type; grey bar: the *rpoS* mutant.

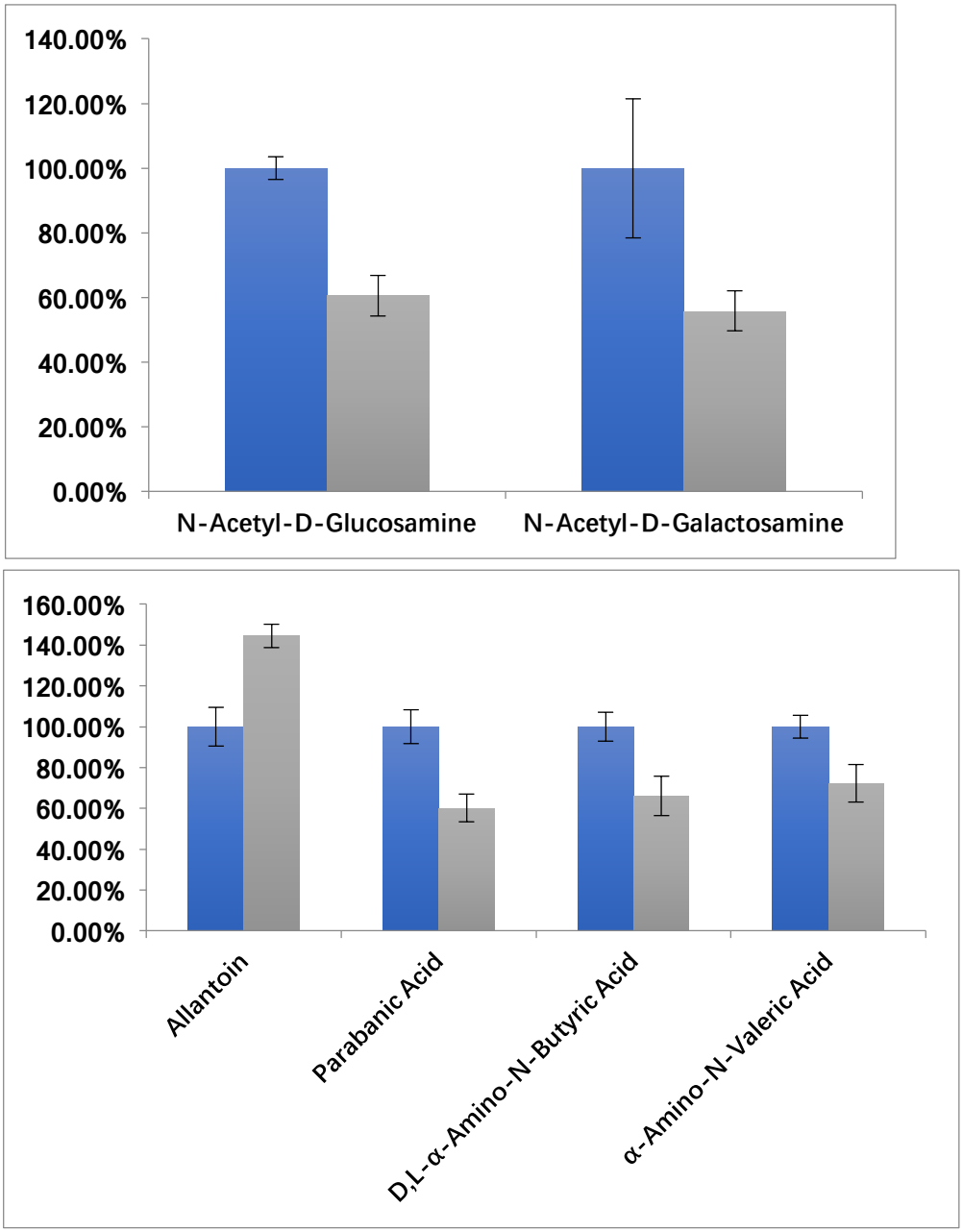

Figure A.12. Utilization of nitrogen sources (48h, part 4). Blue bar: wild type; grey bar: the *rpoS* mutant.