Characterization of tmRNA function and regulation in *Pseudomonas aeruginosa*

By

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Abstract

Transfer messenger RNA (tmRNA) encoded by *ssrA*, is a hybrid RNA with tRNA and mRNA domains that catalyzes recycling of stalled ribosomes. This bioenergetically important process appears to play diverse functions in the physiology of various bacteria including stress response and cell viability. Results from our laboratory demonstrated that tmRNA is required for optimal thermotolerance and osmotic stress in *Pseudomonas aeruginosa*. In addition, tmRNA plays a role in optimal production of several virulence determinants. Although tmRNA has been characterized extensively at the biochemical level, very little is known about how this unique RNA is regulated at the genetic level. Therefore, I hypothesized that tmRNA was controlled by a complex regulatory network and that characterization of this network may provide critical insight into the global role of tmRNA in *P. aeruginosa* physiology. In order to study regulation of tmRNA in *P. aeruginosa*, I used a strain with a *ssrA*::*lacZ* transcriptional fusion integrated into the genome of PAO1, a wound isolate. Expression of tmRNA was measured via β-galactosidase assay of *ssrA*::*lacZ*. I determined that *ssrA* was more highly expressed at 30°C than at 37°C in *P. aeruginosa*. In addition, maximum tmRNA expression required the stationary sigma factor RpoS. Furthermore, tmRNA was needed for expression of full expression of *rpoS*. I also took a non-predicted approach and conducted a transposon mutagenesis with Tn*5*-B30 to isolate insertion mutants with altered *ssrA*::*lacZ* phenotype. Tn*5*-B30 insertion sites were determined via arbitrary PCR and DNA sequencing. Out of approximately 40,000 insertion mutants screened, I

identified 11 genes that affected the *ssrA*::*lacZ* fusion in *P. aeruginosa*. One insertion (*prt2*) was in a gene that encodes for a heat shock protein: ClpB (*PA4542*). Another insertion (*prt10*) was in a gene that encodes for a malic enzyme, *PA5046*. One mutant (*prt5*) was mapped to a putative protein involved in the Type VI secretion system, *PA1658 (hsiC2)*. Two mutants (*prt4* and *prt6*) were defective in putative transcriptional regulators, *PA5431* and *PA0319* respectively. The other six mutants mapped to genes that encode for hypothetical proteins, *PA4929, PA2729, PA3693, PA1761, PA0738* and *PA2721*. I successfully cloned the wildtype alleles of four genes and complemented the original transposon insertion mutations in *PA2729* (*prt3*), *PA1658* (*prt5*), *PA0319* (*prt6*), *PA0738* (*prt7*) by restoring the *ssrA*::*lacZ* expression. My study represents one of the first attempts to characterize regulatory mechanisms of tmRNA in any bacteria. In addition, I demonstrated that tmRNA affects *rpoS* expression via PsrA. Furthermore, my data indicate that tmRNA regulates expression of *psrA* by yet unknown mechanism. This study represents one of the first attempts to characterize regulatory mechanisms of tmRNA expression in bacteria.

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Literature Review

Introduction

Bacterial pathogens have to successfully adapt to changing environmental conditions as they infect the host. Some of the conditions a pathogen may have to adapt to include alterations in temperature, oxygen tension, nutrient availability, host's immune response, and presence of therapeutics such as antibiotics. With the increasing emergence of antibiotic resistant pathogens, it is imperative to better understand pathogens' physiology and metabolism in order to develop better approaches to combat them more effectively. *Pseudomonas aeruginosa* is an ideal model organism for studying mechanisms of bacterial pathogens' stress response because this bacterium can live in a variety of environments and it is an important opportunistic pathogen for humans and animals (10,33). *P. aeruginosa* is a versatile pathogen that causes a variety of acute infections in skin, cornea, cochlea, and urinary tract (22, 23, 41, 56, 94). In addition, *P. aeruginosa* causes chronic infections in the lungs of cystic fibrosis patients and people who are intubated (10, 12). The overall goal of our research is to utilize *P. aeruginosa* as a model system to elucidate the global bacterial stress response mechanisms.

The transfer messager RNA (tmRNA) is part of a quality-control system that recognizes stalled ribosomes (both Nonstop and No-Go pathways) and provides an alternative mechanism for recycling of ribosomes and amino acids in incomplete peptides (46, 89, 97). Our initial characterization of tmRNA function in *P. aeruginosa* suggests that it has a subtle but global effect (Tucker, Wu, and Suh, unpublished data). Although tmRNA has been studied extensively at the biochemical level, very little research has been performed on the physiological function or genetic regulation of this unique molecule in bacteria (9, 39, 80, 85, 95, 98). We are interested in understanding the impact of tmRNA on the pathogenesis and stress response mechanisms of *P. aeruginosa*. Based on our initial characterization of the tmRNA$^-$ mutant phenotype, I hypothesized that a complex regulatory network controls tmRNA gene expression and that characterization of this network will provide critical insight into the global role of this unique hybrid RNA in *P. aeruginosa* physiology.

Pseudomonas aeruginosa

The gram-negative facultative anaerobic bacterium *P. aeruginosa* is found in many different temperate environments including in association with animals and plants, in soil, fresh water habitats, and marine environments (21, 28, 33). The ability of the bacterium to survive and persist in various environments suggests that *P. aeruginosa* is capable of withstanding numerous environmental challenges. Analysis of the *P. aeruginosa* strain PAO1 genome indicated the presence of a large number of genes devoted to the regulators of gene expression in response to environmental stimuli as well as for catabolism, transport, and efflux of organic compounds (87). Thus, the genomic data support that *P. aeruginosa* is a good model system for studying the

relationship between bacterial stress response and pathogenesis (55).

P. aeruginosa is a highly successful opportunistic pathogen that predominantly affects immunosuppressed hosts (26). This bacterial pathogen causes localized infections including otitis media, corneal infections, skin lesions, and urinary tract infections as well as systemic infections (23, 41, 56). *P. aeruginosa* is also the major etiologic agent of chronic pulmonary infections in people who suffer from the genetic disorder cystic fibrosis (CF) (10). The chronic pulmonary infections caused by *P. aeruginosa* are responsible for almost 90% mortality of the CF patients (62). During establishment of infections, *P. aeruginosa* may be exposed to various potentially hostile environmental effects such as temperature change, oxidative and osmotic stress, nutrient deprivation, and human intervention in the form of antimicrobial therapy (84). To successfully establish infections and persist in hostile environments, *P. aeruginosa* must be able to sense changes and react appropriately. Therefore, a large number of regulators of gene expression in *P. aeruginosa* is likely important for facilitating the bacterium's ability to adapt to new environments.

In addition to its ability to withstand environmental stresses, *P. aeruginosa* produces a battery of virulence determinants that aids in the establishment of infections. These include several toxins (exotoxin A, exoenzymes S, U, and T) (65, 90), proteases with a wide range of substrates (LasA protease and elastase) (48), phospholipases (7), and several pigments (pyocyanin, pyoverdine, and pyochelin) (18). These virulence determinants facilitate survival of the bacterium through protection and nutrient acquisition (13). For example, the elastase can degrade immunoglobulins, phospholipases can provide the cell with lipids and glycerol-phosphates as nutrients, pyoverdine and pyochelin are siderophores that acquire iron for

the bacterium, and pyocyanin is a redox-active antimicrobial phenazine compound that can also induce apoptosis of neutrophils (13, 18, 20, 48, 58).

Many of these virulence factors are essential for establishment of acute infections and their productions are tightly controlled by several global regulators of gene expression including cell-cell communication systems of *P. aeruginosa* and alternative sigma factors such as RpoS, RpoN, and AlgU (36, 37, 42). *P. aeruginosa* contains two cell-cell communication systems mediated by acyl-homoserine lactone signaling molecules (LasRI and RhlRI) and one quinolone mediated PQS system(18, 53). These cell-cell communication systems control a large number of genes for coordinated expression in a bacterial population. Because *P. aeruginosa* contains three distinct and yet interconnected cell-cell communication systems, it has become the model for study of intercellular communication systems in bacteria. *P. aeruginosa* also contains several alternative sigma factors that are important for production of virulence factors and adaptation to environments. AlgU is an alternative sigma factor that belongs to the ECF family (77). AlgU is the major regulator of the exopolysaccharide alginate biosynthesis (36). Alginate production results in formation of thick biofilms that protect the organism from predators, desiccation, and antibiotics (14, 15, 83). RpoS is a sigma factor that regulates the general stress response that confers protection against multiple environmental stresses in *P. aeruginosa* and several other gram-negative bacteria (17, 53, 70).

P. aeruginosa *and cystic fibrosis*

P. aeruginosa infections are the leading cause of morbidity and mortality for cystic fibrosis (CF) patients (62). *P. aeruginosa* establishes life-long infections in the CF lung by

utilizing various adaptation strategies to cause chronic infections (10, 11). *P. aeruginosa* is acquired from the environment, and once it colonizes the CF airways, the bacterium persists in the CF lung almost indefinitely until the death of the patient (4, 10, 11). Sometime after colonization but during the establishment of persistence, *P. aeruginosa* acquires many mutations that result in alterations in nutrient metabolism, decreased production of many acute virulence determinants, and in colony morphology (27, 70, 72). A hallmark of CF isolates is the mucoid phenotype due to overproduction of the virulence determinant alginate (69) controlled by the alternative sigma factor AlgU (64). This exopolysaccharide facilitates formation of biofilms that play an important role in protecting the bacterium from antibiotics and phagocytosis, and inhibiting activation of the complement system (27). Once biofilms are established, *P. aeruginosa* is able to persist in the lung by resisting therapeutic measures and the host immune system (27, 63, 74). In addition to facilitating formation of biofilms, alginate also contributes to the airway blockage and interferes with effective clearance of pathogens by pulmonary cilia (69). In addition to alginate, the CF isolates of *P. aeruginosa* also produces increased quantities of several other virulence determinants including HCN (82). In contrast, most of the well-characterized virulence factors that have been demonstrated to be important for establishing acute infections are down regulated in CF isolates (76).

Recent data demonstrate that in addition to alterations in virulence determinant productions, *P. aeruginosa* changes its preference for nutrient catabolism (29). Unlike the acute isolates, the chronic CF isolates of *P. aeruginosa* grow well on fatty acids and glycerol via the glyoxylate pathway in lieu of the TCA cycle (29, 30). In summary, *P. aeruginosa* pathogenesis of the CF lung involves a complex interaction of stress response mechanisms including acquisition of multiple mutations by becoming hypermutable (68).

Transfer messenger RNA

Transfer messenger RNA (tmRNA), encoded by *ssrA*, is a hybrid RNA with tRNA-like and mRNA-like domains. This uniquely bacterial hybrid RNA catalyzes recycling of stalled ribosomes in the cell via a process designated as *trans*-translation (46, 66). When ribosomes stall due to lack of charged tRNA, damaged mRNA, or other interventions such as runaway transcript lacking termination codon, tmRNA that is aminoacylated at the tRNA-like domain (TLD) with alanine enters the ribosome with assistance of the SmpB protein. This action promotes translocation of the ribosome to the mRNA-like domain (MLD) of the tmRNA to translate the degradation tag (ANDENYALAA in *E. coli*) for turnover of the incomplete polypeptide (45, 46, 95). The structure of *E. coli* tmRNA is shown in Fig.1.1. The molecule is composed of 4 pseudoknots, a $tRNA^{ala}$-like domain and a degradation tag-coding MLD (45, 95). $tRNA^{ala}$ secondary structure is shown to compare with tmRNA's $tRNA^{ala}$-like domain. The four pseudoknots are labeled as ψ1 to ψ4. A stop codon is located at the end of the highlighted translatable proteolysis tag coding domain to release the ribosome (45, 95).

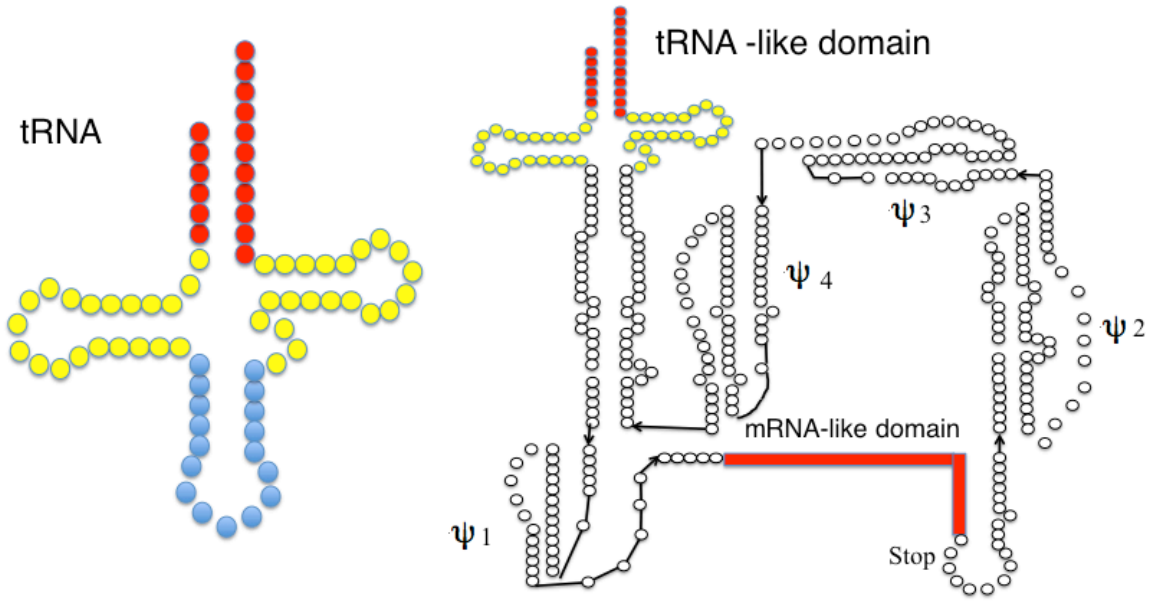

Figure 1.1. Predicted secondary structure of tmRNA in *E.coli.* (Adopted from (45))

tmRNA and trans-*translation.*

The tmRNA is the key factor for *trans*-translation. This simple and yet important role of tmRNA appears to have different physiological effect on various organisms. Fig.1.2 shows how tmRNA helps release the stalled ribosome and tag the unwanted polypeptide. First, tmRNA is aminoacylated by alanyl-tRNA synthetase (AlaR) and the charged $tmRNA^{ala}$ binds to SmpB and EF-Tu:GTP to form the activation complex (box 1). The complex recognizes and enters the A site of the stalled ribosome. Second, the complex transfers from A site to P site during which transpeptidation reaction occurs to elongate the peptide chain with Alanine brought in by tmRNA. Third, the ribosome translates the coding sequence in the MLD of tmRNA instead of original mRNA (message switch). The released mRNA is degraded by a cellular RNase (box 2). The ribosome translates the coding sequence in MLD until the stop codon and release to become free ribosomal subunits. The tagged protein is targeted by proteases such as ClpXP, Tsp, Lon (box 3) for degradation to recycle amino acids (Fig.1.2).

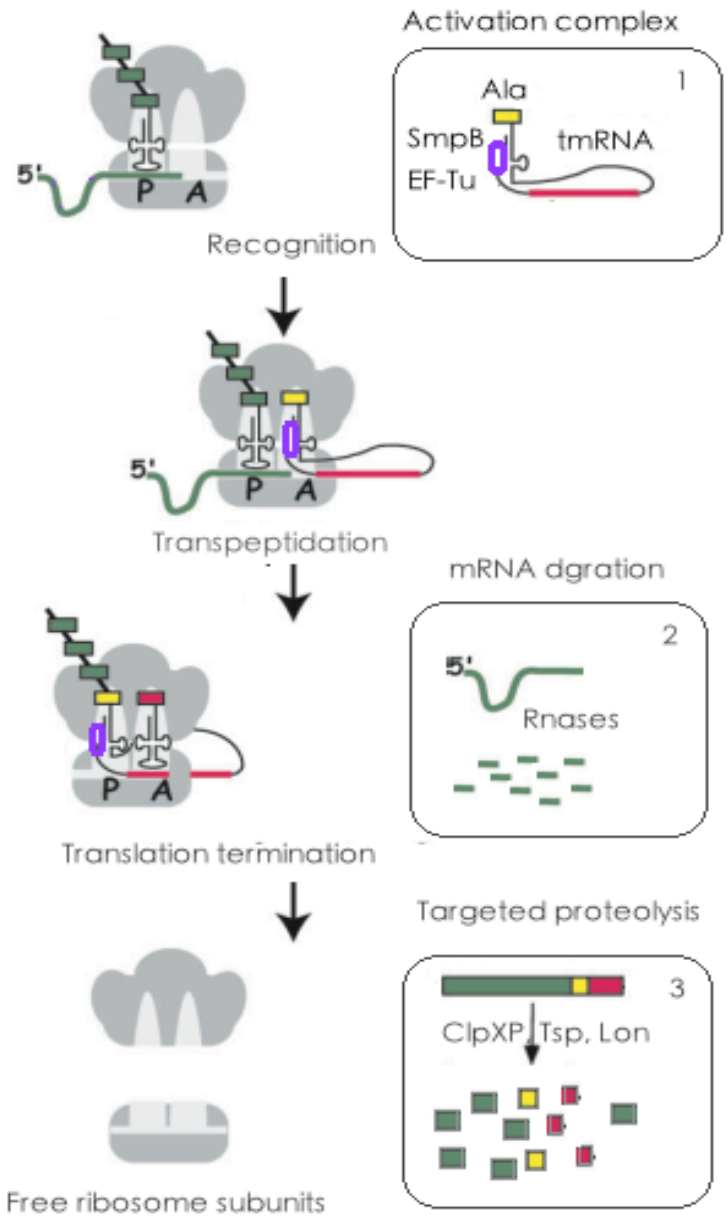

Figure 1.2. The mode of action of tmRNA. (Adopted from (46))

Physiological role of tmRNA.

The relative importance of tmRNA for various bacteria has just started to be studied and the physiological role of this molecule has been characterized only in a few organisms to date. In *Neisseria gonorrhoeae* and *Shigella flexneri*, tmRNA appears to be essential because a null tmRNA mutation could not be isolated in these bacteria (40, 47). However, in other bacteria, tmRNA does not appear to be essential. In *E. coli*, the *ssrA* mutant grows slightly slower than the wildtype and is hypersensite to amino acid starvation, and to heat shock (2, 57, 67, 96). In *Yersinia pseudotuberculos*is, the *ssrA* mutants are hypersensitive to temperature fluctuations and antibiotics (86). For *Bacillus subtilis*, tmRNA is required for growth under stressful conditions (32). Therefore, although the physiological function of tmRNA has been studied only in a few organisms, the available data suggest that tmRNA is involved in stress response but its global effect is different in various organisms.

Regulation of gene expression by tmRNA

Although tmRNA has been highly characterized at biochemical level for *trans*-translation, its role in physiology is just beginning to be studied and almost nothing is known about its genetic regulation. As the key factor of *trans*-translation, tmRNA is required to resolve nonstop or no-go pathways. Nonstop translations lack an in-frame stop codon and therefore the ribosomes fail to terminate at the proper site. No-go pathway is encountered when the mRNA is damaged

prior to the stop codon and results in stalled ribosomes. In both situations, translation is affected and results in production of incorrect polypeptides. In the presence of tmRNA, these incorrect peptides are tagged with the degradation tag encoded by the MLD of tmRNA and efficiently degraded (46). The tmRNA mediated gene expression via *trans*-translation has been studied in the production of two proteins in *E. coli*, RpoS and LacI (71). RpoS is an alternative sigma factor that mediates the general stress resistance in some gram-negative bacteria including *E. coli* and *P. aeruginosa* (88). tmRNA was demonstrated to be important for efficient translation of RpoS under low temperature. By affecting translation of a major stress response regulator, tmRNA may affect many aspects of *E. coli*'s general stress response (6, 71). The most thoroughly studied example of a protein that is regulated by tmRNA is LacI, the repressor for the *lac* operon of *E. coli* (1). The investigators determined that tmRNA was required for *E. coli* to produce full sized LacI. In other organisms, tmRNA was shown to regulate the expression of *kinA* in *B.subtilis* (54), and biosynthesis of a pigment in *Staphylococcus aureus* (59).

The alternative sigma factor RpoS

In nature, bacteria exist mainly in a state of nutrient deprivation (analogous to the stationary phase observed in the laboratory), with periodic spurts of growth when nutrients become available (81). Upon entering the stationary phase of growth in which nutrient is scarce, several gram-negative bacteria, including *P. aeruginosa,* induce the alternative sigma factor, RpoS, mediated general stress response that provides protection against a multitude of stresses (6, 51, 60, 61, 75, 88). General stress response is a protective mechanism that protects the bacterium not only from the stress that triggered the response, but also from other stresses the bacterium

has yet to encounter (35). Some of these stresses include prolonged nutrient starvation, heat-shock, and osmotic stress. Thus, a null-mutation in *rpoS* renders many bacteria unable to adapt to environmental changes, and it can severely decrease the virulence of a pathogenic bacterium (35). The central role of RpoS has been described in several gram-negative bacteria including *E. coli* (6), *Salmonella enterica* serovar Typhimurium (25, 51)*, Vibrio cholerae* (19, 99), *Yersinia enterocolitica* (5), *Legionella pneumophila* (31), and *P. aeruginosa* (88). Beyond its functions in mediating the general stress response, RpoS also regulates genes that response to environmental variations, cell morphology, programmed cell death, cell metabolism, and virulence factor production in some of these bacteria (73).

RpoS and stress response in E. coli

In *E. coli*, RpoS controls expression of the genes involved in cell survival in the stationary phase (6, 35, 91, 93). To date, more than 500 RpoS dependent genes have been identified in *E. coli* which is about 10% of the total number of genes (92). RpoS regulated genes have been demonstrated to be responsible for resistance against oxidative stress, near-UV irradiation, hyperosmolarity, acidic pH, and ethanol (3, 43, 60, 79). Some of the other RpoS regulated gene products have been demonstrated to be involved in changes in the cell envelope and overall cellular morphology, as in the example of BolA that changes the shape of *E. coli* from a rod to a sphere (34).

Regulation of rpoS expression in E.coli

Production of RpoS in *E. coli* is highly complex and involves transcriptional, translational and post-transcriptional controls (91) (Fig.1.3). The major promoter of *rpoS*

transcription is located in the gene of *nlpD*, at 567 nucleotides upstream of *rpoS* (Fig.1.3.a). This untranslated region (UTR) is important for *rpoS* translational control. ArcAB and CRP:cAMP have been reported to repress while BarA-UvrY and (p)ppGpp induce transcription of *rpoS* (52) (Fig.1.3.a). The UTR forms a hairpin loop to prevent ribosome binding until Hfq, Hu and small RNAs (sRNA) including RprA and DsrA relieve the secondary structure to allow translation initiation. Another sRNA, OxyS, inhibits *rpoS* expression by interacting with Hfq and preventing its function (16). These sRNA are produced in response to different stress and mediates *rpoS* translation (100) (Fig.1.3.b). More importantly in regards to my research, tmRNA has been demonstrated to be important for full translation of *rpoS* mRNA by relieving ribosome stalling (71). However, exact mechanism has not yet been deduced. Once RpoS is synthesized, it quickly gets phosphorylated by RssB and degraded by ClpXP in the absence of stress (Fig.1.3.d). However, in the presence of stress, RpoS binds to the core RNA polymerase to express RpoS dependent genes (Fig.1.3.c).

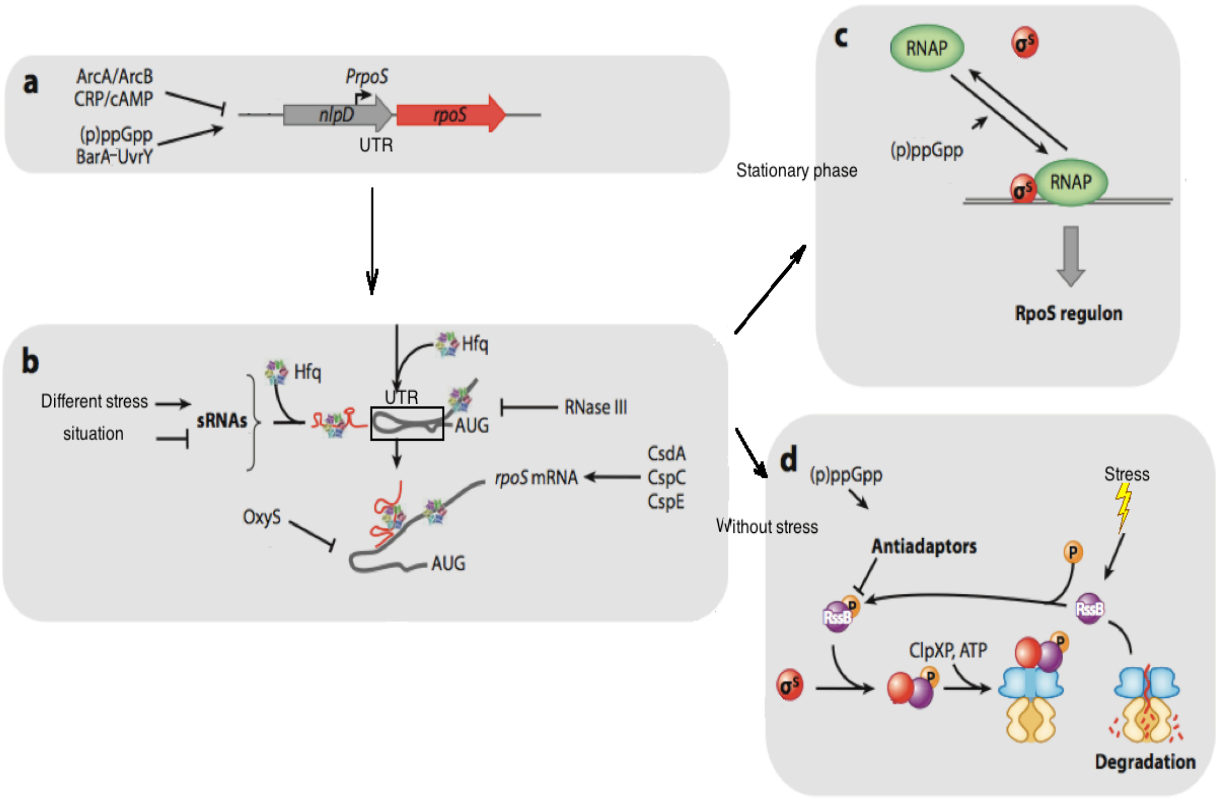

Figure1.3. Regulation of RpoS expression in *E.coli.* (Adopted from (91))

Role of RpoS in P. aeruginosa

In *P. aeruginosa*, we previously demonstrated that in addition to general stress resistance, RpoS mediates optimal production of several important virulence factors, including exotoxin A, elastase, pyocyanin, pyoverdine, and alginate (88). In addition, Foley et al. (24) demonstrated that *P. aeruginosa rpoS* was highly expressed in the sputum of CF patients infected with *P. aeruginosa*. Finally, RpoS is required for proper biofilm formation (38). Thus, RpoS appears to play an important role for *P. aeruginosa* colonization and persistence in the CF lung. In support of this, Schuster et al. (78) identified over 720 RpoS regulated genes via transcriptome analysis, and demonstrated the importance of RpoS in almost every physiological aspect of aerobically growing *P. aeruginosa*, including antibiotic resistance, cell wall biogenesis, and energy metabolism.

Regulation of rpoS *Expression in* P. aeruginosa

In *P. aeruginosa*, the mechanism of *rpoS* expression has not yet been characterized in great detail and only a few putative regulators have been identified. In 2000, Whitely et al. (93) showed that the RhlRI cell-cell communication system affected *rpoS* transcription. However, a detailed mechanism was not described and it is likely that the regulation is indirect as no LuxR box has been found in the *rpoS* promoter region. In 2001, Kojik and Venturi (50) discovered a TetR class of regulator, PsrA, that was responsible for almost 90% of *rpoS* transcription in *P. aeruginosa*. PsrA binds to *rpoS* promoter to activate its transcription (49). Interestingly, PsrA

represses its own transcription during logarithmic growth. In stationary phase, this repression is partially relieved and PsrA activates *rpoS* transcription (49). Although *psrA* expression is induced by long-chain fatty acids (44), exact mechanism of its regulation is not yet known. In addition to the transcriptional regulation, Bertani et al. (8) demonstrated that ClpXP protease was important for RpoS degradation during exponential growth in *P. aeruginosa*.

Summary

Although tmRNA has been characterized in great detail, study of its role in bacterial physiology is just being initiated. To date, tmRNA appears to be involved in stress response in several organisms but the mechanisms have not yet been elucidated. In addition, tmRNA appears to regulate various functions in different bacteria. Thus, the physiological role of tmRNA must be elucidated for each bacterium being studied. In this study, my goal was to characterize the expression of the *ssrA* gene that encodes for tmRNA in *P. aeruginosa*. To achieve my goal, I determined the environmental effect on *ssrA* expression and conducted a transposon insertion mutagenesis to identify putative transcriptional regulators of *ssrA*. Furthermore, I determined there is a regulatory circuit between RpoS and tmRNA, two important stress response regulators in *P. aeruginosa*. To our knowledge, this is the first time a comprehensive study has been performed to characterize the regulation of *ssrA* expression in any bacteria.

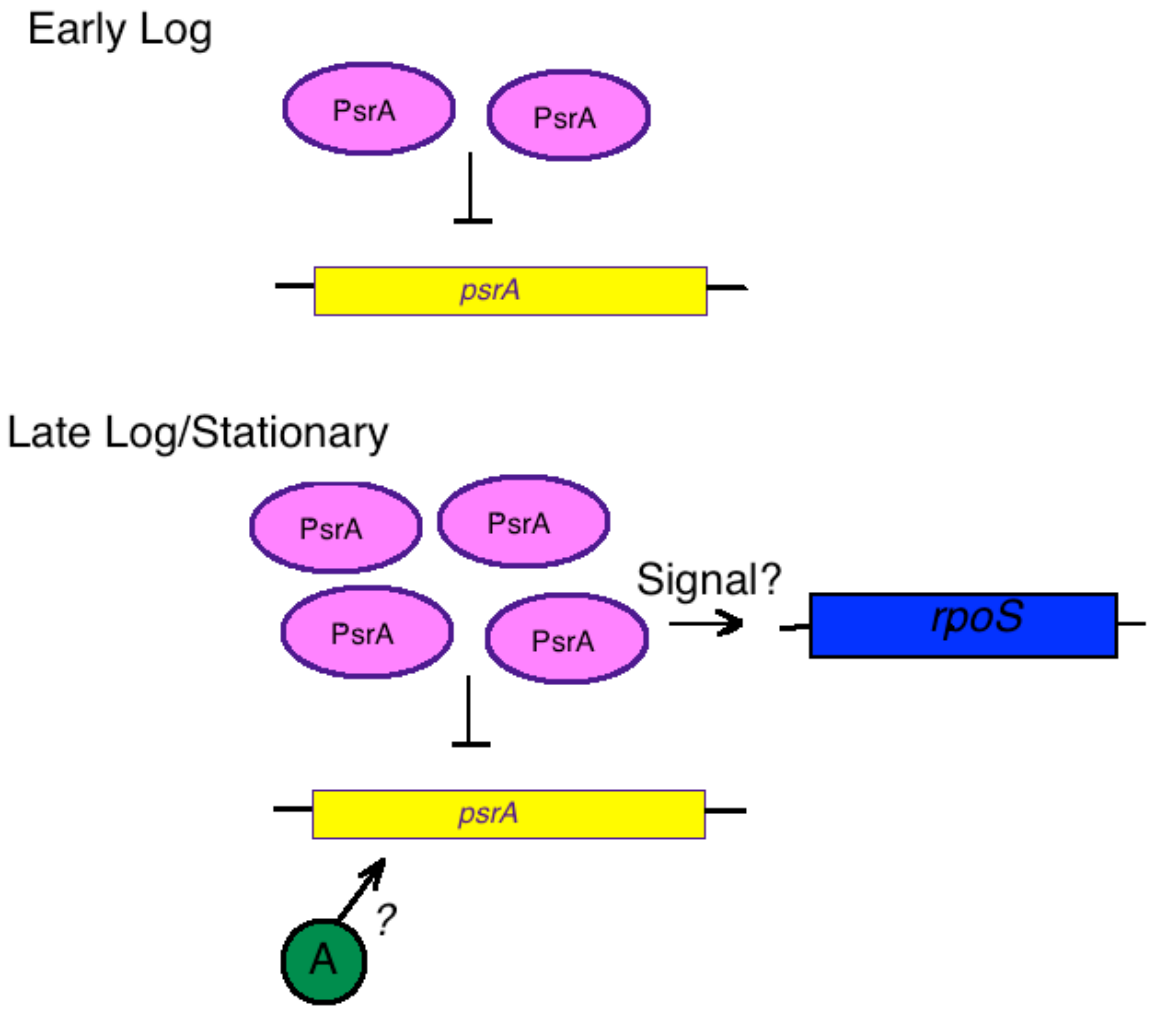

Figure 1.4. Regulation of *rpoS* and *psrA* expression by PsrA. (Adopted from (49))

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Chapter 2

Initial Characterization of tmRNA Function in *Pseudomonas aeruginosa*

Abstract

Transfer messenger RNA (tmRNA) encoded by *ssrA* is a hybrid RNA with a tRNA-like (TLD) and an mRNA-like (MLD) domain. tmRNA catalyzes recycling of stalled ribosome in bacteria by a process designated as *trans*-translation. tmRNA is the key factor of *trans*-translation which appears to play a variety of physiological function, including heat stress response and cell viability, in different bacteria . *Pseudomonas aeruginosa* is the paradigm of a gram-negative opportunistic bacterial pathogen that predominantly affects immunosuppressed hosts. To initiate the study of physiological function of tmRNA in *P. aeruginosa*, I characterized the phenotype of a *ΔssrA* mutant derivative of a wound isolate PAO1 when the bacterium was exposed to temperature changes and osmotic stress. My data demonstrate that tmRNA is important for initial adaptation of *P. aeruginosa* to temperature change and osmotic stress because the *ΔssrA* mutant exhibits a much longer lag phase than does the wildtype strain when it is grown at 30°C in the presence of 0.8M NaCl. This prolonged adaptation in the lag phase was

evident only when the *ΔssrA* mutant was exposed to both low temperature and high osmolarity. In addition, I determined the effect of carbon source, pH, and temperature on the expression of *ssrA* in *P. aeruginosa*. Using a chromosomally inserted *ssrA*::*lacZ* fusion, I determined that *ssrA*::*lacZ* was more highly expressed at 30°C than at 37°C or 42°C in *P. aeruginosa*. In contrast, neither glucose, glycerol, nor succinate affected the *ssrA*::*lacZ* expression. pH of the initial culture medium also did not affect the *ssrA*::*lacZ* expression. Taken together with other preliminary data from our laboratory on the effect of *ΔssrA* mutation on the virulence determinants, it appears that tmRNA has a subtle but global effect on *P. aeruginosa* physiology.

Introduction

Pseudomonas aeruginosa is a gram-negative bacterium that is found in various environment including water, soil, hospital environments, and in association with plants and animals (9, 16, 21). It is an opportunistic human pathogen that predominantly affects immunosuppressed hosts (11, 13, 25, 30, 41). During establishment of infections, *P. aeruginosa* has to adapt to various environmental fluctuations including temperature change, nutrient availability, and oxygen availability (5, 7). *P. aeruginosa*'s ability to flourish in numerous environmental conditions, including inside of human body as a pathogen, is due to the bacterium's natural stress response mechanisms, antibiotic resistance, and possession of numerous virulence determinants (26, 5, 14). The success of *P. aeruginosa* as a pathogen is reflected in its genome that contains an unusually large number of regulatory genes as well as nutrient acquisition and utilization genes (36). Determining how *P. aeruginosa* survives and causes infections will be important for developing better therapeutic approaches.

Transfer messenger RNA (tmRNA or 10Sa RNA) is a hybrid RNA with a tRNA-like and an mRNA-like domain (42, 43). tmRNA catalyzes the quality-control system designated as *trans*-translation (22, 28, 43). This hybrid RNA acts as a tRNA and a mRNA to provide an alternative mechanism to complete unfinished peptide synthesis, releases ribosomes that have stalled, and helps the cells to degrade the nascent polypeptide (1, 28). This simple and yet important role of tmRNA appears to have different physiological effect on various organisms. tmRNA is involved in thermotolerance in *Bacillus subtilis* (1) , and it is essential in *Neisseria gonorrhoeae* and *Shigella flexneri* (24, 34). These data demonstrate not only the importance of tmRNA in bacteria but also that its physiological function varies depending on the bacterium.

Thus, it is important to characterize the specific role of tmRNA in each bacterium.

In the systems that have been studied to date, tmRNA acts in conjunction with SmpB (small protein B), an associated protein factor that binds to the TLD to activate the RNA molecule (20, 27, 35, 43). Sequence analysis demonstrated that SmpB orthologs can be found in all bacterial genomes (38). Although a study suggested that SmpB can bind to ribosome independently of tmRNA (19), the crystal structure result showed that SmpB is in complex with the TLD of tmRNA in *Aquifex aeolicus* (17). Therefore, SmpB may have functions in addition to binding and activating tmRNA. Like tmRNA, the role of SmpB in the physiology of *P. aeruginosa* has not yet been characterized.

In this study, I characterized the role of tmRNA and SmpB in *P. aeruginosa* stress response.

Materials and Methods

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 2.1. Unless otherwise indicated, bacteria were cultured in Luria-Bertani broth (LB broth) or on LB-agar at 37°C supplemented antibiotics when appropriate. LB broth, LB Agar and Pseudomonas Isolation Agar (PIA) were purchased from Fisher Scientific (Pittsburgh, PA). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: ampicillin (Amp) 100 μg/ml for *E. coli*; carbenicillin (Carb) 100 μg/ml for *P. aeruginosa;* gentamicin (Gm) 20 μg/ml

for *E. coli* and 100 µg/ml for *P. aeruginosa*; tetracycline (Tet) 20 µg/ml for *E. coli* and 100 µg/ml for *P. aeruginosa,* and kanamycin (Km) 50 µg/ml for *E. coli* and 1mg/ml for *P. aeruginosa*. No-carbon-E minimal medium (NCE) supplemented with 0.1% (w/v) casamino acids (CAA) was used as the minimal medium (8). A 1:1 mixture of LB-agar and PIA was used to select for *P. aeruginosa* transconjugants and to counter select for *E. coli* following tri-parental mating.

Strains	Genotype and relevant characteristics	Source
E.coli		
DH10B	F$^-$ *mcrA Δ* (*mrr-hsdRMS-mcrBC*) F*80dlacZDM15 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
SS20[#]	HB101 / pRK2013	(12)
SS1365[*]	DH10B/ pEX100T-link	(33)
P. aeruginosa		
PAO1	Prototroph wound isolate	(23)
SS1372	PAO1 *ΔssrA*	Lab Collection
SS1740	PAO1 P*ssrA*::*lacZ1*	Lab Collection
SS2108	PAO1 *ΔsmpB*	This work
SS2101	PAO1 *ΔsmpB; ΔssrA*	This work
Plasmids		
pRK2013	Tra1 (RK2), ColE1; Km^r	(12)
pSW001	Δ*smpB*::pEX100T-link	This work
pEX100T-link	*sacB*, *oriT*; Ap^r	(33)

Table 2.1Bacterial strains and plasmids. [#]: Suh laboratory designation for the strain published by Figurski and Helinski (12); [*]: Suh laboratory designation for the strain published by Quénée (33).

DNA manipulations, transformations, and conjugations

For DNA cloning, *E. coli* strain DH10B was routinely used as the host strain. DNA fragments were introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (37). 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 (Beverly, MA). Either *Pfu* polymerase from Stratagene (La Jolla, CA) or *Taq* polymerase from New England Biolabs was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA)

Construction of P. aerugiosa ΔsmpB *mutants*

To delete *smpB* from *P. aeruginosa*, I first generated a *smpB* deletion allele. Briefly, a DNA sequence containing approximately 500 bp upstream and 630 bp downstream of the *smpB* coding sequence was amplified via Spliced Overlap Extension (SOE) PCR from PAO1 (40) (Fig.2.2.A). Primer sequence were SSO-410/F1: 5'- CAT GAA TTC CGG TCC GTT TTC CAG GTT CAT C-3'; SSO-411/R1: 5'-TTG CCG TGG CGC ATA GGC GGC GCA TTA TAG GGA GTC-3'; SSO-412/F2: 5'-ATA ATG CGC CGC CTA TGC GCC ACG GCA AGG ACG ACT G-3'; SSO-413/R2: 5'-CAT AAG CTT GTG GCG GCA ACT ATG TGA CC-3'. The unique *Eco*RI site built into F1 and the unique *Hin*dIII site built into R2 is highlighted with underline. In the first round, a fragment carrying approximately 500bp of DNA upstream and a second

fragment carrying approximately 630bp of DNA downstream of *smpB* was PCR amplified from PAO1. Between the two fragments is an overlap of 15 nucleotides by which the two fragments can be joined by SOE PCR. *Pfu* thermostable DNA polymerase (Stratgene, La Jolla, CA) was used for amplification to minimize incorporation errors. In the second round of PCR, two fragments were used as the template DNA with the upper primer of the upstream sequence (F1) and the lower primer of the downstream sequence as the two primers (R1). This generated a 1.2 kb fragment which was deleted for *smpB* and joined in the middle by the 15 bp of overlap (Fig.2.1.A).

The *ΔsmpB* DNA fragment was then cloned as an *Eco*RI/*Hin*dIII fragment into pEX100Tlink digested with the same enzymes to form pSW001. The pEX100Tlink is a suicide vector that carries carbenicillin resistance and a counterselectable marker (*sacB*) (33) (Fig.2.1.B). pSW001 was introduced into *P. aeruginosa* strains PAO1 and SS1372 by tri-parental conjugation and the merodiploids with pSW001 integrated into the genome via homologous recombination were isolated as carbenicillin resistant (Cb^r) colonies. The *Δ smpB* mutants were isolated as sucrose resistant but carbenicillin sensitive colonies that have resulted via resolution of the merodiploids via homologous recombination on sucrose selection. Deletion of *smpB* was confirmed by PCR analysis.

Growth curve assays to assess environmental stress response

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 plate reader

(Winooski, VT). To assess growth, overnight culture of cells grown in LB at 37°C was diluted 1:100 into appropriate medium and the cells were grown with shaking at the specified conditions. Cell growth was monitored as A_{600}. The effect of temperature on the growth of a *ΔssrA* was tested by growing *P. aeruginosa* in LB at 30°C, 37°C, or 42°C. The effect of hyperosmolarity on the growth of *ΔssrA* was determined by growing PAO1 and SS1372 in LB supplemented with 0 M, 0.1 M, 0.2 M, 0.4 M, 0.6 M, and 0.8 M of NaCl. The effect of exogenous carbon source was tested by subculturing the overnight cultures in NCE minimal medium supplemented with 10 mM glucose, 20 mM succinate, or 20 mM acetate as the sole carbon source as previously described (37).

Heat shock assay

To measure cells' ability to survive heat shock, cells grown overnight in LB at 37°C with aeration were washed twice and diluted in NCE to a density of approximately 7,000 CFU/ml. One milliliter of the diluted culture was placed in a prewarmed tube at 50°C, and the cell viability was determined by taking periodic aliquots and plating them directly on LB plates to measure the CFU.

β-galactosidase assay

β-galactosidase assays to determine the target gene expression were preformed as described by Miller (31). Absorbance measurements were performed on Shimadzu UV1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Results

Construction of ΔsmpB *and* ΔsmpB ΔssrA *mutants*

The small protein B, SmpB, works in conjunction with tmRNA in many of the systems that have been studied to date (10, 18, 20, 27, 29, 35, 39). In order to verify that SmpB served that same function in *P. aeruginosa*, I constructed a *ΔsmpB* and a *ΔsmpB ΔssrA* double mutant. The clean non-polar deletion allele of *smpB* was generated via SOE PCR (40) as described in Materials and Methods (Fig.2.1). The *ΔsmpB* allele was introduced into the parent strain PAO1 and its *ΔssrA* derivative via conjugation and those cells that have undergone allelic exchange between the wildtype and the mutant allele were selected as sucrose resistant but carbenicillin sensitive isolates. The *ΔsmpB* deletions were confirmed by PCR analysis (data not shown).

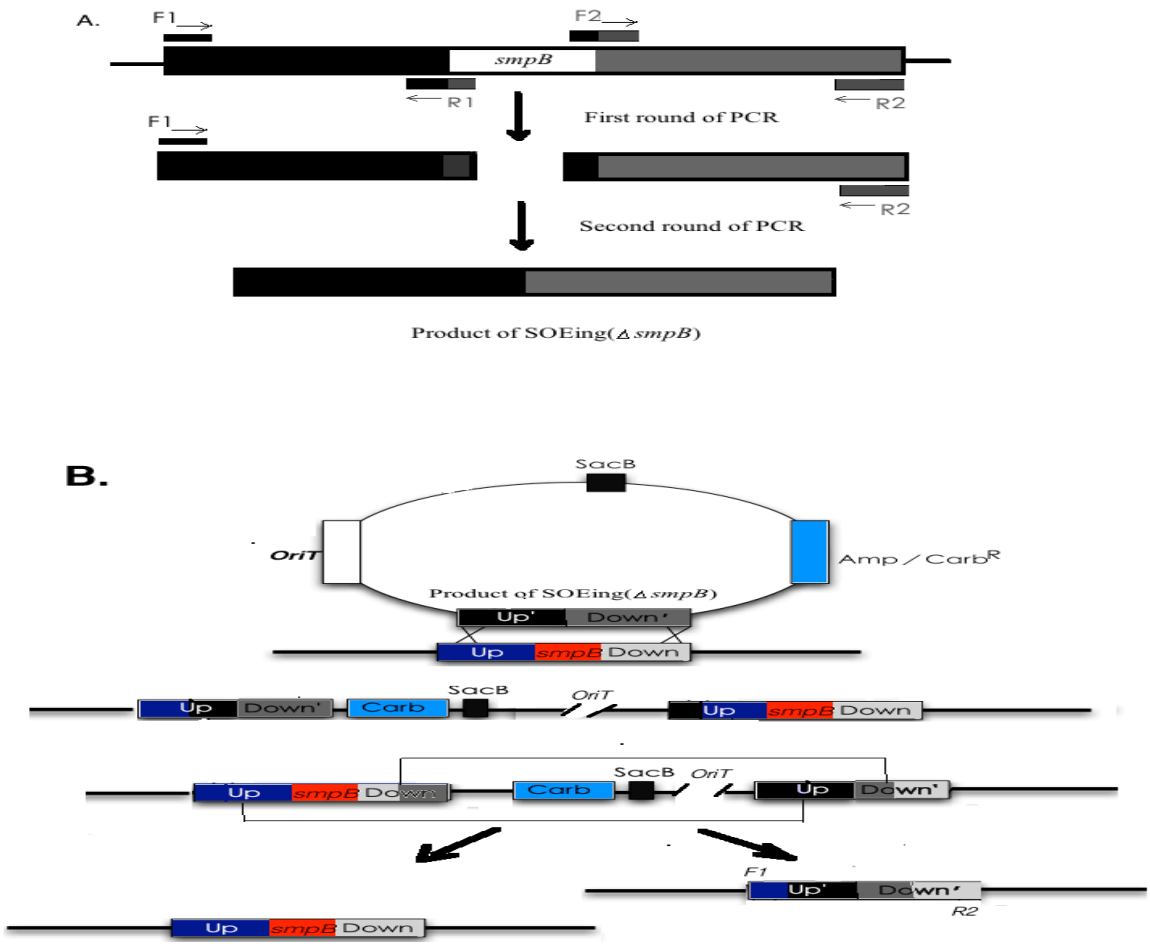

Figure.2.1. Construction of a *ΔsmpB* mutant.

Effect of tmRNA and SmpB on heat shock responss of P. aeruginosa

To determine whether tmRNA and/or SmpB is involved in the thermotolerance of *P. aeruginosa*, all four strains (wt, *ΔssrA, ΔsmpB*, and *ΔssrA ΔsmpB*) were grown in LB to stationary phase (OD_{600} of 2.0), diluted to approximately 7000 CFU/ml and incubated at 50°C. As demonstrated in Fig.2.2, *P. aeruginosa* strains lacking tmRNA, SmpB, or both were more sensitive to heat shock at 50°C. After 8 min of incubation at 50°C, the survival percentage of the wild-type (PAO1) was 60% and all three mutants (*ΔssrA*, *ΔsmpB,* and *ΔssrA ΔsmpB*) 40%.

Effect of tmRNA on P. aeruginosa *growth in different temperatures*

In order to assess a potential role of tmRNA on *P. aeruginosa* growth as function of temperature, I grew the wildtype and the *ΔssrA* mutant in LB with aeration at 30°C, 37°C and 42°C. As demonstrated in Fig.2.3, there was no visible effect of tmRNA on temperature dependent growth of *P. aeruginosa*. Although *P. aeruginosa* exhibited longer lag at 30°C and at 42°C, there was no observable difference between the wildtype and the *ΔssrA* mutant in growth rate and in entrance into and maintenance in stationary phase.

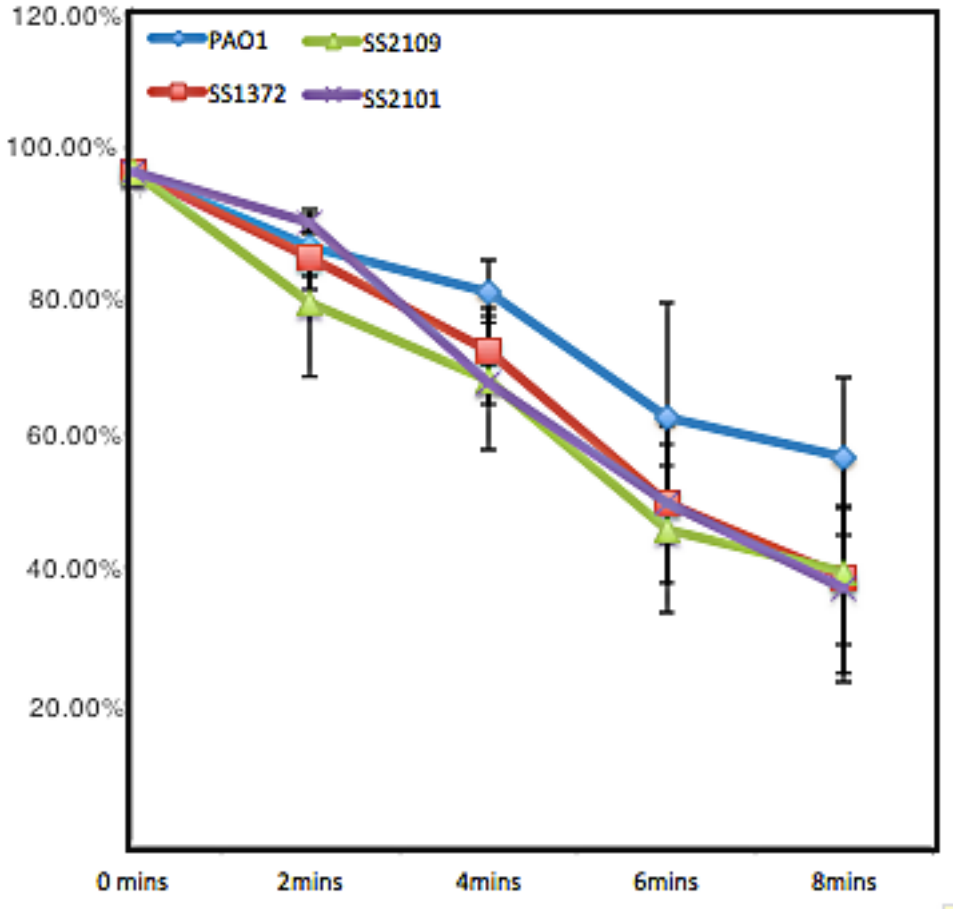

Figure.2.2. Effect of tmRNA and SmpB on thermotolerance of *P. aeruginosa*.

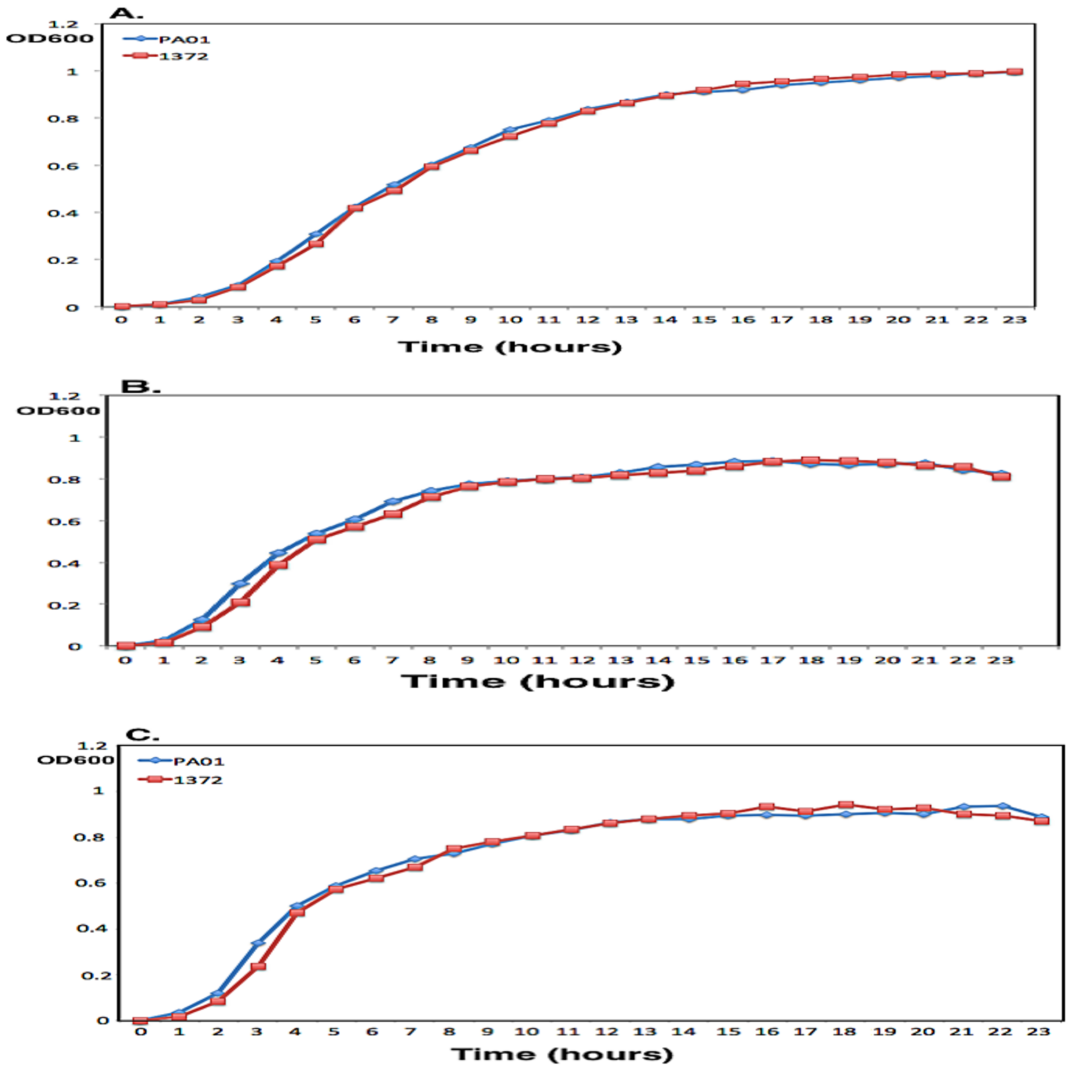

Figure.2.3. Effect of *ΔssrA* mutation on *P. aeruginosa* growth in different temperatures. A: 30°C, B: 37°C, C: 42°C. The cells were grown in a 24-well microtiter plate at 37°C with shaking in a BioTek Synergy HT. The data shown are representative of three independently performed experiments.

Effect of tmRNA on P. aeruginosa growth as a function of osmolarity

A potential role of tmRNA on the response of *P. aeruginosa* to extracellular osmolarity was assessed. I grew the wildtype and the *ΔssrA* mutant in LB supplemented with 0 M (Fig.2.4.A), 0.1 M (Fig.2.4.B), 0.2 M (Fig.2.4.C), 0.4 M (Fig.2.4.D), 0.6 M (Fig.2.4.E), and 0.8 M (Fig.2.4.F) in a water bath at 37°C with aeration. The results are shown in Fig.2.4. At 1.0 M concentration of NaCl, *P. aeruginosa* failed to grow (data not shown). At 0 M NaCl, *P. aeruginosa* grew at a comparable rate but entered the stationary phase relatively early and the cells only grew to approximately 50% density as in 0.1 M NaCl. Starting from 0.2 M NaCl (Fig.2.4 C), *P. aeruginosa* growth rate was slowed and the cells entered the stationary phase at a later time. At 0.6 M (Fig.2.4 E) and 0.8 M (Fig.2.4 F) NaCl concentrations, the hyperosmolarity of the growth medium clearly affected the growth rate as well as the extension of the lag phase. The extended lag and the decreased growth rate are presumable due to the hyperosmotic response of the bacterium including production of proline and potassium glutamate. Significantly, there was no tmRNA effect on *P. aeruginosa* growth characteristics as a function of NaCl concentration in the growth medium.

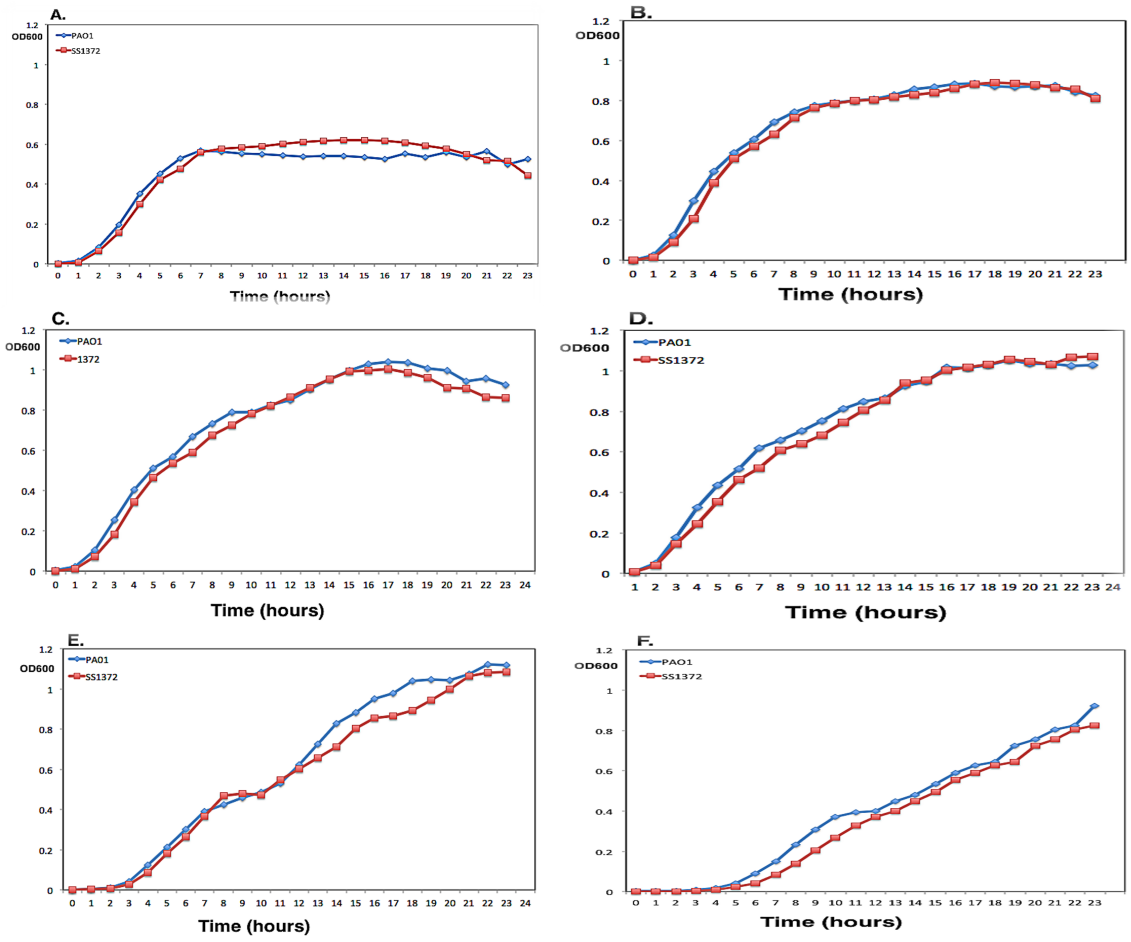

Figure.2.4. Effect of *ΔssrA* mutation on *P. aeruginosa* growth in different salt concentrations. A: 0 M NaCl, B: 0.1 M NaCl, C: 0.2 M NaCl, D: 0.4 M NaCl, E: 0.6 M NaCl, F: 0.8 M NaCl. Cells were grown in LB supplemented with NaCl at 37°C with aeration. Data shown are a representative of three independently performed experiments.

Effect of tmRNA and SmpB on carbon source metabolism

I addressed a potential role of tmRNA on the growth of *P. aeruginosa* using different carbon compound. The wildtype and the *ΔssrA* mutant were grown in the No-carbon E (NCE) minimal medium (8) supplemented with either 20 mM succinate (Fig.2.5.A), 10mM glucose (Fig.2.5.B), or 20mM acetate (Fig.2.5.C) as a sole carbon source. Succinate was chosen because it is considered to be the preferred carbon source for *P. aeruginosa* while glucose is a poor carbon due to the fact that *P. aeruginosa* catalyzes glucose via inefficient Entner-Doudoroff pathway (6). The cells were grown at 37°C with aeration and OD_{600} was measured periodically to determine the culture density. As shown in Fig.2.5, I observed no difference between the wildtype and the *ΔssrA* mutant. Thus, tmRNA does not appear to be important for *P. aeruginosa* growing on these three carbon sources.

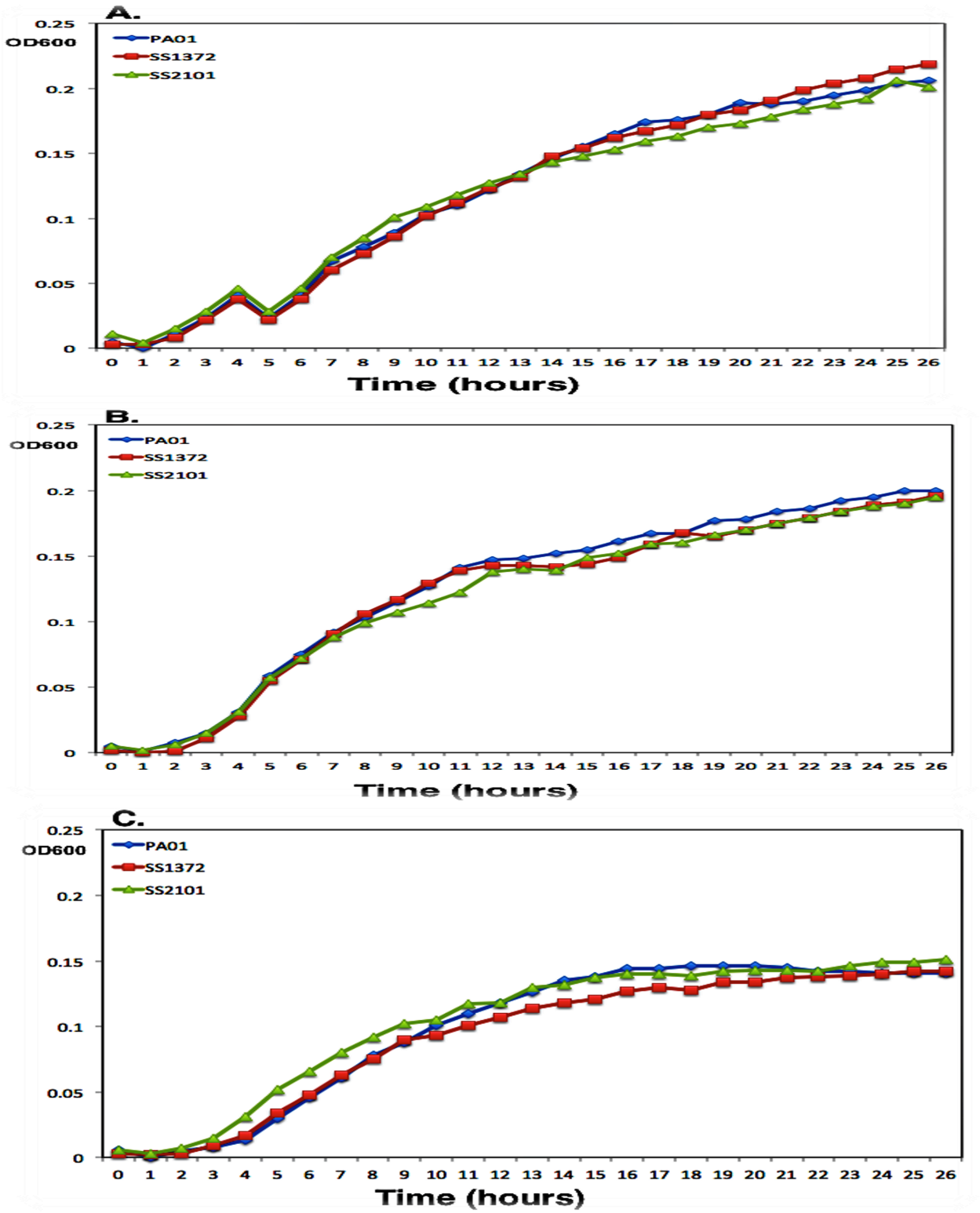

Figure 2.5. Effect of *ΔssrA* and *ΔsmpB* mutations on *P. aeruginosa* growth on different carbon sources. A: 20 mM succinate, B: 10 mM glucose, C: 20 mM acetate. Cells were grown in NCE medium supplemented with the carbon sources at 37°C with shaking. Data shown are a representative of three independently performed experiments.

Effect of tmRNA on P. aeruginosa *growth in high osmotic medium at low temperature*

I tested the potential role of tmRNA when *P. aeruginosa* was exposed simultaneously to osmotic and thermal stresses. PAO1 (wildtype) and SS1372 (*ΔssrA*) were grown in LB supplemented with various concentration of NaCl and grown at 30°C, 37°C, and 42°C with aeration. I tested NaCl concentrations of 0, 0.1, 0.2, 0.4, 0.6, and 0.8 M. As previously shown in Fig.2.4, I observed no effect of NaCl on *P. aeruginosa* growth between the wildtype and the *ΔssrA* mutant when the cells were grown at 37°C and 42°C (data not shown). There was also no difference between the wildtype and the *ΔssrA* mutant when the cells were grown at 30°C with 0, 0.1, 0.2, 0.4, and 0.6 M of NaCl. However, as demonstrated in Fig.2.6, the *ΔssrA* mutant exhibited a prolonged lag phase when the cells were grown at 30°C and 0.8 M of NaCl. Once the *ΔssrA* mutant came out of the lag phase, the mutant actually grew at a faster rate than the wildtype and they both reached the stationary phase at approximately the same time with similar cell density. Thus, tmRNA appears to be important for initial adaptation of *P. aeruginosa* to osmotic stress at 30°C.

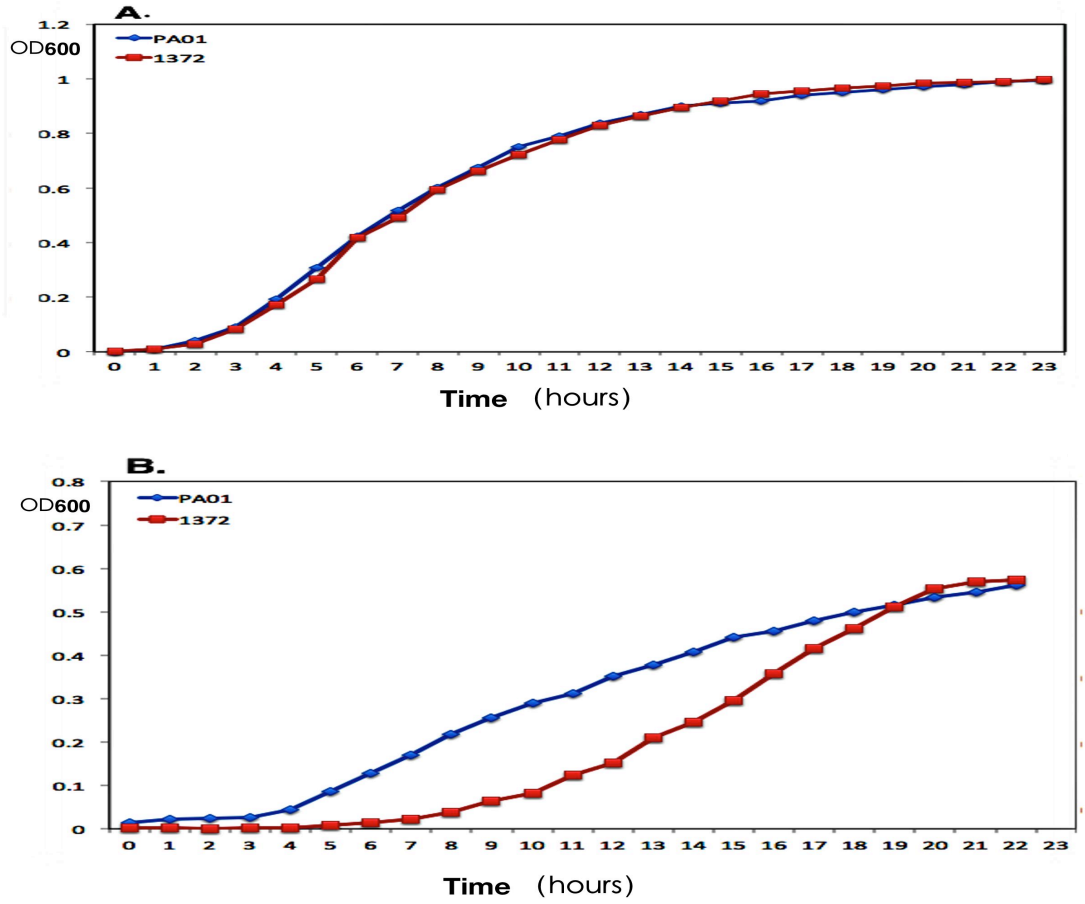

Figure 2.6. Effect of *ssrA* mutation in 30°C and 0.8M NaCl growthcurve. Cells were grown in LB supplemented with NaCl at 30°C with aeration. Panel A: NaCl concentration of 0.1 M. Panel B: NaCl concentration of 0.8 M. The data shown are a representative of three independently performed experiments.

Initial characterization of ssrA::lacZ *expression in* P. aeruginosa

In order to initiate characterization of *ssrA* gene expression in *P. aeruginosa*, I used a *P. aeruginosa* strain that had an integrated copy of *ssrA*::*lacZ* transcriptional fusion (Tucker and Suh, unpublished results, Fig.2.7). This strain contains a wildtype copy of the *ssrA* gene in addition to the *ssrA*::*lacZ* fusion that had been integrated via homologous recombination. I determined the effect of pH, carbon source, and temperature on *ssrA* expression.

In order to assess potential effect of pH on *ssrA* expression, I grew the wildtype carrying the *ssrA*::*lacZ* fusion (SS1740) in LB that had been pH adjusted to pH of 5, 6, 7, 8, and 9. LB broth prepared without preadjusting the pH was used as a control. This broth is assumed to be near neutral pH of 7 according to the manufacturer's instructions. Growth of the cells at 37°C was monitored by OD_{6oo} measurements and the cells were harvested three hours after they had entered the stationary phase. The *ssrA*::*lacZ* expression of *P. aeruginosa* grown in various pH is shown in Fig.2.8. Although the maximum expression was seen when the initial pH was 7, my data indicate that *ssrA* expression is not greatly affected by the initial pH of the growth medium.

I also tested for the potential effect of carbon source on the *ssrA* gene expression. The overnight culture of SS1740 that had been grown in LB was diluted 1:100 in NCE medium (8) supplemented with 10 mM glucose, 20 mM acetate, or 20 mM succinate and grown at 37°C with shaking. As shown in Fig.2.9, the *ssrA*::*lacZ* expression is not affected by the sole carbon sources provided exogenously in the growth medium.

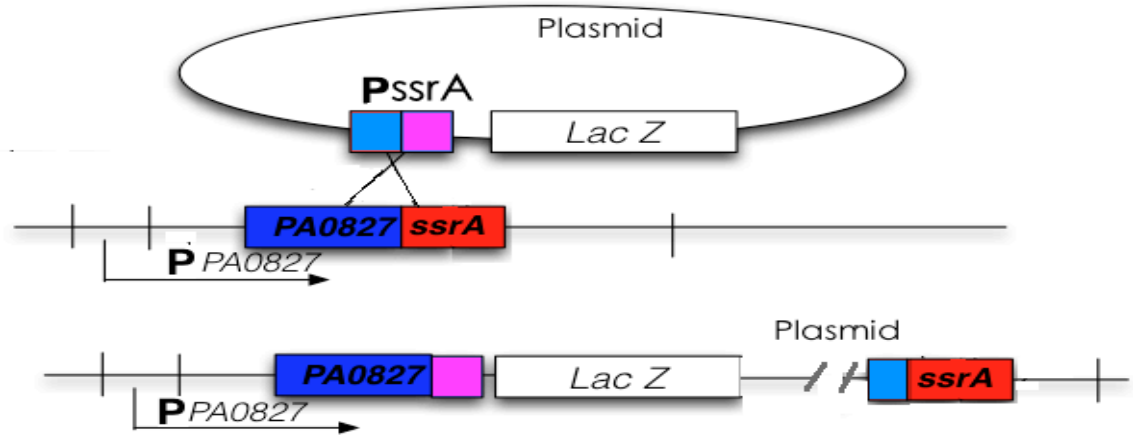

Figure 2.7. Construct a *P. aeruginosa* strain with an integrated copy of *ssrA*::*lacZ* transcriptional fusion.

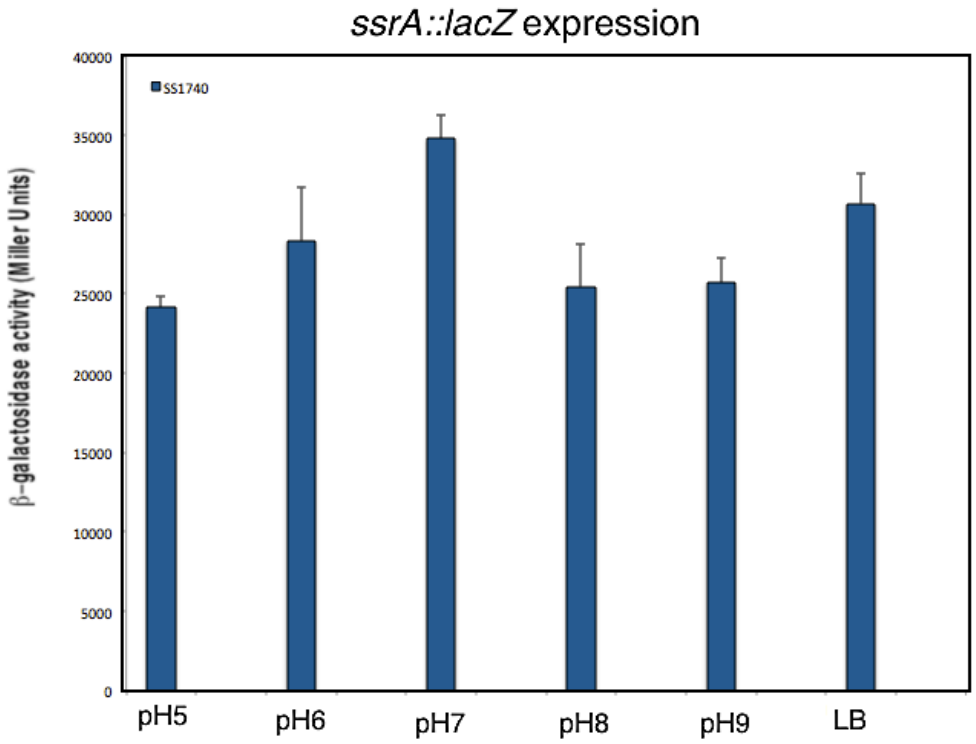

Figure 2.8. *ssrA*::*lacZ* expression as a function of pH of the growth medium. P. aeruginosa was grown initially overnight in LB. The overnight culture was diluted 1:100 in 20 ml of LB adjusted to the shown pH and the cells were grown at 37°C with aeration. Three hours after the culture has entered the stationary phase of growth as determined by OD600 measurement, cells were collected and *ssrA*::*lacZ* expression was determined by β-galactosidase assay as described by Miller (31) in duplicate samples. The data shown are a representative of three independent experiments.

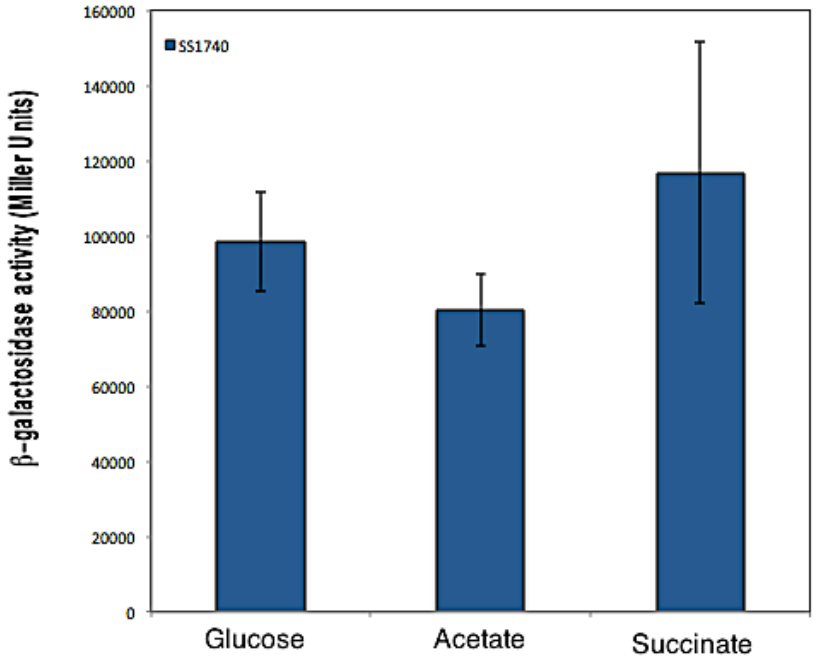

Figure 2.9. *ssrA*::*lacZ* expression is not affected by the carbon source. SS1740 was grown in LB overnight at 37°C with shaking. The cells were washed with NCE (8) and diluted 1:100 in 20 ml of NCE supplemented with 10 mM glucose, 20 mM acetate, or 20 mM succinate. The cells were harvested in stationary phase and β-galactosidase assay was performed as previously described (31) in duplicate samples. The results shown are a representative of three independent experiments.

As *P. aeruginosa* establishes infections in humans, it has to adjust to the difference in temperature between the outside environment and inside of the host. To assess whether the *ssrA* gene expression is affected by temperature, I determined the *ssrA*::*lacZ* fusion expression when *P. aeruginosa* strain SS1740 was grown at 30°C, 37°C, and 42°C. The cells were removed at mid-log, late-log, early stationary, and stationary phase and assayed for β-galactosidase activity. The results are shown in Fig.2.10. My data demonstrate that *ssrA* expression is inversely correlated with temperature increase. The highest expression of *ssrA* was in the stationary phase of cells grown at 30°C. The lowest expression of *ssrA* occurred when the cells were grown at 42°C. At 37°C, the body temperature of humans, *ssrA* gene expression at 50 – 60% of that when *P. aeruginosa* was grown at 30°C. Thus, *ssrA* expression is temperature regulated.

Discussion

The uniquely bacterial tmRNA catalyzes ribosome and amino acid recycling via *trans*-translation (27, 28). In order to initiate characterization of the tmRNA function in *P. aeruginosa*, Alex Tucker in our laboratory constructed a *ΔssrA* null mutant in *P. aeruginosa* (Tucker and Suh, unpublished data). Successful construction of a *ΔssrA* mutant indicated that tmRNA was not essential for viability of *P. aeruginosa*. That distinguished its function from *Neisseria gonorrhoeae* and *Shigella flexneri* (24, 34). Although tmRNA has been studied in great detail biochemically, its physiological function in bacteria has only recently been addressed in few organisms. In several bacteria, including *E. coli*, *B. subtilis,* and *S. coelicola* (32), tmRNA has been demonstrated to be important for stress response.

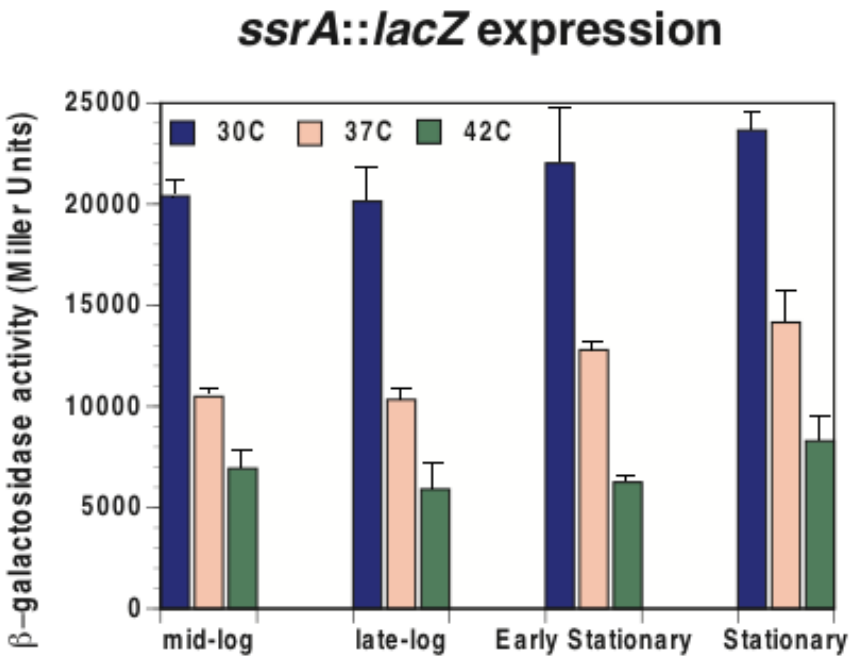

Figure 2.10. *ssrA*::*lacZ* expression is temperature dependent. SS1740 grown overnight in LB at 37°C was diluted 1:100 in 20 ml of fresh LB and grown at indicated temperature with shaking. The cell density was monitored as OD600. Cells were removed at mid-log (OD600 of ~0.8 - 1.0), late-log (OD600 of ~1.5), early stationary (OD600 of ~1.8 - 2.0), and stationary phase (OD600 of ~2.2-2.5). β-galactosidase assay was performed as previously described (31) in duplicate samples. The data presented are a representative of three independent experiments.

Our laboratory is interested in elucidating the stress response mechanisms of the human pathogen *P. aeruginosa*. Therefore, I assessed whether tmRNA was involved in several basic stress response of *P. aeruginosa*. I determined that under standard laboratory growth conditions in a rich medium (LB) and grown 30°C, 37°C, and at 42°C, tmRNA did not appear to affect the the growth of *P. aeruginosa*. This was different from the phenotype of an *E. coli ssrA* mutant (2). In addition, tmRNA did not appear to play a role in efficient utilization of preferred (succinate and acetate) or non-preferred (glucose) carbon sources. Even against hyperosmotic stress, tmRNA did not appear to play an obvious role. However, when *P. aeruginosa* was exposed to high concentration of exogenous NaCl (0.8 M) and grown at 30°C, the $tmRNA^-$ mutant demonstrated difficulty in adapting to the condition as exhibited by a prolonged lag phase. Interestingly, once the *ssrA* mutant adapted, it grew at a faster rate then did the wildtype. These data suggest that tmRNA is only important for initial adaptation. Alternatively, the data may suggest that in the absence of tmRNA and under high osmotic condition and growing at 30°C, *P. aeruginosa* may undergo hypermutability and acquire secondary or suppressor mutations. Another possibility is the presence of a backup system to tmRNA that may take time to be induced. In *E. coli*, ArfA suppresses tmRNA defect to catalyze *trans*-translation (3, 4). It was recently demonstrated that tmRNA is required for ArfA synthesis (15). The prolonged lag observed in our *ΔssrA* mutant may reflect a similar requirement for ArfA in *P. aeruginosa*. I recently identified a putative open reading frame (ORF) that may encode for *P. aeruginosa* ArfA.

The *trans*-translation requires two extra ribosomal factors in tmRNA and SmpB (10, 27,

28, 35). I addressed whether SmpB played a similar role in *P. aeruginosa*. I constructed a *ΔsmpB* and a *ΔssrA ΔsmpB* mutant and compared their phenotype. As shown in Fig.2.2 and 2.5, *P. aeruginosa* appears to require both tmRNA and SmpB for *trans*-translation because I observed no differences between a *ΔsmpB* and a *ΔssrA ΔsmpB* mutant.

In addition to characterizing general phenotype of the *P. aeruginosa ΔssrA* mutant, I initiated a study to characterize the regulation of tmRNA synthesis. Very little information is available about the genetic regulation of tmRNA synthesis in any bacteria. Using a *ssrA*::*lacZ* fusion carrying *P. aeruginosa* (SS1740), I determined that pH and carbon source did not affect *ssrA* expression. However, I discovered that *ssrA* expression was inversely related to increasing temperature. Under the conditions tested, the *ssrA* gene expression was highest at 30°C and was severely decreased at 37°C and at 42°C. At 37°C and at 42°C, the *ssrA*::*lacZ* expression was decreased by 40 – 60% (Fig.2.10). These data raise interesting question about the potential role of tmRNA during *P. aeruginosa* infection versus the importance of this hybrid RNA for survival of the bacterium in the environment where the temperature is lower than 37°C. In addition, because expression of *ssrA* as a function of temperature has not yet been studied in other bacteria, it is difficult to assess whether this temperature regulation is a conserved mechanism in other organisms.

In this chapter, I described my efforts to initiate a study of tmRNA in *P. aeruginosa*. Based on my results, it appears that tmRNA catalyzes some of the similar functions as in other bacteria including a subtle role in stress response. However, this aspect of tmRNA function in *P. aeruginosa* needs to be studied further. However, for future studies, it is evident that *P.*

aeruginosa should be grown at 30°C because *ssrA* is expressed higher at lower temperature than at higher temperature of 37°C, which is the standard growth temperature for *P. aeruginosa* studies. Thus, any phenotype due to the lack of tmRNA is expected to be exaggerated when the bacterium is grown at 30°C rather than the standard temperature of 37°C.

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Chapter 3

Isolation of regulatory mutants of the *ssrA* gene expression

Abstract

Transfer messenger RNA (tmRNA) encoded by *ssrA*, is a hybrid RNA with tRNA and mRNA domains that catalyzes recycling of stalled ribosomes. Although tmRNA has been characterized extensively at the biochemical level, very little is known about how this unique RNA is regulated at the genetic level. *P. aeruginosa* is a bacterial pathogen with a genome that devotes a large number of genes for the regulators of gene expressions. In order to characterize the mechanisms of *ssrA* expression in *P. aeruginosa*, a transposon insertion mutagenesis was conducted to identify putative regulatory genes for *ssrA*::*lacZ*. My previous data demonstrated that *ssrA* expression was temperature controlled with higher gene expression at lower than at elevated temperatures. Therefore the transposon mutagenesis was conducted with cells grown at 30°C. Out of approximately 40,000 Tn*5*-B30 insertion mutants screened, I identified 11 genes that affected the *ssrA*::*lacZ* fusion expression in *P. aeruginosa*. Two mutants (*prt4* and *prt6*) were mapped to genes encoding putative transcriptional regulators, *PA0319* and *PA5431* respectively. One insertion (*prt2*) was in a gene that encodes for a heat shock protein: ClpB

(*PA4542*). Another insertion (*prt10*) was in a gene that encodes for a malic enzyme, *PA5046*. *PA4542* and *PA5046* are the only genes identified whose functions have been demonstrated. One mutation (*prt5*) was mapped to a putative protein involved in the Type VI secretion system, *PA1658* (*hsiC2*). The other six mutations mapped to genes that encode for hypothetical proteins, *PA4929, PA2729, PA1761, PA0738, PA2721, and PA3693*. I successfully cloned the wildtype alleles of four genes and complemented the original transposon insertion mutations in *PA2729* (*prt3*), *PA1658* (*prt5*), *PA0319* (*prt6*), *PA0738* (*prt7*) by restoring the *ssrA*::*lacZ* expression. The other seven transposon insertion mutants have not yet been verified via genetic complemention. My study represents one of the first attempts to characterize regulatory mechanisms of tmRNA expression in any bacteria.

Introduction

The uniquely bacterial process designated as *trans*-translation is a process by which bacteria can recycle ribosomes those are stalled on mRNA transcripts (14). Furthermore, this process tags the incomplete peptide for degradation so that amino acids can also be recycled (14, 38). *Trans*-translation is catalyzed by the small RNA molecule tmRNA that contains both a tRNA-like domain (TLD) and a mRNA-like domain (MLD) (4, 12, 39). tmRNA is a key factor when a bacterium faces problems such as nonstop or no-go pathways. Nonstop translations lack an in-frame stop codon and therefore the ribosomes fail to terminate at the proper site. No-go pathway is encountered when mRNA is damaged before the stop codon and results in stalled ribosomes. In both situations, translation is affected and results in the production of incorrect polypeptides. In the presence of tmRNA, these incorrect peptides are tagged with the degradation tag encoded by the MLD of tmRNA and efficiently degraded (14).

Through *trans*-translation, tmRNA mediates expression of several gene products. tmRNA regulates translation of the general stress response regulator RpoS (24) and the transcriptional repressor LacI (1) in *Escherichia coli*, the *kinA* gene expression in in *Bacillus subtilis* (17), and the biosynthesis of a pigment in *Staphylococcus aureus* (19). In *E. coli*, tmRNA is required for translation of entire length of mRNA for production of RpoS and LacI. Therefore, it is expected that tmRNA functions in a similar fashion in regulation of other genes in bacteria.

Although tmRNA has been the focus of intense investigation by biochemists for many years to understand its unique structure and function (14-16), regulation of its expression remains a mystery. Given that tmRNA's role in bacterial physiology appears to be complex, I

hypothesize that tmRNA is controlled by a complex regulatory network and that characterization of this network will provide critical insight into the global role of tmRNA in *Pseudomonas aeruginosa* physiology.

P. aeruginosa is a gram-negative facultative anaerobic bacteria pathogen. It can be found in many different temperate environments including in association with animals and plants, in soil, fresh water habitats, and marine environments (5, 10, 11). As a pathogen, during establishment of infections, *P. aeruginosa* may be exposed to various potentially hostile environmental effects such as temperature change, oxidative and osmotic stress, nutrient deprivation, and human intervention in the form of antimicrobial therapy (3, 9, 18, 25). To successfully establish infections and persist in hostile environments, *P. aeruginosa* must be able to sense changes and react appropriately (8, 9, 25). The ability of *P. aeruginosa* to survive in so many different environments is because of its genome devoting a large percentage to transcriptional regulators (33). Some of the highly characterized regulatory systems of *P. aeruginosa* include cell-cell communication systems as well as a large number of two-component regulatory systems and alternative sigma factors (6, 23, 27, 32, 33, 36, 37). As a highly successful opportunistic pathogen, *P. aeruginosa* possesses two *N*-acyl-homoserine lactone (LasRI and RhlRI) and one quinolone mediated (PQS) cell-cell communication systems that regulate a large overlapping sets of genes (6, 27, 32, 36). The cell-cell communication systems' regulation of gene expression in *P. aeruginosa* works in conjunction with a highly complex network of other regulatory systems that can integrate or respond to multiple environmental signals (26). Such complex regulation systems lead to the adaptibility of *P. aeruginosa* to survive in diverse environments.

Materials and Methods

Bacterial strains, plasmids, chemicals, and media

Bacterial strains and plasmids used in this study are listed in Tab.3.1. Unless otherwise indicated, bacteria were cultured in Luria-Bertani broth (LB broth) or on LB-agar supplemented with appropriate antibiotics. LB broth, LB premixed Agar and Pseudomonas Isolation Agar (PIA) were purchased from Fisher Scientific (Rockford, IL). Antibiotics and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: ampicillin (Amp) 100 μg/ml for *E. coli*; carbenicillin (Carb) 100 μg/ml for *P. aeruginosa;* gentamicin (Gm) 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; tetracycline (Tet) 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa;* IPTG at 0.5 mM for *P. aeruginosa.* A 1:1 mixture of LB-agar and PIA was used to select for *P. aeruginosa* transconjugants and to counter select for *E. coli* following tri-parental mating as previously described (35). Restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, and *Phusion* DNA polymerase were purchased from New England Biolabs (Beverly, MA).

Strains	Genotype and relevant characteristics	Source
E. coli		
DH10B	F$^-$ *mcrA Δ*(*mrr-hsdRMS-mcrBC*) *Φ80dlacZDM15 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
S17-1	*Pro-82* res$^-$ m$^+$/*RP4-2Tc*::*Mu-km*::T_N*7*	(29)
SS20[#]	HB101/pRK2013	(7)
SS1479[*]	S17-1 pro res$^-$ m$^+$ / *RP4-2Tc*::*Mu-km*::T_N*7* / $(Gm^R)_{102}$(Zk2219) (SMC154 = C_{70}) pSUP::Tn*5*-B30 (Tc)/pSUP::TN*5*-B30	(30)
P.aeruginosa		
SS1740	PAO1 P*ssrA*::*lacZ*	Lab Collection
SS2138 (*prt1*)	PAO1 P*ssrA*::*lacZ PA4929*::Tn*5*-B30	This work
SS2152 (*prt2*)	PAO1 P*ssrA*::*lacZ clpB*:: Tn*5*-B30	This work
SS2149 (*prt3*)	PAO1 P*ssrA*::*lacZ PA2729*:: Tn*5*-B30	This work
SS2621	PAO1 P*ssrA*::*lacZ PA2729*:: Tn*5*-B30/pSW094	This work
SS2623	PAO1 P*ssrA*::*lacZ PA2729*:: Tn*5*-B30/pSW096	This work
SS2027 (*prt4*)	PAO1 P*ssrA*::*lacZ PA5431*:: Tn*5*-B30	This work
SS2658 (prt5)	PAO1 P*ssrA*::*lacZ PA1658*:: Tn*5*-B30	This work
SS2637	PAO1 P*ssrA*::*lacZ PA1658*:: Tn*5*-B30/pSW100	This work
SS2639	PAO1 P*ssrA*::*lacZ PA1658*:: Tn*5*-B30/pSW096	This work
SS2056 (prt6)	PAO1 P*ssrA*::*lacZ PA0319*:: Tn*5*-B30	This work
SS2613	PAO1 P*ssrA*::*lacZ* PA0319:: Tn*5*-B30/pSW088	This work
SS2611	PAO1 P*ssrA*::*lacZ* PA0319:: Tn*5*-B30/pSW056	This work
SS2055 *(prt7)*	PAO1 P*ssrA*::*lacZ PA0738*:: Tn*5*-B30	This work
SS2589	PAO1 P*ssrA*::*lacZ PA0738*:: Tn*5*-B30/pSW080	This work
SS2542	PAO1 P*ssrA*::*lacZ PA0738*:: Tn*5*-B30/pSW056	This work
SS2030 *(prt8)*	PAO1 P*ssrA*::*lacZ PA1761*:: Tn*5*-B30	This work
SS2050 *(prt9)*	PAO1 P*ssrA*::*lacZ PA2721*:: Tn*5*-B30	This work
SS2023 *(prt10)*	PAO1 P*ssrA*::*lacZ PA5046*:: Tn*5*-B30	This work
SS2043 *(prt11)*	PAO1 P*ssrA*::*lacZ PA3693*:: Tn*5*-B30	This work

Plasmids		
pSS213	$P_{T7(A1/04/03)}$, *aacCI* (Gent^r)	(35)
pSU39	Kan^r, MCS	(20)
pSW053	pSU39 Δkan^r::*aacCI*	This work
pSW056	pSU56, mSF	This work
pSW057	pSW056:: *PA00319*	This work
pSW058	pSW056:: *PA0738*	This work
pSW080	pSW056:: *PA0738*, mSF	This work
pSW082	pSS213::*PA2729*	This work
pSW088	pSW056:: *PA00319*, mSF	This work
pSW094	pSS231::*PA2729*, mSF	This work
pSW096	$P_{T7(A1/04/03)}$, *aacCI*, mSF	This work
pSW099	pSS213::*PA1658*	This work
pSW100	pSS213::*PA1658*, mSF	This work

Table3.1Bacterial strains and plasmids. #: Suh laboratory designation for the strain published by Figurski and Helinski (7); *: Suh laboratory designation for the strain published by Simon et al. (30).

DNA manipulations, transformations, and conjugations

For DNA cloning, *E. coli* strain DH10B was routinely used as the host strain. DNA fragments were introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (34). 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 (Beverly, MA). Either *Pfu* polymerase from Stratagene (La Jolla, CA) or *Taq* polymerase from New England Biolabs was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA)

Transposon mutagenesis

A transposon mutagenesis was performed to isolate insertion mutants that altered *ssrA*::*lacZ* expression in *P. aeruginosa.* The mini transposon Tn*5*-B30 (30) was introduced into *P. aeruginosa* strain SS1740 (PAO1 *ssrA*::*lacZ*) via conjugation as previously described (34). Transposon insertion mutants were selected as tetracycline resistant (Tc^r) colonies growing at 30°C on Pseudomonas Isolation Agar (PIA) plates supplemented with 80 μg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The cells were grown at 30°C because *ssrA* is expressed higher at that temperature than at 37°C. The Tc^r colonies with altered expression of *ssrA*::*lacZ* were isolated and rescreened on fresh PIA Tc^r plates as likely candidates to have transposon insertions in genes that encode for regulators of *ssrA* expression. Approximately 40,000 colonies were screened with the intention to cover the

whole genome at least three times (33). Colonies that demonstrated a consistent phenotype on plates were rescreened by β-galactosidase assays in 96-well microtiter plates. Briefly, cells were grown in 96-well microtiter plates in LB supplemented with carbenicillin (to maintain *ssrA*::*lacZ*) with shaking. The following day, β-galactosidase assay was performed as described by Miller (21) to measure *ssrA*::*lacZ* expression in the putative mutants. The *ssrA*::*lacZ* expression in putative mutants were determined for cells grown at 30°C as well as at 37°C in order to identify potential temperature dependent regulators of tmRNA. Those mutants that consistently demonstrated altered *ssrA*::*lacZ* expression in microtiter plate assays were selected and tested again by growing cells in batch cultures in test tubes or in flasks. For test tube β-galactosidase assays, putative *ssrA* regulator mutants were grown in LB broth with 100ug/ml carbenicillin overnight at 30°C and 37°C with aeration. The following day, cells were harvested and assayed for β-galactosidase activity to determine the expression of *ssrA*::*lacZ* fusion. By comparing *ssrA*::*lacZ* expression between 37°C and 30°C grown cells, we expected to isolate mutants that regulate *ssrA* differentially as a function of temperature. Each putative regulatory mutant was assayed at least three independent times to validate expression of the *ssrA*::*lacZ* fusion phenotype.

Southern hybridization

Southern hybridizations were performed to confirm that mutant phenotype was due to a single insertion of Tn*5*-B30 (31). The 1.2 kb *tetAB* fragment of Tn*5*-B30 was used as the probe for the transposon (30). Briefly, genomic DNA from each of the putative *ssrA* regulatory mutant and the wild type strain was digested with *Eco*RI, separated on a 1% agarose gel, transferred to

nitrocellulose membrane by capillary action, and hybridized with the labeled *tetAB* probe. The blots was incubated at 42°C, washed several times, and the probe was detected using the Invitrogen's BrightStar Biodetect kit (Grand Island, NY) according to the manufacturer's instructions.

Arbitrary PCR and DNA sequencing

The insertion site of each Tn*5*-B30 mutant was mapped via arbitrary PCR as previously described (22). Briefly, the first round of PCR was performed with the primers Tn5Ext and Arb6 at 45°C as annealing temperature to increase random priming. The second round of PCR was performed with the primers Tn5Int and Arb2 at 55°C as annealing temperature to increase the specificity of priming. 1 µl of 100-fold diluted sample from the first round of PCR was used as the template. The primers used for arbitrary PCR and other reactions are listed in Tab.3.2. *Taq* DNA polymerase was used for arbitrary PCR. DNA sequencing was performed by the Auburn University Research Instrumentation Facility.

Primer	Sequences (5’ to 3’)	Reference
Tn5Ext	GAA CGT TAC CAT GTT AGG AGG TC	(22)
Tn5Int	CGG GAA AGG TTC CGT TCA GGA CGC	(22)
Arb2	GGC CAC GCG TCG ACT AGT AC	(22)
Arb6	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC	(22)
SSO-501	CAT GAG CTC CTC CGC AAC GCT GGC AAC AAC ACG	This work
SSO-502	CAT GGT ACC CCC GCG GCG AGA GTA AAG	This work
SSO-509	CAT GAG CTC CGC GCC CGG GAA ATC TGG	This work
SSO-510	CAT GGA TCC GAG GGG GCA TCC AGG GGT CAA G	This work
SSO-512	CAT CCC GGG TGG ATA CGG CAG CCT GTT CGA GCG	This work
SSO-517	CAT GAG CTC AGT CCC GTA GCC GTG TGC	This work
SSO-645	CAT GAG CTC CAG GCC GGC GAT GTG GTG	This work
SSO-646	CAT GCA TGC AGC CCG CCC GCA ACT TCA	This work

Table3.2. PCR primers.

Construction of complementing plasmids

To complement the transposon insertion mutants, a new plasmid vector was constructed from pSU39 (20). Briefly, I replaced the kanamycin resistance gene on pSU39 with a gentamicin resistance cassette by inserting the *aacCI* cassette (28) at *Nco*I and *Bcl*I sites.

To complement the transposon mutations, the wild-type gene or operon corresponding to the mutated gene or operon was PCR amplified from PAO1 using the high fidelity Phusion DNA polymerase (New England Biolabs, Beverly, MA). The following primer pairs were used to amplify specific gene or operon: *PA2729* (*prt3*), SSO-509/SSO-510; *PA1658* (*prt5*), SSO-512/SSO-517; *PA0319* operon (*prt6*), SSO-645/SSO-646; *PA0738* operon (*prt7*), SSO-501/SSO-502. The operons were cloned into the multiple cloning site of pSW053 and the single genes were cloned into pSS213 (35). The mini-stabilizing fragment (mSF) that encodes for a *Pseudomonas* plasmid replication origin (35) was then subcloned into the plasmids at the *Hin*dIII site. The plasmids were introduced into corresponding *P. aeruginosa* Tn*5*-B30 insertion mutants by electroporation as previously described (34) and the tranformants were isolated as gentamicin resistant colonies.

Results

Isolation of tmRNA regulatory mutants via transposon mutagenesis

In order to study regulation of tmRNA in *P. aeruginosa*, I conducted a transposon mutagenesis with Tn*5*-B30 (30) to isolate insertion mutants with altered *ssrA*::*lacZ* phenotype. A plasmid vector carrying the transposon was transferred from *E. coli* to a *P. aeruginosa* strain carrying the *ssrA*::*lacZ* fusion via conjugation and the transconjugants were selected as Tc^r resistant colonies. A schematic of the transposon mutagenesis and the genetic screens to identify putative *ssrA* regulatory mutants are shown in Fig.3.1. Approximately 40,000 Tn*5*-B30 insertion mutants were screened by blue white screening on PIA plates containing carbenicillin and X-gal. Subsequent 1440 colonies that showed altered blue color from the original parent strain were inoculated into 96-well plates and tested for β-galactosidase activity. The microtiter screen initially yielded 177 colonies that had altered β-galactosidase activity. After several rounds of rigorous screening (an average of six independent β-galactosidase assays with duplicates at each time), I narrowed the putative mutants from 117 to 60 (Fig.3.2). The Tn*5*-B30 insertion mutants were designated as *prt* for putative regulator of *t*mRNA.

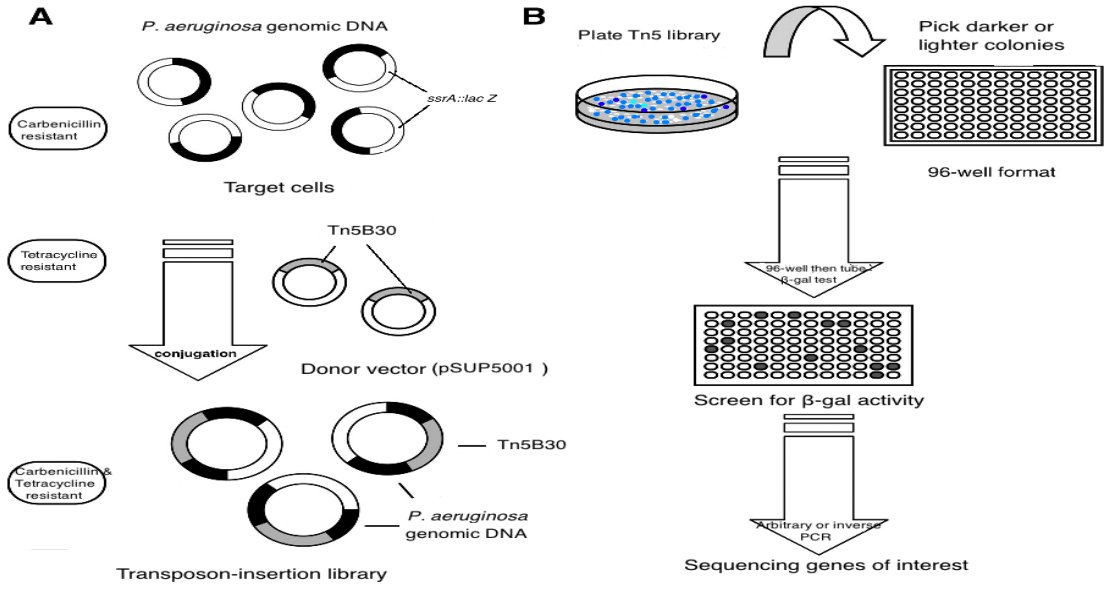

Figure3.1. Tn*5*-B30 mutagenesis and high-throughput screening in *P. aeruginosa.*

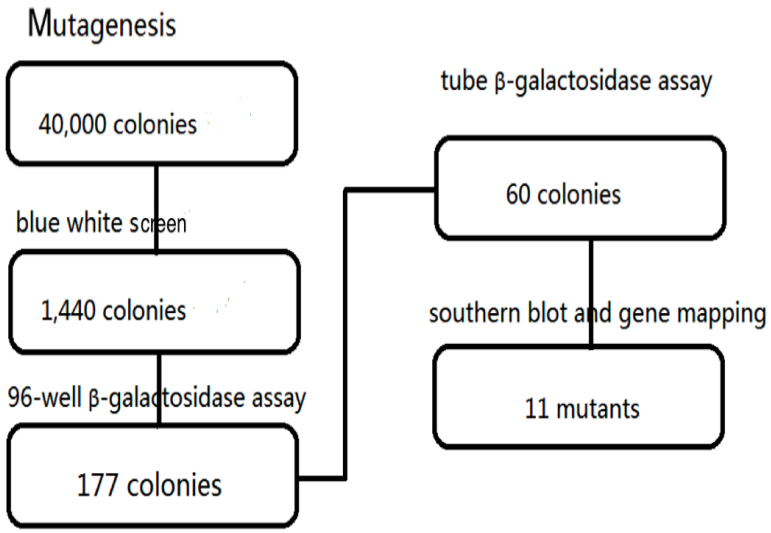

Figure 3.2. Transposon Mutagenesis Summary.

Southern hybridization to identify single transposon insertion mutants

Once the putative *ssrA* regulator mutants were narrowed to 60, I performed southern hybridization experiments to verify that each transposon mutant only had one Tn*5*-B30 insertion. Genomic DNA from all 60 putative mutants and PAO1 digested with *Eco*RI were tested with the *tetAB* gene of Tn*5*-B30 as the probe. The enzyme *Eco*RI was used because it does not cut within *tetAB*. Fig.3.3 shows an example of my southern hybridization results. Lane 1 is the positive control of pSUP::Tn*5*-B30 (30). The mutant *prt10-11* shows two bands indicating two transposon insertions. Of the 60 mutants tested, four mutants consistently showed two bands that hybridized to *tetAB*. These mutants were discarded. The mutant *prt10-12* is an example of those with unclear results. These samples were retested to clarify the results. Those Tn*5*-B30 insertion mutants with a single hybridized band were further characterized.

Genetic mapping of transposon insertion sites

The insertion site of each Tn*5*-B30 mutant was mapped via arbitrary PCR as previously described (22). A schematic of the arbitrary PCR mapping is shown in Fig.3.4. The most prominent DNA fragments from arbitrary PCR were sequenced and compared to the PAO1 genome database (http://www.pseudomonas.com/) to identify the mutated genes. From my analysis of 56 putative mutants, I found 11 genes that were mutated by Tn*5*-B30 insertion. The identities of the mutated genes are shown in Tab.3.3. Two mutants (*prt4* and *prt6*) were mapped to genes encoding putative transcriptional regulators, *PA0319* and *PA5431* respectively. One insertion (*prt2*) was in a gene that encodes for a heat shock protein: ClpB (*PA4542*). Another

insertion (*prt10*) was in a gene that encodes for a malic enzyme, *PA5046*. *PA4542* and *PA5046* are the only genes identified whose functions have been demonstrated. One mutation (*prt5*) was mapped to a putative protein involved in the Type VI secretion system, *PA1658* (*hsiC2*). The other six mutations mapped to genes that encode for hypothetical proteins, *PA4929, PA2729, PA1761, PA0738, PA2721, and PA3693*.

ssrA::lacZ *expression in* prt *mutants*

As illustrated in Fig.3.5, all eleven *prt* mutants affected the *ssrA*::*lacZ* expression by ≥ 40% when the cells were grown at either 30°C, 37°C or both. *prt1*, *prt2* and *prt3* had significantly decreased *ssrA* expression at both 30°C and 37°C. *prt4* and *prt5* decreased the expression only at 30°C. *prt6* and *prt7* affected *ssrA* expression only at 37°C. The last group of *prt8*, *prt9*, *prt10* and *prt11* likely affected repression of *ssrA* at 37°C because these mutants all exhibited overexpression of *ssrA* at that temperature.

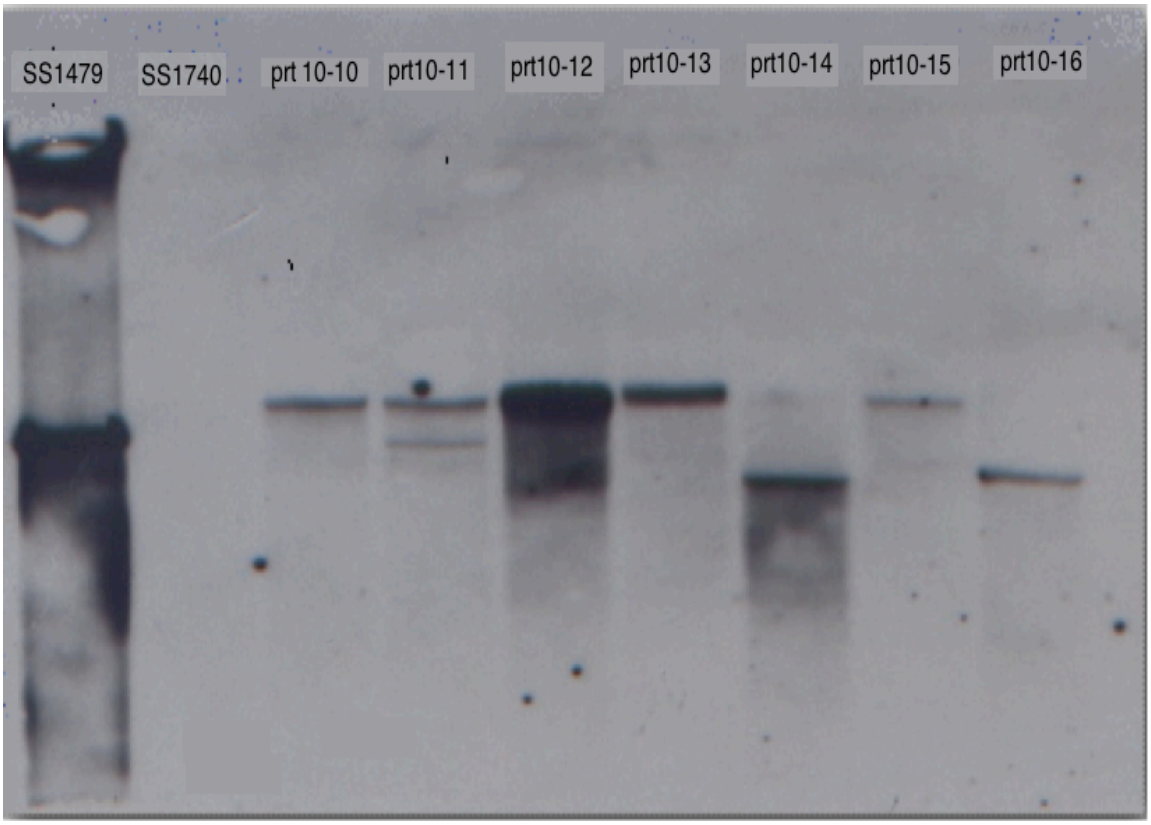

Figure3.3. Southern blot analysis of transposon insertion mutants. Genomic DNA from each of the 60 putative Tn5-B30 insertion mutants and the wildtype (PAO1) were isolated, digested with EcoRI, and run on 0.7% agarose gels. Lane 1 (SS1479) is the positive control of pSUP::Tn5-B30. SS1740 is the PAO1 ssrA::lacZ strain which was used as the negative control. The prt mutant designation for this figure represents the position of the strain in the 96-well microtiter plates.

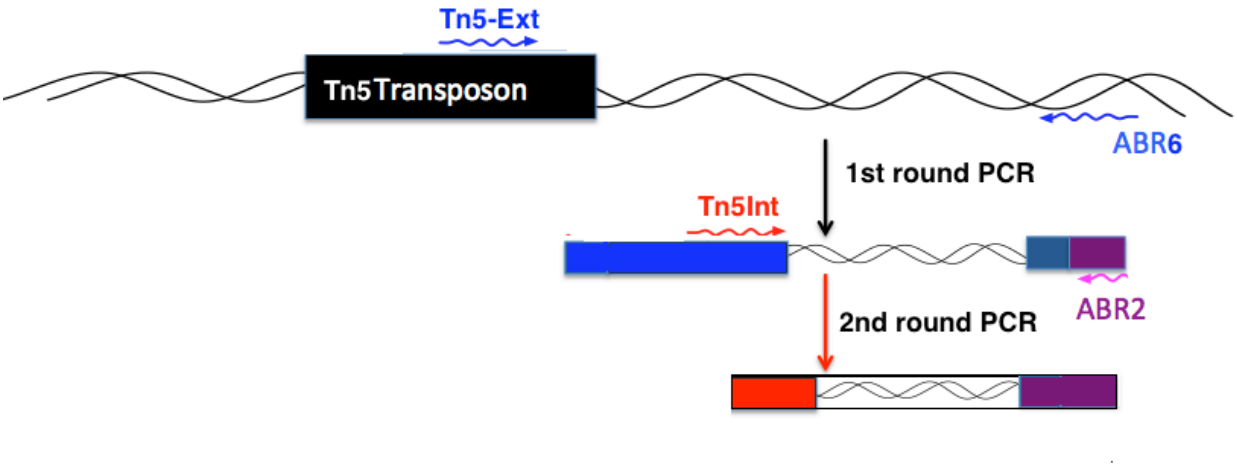

Figure 3.4. Arbitrary PCR

Strain	*ssrA*::*lacZ* expression at 37°C	*ssrA*::*lacZ* expression at 30°C	Gene with Tn insert	Gene Product
prt1	0.2	0.32	*PA4929*	Hypothetical protein
prt2	0.17	0.36	*PA4542 (clpB)*	ClpB protein
prt3	0.18	0.15	*PA2729*	Hypothetical protein
prt4		0.62	*PA5431*	Probable transcriptional regulator
prt5		0.37	*PA1658* (*hsiC2*)	HsiC2 (Type VI export machinery)
prt6	0.22		*PA0319*	Putative transcriptional regulator
prt7	0.6		*PA0738*	Hypothetical protein
prt8	2		*PA1761*	Hypothetical protein
prt9	1.7		*PA2721*	Hypothetical protein
prt10	1.8		*PA5046*	Malic enzyme
prt11	1.6		*PA3693*	Hypothetical protein with homology to OmpA

Table3.3. Summary of 11 *prt* mutants. The crossed cell designates no difference between the mutant and the wildtype in *ssrA* gene expression. Expression of *ssrA*::*lacZ* in the parent strain (SS1740) was set as 1. The genes were identified from the *P. aeruginosa* database **(**http://www.pseudomonas.com/**).**

Complemention of prt *mutants*

To verify the validity of the transposon insertion mutants, I cloned and complemented the mutations with the wildtype copy of the gene or operon. In order to perform complementation experiment, I initially constructed a new plasmid vector that could be stably maintained in *P. aeruginosa* with a selectable marker. Therefore I constructed pSW053 (Fig.3.6), a plasmid vector with the mSF (35) for replication and gentamicin resistance. To date, I have successfully cloned and complemented four out of eleven *prt* mutants. *PA0738* (*prt7*) and *PA0319* (*prt6*) operons were cloned into pSW053 and complemented *in trans*. For *PA2729* (*prt3*) and *PA1658* (*prt5*), I cloned 5' end of the mutated operons and cloned them into pSS213 (35). The mutated operons for *prt3* and *prt5* were 11 kb and 4.5kb, respectively. In lieu of cloning such large operons, I first cloned the affected genes into pSS213 under the control of a regulatable promoter and then subcloned in the mSF into the recombinant plasmids. This resulted in my ability to control expression of the wildtype gene with 0.5 mM IPTG to complement the mutated genes. The results of genetic complementation are shown in Fig.3.7.

To test for complementation, the *prt* mutants and the mutants carrying either the recombinant plasmid or the vector plasmids were grown in LB with appropriate antibiotic selection at indicated temperature with shaking. Fig.3.7 Panel A shows the results from cells grown at 30°C and Panel B shows the results from cells grown at 37°C. When complemented *in trans*, *PA2729* (*prt3*) restored the *ssrA* expression to 68% of the wildtype at 30°C (Fig.3.4.A) and to 78% at 37°C (Fig.3.4.B). *PA1658* (*prt5*) complementation doubled the expression of *ssrA* at 30°C (Fig.3.4.A). *PA0319* (*prt6*) complementation showed similar results but at 37°C (Fig.3.4.B). *PA0738* (*prt7*) complementation increased the *ssrA* expression only by 20% (Fig.3.4.B).

Interestingly, the *prt7* mutant showed a significant negative effect of the vector plasmid on the *ssrA* gene expression. Thus, it is likely that the gentamicin selection to maintain the plasmid may affect the *ssrA* gene expression in *prt7*.

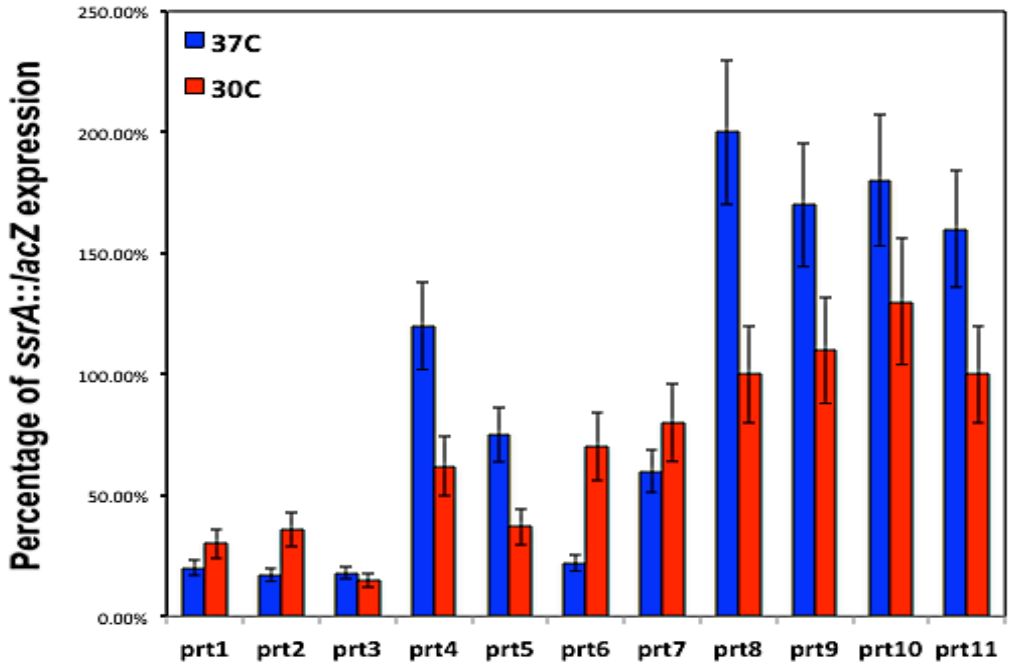

Figure 3.5. *ssrA*::*lacZ* expression in *prt* mutants. *ssrA*::*lacZ* activity was determined from overnight culture of *P. aeruginosa* grown in LB-broth. The expression of mutants grown in 37°C are shown in blue, and the expression in 30°C are shown in red. The expression of the PA01 *ssrA*::*lacZ* control (SS1740) was set at 100%. The data shown are a compilation of six independent experiments performed in duplicate.

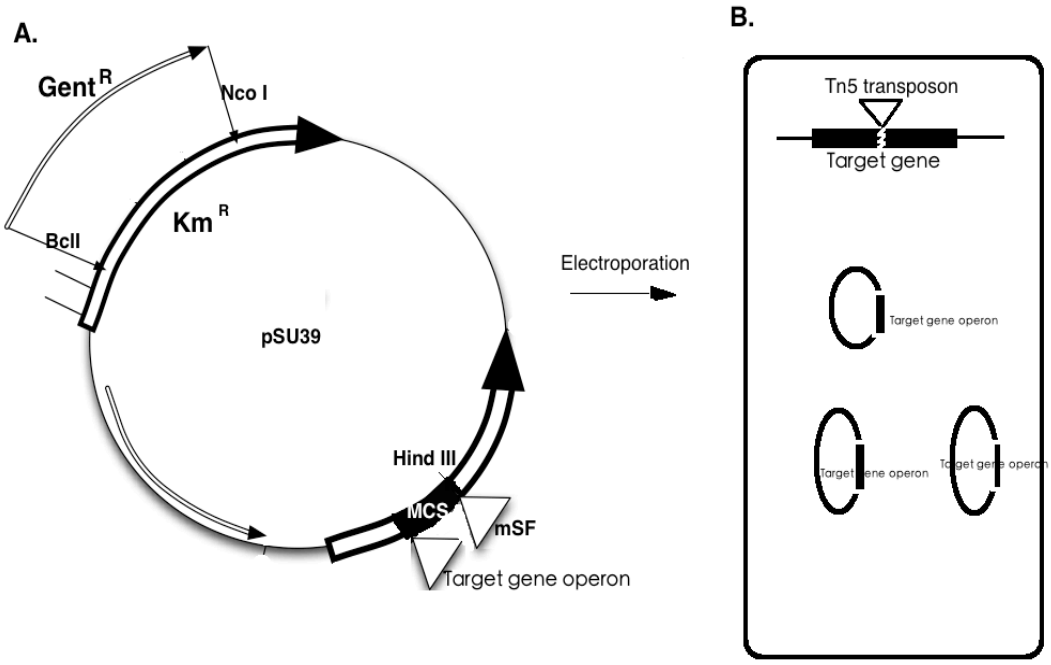

Figure 3.6. Construction of a *prt* mutant complementing plasmid.

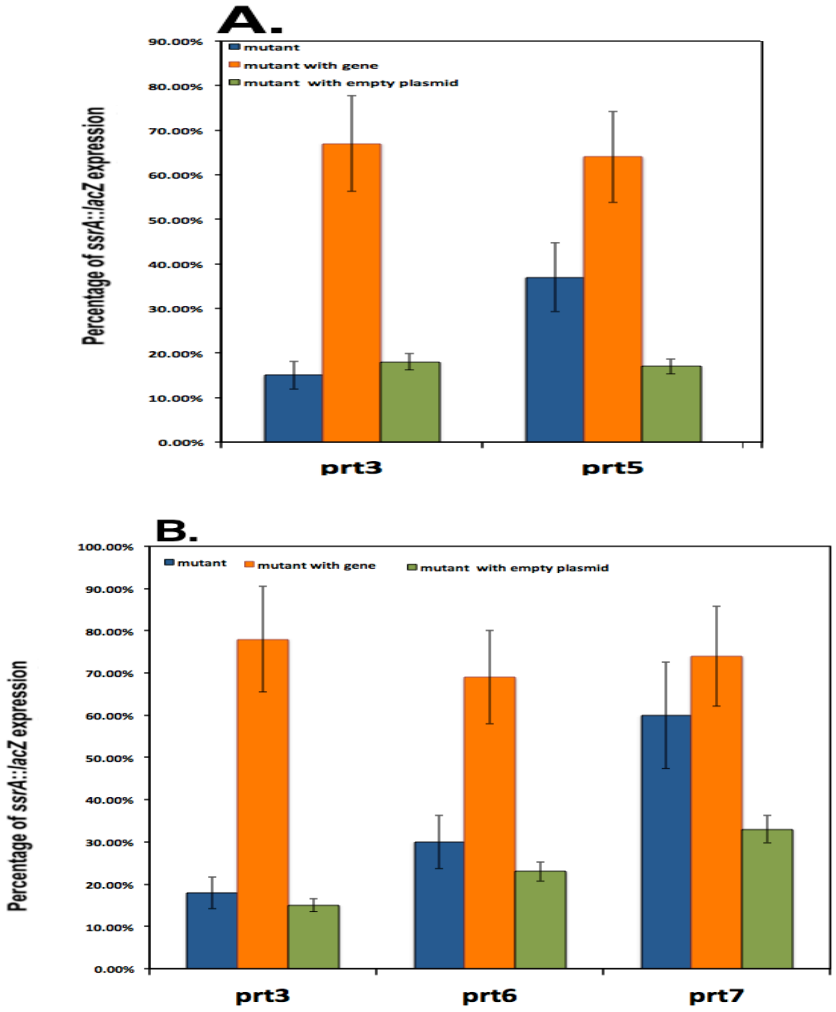

Figure 3.7. Complementation of *prt3, prt5, prt6,* and *prt7.* Panel A, 30°C; Panel B, 37°C. The *ssrA*::*lacZ* activity was assayed from overnight culture of *P. aeruginosa* grown in LB-broth. Transposon mutants are shown in blue, complemented strains are shown in orange, and the mutant strain with vector plasmid control are shown in green. The expression of the PA01 *ssrA*::*lacZ* control (SS1740) was set at 100%. The data shown are a compilation of three independent experiments performed in duplicate.

Discussion

In order to elucidate the genetic regulation of tmRNA biosynthesis, I conducted a transposon insertion mutagenesis to isolate mutant that affected *ssrA* expression. Using a mini-Tn*5* (Tn*5*-B30) (30), I screened approximately 40,000 random transposon insertion mutants and identified a total of eleven putative regulatory mutants for *ssrA* expression. These putative regulatory mutants were designated as *prt* for putative regulator of tmRNA. As demonstrated in Chapter 2, *ssrA* expression is affected by temperature. I observed almost twice the expression of *ssrA* when *P. aeruginosa* was grown at 30°C than at 37°C. Therefore, my mutagenesis was conducted with *P. aeruginosa* grown at 30°C but the mutants were screened for *ssrA*::*lacZ* expression at both 30°C and at 37°C in order to identify putative temperature dependent regulators of *ssrA*. As demonstrated in Fig.3.5, 11 *prt* mutants can be group as decreased *ssrA* expression at both of 30 and 37°C (*prt1*, *prt2* and *prt3*), decreased expression only at 30°C (*prt4* and *prt5*), decreased expression only at 37°C (*prt6* and *prt7*), and increased expression only at 37°C (*prt8*, *prt9*, *prt10* and *prt11*). These data suggest that *prt8, prt9, prt10, and prt11* mutated putative transcriptional repressor that inhibits *ssrA* expression at 37°C. Thus, my mutagenesis data support the observed putative temperature effect on *ssrA* expression.

Of eleven *prt* mutants isolated, only two transposon insertions mapped to known genes. One insertion (*prt2*) was in a gene that encodes for a heat shock protein: ClpB, *PA4542*. Another insertion (prt10) was malic enzyme *PA5046*. Two mutants (*prt4* and *prt6*) were defective in putative transcriptional regulators, *PA5431* and *PA0319* respectively. One mutation (*prt5*) was mapped to a putative protein involved in the Type VI secretion system, *PA1658* (*hsiC2*). The

other six mutations mapped to genes that encode for hypothetical proteins, *PA4929, PA2729, PA1761, PA0738, PA2721, and PA3693*. So far, I successfully cloned and complemented four *prt* mutants (*prt3*, *prt5*, *prt6, prt7*) for *ssrA* expression.

Two putative transcriptional regulators, PA0319 (Prt6) and PA5743 (Prt4), appear to be temperature dependent regulators of *ssrA* expression. PA0319 (Prt6) is a putative transcriptional regulator, which has an SdiA-regulated domain identified by Reversed Position Specific Blast (RPSBLAST). SdiA is an *E. coli* LuxR homologue that can bind to promoter regions of *esp* and *eae* genes *in vitro* (13). PA5743 (Prt4) is a probable transcriptional regulator that contains a DNA-binding helix-turn-helix (HTH) motif and an aminotransferase domain belonging to the MocR family of regulators.

ClpB, a molecular chaperone (2), was found to enhance *ssrA* expression in *P. aeruginosa*. This supports my hypothesis that tmRNA is involved in global stress response in *P. aeruginosa*. All of the *prt* mutants affected *ssrA* gene expression at less than 40%. My data suggest a complex system to control *ssrA* expression instead of a simple mechanism.

This is the first time a comprehensive effort has been made to understand tmRNA expression. Although I screened approximately 40,000 transposon insertion to ensure a complete coverage of the *P. aeruginosa* genome, it is clear that I did not identify every potential regulator of *ssrA* in this bacterium (Chapter 4). However, this is a good start to initiate the elucidation of tmRNA regulation in *P. aeruginosa*. The future study will characterize the mechanisms by which the identified mutants regulate *ssrA* transcription and their effect on *P. aeruginosa* physiology and pathogenesis.

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Chapter 4

Intertactive regulation of tmRNA and RpoS in *Pseudomonas aeruginosa*

Abstract

Transfer messenger RNA (tmRNA), encoded by *ssrA*, has been demonstrated for its role in stress response and pathogenesis in several bacteria. The alternative sigma factor, RpoS, has been characterized in detail in several gram-negative bacteria for its role as the master regulator of the general stress response that protects the organism from multiple stresses. Our efforts to characterize the function and regulation of tmRNA in *Pseudomonas aeruginosa* have suggested a possible overlap between tmRNA and RpoS in stress response and production of virulence factors. I demonstrate in this study that full expression of *ssrA*::*lacZ* requires RpoS in *P. aeruginosa.* Concurrently, the full expression of *rpoS*::*lacZ* requires tmRNA in *P. aeruginosa*. My data suggest that tmRNA regulates *rpoS* expression indirectly via regulating expression of *psrA* that encodes for the major transcriptional activator of *rpoS* in *P. aeruginosa*. tmRNA regulation of *psrA* expression is also indirect because tmRNA controls transcription of *psrA*. It is not yet clear exactly how tmRNA regulates *psrA* transcription. In summary, my data suggest that tmRNA and RpoS are intimately involved in regulating the stress response and pathogenesis of *P. aeruginosa* together.

Introduction

Pseudomonas aeruginosa is a highly successful opportunistic pathogen that can live in a variety environments including marine environment, soil, fresh water, plant and animals (8, 9). This suggests that *P. aeruginosa* is capable of withstanding a number of different environmental challenges. *P. aeruginosa* is a versatile pathogen that can cause various acute infections in skin, cornea, cochlea, and urinary tract (5, 7, 14, 22). In addition, *P. aeruginosa* is the major agent that causes chronic infections in the lungs of cystic fibrosis patients (4). The extra large size of genome (6.3 million bp) that devotes a large number of genes devoted to the regulators of gene expression makes this organism able to regulate stress environments in a timely and effective fashion (26).

tmRNA, encoded by *ssrA*, is a small stable RNA that functions to recycle stalled ribosomes and amino acids via *trans*-translation (18). When ribosomes stall due to lack of charged tRNA, damaged mRNA, or other interventions such as runaway transcript lacking termination codon, tmRNA aminoacylated at the tRNA-like domain (TLD) with alanine enters the ribosome with the assistance of SmpB protein. This action promotes translocation of the ribosome to the mRNA-like domain (MLD) of the tmRNA to translate the degradation tag (ANDENYALAA in *Escherichia coli*) for turnover of the incomplete polypeptide (17, 18, 29). In several bacteria, tmRNA has been implicated with stress response (32, 33).

RpoS is an alternative sigma factor that mediates the general stress response and facilitates adaptation to various environmental stresses in several gram-negative bacteria (16, 31).

The general stress response is induced when a cell experiences nutrient deprivation or other stresses and confers a broad resistance to numerous stresses (2, 15). In *P. aeruginosa*, RpoS mediates the general stress response and protects the bacterium from oxidative stress, osmotic stress, heat-shock, and prolonged starvation when it is grown on glucose as the sole carbon source (27). In addition, RpoS is required for optimal production of virulence factors including exotoxin A, elastase, LasA Protease, rhamnolipids, pyocyanin, and pyoverdine (27). Our previous characterization of a *ΔssrA* mutant on virulence factor production demonstrated that tmRNA had a similar effect on optimal production of elastase, LasA protease, and pyocyanin. These data suggested a possible relationship between tmRNA and RpoS. In *E. coli*, *rpoS* regulation is complex because it is regulated by multiple factors at transcription, posttranscription, translation, and posttranslation levels (3, 10, 11). In *E. coli*, full translation of RpoS requires *trans*-translation mediated by tmRNA (24).

In *P.aeruginosa, rpoS* is regulated mostly at the transcriptional level by PsrA, a TetR family of a transcriptional regulator (19, 30). PsrA negatively regulates its own expression when cells are growing exponentially but this inhibition is released in stationary phase (20) and results in the induction of *rpoS* expression. Because our data suggest a potential overlap in the tmRNA and RpoS function, I addressed whether these two regulatory molecules affect each other's expression in *P. aeruginosa*.

Materials and Methods

Bacterial strains, plasmids, chemicals, and media

Bacterial strains and plasmids used in this study are listed in Table 3.1. Unless otherwise indicated, bacteria were cultured in Luria-Bertani broth (LB broth) or on LB-agar supplemented with appropriate antibiotics. LB broth, LB premixed Agar and Pseudomonas Isolation Agar (PIA) were purchased from Fisher Scientific (Rockford, IL). Antibiotics and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: ampicillin (Amp) 100 μg/ml for *E. coli*; carbenicillin (Carb) 100 μg/ml for *P. aeruginosa;* gentamicin (Gm) 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; tetracycline (Tet) 20 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa;* IPTG at 0.5 mM for *P. aeruginosa.* A 1:1 mixture of LB-agar and PIA was used to select for *P. aeruginosa* transconjugants and to counter select for *E. coli* following tri-parental mating as previously described (27). Restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, and *Phusion* DNA polymerase were purchased from New England Biolabs (Beverly, MA).

DNA manipulations, transformations, and conjugations

For DNA cloning, *E. coli* strain DH10B was routinely used as the host strain. DNA fragments were introduced into *E. coli* by electroporation and *P. aeruginosa* by conjugation as previously described (27). 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 (Beverly,

MA). Either *Phusion* or *Taq* polymerase from New England Biolabs was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Strain constructions

P. aeruginosa strains with the integrated reporter fusion for study of gene expression were constructed by introducing the *gene of interest* (*goi*)::*lacZ* carried on a suicide plasmid to *P. aeruginosa* via tri-parental mating and selecting for the transconjugants that have acquired the fusion via homologous recombination as previously described (28). Briefly, for regulation of *rpoS*, pSS235 (*rpoS*::*lacZ1*) and pSS236 (*rpoS*::*lacZ2*), and for *psrA*, pSM14 (*psrA*::*lacZ1*) and pSM15 (*psrA*::*lacZ2*) were conjugated into PAO1 and SS1372. *lacZ1* denote transcriptional fusion and *lacZ2* denote translational fusion. The transconjugants were selected as carbenicillin resistant colonies.

ssrA *complementation*

To complement the *ΔssrA* mutant, the wild-type operon with its native promoter (*PA0827-ssrA*) was PCR amplified from PAO1 with two primers SSO-618 (5'-CAT GAG CTC CCT CCA GCT TGC CGG CGA AAT ACT-3') and SSO-642 (5'- CAT TCT AGA CAT AGC AAG GCG AGC CGG CAA CAG GAA-3') with the Phusion polymerase to avoid random mistakes. The underlined residues denote a unique *Sac*I site on SSO-618 and a unique *Xba*I site on SSO-642. The

resulting DNA fragment was cloned into the MCS of the suicide vector pSS213 (28) as a *Sac*I/*Xba*I fragment to yield pSW105. The resulting suicide plasmid pSW105 was electroporated into *P. aeruginosa ΔssrA* null strains and the the derivatives that have acquired the integrated plasmid were selected as carbenicillin resistant cells. Plasmid integration event was verified by PCR analysis.

Cell free extract preparation

One ml *P. aeruginosa* cells in stationary phase were harvested by centrifugation at 13,000 rpm in an Eppendorf microcentrifuge. The cells were resuspended in 100 μl of cell lysis buffer (1) and boiled for 10 minutes. DNA in the cell free extract (CFE) was sheared with 30 seconds of sonication with the Model 100 Sonic membrane dismembrator fitted with a microtip (Fisher Scientific). CFE was centrifuged for ten minutes to discard unbroken cells and other precipitates.

Biochemical assays

Protein concentration was determined by the Bradford method using the Bio-Rad reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Approximately 30 μg of proteins were loaded per lane and analyzed on a Bio-Rad miniprotean III.

Immunodetection of PsrA via western.

The proteins separated on a SDS-PAGE were transferred to nitrocellulose membrane (GE Healthcare, Pittsburgh, PA) by electrotransfer on a Bio-Rad miniprotean III. Immunoblotting

was performed as previously described (1). For blocking the membrane, 1% skim milk was used. PsrA was bound with the primary PsrA antibody from rabbit (20) and then probed with the secondary anti-rabbit antibody from mouse that was conjugated with horse radish peroxidase (HRP) (Sigma Aldrich). The membrane was treated with 1 ml of chemiluminescent HRP substrates (PerkinElmer, Downers Grove, IL) and visualized using the ImageQuant 4000 (GE Healthcare).

Strains	Genotype and relevant characteristics	Source
E. coli		
DH10B	F⁻ *mcrA Δ*(*mrr-hsdRMS-mcrBC*) *Φ80dlacZDM15 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
SS20[×]	HB101/pRK2013	(6)
SS208[#]	HB101 / pLAFR3	(25)
SS622	DH10B P*rpoS*::*lacZ2*	Lab Collection
SS1708	DH10B P*psrA*::*lacZ1*	Lab Collection
SS1709	DH10B P*psrA*::*lacZ2*	Lab Collection
P. aeruginosa		
PAO1	Prototroph wound isolate	(13)
SS24	PAO1 *ΔrpoS101*::*aacC1*	(27)
SS638	PAO1 P*rpoS*::*lacZ1*	Lab Collection
SS710	PAO1 P*rpoS*::*lacZ2*	Lab Collection
SS761	PAO1 P*vfr*::*lacZ1*	Lab Collection
SS948	PAO1 P*algT*::*lacZ1*	Lab Collection
SS1372	PAO1 *ΔssrA*	Lab Collection
SS1711	PAO1 P*psrA*::*lacZ1*	Lab Collection
SS1712	PAO1 P*psrA*::*lacZ2*	Lab Collection
SS1740	PAO1 P*ssrA*::*lacZ1*	Lab Collection
SS1742	PAO1 *ΔrpoS101*::*aacC1* P*ssrA*::*lacZ1*	Lab Collection
SS1746	PAO1 *ΔrpoS101*::*aacC1* P*ssrA*::*lacZ1*/pSS116	Lab Collection

SS1736	PAO1 *ΔssrA* P*rpoS*::*lacZ2*	Lab Collection
SS1744	PAO1 *ΔrpoS101*::*aacC1* P*ssrA*::*lacZ1*/pLAFR3	Lab Collection
SS 2110*	PAO1 *psrA*::Tn*5*-IS*phoA*/*hah*	*P. aeruginosa* genetic stock center
SS2281	PAO1 *ΔssrA* P*vfr*::*lacZ1*	Lab Collection
SS2283	PAO1 *ΔssrA* P*algT*::*lacZ1*	Lab Collection
SS2311	PAO1 *ΔssrA* P*rpoS*::*lacZ1*	This work
SS2573	PAO1 *ΔssrA* P*psrA*::*lacZ1*	This work
SS2603	PAO1 *ΔssrA* P*psrA*::*lacZ2*	This work
SS2633	PAO1 *ΔssrA* P*rpoS*::*lacZ1*/*ssrA*$^+$	This work
SS2635	PAO1 *ΔssrA* P*rpoS*::*lacZ2*/*ssrA*$^+$	This work
SS2639	PAO1 *ΔssrA* P*psrA*::*lacZ2*/*ssrA*$^+$	This work
SS2641	PAO1 *ΔssrA* P*psrA*::*lacZ1*/*ssrA*$^+$	This work
Plasmids		
pLAFR3	Broad host range vector plasmid, Tc^r	(25)
pRK2013	Tra1 (RK2), *ColE1*; Km^r	(6)
pSM14	P*psrA*::*lacZ1*	Lab Collection
pSM15	P*psrA*::*lacZ2*	Lab Collection
pSS116	pLAFR3::*nlpD-rpoS*	Lab Collection
pSS213	$P_{T7(A1/04/03)}$, *aacCI* (Gent^r)	(28)
pSS235	P*rpoS*::*lacZ1*	Lab Collection
pSS236	P*rpoS*::*lacZ2*	Lab Collection
pSW103	$P_{T7(A1/04/03)}$::*PA0827-ssrA, aacCI* (Gent^r)	This work

Table.4.1. Bacterial strains and plasmids. #: Suh laboratory designation for the strain published by Staskawicz (25); *: Suh laboratory designation for the strain purchased from P.aeruginosa stock center. ×: Suh laboratory designation for the strain published by Figurski and Helinski (6).

Results

A potential regulatory circuit between RpoS and tmRNA

Our previous data suggested a potential overlap between tmRNA and RpoS in virulence factor production in *P. aeruginosa* (Tucker and Suh, unpublished data) (Tab.4.2). I tested a potential regulatory relationship between tmRNA and RpoS. The expression studies were conducted with reporter fusions integrated into the genome of appropriate strains. As demonstrated in Fig.4.1, *ssrA*::*lacZ1* required RpoS for full expression. In the *ΔrpoS101*::*aacCI* mutant, *ssrA*::*lacZ* expression was decreased by almost 60%. When the *ΔrpoS101*::*aacCI* mutant was complemented with pSS116, the *ssrA*::*lacZ1* gene expression was partially restored. This restoration of *ssrA*::*lacZ1* expression was not observed with the vector plasmid pLAFR3.

I also tested a potential effect of tmRNA on *rpoS* expression in *P. aeruginosa*. In *E. coli*, tmRNA affects full translation of RpoS by *trans*-translation (24). I determined the potential effect of tmRNA on *rpoS*::*lacZ1* and *rpoS*::*lacZ2* expression to test for potential transcriptional and/or translational effect, respectively, in *P. aeruginosa*. The data demonstrate that tmRNA affects *rpoS* expression at all growth phases (Fig.4.2). However, unlike in *E. coli*, tmRNA affected *rpoS* expression at the level of transcription. The tmRNA effect on *rpoS*::*lacZ2* is likely due to its effect on *rpoS* transcription rather than a direct effect on the translation of *rpoS* mRNA. Complementation of the *ΔssrA* mutation with a copy of the wildtype gene restored the *rpoS*::*lacZ1* and *rpoS*::*lacZ2* expression.

One possibility for the observed phenotype of tmRNA's effect on *rpoS*::*lacZ* expression is its effect on translation of the reporter (LacZ) protein. In order to eliminate that as a possibility, I tested the potential effect of tmRNA on the expression of *algT*::*lacZ1* and *vfr*::*lacZ1* fusions (Fig.4.3). As clearly demonstrated by a significant increase in the *vfr*::*lacZ1* fusion in the *ΔssrA* mutant, it is highly unlikely that tmRNA affects translation of LacZ. Thus, my data suggest a putative regulatory interaction between tmRNA and RpoS in *P. aeruginosa*.

During the regulation studies, we discovered that although tmRNA is not essential for *P. aeruginosa* viability, overproduction of tmRNA is detrimental for the bacterium. Although we tried plasmid vectors with varying copy numbers from 5-8 copies to 20-30 copies per cell, we were unable to introduce *ssrA* to *P. aeruginosa* on multicopy plasmids. Thus, all of my *ssrA* complementation studies shown in Fig.4.2 were conducted by integrating a wildtype copy of the *PA0827-ssrA* operon into the *P. aeruginosa* genome via homologous recombination. The schematic of *ΔssrA* complementation is shown in Fig.4.4.

	Elastase	**LasA protease**	**Pyoverdine**	**Pyocyanin**	**Exotoxin A**
RpoS	Yes	Yes	Yes	Yes	Yes
tmRNA	Yes	Yes	Yes	Yes	N.D.

Table 4.2. Effect of *rpoS* /ΔssrA mutation on *P. aeruginosa* virulence factors. (Tucker and Suh, unpublished data)

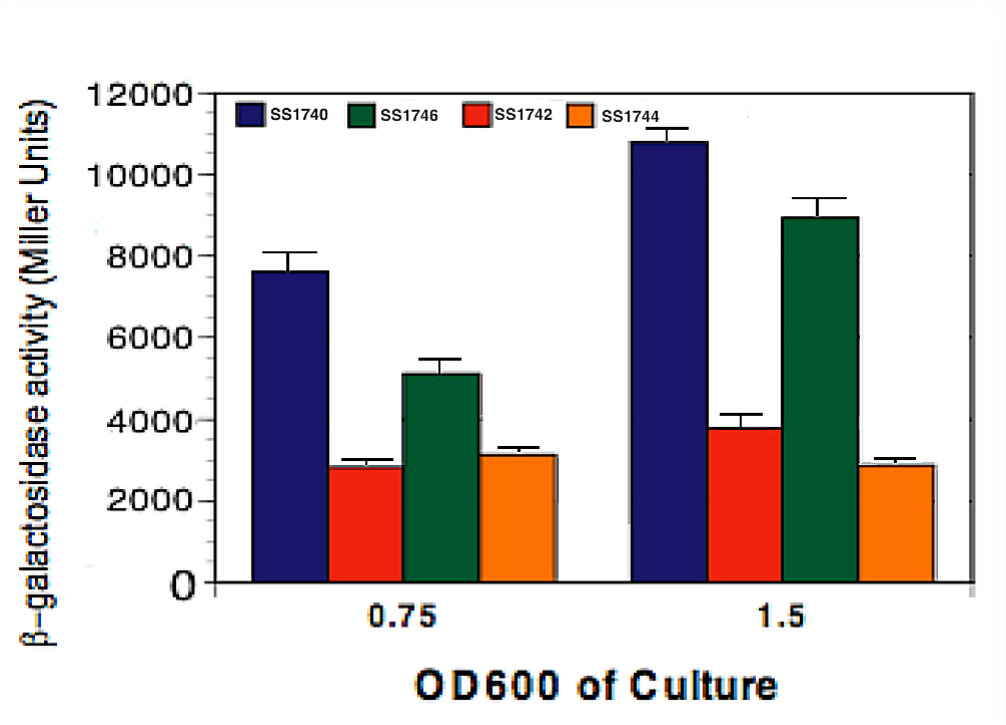

Figure 4.1. Effect of *rpoS* mutation on *ssrA*::*lacZ1* activity. *P. aeruginosa* was grown initially overnight in LB. The overnight culture was diluted 1:100 in fresh LB and grown at 37°C with aeration. Cell growth was monitored and cells were harvested at indicated OD600 and *ssrA*::*lacZ* expression was determined by β - galactosidase assay (23). The data shown are a representative of three independent experiments performed in duplicate.

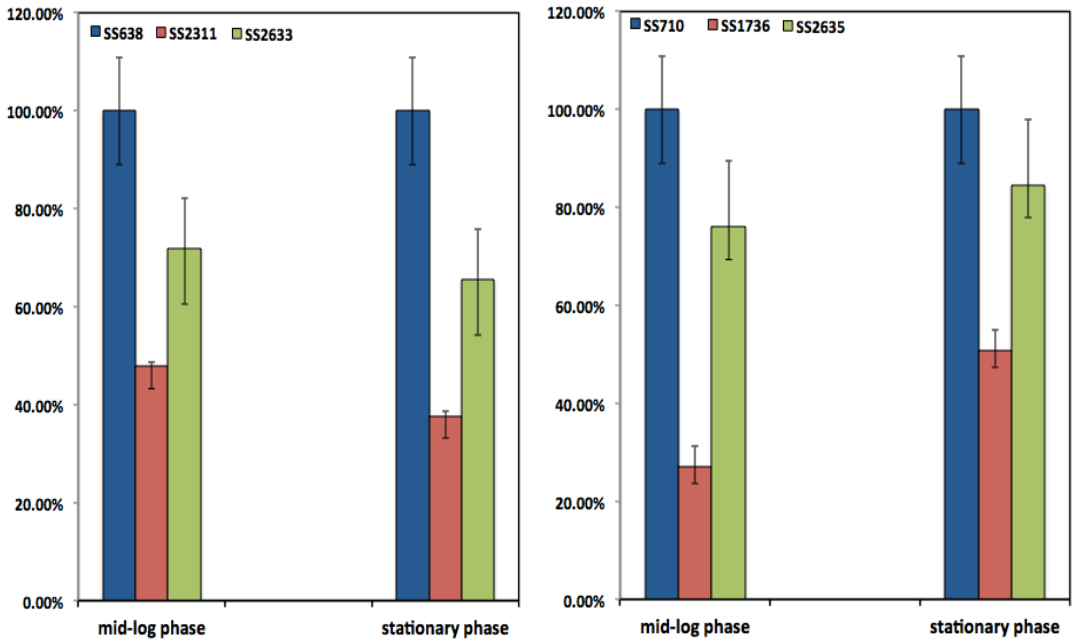

Figure 4.2. Effect of *ssrA* mutation on *rpoS*::*lacZ1* and *rpoS*::*lacZ2* expression. Mid-log, OD_{600} of 1.2-1.4; stationary, OD_{600} of 2.2-2.5. A: *rpoS*::*lacZ1* transcriptional fusion. B: *rpoS*::*lacZ2* translational fusion. *P. aeruginosa* was grown in LB at 37°C until the desired cell density and the gene expression was determined by β - galactosidase assay (23). The expression of the wild type was set as 100%. The data represent the average of at least of three independent experiments.

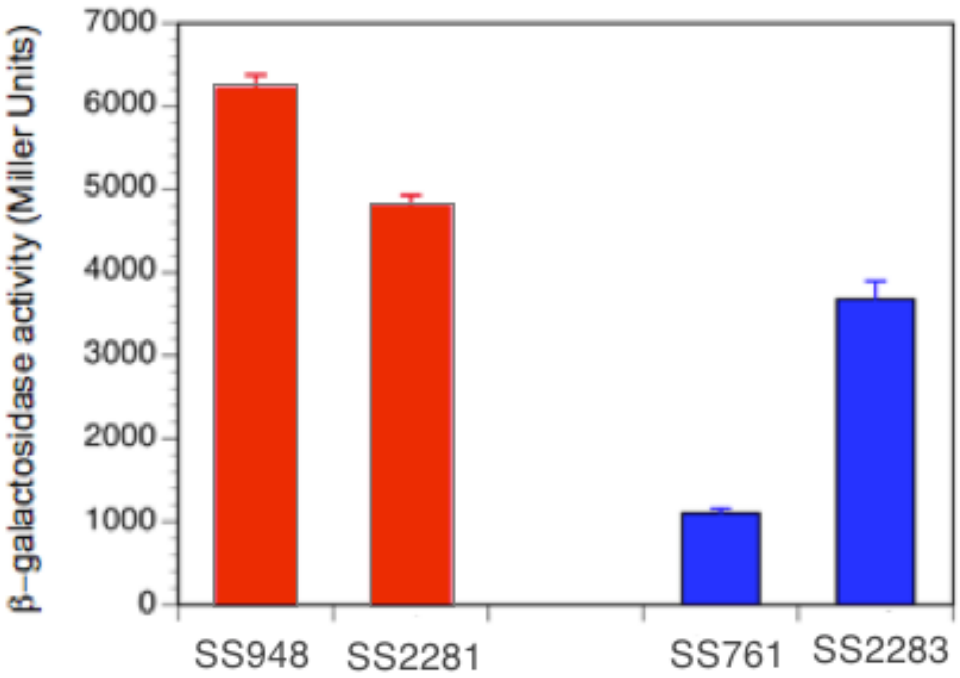

Figure 4.3. Effect of *ssrA* mutation on *algT*::*lacZ1* and *vfr*::*lacZ1*expression. *P. aeruginosa* was grown at 37°C with aeration. Three hours after the culture has entered the stationary phase of growth, the cells were collected and the gene expression was determined by β-galactosidase assay as described by Miller (23) in duplicate samples. The data shown are a representative of three independent experiments.

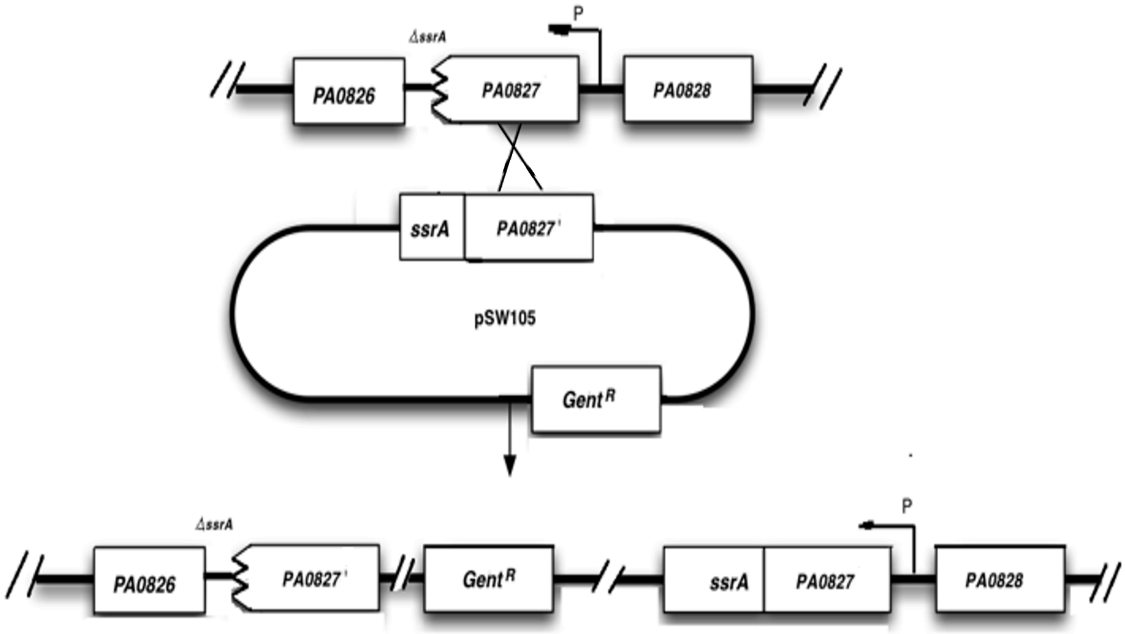

Figure 4.4. *ΔssrA* complementation by integration of the wildtype *PA0827-ssrA* operon in the genome by homologous recombination.

Effect of tmRNA on psrA *expression*

PsrA is a member of the TetR family of transcriptional regulators that had been identified as the major activator of *rpoS* transcription in *P. aeruginosa* (16). Therefore, tmRNA may activate the *rpoS* transcription indirectly by regulating the expression of *psrA*. I determined whether tmRNA affects the expression of *psrA*::*lacZ1* and *psrA*::*lacZ2* fusions. The data are shown in Fig.4.5. I determined that tmRNA affects *psrA* expression in a very similar fashion to the way it does *rpos* expression. My data suggest that tmRNA affects transcription of *psrA* (Fig.4.5.A). When the *ΔssrA* is complemented with integration of a wildtype copy of the *PA0827-ssrA* operon, *psrA* expression was partially restored.

Effect of tmRNA on PsrA production

The reporter fusion data on the effect of tmRNA on *psrA* expression were verified by immunohybridization. The western blot analysis was performed with approximately 30 μg of total cellular protein per lane prepared from PAO1, SS1372 (*ssrA*$^-$), and SS2110 (*psrA*$^-$). The anti-PsrA antibody was a generous gift from Professor Venturi of the International Centre for Genetic Engineering and Biotechnology, Triste, Italy. The relative quantity of PsrA in each cell free extract was measured with ImageQuant (GE Healthsciences, Pittsburgh, PA). As demonstrated in Fig.4.6, the *ΔssrA* mutant had approximately 40% less PsrA than did the PAO1. These data corresponded closely with the gene expression data acquired with reporter fusions.

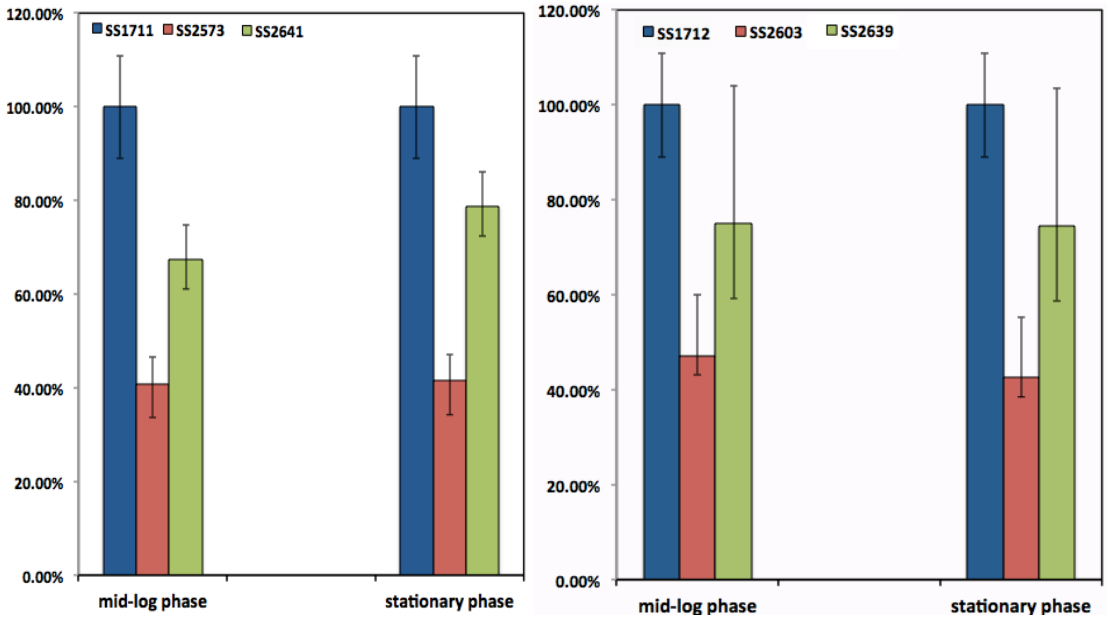

Figure.4.5. Effect of *ΔssrA* mutation on *psrA* expression in *P. aeruginosa*. **A**: *psrA*::*lacZ1* transcriptional fusion. B: *psrA*::*lacZ2* translational fusion. *P. aeruginosa* was grown at 37°C with aeration. Three hours after the culture has entered the stationary phase, the cells were collected and *ssrA*::*lacZ* expression was determined by β-galactosidase assay as described by Miller (23). The expression of the wild type was set to be 100%. The data represent the average (standard deviations) of three independent experiments.

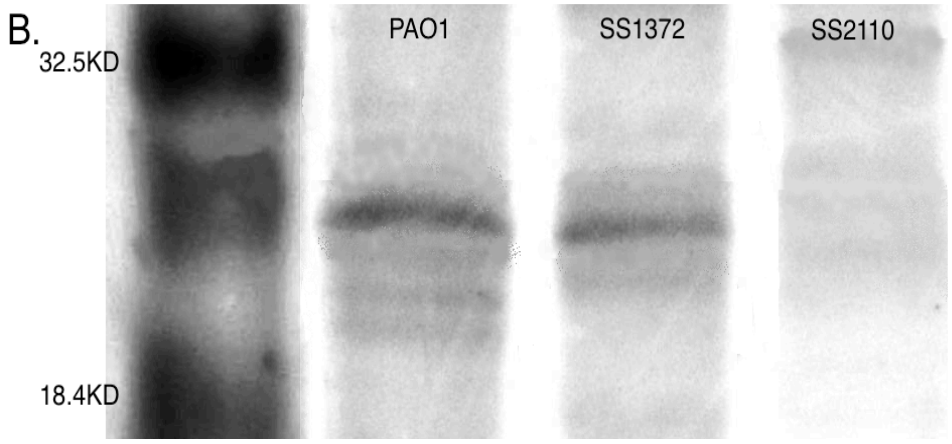

Figure 4.6. Immunohybridization of PsrA. Approximately 30 μg of total cellular protein was probed with anti-PsrA antibody, followed by secondary antibody conjugated with horse radish peroxidase. The image was acquired with the ImageQuant. The data shown are a representative of two independent experiments.

Discussion

Our preliminary data suggested a potential overlap between tmRNA and RpoS in *P. aeruginosa* for stress response and virulence factor productions (Chapters 2 and 3). In this study, I determined that this apparent overlap in phenotype is due to a complex regulatory mechanism in which tmRNA and RpoS regulate each other's expression in *P. aeruginosa*. Unlike in *E. coli* in which the RpoS translation requires the tmRNA mediated *trans*-translation (24), my data suggest that tmRNA is required for transcription of *rpoS* in *P. aeruginosa*. Translation of RpoS was also decreased in the *ΔssrA* mutant. However, it is unclear whether this decrease in translation was due to a direct effect of *trans*-translation on RpoS or a secondary effect of decreased mRNA. Addressing this issue will require a future experiment in which *rpoS* is expressed independently of tmRNA from a regulatable promoter and looking at the translation of the RpoS peptide.

The effect of tmRNA on *rpoS* transcription was demonstrated to be indirect because tmRNA was required for transcription of *psrA*. PsrA is the major transcriptional activator of *rpoS* in *P. aeruginosa* (21). Using a transcriptional and a translational fusions to *psrA*, I showed that transcription of *psrA* was severely decreased in the *ΔssrA* mutant. Therefore, there is yet another layer of complexity in tmRNA regulation of the *rpoS* expression in *P. aeruginosa*. This is in stark contrast to the mechanism of tmRNA being required for full translation of RpoS via *trans*-translation in *E. coli*.

The complex regulatory circuit is completed by RpoS being required for the full

transcription of *ssrA*. The promoter of the *PA0827-ssrA* operon does not appear to contain a putative RpoS recognition site that had been deduced for *E. coli* RpoS promoters (10, 12). However, because the RpoS promoters have not yet been characterized in detail in *P. aeruginosa*, it is unclear whether RpoS affects *ssrA* transcription directly or indirectly.

It is yet unclear why my transposon mutagenesis did not identify RpoS as a regulator of *ssrA* (Chapter 3). I screened approximately 40,000 transposon insertion mutants to make certain that I covered the entire genome at least three to four times. One possibility is that Tn*5* transposition in *P. aeruginosa* is not as random as assumed. This hypothesis appears to be supported by isolation of many sibling mutants in my mutagenesis.

In this study, I discovered a very exciting, complex, and circuitous interactive regulatory pathway between tmRNA and RpoS in *P. aeruginosa*. This pathway is highly complex and includes an indirect effect of tmRNA on the production of RpoS and PsrA as well as the regulation of tmRNA production by RpoS. It still remains to be seen how other putative regulators of *rpoS*/RpoS are tied into the tmRNA mediated pathway. What is clear that understanding the molecular mechanisms of bacterial stress response is even more complex than previously imagined.

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Chapter 5

Conclusion and Future Directions

Conclusion

With increasing emergence of antibiotic resistant pathogens, it is imperative to understand more about pathogens' physiology and stress response way to develop better approaches to combat them more effectively. *Pseudomonas aeruginosa* is a good model system for studying mechanisms of bacterial stress response because this bacterium can live in a variety of environments and it is an important opportunistic pathogen (4). The overall goal of our research is to utilize *P. aeruginosa* as a model system to identify global bacterial persistent strategies. The improved effectiveness and development of novel therapies used to treat such bacterial infections.

Initiate characterization of the tmRNA function in P. aeruginosa

The major goal of this study was to initiate characterization of the tmRNA function and regulation in *P. aeruginosa*. Our previous characterization of the *ssrA* deletion mutant indicated that tmRNA was not essential for the viability of *P. aeruginosa*. Under standard laborator growth conditions in rich medium (L-broth) and at 37°C/30°C, the *ΔssrA* mutant did not show a growth

defect, until it faced double stress of low temperature (30°C) and high osmotic (0.8 M NaCl) growth condition (Fig.2.6). In this case, the *ΔssrA* mutant exhibited an extended lag phase. This implied that tmRNA is involved in stress response. Such data had been previously acquired by Alex Tucker in Dr. Sang-Jin Suh's laboratory when he demonstrated that the *ΔssrA* mutant was hypersensitive to heat-shock and osmotic stress when the cells were grown at 37°C (Tucker and Suh, unpublished data). In this study, I demonstrated that *ssrA* gene expression was almost doubled when *P. aeruginosa* was grown at 30°C than at 37°C (Fig.2.10). The *ssrA* expression was even lower when the bacterium was grown at 42°C. These are the first data to demonstrate temperature regulation of tmRNA expression in any bacteria.

Identification of putative regulators of ssrA *expression in* P. aeruginosa

I conducted a transposon insertion mutagenesis to isolate putative regulatory mutants of *ssrA* expression in *P. aeruginosa*. After screening approximately 40,000 transposon insertion mutants, I identified a total of eleven mutants that affected *ssrA* expression. These mutants were designated as *prt* for putative regulator of tmRNA. Many of my *prt* mutants demonstrated temperature dependent regulation of *ssrA* to confirm my previous data. Of the eleven genes, only two have been characterized for their function (*prt2, PA4542* encoding ClpB; and *prt10, PA5046* encoding a malic enzyme). Most interesting genes identified were *prt4* (*PA5431*) and *prt6* (PA0319) both which encode for putative transcriptional regulators. Unfortunately, neither gene product's function has been demonstrated. One gene had (*PA1658, hsiC2*) homology to a protein participating in the Type VI secretion system. The other six insertion mutations all mapped to open reading frames that encode for hypothetical protein with no known function.

Discovery of a regulatory circuit between tmRNA and RpoS

As demonstrated in Table 4.2, there was an overlap between tmRNA and RpoS in the optimal production of several virulence factors in *P. aeruginosa*. Additionally, they both responded similarly to several stress response tests (data not shown). These data suggested a potential overlap in their physiological function. This was enhanced by the discovery that in *Escherichia coli*, tmRNA is required for full translation of RpoS peptide (7). Thus, I assayed a potential role of tmRNA on the regulation of RpoS in *P. aeruginosa*. My data demonstrated that tmRNA affects *rpoS*/RpoS expression at the transcriptional level as well as potentially at the translational level (Fig.4.2). These data clearly demonstrated that tmRNA's affect on *rpoS* expression in *P. aeruginosa* is different than in *E. coli*. In *P. aeruginosa*, the major regulation of *rpoS* is done at the transcriptional level by PsrA that binds to *rpoS* promoter to activate gene expression (5, 6). Thus, I tested and discovered that tmRNA affects *psrA* expression. Thus, tmRNA's effect on *rpoS* regulation appears to be indirect. In addition, because tmRNA affected *psrA* transcription, this also appears to be indirect since *trans*-translation is involved in translation and not transcription.

Future Directions

Identify back up systems for trans-*translation mechanism*

It was somewhat disconcerting that tmRNA, which had been described for various physiological functions including cell viability, heat-shock, and pathogenesis in other bacteria, appeared to have such subtle effect in *P. aeruginosa*. This implied either tmRNA was really not important or that there is a backup system that compensates for lack of tmRNA in *P. aeruginosa*. Recently, such a backup system was identified in *E. coli* and has been designated as Arf (1, 2).

ArfA can rescue ribosomes when tmRNA is lacking in *E. coli*. Attempts to construct a double mutant of *ssrA* and *arfA* were unsuccessful in *E. coli* (3). Through a database search, I identified a potential homologue of *arfA* in *P. aeruginosa*. It remains to be elucidated whether this putative *P. aeruginosa arfA* homologue suppresses tmRNA defect in the bacterium and whether *trans*-translation is essential in *P. aeruginosa* as it is in several other bacteria.

Characterization of prt *mutants*

Clearly, all eleven of the *prt* mutants should be characterized to determine their function in regulating *ssrA*. This characterization should be done to define both overall physiology of the bacterium with a focus on the temperature dependent regulation of *ssrA*. Most of *P. aeruginosa* studies have focused on its role as an opportunistic pathogen of humans and therefore almost all of the studies have been carried out at 37°C. However, as demonstrated by my data on *ssrA* regulation, it is apparent that we need to better understand temperature dependent gene expression in *P. aeruginosa*, especially since this bacterium survives and persists out in nature in ambient temperature of its environmental niche. Such an understanding may suggest better approaches to minimize *P. aeruginosa* contamination in hospital settings to prevent nosocomial infection by this important pathogen. Of the eleven genes identified from my mutagenesis, the most two intriguing are the putative transcriptional regulators. Thus far, no transcriptional regulators of *ssrA* have been identified in any organism. Thus, it would be high priority characterize these putative regulators for their role in *ssrA* expression. It is also very interesting that they affect *ssrA* expression at different temperatures. PA5431 affects *ssrA* expression at 30°C while PA0319 affects it at 37°C. The most direct approach is to purify the gene products from *PA5431* and *PA0319* and demonstrate their binding to the promoter of *PA0827-ssrA* operon. It is also imperative to map the start site of *ssrA* transcription at 30°C versus 37°C to determine whether

two different promoters used or simply the presence of different transcriptional activator determine the temperature dependent expression. Elucidating functions of the six identified genes that encode for hypothetical proteins will be much more difficult. It is important to approach this from genetic, biochemical, and physiology perspectives. Each mutant should be studied for its effect on *P. aeruginosa* physiology related to tmRNA function. In addition, regulation of those genes should be characterized to determine more complete regulatory pathway of *ssrA* expression in *P. aeruginosa*.

Regulatory circuit between tmRNA and RpoS

Based on my data, it is clear that tmRNA regulates *rpoS* expression indirectly at the transcriptional level by activating transcription of PsrA, the major activator of *rpoS* expression in *P. aeruginosa*. It is not yet clear whether tmRNA also affects RpoS translation as it does in *E. coli* because of its initial effect on mRNA production in *P. aeruginosa*. This can be addressed by transcribing *rpoS* from a foreign promoter and then to measure RpoS protein synthesis. We have such a construct already made to perform this experiment. More interesting question is how tmRNA regulates *psrA* expression. Since this is also done at the transcriptional level, it is also an indirect regulation. Thus, in order to characterize the tmRNA effect on *psrA* regulation, we first have to identify transcriptional regulators of *psrA* and determine whether any of those regulators require *trans*-translation for full translation of the peptide. In order to characterize the mechanism of RpoS regulation of *ssrA*, we have to first map the transcript start site of *PA0827-ssrA* operon. However, to really decide whether RpoS effect is direct or indirect, we have to first compose a consensus sequence of RpoS promoters in *P. aeruginosa*. Alternatively, RpoS effect may be indirect through its regulation of *PA5431* and *PA0319*. There is much study yet to be done to

understand the function and regulation of tmRNA in *P. aeruginosa*. My study represents the first comprehensive approach to identify tmRNA regulation in any bacteria. As such, it sets the ground for future studies for clarifying this complex regulatory circuit between tmRNA and RpoS and their role in *P. aeruginosa* physiology and pathogenesis.

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