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# Post-transcriptional Regulation of RsmA In Pseudomonas aeruginosa

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# Post-transcriptional Regulation of RsmA In *Pseudomonas aeruginosa*

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ABSTRACT

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*Pseudomonas aeruginosa* is an infectious Gram-negative bacillus that is found in a variety of environments. Gene regulatory mechanisms control the expression of virulence factors that allow *P. aeruginosa* to initiate acute infections and persist as a chronic infection of its host. Two-Component Systems (TCS), transcriptional and post-transcriptional regulators, small non-coding RNAs (sRNA), mRNA secondary structure, and others are involved in the coordination of specific virulence genes. A significant post-transcriptional regulator involved in this regulatory network is the Regulator of Secondary Metabolites (RsmA). In this study, we investigated the contribution of a putative stem-loop has on expression of RsmA using bioinformatics and molecular techniques. We constructed *rsmA* leader fusions to measure translation with and without the secondary structure present. Secondly, we introduced point mutations in the stem of the stem-loop of the leader fusions to disrupt the formation of the stem-loop. Finally, we performed Site-Directed Mutagenesis on the *rsmA* leader to examine protein levels *in vivo* by western blot analysis using an HA-tagged *rsmA*. Our data suggests that the segment of RNA that contains the putative stem-loop structure serves some function in post-transcriptional regulation of RsmA.

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# CHAPTER 1

# INTRODUCTION

## *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a metabolically versatile, infectious Gram-negative bacillus that is found in environments ranging from aerobic to anaerobic, soil to water, plant tissues to human tissues, and even found thriving on medical implant devices (Stover et al. 2000; LaBauve and Wargo 2012). *P. aeruginosa* is ubiquitous in our environment and can infect anyone. Infections of healthy individuals are less common and are generally acute in nature. Largely an opportunistic pathogen, *P. aeruginosa* is a major concern for immunocompromised individuals, where infections are more severe and can become life-threatening (LaBauve and Wargo 2012). This population includes those with; diabetes, chronic obstructive pulmonary disease, a recent surgery, a severe burn, or other ailments that result in a compromised immune system, such as Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS), and cancer (Rizzi et al. 2006; Dubern et al. 2015; Mulcahy et al. 2015). Additionally, individuals living with cystic fibrosis(CF) experience the highest rate of morbidity and mortality from chronic lung infections with *P. aeruginosa* (Mathee et al. 1999). Due to its adaptability and numerous virulence factors, this bacterium can infect most tissues of the human body (Dubern et al. 2015). Intrinsic and acquired antibiotic resistance of *P. aeruginosa* has become a major concern for hospital acquired infections in the United States, with 51,000 nosocomial infections per year; based on most recent estimates (CDC 2013). To develop a more efficacious anti-pseudomonad therapy, many researchers are investigating the possibility of developing anti-virulence factor therapies (Fernebro 2011). Understanding gene regulation and the many regulatory networks of *P. aeruginosa* is necessary to aid in the development of anti-virulence

therapies.

*Pseudomonas aeruginosa* Virulence

Pathogenic organisms utilize virulence factors to initiate colonization of the host, to cause disease, or persist as an on-going infection. *P. aeruginosa* possesses an abundance of virulence factors aiding its ability to cause disease. It can colonize and cause acute infections; such as keratitis, otitis externa, folliculitis, and pneumonia in relatively healthy individuals (Gellatly and Hancock 2013). When acute infections are unsuccessfully treated, *P. aeruginosa* can modify the expression of virulence factors to produce those that are more suitable for maintaining a chronic infection (Veesenmeyer et al. 2009). For this reason, virulence factors are often divided into two categories: those responsible for acute infections, and those responsible for chronic infection. Thus, a paradigm exists between acute and chronic lifestyles of *P. aeruginosa.*

In general, acute infections are characterized by a bacteria that are motile, by means of a single polar flagellum and Type Four Pili(TFP) and secrete effector proteins into host cells by the Type III Secretion System(T3SS). The flagellum is commonly associated with swimming motility, but has also been implicated in adhesion to the basolateral surface of host cells, migration through mucin layers, and biofilm formation (Bucior et al. 2012; Hayashi et al. 2015). TFP are responsible for twitching motility, attachment to apical surfaces of host cells, and biofilm formation (Bucior et al. 2012). Additionally, TFP possess a mechanosensory function that can indirectly increase levels of cyclic adenosine monophosphate (cAMP) (Persat et al. 2015). cAMP is known to be involved in several cellular functions. One function is the activation of the transcription factor Vfr(Virulence Factor Regulator) by direct binding (Fuchs et al. 2010). Vfr-cAMP has several downstream targets; one of which is positive regulation of the T3SS (Fuchs et al. 2010; Persat et al. 2015). The T3SS is a needle-like projection composed of several

proteins that is used to secrete the effector proteins ExoS, T, U, and Y into the host cell (Hauser 2009). The secretion of these toxins into the host cell results in cessation of DNA synthesis, disruption of the actin cytoskeleton, degradation of plasma membranes, and many other damaging effects. Intoxication by these enzymes allows for quicker dissemination of the pathogen, decreased innate immune function, and host cell death (Hauser 2009). Given this information, it is apparent how these three virulence factors play a significant role in the initial colonization and subsequent acute infections by *P. aeruginosa*

To persist as a chronic infection, such as those found in the CF lung, *P. aeruginosa* must adapt to survive and exploit the new niche. Biofilm formation is a tactic used to establish a lasting infection. *P. aeruginosa* biofilms are composed of extracellular polymeric substances(EPS) such as the exopolysaccharides Pel, Psl, and alginate. Other substances include lipids, proteins, and extracellular DNA. The presence and levels of each component can vary between different environments, the stresses imposed on the biofilm, and the stage of biofilm development (Moradali et al. 2017). It is worth noting that Pel and Psl are expressed by both acute and chronic infecting strains. Involvement of Pel and Psl in biofilm formation is seen in the early stages of infection aiding adhesion, and is maintained as a structural component of the mature biofilm seen in chronic infections (Jones and Wozniak 2017). Longitudinal studies of *P. aeruginosa* isolates from CF patients have established common genotypes associated with chronic infection. One particular genotypic variation is the mutation of the anti-sigma factor gene *mucA* (Winstanley et al. 2016). Thought to be a result of environmental stresses found in the CF lung, mutation of *mucA* results in freeing of the sigma factor AlgU (Figure 1). AlgU, as well as AlgR, are involved in the activation of the alginate biosynthetic pathway leading to the production of alginate (Sautter et al. 2012). The cell adopts the mucoidy phenotype, a hallmark

characteristic of chronic CF isolates (Wu et al. 2004). The importance of biofilms in a chronic infection are protection from  host immune defenses and antibiotic resistance (Gellatly and Hancock 2013).

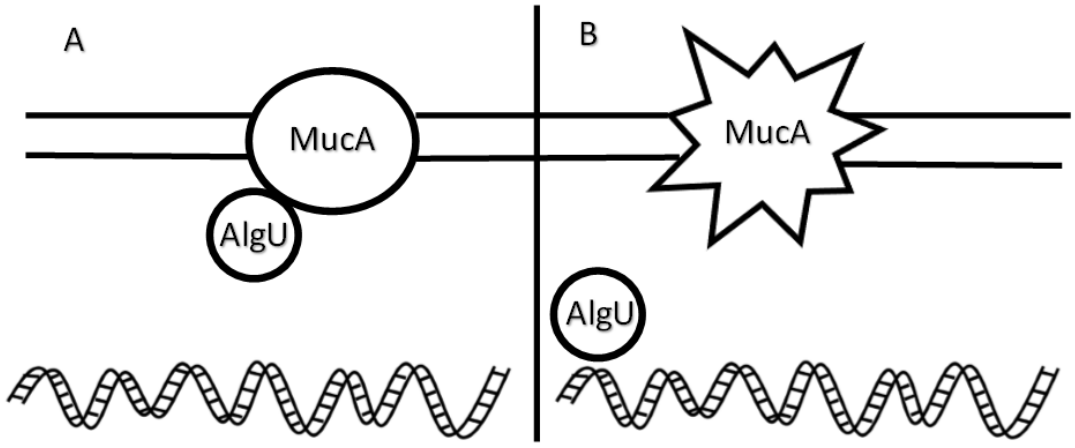



Figure 1. Mutation of MucA leads to alginate production and the mucoidy phenotype. A.) The sigma factor AlgU is sequestered by the anti-sigma factor MucA at the inner membrane. B.) Mutation of *mucA* leads to activation of the alginate biosynthetic pathway by AlgU.

An additional chronic state virulence factor extensively studied is the Type VI Secretion System (T6SS). Resembling the bacteriophage puncturing apparatus, the T6SS secretes effector proteins directly into target cells. *P. aeruginosa* has three known types of T6SSs originating from three genes clusters. They are H1-T6SS, H2-T6SS, and H3-T6SS (Chen et al. 2015). These secretion systems have utility in both bacteria-bacteria and bacteria-host cell interactions. While there are numerous effector proteins, for brevity, only a few examples will be mentioned. Type Six Exported (Tse) 1, 2, and 3 are translocated by the H1-T6SS. Tse1 and 3 cause degradation of peptidoglycan in bacteria, while injection of Tse-2 causes cessation of cellular growth in prokaryotes (Russell et al. 2011). H2 and H3-T6SS secrete the effectors PldA and PldB, which are involved in the invasion of *P. aeruginosa* into eukaryotic cells and the killing of other bacterial cells (Sana et al. 2015). Altogether, this system has implications in establishing

dominance in polymicrobial environments and innate immune evasion (Hood et al. 2010; Sana et al. 2015).

Motility by TFP and flagellum, and T3SS are characteristic of acute infections. Becoming sessile and expressing T6SS has been associated with chronic infections. The virulence factors described here are only a small representation of the arsenal that *P. aeruginosa* possesses. Cues that drive this transition between acute and chronic phenotypes vary from numerous environmental stresses to cell population density, and at the root of it all are various complex mechanisms that shift the continuum of gene expression.

RsmA and Post-Transcriptional Regulation

To thrive, pathogenic and non-pathogenic bacteria must be able to respond quickly to changes in their environment. This is achieved through rapid modulation of gene expression that can result in adaptive phenotypic changes (Jean-Pierre et al. 2017). At the time of its complete genome sequence, *P. aeruginosa* had the highest proportion of predicted regulatory genes of all sequenced bacterial genomes (Stover et al. 2000). Housekeeping and virulence associated genes, are controlled by complex regulatory systems composed of Two-Component Systems (TCS) and transcriptional and post-transcriptional regulators. These regulators specifically include, DNA and RNA binding proteins, small non-coding RNAs (sRNA), intrinsic RNA secondary structures (stem-loops and riboswitches), and others (Romeo 1998; Svensson and Sharma 2016). Post-transcriptional regulation is one scheme bacteria use to rapidly alter gene expression in a changing environment.

A significant post-transcriptional regulator involved in global gene expression in *P. aeruginosa* is Repressor of Secondary Metabolites (RsmA) (Pessi et al. 2001). RsmA is a homologue to Carbon Storage Regulator (CsrA), belonging to the Carbon Storage Regulator

(Csr) family of mRNA binding proteins found in both Gram-positive and Gram-negative bacteria (Yakhnin et al. 2007). In bacteria, the first step in protein synthesis, after mRNA transcription, is recognition of the Ribosomal Binding Site(RBS) or Shine Dalgarno(SD) sequence by the 16s rRNA within the 30s ribosomal subunit. After binding of the RBS by the 30s subunit, the 50s ribosomal subunit will join the 30s subunit making a complete 70s ribosome. Translation can begin once the 70s ribosome has assembled (Laursen et al. 2005). This process is interrupted when CsrA/RsmA bind their recognition sites within the 5' Untranslated Region (UTR) of mRNAs. It is generally accepted that CsrA/RsmA recognize GGA motifs located in the loop of stem-loops and impose their activity by binding GGAs that overlap the RBS (Figure 2) thus, inhibiting interactions of the 30s ribosomal subunit to the target mRNA (Baker et al. 2002).

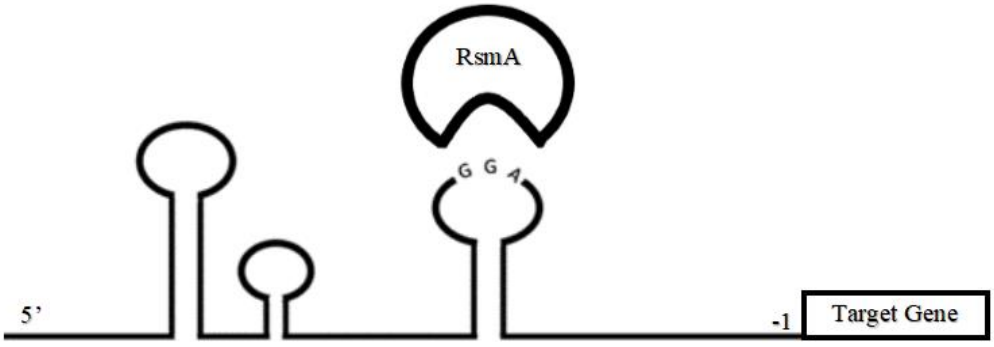



Figure 2. RsmA inhibits translation by recognizing and binding GGA motifs presented in the loop of stem-loops present in 5' Untranslated Regions of target mRNAs.

The example just described is known as repression of translation initiation. This was first described for CsrA in the repression of *glgC*, a gene involved in glycogen biosynthesis (Baker et al. 2002). Since then, other mechanisms whereby CsrA represses or activates gene expression have been described. For example, CsrA mediates expression of the *pgaABCD* operon; necessary for biofilm formation, by translation repression, transcription attenuation, and indirectly represses expression by targeting an mRNA that encodes an activator of the *pgaABCD* operon (Romeo and Babitzke 2018). Conversely, the transcript for the *flhDC* operon; which is

responsible for flagella biosynthesis, is stabilized by bound CsrA, leading to flagellar motility (Wei et al. 2001).

Like CsrA, RsmA has been implicated in the regulation of several hundred genes in *P. aeruginosa*. A microarray study performed in 2006 determined that RsmA was involved in the regulation of 506 out of 5570 genes analyzed in the PAO1 reference strain versus an *rsmA* mutant (Burrowes et al. 2006). Another study in 2009 examined strain PAK and found that 528 of 5678 gene were affected by RsmA (Brencic and Lory 2009). Interestingly, only 67 genes overlapped between the two strains, which was partially attributed to varied growth conditions (Brencic and Lory 2009).

In both studies, transcript levels of T3SS and TFP operons were decreased in the *rsmA* mutant. In their 2009 study, Brencic and Lory analyzed their findings by transcriptional and translational fusions using genes found in both operons. Of the genes analyzed, all showed decreased levels of transcription in the *rsmA* mutant but showed no difference in the translational fusions. In addition, purified RsmA did not bind the mRNA of the selected genes in their gel mobility assay (Brencic and Lory 2009). These data suggest RsmA is involved in the positive expression of type three secretion and twitching motility by TFP, albeit indirect. In this case, it is possible that RsmA is inhibiting translation of a transcriptional regulator of both T3SS and TFP.

The *rsmA* mutation had a positive effect on components of the H1-T6SS, PA0081 and PA0082. In light of this finding, Brencic and Lory set out to determine if RsmA had direct post-transcriptional regulation on the H1-T6SS. mFold analysis revealed RsmA binding sites within the 5'UTRs of PA0081 and PA0082 and both mRNAs co-purified with RsmA. Also, RsmA binding to the mRNA was confirmed with gel mobility assays, concluding that RsmA directly inhibits translation of these messages (Brencic and Lory 2009).

Biofilm biosynthesis is repressed by RsmA indirectly and directly. Increased levels of c-di-GMP result in a less motile phenotype and one that produces biofilms. The diguanylate cyclase, SadC, is controlled by RsmA. Western blot analysis determined that when RsmA is sequestered by RsmY, SadC is present. However, when RsmA is abundant SadC is not detectable (Moscoso et al. 2014). Therefore, RsmA represses biofilm production indirectly by controlling c-di-GMP levels through SadC. Psl is negatively regulated by RsmA. A study has shown that RsmA is involved in stabilizing an anti-SD and SD interaction by binding the single-stranded region of a stem-loop with a GGA motif whereby translation is directly repressed (Irie et al. 2010).

Since RsmA is an mRNA binding protein that functions post-transcriptionally and these experiments examined the transcriptional difference between wildtype and a *rsmA* mutant, the results only suggest that RsmA indirectly affects a broad range of genes. The majority of the targets have not been confirmed by direct RsmA binding; nonetheless, these two studies demonstrated the expansive role RsmA has on global gene expression in *P. aeruginosa*. Categories affected included iron acquisition and storage, antibiotic resistance, stress response, and quorum sensing, and others. More importantly, RsmA is involved in the regulation of virulence factors, which include: TFP, T3SS, T6SS, and biofilm biosynthesis (Brencic and Lory 2009). Recall, TFP and T3SS are associated with acute infections and T6SS and biofilms are characteristic of chronic infections. Thus, RsmA plays a role in shifting *P. aeruginosa's* phenotypes between acute and chronic infections.

Gene Regulation by RNA & RNA Secondary Structures

Beyond gene regulation at the transcriptional and post-transcriptional level by DNA and mRNA binding proteins, RNA and their secondary structures have emerged as crucial elements in gene expression. RNA regulation comes in many forms. They can be small non-coding RNAs(sRNAs) that are *cis*- or *trans*-acting elements, they can exist as intrinsic secondary structures in the 5' and 3'UTRs of mRNAs, or as dual-functioning mRNAs that have a coding region and also function as *trans*-acting elements. These RNAs are gaining more attention for their involvement in gene expression as many are associated with virulence and survivability in various environments (Svensson and Sharma 2016). Most known examples of gene regulation by RNAs are found at the post-transcriptional level; however, regulation at the transcriptional level does occur. Demonstrated in *E. coli*, when the $\sigma^{70}$ subunit of RNA Polymerase(RNAP) is bound by the 6S sRNA, transcription for genes that have promoters recognized by the $\sigma^{70}$ sigma factor were reduced (Wassarman and Storz 2000). sRNAs have multiple functional mechanisms when it comes to post-transcriptional regulation. In the simplest form, sRNAs can bind target mRNAs with great complementarity using *cis*-acting sRNAs, or to a lesser degree of complementarity with *trans*-acting sRNAs. Using their base-pairing affinity, the sRNAs aid in the inhibition or activation of protein translation. Additionally, protein activity can be altered by sRNA interactions (Svensson and Sharma 2016). For example, the sRNAs RsmY and RsmZ are known to sequester RsmA, thus imposing indirect post-transcriptional regulation (Lapouge et al. 2008).

Intrinsic secondary structures within UTRs provide numerous modes of gene expression. It is largely accepted that mRNAs among bacterial species have relatively short 5'UTRs, generally 20-40 nucleotides in length. mRNAs with longer 5'UTRs typically show greater potential for formation of secondary structures and post-transcriptional regulation (Svensson and

Sharma 2016). Secondary structures possess regulatory functions in the form of mRNA stability, thermosensors (RNA thermometers), and riboswitches, to name a few examples (Svensson and Sharma 2016).

Protein synthesis requires time and energy. First, mRNA must be transcribed from DNA. Then, mRNA must be translated into protein. At the time of an environmental shift, such as infecting a host, there are many cues received that induce a shift in gene expression. Therefore, regulating gene expression at a post-transcriptional level afford bacteria a more rapid and efficient response. The presence and abundance of mRNA in a cell can dictate the level of expression of the gene product. Therefore, the degradation or stability of mRNA is important in controlling levels of gene expression (Hui et al. 2014).

Bacteria use a panel of nucleases to degrade unwanted mRNA, thus decreasing gene expression. These nucleases can be 5' or 3' exonucleases, endonucleases, and oligonucleases (Hui et al. 2014). Nucleases have various requirements to inflict their function on mRNAs. For example, the phosphorylation state, monophosphorylated vs. triphosphorylated 5' ends of target mRNA allows endolytic cleavage by RNaseE (Celesnik et al. 2007). Additionally, RNaseE preferentially cleaves A/U rich single-stranded RNA. Some nucleases, like RNase III, cut double-stranded RNA like that found in stem-loops (Hui et al. 2014). Some nucleases are not able to completely degrade their targets, so oligonucleases finish degrading short fragments of RNA (2-5 nucleotides) to mononucleotides. Degrading RNAs down to single nucleotides is energetically beneficial to the organism as it replenishes the RNA precursor pool (Hui et al. 2014). Just as it is important to degrade unwanted messages, it is also important for some mRNAs to possess longevity. mRNAs are stabilized through 5' and 3' secondary structures. Take for example RNaseE, which again prefers mRNA with a 5' monophosphate and cleaves

single-stranded A/U rich regions. In this example a 5’ stem-loop provides protection from RNaseE endolytic attack (Hui et al. 2014). In another example, stem-loops can provide binding sites for *trans*-acting elements that prevent nucleolytic cleavage. CsrA has been shown to increase the half-life of the *flhDC* mRNA by binding two sites upstream of an RNase E cleavage site and preventing degradation (Yakhnin et al. 2013). Stem-loops found at the 3’ end of full length mRNAs protect the messages from 3’exonucleases which will require nucleases to begin degradation in other locations (Hui et al. 2014). Degradation of mRNAs by nucleases is an indiscriminate process. Structured segments within 5’ and 3’ UTRs of mRNAs can provide protection from some nucleases while providing a target for others.

Some human pathogens have developed genetic mechanisms for regulating the expression of certain genes in a temperature dependent manner (Grosso-Becerra et al. 2014). Many of these genes are involved in the positive regulation of virulence factors or encode heat shock proteins (Grosso-Becerra et al. 2014). There are two well-characterized RNA thermometers identified in several bacteria. They are the Repression Of heat-Shock gene Expression (ROSE) element and the Four-U RNA thermometer (Svensson and Sharma 2016). The ROSE element is an RNA thermometer that can range in length, from 60 to over 100 nucleotides, and composed of several (2-4) stem loops (Figure 3). The stem-loops proximal to the translation initiation site sequester the Ribosomal Binding Site (RBS). They are stable at low temperatures and prevent translation of the mRNA. At higher temperatures, like those experienced during infection of the human host, translation of the message can occur because the stem-loop occluding the RBS will melt (Grosso-Becerra et al. 2014). The other RNA thermometer, Four-U (Figure 3), uses a UUUU motif to bind the RBS and forms a stem-loop

(Waldminghaus et al. 2007). Like the ROSE, the stem-loop is stable at lower temperatures but melts at higher temperatures to allow expression of the gene under its control.

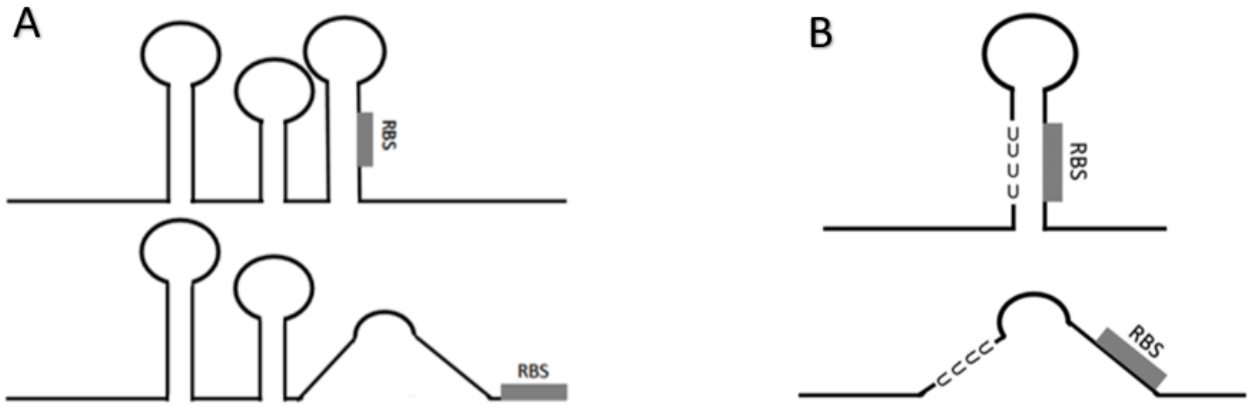



Figure 3. RNA thermometers regulate mRNA translation in a temperature dependent manner by occluding the Ribosomal Binding Site (RBS) at lower temperatures. Temperature increases lead to exposure of the RBS. A.) The Repression Of heat-Shock gene Expression (ROSE) consists of several stem-loop structures in the 5' Untranslated Region. The most 3' stem-loop will occlude the RBS. B.) The Four-U RNA thermometer uses four Uracil ribonucleotides to loosely base-pair with the RBS.

Riboswitches are genetic regulators of various biosynthetic pathways found in the 5'UTR of mRNAs(Figure 4). These structured regulatory elements affect transcription or translation of the mRNA, generally by sensing metabolites, pathway intermediates, or products related to the translated mRNA (e.g. vitamins, purines and derivatives, amino acids, some metals ($Mg^{2+}$), some anions ($F^-$), and others) (Serganov and Nudler 2013). There are two components of a riboswitch; the aptamer and the expression platform. The main mechanism for riboswitch activity is the conformational change that occurs at the expression platform when the aptamer binds its ligand (Svensson and Sharma 2016). Transcriptional regulation by riboswitches occurs in the formation of Rho-dependent and independent terminators and antiterminator hairpins. Translational regulation is achieved by RBS accessibility or exposure to various enzymatic cleavage sites (Serganov and Nudler 2013; Svensson and Sharma 2016).

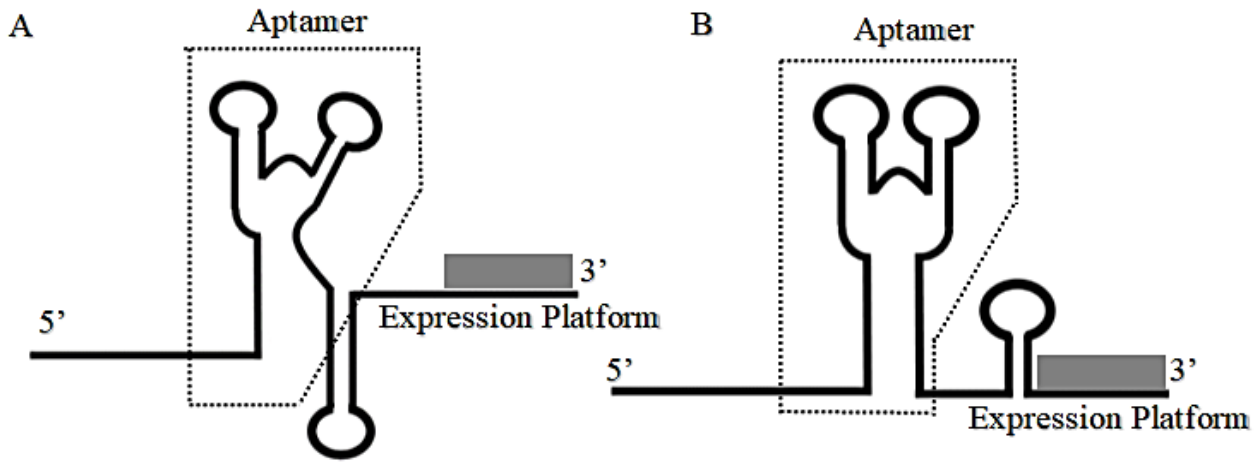



Figure 4. Riboswitches are structed elements in the 5'UTR of mRNAs. A.) The aptamer has not bound its ligand and the expression platform is available and translation of the message can proceed. B.) The aptamer has bound its ligand resulting in a conformational change of the riboswitch. The expression platform is now occluded, and translation is inhibited.

Bacteria possess many mechanism for controlling the expression of their genes. Stability or degradation of mRNA after transcription allows the bacterium to efficiently modulate gene expression at a post-transcriptional level. Intrinsic secondary structures within the 5' UTR can provide binding sites for *cis* or *trans*-acting elements like sRNAs and mRNA binding proteins. They can also provide protection or enhance nucleolytic activity (Hui et al. 2014). These mechanisms are important to understand and further investigate as they are involved in many cellular processes, including virulence gene regulation.

Regulation of RsmA

The GacS/GacA Two-component systems (TCSs) has been established as a major influence on post-translational regulation of RsmA. The main components of a TCS are the sensor histidine kinase (SK) and response regulator (RR). *P. aeruginosa* has 64 SKs, 72 RRs (Francis et al. 2017). The inner membrane bound SK senses the environment for numerous signals (e.g. ions, antimicrobial peptides, kin cell lysates, c-di-GMP, and others). Under appropriate conditions the SK is autophosphorylated on a conserved histidine residue and will

trigger a phosphorelay directly to its response regulator, or in some cases via a Histidine Phosphotransfer(Hpt) protein (Francis et al. 2017). The GacS/GacA system works as a multikinase network involving the SKs LadS, PA1611, and the GacS inhibitor, RetS. Levels of phosphorylated GacA are controlled through the various SKs (Francis et al. 2017). Once phosphorylated, GacA acts as a transcriptional regulator and positively regulates two small non-coding RNAs, RsmY and RsmZ (Figure 5). RsmY/Z possess multiple binding sites for RsmA and sequester RsmA (Lapouge et al. 2007). Therefore, the GacS/GacA TCS is capable of titrating levels of RsmA through  increasing or decreasing RsmY/Z levels in the cell.

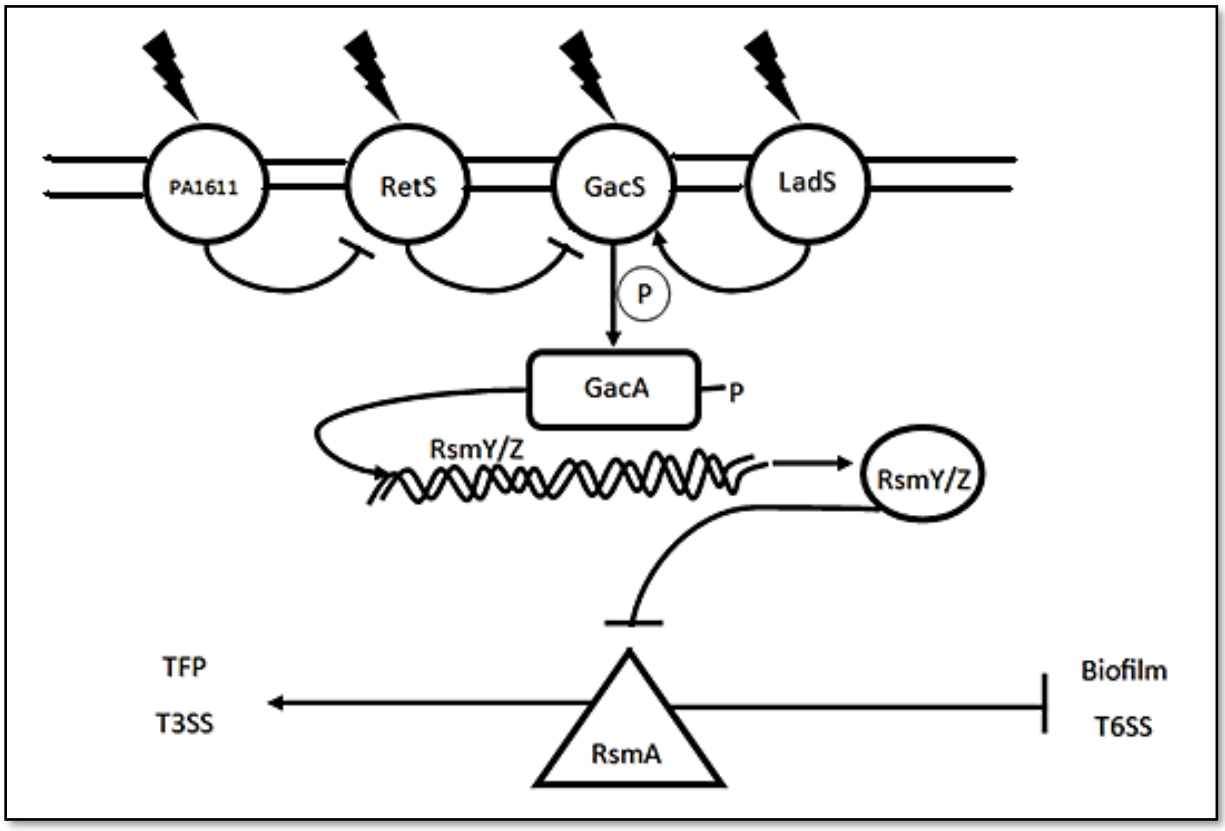



Figure 5. The GacS/GacA Two Component System is a multi-sensor histidine kinase system with the central response regulator, GacA, being phosphorylated by GacS. The small non-coding RNAs RsmY and RsmZ are activated by GacA. RsmY/Z titrate levels of RsmA in a Post-translational manner by presenting multiple GGA motifs in the loops of stem-loops.

Recent studies have established a mechanism by which *rsmA* is regulated at a transcriptional level. Through primer extension experiments, a previous study established the existence of an additional Transcriptional Start Site(TSS) for *rsmA* 46 nucleotides upstream of a previously identified putative RpoD dependent TSS and the presence of two different length *rsmA* mRNA transcripts via RNase protection assay(RPA) (Stacey and Pritchett 2016). Interestingly, more mRNA is observed in the mucoid (*mucA22*) background. The information gained from the primer extensions and RNase protection assay prompted the examination of the intergenic region between *rsmA* and the upstream gene *lysC.* Initially, a transcriptional fusion spanning the entire intergenic region was constructed and assayed in PAO1 and *mucA22.* The fusion data was in alignment with the data obtained from the RPA indicating more RsmA transcript in *mucA22*. Since two TSSs and two messages were observed, two more TFs were constructed in conjunction with the observed TSSs with additional upstream nucleotides to encompass promoter regions. Reporter activity from the more distal fusion was significantly increased over PAO1 in the *mucA22* background, whereas no difference was seen between PAO1 and *mucA22* in the more proximal fusion. Conserved AlgU binding motifs upstream of the distal TSS prompted the examination of the TFs in an AlgU mutant. In both fusions containing the distal TSS and promoter region, significant decreases in reporter activity were observed in the *mucA22 algU* double mutant. Western blot analysis confirmed that there are higher levels of RsmA in *mucA22* relative to PAO1, and deletion of *algU* significantly reduced intracellular levels of RsmA. Lastly, mutagenizing the AlgU -35 binding site significantly reduced reporter activity in *mucA22* (Stacey and Pritchett 2016). Thus, these data provide evidence that RsmA is under the control of the alternative sigma factor AlgU.

In a similar fashion, using the same TFs a later study determined that the transcription factor AlgR is also involved in the expression of RsmA in *Pseudomonas aeruginosa.* AlgR is the RR in the AlgZ/R TCS. To test if *rsmA* was under the transcriptional control of this TCS, the TFs were assayed in an *algZ* mutant. No difference was seen between *mucA22* and the *mucA22 algZ* double mutant (Stacey et al. 2017). Also, since AlgR can be phosphorylated by AlgZ, AlgR phosphomimetic and phosphodeficient strains were engineered so that AlgR structural conformation in a constant phosphorylated or unphosphorylated could be analyzed. The study concluded that *rsmA* is activated at a transcriptional level by unphosphorylated AlgR and RsmA is not under the control of the AlgZ/R TCS (Stacey et al. 2017). These two studies have furthered our understanding of the transcriptional controls overlying the expression of *rsmA.* The microarray data by Brencic and Lory, and Burrowes and colleagues has given insight into the RsmA regulon, yet there are still gaps in our knowledge of *rsmA* expression.

Due to the discoveries described above, in this study I took a post-transcriptional approach to further investigate the regulation of *rsmA* in *Pseudomonas aeruginosa.* Using bioinformatics, translational leader fusion analysis, site-directed mutagenesis, and western blot analysis, I provide evidence that the RsmA transcript is under post-transcriptional regulation.

# CHAPTER 2

# MATERIALS AND METHODS

## Bacterial Strains and Growth Conditions

*Pseudomonas aeruginosa* strain PAO1 was selected to represent the nonmucoid wildtype strain. *mucA*22 was used to represent mucoid strains commonly isolated from the CF lung. New England Biolabs 5-*alpha* (NEB®5-α) *Escherichia coli* competent cells were used for plasmid propagation and conjugation. When necessary, pRK2013 was used for triparental mating. Unless stated otherwise, all strains were incubated at 37$^{\circ C}$ with shaking at 250rpm under aerobic conditions. *E. coli* strains were grown and maintained on Luria-Bertani (LB). Pseudomonas Isolation Agar (PIA) was used for the growth and maintenance of *P. aeruginosa* strains. Antibiotics were used for selection, at appropriate concentrations, when necessary (Appendix A). All strains were stored in cryo-tubes at -80$^{\circ C}$ in 30% (v/v) glycerol (Appendix B).

## Colony PCR

In this study, site-directed mutagenesis and fusion construction was performed. In order to screen for the mutations and fusions, colony PCR was performed. From an overnight streak plate, a single colony was selected and resuspended in 50µL of sterile dd$H_2O$ in a microfuge tube. The colony was vortexed and then placed in a heat block set to 65$^{\circ C}$ for 10 minutes. While the cell suspension was heating, the master mix was prepared on ice. After 10 minutes of heating, 2µL of the cell suspension was added to a thin-walled PCR tube followed by 23µL of master mix. The PCR reaction tube was placed in a thermocycler with a heated lid and the program was adjusted to the appropriate settings.

Table 1. Standard Colony PCR Protocol

| Mater Mix | |
|---|---|
| Forward Primer 10µM | 1µL |
| Reverse Primer 10µM | 1µL |
| DMSO | 1µL |
| DNTPs 10µM | 1µL |
| Thermopol Reaction Buffer 10X | 2.5µL |
| Sterile ddH2O | 16.5µL |
| Taq DNA Polymerase | 0.1µL |
| Bacterial Sample Suspension | 2µL |

| Thermocycler Protocol | | |
|---|---|---|
| Step | Temperature | Time |
| Initial Denaturation | 95°C | 5 min |
| 25 Cycles | 95°C | 30s |
| | 50-72°C | 30s |
| | 72°C | 1 min per kb |
| Final Extension | 72°C | 10min |
| Hold | 4°C | ∞ |

Genomic DNA Isolation

Overnight cultures were prepared in 5ml of LB broth. On day two, the cells were pelleted using a centrifuge set to 8000XG for 10 minutes. The spent LB was removed and the cells were resuspended in 400µl of 1X TNE and transferred to a microfuge tube. Next, 17µl of sarkosyl 30% (w/v) and 5µl Proteinase K 10mg/ml were added and the suspension was incubated at 37°C for 1 hour. After incubation, 400µl of 4M Ammonium Acetate ($NH_4OAc$) was added and the suspension was mixed by inversion. Cell debris were pelleted using a centrifuge set at 14,000XG for 10 minutes. The supernatant was carefully removed and placed in a new microfuge tube. Two washes were performed with 1:1 phenol-chloroform; centrifuging and transferring the aqueous phase to a new microfuge tube each time. One wash with 600µl of chloroform was performed, mixed by inversion, and centrifuged. The aqueous layer was transferred to a new microfuge tube and the genomic DNA was precipitated using 2.5 volumes of chilled absolute ethanol and incubated at -20°C for no less than 10 minutes. The DNA was pelleted using a centrifuge set at 13,000XG for 10 minutes. The absolute ethanol was removed, and the DNA pellet was washed

with 1 ml of 70% ethanol and pelleted again. The 70% ethanol was carefully removed so not to dislodge the pellet. Finally, the DNA was resuspended in 50µl of 1X TE and stored at $4^{\circ C}$.

Plasmid DNA Isolation

Overnight cultures were prepared in 5ml of LB broth with suitable antibiotics. On day two, the cells were pelleted using a centrifuge set to 8000XG for 10 minutes. The spent LB was removed and the cells were resuspended in 150µl of 1X TE. The suspension was transferred to a microfuge tube and 10µl of 10mg/ml RNase A was added. To lyse the cells, 300µl of 1% SDS / 0.2M NaOH was added to the suspension and then incubated on ice for five minutes. A volume of 225µl of 5M potassium acetate / 11.5% acetic acid added to the mixture, mixed by inversion, and returned to ice for five minutes. Cellular debris were pelleted via centrifugation (13,000XG for 10 minutes) and the supernatant was transferred to a new microfuge tube. 600µl of Phenol-Chloroform-Isoamyl alcohol (24:25:1) was added to the supernatant, vortexed, and then centrifuged for 5 minutes. The aqueous phase was removed and transferred to a new tube, followed by two washes with 600µl of Chloroform, removing the aqueous layer and transferring to a new microfuge tube each time. The plasmid DNA was precipitated using 1ml of chilled absolute ethanol. The solution was incubated on ice for 10 minutes and then the plasmid DNA was pelleted by centrifugation. The absolute ethanol was removed, the pellet was washed with 70% ethanol and re-pelleted. The 70% ethanol was removed, and the plasmid DNA was left to air dry. The plasmid DNA was resuspended in $ddH_2O$ (20-50µl) and stored at $-20^{\circ C}$ or $4^{\circ C}$.

Site-Directed Mutagenesis

For deletion:

The suicide vector pEX18gm containing the *rsmA* coding region with a 3' hemagglutinin (HA) tag (pEX18gm*rsmA*HA) with 1kb flanking either side of *rsmA* was used as template for the construction of stem-loop deletion mutants. *E. coli* harboring pEX18gm*rsmA*HA was grown on LB gentamycin[15]. A single isolate was selected to inoculate a 5ml LB gentamycin[15] broth and grown overnight at 37°C with shaking. The plasmid DNA was isolated and quantified via nanodrop. Forward and reverse primers were designed with the 5' ends back-to-back, flanking the desired nucleotide deletions (Table 5, Appendix C). A separate 25µl inverse PCR reaction was assembled on ice for each desired stem-loop deletion, following the protocol in (Table 2).

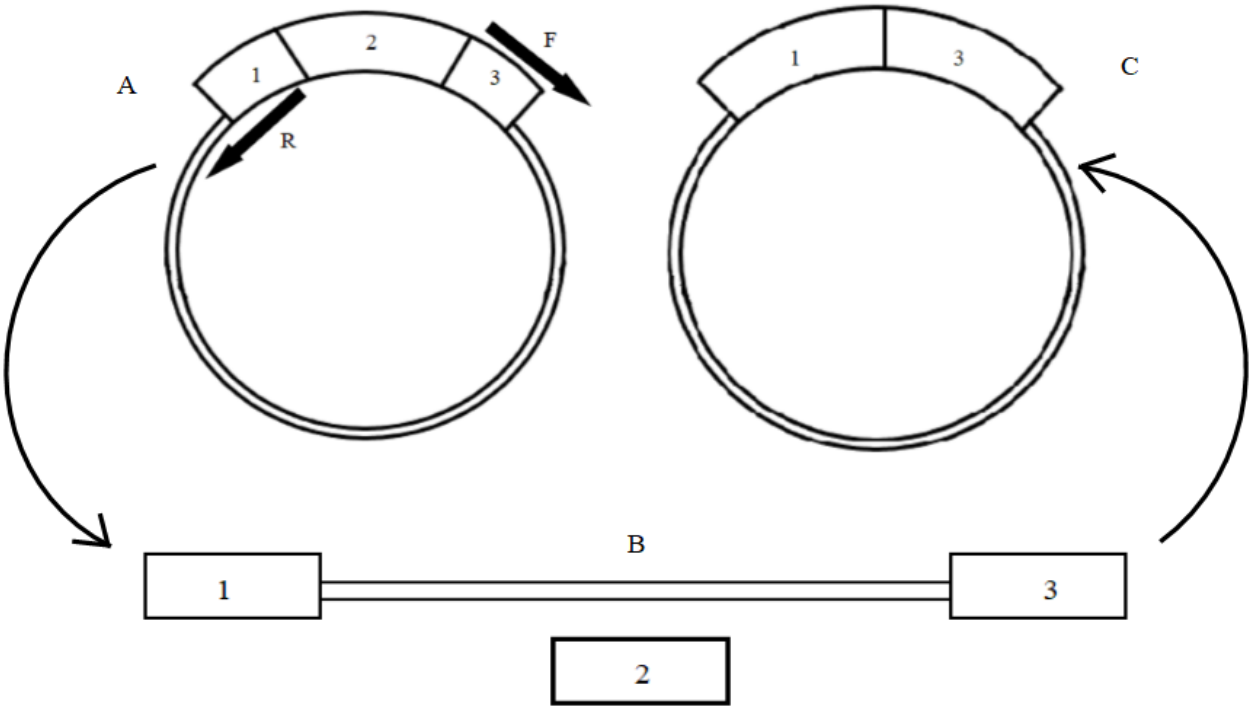



Figure 6. Concept for site-directed mutagenesis of 5' UTR deletion mutations by inverse PCR. A.) A Forward (F) primer with complementarity to zone 3 and a reverse (R) primer with complementarity to zone 1 amplify the entire plasmid in opposite directions resulting in linear double stranded plasmids. B.) Linear plasmids maintain zones 1 and 3, while zone 2 is not

amplified. Endonuclease DpnI is added to the tube to digest template plasmid. C.) Mutagenized plasmids are circularized, joining zones 1 and 3, using the standard ligation protocol (Table 3).

After completion of PCR, each reaction was digested for one hour with the methylation sensitive endonuclease, DpnI, to rid the reaction of template plasmid. Next, a ligation reaction was set up following standard protocol (Table 3) to re-circularize and ligate the ends of the plasmid (Figure 6). Plasmids were transformed into NEB®5-α competent cells following standard protocol. Mutant transformants were incubated overnight on LB gentamycin[15]. The following day, transformants were picked from the LB gentamycin[15] and patched onto the same media. Newly patched colonies were allowed to incubate overnight at 37 $^{\circ C}$. PCR was used to amplify a region of the plasmid approximately 400bp upstream of the *rsmA* start codon through the HA allele at the end of *rsmA*, resulting in an amplified fragment of approximately 600bp (Table 5, Appendix C). In each case, a native BamHI endonuclease site was deleted from the amplified region and was used as a marker for proper deletions (Figure 11. A). All mutated plasmids were sent to the ETSU Molecular Biology Core for sequencing.

Table 2: Site-Directed Mutagenesis Thermocycler Protocol

| Master Mix | | | |
|---|---|---|---|
| | | 25µl Reaction | Final Concentration |
| Forward Primer | 10µM | 1.25µL | 0.5 µM |
| Reverse Primer | 10µM | 1.25µL | 0.5 µM |
| Template DNA | (1-25ng/µl) | 1µL | 1-25ng |
| DNTPs | 10µM | 1.25µL | 0.5 µM |
| Q5 Reaction Buffer | 5X | 5µL | 1X |
| High GC Enhancer Buffer | 5X | 5µL | 1X |
| Sterile $ddH_2O$ | | 10.25µL | NA |
| Q5 Polymerase (2000U/ml) | | 0.5-1.0µL | 1-2 U |

| Thermocycler Protocol | | |
|---|---|---|
| Step | Temperature | Time |
| Initial Denaturation | 98°C | 30s |
| 25 Cycles | 98°C | 10s |
| | 50-72°C | 10-30s |
| | 72°C | 4min |
| Final Extension | 72°C | 2min |
| Hold | 4-10°C | ∞ |

For substitution:

To mutate the stem-loop by nucleotide substitution, forward primers with the desired substitutions were designed and paired with a common reverse primer (Table 5, Appendix C). Using the standard PCR protocol (Table 1) each substitution was made by amplifying the *rsmA* leader region from the distal transcriptional start site through the second codon of *rsmA.* After amplification, each fragment and the cloning vector, *lacUV*5CTX.CP, was double digested with endonucleases EcoRI and ScaI overnight, following standard protocol. The next day, the fragments were individually ligated into *lacUV*5CTX.CP, creating the *lacUV*5*rsmAlacZ* translational leader fusions with nucleotide substitutions. Plasmids were transformed into NEB®5-α competent cells following standard protocol. Plasmids were sent to the ETSU Molecular Biology Core for sequencing.

Vector Digest and Ligation

The expression vector *lacUV*5CTX.CP and desired inserts were individually double digested with appropriate enzymes to obtain the proper ends desired. Double digest reactions were assembled (Table 3) and then incubated for at least 1 hour at 37$^{\circ C}$. Enzymes were heat inactivated, if possible, and products to be cloned were purified using the New England Biolabs Monarch® DNA Gel Extraction Kit. After purification, DNA quantities of insert and vector were determined using a Thermo Fisher Scientific NanoDrop™ spectrophotometer. Using Promega Biomath Calculators for ligation, a 1:3 molar ratio of vector to insert was calculated. The ligation reaction was assembled (Table 3) and then incubated at room temperature for 2 hours or overnight at 4$^{\circ C}$.

Table 3: Double Digest & Ligation Mix

| Double Digest Mix | |
|---|---|
| Enzyme #1 (20 Units) | 1µL |
| Enzyme #2 (20 Units) | 1µL |
| Vector/Insert | 0.5µg |
| 10X NEB Cutsmart Buffer | 2µL |
| $ddH_2O$ | to 20µL |
| | |
| Incubate at 37˚C for at least 1 hour | |

| Ligation Mix | |
|---|---|
| Digested Vector (100ng) | N/A |
| Digested Insert (Xng) | N/A |
| 10X T4 DNA Ligase Buffer | 2µL |
| $ddH_2O$ | to 20µL |
| Maintain 1:3 Vector:Insert ratio | |
| | |
| Incubate overnight at 4˚C | |

Transformation

NEB®5-α competent cells were thawed on ice. In each transformation, 50µl of thawed competent cells were transferred to a thin walled PCR tube and mixed with 5µl of post-ligation (plasmid/insert) by flicking the tube 3-5 times and incubated on ice for 30 minutes. The mixture was heat shocked using a heat block set at 42$^{\circ C}$ for 30 seconds and then immediately placed back on ice for 5 minutes. The mixture and 950µl of room temperature LB (2 per transformation) were combined in a microfuge tube and incubated with shaking for 60 minutes for recovery. LB plates supplemented with appropriate antibiotics were incubated at 37$^{\circ C}$ for the duration of the recovery. After 60 minutes, 100µl of the transformed cells were spread on a selection plate. The remaining 900µl were centrifuged at 3000XG for 5 minutes. 800µl of the spent LB was removed and the cells were resuspended and plated with the remaining 100µl of LB. The plates were then incubated overnight at 37$^{\circ C}$.

Triparental Mating

Triparental mating was used to efficiently transfer vector DNA from *E. coli* to *P. aeruginosa* for the purpose of introducing translational leader fusions or mutations into the

chromosome. PAO1 or *mucA22* was grown overnight at 42$^{\circ C}$ on PIA. NEB5α *E. coli* with desired vector was grown overnight at 37 $^{\circ C}$ on LB with appropriate antibiotics. The helper strain pRK2013 was grown overnight at 37 $^{\circ C}$ on LB Kan$^{50}$. After incubation, the three strains were removed from the plates with an inoculation loop and resuspended together in a microfuge tube containing 500µL of 0.85% (w/v) saline. The bacterial suspension was plated on LB and incubated overnight at 30 $^{\circ C}$. The following day, the bacteria was collected from the plate with an inoculation loop and resuspended in 500µL of 0.85% (w/v) saline. To select for the *Pseudomonas* strain that successfully took in the vector, 100µL of the suspension was spread plated onto PIA with the appropriate antibiotics. The vector will provide *Pseudomonas* with antibiotic resistance; therefore, the *Pseudomonas* that did not take up the vector will be removed from the pool. The spread plate was incubated overnight, or up to 48hours, at 37 $^{\circ C}$. If colonies did not appear after 48 then the plate was discarded and the triparental mating was repeated.

Insertion of Translational Leader Fusion

The translational fusion vector used is a derivative of the mini-CTX integration vector. This vector contains Flp recombinase recognition sites (FRT) flanking the desired insertion sequence and the φ CTX attachment site, *attP*. After integration of the plasmid into the *attB* site( φ CTX attachment site) in the chromosome of *Pseudomonas*, the extra unwanted portion of the plasmid must be removed. This will leave behind the fusion, and remove the integrase, tetracycline resistance, and origin of replication.

To begin, a *Pseudomonas* colony grown on tetracycline supplemented PIA was picked and streaked onto YT tetracycline$^{50}$irgasan$^{25}$, and grown overnight at 42 $^{\circ C}$. Meanwhile, *E. coli* with the plasmid encoding Flp recombinase(pFLP$_2$) was grown overnight at 37 $^{\circ C}$ on LB ampicillin$^{100}$. Both strains were scraped from their plates with an inoculation loop and

resuspended in 500µL of 0.85% (w/v) saline. The suspension was plated on LB and incubated overnight at 30 $^{\circ C}$. The bi-parentally mated bacteria were removed from the plate and resuspended in 500µL of 0.85% (w/v) saline. *Pseudomonas* colonies were isolated by streaking 50 µL of the suspension onto Vogel-Bonner Minimal Media (VBMM) carbenicillin$^{300}$. After overnight incubation at 37 $^{\circ C}$, the colonies were patch plated onto PIA and YT tetracycline$^{50}$irgasan$^{25}$. Successful removal of the unwanted plasmid is determined by loss of the tetracycline resistance gene, resulting in tetracycline sensitive colonies. Tetracycline sensitive colonies were screened for the fusion via PCR.

Insertion of Deletion Mutation

During triparental mating with a suicide vector harboring a deletion mutation, homologous recombination occurs. This results in the same cell having two alleles for the same gene. This cell is referred to as a merodiploid. In this state, the cell must discard one copy of the gene; as it is undesirable to have multiple copies of the same gene. Therefore, after selecting for *Pseudomonas* colonies with appropriate antibiotic resistance, the colonies will have a period of recovery in LB broth without antibiotic pressure for a second cross-over event. During this time, the cell will discard either the wild-type gene or the mutated genetic sequence and keep the other. The suicide vector used, pEX18, contains the levansucrase encoding gene, *sacB.* This gene is used, in this case, as a counter-selectable marker. Bacteria that do not succeed at this second cross-over still encode the *sacB* gene or are wild-type. The production of levansucrase cleaves the sucrose present in the media. This results in the buildup of levans in the cells which becomes lethal.

A *Pseudomonas* colony was picked from the antibiotic supplemented PIA plate and placed in a microfuge tube containing 1mL of LB. The suspension was incubated at 37$^{\circ C}$ with

shaking at 250rpm for greater than one hour. Next, 50µL of the bacterial suspension was streaked for isolation on YT 10% sucrose and grown overnight at 30 $^{\circ C}$. Colonies that survive selection on YT 10% sucrose are patch plated on PIA and antibiotic supplemented PIA. Antibiotic sensitive colonies were PCR screened for the desired mutation. Given that a BamHI endonuclease site was removed from the mutated region in this study, subsequent restriction digests using BamHI was used to distinguish wild-type from mutant.

β-Galactosidase Assay

A Pseudomonas isolate was inoculated into 5ml of LB broth and incubated for 12-18 hours at 37$^{\circ C}$ with shaking. A 1% inoculum was used to subculture the strain into 5ml of fresh LB broth and grown for an additional 8 hours at 37 $^{\circ C}$ with shaking. Assays were performed by first, collecting and pelleting 500µl of the subculture at 8000XG for 10 minutes. The cell pellet was resuspended in 1ml of Z-buffer/2.7% β-mercaptoethanol and the optical density was measured at a wavelength of 600nm using a spectrophotometer. Permeabilization of the cells was achieved by adding 50µl of 0.1% SDS and 100µl of chloroform. The suspension was vortexed and incubated at room temperature for 10 minutes. Cell lysate was removed, 20-500µl, placed in a new microfuge tube and brought to 1ml with Z-buffer-2.7% β-mercaptoethanol. A 4mg/ml solution of 2-nitrophenyl-β-D-galactopyranoside (ONPG) in Z-buffer was prepared. For every reaction in each assay, 200µl of ONGP solution was added and a stopwatch was started. Once a pale-yellow color developed, 500µl of 1M $Na_2CO_3$ was added to end the reaction, the stopwatch was stopped, and the time recorded. The optical density was measured once more at wavelengths of 420nm and 550nm. The time (*t*), volume of lysate used (*v*), and the three optical density values were used to calculate Miller Units using the following equation:

$$\textbf{Miller Unit} = 1000 \times \frac{OD\ 420nm - (1.75 \times OD\ 550nm)}{t \times v \times OD\ 600nm}$$

Western Blot Analysis

Three mutation constructs were created using site-directed mutagenesis. The vector pEX18GM*rsmA*HA was used as template. Each construct deletes different lengths of the 5' UTR of *rsmA* within the proposed stem-loop region (Figure 11). Strains PAO1 and *mucA*22 were conjugated via triparental mating with each of the three mutant constructs. Strains were confirmed by first amplifying a region of approximately 600bp using primers *rsmA*SDMFcheck and HAR. This colony PCR confirms the presence of the *rsmA* HA epitope tagged allele. Secondly, the PCR fragments were digested using the endonuclease BamHI. Fragments that do not digest into two pieces indicate that the deletion mutation is present.

PAO1 and *mucA*22 strains harboring the deletion mutation were grown overnight in 5mL LB broth cultures at 37$^{\circ C}$ with shaking. Using a 1% inoculum (100µL) of the overnight culture, 10mL LB broths were setup for growth at 37 $^{\circ C}$ with shaking for 8 hours. Cells were pelleted by centrifugation at 8000XG for 10 minutes. The supernatant was removed, and the cell pellets were frozen at -20$^{\circ C}$ for at least 4 hours. All subsequent steps were performed on ice. Cell pellets were thawed and resuspended with 1mL of 0.85% saline. The suspensions were transferred to 2mL microfuge tubes. Thirty microliters of 20mg/mL lysozyme were added to the suspension, and then the total volume was brought up to 2mL with 0.85% saline. Enzymatic lysis of the cells lasted for 10 minutes. Three rounds of sonication, 20 seconds each, with at least 1 minute of rest in between each step was performed on every sample. After sonication, whole cell lysates were centrifuged at 8000XG for 10 minutes. Supernatant was transferred to fresh 2mL microfuge tubes and the protein content was quantified via Bradford Assay following manufactures protocol.

Ten nanograms of protein was mixed with 4X SDS PAGE loading dye (Appendix B) to a total volume of 10µL. The mixtures were heated to 92$^{\circ C}$ for 5 minutes and then chilled on ice for 5 minutes. A 4% stacking 15% separating, discontinuous SDS-PAGE gel was cast and pre-run for 30 minutes in 500mL 1X glycine buffer (diluted from 10X glycine buffer, Appendix B) at 165V(Table 4). The samples were loaded and ran at 180V until the dye-front was ¼ the length of the gel from the bottom.

Table 4: 4% Stacking 15% Resolving Discontinuous SDS-PAGE Gel

| Stacking Gel | |
|---|---|
| 30% (w/v) Acrylamide Solution | 5mL |
| 1.5 mol/L Tris/HCl Solution, pH 8.8 | 2.5mL |
| 10% (w/v) SDS Solution | 100µL |
| TEMED (tetramethylethylenediamine) | 10µL |
| Distilled Water | 2.39mL |
| 10% (w/v) Diammonium Peroxydisulfate Solution (APS) | 40µL |

| Resolving Gel | |
|---|---|
| 30% (w/v) Acrylamide Solution | 300µL |
| 0.5 mol/L Tris/HCl Solution, pH 6.8 | 500µL |
| 10% (w/v) SDS Solution | 20µL |
| TEMED (tetramethylethylenediamine) | 2µL |
| Distilled Water | 1.7mL |
| 10% (w/v) Diammonium Peroxydisulfate Solution (APS) | 6µL |

Polyvinylidene fluoride (PVDF) membrane was cut wide enough for all protein-loaded wells to fit; and long enough to ensure that RsmA is transferred to the membrane. The membrane was soaked in 100% methanol for 10 minutes before transferring. Meanwhile, 1X Towbin Electroblotting buffer prepared (Appendix B). Four sheets (two thick and two thin) of electroblotting paper were cut slightly larger than the PVDF membrane and soaked in electroblotting buffer. Once the gel had run a sufficient time, it was removed from the casting cassette and trimmed with a razorblade to remove excess gel. The electroblotting paper, PVDF membrane, and gel were assembled on a semi-dry electroblotting apparatus to transfer the proteins from the gel to the PVDF membrane. The proteins were transferred at 115mA for 90 minutes.

After transferring, the electroblotting apparatus was disassembled and the membrane was placed in 50mL of Blocking Buffer (Appendix B) and rocked for 2 hours at room temperature or overnight at 4$^{\circ C}$. The Blocking Buffer was poured off and replaced with 50mL of the Primary Antibody(1˚Ab) solution (Appendix B). The 1˚Ab was rocked for at least one hour at room temperature or overnight at 4$^{\circ C}$. The 1˚Ab was removed, and the membrane was washed with 50mL with Washing Buffer (Appendix B) for 15 minutes, three times. The Secondary Antibody(2˚Ab) solution was added and rocked for 1 hour at room temperature. The 2˚Ab was removed and the membrane was washed again following the same procedure.

In order to visualize the HA tagged proteins on the membrane a substrate must be added for the horseradish peroxidase to cleave. The Pierce™ ECL Western Blotting Substrate Kit was used to provide that substrate. A 3mL solution was made by mixing Detection Reagent 1 (peroxide solution) and Detection Reagent 2 (luminol enhancer) in a 1:1 ratio. The solution was pipetted evenly over the membrane and allowed to react for 10 minutes. Finally, the RsmA HA epitope tagged proteins were visualized using the Bio-Rad ChemiDoc™ Imaging System.

## Statistical Analysis

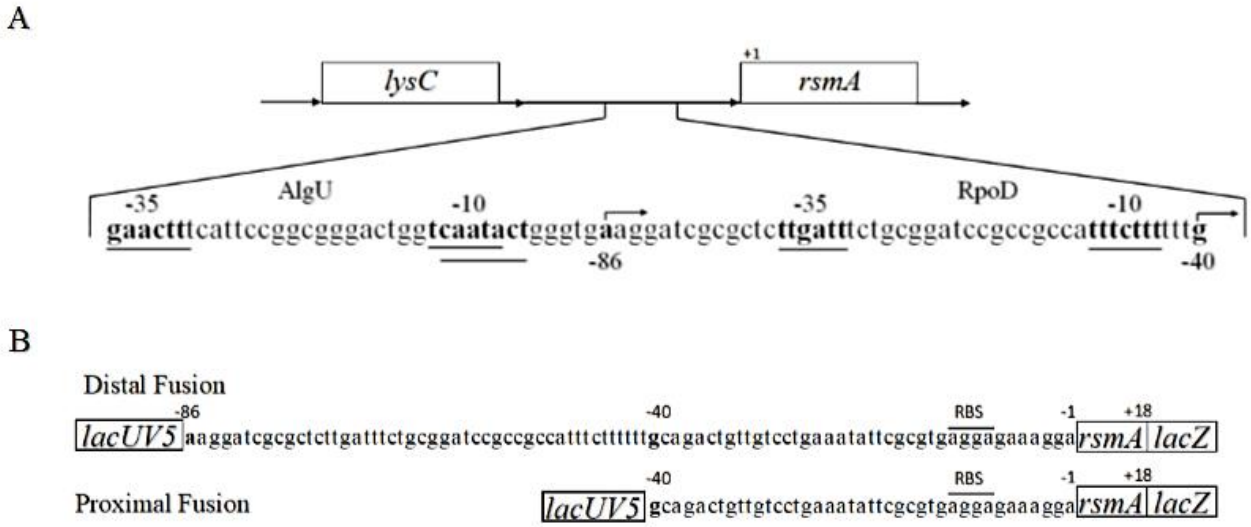
B-galactosidase assays were performed in technical and biological triplicate, resulting in a sample size of nine (n=9) for each strain in each assay. Western Blots were performed in biological triplicate (n=3). Values determined from assays were averaged and the standard error of the mean was determined to produce error bars. The Student's T-Test was used to determine statistical significance among experimental strains and are reported in figure legends.

# CHAPTER 3

# RESULTS

## The *rsmA* mRNA Transcript is Under Post-Transcriptional Regulation

The aim of this study was to investigate the possibility that RsmA is post-transcriptionally regulated. Initially, two *lacZ* translational leader fusions were constructed to establish the reporter activity from the two *rsmA* promoters. In both cases, the native promoter regions were replaced with the constitutively active *lacUV*5 promoter. Replacing the native promoters with *lacUV*5 removes variation caused by differing strengths of the native promoters (i.e. normalizing transcription by removing variation due to levels of AlgU and RpoD). Each fusion contains the native *rsmA* RBS, start codon (plus six additional codons), and the *lacZ* gene. For the first fusion, the *lacUV*5 promoter was placed upstream of the AlgU dependent TSS; henceforth referred to as the distal fusion (Figure 7. B.). The second fusion was constructed with the *lacUV*5 promoter upstream of the RpoD dependent TSS (Figure 7. B.). This will be referred to as the proximal fusion. Both fusion constructs were introduced into the chromosomes of PAO1 and *mucA22* at the *attB* site and analyzed via β-galactosidase assay.



C

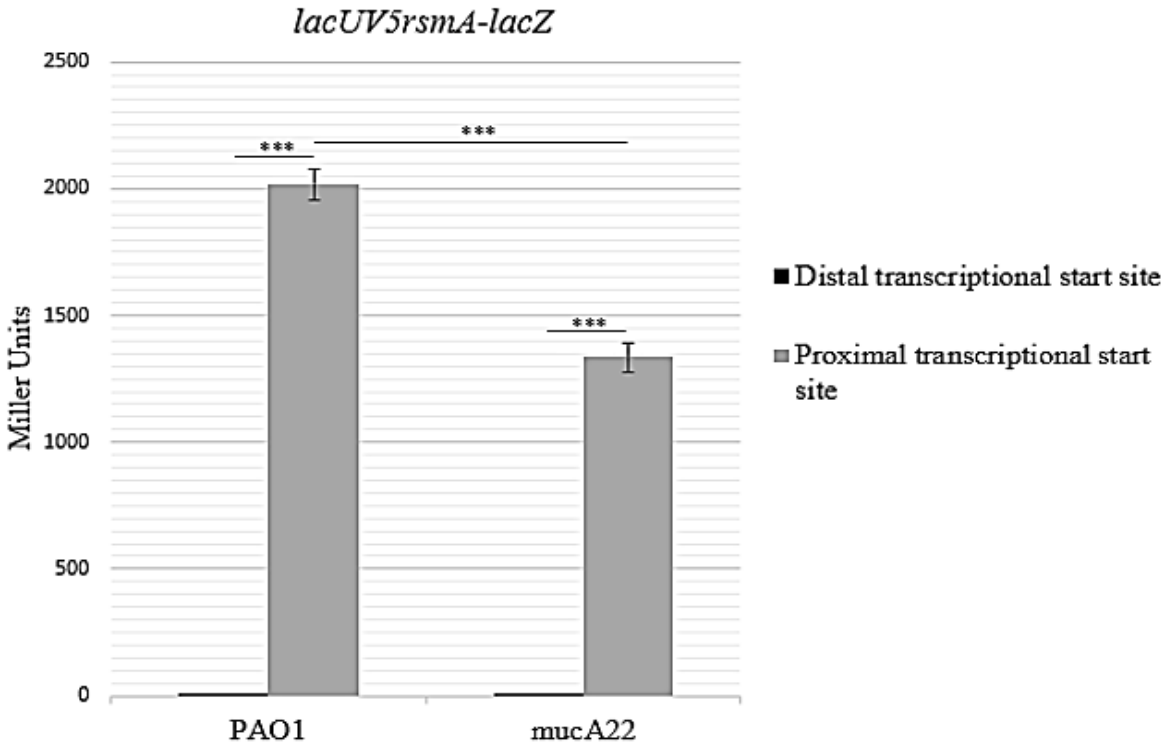



Figure 7. The RsmA mRNA Transcript is Under Post-Transcriptional Regulation. A.) Schematic of the intergenic regions between *lysC* and *rsmA.* The AlgU and RpoD dependent promoters are indicated by the underlined sequences. Transcriptional start sites are bolded with bent arrows overtop at -40 and -86. B.) Schematic of the Distal and Proximal leader fusions with the *lacUV5* promoter replacing the native promoters. Transcriptional start sites are bolded at -40 and -86 and the Ribosomal Binding Site is designated RBS. C.) Leader fusions were designed and introduced into the PAO1 and *mucA22* chromosomes. Both fusions were analyzed by β-Galactosidase assay. Assays were performed in triplicate with 8 hour LB broth cultures. Statistical analysis of expression: *mucA22* Distal vs. *mucA22* Proximal: $p<0.001$, PAO1 Distal vs. PAO1 Proximal: $p<0.001$, *mucA22* Proximal vs. PAO1 Proximal: $p<0.001$.

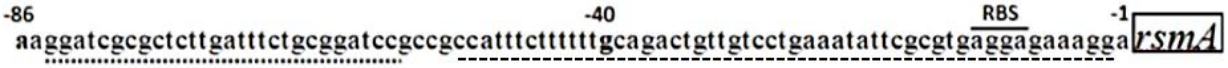
The distal fusion in PAO1 exhibited no reporter activity, while the proximal fusion expressed 2017 Miller Units (Figure 7.B). The same trend was seen between the two fusions in *mucA22.* Although, the proximal fusion expressed fewer Miller Units, 1335, than what was observed in PAO1 (Figure 7.B). The increase in activity of PAO1 over *mucA22* is statistically significant, $p<0.001$, and may suggest that RsmA is needed in higher abundance in PAO1. This result also calls into question the existence of a putative RpoD promoter since the distal fusion contains the RpoD promoter, yet expression cannot be detected. Previously, TFs designed to

investigate transcription from the distal promoter suggested that transcription does indeed occur from the distal promoter (Stacey and Pritchett 2016). The lack of expression from the distal fusion, coupled with previous transcriptional analysis, provide evidence that the mRNA transcript of RsmA is under post-transcriptional regulation.

The 5' Untranslated Region of the RsmA Transcript Contains Secondary Structures

Due to the length of the 5' UTR and the results obtained from the translational leader fusion analysis, the leader sequence was further analyzed. Bioinformatics obtained using the DNA and RNA folding software, mFold, predicted two secondary structures to form in the 5' UTR of the mRNA. The more 5' secondary structure lies between the two TSSs, starting at the third nucleotide after the distal transcriptional start site (-86) of the RsmA transcript (Figure 8.A). The structure forms a stem-loop consisting of 28 nucleotides. Seventeen nucleotides comprise the stem and the loop is 11 nucleotides in length; with the stem having 100% complementarity, the only exception being a one nucleotide bulge(Figure 8.B). The second structure also forms a stem-loop. It starts 13 nucleotides upstream of the proximal TSS and terminates immediately before the final nucleotide of the leader. The mFold output reveals that the RBS is occluded by four uracil's on the 5' stem of the second stem-loop. Low complementarity of the bases in the stem of the second stem-loop raises doubts on whether the second stem-loop is stable (Figure 8.B). The bioinformatics data cannot stand alone and will have to be further investigated using other methods.

A



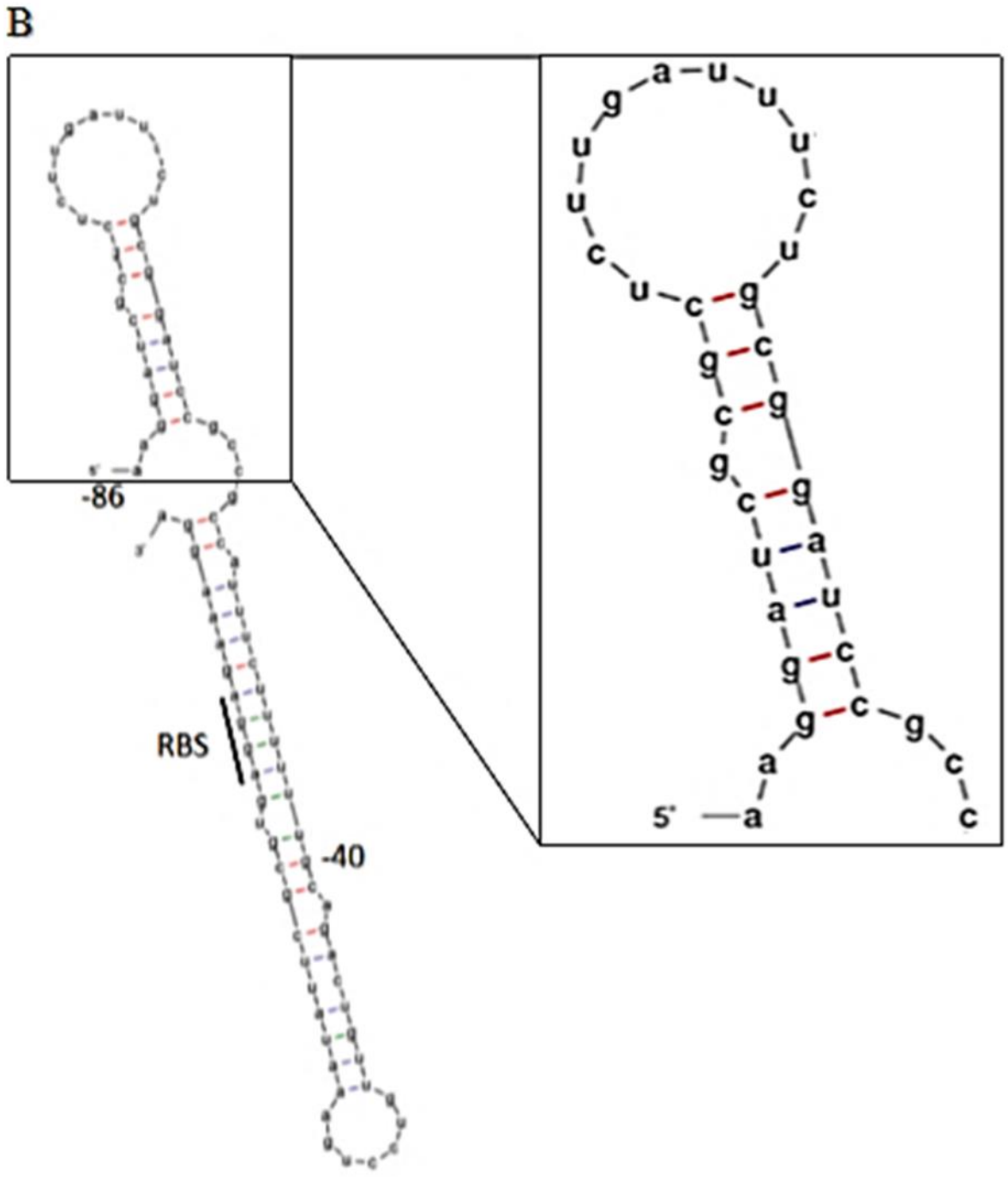



Figure 8. mFold Predicts the Formation of Two Stem-loops in the 5' Untranslated Region(UTR) of the RsmA Transcript. A.) Schematic of the 5' UTR. Proximal and distal transcriptional start sites(TSS) are bolded at -40 and -86, respectively. Underlined nucleotides indicate the location of the secondary structures predicted by mFold. B.) mFold output of the RsmA 5'UTR. Transcriptional start sites are marked at the -86 adenine and the -40 guanine. The ribosomal binding site is underlined and labeled RBS.

The Putative Distal Stem-loop Contributes to the Post-transcriptional Regulation of *rsmA*

To investigate the role of the putative distal stem-loop uncovered through bioinformatics, non-complementary nucleotide substitutions were made within the stem using site-directed mutagenesis. The first substitution was made at the third nucleotide of the mRNA transcript; the most 5' guanine at the base of the stem. The guanine was changed to an adenine and is referred to as G3A (Figure 9. A). An increase from zero to 72 Miller Units are observed with the substitution of just one nucleotide (Figure 9. B). Next, the first two 5' guanines were replaced with adenines (G3A/G4A) (Figure 9. A) and a 5-fold increase (361 Miller Units) over the initial single nucleotide substitution was observed (Figure 9. B). The final substitutions made were G3A/G4A/C7A/C9A (Figure 9. A). The result was a 6-fold increase (450 Miller Units) over the initial single nucleotide substitution (Figure 9. B). The increase of expression from the distal fusion with the nucleotide substitutions resembles a dose response curve (i.e. destabilizing the putative distal stem-loop results in increased expression of the fusion with the build-up nucleotide substitutions). These results suggest that the nucleotides substituted are involved in the regulation of the RsmA transcript, although the exact mechanism cannot be determined from this data.

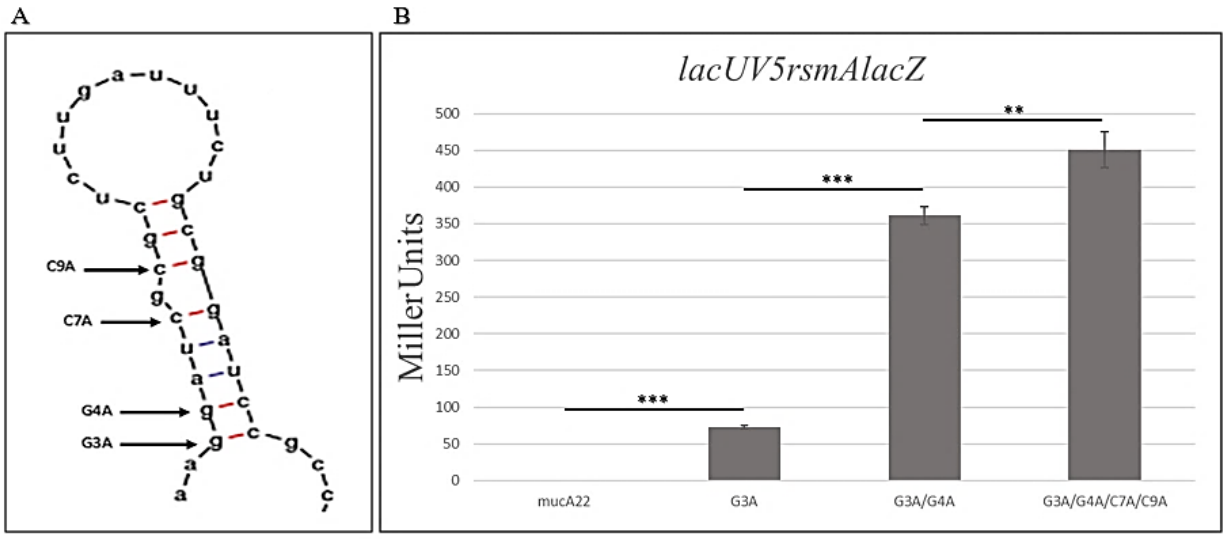



Figure 9. The Putative Distal Stem-loop Contributes to the Post-transcriptional Regulation of RsmA. A.) mFold output of the putative stem-loop. Arrows indicate the nucleotide substituted to adenines. B.) Distal fusions with substitutions were introduced into *mucA22* and analyzed by β-galactosidase assay. (*** $p<0.001$) (** $p<0.01$)

Translation of the RsmA Transcript is Not Attributed to the GacS/GacA TCS or RsmA

To determine if translation was inhibited by the GacS/GacA TCS or if RsmA was in a self-regulated feedback loop, I examined the distal fusion in *gacA* and *rsmA* single mutants in PAO1 and *mucA22.* The data obtained from the distal fusion in the *gacA* and *rsmA* single mutants mirrored the observation of the distal fusion in the wildtype strains. Since the Miller Unit equation uses time (*t*) as a variable, assays that take several hours and never produce a yellow pigment can result in reduced, or in this case, negative Miller Unit values (Giacomini et al. 1992). These data suggest that translation of the RsmA transcript is not influenced by the GacS/GacA TCS and that RsmA does not act as a translational repressor of its own message based on fusion design in this study. Tabulated raw data can be view in Table 7 of Appendix C.

The Putative Stem-loops are Not RNA Thermometers

To evaluate whether the putative stem-loops were temperature sensitive, PAO1 and *mucA22* harboring the distal fusion was grown at 30˚C, 37˚C, and 42˚C and evaluated by β-galactosidase assay (Figure 10). In both PAO1 and *mucA22* report activity was near undetectable levels at all temperatures tested. Reporter activity was completely undetectable in *mucA22* at 42˚C. These data suggest that the putative stem-loops are not RNA thermometers under the growth conditions that they were tested.

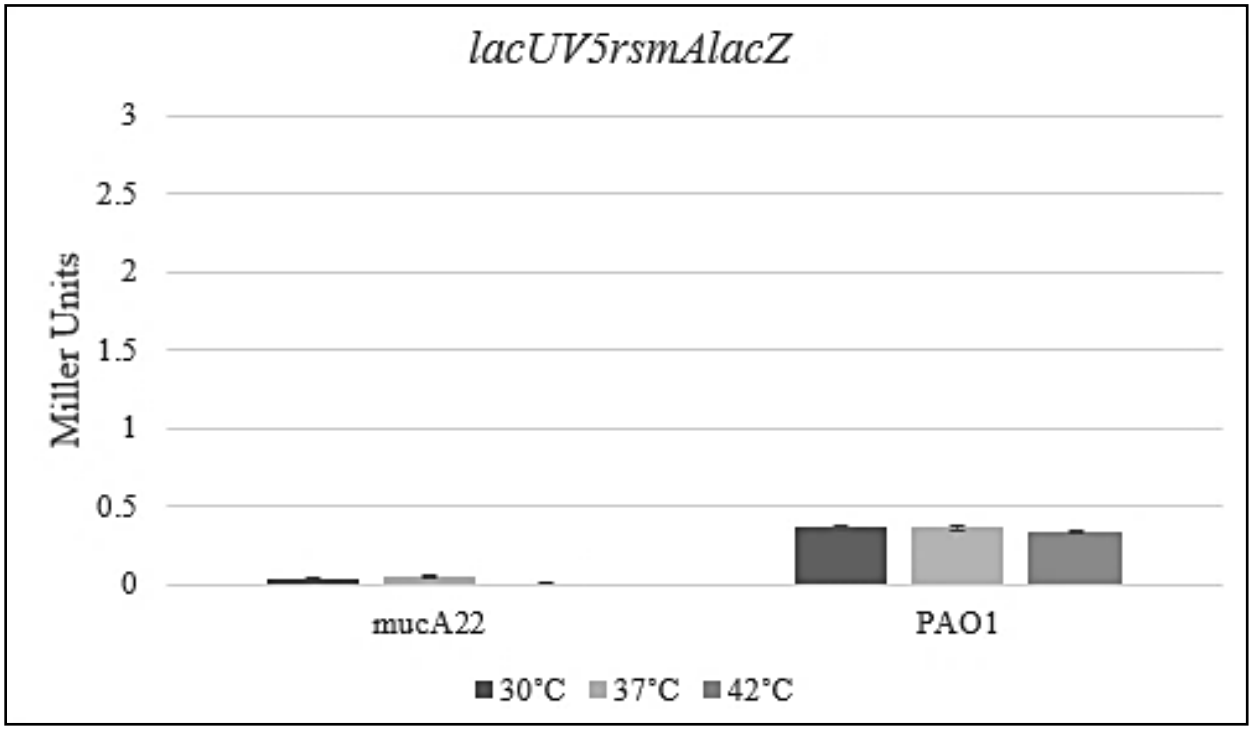


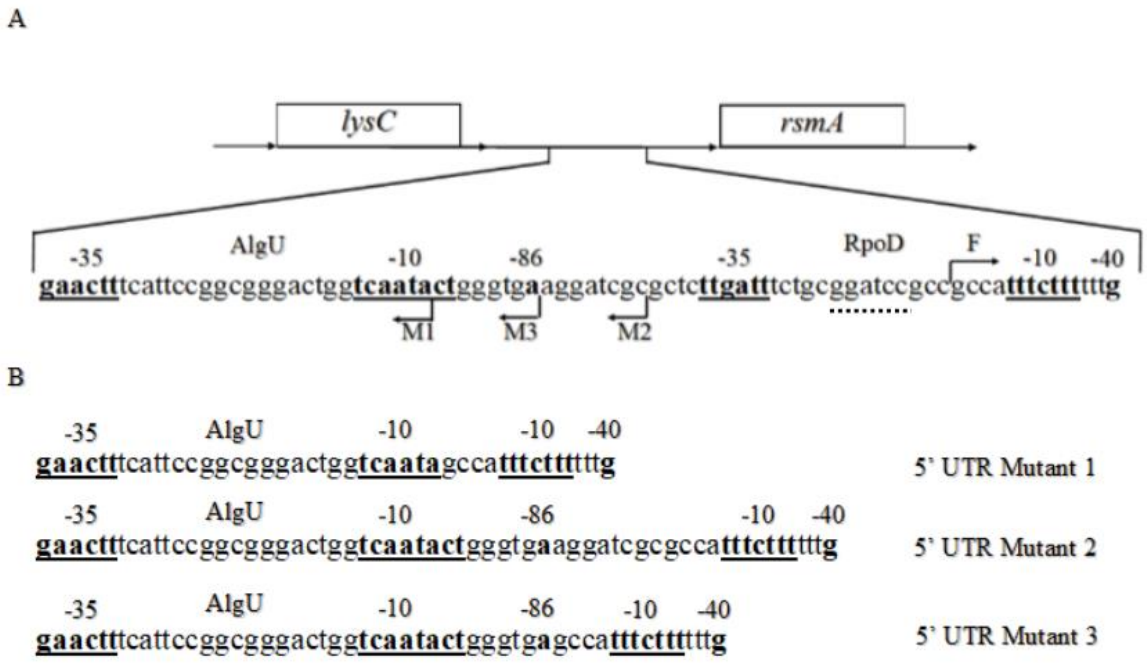

Figure 10. The Putative Stem-loops are Not RNA Thermometers. PAO1 and *mucA22* were grown at 30˚C, 37˚C, and 42˚C and examined via β-galactosidase assay. Reporter activity was near undetectable levels in both strains at all temperatures tested. No significant difference was found between the different growth temperatures.

Western Blot Analysis Provides Insight into RsmA Levels Among 5' UTR Mutants

The 5' Untranslated Region (UTR) was mutated by Site-Directed Mutagenesis (SDM) in the region of the putative distal stem-loop and flanking region. Three different mutations were designed to investigate if the UTR between the two putative promoters affected RsmA levels *in vivo.* The suicide vector pEX18gm containing the *rsmA* coding region with a 3' hemagglutinin (HA) tag (pEX18gm*rsmA*HA) with 1kb flanking either side of *rsmA* was used as template for the construction of the 5' UTR deletion mutants. All three mutant constructs used a common forward primer. As a consequence, the putative RpoD -35 has been deleted in all mutants but maintain the putative RpoD -10 plus four nucleotides directly upstream (gcca). Three different reverse primers were used to make various mutations (Figure 11. A). Deletions made in Mutant 1 include: the last two nucleotides of the AlgU -10 promoter, the distal TSS, the stem-loop, and finally the RpoD -35 (Figure 11. B). Mutant 2 restores the AlgU -10 and distal transcriptional start site and includes nucleotides -85 to -77 (5' stem of the stem-loop). Mutant 2 lacks the loop, the 3' stem portion of the stem-loop, and the RpoD -35 (Figure 11. B). Mutant 3 removes every nucleotide after the distal TSS through the forward primer annealing location at -54. This mutation removes the stem-loop and the RpoD -35 (Figure 11. B). Once the constructs were made and the correct sequences were verified, the constructs were introduced into the chromosomes of PAO1 and *mucA22* via homologous recombination, replacing the wild-type *rsmA* allele. All six mutants, PAO1*rsmA*HA wild-type, *mucA22rsmA*HA wild-type, and a negative control, PAO1 wild-type, were analyzed by western blot.

The western blot shows that RsmA is expressed to a greater extent in the *mucA22* relative to PAO1, which has been previously demonstrated in the literature (Stacey and Pritchett 2016). PAO1 and *mucA22* with the 5' UTR mutation 1 do not express RsmA. Expression is partially

restored in 5'UTR mutation 2 in *mucA22*, but RsmA remains undetectable in PAO1. RsmA in *mucA22* is restored to levels comparable to that seen in the wild-type strain the 5' UTR mutant 3, while PAO1 remains well below wild-type levels. The regions mutated are having various influences on the expression of RsmA in the two backgrounds tested. The increased levels of AlgU in the *mucA22* strain can explain why there is increased expression of RsmA compared to PAO1. Modification of the AlgU and/or RpoD promoter is likely the reason for the loss of expression found in Mutant 1. These data also suggest that the region between the two promoters is having a stronger influence on RsmA expression in PAO1.




(Figure 11 continued on next page.)

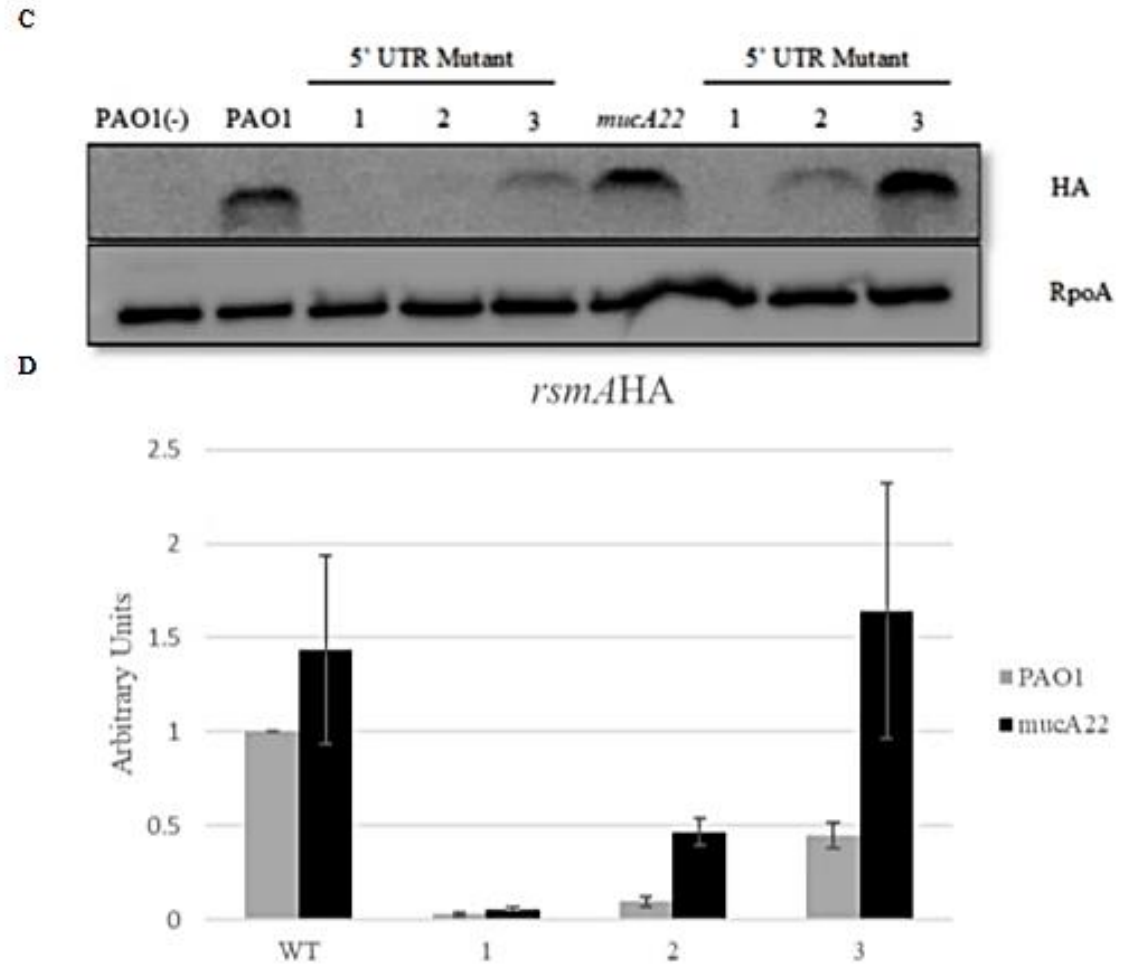



Figure 11. Western Blot Reveals that Regulation of RsmA is Multi-tiered. A.) Schematic of the 5' UTR of RsmA. The AlgU and RpoD -10 and -35 elements are underlined. The location of the three reverse primers used are indicated by M1, M2, and M3. The location of the common forward primer is indicated by "F." The BamHI restriction enzyme site is marked with a dotted underline. B.) Mutant 1, 2, and 3 genomic sequences. C.) Top panel: Western blot of PAO1 and *mucA22* wildtypes and mutants 1, 2, and 3 in both backgrounds. Bottom panel: RpoA loading control. D.) Densitometry of PAO1 and *mucA22* labeled as wildtype. Labels 1, 2, and 3 correspond to the three mutants.

# CHAPTER 4

# DISCUSSION

*Pseudomonas aeruginosa* is an infections Gram negative bacterium found in numerous environments and adhering to various surfaces. *Pseudomonas aeruginosa* has become a major health threat over the past century because of its adaptability, intrinsic antibiotic resistance, and its suite of virulence factors. Understanding the complex regulatory networks that control the expression of the genes used by *Pseudomonas* to cause disease is necessary to develop new treatments against this organism. Prokaryotes, as well as Eukaryotes, modify gene expression at the transcriptional, post-transcriptional, and post-translational level. In *Pseudomonas aeruginosa,* and other *Pseudomonads,* the post-transcriptional regulator RsmA, has implications in regulating carbon metabolism, virulence, motility, biofilm formation, and others. Studies have taken several approaches to determine the targets of RsmA and how RsmA is regulated at the post-translational level; however, very few researchers have investigated the transcriptional control of RsmA. Additionally, to my knowledge no one has investigated the possibility of post-transcriptional regulation of the RsmA transcript and this is the first study to do so. This investigation provides evidence that the RsmA transcript is under post-transcriptional regulation by using translational leader fusions. Using bioinformatics, this study identifies a secondary structure in the 5' UTR of the transcript that may provide the mechanism whereby the message is regulated. Western blot analysis, although inconclusive, provides insight into RsmA levels *in vivo* among the various 5' UTR mutants. Analyzing the distal fusion at 30˚C, 37˚C, and 42˚C suggests that the 5' UTR is not an RNA thermometer. Finally, my data rules out RsmA and the sRNAs, RsmY and RsmZ, as regulators of the RsmA transcript by analyzing the distal leader fusion in a Δ*rsmA* mutant and a Δ*gacA* mutant in both PAO1 and *mucA22.*

In the RNase protection assay (RPA) from a previous study (Stacey and Pritchett 2016), two RsmA transcripts of two lengths can be observed. The quantity of long to short RsmA transcripts appear to be equal in PAO1 and also in *mucA22*. However, there is a proportional increase in the amount of the shorter transcript relative to the longer transcript in the *mucA22* strain, which would not be expected if the proximal TSS was strictly under the control of the putative RpoD promoter. This observation was the first clue that the RsmA transcript could be under post-transcriptional regulation and that the short transcript could be a processed message.

Leader Fusion Analysis and Bioinformatics Provide Evidence of Post-transcriptional Regulation.

The translational leader fusions provide compelling evidence of post-transcriptional regulation of the RsmA transcript. The distal fusion contains the *lacUV5* constitutively active promoter in place of the AlgU dependent promoter. This allows for continuous transcription of the message because it functions independently of activator proteins (Noel and Reznikoff 2000). In addition to the *lacUV5* promoter, the distal fusion maintains the putative RpoD promoter, and yet, reporter activity in this fusion is undetectable in both PAO1 and *mucA22*. If transcription and subsequent translation was occurring from the proximal TSS it should have been detectable in this distal fusion. Interestingly, reporter expression is only seen in the proximal fusion. These data and the two transcript observation in the RPA (Stacey and Pritchett 2016) support the notion of a post-transcriptional processing event that leads to expression of RsmA.

Given the length of the UTR, at least 86 base-pairs, the possibility of post-transcriptional regulation is greater than that of shorter (<40nt) UTRs (Svensson and Sharma 2016). mFold predicts the formation of two stem-loops in the 5' UTR of the RsmA transcript. The more distal one lies between the two putative promoter. The second starts 13 nucleotides upstream of the putative RpoD promoter and extends to the -2 nucleotide. The proximal stem-loop has low

complementarity; however, it occludes the RBS with four uracil's. Given that, I investigated whether the proximal stem-loop was acting as a Four U RNA thermometer. B-galactosidase assays were performed using cultures of PAO1 and *mucA22* grown at 30˚C, 37˚C, and 42˚C. No difference was seen between these growth conditions. The stem-loop acting as RNA thermometer was ruled out as a possible function.

Knowing that secondary structures have regulatory functions, I decided to perform site-directed mutagenesis within the region of the putative distal stem-loop. Substituting nucleotides resulted in a dose-dependent effect. An increase in reporter expression occurred with the addition of a single and with multiple nucleotide substitutions in the distal fusion. Since changing the nucleotide sequence led to reporter activity I questioned whether the sRNAs, RsmY and RsmZ, or RsmA was recognizing and binding this region. Since GacA activates RsmY/Z transcription, I analyzed the distal fusion in a Δ*gacA* mutant in both PAO1 and *mucA22.* Neither PAO1Δ*gacA* or the *mucA22*Δ*gacA* double mutant had β-galactosidase activity, thus ruling out another possible mechanism for post-transcriptional regulation of RsmA. To determine if RsmA was regulating itself, I performed β-galactosidase assays on PAO1Δ*rsmA* and *mucA22*Δ*rsmA.* Again, like the Δ*gacA* mutant, no change was seen between the mutants and wild-type strain. One study has shown RsmA autoregulation by translational fusion analysis and RsmA-*rsmA* interaction by RNA mobility shift assay (Jean-Pierre et al. 2015). However, there results indicate that RsmA inhibits translation by binding within the *rsmA* coding region. Their translational fusions extend further into the coding region than the one used in this study. This can explain the discrepancy between the two results. Together, these data provide compelling evidence that the RsmA transcript is under post-transcriptional regulation and suggests that the region between the two promoters provides a regulatory function unaffected by RsmY/Z or RsmA.

Further experiments will have to be performed to confirm these data. For example, the mFold output will have to be confirmed by RNA structural studies. There are several chemical and enzymatic techniques that have been developed to analyze secondary structures in RNA. An RNA footprinting technique using ribonuclease T1 can be employed to establish the existence of any RNA secondary structures within the 5' UTR, as ribonuclease T1 can only cleave single stranded RNA (Ziehler and Engelke 2001).

Primary transcripts will have a 5' triphosphate. If the shorter transcript observed in the RPA is due to a post-transcriptional cleavage event, then the shorter transcript will have a 5'monophosphate. An assay like the Phosphorylation Assay by Ligation of Oligonucleotides (PABLO) can be used to determine the 5' phosphorylation state of the transcript (Celesnik et al. 2007).

Western Blot Reveals that Regulation of RsmA is Multi-tiered

To look at protein expression and the effect of mutations in the 5' UTR *in vivo,* I performed western blot analysis. The western blot made the role of the 5' UTR in post-transcriptional regulation less clear as it cannot distinguish between transcriptional or post-transcriptional effects. My data supports previous observations of increased RsmA in the *mucA22* background (Stacey and Pritchett 2016) suggesting that RsmA may have a significant role in chronic infections.

Removing the AlgU and RpoD -35 element and distal TSS in mutant one abolished RsmA expression. This result was expected and further supports a previous experiment in our lab (Stacey and Pritchett 2016), concluding that the interrupted region is required for RsmA expression. The results from mutant two are more difficult to assign an explanation. This mutant has a complete AlgU promoter, distal TSS, and the 5' stem nucleotides of the putative stem-loop.

Although expression of RsmA is not completely abolished in *mucA22*, it is still well below wildtype expression and expression in the PAO1 strain was still undetectable. Interestingly, both mutant one and mutant two resemble a Δ*rsmA* mutant phenotype when grown on PIA. Δ*rsmA* mutants secrete more pyocyanin resulting in blue pigmented colonies and media. The observation that mutant one and mutant two were blue when grown on PIA supports the western blot data, suggesting that RsmA is not expressed in these strains. Possibly the most intriguing results were those obtained from mutant three. The entire stem-loop and every nucleotide upstream of the forward primer annealing location are deleted. There is only a slight difference in expression of RsmA between wildtype and mutant three in the *mucA22* background, suggesting that the distal promoter is sufficient in activating RsmA expression in this strain. However, the regulatory function of the putative distal stem-loop still cannot be assigned based on this observation. RsmA expression in mutant three of PAO1 was half of that observed in the wildtype strain and does not mirror what was seen in *mucA22*. This suggests that the putative distal stem-loop has a very important function in PAO1.

The western blot data is preliminary. The mutant constructs should be redesigned to provide consistency between the leader fusion nucleotide substitution experiment and western blot analysis. If RsmA expression is different than wildtype, this experiment would provide even more support that the region of the putative distal stem-loop plays a vital role in RsmA expression *in vivo*. Additionally, mutant constructs using the pEX18GM*rsmAHA* suicide vector should be made that would include the entire RpoD promoter to prove or disprove its existence. SDM mutants should be made in the region that composes the proximal stem-loop to investigate its role in RsmA expression.

The importance of RsmA has been demonstrated in several studies. The comparison of the transcriptome in wildtype versus a *rsmA* mutant has been conducted in PAO1 and PKA strains of *Pseudomonas aeruginosa* (Burrowes et al. 2006; Brencic and Lory 2009). In both cases, the regulon of RsmA has consisted of over 500 genes. However, only 67 genes were common between the two strains in those studies. Given the observation that there is more RsmA in the *mucA22* strain begs the question of its role in chronic infections. Conducting a transcriptomic or proteomic analysis of RsmA in the *mucA22* background could provide additional targets of RsmA and potentially new targets for treatments in chronic infecting strains. Developing a treatment for chronic infecting strains would have a positive impact on the 70,000 individuals living with Cystic Fibrosis around the world.

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# APPENDICES

## Appendix A: Media

### 1% Agarose Gel (yields two gels)

0.5g Agarose
5mL 10X TBE (see 10X TBE)
45mL $ddH_2O$

Heat, using microwave, for 1 minute

Add 2.7µL Ethidium Bromide before pouring into molds and container is warm to the touch

### 10X Vogel-Bonner Minimal Media (VBMM) Salts – 500ml

15g Trisodium Citrate
10g Citric Acid
50g $K_2SO_4$
17.5g $NaNH_4PO_4 \cdot 4H_2O$
$ddH_2O$ to 500ml
pH to 7  autoclave

### Luria-Bertani (LB) 1-Liter

15g Agar
10g NaCl
10g Tryptone
5g Yeast Extract
1L $ddH_2O$

Autoclave

Antibiotic Concentrations:
100µg/mL Ampicillin
15µg/mL  Gentamycin
50µg/mL  Kanamycin
10µg/mL  Tetracycline

### Yeast-Tryptone 10% Sucrose 1-Liter

15g Agar
10g Tryptone
5g Yeast Extract
800mL $ddH_2O$

Autoclave – Allow to cool

200mL 50% D-Sucrose

Yeast-Tryptone $tet^{50}$ $irg^{25}$ 1-Liter

15g Agar
10g Tryptone
5g Yeast Extract
25mg Irgasan

Autoclave

50µg/mL Tetracycline

Pseudomonas Isolation Agar (PIA) 1-Liter

13.6g Agar
20mL Glycerol (2%)
25mg Irgasan
10g $K_2SO_4$
1.4g $MgCl_2$
20g Peptone
980mL $ddH_2O$

Autoclave

Antibiotic Concentrations:
150µg/mL Gentamycin

Vogel-Bonner Minimal Media (VBMM) 1- Liter

450ml $ddH_2O$
7.5g Agar

Autoclave then allow to cool to ~$50^{\circ C}$

50ml 10X VBMM salts
500µl 1M $MgSO_4$
50µl 1M $CaCl_2$

Antibiotics:
300µg/ml Carbenicillin

# Appendix B: Buffers

0.5M Tris pH 6.8
6.1g Tris base
90ml $ddH_2O$
pH to 6.8 with 12N HCl

$ddH_2O$ 100ml

1.5M Tris pH 8.8
23.6g Tris base
90ml $ddH_2O$
pH to 8.8 with 12N HCl

$ddH_2O$ to 100ml

10X Glycine Running Buffer
144.1g glycine
10g Sodium dodecyl sulfate (SDS)
30.3 Tris base
$ddH_2O$ to 1 liter

10X TBE
55g Boric Acid
40ml 0.5M EDTA
108g Tris base
$ddH_2O$ to 1 liter

10X TBS
8.76g NaCl
2.42g Tris base
$ddH_2O$ to 100ml

10X TE
55g EDTA
108g Tris base
$ddH_2O$ to 1 liter

10X TNE
3.7g EDTA
116.8g NaCl
12.1g Tris base
$ddH_2O$ to 1 liter

10X Towbin Electroblotting Buffer
144g glycine

30.3g Tris base
dd$H_2O$ to 1 liter

4X SDS Loading Buffer
0.5mL β-mercaptoethanol
0.5mL 1% Bromophenol Blue
2.2mL 20% SDS
4.4mL 0.5M Tris HCL pH6.8
4.4mL Glycerol

Store at -20$^{\circ C}$

1X TBS-T
100ml 10X TBS
500µl of Tween 20
dd$H_2O$ to 1 liter

1X Towbin Electroblotting Buffer
10ml 10X Towbin Electroblotting buffer
20ml Methanol
70ml dd$H_2O$

1˚ Antibody Wash
5ml TBS-T buffer
45ml dd$H_2O$
2.5µl mouse/anti-HA antibody (1: 10,000)

2˚ Antibody Wash
5ml TBS-T buffer
45ml dd$H_2O$
2.5µl goat/anti-mouse antibody with conjugated horseradish peroxidase (1:10,000)

Blocking Buffer
2.5g Skim milk powder
5ml 1X TBS-T buffer
45ml dd$H_2O$

Washing Buffer
5ml 1X TBS-T
45ml dd$H_2O$

Z buffer
750mg KCl
246mg $MgSO_4 \cdot 7H_2O$
16.1g $Na_2HPO_4 \cdot 7H_2O$
5.5g $NaH_2PO_4 \cdot 4H_2O$

$ddH_2O$ to 1 liter
pH to 7

*β-galactosidase assay: add 2.7µl/ml β-mercaptoethanol before use.

<u>30% (v/v) Glycerol</u>

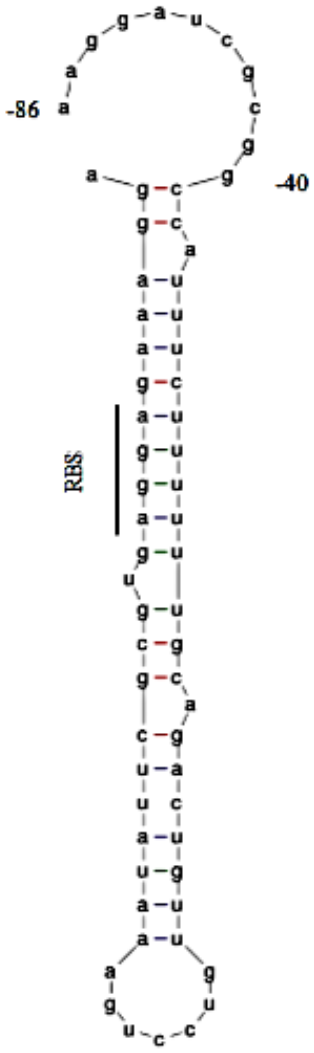
30mL Glycerol
70mL $ddH_2O$
Autoclave

Appendix C: Supplemental Material

5’ UTR Mutant 1

N/A

5’ UTR Mutant 2



5’ UTR Mutant 3

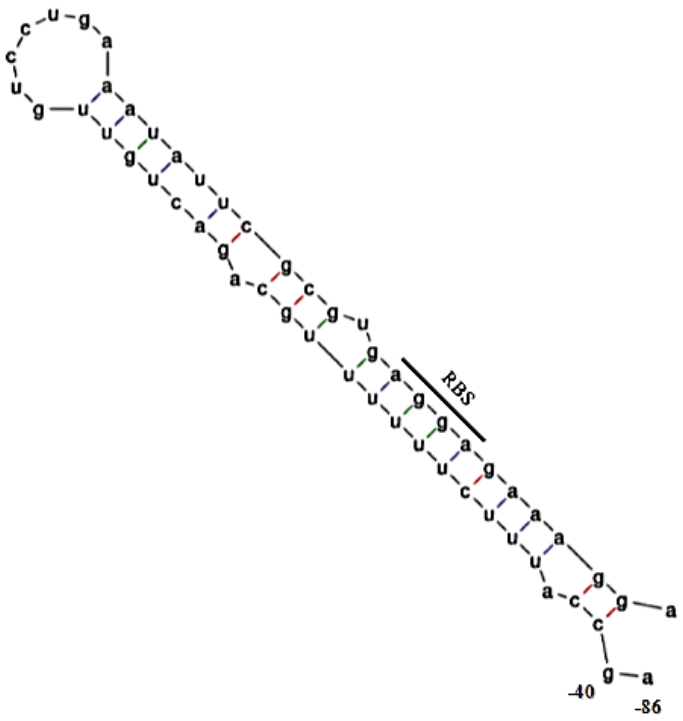



Table 5. Primers

| Primer Name | Sequence | Use | Notes |
|---|---|---|---|
| rsmAtransIR2 | GCGCAGTACTACGAGTCAGAATCAGCATTCCTTTC | Leader Fusion | Translational leader fusion common reverse primer |
| rsmAstemloopmut1F | GCGCGAATTCGAAAAATCGCGCTCTTG | Leader Fusion | G3A/G4A |
| rsmAstemloopmut2F | GCGCGAATTCGAAAAATAGAGCTCTTG | Leader Fusion | G3A/G4A/C7A/C9A |
| rsmAstemloopmut4F | GCGCGAATTCGAAAGATCGCGCTCTTG | Leader Fusion | G3A |
| rsmASDMcheckF | GCCAAGGTTTCCATCGTCGG | PCR Check | |
| HAR | GCGTAATCTGGAACATCGTATG | HA Allele Check | |
| lacZRforTF | GATGTGCTGCAAGGCGATTAAG | PCR Check | |
| CTXlacZF | CATCCACCGGCGCGCAATTAAC | PCR Check | |
| rsmAstemloopSDM3 | CGCGATCCTTCACCCAGTATTG | SDM | 5' UTR mutant 2 |
| rsmAstemloopTSSR | TCACCCAGTATTGACCAGTCCC | SDM | 5' UTR mutant 3 |
| B2BstemloopSDMR | TATTGACCAGTCCCGCCGGAAT | SDM | 5' UTR mutant 1 |
| SDMrsmAStemloopF | GCCATTTCTTTTTTGCAGACTGTT | SDM | 5' UTR mutant common forward primer |

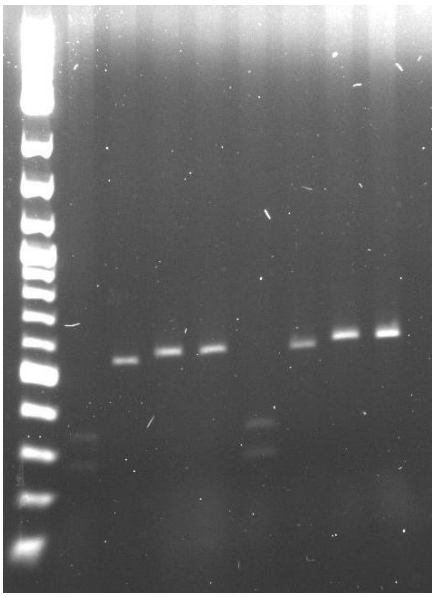
Table 6. Plasmids

| Plasmid | Use |
|---|---|
| *lacUV5*CTX.CP | Translational leader fusions |
| pEX18gm*rsmAHA* | 5' UTR mutants |
| pRK2013 | used for triparental mating |
| pFLP2 | encodes FLP recombinase |

Table 7. *gacA* and *rsmA* Single Mutant Leader Fusion Tabulated Raw Data

| | Δ*gacA* | Δ*rsmA* |
|---|---|---|
| *mucA22* | -0.3443 | -4.4341 |
| PA01 | 2.37254 | -0.1949 |

Site-Directed Mutagenesis Conformation Agarose Gel



All PCR products were digested with BamHI prior to visualizing on the agarose gel. Lanes 2 and 6 contain PAO1*rsmA*HA and *mucA22rsmA*HA, respectively, and digest into two bands because the BamHI site has not been deleted from the construct. *Lane* 1: 2-log Ladder. Lanes 2-5: PAO1*rsmA*HA, PAO1*rsmA*HA 5' UTR Mutant 1, PAO1*rsmA*HA 5' UTR Mutant 2, PAO1*rsmA*HA 5' UTR Mutant 3, respectively. Lane 6-9: *mucA22rsmA*HA, *mucA22rsmA*HA 5' UTR mutant 1, *mucA22rsmA*HA 5' UTR mutant 2, *mucA22rsmA*HA 5' UTR mutant3, respectively.

# VITA

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| | |
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