Electronic Theses and Dissertations   Student Works

8-2013

Regulating rsmA Expression in Pseudomonas aeruginosa

Sean D. Stacey
East Tennessee State University

Regulating *rsmA* Expression in *Pseudomonas aeruginosa*

_____

A thesis

presented to

the faculty of the Department of Biology

East Tennessee State University

in partial fulfillment

of the requirements for the degree

Master of Science in Biology

_____

by

Sean Denver Stacey

August 2013

_____

Dr. Christopher Pritchett, Chair

Dr. Eric Mustain

Dr. Bert Lampson

Keywords: *Pseudomonas aeruginosa*, RsmA, mucoid

ABSTRACT

Regulating *rsmA* Expression in *Pseudomonas aeruginosa*

by

Sean D. Stacey

Pseudomonas aeruginosa, a Gram-negative bacillus, commonly infects immunocompromised individuals and uses a variety of virulence factors to persist in these hosts. The posttranscriptional regulator, RsmA, plays a role in the expression of many virulence factors in *P. aeruginosa*. RsmA up regulates virulence factors used in colonizing hosts. However, regulation of *rsmA* is not well elucidated. Transposon mutagenesis was performed on *P. aeruginosa* containing a transcriptional *rsmA-lacZ* fusion to answer this question. Mutants were screened via β-galactosidase assay and transposon insertions identified via arbitrary PCR. A probable MFS transporter, we named *mtpX*, was one significant transposon mutant identified. A Δ*mtpX* mutant containing the *rsmA-lacZ* transcriptional fusion was constructed to confirm our results. Further analysis of *rsmA,* looking at RNA and protein levels, revealed varying results in nonmucoid versus mucoid backgrounds. Phenotypic assays were performed to characterize this unknown transporter and develop a putative mechanism as to how MtpX affects *rsmA* expression.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacillus capable of colonizing a wide variety

of environments in part because of its ability to metabolize an array of carbon sources (1).

However, in human hosts the bacterium is an opportunistic pathogen. *P. aeruginosa* most

commonly infects the immunocompromised hosts. Individuals with Human Immunodeficiency

Virus (HIV) infections, severe burns, or cystic fibrosis (CF) are at an increased risk for *P.

aeruginosa* infection (5,40). Additionally, *P. aeruginosa* is frequently associated with infections

of the ears, eyes, and the urinary tract (1,10,40). Indeed, *P. aeruginosa* is capable of infecting

any tissue in the human body owing to the myriad of virulence factors produced by this

bacterium. In addition, *P. aeruginosa* biofilms are recalcitrant to antibiotic therapy and treatment

of *P. aeruginosa* infections is difficult.

Virulence Factors

Colonization is a pivotal step for the bacterium in a human host. *P. aeruginosa* has 2

virulence factors that aid in the motility of the organism, a single polar flagellum and type IV

pili(1,10). A flagellum is a tail-like propeller on the cell. An ATP motor in the inner cell

membrane powers the flagellum. The flagella can be examined in vitro using swimming or

swarming assays on agar plates of various agar concentrations. While flagella are an important

virulence factor, flagella are recognized by the innate immune system. Toll-like receptor

5(TLR5) specifically recognizes the flagellum protein flagellin and stimulates inflammation

(8,10). *P. aeruginosa* uses the AprA enzyme to evade the immune response by cleaving the

flagellum into units TLR5 cannot bind (2). However, without flagellar motility *P. aeruginosa* is

known to be less virulent or attenuated (10).

Type IV pili are also used for motility as well as adhering to epithelial cells inside the host. These small appendages are composed of multiple proteins that can extend and retract in to the bacterial cell (14,25). This type of cellular movement is termed twitching. Like flagella, type IV pili are also recognized by the immune system and can activate the inflammasome, a group of proteins that produce inflammatory cytokines (23). Pili are important for adeherence to epithelial cells as well as form biofilm formation and development (29).

P. aeruginosa also possesses the type III secretion system that is important in acute infections. This virulence factor is a needle-like projection from the bacterium that directly injects bacterial toxins into the host cells leading to cell death (23). *P. aeruginosa* uses the type III secretion system to inject several toxins, like ExoS and ExoT, into the host cell (12). Like type IV pili, this virulence factor can also activate the host's inflammasome (23). The type III secretion system is important in acute infections but plays little or no role in chronic infections (6).

When an acute infection persists or is left untreated, *P. aeruginosa* can become a chronic infection. The change from acute to chronic infection involves a change in the virulence factors expressed. For example, type IV pili and the type III secretion system are down regulated when *P. aeruginosa* begins expressing a type VI secretion system and alginate production increases (6,29). The type VI secretion apparatus is still not completely understood, but it functions similarly to the type III secretion system by infecting enzymatic compouds into host cells or unrelated bacteria (6). The down regulation of virulence factors such as type IV pili and the type III secretion system may allow *P. aeruginosa* to persist in the host by preventing its detection from the immune system.

A major factor allowing *P. aeruginosa* to evade the host's immune system is its ability to form biofilms. *P. aeruginosa* secretes exopolysaccharide in both acute and chronic infection states (13). The biofilm creates a protective barrier around the bacterial growth and inhibits chemotherapeutic agents from reaching the cell as well as prohibiting phagocytosis by immune cells (13). The lungs of individuals with cystic fibrosis create thick, sticky mucus, which acts like a biofilm because of a mutation in the cystic fibrosis transmembrane conductance regulator protein (7,36). However, when strains of *P. aeruginosa* are isolated from these individuals, they begin to make their own biofilm of alginate similar to that found in the CF lung (6). Alginate has been shown to inhibit the activity of reactive oxygen species and to prevent phagocytosis (7,13,40). Several regulators of biofilm formation and development have been described (6, 18, 19, 29) One important regulator of biofilm development and virulence factor regulation is RsmA, a posttranscriptional regulator(4).

RsmA

In *P. aeruginosa*, RsmA, or regulator of secondary metabolites, is a crucial protein that regulates many genes including those important for virulence and biofilm formation (4,5). It has a predicted molecular weight of 6.9 kilodaltons (kDa)(4). RsmA recognizes the sequence GGA on messenger RNA (mRNA) and binds this sequence (4,19). By binding mRNA, RsmA inhibits ribosomal attachment and the bound message is not translated (Figure 1). This is a form of direct negative regulation. By posttranscriptional regulation RsmA is able to regulate multiple genes, most importantly virulence factors. Previous studies have shown that in PAO1 and PA14, RsmA regulates virulence factors that initiate colonization like type IV pili, type III secretion system, as well as iron acquiring compounds by increasing the expression of these genes (4,5). By binding mRNA to be translated to make proteins that would suppress these genes, RsmA is able to

increase these acute infection genes' expression. Conversely, RsmA will also down regulate the expression of virulence factors that appear in the chronic infection state (4,5). RsmA also shares homology with the *Escherichia coli* protein CsrA (4,26).
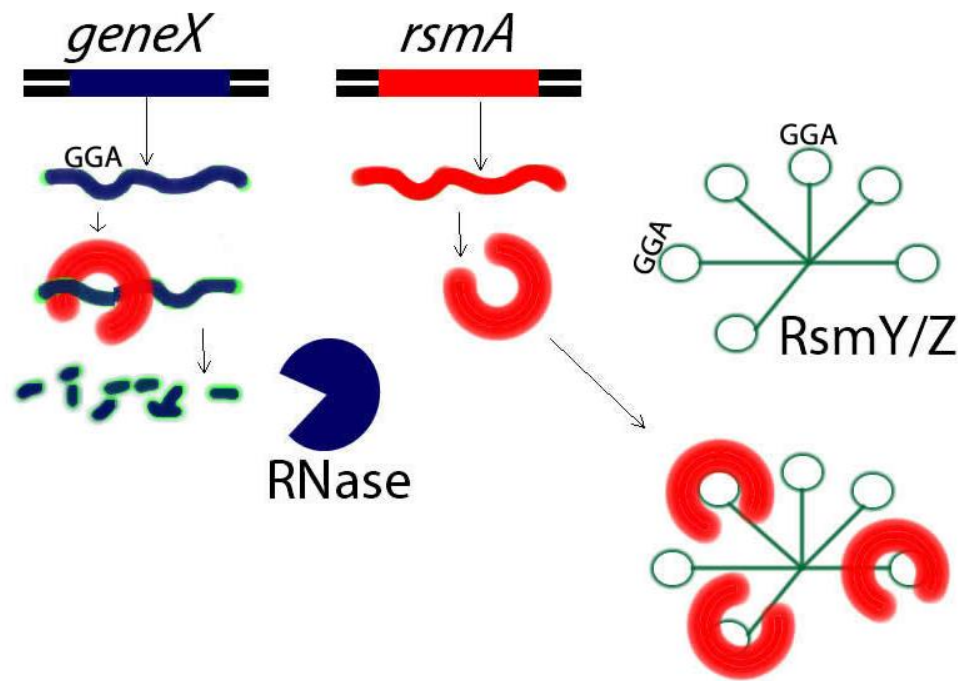
Figure 1. Diagram of RsmA function

RsmA has 2 known antagonists, the small RNAs, RsmY and RsmZ, homologous to CsrB and CsrC in *E. coli* (4). These small RNAs have numerous GGA repeats that can bind more than one RsmA at a time (4,19). RsmY and/or RsmZ bind to RsmA and prevent RsmA's regulatory activity (4,19). These antagonists are the downstream effect of a larger system that is affected by quorum sensing (QS), one way for cells to respond to environmental stresses (18). The GacA/S 2-component system, a part of QS, positively regulates the small RNAs that in turn suppress RsmA's functioning(18).

Previous work and our own have demonstrated that RsmA expression is maximal in the log

phase of growth (4, Figure 2). *mucA22* has a single guanine missing in the antisigma factor gene,

mucA, causing increased alginate production and a mucoid strain phenotype (24). To begin to

better understand the regulation of RsmA in both the wildtype strain of *P. aeruginosa*, PAO1,

and a strain representing a chronic infection state, *mucA22* (Figure 2), Western blot analysis was

performed. The Western Blot analysis shows that RsmA's presence increases in log phase and

begins to diminish in stationary phase. We also saw that RsmA is present longer and in greater

amounts in PAO1 than *mucA22*(Figure 2).
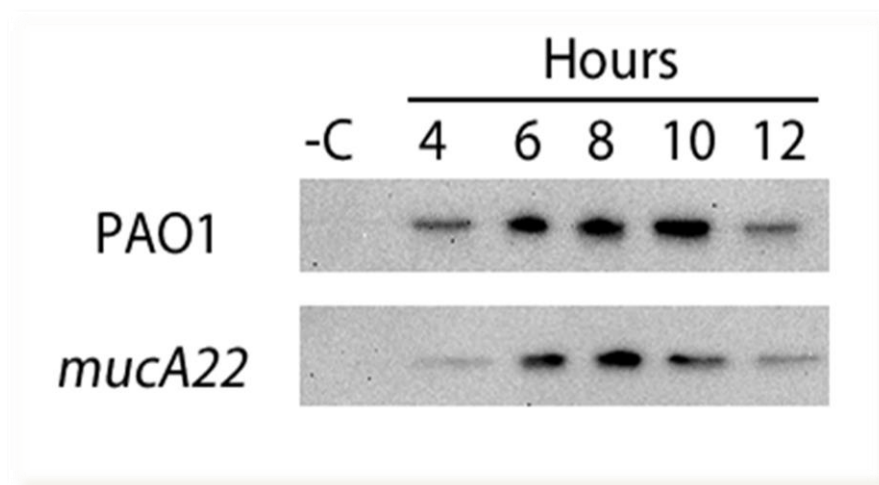
Figure 2. RsmA-HA tagged Western Blot Analysis over 12 hours with 10 μg of protein. We
analyzed both the nonmucoid strain PAO1 and the mucoid strain *mucA22.* A negative of control
of PAO1 without HA-tagged RsmA was used. Samples were taken from a 500 ml broth culture
seeded with 1% of an overnight culture at 0 time point.

These data sparked our interest in studying RsmA in the *mucA22* strain. Another question

we wanted to answer was, "What genes are regulating *rsmA* expression?" Previous microarray

and and transcriptome analysis in PAO1 and PA14 strains (representations of nonmucoid strains)

have better defined the role of RsmA in the cell (4,5).

Identifying *rsmA* Regulators

Two orphan sensor histidine kinases, RetS and LadS, are important in controlling the amount of free RsmA in the bacterial cell (19,27). However, little is known about the regulators of *rsmA*. Additionally, no studies have examined the role of RsmA in the mucoid background.

An *rsmA-lacZ* transcriptional fusion was assayed in the *mucA22* strain to begin to understand the role of RsmA in chronic-infecting strains. This fusion was designed based on a primer extension of *rsmA* (Figure 3). This extension showed that there are 2 transcriptional start sites for *rsmA*. Both of the promoters for these start sites were included in the fusion. One of the putative promoters fits the consensus sequence as an AlgU-dependent promoter (33). This is the promoter that is 130 bp upstream of the translational start site of *rsmA*. The other sigma factor regulating the second promoter has yet to be identified.
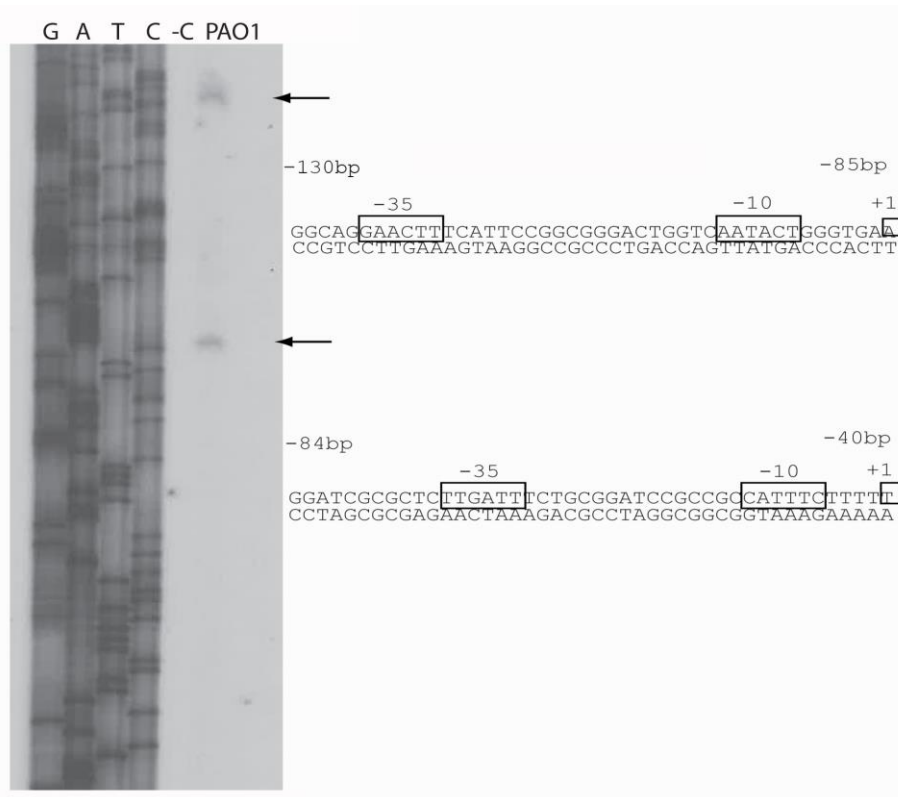
Figure 3. Primer Extension of *rsmA* and genomic sequence showing promoters. There are 2 promoters for *rsmA* in PAO1. The distal promoter is an AlgU promoter while the second promoter is still unidentified.

The fusion strain was conjugated with an *E. coli* strain bearing the pBT20 plasmid that contains gentamicin resistance (Gm^R) and a transposon. Gentamicin-resistant colonies were examined for β-galactosidase activity. X-gal containing plates were used to screen colonies and those identified as having differential *rsmA* expression were used in quantitative β-galactosidase assays and compared to the parent strain.

Confirming Transposon Mutant Results

After identifying several strains exhibiting differential *rsmA* expression compared to the parent strain, our next goal was to identify the gene interrupted by the transposon. When the transposons insert into the genome, they can affect more than one gene if the genes are in the same operon. This can cause polar effects downstream of the operon, skewing the β-galactosidase assay results, and, therefore, clean knockout mutants of the interrupted genes had to be constructed.

We make knockout mutants in the nonmucoid background, PAO1, and the mucoid background, *mucA22*. These mutants were subject to growth curve analysis in various broths to look for possible defects. Knockouts were also constructed for 2 CF clinical isolate strains, FRD1 and 2192, to confirm the results of the *mucA22* mutant. The *rsmA-lacZ* fusion was inserted into our knockout mutants and β-galactosidase assays were performed to confirm our transposon mutant results and refute downstream effects of the inserted transposon. Because transcriptional fusions indirectly monitor gene expression, RNase Protection Assays and Western Blot analysis were performed to not only confirm our β-galactosidase results but also to examine RsmA protein levels. This would allow us to determine *rsmA* regulators and further our understanding of *rsmA* regulation.

Characterizing the Identified *rsmA* Regulators

      Our last aim was to better characterize *rsmA* regulators identified in our study. In particular, we focused on *mtpX*. We conducted several phenotypic assays based on the visual traits of the knockout mutants as well as determining their cellular appearance via electron microscopy. As we characterized the phenotype of the knockout mutant, we were able to gain a better understanding of the gene's role of *rsmA* regulation and describe its mechanisms in both nonmucoid and mucoid backgrounds.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa strain PAO1 was used as the wildtype stain and example of a

nonmucoid background. A strain of PAO1 with the defective antisigma factor *mucA*,

mucA22/PDO300, was used to represent a mucoid background. Two clinical isolates, FRD1 and

2192, isolated from cyctic fibrosis patients, were also used as mucoid strains to demonstrate

clinical relevance. *Escherichia coli* SM10 and JM109 strains were used for conjugating various

plasmids with *P. aeruginosa* strains. JM109 strains had to be triparentally mated with *E. coli*

contain the pRK2013 vector. All strains were grown at 37℃ unless otherwise stated. *P.*

aeruginosa strains were inoculated on *Pseudomonas* Isolation Agar(PIA) with the possible

addition of antibiotics (Appendix) while *E. coli* strains were inoculated on Luria-Bertani(LB)

Agar with possible antibiotic variations(Appendix). Fifteen percent Glycerol stocks were made

for storage of each strain with 500μl of sterile LB broth and 500μl of sterile 30% glycerol.

Strains were scraped from a plate with a sterile stick and placed into the stock tube. These stocks

were stored at -80℃. Various plasmids used can be found in the appendix.

Genomic DNA Isolation

For genomic DNA isolation cultures were grown on PIA plates overnight. The cells were

then scraped from the plate with a sterile stick and washed with 1X TNE(Appendix). Cells were

placed in 1.7 ml microcentrifuge tubes then pelleted by centrifugation at 13.2x1000 rpm for 2

minutes. The cells were resuspended in 1.5 ml of 70% ethanol (EtOH) and placed on ice for 15

minutes. Cells were then centrifuged for 5 minutes and EtOH was poured off and the pellet was

left to air dry. Cells were resuspended in 446μl of TEST buffer (Appendix), 30μl of 20mg/ml of

lysozyme, and 4μl of 10mg/ml RNase A. Cells then were incubated for an hour on ice and then

frozen at -20℃ for 15 minutes. The cells were then incubated at 68℃ for 10 minutes. Fifty- three

μl of 10% SDS(sodium dodecyl sulfate) was added and left at room temperature(RT) for 15

minutes. Then 87μl of 5M NaCl and 69μl of CTAB(Cetyl trimethylammonium bromide)/NaCl

solution was then added, vortexed, and left to sit for 15 minutes. Tubes were then placed in -

20℃ for 30 minutes. After thawing, Phenol-Chloroform Extraction was performed. We added

650μl of 24:25:1 chloroform:phenol:isoamyl alcohol(C:P:I) was added and vortexed. Tubes were

centrifuged at 13.2x1000 rpm for 10 minutes. The aqueous layer was removed and placed in a

new microcentrifuge tube with 600 μl TE buffer(Appendix) and 600 μl 24:1 chloroform:isoamyl

alcohol(C:I). Tubes were vortexed and the spun at 13.2x1000 rpm for 10 minutes. The aqueous

later was removed again and placed into a new microcentrifuge tube. Salt and nucleic acid was

precipitated with 1 ml of 95% EtOH and put on ice for 15 minutes. Tubes were centrifuged at

13.2x1000 rpm for 10 minutes and EtOH was poured off. One and a half milliliters of 70% EtOH

was then added, mixed, and centrifuged for 10 minutes. The remaining EtOH was poured off and

pellets were left to air dry. After the EtOH had evaporated completely, DNA pellets were

resuspended in 50 μl of double distilled H_2O(ddH_2O). DNA extracted was confirmed via

electrophoresis on 1% agarose gel (Appendix) and samples were stored at 4℃.

<div align="center">Transposon Mutagenesis</div>

A transposon mutant library was created by conjugating the pBT20 vector containg the

mariner transposon and Gm[R] with a *mucA22* strain contain an *rsmA-lacZ* fusion (3,34). The *E.*

coli strain was grown up overnight in 5ml of LB broth with 15 μg/ml of gentamicin while the *P.*

aeruginosa strain was grown in 5ml of LB broth. After overnight growth, the *P. aeruginosa*

culture was incubated at 42℃ for 2 hours. Both cultures were then centrifuged at 7000 rpm for 5

minutes and resuspended together in 500 µl of 0.85% NaCl. The mixed strains were then

vortexed and dispensed into the middle of a LB plate for 1.5 hours at 30℃. The plate was then

scraped and the cells were again resuspended in 500 µl of 0.85% NaCl. These cells were then

spread-plated over four 150 µg/ml gentamicin PIA plates and incubated at 37℃ overnight.

Gentamicin-resistant colonies appeared 1 to 2 days after incubation at 37oC.

Patch Plating

Individual colonies were picked from the PIA gentamicin plates with a sterile toothpick

and patch-plated on to a 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside(X-gal)

PIA gentamicin plate. There were 52 colonies patched per plate. These plates were then

incubated at 37℃ overnight. A blue color was evident from the *mucA22* strain carrying the

rsmA-lacZ fusion after 24 hours. This strain was used to compare the transposon mutant library.

Transposon mutant patches with lighter or darker blue appearance compared to the parent strain

were then further analyzed.

β-Galactosidase Assay

Transposon mutants identified in the patch plate assay for differential *rsmA* expression

were tested in a quantitative β-galactosidase assay (28). Strains were grown overnight on

gentamicin PIA plates or *mucA22 rsmA-lacZ* on PIA plates or in 16 hour LB broth culture

(gentamicin added for transposon mutants to 20µg/ml). Pellets of cultures were collected either

with 1ml of 1X TNE and scraping from a plate or 350 µl of broth culture and centrifuged. Cells

were resuspended in 1 ml of Z Buffer (Appendix) and optical density (O.D.) 600nm was

measured. Samples needed to be between 0.4 – 1.0. Fifty µl of 0.1% SDS and 100 µl of

chloroform were then added to each sample and vortexed for 15 seconds. Samples were left to

set for 10 minutes. One hundred microliters of supernatant was removed and placed in a new

microcentrifuge tube with 800 µl of Z buffer. 200 µl of ortho-Nitrophenyl-β-galactoside(ONPG) solution (4 mg/ml ONPG in Z buffer) was added to each sample and reaction time was recorded until samples turned a yellow color. At the time point of yellow color, 500 µl of 1M Na_2CO_3 was added to stop the reaction. O.D. 420nm and 550nm was then recorded. Values were then substituted into the Miller Units.

Equation:

$$\frac{1000 \times (O.D.420nm - 1.75 \times O.D.\ 550nm)}{Time \times Volume\ of\ sample \times O.\ D.\ 600nm} = \text{Miller units of β-galactosidase activity}$$

Arbitrary PCR

Transposon mutants having differential β-galactosidase activity were then subject to arbitrary PCR to amplify flanking regions of the mutant chromosome adjacent to the transposon (30). *Taq* DNA polymerase and Thermopol Buffer were purchased from New England Biolabs (NEB). A revised version of the George O'Toole and Kolter protocol is as follows (Table 1). Perform genomic DNA isolation on mutant and digest with either EcoR1 or BamH1 restriction enzyme.

Table 1: Arbitrary PCR #1

Reagents	Thermocycler:
0.05-0.5 µg of digested genomic DNA with EcoR1/BamH1	95˚C for 5'
	5x 95 ˚C for 30"
1.0 µl 10mM dNTPs	30 ˚C for 30"
5.0 µl 10X Thermopol Buffer	72 ˚C for 1'
2.0 µl 50mM MgCl$_2$	30x 95 ˚C for 30"
2.0 µl 10 µM arbitrary primer(Appendix)	55 ˚C for 30"
2.0 µl 10 µM internal specific primer	72 ˚C for 1'
0.25 µl Taq Polymerase	72 ˚C for 5'
36.25 µl of ddH$_2$O	
50 µl total volume	

Table 2: Arbitrary PCR#2

Reagents	Thermocycler:
1.5 µl of PCR#1 product	95˚C for 5'
1.0 µl 10mM dNTPs	30x 95 ˚C for 30"
5.0 µl 10X Thermopol Buffer	55 ˚C for 30"
2.0 µl 50mM MgCl$_2$	72 ˚C for 1'
10 µl 10 µM arbitrary primer(Appendix)	72 ˚C for 5'
2.0 µl 10 µM external specific primer	
0.25 µl Taq Polymerase	
28.25 µl of ddH$_2$O	
50 µl total volume	

The PCR#1 product in Table 2 is simply 1.5 µl of the 50 µl volume without clean up or gel extraction. Products of PCR#2 were run on a 1.5-2% agarose gel to confirm amplification then cleaned up via Promega Wizard® SV Gel and PCR Clean-Up System. Samples were then sent to the ETSU Molecular Biology Core for sequencing.

<div align="center">Mutant Construction</div>

To confirm transposon mutant results knockout mutants were constructed. This was performed so that any polar effects due to transposon insertion in the mutant library were nullified.

Splicing by Overlap Extension

Two sets of primers, F1 & R2 and R1 & F2, (Appendix) were designed around the gene of interest (17). F1 is at a region 1000 bases upstream from the gene while R1 is 1000 bases downstream. The F1 and R1 primers both have 4 random nucleotides at their 5' end and then an endonuclease site of choice. The endonuclease chosen usually is found in not only a cloning vector but a suicide vector as well and cannot cut the sequence being amplified. F2 and R2 are slightly more complex. R2 is on the bottom strand under the translational start of the gene usually between an 18 to 20-mer. This sequence is then reverse complemented and starts the 5' piece of the F2 primer. F2 is on the top strand including the translational stop of the gene and this 18 to 20-mer is also reverse complemented and starts the 5' of the R2 primer. These reverse complements will create overhangs on the first fragments that will aid in annealing the segments together in the second PCR. A 1000 base pair (bp) fragment was amplified upstream(5' segment) and downstream(3' segment) of the gene. In a second PCR, both of these fragments were spliced together via regions of homology on the F2 & R2 primers and then amplified to create a knockout construct. The F1 & R1 primers also had restriction enzyme sites on the 5' end

so that ligating with a suicide vector, pEX18Tc, would be easier. After each PCR the amplified segment was gel extracted and put through the Promega Wizard® SV Gel and Clean- Up System. This clean up system removes any debris from the agarose gel and purifies our amplified segments.

Plasmid DNA Isolation

Cultures were grown overnight with appropriate antibiotics in 5ml of LB broth. On the second day the culture was spun down in a centrifuge at 7000 rpm for 10 minutes. The LB broth was poured off and the pellet was resuspended in 150 μl TE. This suspension was transferred to a microcentrifuge tube and 300 μl of 1% SDS/ 0.2M NaOH was added and mixed. The tube was incubated on ice for 5 minutes. 225 μl of potassium acetate/acetic acid (5M potassium acetate and 11.5% acetic acid) was then added along with 4μl of 10mg/ml RNase A, mixed, and then incubated on ice for 5 minutes. The tubes were then centrifuged at maximum speed for 10 minutes in a microcentrifuge and the supernatant transferred to a new tube. Phenol-Chloroform-Isoamyl alcohol was added in equal volume, vortexed, and centrifuged for 5 minutes. The aqueous phase was removed to a new tube and the plasmid DNA precipitated using 95% EtOH and incubation on ice for 10 minutes. The plasmid DNA pellet was washed with 70% EtOH, the pellet air dried, and the plasmid DNA was resuspended in 50 μl of ddH$_2$O and stored at -20 or 4℃.

Double Digest & Ligation

Both the knockout construct and suicide vector were individually double digested with the necessary restriction enzymes to produce sticky ends on both products (Table 3).

Table 3: Double Digest Mix

```
1.0 µl of Enzyme #1 (20 units)
1.0 µl of Enzyme #2 (20 units)
2.0 µl of plasmid or knockout construct(0.5 µg)
2.0 µl of Buffer 4 (NEB)
4.0 µl of ddH₂O
10.0 µl total volume

Incubate @ 37°C for 1 hour
```

If possible, restriction enzymes were inactivated using heat inactivation or using Promega enzymatic cleanup protocol. After double digest was performed, a new microcentrifuge tube was used for the ligation with a 1:3 molar ratio of plasmid to knockout construct. The molar amount of a DNA sample can be calculated by quantifying it using the spectrophotometer at A260. Calculating the µg/ml by multiplying the value from A260 * 50 * 100. Then dividing this amount by 0.66*size of DNA segment. 0.66 is the average weight of 1 base pair of nucleotides (38). ddH₂O, T4 DNA ligase buffer, and T4 DNA ligase were added to the tube and put at 4°C overnight (Table 4). To confirm the ligation

Table 4: Ligation Mix

```
1 digested plasmid : 3 knockout construct
2.0 µl T4 DNA ligase buffer (NEB)
1.0 µl T4 DNA ligase (NEB)(400 units)
X   µl ddH₂O
10.0 µl total volume
```

was successful, 3 µl of the volume was removed before adding the T4 DNA ligase and run on a 1% agarose gel with 3 µl of the post ligation volume. The preligation lane should contain 2 bands, the insert and the plasmid, while the postligation lane can contain a smear and no insert

band.

Transformation

After ligation, 100-150 μl of competent *E. coli* SM10 or JM109 cells were placed on ice and 5 μl of the post ligation mix was added and incubated for 10 minutes. This mixture was placed at 42℃ for 45 seconds to 1 minute and then placed on ice for 2 minutes. One milliliter of LB broth was added to the microcentrifuge tube and placed on a shaker at 37℃ for 1 hour to rescue. The cells were pelleted and spread-plated on to an LB plate with the appropriate antibiotic for the suicide vector to be expressed as a form of positive selection. Colonies were screened using blue-white selection. White colonies were grown up overnight in LB with the appropriate antibiotic and plasmid DNA isolated and digested with the appropriate restriction enzymes to confirm insertion of the amplified construct into the suicide vector.. The final suicide construct was verified by sequencing at the ETSU Molecular Biology Core facility.

Homologous Recombination

In order to perform allelic exchange, the suicide construct was conjugated into various *P. aeruginosa* srains using tri-parental or bi-parental mating. Merodiploid colonies were obtained by selecting for antibiotic resistant colonies postconjugation indicating that the suicide construct had inserted into the bacterial chromosome. Merodiploids placed in 5 ml of LB broth overnight without selection in order to allow for a second cross-over event. Due to counter-selectable marker *sacB,* gene on the suicide vector, coding for the protein levansucrase, sucrose counter-selection was used to detect bacteria that had undergone the second recombination event. Serial dilutions of 10^{-4}, 10^{-6}, and 10^{-7} were performed on LB 10% Sucrose plates. Sucrose-resistant colonies were patch plated onto antibiotic plates and PIA plates. Bacteria only growing on PIA were screened via internal primers to detect wildtype or mutant variation.

Growth Curve

Cultures of *P. aeruginosa* were initiated in a 5 ml LB broth overnight. On day 2, the absorbency at O.D. 600nm was measured and diluted to 0.1. A 99 ml broth, either LB or minimal media, was seeded with 1 ml of the diluted culture. O.D. 600nm reading was taken every 3 hours. The experiment was performed in triplicate. Minimal media consisted of M9 salts (Appendix) and either 1% glucose or 1% succinate as a carbon source.

Construction of *rsmA-lacZ* Transcriptional Fusion Strains

Mutant strains were conjugated, in the same fashion as the suicide vector in the mutant construct section, with the mini-CTX-lacZ plasmid containing the *rsmA-lacZ* fusion (39). mini-CTX-lacZ uses the attB site on the *P. aeruginosa* chromosome for insertion. By doing so, it is inserted as a single copy, unlike other complementation vectors that have multiple copies in the cell. Colonies with tet^R were than grown up overnight and conjugated with an *E. coli* strain containing the plasmid pFLP2 to flip out the mini-CTX plasmid vector backbone and leave the *rsmA-lacZ* fusion. This conjugation was then streaked for isolation on VBMM $_{Carb300}$ plates (Appendix). The isolated colonies were patch plated on to LB tet irg (Appendix) and PIA plates to confirm the loss of the tet^R gene. Growth overnight at 37°C without selection was used to cure the *P. aeruginosa* strains of pFLP2. Colonies grown on PIA and killed on VBMM $_{Carb300}$ were then screened for the *rsmA-lacZ* insert via rsmA forward and lacZ reverse primers (Appendix).

RNA Isolation

Cultures of *P. aeruginosa* were grown in 5 ml of LB broth for 6 to 8 hours (37). Three hundred microliters of the culture was aliquoted into a microcentrifuge tube and pelleted at 7000 rpm for 5 minutes. LB broth was poured off and 1 ml of TRIzol® (Invitrogen) was used to resuspend the pellet. Five hundred µl of glass beads were added and beat for 3 intervals at 20

seconds. Tubes were intermittently placed on ice. Tubes were left to set for 10 minutes. The

TRIzol®/cellular mix was then removed and added to a clean microcentrifuge tube with 200μl of

chloroform. Tubes were inverted for 2 minutes and then centrifuged for 10 minutes. Supernatant

was then added to the column of the Promega SV Total RNA Isolation System. The Promega SV

Total RNA Isolation System protocol was then followed. After 2 elutions, 20μl of sodium acetate

was added and 500μl of 95% EtOH was followed. Tubes were incubated at -20℃ for 15 minutes.

Tubes were then centrifuged for 10 minutes and EtOH was poured off. 1 ml of 70% EtOH was

placed on top of the RNA/salt pellet and tubes were stored at -80℃ until used. All reagents used

DEP-C treated or nuclease free water. Successful RNA isolation was confirmed via

electrophoresis with an 1% agarose/formaldehyde gel(). Quantifying RNA was done with 5 μl of

RNA sample in 495 μl of TE. Absorbance was measured at O.D. 260 and 280. Purity was

confirmed by the ratio A260/A280, while amounts were estimated by multiplying A260 x

40(representative of weight) x 100 (dilution factor).

<div align="center">RNAse Protection Assay</div>

The RPA IIITM Ribonuclease Protection Assay Kit (Ambion®) was used for this assay.

The protocol with this kit was followed and used to design and make a biotinylated probe for

rsmA mRNA. The *rsmA* probe is a single strand of antisense RNA to the mRNA of interest, in

this case *rsmA's* mRNA. The probe was made via primers (Appendix) for *rsmA* with a reverse

primer also containing a promoter for T7 phage RNA polymerase (Ambion RPAIII Kit). The

sequence was transcribed via the RNA polymerase and then gel extracted from an 8M urea/

5%acrylamide gel (Table 5). The probe was aliquoted and stored at -20oC. After aliqoting the

probe, the 5 μg of RNA samples and a negative control of yeast RNA were added to a clean

microcentrifuge tube and 3 μl of a 200-fold diluted probe were hybridized with hybridization

buffer over night at 42°C. A solution of RNase Digestion Buffer 3 and RNase A/T1 was made

with 150µl Digestion Buffer 3:1µ RNase per tube. The RNase was not added to the probe by

itself. These samples were then incubated for 30 minutes at 37°C. After, 225µl of Inactivation

Solution was added and the samples were incubated at -20°C for 15 minutes. The tubes were

centrifuged for 15 minutes at maximum speed. The supernatant was removed and 10 µl of

loading buffer was added. The samples were heated at 92°C for 5 minutes and then placed in

slushy ice. Samples of RNA and probe were run on a 5% acrylamide / 8M urea gel (Table 5) and

run

Table 5. 5% acrylamide/8M urea gel mix

7.2 g Urea
1.5 ml of 10X TBE
1.9 ml 40% Acrylamide/bis-Acrylamide
X   ml of ddH$_2$O
15 ml total volume

Stir at room temperature.

Add 120 µl of 10% ammonium persulfate(APS)
and 16 µl of TEMED

Mix and pour gel into 1 mm x 8cm x 8cm
cassette

in 1X TBE buffer (Appendix) at 200 V. The gel was electroblotted in 0.5X TBE for 1 hour at

150 mA on to a nitrocellulose membrane. The membrane was then UV crosslinked for 30

seconds. The BrightStar® BioDetectTM Kit (Ambion®) was then used detect protected RNA

samples on the membrane. The kit's protocol was followed. To visualize the samples a

ProteinSimple FluorChem M system was used with the chemiluminescence option.

Insertion of *rsmA*-HA Tag

The suicide vector pEX18Gm was used to clone the *rsmA* gene with hemaglutinin(HA) tag added to the 3' end of the *rsmA* gene. This vector was conjugated as described previously into various *P. aeruginosa* strains. Primers rsmA SDM check F(forward) and rsmA R check(reverse) were used to detect the addition of the HA portion on to *rsmA* (Appendix).

Western Blot Analysis

Cultures of *P. aeruginosa* were either grown in LB broth or harvested from LB plates after 12 hours. Cells were pelleted in 2 ml tubes and resuspended in 0.85% NaCl to a final volume of 1.7 ml. Tubes were left on ice. Thirty microliters of 20mg/ml of lysozyme was added to help lyse of the cells. Sonication was performed for each tube for 20 seconds twice. Intermittently, tubes were left on ice between sonication. Tubes were centrifuged at 7000 rpm for 10 minutes to pellet debris. Supernatant was removed and placed into a new microcentrifuge tube. A standard curve was created using the Bio-Rad Protein Assay and BSA at concentrations of 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml. Ten milliliters of colorimetric assay dye was added to 40 ml of ddH$_2$O. Thirty microliters of each BSA sample was added to 3.5 ml of the assay dye solution. Thirty microliters of each HA-tagged sample's supernatant was also added to 3.5 ml of assay dye solution to quantify the amounts of protein against the standard curve. All samples were measured at O.D. 595nm. The BSA samples were then analyzed in Excel and a standard curve generated. The amount of protein in the cellular samples was calculated from the trend line equation. Only R^2 values of 0.98 or higher were used. Ten micrograms of cellular samples were mixed with a 4X SDS PAGE loading dye (Appendix). The samples were heated for 5 minutes at 92℃, briefly centrifuged, and put in slushy ice. A discontinuous SDS-PAGE gel of 4% stacking/15% separating (Table 6) was used for the RsmA-

HA Western Blot. Samples were run at 180 V until the dye front

Table 6. SDS-PAGE for Western Blot

15% Separating Gel	4% Stacking Gel
3 ml of 40% Acrylamide/Bis 2 ml of 1.5M Tris pH 8.8 2.88 ml of ddH$_2$O 80 µl of 10% SDS 8 µl of TEMED 80 µl of 10% APS Pour gel into cassette and add 1.5 ml of 0.1% SDS to allow for level surface. Allow to polymerize.	500 µl of 40% Acrylamide/Bis 1.25 ml of 0.5M Tris pH 6.8 3.1 ml of ddH$_2$O 50 µl of 10% SDS 5 µl of TEMED 25 µl of 10% APS Pour off 0.1% SDS and add stacking gel to top of separating gel. Put in comb and allow to polymerize.

reached the end of the separating gel. The gel was run in 500 ml of 1X Glycine Running Buffer

(Appendix). To prepare for transferring to a polyvinylidene fluoride (PVDF) membrane, 1X

Towbin Buffer (Appendix) and 10% methanol were used for transfer to the PVDF membrane

after soaking in methanol for 10 minutes The transfer apparatus was assembled and run at 150

mA for 1.5 hours.

After transferring, the membrane was removed from the sandwich and placed in Blocking

Buffer (Appendix). This was rocked for 2 hours at RT. The Blocking Buffer was poured off and

1˚antibody for HA was added (Appendix) at a 1:10,000 dilution. This was rocked at 4℃

overnight. On day 2, the antibody wash was poured off and washed 3 times with Washing Buffer

(Appendix) for 15 minutes each time. The 2˚antibody (goat anti-mouse) containing conjugated

horseradish peroxidase was added (Appendix) at a 1:10,000 dilution for 1 hour and rocked at RT.

The antibody wash was poured off and washed again 3 times for 15 minutes each time. Lastly,

the Pierce ECL Western Blotting Substrate kit was used to visualize the proteins tagged on the

membrane. 3 ml of Substrate A and 75 µl of Substrate B were mixed and poured over the

membrane. The membrane was hand rocked for 5 minutes. The membrane was then transferred to the ProteinSimple FluorChem M visualizer for data analysis.

Swimming Assay

LB plates (0.3% agar) were used in this experiment (22). Cultures of *P. aeruginosa* were grown up overnight and transferred to swimming plates via a sterile toothpick. Plates were stabbed with the inoculated sterile toothpick and incubated at 30℃ for 48 hours. The zone of growth was measured in centimeters. Experiments were performed in triplicate.

Twitching Assay

LB plates (1.5% agar) were used in this experiment (22). Cultures of *P. aeruginosa* were grown up overnight and transferred to twitching plates via a sterile toothpick. Plates were stabbed with the inoculated sterile toothpick and incubated at 30℃ for 48 hours. Agar was removed with a sterile stick and 1% crystal violet was used to flood the plate and left to set for 5 minutes. This stain was used to visualize the twitching zones. The crystal violet was washed with water and twitching zones were measured in mm. Experiments were performed in triplicate.

Transmission Electron Microscopy (TEM)

Cultures were streaked for isolation and grown up on PIA plates overnight. 5 µl of ddH$_2$O was added to a Formvar/Carbon film coated, 200 Mesh, Cu, TEM grid. To this drop of water, a single colony was picked with a sterile toothpick and touched for 5 minutes to the water(14). Five µl of 1% uranyl acetate (UA) was added to the drop of water and left to set for 45 seconds. The drop was then wicked with sterile chromatography paper from the other side of the grid where the drop was placed. Grids were stored in a carrier and taken to the TEM facility. Microscopy was performed with a Philips Tecnai 10 transmission electron microscope with an acceleration of 80 kV.

Iron Acquisition on Chrome Azurol S Plates

Chrome Azurol S(CAS) plates were used to study the degree of iron acquisition of wildtype versus mutant strains (21). Iron III solution consisted of 27 mg $FeCl_3 \cdot 6H_2O$, 83.3 µl of 12M HCl, and 100 ml ddH_2O. Basal Agar Medium consisted of 415 ml ddH_2O, 15g of 3-(N-morpholino)propanesulfonic acid (MOPS), 0.25 g NaCl, 0.15g KH_2PO_4, 0.05g NH_4Cl, 2.5g of L-asaparagine. The pH of solution was measured to 6.8 with 5M NaOH and 7.5g of agar was added and then autoclaved. CAS indicator solution consisted of: Solution A, 60.5mg Chrome azurol S and 50ml of ddH_2O, after dissolving, 10ml of Iron III solution was added, Solution B, 72.9mg hexadecyltrimethyl ammonium bromide (HDTMA) and 40ml of ddH_2O. Solution B was slowly added to Solution A and autoclaved. After autoclaving and allowing the basal agar medium to cool, 10ml of 50% succinate and 50ml of the CAS indicator solution were added and mixed. Cultures of *P. aeruginosa* were grown overnight and transferred to CAS plates via a sterile toothpick. Plates were stabbed with the inoculated sterile toothpick and incubated at 30℃ for 48 hours.

Biofilm Assay

The microtiter dish biofilm formation assay described by O'Toole was performed on wildtype and Δ*mtpX* mutants as well as Congo Red media (22,31). LB broth cultures were grown overnight and the next day diluted 1:100 in M63 broth (Appendix). One hundred microliters of the dilution was added to a row of wells on a 96-well flat bottom plate. The dish was incubated for 24 hours at 37℃. Plates were washed 2 times with ddH2O and then 125 µl of 1% CV was added to each well and set for 10 minutes. The plate was washed 3 times with ddH2O to remove excess CV and left to dry overnight. 125 µl of 33% acetic acid was added to each well to solubilize the CV. The plate was then read in a microtiter plate reader at O.D. 620nm. This

experiment was performed in triplicate.

Alginate Assay

Pseudomonas cultures were grown on PIA plates at 37℃ for 48 hours. Plates were scraped

with a sterile stick and contents added to 0.85% NaCl. Cells were centrifuged to separate alginate

from the cell. Supernatant was removed and stored in a separate tube. Three milliliters of

Sulfuric Acid/borate solution (Appendix) was transferred to glass tubes on ice and 350 μl of the

supernatant was layered on top. A standard curve was made from alginic acid in amounts of: 5,

50, 100, 200, 300, 600, and 1000 μg/ml. Tubes were vortexed for 1 second and put back on ice.

One hundred microliters of 0.1% Carbazole in EtOH was added and vortexed. Tubes were then

placed in a 55℃ water bath for 30 minutes (20). Tubes were removed and allowed to set at RT

for 5 minutes. Absorbency was measured at O.D. 530nm. This experiment was performed in

triplicate.

Reverse Transcriptase PCR(RTPCR)

The GoScriptTM Reverse Transcription System by Promega was used to produce cDNA.

Both random primer (data not shown) and specific primer were used to generate cDNA. Isolated

RNA from PAO1 was quantified and 5μg was used. The RNA, 1 pmol of primer, nuclease free

water to a volume of 5 μl were added to a PCR tube. This tube was incubated at 70°C for 5

minutes and then placed on ice. The tube was incubated at RT for 5 minutes with the addition of

4 μl of GoScriptTM 5X Reaction Buffer, 2 μl of MgCl$_2$, 1 μl of PCR Nucleotide Mix, 20 units of

Recombinant RNasin® Ribonuclease Inhibitor, 1 μl of GoScriptTM Reverse Transcriptase, and

1.5 μl Nuclease-Free water. The extension step was incubated at 42°C for 1 hour. The Reverse

Transcriptase was incubated at 70°C for 15 minutes to inactivate the enzyme. cDNA samples

were incubated at -20°C until time for PCR. A negative control was also performed without

Reverse Transcriptase. The PCR that followed had a shorter series of cycle repeats, 26, versus the PCR used to generate the genomic DNA control with 30 cycle repeats. Results were run on a 1% agarose gel and performed in triplicate with 2 different RNA preps.

<div align="center">Statistical Analyses</div>

Where applicable, experiments performed in triplicate were subject to mean, standard deviation, and standard error analysis. Graphs show means and standard error via error bars. Student's *t* test was also performed to confirm significance within each experiment. Stars show statistical significance with 1 star having a $p<0.05$ and 2 stars having a $p<0.01$.

CHAPTER 3

RESULTS

Transposon Mutant Screen

By using the *rsmA-lacZ* transcriptional fusion designed from the primer extension data

(Figure 2), we looked at *rsmA* expression in the mucoid strain, *mucA22*, versus the wildtype

strain PAO1(Figure 4). After seeing a significant increase in *rsmA* expression, we set out to

identify possible regulators of *rsmA* causing this significant increase in expression. To identify

regulators of *rsmA*,
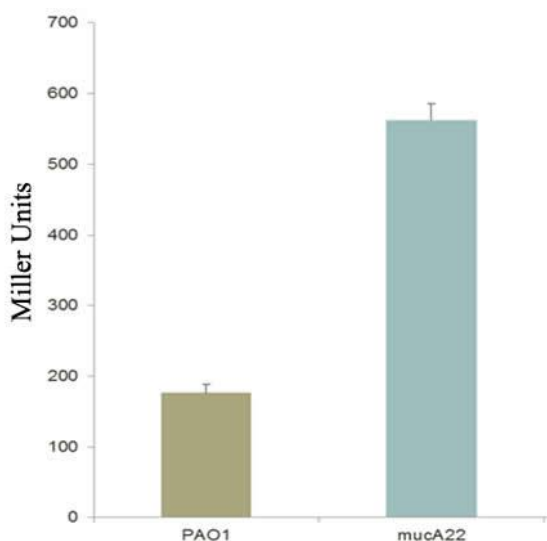
Figure 4. *rsmA* expression is increased in a *mucA22*. PAO1 and the isogenic strain *mucA22* were
both analyzed via β-galactosidase assay with the transcriptional fusion *rsmA-lacZ*.

we created a transposon mutant library with the parent strain *mucA22 rsmA-lacZ* and the

transposon vector pBT20 (Figure 5).
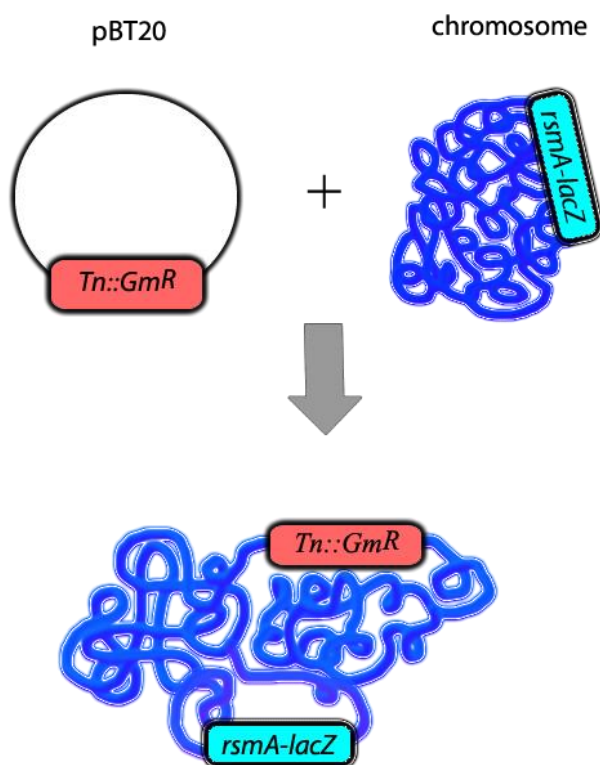
Figure 5. A representation shows conjugation between transposon vector with Gm^R gene and *mucA22 rsmA-lacZ* to visualize the creation of transposon mutants.

Patch Plating

Transposon mutants were picked from PIA gentamicin plates with a sterile toothpick. Only colonies that were isolated as a single colony were chosen to avoid contamination. Growing colonies on PIA gentamicin plates ensured that there would be no *E. coli* due to irgasan and no parent *mucA22 rsmA-lacZ* without the transposon and Gm^R genes. X-gal was added to visualize differences in *rsmA* expression levels. Because it can take up to 48 hours for alginate to accumulate from the patched colony, plates were observed over the course of 2 days to look for a mucoidy appearance. In terms of β-galactosidase activity on the plates, after incubating overnight, the plates were left at room temperature for an hour to let blue color develop further (Figure 6). The patched mutants were compared to a patched colony of *mucA22 rsmA-lacZ*. Those colonies that were drastically darker or lighter than the parent strain with the

transcriptional fusion were then streaked for isolation on PIA gentamicin plates. The plates were incubated overnight. Freezer stocks were made for storage of the transposon mutants of interest and those mutants were also subject to β-galactosidase assay.
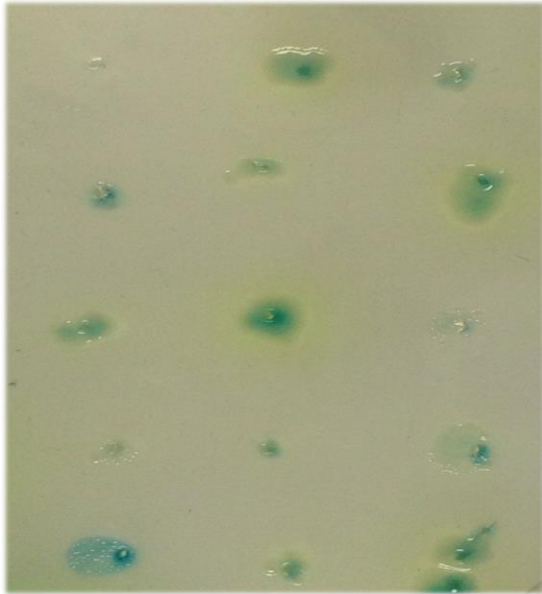
Figure 6. Patched transposon mutants on a PIA 80 μg/ml X-gal 150 μg/ml gentamicin plate. The blue pigment is a result of *rsmA* expression from the *rsmA-lacZ* transcriptional fustion.

β-Galactosidase Assay

Over 50 transposon mutants have been screened via the β-galactosidase assay since the start of our project looking for regulators of *rsmA*. The assay was performed with 16-hour cultures. The mutants that we analyzed were performed in triplicate to assure that the assay was consistent. We also compared results of broth versus cultures grown on a plate to look for variances in *rsmA* expression between the 2 environments. Student's *t* test was performed on the values of the individual transposon mutants and *mucA22 rsmA-lacZ* to look for significant differences between the 2 (Figure 7). If the p value was less than 0.05, we considered it a significant difference to further study the transposon mutant by identifying the interrupted gene. We have identified 50 signficiant mutants by this method, 20 of which have exhibited very significant differences. The most drastic difference we have seen is a fourfold decrease in *rsmA*

expression. Some mutants were identified by arbitrary PCR for their interesting appearance or

odd growth patterns on plate medium.
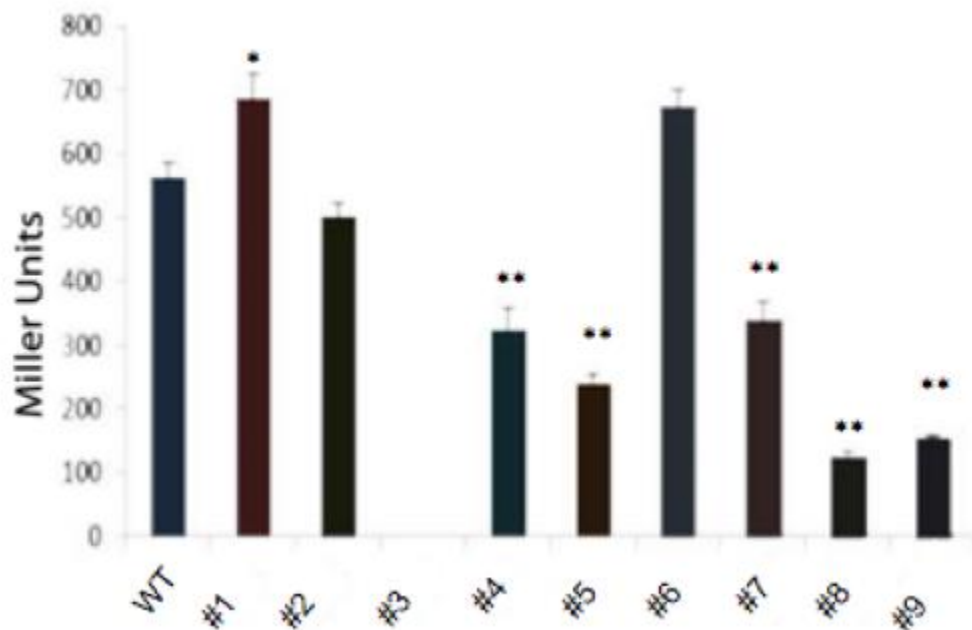
Figure 7. β-Galactosidase Assay results with *t* test analysis. This experiment was performed in triplicate to ensure the least amount of error. *mucA22 rsmA-lacZ* was our standard we compared the transposon mutants to in this experiment. One star signifies $p<0.05$ and 2 stars signify $p<0.01$.

Arbitrary PCR

The original arbitrary PCR protocol called for adding all 3 primers to amplify the

transposon interrupted region (30). However, we found that we had more success using the

arbitrary primers individually instead of used together as the protocol suggests. We also

discovered that Arb1 and Arb2 primers were more successful at amplifying segments to be

sequenced than Arb3. Samples were sent to the ETSU Molecular Biology Core to be sequenced.

We used the program Chrome to analyze the results and then BLAST the sequence on

http://www.pseudomonas.com to identify which gene was interrupted (41). Based on our β-

galactosidase assay, Table 7 lists 9 genes that have been identified to regulate *rsmA* expression in

P. aeruginosa, one previously identified in another study. *pilW* was the first to be identified in

this experiment. An individual project revolving around the minor pilin gene was begun at that time, but we continued to identify genes. Another important gene identified by arbitrary PCR was *retS*. RetS is a negative regulator of the GacA/S 2 component system, which in turn favors production of RsmA (27).

Table 7. Identified Genes from Arbitrary PCR

Identified Genes and Function	
#7............*pilW*.........................	minor pilin protein
#5.............*PA1626(mtpX)*.............	probable major facilitator superfamily transporter
#4.............*retS*.........................	regulator of exopolysaccharide and Type III Secretion
#2.............*PA4671/72*.................	probable ribosomal protein L25/ peptidyl-tRNA hydrolase
#1............*dsbA*.........................	thiol:disulfide interchange protein
#6............*nuoD*.........................	NADH dehydrogenase I chain C,D
#3............*lacZ*.........................	β-galactosidase
#9...........*PA2028*......................	probable transcriptional regulator
#8...........*PA1192*......................	conserved hypothetical protein

PA1626 is another gene of interest from this study, which we named *mtpX*. Our β-galactosidase assay showed a 4-fold decrease when compared to *mucA22 rsmA-lacZ*. Other mutants ranged from a two-thirds increase (*dsbA)* to almost fivefold decrease (*PA1192)* in terms of Miller units measuring *rsmA* expression. The mucoid strain became nonmucoid as well as had very decreased levels of *rsmA* expression via the *lacZ* reporter. After identifying this gene via

sequencing, we set out to make a knockout of *mtpX* in order to confirm our results from the

transposon insertion. Like *pilW*, *mtpX* was in an operon, but it is located next to a transcriptional

regulator. This transposon insertion could have complicated our results by interrupting the

message of both genes. We then began to make a knockout mutant of *mtpX*.

Mutant Construct

Two genes, *pilW* and *mtpX*, were selected for making mutants to compare results with the

transposon mutants. Primers were designed to amplify segments surrounding the gene of interest

(Figure 8). These segments were then spliced together to create the deletion construct.
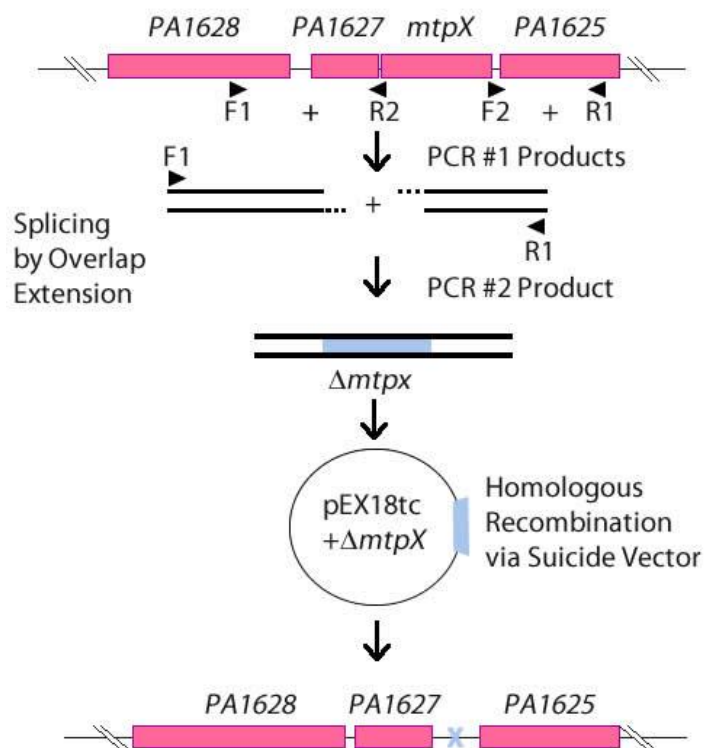

Figure 8A. Diagram showing Construction of mutant strains

The individual 5' and 3' fragments were successfully amplified as a single amplicon of

approximately 1000bp and were gel purified without UV exposure as well as ethidium bromide

that might have damaged DNA. The 5' and 3' pieces were successfully used as templates in a

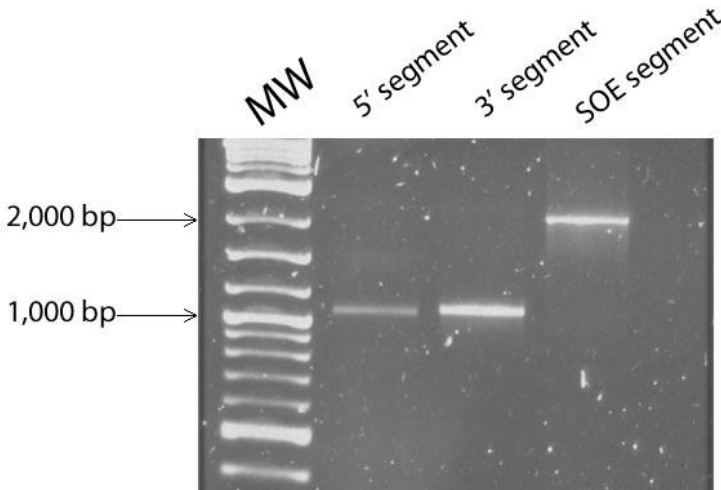second PCR using primers, F1 and R1 (Figure 8B).

Figure 8B. 1% agarose gel used to show amplified 5' and 3' segments along with SOE amplified segment. MW is molecular weight marker used to measure amplified segments of DNA.

This mutation construct was digested along with both a cloning vector and suicide vector. We used a cloning vector as a precaution in case more knockout constructs were required. The mutant construct was then ligated into the suicide vector and then transformed in *E. coli*. The mutant construct was conjugated into various *P. aeruginosa* strains. After making merodiploids, which are cells with both mutant and wildtype genes, we left them to perform homologous recombination in LB broth overnight. This broth was serially diluted on 10% sucrose LB plates. The second crossover was successful as determined by detection of the mutant allele on sucrose-resistant colonies after PCR analys using primers (Appendix). After completing the mutant screening successfully and confirming the mutation with internal detection primers, freezer stocks were made of the mutants. We made mutants in both mucoid and nonmucoid backgrounds.

Growth Curves of Δ*mtpX* Mutants

One possible explanation for the differential *rsmA* expression in the transposon mutants is that these mutants have a growth defect due to the transposon insertion. To address this possibility, we performed growth curve analysis of the constructed mtants compared to the wild

type strains. As the *mtpX* mutant may have a arole in supplying nutrients to the cell, we analyzed

the growth kinetics of this strain in several media, both complex and minimal media.

In LB Media

In LB broth, there was no difference between the mutants and the parent strains. PAO1 had

a spike in the O.D. reading at 12 hours, but overall there was no difference. This growth curve

analysis was performed in triplicate to ensure that there was no difference between growth of the

mutants and PAO1 or *mucA22*. These results can be seen in Figure 9. In addition our results

demonstrate that in complex media there is both the nonmucoid PAO1 and the mucoid strain,

mucA22, have similar growth kinetics.
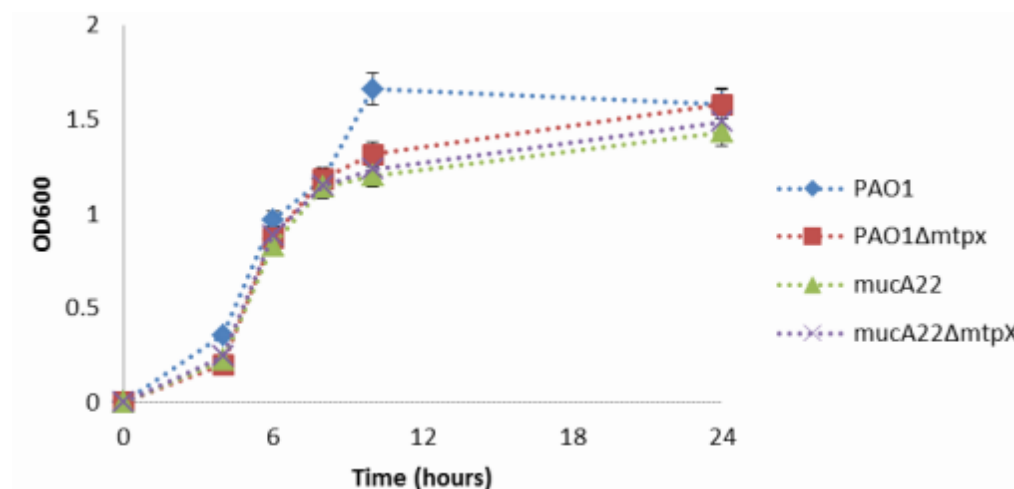
Figure 9. Growth Curve analysis of *mtpX* mutants versus PAO1 and *mucA22* show no variation
in growth. PAO1(blue diamond), PAO1Δ*mtpX*(red square), *mucA22*(green triangle), and
*mucA22*Δ*mtpX*(purple X) were grown in LB broth over 24 hours. Standard error bars are shown
at each time point.

In Minimal Media

Because MtpX may play a role in the metabolism of *P. aeruginosa*, we compare the

growth of the mtpX mutant to the parent strain in minimal media using different carbon sources.

M9 salts minimal media and an added carbon source; either glucose or succinate was used.

1% Glucose. *P. aeruginosa* does not readily metabolize sugars like glucose, sucrose, and lactose (9). However, it can still grow using glucose as the sole carbon source. There again was no difference in growth between the mutants and parent strains (Figure 10). However, there was a visual difference in growth. Around the 12 hour time point, PAO1, PAO1Δ*mtpX*, and *mucA22*Δ*mtpX*, all changed color of their media to a red pigment, which we predict is secreted pyorubin, while *mucA22* remained yellow in color. It is interesting to note the difference in the *mucA22*Δ*mtpX*. This mutant was able to secrete pyorubin with the inner membrane protein MtpX removed from the cell in the mucoid background.

Figure 10. Growth Curve Analysis in 1% Glucose minimal media shows no variations between mutants and parent strains. PAO1(blue diamond), PAO1Δ*mtpX*(red square), *mucA22*(green triangle), and *mucA22*Δ*mtpX*(purple X) were grown in minimal media broth over 24 hours. Measurements were recorded at O.D. 600nm.

1% Succinate. Succinate is readily metabolized by *P. aeruginosa*. This carbon source also increased pyoverdine secretion. There was no difference in growth analysis (Figure 11). The strains also exhibited pigment change at the same time.
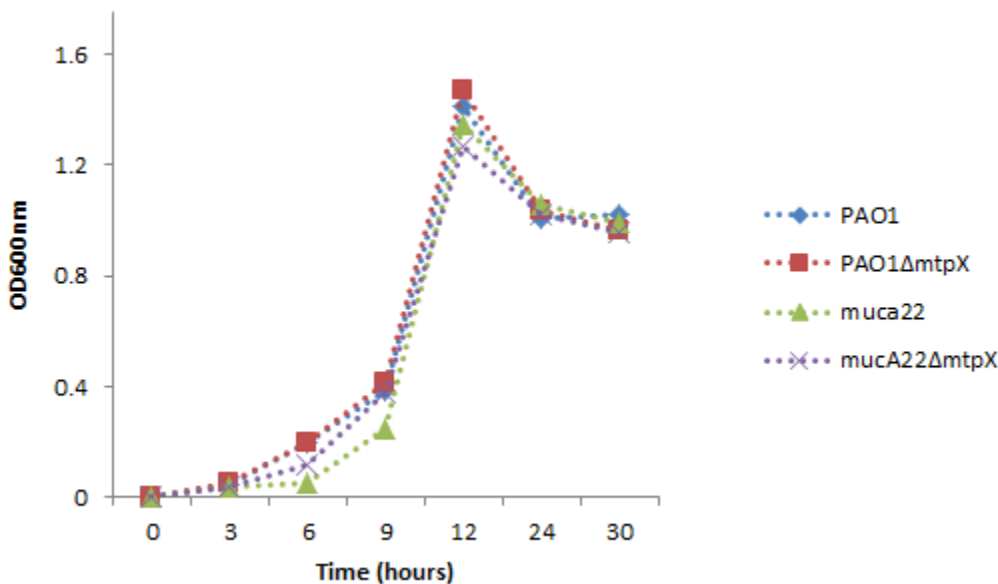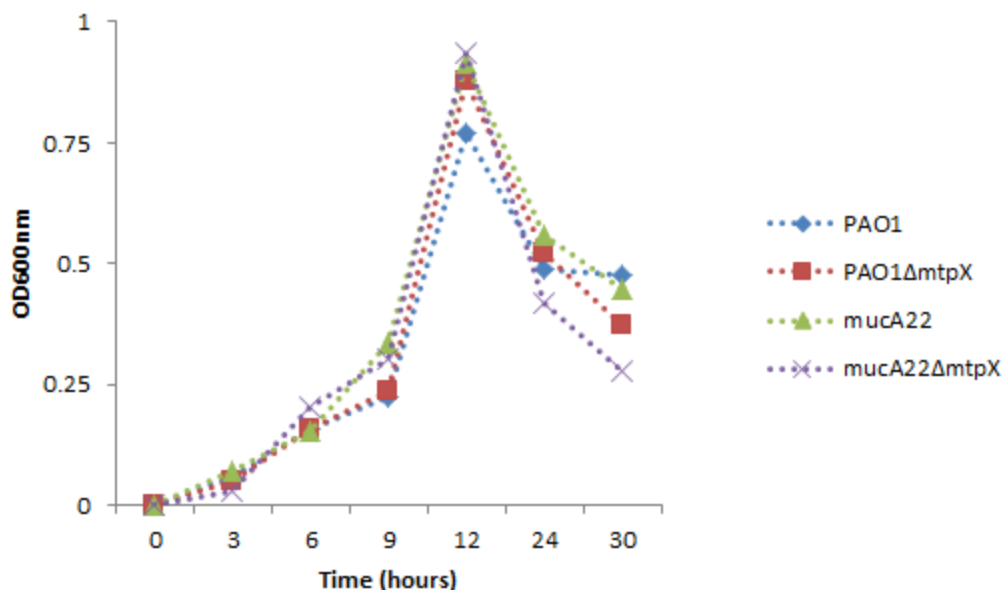
Figure 11. Growth Curve Analysis in 1% Succinate minimal media shows no variations between mutants and parent strains. PAO1(blue diamond), PAO1Δ*mtpX*(red square), *mucA22*(green triangle), and *mucA22ΔmtpX*(purple X) were grown in minimal media broth over 24 hours. Measurements were recorded at O.D. 600nm.

β-Galactosidase Assay of Δ*mtpX* Mutants with *rsmA-lacZ* Fusion

In order to confirm our transposon mutant results from the β-galactosidase assay, we inserted the *rsmA-lacZ* fusion into the knockout mutant's chromosome of both backgrounds. These were performed from broth cultures and done in triplicate (Figure 12) with *t* tests to show significance. We not only repeated our transposon mutant results in this knockout mutant but also showed a significant decrease in the nonmucoid background strain PAO1. This confirms the transposon screen results and suggests that MtpX affects *rsmA* expression by some undefined mechanism.
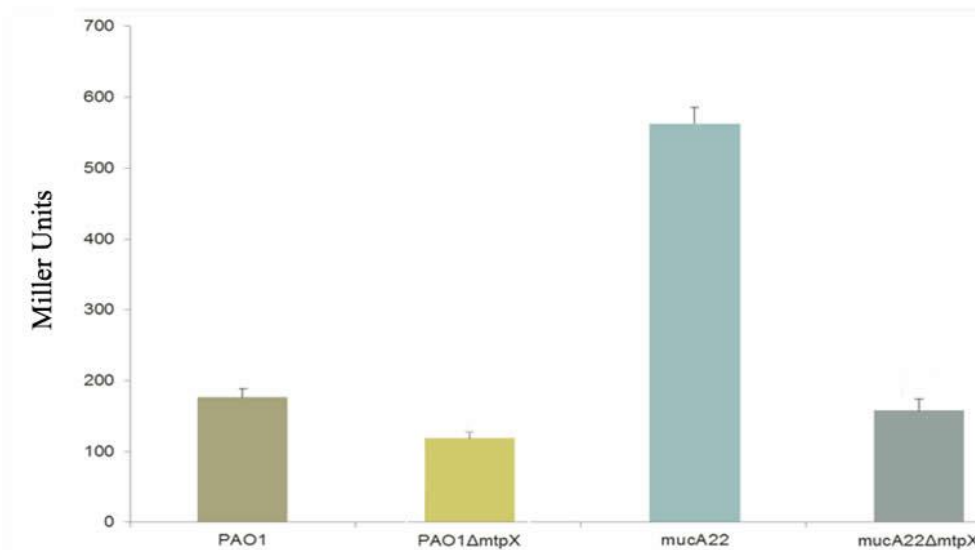
Figure 12. B-galactosidase assay shows significant decreases in *ΔmtpX* mutants' *rsmA* expression. Miller units were calculated from recording O.D. 600nm, 420nm, and 550nm during the course of the assay using Miller's equation. One star represents a $p < 0.05$ and two stars, a $p < 0.01$.

RNase Protection Assay of Δ*mtpX* Mutants with *rsmA* Probe

As transcriptional fusion analysis is one way to analyze gene expression, it uses a reporter gene controlled by the promoter of the gene of interest. This provides an indirect measurement of gene expression. In order to directly measure the amount of *rsmA* message, we performed an RNase protection assay. A probe consisting of biotinylated *rsmA* antisense message was produced by invitro transcription, gel purified, and used in a hybridization experiment using total RNA isolated from various strains. The RNA probe was combined with RNA isolated from each of the 4 strains (Figure 13). RNA was isolated at 6 to 8 hours from broth cultures, so there is potential for change in results when compared to *rsmA* expression using a transcriptional fusion measured at 16 hours. However, when comparing PAO1 and *mucA22* we see darker bands in the mucoid background versus the nonmucoid. The protein simple Fluoro Chem M imager was used to confirm analysis of the intensity of the bands present (data not shown).

Figure 13. RNase Protection Assay with Δ*mtpX* mutants. These results show that the nonmucoid mutant is actually increasing *rsmA* expression via the AlgU promoter while the muc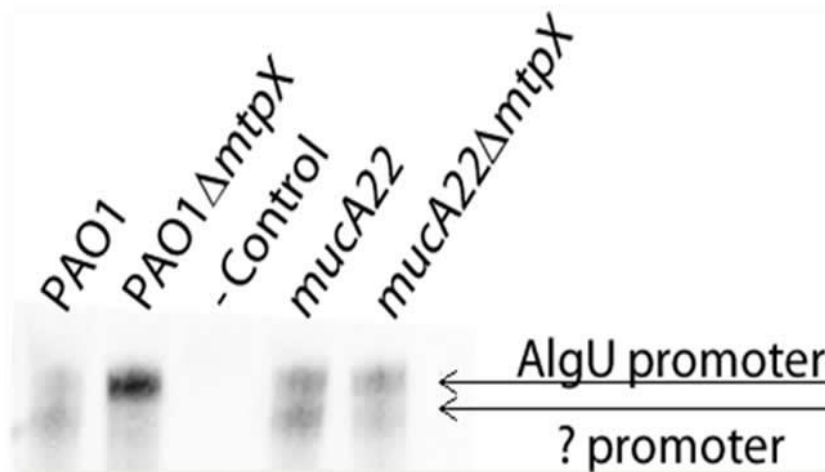oid background mutant has less expression than that of *mucA22*. A negative control was used with yeast RNA and the *rsmA* probe. The top arrow marks the band transcribed from the AlgU promoter while the lower arrow shows the unknown promoter.

We see 2 bands as well, which indicates that there are 2 *rsmA* messages. This result is supported by the primer extension results demonstrating that there are 2 transcriptional start sites (Figure 3). The longer message is from the AlgU-dependent promoter while the lower band is controlled by an unknown sigma factor.

These results vary from the β-galactosidase assay, which showed decrease in *rsmA* expression in the nonmucoid mutant. The only variable here is time of experiment. We showed (Figure 2) that RsmA is diminished greatly between 6 hours and 12 hours in PAO1 and this may explain our RNase protection assay results. Two bands can be seen in the 2 parent backgrounds, PAO1, and *mucA22*. The messages appear more intense in the mucoid background supporting our β-galactosidase assays. However, the *mtpX* mutant has a great deal more message from the AlgU-dependent promoter and a very faint band representing the second message. This result suggests that there is more *rsmA* message from the AlgU promoter and this may be due to different RNA concentrations or different levels of RNA degradation.

In the mucoid background, we confirm our β-galactosidase results. The top band in the lane

with the *mucA22ΔmtpX* is slightly lighter in appearance when compared to *mucA22*. The second band, however, is significantly lighter than that of *mucA22*. The correlation between both these messages has not been elucidated and how much they effect the β-galactosidase results is unclear. Another time course experiment using the RNAse protection assay may be useful in seeing the expression of the various messages in both mucoid and nonmucoid background. We also have a construct consisting of a site-directed mutation of the AlgU promoter of *rsmA* and would like to repeat this experiment to confirm that these results are consistent with our primer extension data showing the presence of 2 messages as well to prove the results of the PAO1 *mtpX* mutant.

<div align="center">Western Blot Analysis of Δ*mtpX* Mutants with HA Tagged RsmA</div>

An alternative way to look at *rsmA* expression is to determine the levels of RsmA in the cells. We confirmed the presence of the HA tag via PCR and running the amplified products on a 1% agarose gel. The *mtpX* mutants were made by conjugating the Δ*mtpX* construct with PAO1 and *mucA22* strains with the HA-tagged RsmA already inserted on the chromosome. Using an HA- tagged RsmA, we were able to analyze the abundance of RsmA in the various strains. These cultures were taken from LB plates to reduce the amount of alginate produced by *mucA22*. The strains were grown for 12 hours, lysed, and 12 μg of protein was loaded into each well. We saw a very different result in our Western Blot (Figure 14). PAO1 showed more RsmA present than that of *mucA22*. This is
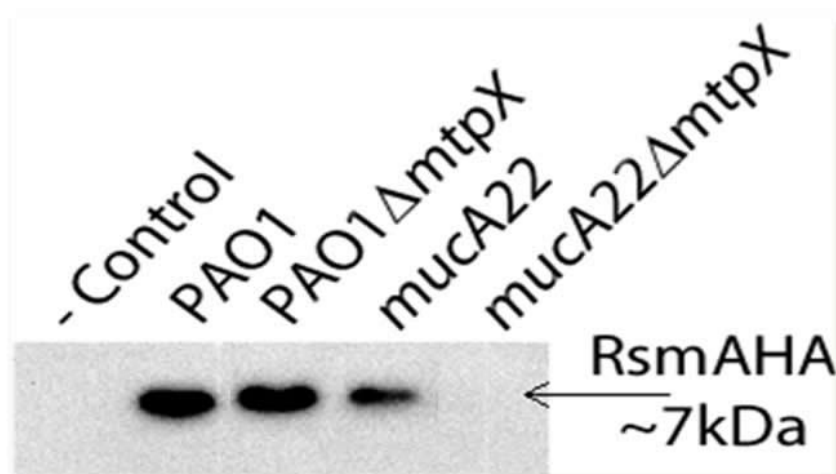
Figure 14. Western Blot of Δ*mtpX* mutants with HA-tagged RsmA. 20μg of protein was used. PAO1 without HA-tagged RsmA was used as a negative control. The arrow marks the HA-tagged RsmA and its size of approximately 7 kDa. When 20 μg of protein is loaded, PAO1Δ*mtpX* appears to have more HA-tagged RsmA(data not shown).

contrary to our β-galactosidase and RNAse protection assays. The nonmucoid background does not seem to be affected by the removal of *mtpX*; however this may be because 20 μg were loaded on the SDS-PAGE. For the mucoid background, we see the same decrease in the *mtpX* mutant as reported before in other assays. This experiment was repeated twice to confirm the results because there was no presence of RsmA detected in the *mucA22ΔmtpX* strain.

<div align="center">Motility Assays</div>

Having patched the *mtpX* mutants multiple times on various plates, we noticed that there were distinct hazes around the regions of growth that looked similar to twitching zones. *P. aeruginosa* is equipped with the ability to swim via flagella and twitching via type IV pili. Because mucoid strains aren't supposed to be as motile as nonmucoid strains, we decided to screen the *mtpX* mutants in various motility assays.

Swimming Assay Shows Increase in Mucoid Background Δ*mtpX*

The swimming assay was performed on LB plates with 0.3% agar. After incubating for 48 hours at 30°C, the zones of swimming were measured. Because the medium is not as thick,

bacteria use their flagella to "swim" through the medium. *P. aeruginosa's* ability to swim varies

upon the strain. Here we looked at the mutants in both backgrounds (Figure 15). PAO1 and its

mtpX mutant showed no real difference in swimming. The *mucA22* strain is not supposed to

express flagella; instead it is supposed to be more stationary. However, the *mucA22ΔmtpX*

mutant swam more than *mucA22* and PAO1. At the time we did not know why this occurred or

how it may relate to RsmA levels in the cell. We decided to look at twitching in these mutants as

well.
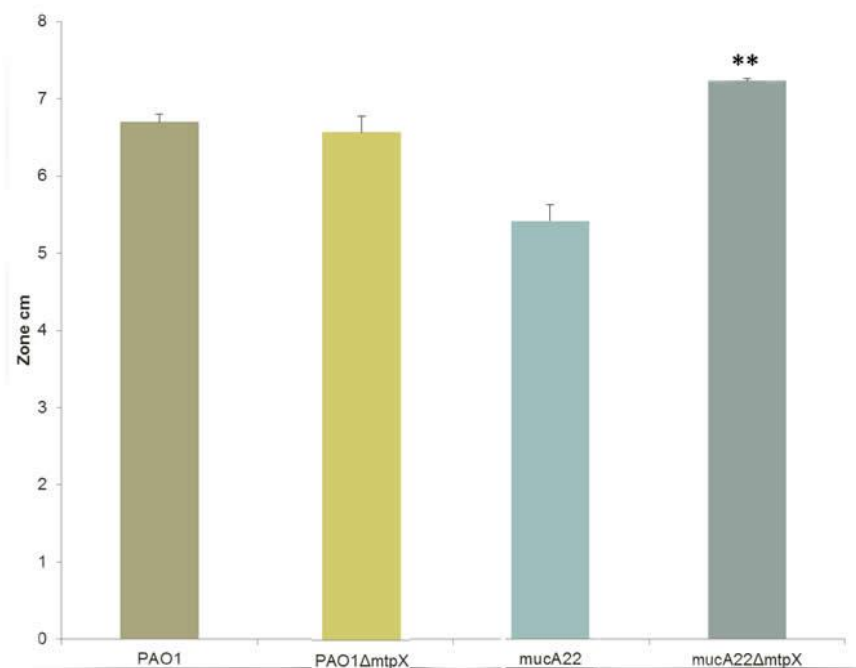
Figure 15. Swimming Assay of *mtpX* mutants and parent strains. *mucA22ΔmtpX* was the only
significant result in the swimming assay. Zones of growth were measured in centimeters. Two
stars represent a $p < 0.01$.

Twitching Assay Shows Increase in Mucoid Background While Decreased in Nonmucoid

Background

The twitching assay looks at the ability of bacteria to propel themselves through a thicker

media, 1.5% agar media. We showed that the inner membrane protein MtpX has some effect on

twitching in both backgrounds (Figure 16A). Zones are measured after the agar is removed and have been stained with crystal violet (Figure 16B). PAO1Δ*mtpX* showed a decrease in twitching ability, almost to the level of *mucA22,* which is not able to twitch. However, similarly to the swimming assay the *mucA22ΔmtpX* strain was able to outperform PAO1 in twitching zones and was drastically different from *mucA22*.
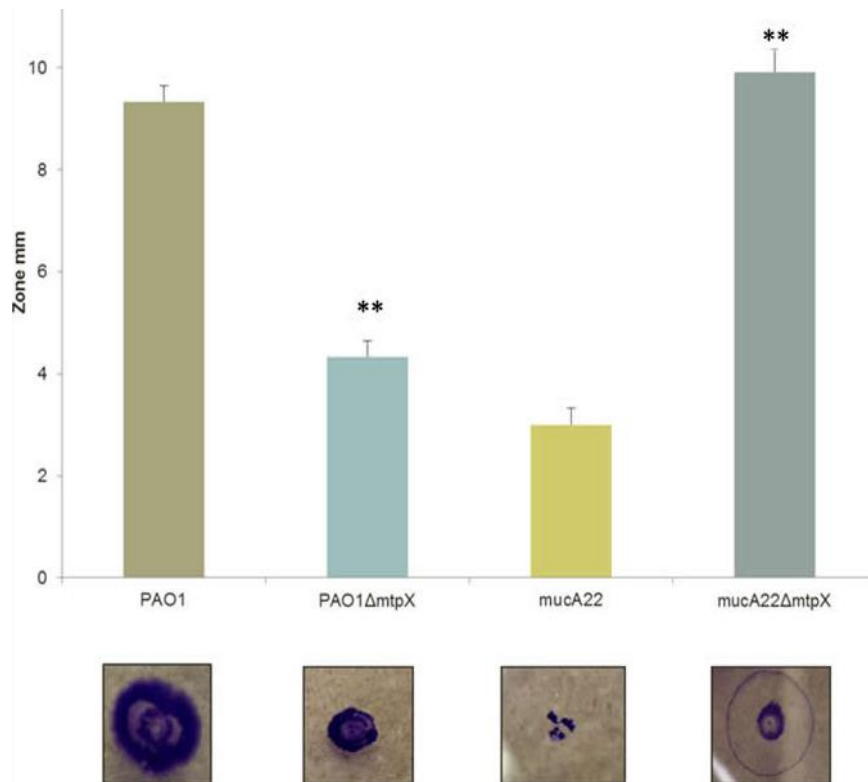
Figure 16. Twitching Assay showing the effects of Δ*mtpX* on type IV pili in both mucoid and nonmucoid backgrounds. A. Measurements in millimeters of twitching zones. B. Images of crystal violet stained twitching zones under their respective strain.

Transmission Electron Microscopy (TEM) of Δ*mtpX* Mutants

Because the motility assays were presenting such interesting results, we set out to visualize the cell surface via TEM. We found that 2% UA provided the best results in visualizing the cells with the TEM when compared to 2% phosphotungstic acid (PTA). The most alluring data, the *mtpX* mutants, show the presence of flagella on their cells. PAO1 (Figure 17A) is our wildtype strain that can express either flagellum or type IV pili. We have shown through the motility assay

that both conditions are possible depending on the type of media used. On the PAO1Δ*mtpX* cell

(Figure 17C), we see a single polar flagellum. This image explains the mutant strains ability to

swim but not twitch. In the mucoid background, we see *mucA22* (Figure 17B) secreting

exopolysaccharide and the cell also has cellular appendages. However, the
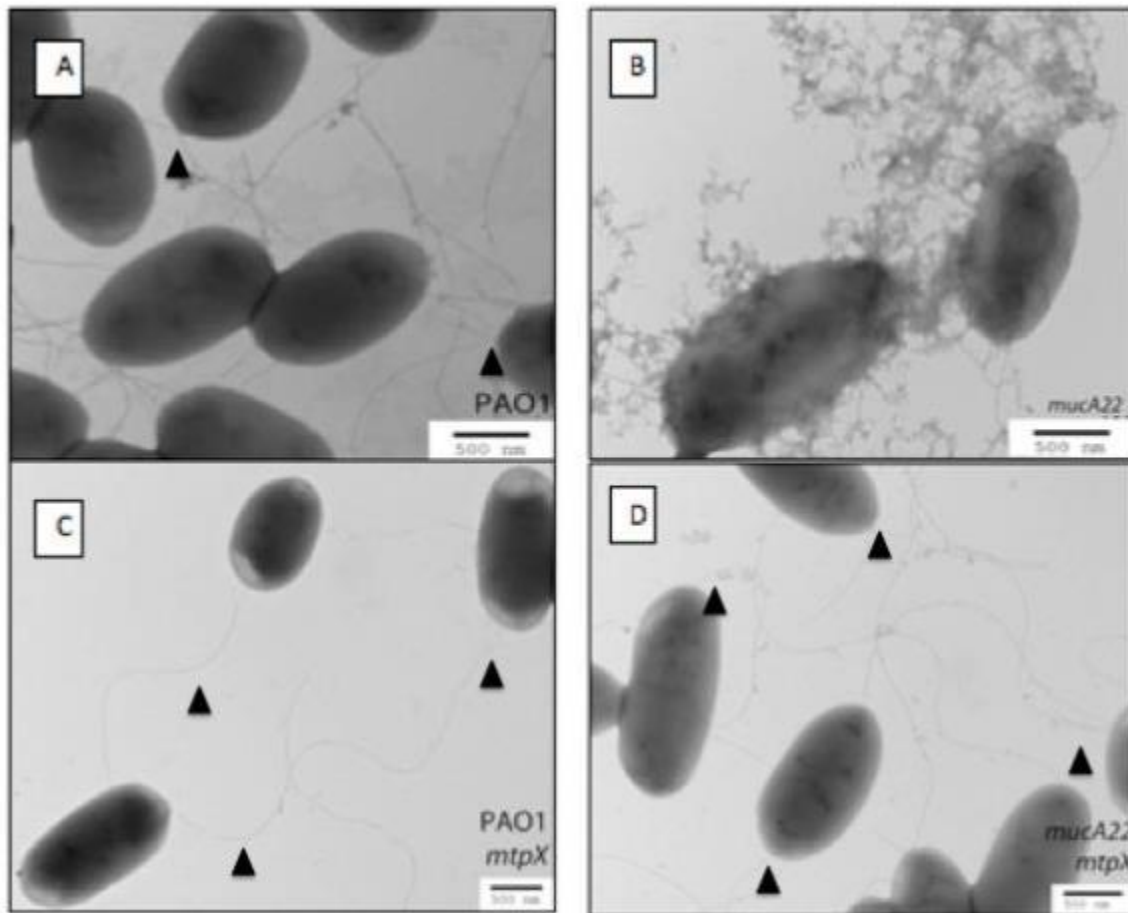
Figure 17. Transmission Electron Microscopy of PAO1(A), *mucA22*(B), PAO1Δ*mtpX*(C), and *mucA22ΔmtpX*(D). Black arrows designate single polar flagellum.

mucA22ΔmtpX cell (Figure 17D) shows small amounts of exopolysaccharide and, like the

PAO1Δ*mtpX* cell, a single flagellum. This explains our swimming results.

Siderophore Production Increases in Mucoid Background Δ*mtpX*

Mutant strains growing on both LB and PIA plates were producing an unusual amount of

yellow-green pigment extending from areas of growth. Under the presence of ultraviolet(UV)

light, the green pigment fluoresced, which is a trademark of pyoverdine, an iron-binding compound produced by *P. aeruginosa*. We made CAS plates in order observe the production of pyoverdine. When iron is sequestered by siderophores, the iron-dye complex of the plate turns from blue to orange when pyoverdine is produced (21). Here we measured the orange zones to see if the *mtpX* mutants were producing more pyoverdine than PAO1 and *mucA22* (Figure 18A). We also exposed the patched strain to UV light to confirm we were actually seeing pyoverdine (Figure 18B). *mucA22ΔmtpX* showed a significant increase in siderophore production when compared to *mucA22* and was statistically significant in this experiment.
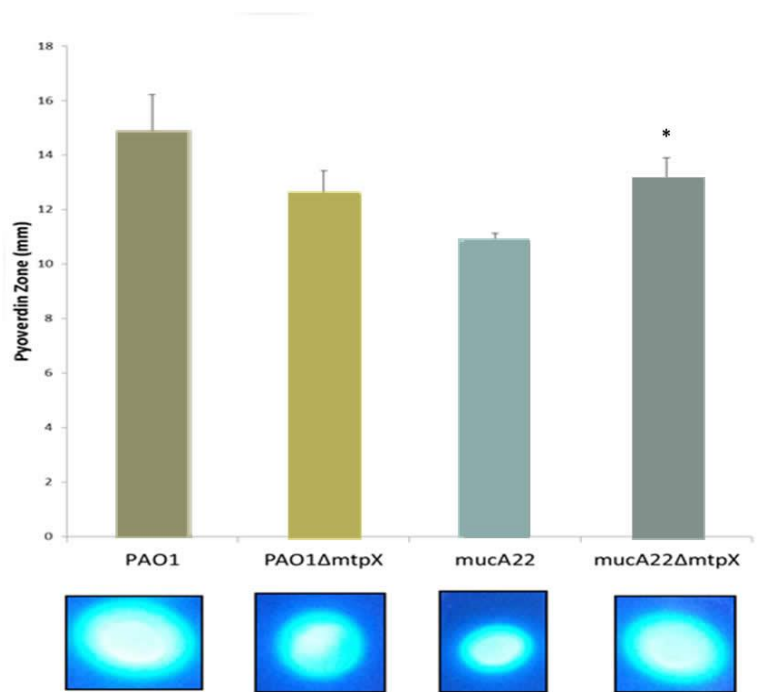
Figure 18. Measured zones of pyoverdine in millimeters (A). Plates exposed to UV light (B) under their respective strain.

Biofilm Assay Shows Decrease in Nonmucoid Background Δ*mtpX* Mutant

P. aeruginosa is well known for its production of biofilm by secreting exopolysaccharide on both biotic and abiotic surfaces. Our mucoid strain *mucA22* produces a very thick biofilm of alginate. We set out to see if our *mtpX* mutants have decreased production of biofilm. When *P.*

aeruginosa is expressing flagella, it is not as likely to be producing biofilm. We also saw a drastic phenotypic change in the *mucA22ΔmtpX* strain, which we assumed affected biofilm. We performed a microtiter biofilm assay (31) and determined the amount of biofilm that adheres to the polystyrene sides using crystal violet and spectrophotometry (Figure 19A). We also looked at patch plating the strains on to Congo Red media (Appendix) (Figure 19B) for any difference in appearance on this media. The Congo Red dye will bind exopolysaccharide secreted by cells, with red or purple strains expressing more biofilm (22).
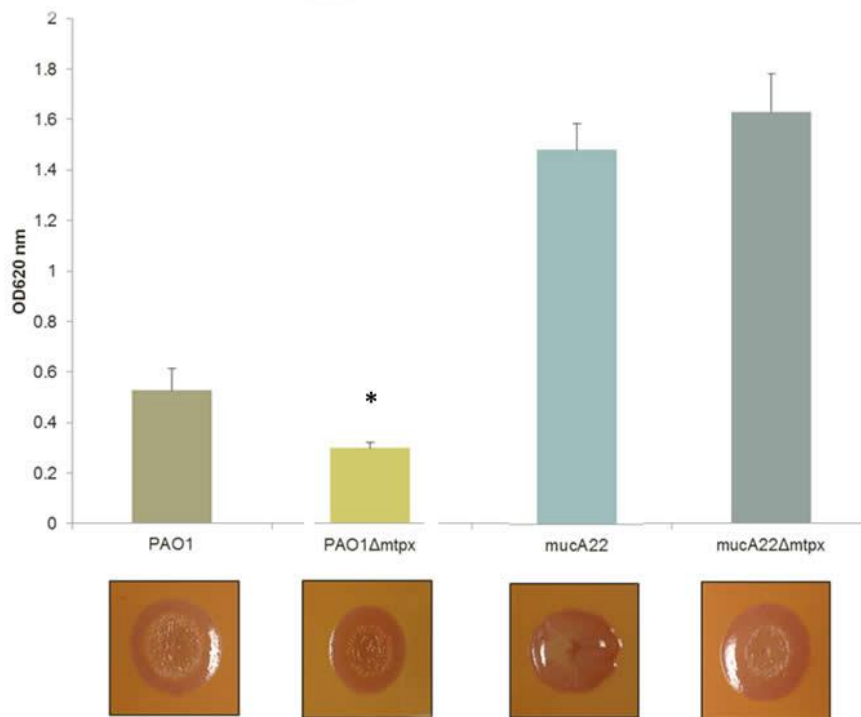
Figure 19. Microtiter Biofilm Assay of ΔmtpX mutants, measured at O.D. 620nm(A). PAO1ΔmtpX was the only strain to show any difference in biofilm production. One star represents a $p < 0.05$. Strains patch plated on Congo Red media (B) show no drastic color appearance.

The PAO1ΔmtpX strain exhibited a decrease in biofilm production, while the *mucAΔmtpX* showed no difference in biofilm production.

Alginate Assay Shows Decrease in Mucoid Background ΔmtpX

The biofilm assay did not give us the results we expected, so we decided to perform an

alginate assay because of the visual appearance of the *mucAΔmtpX*. The *mucAΔmtpX* showed a significant decrease in alginate production (Figure 20). These results confirmed that MtpX is required for alginate production. The amount of alginate was quantified via a standard curve. This experiment was performed in triplicate. As indicated in Figure 20, the *mucA22ΔmtpX* mutant had an almost 2000-fold reduction in alginate. This is the first description of this transporter playing a role in alginate production.
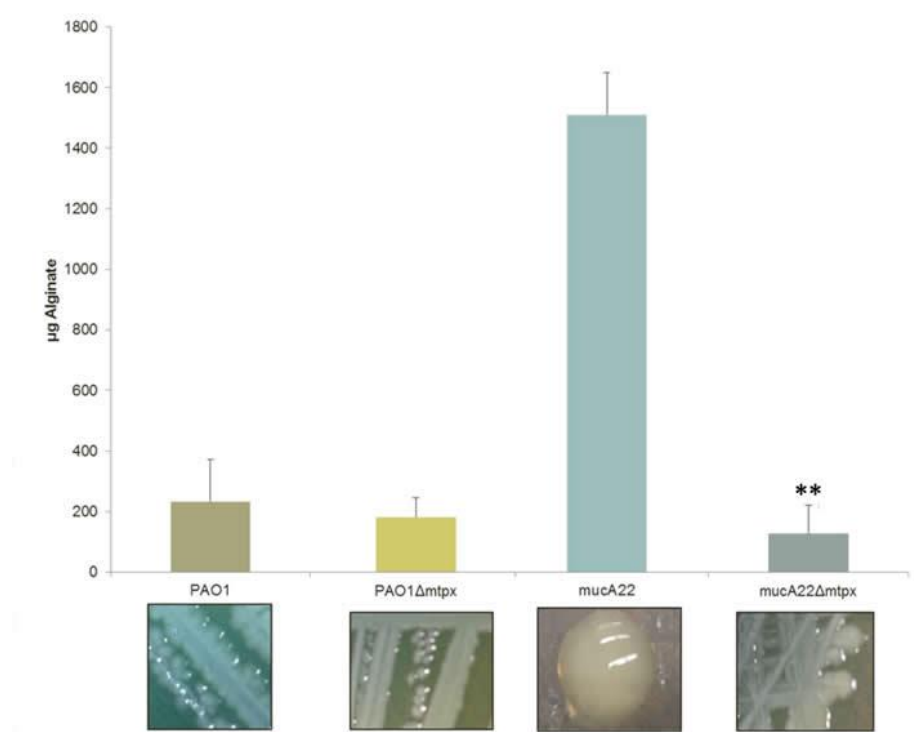
Figure 20. Quantification of alginate produced by Δ*mtpX* mutants (A) and images of strains on PIA plates (B). *mucA22ΔmtpX* showed a significant decrease in alginate production. Two stars represent a $p < 0.01$.

Reverse Transcriptase PCR on *mtpX*/PA1627 Operon

Besides phenotypically analyzing the Δ*mtpX* mutants, we wanted to characterize the *mtpX* messenger RNA. Bioinformatic data suggest that *mtpX* is in an operon with the upstream gene, PA1627, a probable transcriptional regulator. Using gene sequence from pseudomonas.com, we designed primers to produce cDNA for the mRNA containing both *mtpX* and *PA1627* (41).

Forward primers were located at the start of the intergenic space between *PA1628* and *PA1627*, the start of *PA1627*, and the middle of *PA1627*. Our results (Figure 21) showed that *PA1627* and *mtpX* were indeed transcribed in the same message. However, the significance of these results has not been elucidated.
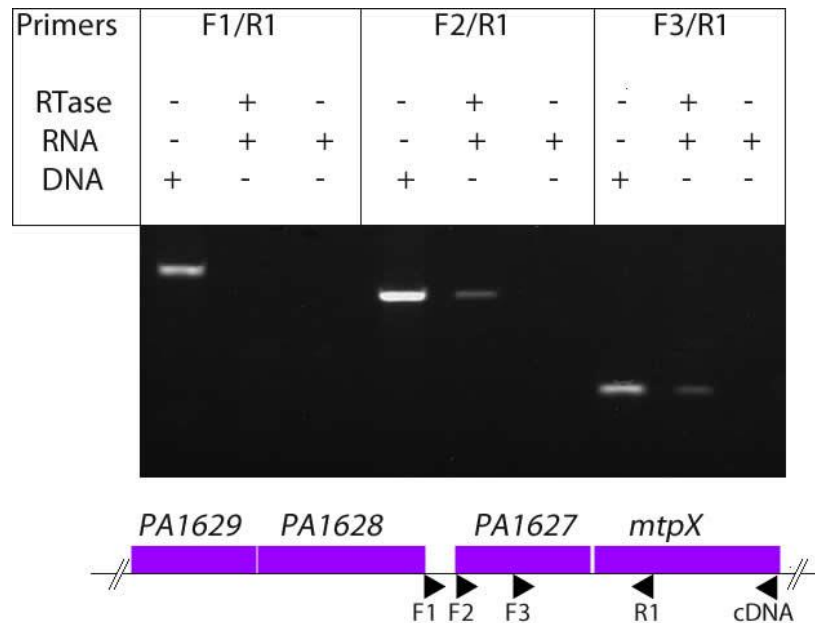
Figure 21. RT-PCR of *PA1627/mtpX* operon to confirm these 2 genes were in an operon. Genomic DNA was used as a positive control. RNA without reverse transcriptase was used as a negative control to ensure there was no genomic DNA contamination. Amplified products were run on a 1% agarose gel. Location of primers on a genomic model is shown below the gel.

CHAPTER 4

DISCUSSION

P. aeruginosa is a virulent opportunistic pathogen notorious for causing complications in immunocompromised individuals and nosocomial infections. An understanding of how *P. aeruginosa* uses its virulence factors to cause infections could lead to better chemotherapeutic treatment of this pathogen. By studying the global regulator RsmA, we have not only learned more about the network of virulence factors it controls but also the regulators of *rsmA*'s expression.

rsmA Regulators

The screen to identify rsmA regulators has increased our knowledge of how this important posttranscriptional regulatory system is controlled. *pilW* was interrupted in the mucoid strain *mucA22*, which is thought to not express type IV pili(4). However, when the transposon interrupted the minor pilin gene, we saw a significant decrease in *rsmA* expression. Because *pilW* is in an operon with 6 other pilin genes, we made a knockout mutant of *pilW* to confirm our transposon results. We found PilW does affect *rsmA* expression in this background (data not shown) and have set out further to study the effects it has on RsmA. Another interesting gene to study will be the probable transcriptional regulator *PA2028*. We still have to knockout this gene and to determine its effects on RsmA's antagonists, RsmY and RsmZ. Lastly, there is *mtpX* the probable major facilitator superfamily transporter. MtpX shows weak homology to CynX, a cyanate permease in *E. coli* with 33% identical residues and 54% similar residues (15,16,32). In *P. aeruginosa* there are 2 other genes that are more homologous to CynX. Since only a few parts of MtpX's cellular function have been elucidated (Figure 22) in both mucoid and nonmucoid backgrounds of *P. aeruginosa*, we are looking to further study this inner membrane transporter.

We have shown that there is a definitive relationship between the expression of *rsmA* and MtpX. After confirming the transposon mutant with β-galactosidase assays of a Δ*mtpX* mutant, we discovered divergent results in our 2 backgrounds (Figure 20). In the mucoid background, we saw a slight decrease in the 2 mRNAs of *rsmA* via RNase Protection Assay.
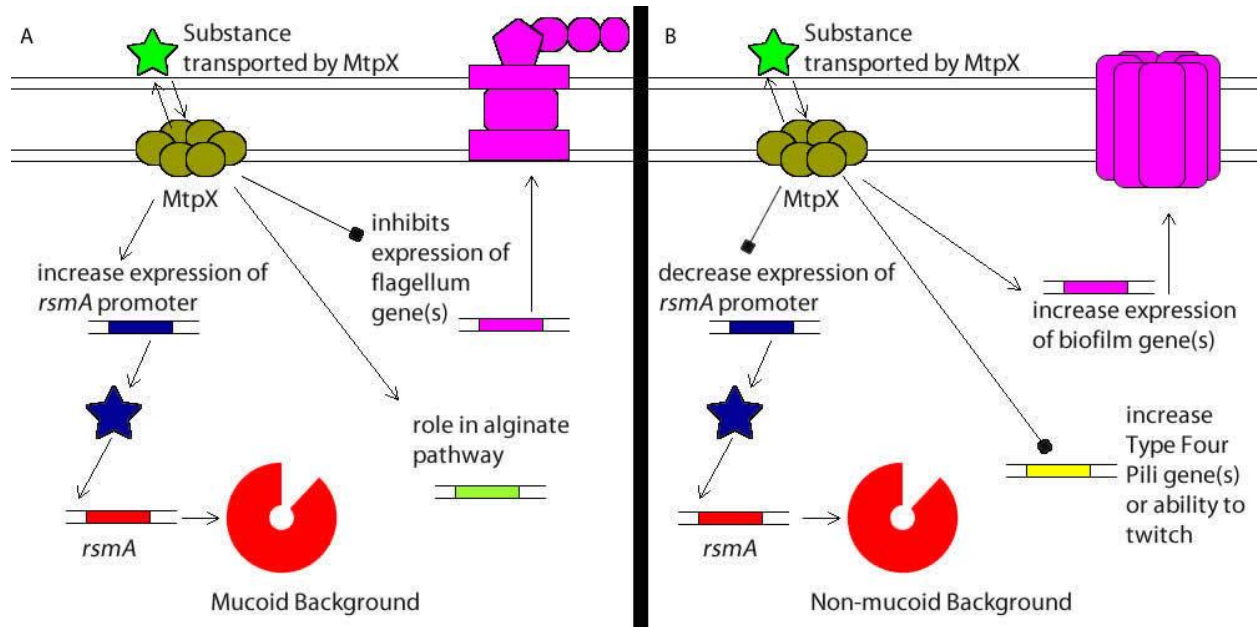
Figure 22. Mechanism proposal of MtpX representing the mucoid strains (A) and nonmucoid strains (B). Arrows denote an up-regulation while squares represent down-regulation of genes.

Our Western Blot analysis also confirmed the loss of RsmA in the Δ*mtpX* mutant. We have seen through β-galactosidase assays that RsmY and RsmZ greatly increase in the mucoid background. This may explain the loss of RsmA from the Western Blot in the *mucA22 ΔmtpX* strain as the decreased levels are bound by RsmA's antagonists. We can pursue this answer by using a *rsmY- lacZ* and *rsmZ-lacZ* fusion and confirming the results with RNAse Protection Assays. We can also look at RsmA with a Western Blot in *mucA22ΔrsmYΔrsmZ* and *mucA22ΔrsmYΔrsmZΔmtpX* mutants with HA-tagged RsmA. If RsmY and RsmZ are factoring into the levels of RsmA in the Δ*mtpX* mutant, either of these experiments would explain the results. Contrarily, MtpX may regulate RsmY and RsmZ. Because we saw a disappearance of RsmA levels via the Western blot analysis in the mucoid background, MtpX could down regulate

the expression of *rsmY* or *rsmZ*. By looking into *rsmY* and *rsmZ* expression in the mucoid background, increases in expression of these 2 small RNA may explain our *rsmA* results. We can also overexpress *rsmY* and *rsmZ* in the nonmucoid background, to see if the results are opposite and RsmA levels decrease (35). In the RNase Protection Assay and Western blot analysis, we saw contrasting results between nonmucoid and mucoid backgrounds.

For the case of the nonmucoid background, we saw a marked increase in the *rsmA* mRNA with AlgU promoter. We can confirm that MtpX affects AlgU by knocking out AlgU and performing another RNase protection assay in this double mutant with the *rsmA* probe, as well as looking examining RsmA levels in an *algU* mutant strain. After completing this series of experiment, we may be able to better understand the role MtpX shares with *rsmA* expression.

<u>Characterizing MtpX</u>

Seeing the transposon mutant revert from the mucoid *mucA22 rsmA-lacZ* strain to a nonmucoid variant was concerning at first. After repeatedly making the knockout mutant in *mucA22* and also our 2 mucoid CF clinical isolates, FRD1 and 2192, it was clear that the MtpX transporter plays an important role in alginate production. This may provide a new chemotherapeutic target that may help eradicate *P. aeruginosa* infections in cystic fibrosis patients (7). The most interesting effect of knocking out the *mtpX* gene, besides loss of alginate production, is the appearance of flagella on the *P. aeruginosa* cells. Dispersion mechanisms are *en vogue* currently in *Pseudomonas* pathogenesis research and MtpX may play a role in biofilm dispersion given that the *mucA22ΔmtpX* demonstrated increased swimming compared to the *mucA22* parent. Even though our biofilm assays did not detect a difference in biofilm formation using the microtiter plate assay, we did not specifically examine biofilm dispersal. This will be the subject of further investigation. Additionally, the overespression of pyoverdine suggests that

the *mucA22ΔmtpX* mutant may have a problem acquiring iron or in overproducing exzymes requiring iron. Further investigation of this mutant will provide important insights into the pathogenic mechanisms used by *P. aeruginosa*.

Further investigations analyzing the transcriptional regulation of the *mtpX* genomic region are underway. This will include mapping the transcriptional start site of *PA1627* by primer extension. In doing so, we will be able to possibly identify the promoter of this operon. It is also in our interest to make a knockout mutant of PA1627 in both mucoid and nonmucoid backgrounds and look at the effects on *rsmA* expression. Because PA1627 is a transcriptional regulator, it could possibly auto regulate the *mtpX-PA1627* operon. Additional studies into the regulation of this system are needed to identify new possible drug targets. Isolating the MtpX protein and clarifying the substance it transports will also be crucial to closing the chapter on this regulator of *rsmA* expression. Although there are still more questions to answer, we feel that MtpX may prove to be an important resource not only in RsmA's cellular role but also the pathogenicity of *Pseudomonas aeruginosa.*

REFERENCES

1. Balasubramanian D, Schneper L, Kumari H, Mathee K. 2012. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. Nucleic Acids Research 41(1):1-20.

2. Bardoel BW, van der Ent S, Pel MJ, Tommassen J, Pieterse CM, van Kessel KP, van Strijp JA. 2011. *Pseudomonas* Evades Immune Recognition of Flagellin in Both Mammals and Plants. PLoS Pathog. 7(8): 1-11

3. Becher A, Schweizer HP. 2000. Integration-Proficient *Pseudomonas aeruginosa* Vectors for Isolation of Single-Copy Chromosomal *lacZ* and *lux* Gene Fusions. Biotechniques 29:948-952

4. Brencic A, Lory S. 2009. Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol. 72(3):612-632.

5. Burrowes E, Baysse C, Adams C, O'Gara F. 2006. Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiol. 152:405-418.

6. Cattoir V, Narasimhan G, Skurnik D, Aschard H, Roux D, Ramphal R, Jyot J, Lory S. 2012. Transcriptional Response of Mucoid *Pseudomonas aeruginosa* to Human Respiratory Mucus. 3(6): 1-10.

7. Childers M, Eckel G, Himmel A, Caldwell J. 2007. A new model of cystic fibrosis pathology: Lack of transport of glutathione and its thiocyanate conjugates. Medical Hypotheses. 68:101-112.

8. Cohen TS, Prince AS. 2013. Activation of inflammasome signaling mediates pathology of acute *P. aeruginosa* pneumonia. J Clin Invest 123(4):1630-1637.

9. Daddaoua A, Krell T, Ramos J. 2009. Regulation of Glucose Metabolism in Pseudomonas: The phosphorylative branch and entner-doudoroff enzymes are regulated by a repressor containing a sugar isomerase domain. J Biol Chem. 284:21360-21368.

10. Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S, Ramphal R. 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol. Micro. 50(3):809-824.

11. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. 76(4): 1648-1652.

12. Galle M, Carpentier I, Beyaert R. 2012. Structure and Function of the Type III Secretion System of *Pseudomonas aeruginosa*. Current Protein and Peptide Science 13(8): 831-842

13. Ghafoor A, Hay ID, Rehm BH. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl Environ Microbiol. 77(15): 5238- 46.

14. Giltner CL, Habash M, Burrows LL. 2010. *Pseudomonas aeruginosa* Minor PIlins Are Incorporated into Type IV Pili. J Mol Biol. 398: 444-461.

15. Geer LY, Marchler-Bauer A, Geer RC, Han L, He J, He S, Liu C, Shi W, Bryant SH. 2010. The NCBI BioSystems database. *Nucleic Acids Res.* 38(Database issue):D492-6.

16. Guilloton MB, Lamblin AF, Kozliak EI, Gerami-Nejad M, Tu C, Silverman D, Anderson PM, Fuchs JA. 1993. A physiological role for cyanate-induced carbonic anhydrase in Escherichia coli. J Bacteriol. 175(5):1443-1451

17. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 77(1): 51-59.

18. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. 2012. The Multiple Signaling Systems Regulating Virulence in *Pseudomonas aeruginosa*. Micro. and Molecular Bio Rev.76(1):46-65.

19. Kay E, Humair B, Dénervaud V, Riedel K, Spahr S, Eberl L, Valverde C, Haas D. 2006. Two GacA-Dependent Small RNAs Modulate the Quorum-Sensing Response in *Pseudomonas aeruginosa*. J Bacteriol 188(16): 6026-6033.

20. Knutson CA, Jeanes A. 1968. A New Modification of the Carbazole Analysis: Application to Heteropolysaccharides. Analytlical Biochem. 24: 470-481.

21. Kronda JM, Cooper RA, Maddocks SE. 2013. Manuka honey inhibits siderophore production in *Pseudomonas aeruginosa*. J Appl Microbiol 115(1):86-90.

22. Kuchma SL, Griffin EF, O'Toole GA. 2012. Minor PIlins of the Type IV PIlus System Participate in the Negative Regulation of Swarming Motility. J Bacteriol 194(19):5388- 5403

23. Lindestam-Arlehamn CS, Evans TJ. 2011. *Pseudomonas aeruginosa* pilin activates the inflammasome. Cellular Microbiology 13(3):388-401.

24. Martin DW, Schurr MJ, Mudd MH, Govan JRW, Holloway BW, Deretic V. 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. 90:8377-8381.

25. Mattick JS. 2002. Type IV Pili and Twitching Motility. Annu Rev Microbiol. 56:289- 314.

26. Mercante J, Edwards AN, Dubey AK, Babitzke P, Romeo T. 2009. Molecular geometry of CsrA(RsmA) binding to RNA and its implications for regulated expression. J Mol Biol. 392(2): 511-528.

27. Mikkelsen H, Sivaneson M, Filloux A. 2011. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. Environmental Microbiol. 13: 1666-1681.

28. Miller JH, 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press.

29. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm Formation as Microbial Development. Annu. Rev. Microbiol. 54:49-79.

30. O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol 28(3): 449-461.

31. O'Toole GA, 2011. Microtiter dish biofilm formation assay. J Vis Exp. 30(47):2437- 2443

32. Pao SS, Paulsen IT, Saier Jr. MH. 1998. Major Facilitator Superfamily. Microbiol Mol Biol Rev. 62(1): 1-34.

33. Potvin E, Sanschargrin F, Levesque RC. 2007. Sigma factors in *Pseudomonas aeruginosa*. FEMS Microbiol Rev. 32: 38-55.

34. Rubin EJ, Akerley BJ, Novik VN, Lampe DJ, Husson RN, Mekalanos JJ. 1999. In vivo transposition of mariner-based elements in enteric bacteria and mycobacteria. Proc Natl Acad Sci. 96: 1645-1650.

35. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. 2008. P_{BAD}-Based Shuttle Vectors for Functional Analysis of Toxic and Highly Regulated Genes in *Pseudomonas* and *Burkholderia* spp. and Other Bacteria. Applied and Environmental Microbiology 74(23):7422-7426.

36. Ramsey DM, Wozniak DJ. 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol Microbiol. 56(2):309-322.

37. Rashid MH, Kornberg A. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA. 97(9):4885-4890.

38. RNA Analysis Notebook Promega Corporation

39. Schweizer HP. 2001. Vectors to express foreign genes and techniques to monitor gene expression in Pseudomonads. Current Opinion in Biotechnology. 12:439-445.

40. Wagner VE, Iglewski BH. 2008. *P. aeruginosa* Biofilms in CF Infection. Clin Rev Allergy Immunol. 35(3):123-34.

41. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS (2011).Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes.Nucleic Acids Res. 2011 Jan;39(Database issue):D596-600.

Appendix A: Media

<u>PIA (1 Liter)</u>

935 ml ddH$_2$O
20 ml Glycerol
bring to boil

Add:
20g Peptone
10g Potassium Sulfate
13.6g Agar
1.4g Magnesium Chloride
25mg Irgasan(irg)

Autoclave
Antibiotic concentrations:
150 µg/ml gentamicin
80 µg/ml X-gal

<u>LB (1 Liter)</u>

940 ml ddH$_2$O
15g Agar
10g NaCl
5g Yeast Extract
10g Tryptone

Autoclave

Antibiotic concentrations:
15 µg/ml gentamicin
10 µg/ml tetracycline
100 µg/ml ampicillin
50 µg/ml kanamycin

<u>LB no NaCL tet^{50} irg^{25} (1 Liter)</u>
same as LB, just no salt added
25 mg Irgasan

Autoclave

50 µg/ml tetracycline

<u>1% agarose gel (for 2 gels)</u>

0.5 g Agarose
5 ml 10X TBE
45 ml ddH$_2$O

Microwave for 1 minute

Add 2.7 µl Ethidium Bromide when container is warm to touch

M9 salts
10X M9 salts (500 ml)
30g Na$_2$HPO$_4$
15g KH$_2$PO$_4$
2.5g NaCl
5g NH$_4$Cl
pH to 7.4

For 100ml of broth:
10ml 10X M9 salts
0.2 ml 1M MgSO$_4$
0.1 ml 0.1M CaCl$_2$
0.1 ml 1M thiamine HCl

Add 1% carbon source of interest.

VBMM
500 ml 10X VBMM stock

400 ml ddH$_2$O
15g Trisodium Citrate
10g Citric Acid
50g K$_2$HPO$_4$
17.5g NaNH$_4$PO$_4$ · 4H$_2$O
pH to 7 and autoclave

plates (500ml):

400 ml ddH$_2$O
7.5g Agar
autoclave and allow to cool to 50°C
50 ml 10X VBMM
500 µl 1M Magnesium Sulfate
50 µl 1M Calcium Chloride

Antibiotics:
300 µg/ml Carbenicillin

M63 minimal media broth(31)
5X M63 stock: (1 Liter)
15g KH_2PO_4
35g K_2HPO_4
10g $(NH_4)_2SO_4$ ddH_2O to 1L

Broth final concentrations:
1X M63
1% Glucose
1mM $MgSO_4$
1% Glycerol
0.5% Casamino acids
0.4% Arginine
sterile ddH_2O to final volume desired

Congo Red(22)
5g Tryptic Soy Broth
6.7g Agar
460 ml ddH2O

Autoclave and cool to 50ºC

Congo Red 40μg/ml Coomassie Brilliant Blue 20μg/ml

Appendix B: Buffers

10X TNE
830 ml ddH$_2$O
12.1g Tris Base
3.7g EDTA
116.8g NaCl

1X TBS-T
10X TBS:
12.1g Tris base
43.8g NaCl
ddH$_2$O to 500 ml

1X TBST:
100 ml 10X TBS
500 µl of Tween 20
ddH$_2$O to 1 L

10X TE
108g Tris Base
55g EDTA
900 mL ddH$_2$O

Z Buffer
900 ml ddH$_2$O
16.1g Na$_2$HPO$_4$ · 7H$_2$O
5.5g NaH$_2$PO$_4$ · H$_2$O
0.75g KCl
0.246g MgSO$_4$ · 7H$_2$O
pH to 7 and autoclave

Add 2.7 µl/ml of β-mercaptoethanol when ready to use

10X TBE
108g Tris Base
55g Boric Acid
40 ml 0.5M EDTA
900 ml ddH$_2$O

TEST Buffer
500 µl 1M Tris
120 µl 0.5M EDTA
1.5 ml 50% sucrose
500 µ 10% Triton X-100

4x SDS Loading Buffer
4.4 ml 0.5M Tris HCl pH6.8
4.4 ml Glycerol
2.2 ml 20% SDS
0.5 ml 1% Bromophenol Blue
0.5 ml β-mercaptoethanol
Store at -20ºC

10X Glycine Running Buffer
30.3g Tris Base
144.1g Glycine
10g SDS
ddH2O to 1L

1.5M Tris HCl pH 8.8
27.23g Tris base
80ml ddH2O
adjust pH to 8.8 with 6N HCl

ddH$_2$O to 150 ml

0.5M Tris HCl pH6.8
6.1g Tris base
80ml ddH$_2$O
pH to 6.8 with 6N HCl

ddH$_2$O to 100 ml

Towbin Buffer
10X Towbin Buffer:
30.3g Tris base
144g Glycine
ddH$_2$O to 1 L

1X Towbin buffer for Electroblotting:
10 ml 10X Towbin buffer
20 ml Methanol
70 ml ddH$_2$O

Blocking Buffer
2.5g Skim Milk powder
5 ml TBS-T buffer
45 ml ddH2O

1° antibody Wash
2.5g Skim Milk powder

5 ml TBS-T buffer
45 ml ddH2O
2.5 μl of HA-tag antibody

Washing Buffer
5 ml TBS-T buffer
45 ml ddH2O

2° antibody Wash
2.5g Skim Milk powder
5 ml TBS-T buffer
45 ml ddH2O
2.5 μl of anti-goat/anti-mouse antibody

Sulfuric Acid/Borate Solution
975 ml Sulfuric Acid
25 ml Borate Solution

Borate Solution
10.09g KOH in 45 ml of ddH$_2$O
24.74g Boric Acid
ddH$_2$O to 100 ml

Primers

```
Arbitrary PCR
RdPa1        GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
RdPa2        GGCCACGCGTCGACTAGTACNNNNNNNNNACGCC
R1TnM        TATAATGTGTGGAATTGTGAGCGG
Rd2Pa        GGCCACGCTCGACTAGTAC
R2TnM        ACAGGAAACAGGACTCTAGAGG
TnMSq        CACCCAGCTTTCTTGTACAC


ΔpilW
F1EcoR1      GCGCGAATTCATCGTCGTCCTGCTGGCCATC
R1Sac1       GCGCGAGCTCGTTCGACAGATTGATACTCCCGC
F2           CTCAGGGTCGAGCCATGAGCAACTCAGGAATCTCGTGCCATGAAC
R2           GTTCATGGCACGAGATTCCTGAGTTGCTCATGGCTCGACCCTGAG
internalF    GATCGAAGTGCTGGTCGCCC
internalR    GCGACGCTATTGGTCTCGTAG

ΔmtpX
F1EcoR1      GCGCGAATTCCTGCTGAGCATTCTCCAGCG
R1BamH1      GCGCGGATCCCTGATGCGCATGCTCACGCG
F2           CCATGACTCACGCCTTGAATGGCAGCGAACACCTGTAGCC
R2           GGCTACAGGTGTTCGCTGCCATTCAAGGCGTGAGTCATGG
internalF    CGACCTGCAGGACTACATAG
internalR    GCTGACCTTCGCCTGCAGC

rsmA-lacZ detection primers
Forward      GCCAAGGTTTCCATCGTCGG
Reverse      GATGTGCTGCAAGGCGATTAAG

rsmA-HA tag detection primers
Forward      GCCAAGGTTTCCATCGTCGG
Revese       CTTAGTTACCAAGATGGCTTTCG

RNase Protection Assay Primers for rsmA probe
Forward      TCTCGCGGAATGCTGATTCTGACTCGTCG
Reverse      TAATACGACTCACTATAGGGAGAGATAAAAATTAATGGTTTGGCTCTTG

RTPCR for PA1627/mtpX
cDNA         GATGAAGATATAGCCGACCGCG
F1           GCGCATTGCGCGAACATCTCG
F2           GTGACCATTCCCCTCAAGCGC
F3           CGCATCGGCGACCTGGCGG
R1           GAACTGGTTCGTCGTCGACTTC
```

Plasmids

plasmid	Relevant Characteristics	Source or reference
pBT20	transposon, Gm^R	(33)
pMX19	ΔmtpX, Amp^R	this study
pRHA18	rsmA-HA, Gm^R	this study
pMX18	ΔmtpX, Tet^R	this study
mini-CTX-AlacZ	rsmA-lacZ, Tet^R	this study
pRK2013	used for triparental mating, Kan^R	(11)
pFLP2	used for removing mini-CTX, Amp^R	(39)

VITA

SEAN DENVER STACEY

Personal Data: Date of Birth: July 20, 1985

Place of Birth: Fort Walton Beach, Florida

Favorite Things: Artemis, Kayaking, Running, Dancing, Singing,

Nature, Music, and Plants

Education: M.S. Biology – Microbiology, East Tennessee State University,

Johnson City, Tennessee 2013

B.S. Biochemistry & Cellular/Molecular Biology;

B.A. Classics – Latin, University of Tennessee Knoxville,

Knoxville, Tennessee 2008

Farragut High School, Knoxville, Tennessee

Professional Experience: Graduate Research Assistant, East Tennessee State University,

Department of Health Sciences, Dr. Chris Pritchett, 2011-2013

Teaching Assistant, East Tennessee State University, Department

of Health Sciences, 2011-2013

Guest Services Representative, Hampton Inn of Johnson City;

Johnson City, Tennessee, 2010-2013

Undergraduate Researcher, University of Tennessee Knoxville,

Department of Biochemistry & Cellular/Molecular Biology,

Dr. Barry Bruce, 2008

Honors & Awards: Excellence in Teaching Award, East Tennessee State University

Quillen Scholarship, East Tennessee State University