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East Tennessee State University

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AlgR Directly Controls *rsmA* in *Pseudomonas aeruginosa*

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A thesis

presented to

the faculty of the Department of Biology

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In partial fulfillment

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Master of Science in Biology

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by

Tyler Speaks

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Dr. Christopher Pritchett, Chair

Dr. Bert Lampson

Dr. Zachary Walls

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ABSTRACT

AlgR Directly Controls *rsmA* in *Pseudomonas aeruginosa*

by

Tyler Speaks

Pseudomonas aeruginosa is a bacterial pathogen that can infect any human tissue. The lungs of cystic fibrosis patients become chronically infected with *Pseudomonas aeruginosa*. Virulence factor gene expression is under elaborate regulatory control that remains poorly characterized. Understanding the regulatory hierarchy involved during infection is essential for identifying novel drug targets. RsmA is a post-transcriptional regulatory protein that controls expression of several virulence factors. Previous studies demonstrated alginate regulatory components AlgU and AlgR as regulators of *rsmA* expression. The aim of this study was to determine how AlgR controls *rsmA* expression. Western blot analysis of HA-tagged RsmA confirmed lower RsmA levels in an *algR* mutant. An electrophoretic mobility shift assay using purified AlgR demonstrated direct binding of AlgR to the *rsmA* promoter. These results indicate AlgR directly controls *rsmA* expression. We propose a mechanism whereby AlgR and AlgU work together to regulate *rsmA*.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative, opportunistic pathogen capable of surviving in a diverse array of environmental and host reservoirs. *P. aeruginosa* exploits a compromised host immune system to cause opportunistic infections, and is able to infect any tissue within the human body (3, 42). *P. aeruginosa* commonly causes nosocomial infections and is a major agent of sepsis in burn patients, eye infections, urinary tract infections, ventilator associated pneumonia and fatal infection in lungs of cystic fibrosis patients (3, 29, 42). *P. aeruginosa* is able to infect such diverse tissues because of a wide array of virulence factors under elaborate genetic control (26, 62). The pathogen's ability to persist in a biofilm is also a major virulence determinant that results in immune evasion and persistence (13, 29, 39). *P. aeruginosa* infects cystic fibrosis patients at an early age and eventually sets up a chronic infection that becomes the leading cause of mortality for these patients (23).

Pseudomonas aeruginosa Infection in Cystic Fibrosis Patients

Cystic fibrosis (CF) is the most commonly inherited lethal disorder among Caucasians, occurring in roughly 1 per 2,500 individuals (10). The disorder results from a loss of function mutation in *CFTR*, which encodes an ion channel transfer protein (20). Individuals possess lungs with sticky mucus and diminished mucociliary clearing capacity. The patient's diminished respiratory innate defense results in colonization and infection with a variety of bacteria (8). In previous years, patients succumbed at an early age to infections from *Staphylococcus aureus* and *Haemophilus influenzae*. With the advent of new antibiotic therapies came the infiltration and

dominance of new infecting strains, with *Pseudomonas aeruginosa* emerging as the deadliest pathogen (11, 23). Patients become colonized with *P. aeruginosa* from the environment at an early age, and the lungs become subject to repeat acute infections, causing inflammation related lung damage (8, 23, 29). Clinical isolates from acute lung infections resemble strains found infecting other areas of the body (4, 66). Isolates are typically motile, by mechanisms involving flagellar swimming, twitching by Type IV pili, swarming as a combination of both, and finally gliding motility enabled by production of biosurfactants (4, 5, 9, 53). Acute infecting strains also possess a Type III secretion system that targets toxins to human cells, resulting in damage to host cell machinery and cell death (60). *P. aeruginosa* is also capable of producing other secreted toxins, proteases, elastases and hemolysins to damage the host (50, 54). Though biofilms are a hallmark of chronic infecting strains, acute strains are also capable of such a lifestyle. Biofilms are regulated by the *pel* and *psl* genes, which produce a different polysaccharide than the alginate typical of chronic isolates (53, 58). During the course of acute infection, repeated stress to the bacteria within the lungs results from antibiotics, immune reactive oxygen species, and osmotic/cell membrane stress. These stressors place selective pressure on the virulent bacteria that eventually lead to the emergence and prevalence of sessile, chronic infecting strains (29, 41, 61). Chronic infecting strains possess different genetic and phenotypic markers from their acute counterparts, and can often be designated by attenuated virulence and heavy alginate biofilms (13, 29, 66). Commonly, chronic isolates overproduce the exopolysaccharide alginate, resulting in the mucoid colony morphology (13, 29, 65 and *Figure 1*). The conversion to mucoidy is associated with declining lung function and poor prognosis for the patient (23, 29). The alginate produced by mucoid strains protects *P. aeruginosa* from phagocytic pseudopods, opsonin deposition on the cell surface, and free radicals produced by immune cells (29, 30, 57). A

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damaging feedback loop results within the lung, where neutrophils are unable to clear the

bacteria but continually secrete inflammatory cytokines for further phagocyte recruitment. This

inflammation, along with damage caused by the bacteria, leads to severe lung damage and

eventual death to the patient from loss of lung function (23, 29, 57). This serious pathology has

resulted in a large amount of study over the last several decades in elucidating the means by

which *P. aeruginosa* converts to a mucoid strain.

<u>Mucoid Phenotype and Alginate Regulation</u>

Mucoid clinical isolates differ from acute infecting strains as a result of genetic mutations

that lead to production of different virulence factors (66). To fully understand the reasons for

this, it is necessary to review the main regulatory system involved in conversion to a mucoid

strain. At the center of this switch are the products of the *algUmucABCD* operon, which

illustrate a system involved in responding to stresses on the cell membrane (36, 40).

In a typical acute infecting strain, the MucA anti-sigma factor bound within the

cytoplasmic membrane sequesters the sigma factor AlgU, also known as AlgT or σ^{22} (40, 51,

66). AlgU bears homology to *E. coli* σ^E, a membrane stress response sigma factor (1, 29). AlgU

controls a large regulon of genes involved in osmotic stress responses, metabolite production and

alginate biosynthesis (22, 56). Activation of this regulon by the normally bound AlgU can occur

in two ways. First, membrane or oxidative stress can result in direct or proteolytic degradation

of MucA, which releases AlgU. AlgU then increases its own expression and controls expression

of genes to combat the environmental stresses (29, 40, 56). In the CF lung, the bacteria are

subject to hypermutation that results in a wide variety of phenotypic variants to increase

survivability (17). Mutations in DNA mismatch repair systems can lead to *mucA* loss of function

mutations (41). In addition, peroxide stress has been shown to increase the recruitment of DinB,

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an error-prone *Pseudomonas* DNA polymerase (59). The mucoid phenotype eventually emerges

through a chromosomal mutation in *mucA*, which results in a protein unable to bind AlgU and

thus overexpression of the AlgU regulon (15, 22, 40). AlgU controls the production of alginate

through a large 12 gene operon responsible for manufacture and transport of alginate

biosynthetic products (36, 40). In addition, AlgU regulates other genes like *amrZ* and *algR*,

which encode transcriptional regulators that directly control the *alg* operon (40, 56, 66 and

Figure 1).
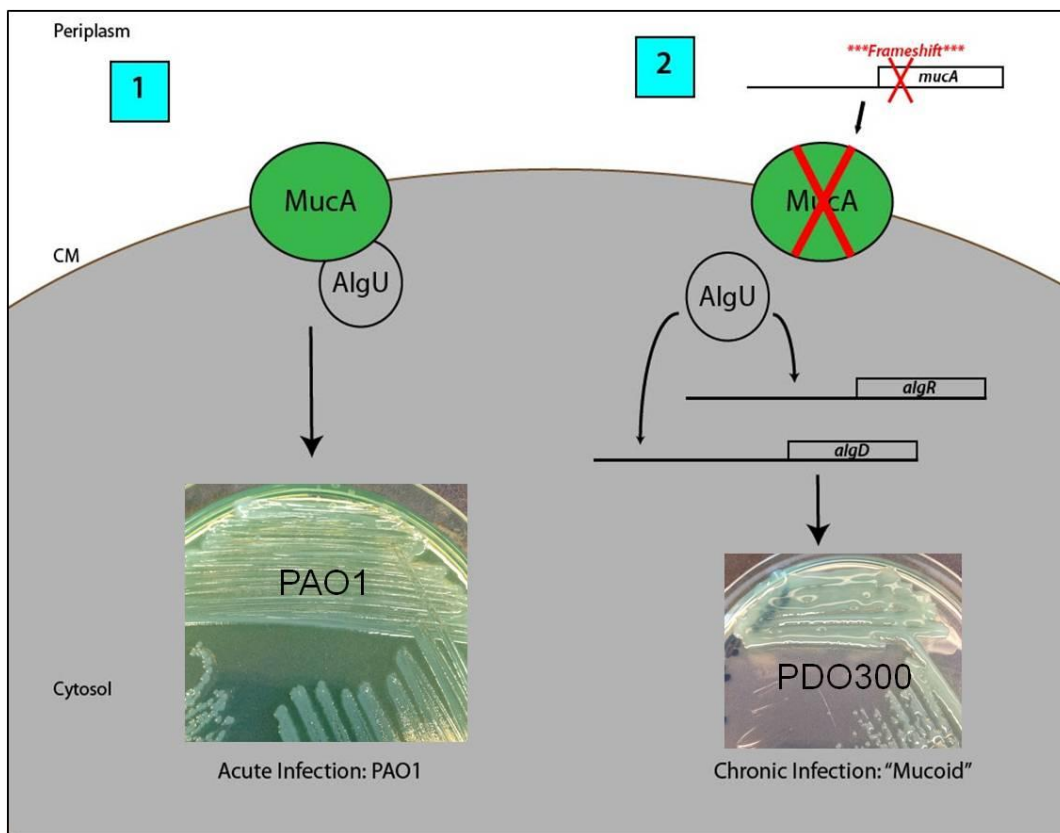
Figure 1: A model depicting a genetic mutation that leads to mucoidy. In strain mucA22 (PDO300), AlgU is no longer bound by MucA and can regulate expression of genes leading to overproduction of alginate.

The transcriptional regulator AlgR is required for alginate production, but also controls

additional virulence factors used during acute or chronic infections (32, 37, 51, 52). *P.*

aeruginosa and many pathogens use two-component systems (TCS) to translate extracellular

signals into changes in genetic expression (26). Typical TCS possess a transmembrane sensor

kinase and a cognate response regulator. AlgR is part of one such TCS, though it has been

shown to regulate some genes independent of its sensor kinase AlgZ (19, 68). AlgR directly

binds the promoter regions of genes it controls at a conserved CCGTTCGTC sequence (25, 35).

AlgR frequently binds multiple sequences upstream of controlled genes, and may control

expression through multimerization and subsequent alterations of chromosomal topography (25,

35).

AlgR was initially identified as an alginate regulator, but further studies illustrate its role

in initial attachment during infection (30, 44, 45). *P. aeruginosa* uses Type IV pili for twitching

motility, loose attachment, and biofilm formation (5, 9, 29, 53). AlgR is required for twitching

motility, and enacts its control by binding upstream of the *fimU* operon, which encodes several

proteins involved in construction and extrusion of Type IV pili (38, 64).

AlgR also influences quorum sensing. Quorum sensing is a population dependent cell

signaling mechanism used by bacteria to coordinate expression of collective virulence factors,

such as biofilm formation, pyocyanin production, and swarming motility (49, 50, 66). AlgR

suppresses quorum sensing directly at the *rhlI* promoter, preventing production of a C4-

homoserine lactone autoinducer (14, 45, 51). Rhamnolipids are secreted to facilitate swarming

motility and micro-colony formation, and are repressed by AlgR through direct binding at the

rhlA promoter (51, 52). AlgR's control of quorum sensing components is important for biofilm

formation, and an *algR* mutant is defective in 6-day biofilm formation (45).

AlgR is also a negative regulator of Type III secretion (T3SS), and works in two separate

but coordinate ways to downregulate Type III gene expression (32, 34). First, AlgR represses

Vfr, a cAMP- binding protein that positively regulates Type III secretion (34). Additionally,

AlgR negatively regulates Type III secretion by controlling expression of the RsmA/Y/Z system, resulting in a net decrease of RsmA, which itself is required for full expression of the T3SS (32). Further understanding of AlgR's role in controlling *rsmA* expression is a current goal of this lab.

AlgR is a major virulence determinant, and *algR* mutants are attenuated for virulence in acute septicemia and pneumonia models in mice (37). Interestingly, overexpressed *algR* attenuates virulence and cell growth, suggesting that AlgR levels are tightly maintained within the cell (37). The diverse array of virulence factors controlled by AlgR illustrates the protein's application in both acute and chronic infection backgrounds.

Gac/Rsm Regulatory System

Another important regulatory system sits at the intersection of acute and chronic infection, and has been the source of much study and characterization in recent years. The GacS/A two-component system is a phosphorelay system using posttranscriptional regulation to affect gene expression. The GacS/A system is controlled by ancillary proteins. GacS, a hybrid sensor kinase, is inhibited through dimerization with an orphan sensor kinase RetS, and inversely supported through interaction with a second sensor kinase LadS (27, 28). LadS causes autophosphorylation of GacS, followed by phosphotransfer to the response regulator GacA. Phosphorylated GacA then directly controls the expression of two non-coding sRNAs, *rsmY* and *rsmZ*. These are the only known genes GacA controls, and the sole purpose of the sRNAs appears to be regulation of RsmA (6, 27, 28). RsmA, a post-transcriptional regulator in the CsrA family of proteins, reciprocally controls expression of genes involved in acute and chronic infection (6, 7, 26, 64). RsmA's full characterization in *P.aeruginosa* is ongoing. RsmA binds the GGA motifs present on the single stranded, exposed portions of RsmY/Z stemloops (27, 28). Each sRNA is capable of binding 4-6 RsmA dimers, resulting in a net decrease of free RsmA in

the cell (6, 53). When RsmA is not bound by the small RNAs it is free to enact its genetic

control at the post transcriptional level. A representation of components of the GacR/S/M

system is illustrated in *Figure 2*.
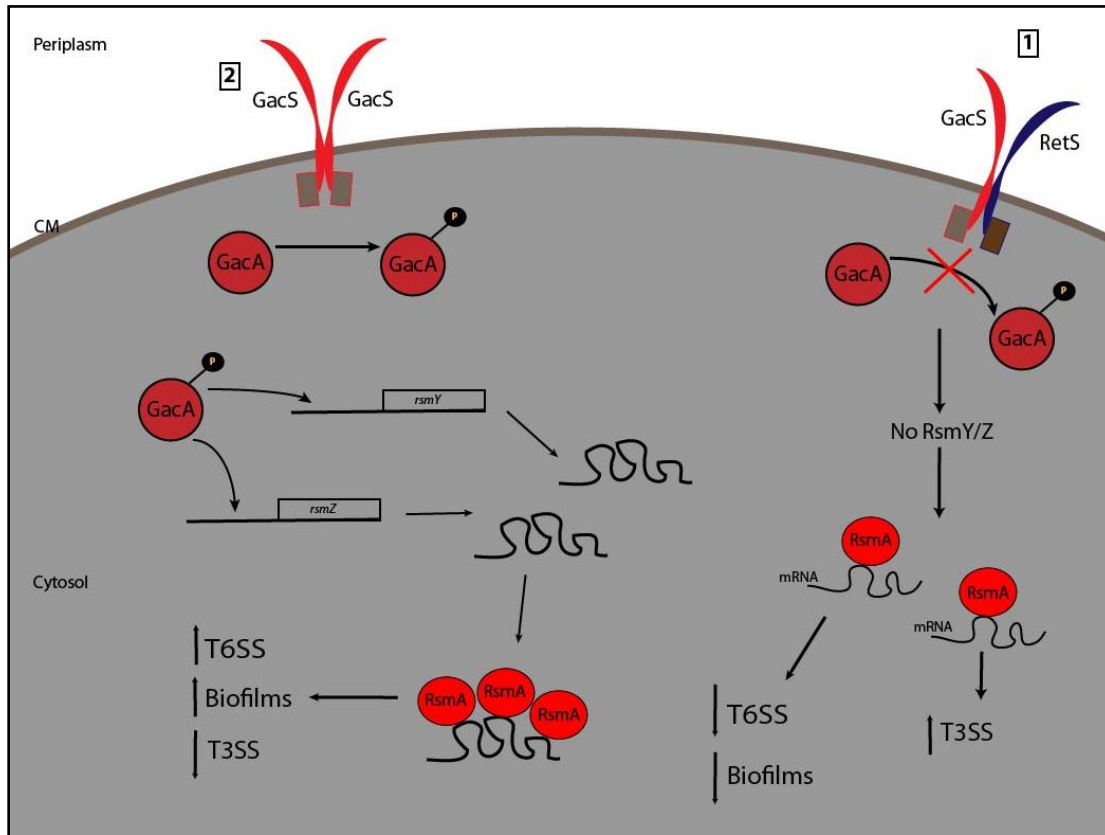
Figure 2: A model depicting the Gac/Rsm regulatory system. In acute strains (1), RsmA is free to bind messages leading to negative regulation of Type VI Secretion and biofilms. In chronic strains (2), phosphorylated GacA controls expression of rsmY/Z, which bind and inhibit RsmA.

RsmA and other CsrA family proteins control translation by directly binding mRNA at

conserved GGA motifs in the 5' untranslated region of target genes (6, 64). Though direct,

positive regulation of messages occurs in *E. coli* CsrA, RsmA in *P. aeruginosa* has only been

shown to negatively regulate mRNA (6, 53, 64). RsmA dimers bind in close proximity to the

Shine-Dalgarno (SD) sequence and prevent translation through occlusion of the ribosome

binding site (2, 33, 43). Additionally, RsmA influences mRNA secondary structure to prevent

ribosomal recruitment (55, 64). A microarray performed on *P. aeruginosa* strain PAK showed

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several genes are downregulated in an *rsmA* mutant (6). RsmA failed to bind these messages in an mRNA binding assay, and positive regulation of gene expression by RsmA in *P. aeruginosa* is believed to be indirect, perhaps by directly repressing expression of negative regulators for the genes shown to be downregulated in the microarray..

RsmA directly represses several chronic virulence factors (21, 55, 64, 67). Type VI Secretion (T6SS) is a chronic associated virulence factor used to attack neighboring bacteria with a phage-tail like syringe. Type VI in *P. aeruginosa* damages competing bacteria through toxin secretion as a retaliation for initial attack from competitors (6, 31, 46). RsmA represses expression of the T6SS by direct binding to mRNA for multiple genes involved in formation of the Type VI syringe (6). RsmA is also a negative regulator of genes involved in Psl polysaccharide formation, and an *rsmA* mutant was able to hypersecrete Psl (24, 33).

RsmA is required for full expression of acute associated virulence factors like Type IV pili and Type III Secretion, which it regulates in a currently unknown manner (6, 48). Since direct positive regulation of mRNA has not been shown by RsmA, it is possible that RsmA positively influences Type IV pili and Type III Secretion by directly inhibiting translation of a negative transcriptional regulator of genes for Type IV Pili and Type III Secretion.

These lines of evidence suggest RsmA transitions the cell from expressing acute to chronic virulence factors through genetic regulation at the post-transcriptional level. Further evidence of this is the fact that an *rsmA* mutant was attenuated in a murine model of acute pneumonia, but was able to better persist in a chronic model of infection (47). Given RsmA's global role in control of virulence factor gene expression, understanding the molecular and biochemical signals involved in regulating *rsmA* expression may provide insight into the way *P. aeruginosa* transitions from acute to chronic infection.

Transcriptional Regulation of *rsmA*

      The GacS/A proteins and the small RNAs RsmY and RsmZ are known to modulate levels

of free RsmA protein, but transcriptional regulation of *rsmA* in *P. aeruginosa* has not yet been

defined. A study of the RsmA homolog in *E.coli*, CsrA, identified five promoters (70). Two

promoters were shown to be under control of RpoS (σ^s), a stress-response sigma factor.

Expression from one of these promoters was shown to be indirectly dependent on CsrA itself.

Additionally, CsrA negatively regulates its own translation, similar to RsmA (64, 70). Given the

elaborate transcriptional control of *csrA* in *E.coli*, *rsmA* regulation in *P. aeruginosa* is likely to

be complex. Understanding *rsmA* regulation is a current goal of this lab. A primer extension on

rsmA was conducted previously to identify transcriptional start sites (69). An upstream promoter

was shown to be under the control of AlgU by showing reduced *rsmA* transcription and lower

RsmA protein levels in an *algU* mutant (69). Sequence adjacent to the AlgU dependent

promoter also showed a possible AlgR binding site (*Figure 3*).

5'-TTGA**CCGTTTGGC**AGGAACTTTCATTCCGGCGGGACTG
GTCAATACTGGGTGAAGGATCGCGCTCTTGATTTCTGCGGATCCGCCGCCATTTCT
TTTTTGCAGACTGTTGTCCTGAAATATTCGCGTGAGGAGAAAGGA**ATG**-3'

Figure 3: A possible AlgR binding site (underlined) exists on the rsmA promoter. Red sequence denotes the -35 and -10 of the AlgU promoter. The blue ATG is the rsmA start codon.

      Data from this lab and a study conducted by Intile, et al (32) suggests AlgR is responsible

for regulating *rsmA* expression, but a mechanism is currently unknown. One aim of this study

was to confirm AlgR regulation of *rsmA* transcription and to assess RsmA protein levels in *algR*

mutant strains. Overexpression of *algR* was attempted using a single copy chromosomal insert

under control of an inducible promoter as a tool to further analyze *rsmA* control by AlgR. The

second aim of this study was to describe a mechanism by which AlgR regulates *rsmA*, which

resulted in the hypothesis that AlgR binds the putative AlgR consensus sequence upstream of

rsmA. To show *in vivo* relevance of this control, we attempted site-directed mutagenesis on the

AlgR binding site within an *rsmA-lacZ* transcriptional fusion. Understanding the mechanism for

AlgR control of *rsmA* expands upon the novel regulatory interaction between AlgU and *rsmA*

described previously, and helps to illuminate the complex feedback patterns the cell uses to keep

RsmA levels controlled. The signals processed by many of these global regulatory systems are

unknown, and understanding the biochemical and genetic control of virulence factor gene

expression may help identify potential drug treatment targets. New drug targets capable of

eliminating *P. aeruginosa* infection will yield a better prognosis for the CF patient.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All strains and their sources are described in Appendix C. PAO1 was used as the acute

infecting wild type lab strain. A strain possessing a frameshift mutation in the *mucA* gene has a

defective anti-sigma factor and is mucoid. This strain, denoted *mucA22* in the text was used as

the wild type mucoid strain. All *Pseudomonas* strains were maintained on PIA plates and grown

at 37˚ unless otherwise noted. In broth, *Pseudomonas* strains were grown in LB at 37˚ with ~200

RPM shaking for aeration unless otherwise noted. *E. coli* strains SM10, JM109 and NEB5α

were used to maintain and conjugate all plasmids for mating. Plasmids are listed in Appendix C.

SM10pRK2013 was used as a donor of the sex pilus when conjugating JM109. BL21 (DE3)

from New England Biolabs was used for protein expression and purification. *E. coli* strains were

grown on LB plates or broth at 37˚ unless stated otherwise. Antibiotic concentrations for

Pseudomonas and *Escherichia* growth media are available in Appendix A.

For long term storage of strains, a sterile loop was used to add the desired strain to 1.5 ml

10% skim milk or 20% glycerol in a cryotube and then stored at -80˚C.

Strains used in Western blot analysis possess a hemagluttinin epitope tag fused to RsmA

and were constructed previously by allelic exchange.

Quadparental Mating

For AlgR overexpression and complementation, quadparental conjugation was used to

introduce inducible *algR* into a neutral site on the *P. aeruginosa* chromosome downstream of the

glmS gene. The coding region of *algR* was cloned in frame downstream of the P_{BAD} promoter of

pTJ1 previously in this lab (Appendix C). *P. aeruginosa* recipient strains were grown overnight

on PIA at 43˚ C. *E. coli* strains bearing pTJ1 or pTJ1algR were grown at 37˚ C. A strain bearing pTNS3 helper plasmid was used to provide the integrase and was also grown at 37˚ C. pRK2013 was used to provide a sex pilus for pTJ1 and was grown at 30˚ C. Following overnight growth in broth, 500 ul of each *E. coli* strain was pelleted at 4000 x g and resuspended in 100 ul LB. Resuspended cells were combined in a single tube and a sterile stick was used to add *P. aeruginosa*. The suspension was pipetted up and down to mix and spotted on LB overnight. Successful integration of pTJ1 confers trimethoprim resistance, so $LB_{tmp1500}$ (Appendix A) was used to select for strains possessing successful integration. Strains were then screened via colony PCR to confirm insertion.

β-galactosidase Assay

Strains to be assayed were grown overnight on PIA and a single colony was inoculated into 5 ml LB broth and grown overnight at 200 RPM. Overnight culture (50 ul) was used to inoculate another 5 ml LB, which was grown for 8 hours. From this 8-hour culture, 700 ul was pelleted at 5000 x g and resuspended in 1 ml Z buffer + BME (Appendix B). Optical density (O.D.) at 600 nm was taken and sample concentration was adjusted so OD_{600} was 0.4-1.0. SDS (50 ul of 0.1%) and 100 ul chloroform were added and the sample was vortexed for 15 seconds and allowed to sit 10 minutes. 500 ul of the lysate was added to a fresh tube with 300 ul Z buffer + BME. Z buffer with ortho-nitrophenyl-β-galactoside (200ul) was added to start the colorimetric reaction. After a deep yellow color developed, the reaction was stopped with 500 ul 1 M Na_2CO_3 and the reaction time recorded. O.D. 420nm and 550nm were taken and used to determine Miller Units as follows:

$$\frac{1000 \times (O.D.420nm - 1.75 \times O.D. 550nm)}{Time \times Volume\ of\ sample\ (ml) \times O.D.\ 600nm} = Miller\ Units$$

Western Blotting

For RsmAHA: strains to be assayed were grown overnight in 5 ml LB. From the

overnight culture, 250 ul were used to inoculate 25 ml LB and grown 8 hours at 37° C at ~200

RPM. Entire cultures were spun at 5000 x g for 10 minutes and pellets were resuspended in 2.0

ml 0.85% NaCl in a 2.0 ml tube. For the reminder of the procedure, samples were kept on ice.

Samples were sonicated in 20 second bursts for a total of 1 minute each with 3-5 minute breaks

on ice between bursts. Lysates were centrifuged at 14000 x g for 10 minutes and 1 ml lysate was

removed to a new tube for quantification using the Bradford assay. Bradford reagent (5X) was

diluted to 1X in ddH$_2$O and 3.5 ml were aliquoted into test tubes. Bovine serum albumin (BSA)

was used as a protein standard in the following concentrations: 2 mg/ml, 1 mg/ml, 0.5 mg/ml,

0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml. A standard curve was generated using 30 ul of

BSA or protein sample, which were added to 3.5 ml 1X Bradford reagent and OD 595nm was

measured. A standard curve was generated in Microsoft Excel and trend line equation was used

to determine protein concentration. Only curves with R^2 values of 0.98 or higher were used. 10

ug protein was mixed with 4X SDS-PAGE loading dye and boiled for 10 minutes before loading

into 15% SDS-PAGE with 4% stacking gel. The gel was run in Tris-glycine running buffer

(Appendix B) at 180 V for ~ 1.5 hours. Gel was removed from cassette and sandwiched between

double layer blotter paper soaked in transfer buffer (Appendix B) adjacent to a

polyvinylidenediflouride membrane that had been activated in methanol. Proteins were

transferred to the membrane at 150 mA for 1.5 hours. Membrane was blocked for ~2 hours in

blocking buffer and then split based on MW marker for separate staining of HA and OmlA

loading control. Primary antibody solution with either anti-HA or anti-OmlA (1:20,000) was

used overnight at 4° C on a rocker. The membrane was washed for 15 minutes 3 times in

TBS-T. Anti-rabbit (OmlA) or anti-mouse (HA) secondary-antibody (1:20,000) solution

containing conjugated horseradish peroxidase was used for 1 hour at room temp. The membrane

was again washed 3 times and placed on a single layer of plastic wrap. Pierce ECL Western

Blotting Detection was used by mixing 3 ml of Substrate A with 75 ul Substrate B and treating

membrane for 5 minutes before visualization in ProteinSimple FlourChem M CCD camera.

Twitching Assay

A sterile toothpick was dabbed onto a single colony and stabbed into 1% LB agar with or

without arabinose (Appendix A). Plates were grown upside down at 30° C for 48 hours. Agar

was removed and the bottom of plate was flooded with 0.1% crystal violet for 1 minute, then

rinsed and allowed to dry. Twitching ability was measured as the diameter (mm) that each strain

had migrated from the initial stab line.

Colony PCR

To confirm the presence of fusions or expression vectors, PCR was performed as follows:

A toothpick was used to dab a single colony which was dispersed into 50 ul sterile, double

distilled (sdd) H$_2$O in a microtube. For a 25 ul reaction, the following was assembled (*Table 1*):

Table 1: PCR master mix

Amount per 25 ul rxn:
2.5 ul 10X ThermoPol Buf.
0.5 ul 10 mM dNTP
0.5 ul 10 uM F primer
0.5 ul 10 uM R primer
1 ul DMSO
18 ul sddH2O
0.11 ul Taq Polymerase (add last)
Mix components in order and keep on
ice. Add 23 ul Master Mix to 2 ul template
in a PCR tube. Cover w/ mineral oil and
place into preheated block.

The master mix (23 ul) was aliquoted into PCR tubes containing 2 ul template. 30 ul mineral oil

was added and tubes were placed in a pre-heated thermal cycler with following run settings (see

Table 2):

Table 2: PCR thermal cycler settings

Step	Temp.	Time
1) Initial Denature	95 C	5"
2) Denaturation	95 C	1'
3) Annealing	62 C	30"
4) Extension	72 C	1'/kb
Repeat Steps 2-4 30X		
5) Final Extension	72 C	10'

Following the Final Extension, tubes were stored at 4° and a 4-6 ul sample was analyzed on an

agarose gel.

AlgR Purification

Transformation

Prior to this study, the complete sequence for *algR* was cloned in frame within the pGEX-

4T-2 expression vector (Appendix C) and its sequence was verified. A plasmid prep was

completed previously and was used for transformation into BL21 expression cells per New

England Biolabs manufacturer's protocol. One tube containing 50 ul NEB BL21 cells was

removed from -80° storage and placed on ice for 8 minutes. The plasmid prep was quantified

using absorbance at O.D.$_{600}$ nm and 100 ng of plasmid was added to the tube and flicked 3-4

times to gently mix. The tube was placed back on ice for 20 minutes and then heat shocked in a

42˚ C water bath for exactly 10 seconds. In subsequent cases where lab-prepared chemically

competent BL21 cells were used, the heat shock was carried out for 45 seconds to 1 minute. The

tube was placed back on ice for 2 minutes and 950 ul SOC (Appendix A) was added. Cells were

rescued at 37˚ C with high aeration for one hour. Several dilutions of the cells were plated onto

LB_{amp100} plates and grown overnight at 37˚ C.

Plasmid Prep Using Alkaline Lysis

To confirm correct transformation, a single colony transformant was used to inoculate 5

ml LB_{amp100} broth and grown overnight. This culture was used to obtain a purified plasmid for

further analysis to confirm the *algR* insert. The entire 5 ml culture was pelleted at 5000 x g for

10 minutes and resuspended in 150 ul TE with 2 ul RNAse A. Just prior to use, a solution of 0.2

N NaOH/ 1% SDS was prepared. Lysis solution (300 ul) was added to the cells and mixed by

inverting the tube 4-6 times. Tubes were placed on ice for 5 minutes, then 250 ul 3M potassium

acetate was added and tube inverted 6 times, then again incubated on ice for 5 minutes. To

separate the fractions, tubes were spun in centrifuge at 14,000 x g for 10 minutes. The

supernatant was removed to a new tube and an equal volume of 24:25:1 chloroform, phenol and

isoamyl alcohol (CPI) was added to denature and remove the protein before spinning again for

10 minutes. The top phase was removed to a new tube and equal volume of chloroform/isoamyl

alcohol (24:1) was added to extract the phenol. The tubes were again centrifuged for 10 minutes

and the supernatant was removed to a new tube. Cold 95% ethanol (EtOH) was added to

precipitate the plasmid and the tube was incubated 10 minutes on ice before again spinning for

10 minutes. The EtOH was carefully extracted and the pellet was allowed to dry briefly before

adding 1 ml 70% cold EtOH. The tube was centrifuged a final time for 5 minutes before

removing EtOH and allowing pellet to dry by laying tube open on a kimwipe. Pellets were

resuspended in 50 ul sddH$_2$O and quantified spectrophotometrically based on absorbance at O.D.
260nm.

Restriction Digest

Digestion of purified plasmid was performed to confirm presence of *algR* sized insert.
Purified plasmid was diluted to ~100 ng/ul and 2 ul was added to a centrifuge tube. 2 ul NEB
10X CutSmart Buffer and 14 ul sddH$_2$O were added. Restriction enzymes (1 ul) Not1-HF and
Sal1-HF were added and tube was flicked to mix. The 20 ul reaction was incubated at 37° C at
least two hours before inactivation of enzymes in 65° C water bath for 5 minutes. Digests were
run on agarose gel to observe an *algR* insert from purified pGEXalgR (Appendix F).

Inducing Protein Expression

A single colony transformant was used to inoculate two separate 1.5 ml LB$_{amp100}$ broths
and was grown overnight at room temperature with high aeration. The next day, the entire
cultures were used to inoculate two 150 ml LB$_{amp50}$ in a 500 ml flask. These cultures were
placed at 37° C under high aeration until growth reached O.D.$_{600}$ of ~0.45. IPTG at 0.5 uM was
then used to induce one of the cultures, then cultures were moved to a room temperature shaker.
Samples were taken at different time points to determine optimal expression. The samples (10
ml) were taken from the cultures at the following times: Pre-induction, followed by 2, 4 and 8
hours post-induction. These 10 ml samples were pelleted at 4000 x g for 5 minutes before
resuspension in 0.85% NaCl. Sonication was conducted the same as for Western Blotting
samples. Lysates were added in equal volumes to a 7% SDS-PAGE and run at 180 V for 1 hour.
The gel was removed and stained in a Pyrex dish with 25 ml 1% Coomassie staining solution
(Appendix B) overnight. The gel was destained using SDS-PAGE destaining solution

(Appendix B) for 1 hour with several kimwipes added to absorb the stain, then was visualized on a ProteinSimple FlourChem M CCD camera.

Protein Purification Using GST Spin Columns

Culture conditions above were used with 0.5 uM IPTG induction and cells were allowed to grow for 4 hours. Four separate 20 ml samples were taken from the flask and pelleted at 4000 x g for 5 minutes. Each pellet was washed with 1X cold PBS and resuspended in 2 ml cold lysis buffer (Appendix B). For the remaining steps, cells were kept on ice. Samples were sonicated in 2 ml tubes using one second sonication bursts at 40% amplitude for a total of thirty seconds sonication. This was repeated three times and tubes were spun at 15,000 x g for 10 minutes. Supernatant was removed to a new tube and used to pass over the GST spin columns. Pierce (0.2 ml resin bed volume) GST-spin columns were used to bind the AlgR-GST fusion. Two columns were used for each round to balance out the centrifuge and increase yield. All further steps were performed at room temperature while lysates were kept on ice. The lysate obtained above (usually about 4 ml from two samples) was passed in 500 ul aliquots (of 400 ul lysate and 100 ul GST buffer) into each column. Columns were plugged and taped to a rocker for 30-45 minutes before spinning out the lysate at 700 x g for 2 minutes. Fractions were saved for downstream analysis. After the final aliquot was passed over column, the column was washed 3-5 times with 400 ul GST wash buffer and plugged. A 50 ul tube of thrombin (1 unit/ul) was thawed on ice and 950 ul cold PBS (Appendix B) was added. Each column was washed 2x with cold PBS before 400 ul of the thrombin mixture was added to each tube. Tubes were kept at gentle rotation at room temp overnight (about 12 hours). The flow through was collected and immediately aliquoted into microtubes in 10 ul volumes. 30 ul 4X protein storage buffer

(Appendix B) was added and tubes were immediately placed at -20˚ C for storage. Several fractions were analyzed on Coomassie stained SDS-PAGE and are shown in the Chapter 3.

Quantification of the Purified Lysate

Purified diluted fractions were quantified using the Bradford assay as well as absorbance at $O.D._{280}$nm and concentration was found to be ~1 mg/ml in 800 ul total volume purified. The purified flow-through (10 ul) was diluted into 30 ul 4X Storage Buffer (Appendix B) leaving 10 ug per stock. According to visual estimation from SDS-PAGE, about 75% percent of the fraction was AlgR, giving a dilution factor of 0.75, or 7500 ng AlgR per 40 ul stock. Since the diluted stocks (to eliminate unspecific proteins) were diluted 1:10, this gave ~750 ng AlgR per stock. As AlgR is 26.7 kDa this gives a 700 nM concentration of AlgR in each 40 ul stock. For binding reactions: (700nM stock)(2 ul) = (x nM)(20 ul binding volume) = 70 nM AlgR/ 2 ul for use in binding reactions.

<div align="center">Electrophoretic Mobility Shift Assay (EMSA)</div>

Generating Unlabeled Probes

For the *pscE/F* gene, 50 base-pair (bp) annealed oligos were used as the binding target. *pscE/F*GS1F (Appendix C) was 3'-biotinylated (see below) and then mixed in an annealing reaction with the unlabeled complementary oligo to generate a double stranded probe as follows: Each probe was adjusted to 1 uM in TNE buffer (Appendix B). A 20 ul volume of each probe was combined in a microcentrifuge tube and placed in a 95˚ heat block for ten minutes. Power to the heat block was removed until it cooled to room temperature, then annealed probes were stored at -20˚ C.

The *rsmA* and *algD* probes were generated by PCR amplification of ~160 bp regions of their respective promoters, followed by gel extraction and 3' biotin end labeling of the DNA.

Sequences of annealed oligos for *pscE/F* and primers used to amplify promoters for *algD* and

rsmA are listed in Appendix C.

Biotin 3' End Labeling

Gel extracted PCR products and single oligos were labeled for visualization in the

EMSAs using ThermoScientific 3' Biotin End DNA Labeling Kit. For labeling reactions: All kit

components (except TdT) were thawed on ice. Immediately prior to making reaction mix,

terminal-deoxynucleotidyl-transferase (TdT) was removed from freezer and diluted to a conc. of

2 U/ul (ex.: 1 ul TdT Buffer + 3.5 ul $sddH_2O$ + 0.5 ul TdT = 5 ul TdT at 2 U/ul). The following

components were assembled in a centrifuge tube (see *Table 3*):

Table 3: Biotin 3' End Labeling reaction mix

Combine in tube:
25 ul sddH20
10 ul TdT Rxn Buffer
5 ul 100 nM DNA
5 ul Biotin-11-UTP(5uM)
5 ulDiluted TdT (2U/ul)

_____

Incubate in 37 degree bath
for 30 minutes.

After 30 minutes, the reaction was halted by adding 2.5 ul 0.5M EDTA. The TdT was extracted

by adding 50 ul CI (24:1) and vortexing, followed by centrifugation to separate the phases. The

aqueous layer was decanted, quantified on ThermoFisher NanoDrop (O.D.$_{260}$nm) and stored at -

20˚C until use in EMSA.

<u>Quantifying Probes for Use in Binding Reactions</u>

For determining the concentration of annealed oligos:

100 nM conc. of the forward oligo was used in the biotinylation reaction. Manufacturer estimates ~80% labeling efficiency with this method, leaving 80 nM concentration following labeling reaction. An 80 nM stock was made of complementary R oligo and annealing reaction was set up using equal amounts of each primer to give final concentration of 80 nM following annealing. Following absorbance reading at O.D. $_{260}$ nm, dilutions were made at 10 ng/ul in 20 ul stocks, or 200 ng/ stock. 200 ng/ 20 ul stock = 6 pmoles (for 50 bp) / 20 ul stock, or 0.3 pmoles/ul. In each binding reaction, 0.25 ul was used, so ~225 fmols/ 20 ul reaction was present.

Concentration of PCR generated probes:

PCR amplicons were gel extracted and quantified, then dilutions were created as above to generate a ~10 ng / ul stock in 20 ul. For this 200 ng at 160 bp, there are roughly 2 pmoles/ 20 ul or 100 fmoles / ul. Since 0.25 ul was used in each binding reaction, ~25 fmoles labeled probe were used per assay.

<u>Gel Shift (EMSA)</u>

For all EMSAs, components of the ThermoScientific LightShift EMSA Kit were used for binding reactions and visualization. Following successful use of the control components, purified AlgR was used in a binding reaction with different labeled probes. All components except AlgR stocks were thawed on ice and the following reaction mix was added to each tube:

Table 4: EMSA Binding Reaction components

<u>Combine in tube:</u>
2 ul 10X Binding Buffer
13 ul Ultrapure Water (variable)
1 ul Poly dI:dC (1ug/ul)
1 ul 50% glycerol
1 ul 100 mM MgCl2
2 ul Protein Extract (variable)
0.25 ul (~20 fmol) probe
_____

Total Volume: 20 ul
Incubate 30 min at room temp.

Reagents were added in the order listed above and allowed to incubate at room temperature for 30 minutes. Meanwhile, a prepared 5% polyacrylamide TBE gel (Appendix B) was placed in a chamber with cold 0.5X TBE (Appendix B) as a running buffer. The gel was pre-run for 30 minutes during the binding reaction at 100V. 5 ul of 5X Blue Juice (Appendix B) was added to each reaction tube and slowly pipetted to mix. The entire reaction mix was loaded onto the gel and run for an hour. The probes were then transferred to a nylon membrane that was soaked in 0.5X TBE using a semi-dry apparatus. The gel was sandwiched on top of the nylon and between a soaked blotter paper bilayer. The probes were transferred for 30 minutes at 380 mA. The membrane was removed, dabbed on a kimwipe and crosslinked by exposure to 120 mJ of UV

light in a crosslinker at 254 nm for ~60 seconds. The membrane was then stored or immediately visualized.

EMSA Chemiluminescent Detection

All components for nucleic acid detection came with the ThermoScientific Chemiluminescent Detection Kit. Blocking buffer and 4X Wash buffer were warmed in a 42° water bath until all particulate had dissolved. All other components were kept at 4° C until ready for use. To block the membrane, 15 ml Blocking buffer was added to a clean tray and the membrane was rocked for 15 minutes. A conjugate/blocking buffer solution was prepared by adding 50 ul Streptavidin-HRP Conjugate to 15 ml Blocking buffer. This was added to the membrane and rocked for another 15 minutes. Membrane was then removed to a clean tray and rinsed briefly with 15 ml 1X Wash buffer. Four additional wash steps were performed for 5 minutes each. 30 ml Substrate Equilibration Buffer was added to a clean tray and the membrane was treated for 5 minutes. During this treatment, a substrate visualization solution was prepared by adding 3 ml Luminol Enhancer to 3 ml Stable Peroxide. The membrane was removed from buffer, dabbed on a kimwipe and placed on plastic wrap. Visualization solution was added for 5 minutes. The membrane was sealed in plastic wrap and visualized using ProteinSimple FlourChem M CCD on the chemiluminescent setting.

Statistical Analysis

For the transcriptional fusions, experiments were performed in triplicate and subject to mean, standard deviation, and standard error analysis in Microsoft Excel. Standard error is depicted on each graph using positive error bars. To determine statistically significant difference between more than two groups, one-way ANOVA analysis was performed in Excel. To determine significance between two groups, a post-hoc two-tailed unpaired t-test with Bonferroni correction was performed in Excel . "***" denotes p value < 0.001 between respective group means.

CHAPTER 3

RESULTS

Induced *algR* Expression Restores Twitching Motility to an *algR* Mutant

Previous studies by this lab and others identified AlgR as a regulator of *rsmA* expression (32). One goal of this study was to use inducible *algR* expression to further understand AlgR's effect on *rsmA*. Expressed *algR* was used by Intile, et al to show an increase in *rsmA* activity using transcriptional fusions (32). To measure *rsmA* activity, this study used transcriptional fusions with the *rsmA* promoter containing the putative AlgR binding site fused to *lacZ*. Another way to measure RsmA is through Western blotting to observe changes in RsmA protein levels. Overexpression was attempted by placing an inducible Tn7-based *algR* expression construct into the chromosome of strains possessing either an *rsmA-lacZ* transcriptional fusion or HA-tagged RsmA.

Previously in this lab, the *algR* open-reading frame was cloned into the pTJ1 expression vector (Appendix C), which can be integrated into an att:Tn7 site on the *P. aeruginosa* chromosome downstream of the highly conserved *glmS* gene. Quadparental mating (Chapter 2) was used to introduce pTJ1*algR* into strains bearing the *rsmA-lacZ* transcriptional fusion, as well as strains with an epitope-tagged RsmA-HA generated previously. Colony PCR was used to verify correct insertion of the complementation vector and is shown in *Figure 4*.
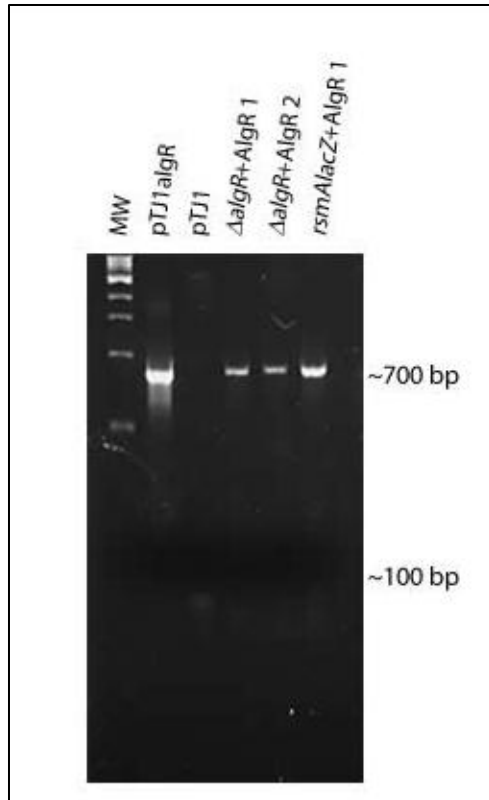
Figure 4: Conjugated strains possess pAlgR. Colony PCR was performed using primers pHERDSF and pTJ1R (Appendix C), which flank regions of the multiple cloning site for pTJ1.

The second lane of the gel in *Figure 4* represents amplification from JM109 cells possessing the *pAlgR* construct and amplifies a ~700 bp band. Cells bearing the empty vector produce a much smaller (~50 bp) band in the next lane. Successfully conjugated *P. aeruginosa* strains produce a band that corresponds in size to the positive control, indicating *pAlgR* is present in these strains.

After successful conjugation, it was necessary to determine whether or not the *algR* overexpression system was able to restore an AlgR dependent phenotype to an *algR* mutant, thus confirming that *algR* was correctly being induced in the presence of arabinose. AlgR regulates twitching motility/Type IV pili by direct binding upstream of the *fimUpilVWXY1Y2E* operon, and the twitching phenotype is lost in an *algR* mutant (38, 64). Production of alginate in the mucoid strains is another phenotype dependent on AlgR, but complementation and

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overexpression studies have shown that this phenotype is sensitive, and that *algR* overexpression will not result in restoration of alginate production (18, 38). A subsurface twitching assay was thus performed to investigate the ability of the *algR* overexpressing strains to restore twitching motility.
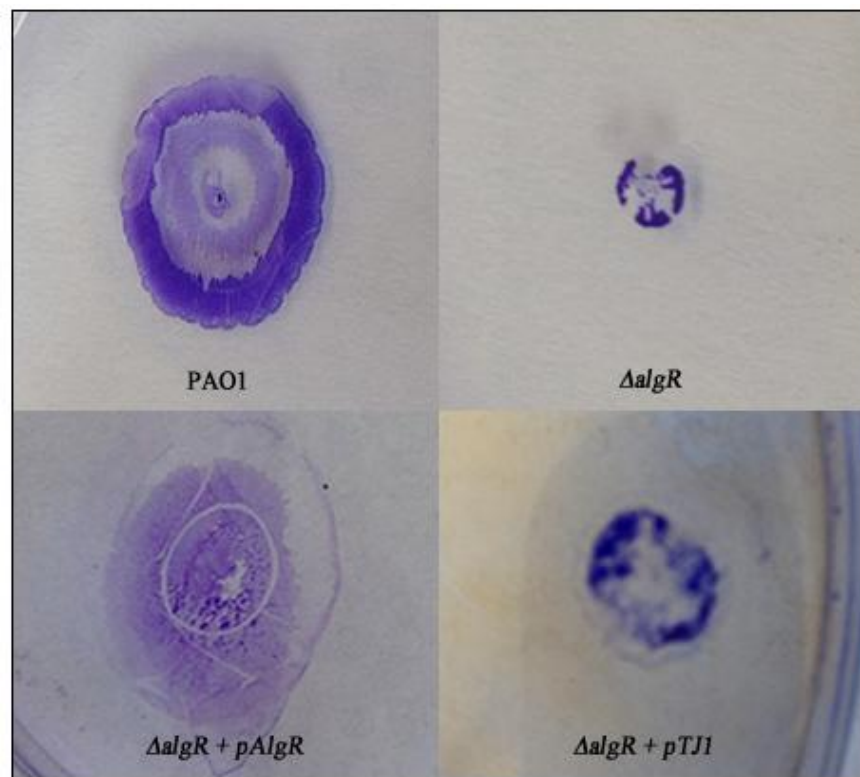
Figure 5: pAlgR restores twitching motility to an algR mutant. Strains above were grown on 1% LB agar with 1% arabinose for 48 hours and twitching zones were stained with crystal violet.

Table 5: Twitching diameter in wild type, mutant and complement strains

Strain	Twitching (mm) on 1% LB	Twitching (mm) w/1% arab.
PAO1	13 ± 1	11 ± 0.3
PAO1*ΔalgR*	2	2 ± 0.16
ΔalgR + pAlgR	6 ± 0.7	16 ± 2
ΔalgR +pTJ1	6 ± 0.3	5

The results of the assay (*Figure 5* and *Table 5*) show that the wild-type PAO1 is capable of twitching motility on both media with and without 1% arabinose. PAO1Δ*algR* is defective for twitching motility, and this is restored by induced expression of *pAlgR*. The complemented strain (*ΔalgR₊pAlgR*) also shows twitching on medium without arabinose, indicating transient induction of *pAlgR*. Some twitching motility is also restored to Δ*algR+pTJ1* on media with and without arabinose. This may indicate that the empty vector alone restores twitching motility. However, the pTJ1 vector inserts into a chromosomally neutral location and this has not been shown to disrupt expression (12, 16). It is possible that the pTJ1 empty vector was conjugated into a mixed culture of *ΔalgR* and an unknown contaminant that was capable of twitching motility, thus explaining the erroneous twitching results seen in *ΔalgR+pTJ1*. This would explain the unexpected twitching despite PCR verified presence of only the empty vector. Restoration of twitching motility is observed in the *algR* mutant bearing *pAlgR*. In addition, this increase is responsive to arabinose, which indicates that despite transient expression and apparent partial restoration by the empty vector, *pAlgR* may be useful for investigating AlgR control of *rsmA*. Strains with *algR* deletions also possess a defect in swarming motility (52, 64). A swarming assay on M9 Minimal Media with the strains above, combined with re-conjugation with the empty vector could provide clarification for the misleading ability of *ΔalgR+pTJ1* to twitch.

mucA22ΔalgR Has a Defect in *rsmA* Expression That is Not Restored by *pAlgR*

This lab and Intile, et al (32) used transcriptional fusions to show that *rsmA* expression

decreased in an *algR* mutant. The fusions used by this lab include both *rsmA* promoters

identified in the primer extension of *rsmA* completed in a previous study (69). First, we sought

to confirm an increase in *rsmA* expression previously seen in *mucA* defective strains (32, 69). In

addition, *rsmA* expression was measured in *algR* mutants in PAO1 and *mucA22* and compared to

wild type (*Figure 6*).
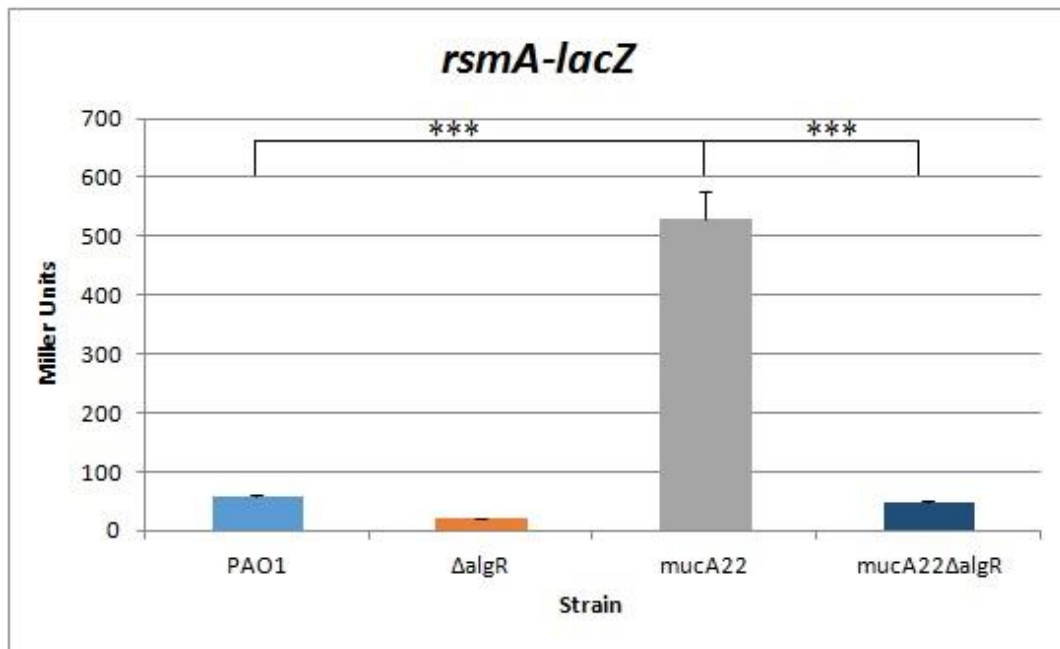
Figure 6: algR mutants have decreased rsmA expression. Cultures were grown for 8-hours and β-galactosidase assay was performed with ONPG and quantified in Miller Units. "***" denotes p-value < 0.001.

The results of *Figure 6* show that *rsmA* expression is increased in the mucoid

background, which agrees with previous studies (32, 69). This increase seen in *mucA22* is

abrogated in the *mucA22ΔalgR* strain, suggesting that AlgR may be required for full *rsmA*

expression in *mucA*22. A slight decrease in *rsmA* expression was seen in *ΔalgR* compared to

PAO1, though these results have yet to be confirmed in further study. Since it appeared AlgR

was required for full *rsmA* expression in *mucA22*, expression of *pAlgR* was hypothesized to result in an increase in *rsmA*. *pAlgR* was integrated into PAO1*rsmA-lacZ* as described in Chapter 2, and β-galactosidase activity was measured to determine inducible *algR*'s effect on *rsmA* transcription (*Figure 7*)..
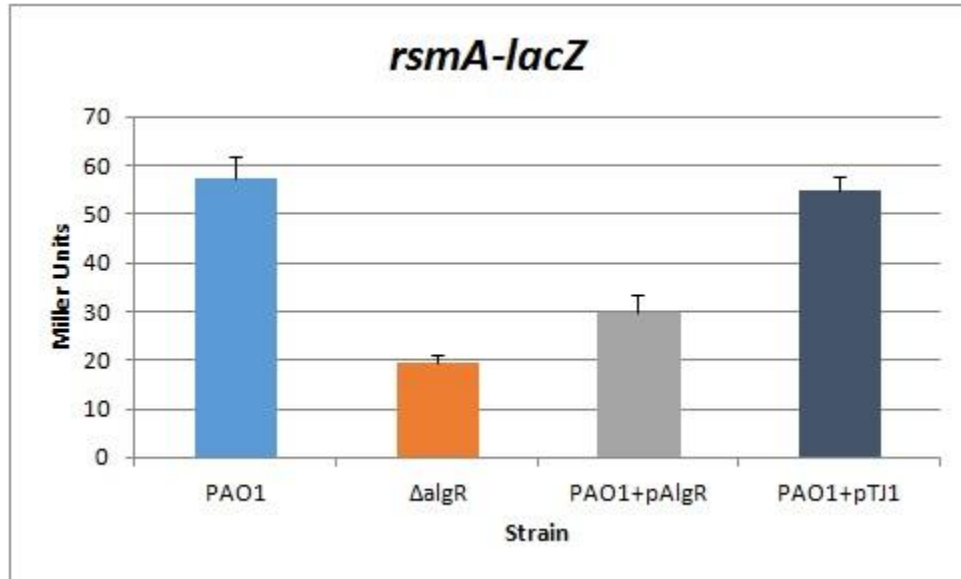
*Figure 7: pAlgR does not increase rsmA expression in PAO1. Cultures were grown as in Figure 6, and pAlgR was induced with 1% arabinose at log phase and grown to 8 hours before measuring β-galactosidase. "***" denotes p-value < 0.001.*

The results in *Figure 7* show that no increase in *rsmA* expression occurs with induced expression of *algR* in PAO1. In fact, expression is slightly reduced from wild-type. If overexpressed *algR* is unable to increase *rsmA* expression in PAO1, it is possible that *rsmA* may be sensitive to optimal levels of AlgR that are disrupted with overexpression. In addition, this promoter may require a combined effect of AlgU and AlgR. To examine this further, complementation studies were attempted in *mucA22ΔalgRrsmA-lacZ* to look for restoration of *rsmA* expression (*Figure 8*).
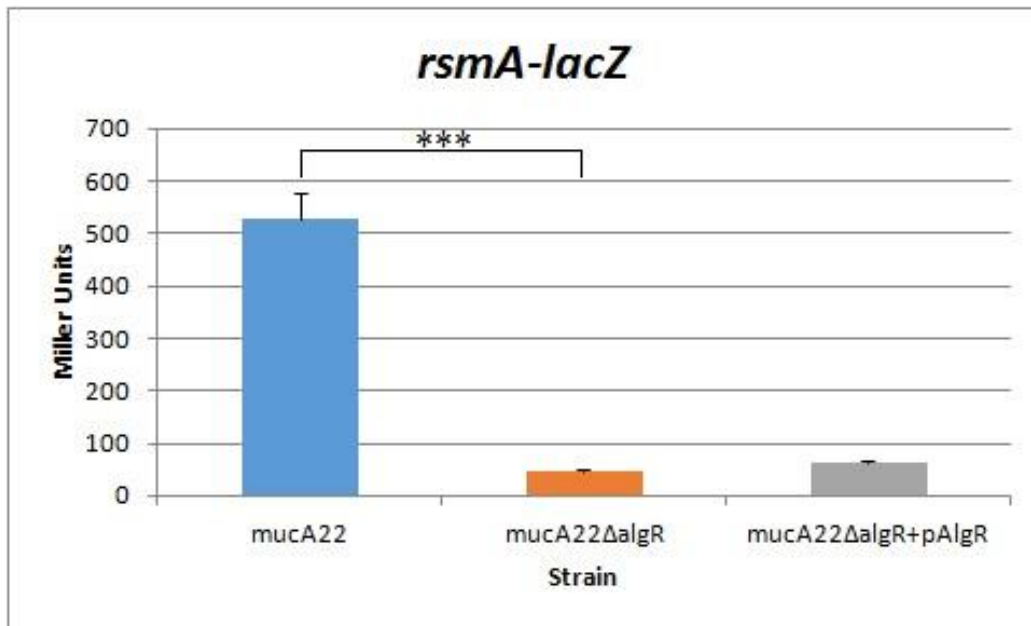
Figure 8: pAlgR does not complement rsmA expression loss in mucA22ΔalgR. pAlgR was induced with 1% arabinose at log phase and grown to 8 hours before measuring β-galactosidase. "***" denotes p-value < 0.001.

The results of *Figure 8* show that there is a drastic loss of *rsmA* expression in *mucA22ΔalgR* that is not restored by *pAlgR*. Taken together, the results of *Figures 6, 7* and *8* suggest that an *algR* mutant in the mucoid background has diminished *rsmA* expression. This may indicate AlgR control of *rsmA*, but overexpression and complementation with *pAlgR* was unable to increase *rsmA* activity in our strains, indicating further study is needed to determine how AlgR is controlling *rsmA* expression in *mucA22*. The inability of *pAlgR* to increase *rsmA* is addressed further in Chapter 4.

algR Mutants Possess Decreased Levels of RsmA-HA

The results of the transcriptional fusions suggest *algR* mutants have decreased *rsmA* transcription, and that overexpressing *algR* does not increase *rsmA* expression in PAO1 or complement the loss in *mucA22ΔalgR*. To determine if protein levels of RsmA are also affected by *algR* mutation, an epitope-tagged RsmA-HA constructed previously was used to analyze RsmA in various strains. The epitope tag has no adverse effect on RsmA's function because *mucA22rsmAHA* remains mucoid, a phenotype lost in *mucA22ΔrsmA* (unpublished, data not show). Culture conditions used in *Figures 6,7* and *8* were replicated to perform Western blotting and observe RsmA-HA. Overexpression of *algR* in PAO1 and complementation in *mucA22ΔalgR* was again investigated for an ability to increase or restore RsmA levels, thus directly implicating AlgR in control of *rsmA*.
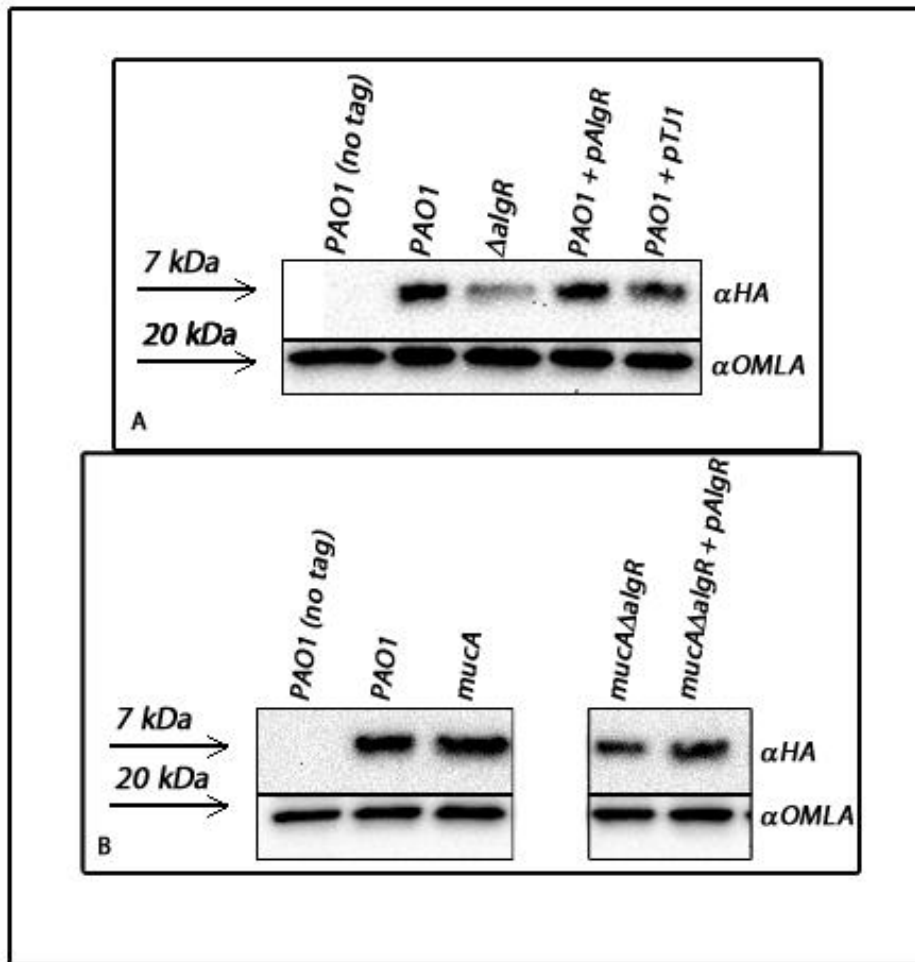
Figure 9: algR mutants have decreased RsmAHA protein levels. Strains were grown in LB broth for 8 hours and levels of RsmAHA were measured by Western blot. A membrane protein (OmlA) was used as a loading control.

Two separate membranes were used to accommodate all of the strains used for analysis of RsmA-HA. In *Figure 9 (A)* there was evidence that PAO1Δ*algR* has diminished RsmA-HA levels compared to wild-type. This loss is more drastic than the decrease in *rsmA* expression seen in *Figure 6*. This result is being investigated further and has yet to be confirmed. A previous study indicates *mucA22* possesses higher levels of RsmA-HA compared to PAO1 (62). The results in *Figure 9 (B)* show a small increase of RsmA-HA in *mucA22,* but at a less drastic level than was previously observed and certainly less than is shown by the fusions in *Figure 6*. *mucA22Δ algR* possessed a decrease in RsmA-HA compared to *mucA22*, but more accurate

controls and stringent protein loading must be used in the future, and at this time this remains preliminary data. In addition, densitometry could be performed on the blot to obtain quantitative results for the protein levels standardized to the OmlA loading control. This may clarify some of the perceived differences in RsmA-HA between the strains. Induced expression of *pAlgR* had no significant effect on RsmA-HA protein levels in either strain, which mimics the fusions and is addressed in Chapter 4.

<div align="center">An AlgR Extract Binds the *rsmA* Promoter</div>

The defect in *rsmA* expression seen by Intile et al (32) and this lab indicate AlgR has an effect on *rsmA* in the cell. However, the mechanism of control remained to be elucidated, and it was unclear whether the AlgR effect on *rsmA* was direct or indirect. The primer extension completed by Stacey, et al identified a putative AlgR binding site adjacent to an AlgU dependent promoter for *rsmA* (62). It was thus hypothesized that AlgR may bind the promoter and regulate *rsmA* directly. To test this, an extract containing AlgR was obtained and used in an electrophoretic mobility shift assay (EMSA) on a labeled section of the *rsmA* promoter containing the putative AlgR binding site.

Expression of an AlgR-GST Fusion is Induced in BL21(DE3)

To determine if AlgR bound the *rsmA* promoter, gel shift studies were initiated. First, purified AlgR was obtained for use in gel shift experiments. Previous work in this lab cloned the complete open reading frame for *algR* into the pGEX4T-2 expression vector (Appendix C). A purified plasmid prep of this was created and sequence verified, then used in this study to overexpress an AlgR-GST fusion. AlgR was then separated from GST using thrombin cleavage.

The plasmid prep above was quantified and 100 ng (determined using absorbance at

OD_{600}nm) was transformed into BL21 (DE3) cells from New England Biolabs, described in

detail in Chapter 2. Expression conditions listed in Chapter 2 were utilized and SDS-PAGE was

conducted followed by Coomassie staining to determine if the AlgR-GST fusion was being

expressed in a quantity sufficient to warrant further purification.
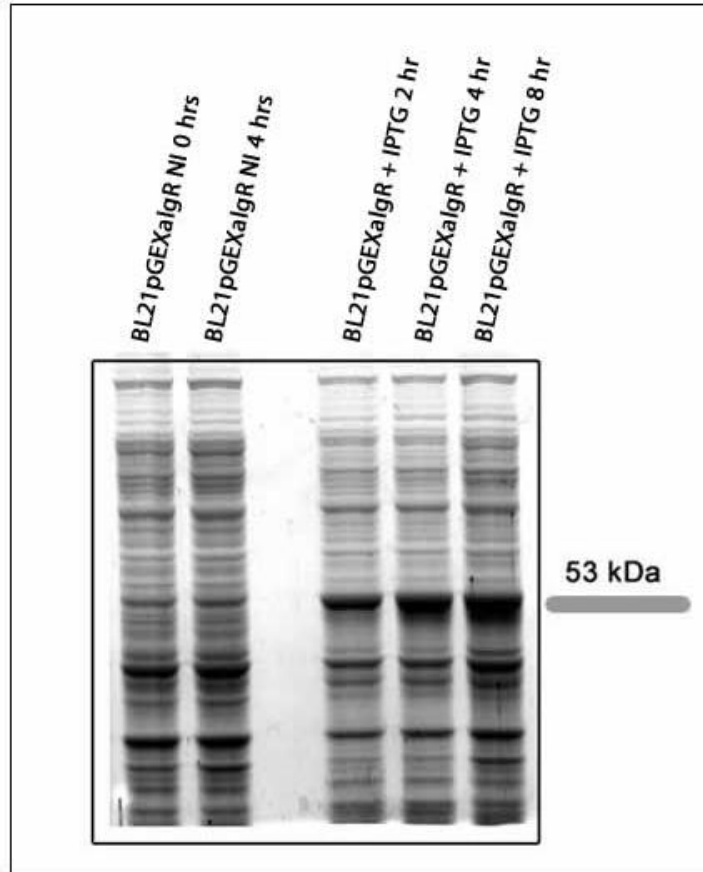
Figure 10: A 53 kDa protein is present in induced cultures. Lysates containing 10 ug total cell protein from induced and non-induced cultures were resolved on 7% SDS-PAGE and stained with Coomassie Brilliant Blue.

Protein lysates from induced cultures displayed specific, increasing expression of a 54-

kDa protein. AlgR and glutathione-s-transferase were fused for purification, and each is ~27

kDa. Induction of cells containing the empty vector was not conducted, but the increase in

quantity of a protein corresponding to the size of an AlgR-GST fusion was not present in uninduced cultures, indicating tight control over transient expression from this vector.

A 27 kDa Protein is Present Following Column Purification

The results of *Figure 7* were enough evidence to proceed with further purification, using GST-Spin Columns from ThermoScientific as described in Chapter 2. Multiple fractions from this process are indicated in *Figure 8*.
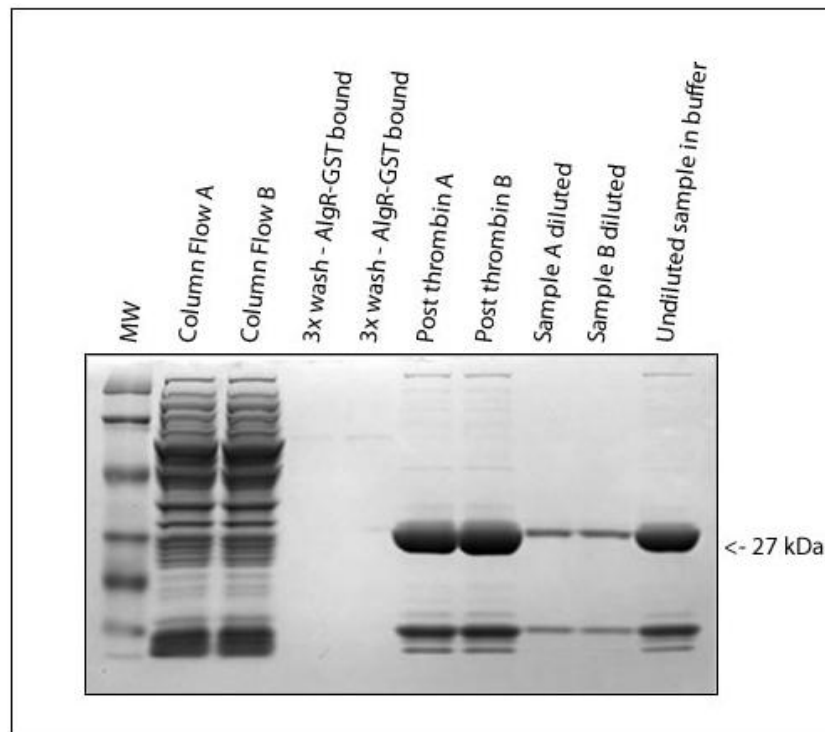
Figure 11: A lysate containing a 27 kDa protein is purified from the spin columns. Various fractions from the column purification process were resolved on 10% SDS-PAGE and Coomassie stained to view proteins present from each stage.

Lysates from induced cultures were passed over the columns and the flow-through is shown in the first two lanes following the molecular weight marker. Following three washes, little protein was observed. A fourth wash was performed before incubation with thrombin overnight to cleave AlgR from the GST moiety. Following thrombin treatment, a large amount of protein at 27 kDa was observed, in addition to several other proteins of multiple sizes. A

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significant amount of protein was present at 20 kDa, and other proteins were present in trace

amounts. This 20 kDa protein may represent enzymatic breakdown of AlgR that occurred in the

column overnight. To reduce the amount of proteins not corresponding to the size of AlgR, the

eluate was diluted 1:10 in sterile PBS then stored in 4X Protein Storage Buffer (Appendix B) at

-20° C. Lanes 8 and 9 in *Figure 8* show that following the dilution, the two proteins in

abundance were at 27 and 20 kDa, with the 27 kDa protein in excess. Lane 10 indicated no

breakdown of the 27 kDa occurred after addition of 4X Storage Buffer.

<u>Western Blotting Confirms AlgR is Purified</u>

Western blot analysis was performed on the purified fraction in order to show that AlgR

was the 27 kDa protein.
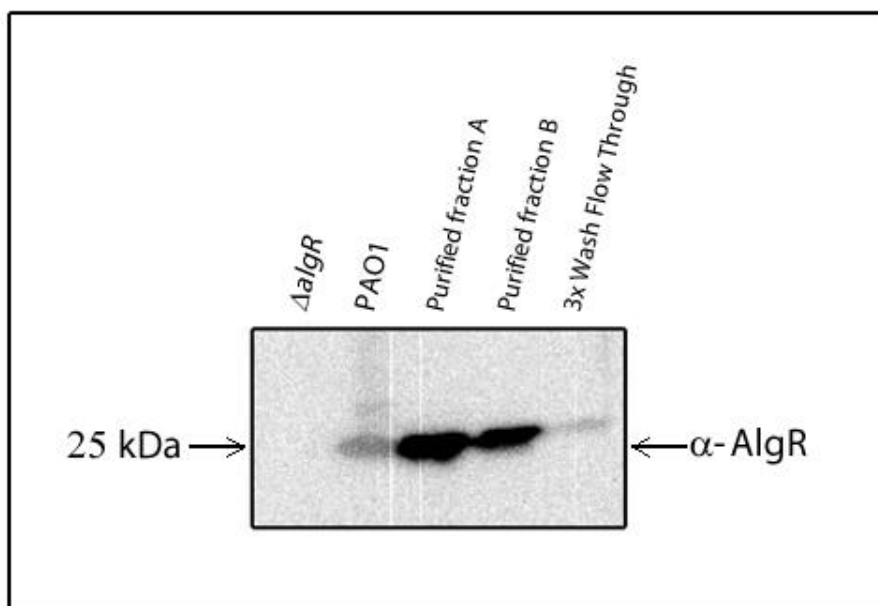
Figure 12: Western blot confirms AlgR was the major protein purified. Following thrombin cleavage, 10 ul of eluate and 10 ul post-wash flow-through were resolved on 10% SDS-PAGE and treated with polyclonal antibody to AlgR. 10 ug whole-cell lysate from PAO1 and PAO1ΔalgR were also run as a reference for AlgR.

The results above show that polyclonal antibody for AlgR did not bind anything in a lane

containing a lysate from PAO1ΔalgR but bound a protein present at ~27 kDa in PAO1 and

within the purified fraction. These results indicate AlgR was the 27kDa protein. Since AlgR was shown to be present in the purified stocks, gel shift studies were pursued.

A Purified Fraction Containing AlgR Binds *algD* and *rsmA* but not *pscE/F*

Before testing the AlgR extract's ability to bind the *rsmA* promoter, it was necessary to ensure that the AlgR extract did not bind a probe nonspecifically. In addition, the extract was tested for binding to a section of DNA previously shown to be controlled by AlgR (44). Since the purified extract contained proteins not corresponding to the size of AlgR, results of these control gel shifts could provide evidence that AlgR was binding correctly, and thus warrant further investigation of the extract's ability to bind *rsmA*.

The purified containing AlgR was mixed in a binding reaction with annealed oligos or PCR probes that were 3' end labeled with biotin by TdT (Chapter 2). AlgR was shown in a previous study not to bind to a site overlapping the *pscE/F* genes (52), and a labeled, 50-base pair probe (sequence available in Appendix C) representing this region was used as a negative control to eliminate the possibility that the protein extract bound nonspecifically. The *algD* gene of the alginate biosynthetic operon has been shown to be bound by AlgR and was thus used as positive control (44). PCR was used to generate probes for *algD* and *rsmA* with primers listed in Appendix C, and this yielded a larger probe (~160 bp) than the negative control. Using PCR to generate these probes afforded the ability to better specify the region bound by AlgR by narrowing the size of the probe in future assays using primers already available to the lab. The *rsmA* probe contained both promoters and the putative AlgR binding site. This probe was generated and labeled as depicted in *Figure 13* below:
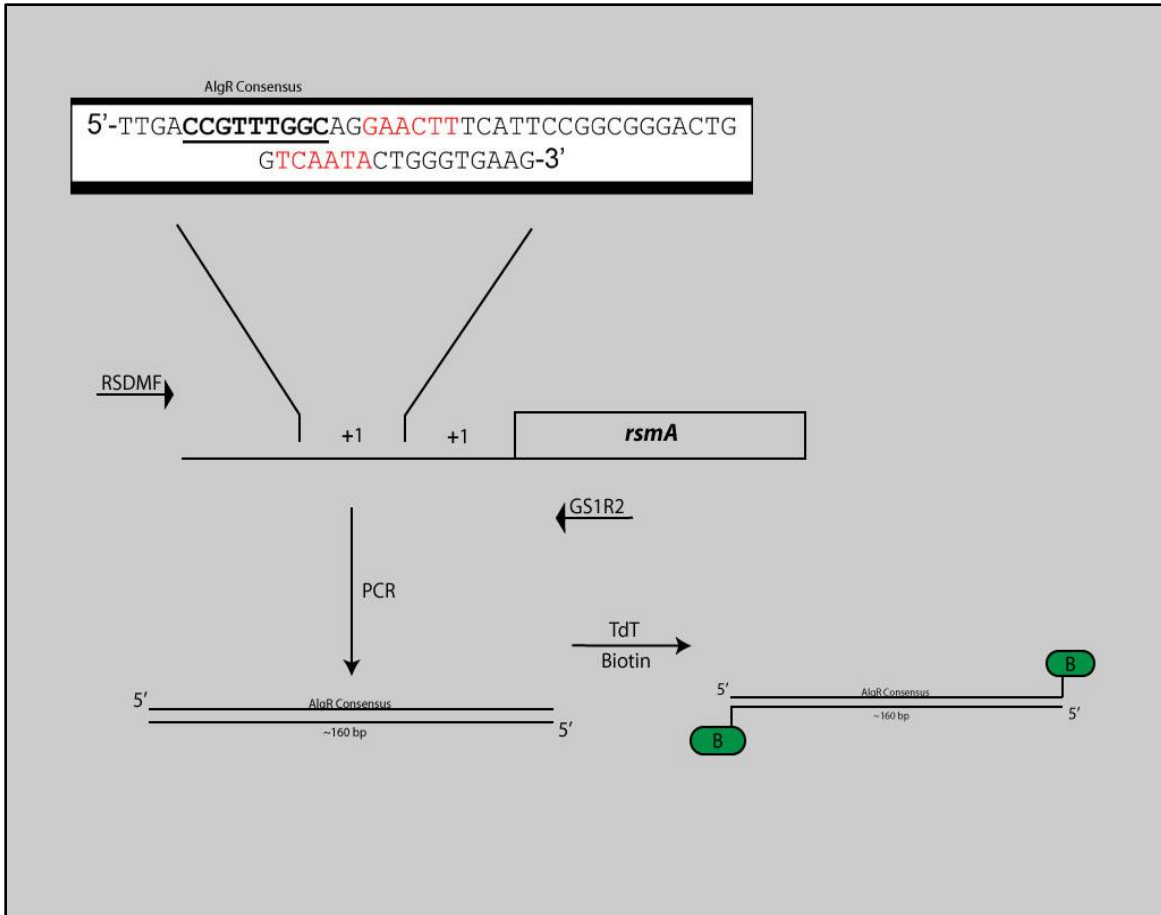
Figure 13: PCR amplification and end-labeling generate probes for EMSA. Primers flanking the rsmA promoter (RSDMF and rsmAGS1R2) were used to create a PCR product that was biotinylated using terminal-deoxynucleotidyl transferase.

To make sure that non-specific proteins present in the purified extract did not cause binding to non-specific probes, a gel shift was performed on *pscE/F* and is depicted below in *Figure 11*.
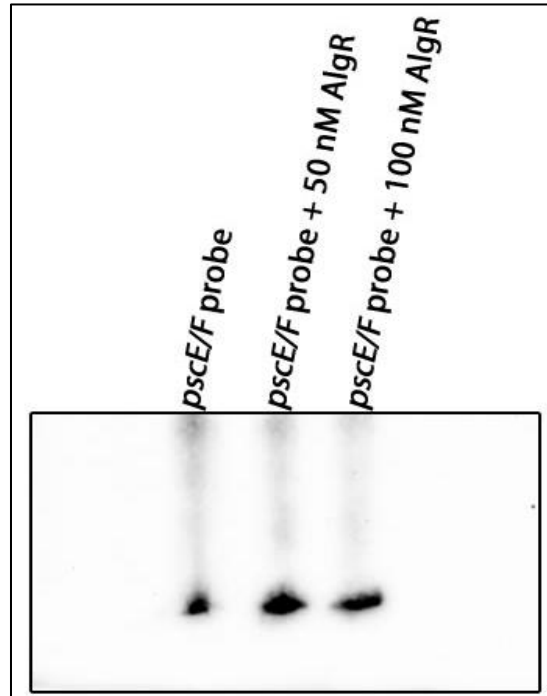
Figure 14: AlgR does not bind the pscE/F probe at 100 nM. ~200 fmols pscE/F probe were mixed with 50 and 100 nM AlgR purified fraction to determine the extract's ability to bind a gene known not to be regulated by AlgR.

The *pscE/F* probe (~200 fmols) was not bound by AlgR at 50 or 100 nM (conc. determined using methods in Chapter 2). These binding reactions were run on a separate gel from the *algD* and *rsmA* reaction due to the size difference in the probes, which necessitated construction of a higher percentage polyacrylamide gel for proper resolution of the ~50 bp *pscE/F* probes. The concentration of AlgR in *Figure 11* is less than that shown to bind *rsmA* (*Figure 12*), but other binding reactions conducted in the lab displayed the extract's inability to bind this probe at higher concentrations, and 100 nM was deemed relevant because that is the

concentration seen binding *algD* below. The inability of the AlgR containing fraction to bind

pscE/F warranted its use in further assays to test its ability to bind the *rsmA* promoter.

The extract was next tested for its ability to bind the *rsmA* probe containing the potential

AlgR binding site. To ensure that the AlgR containing fraction was capable of binding a gene

known to be directly regulated by AlgR, the *algD* probe was included on the same gel as *rsmA* to

verify the extract's correct binding ability.
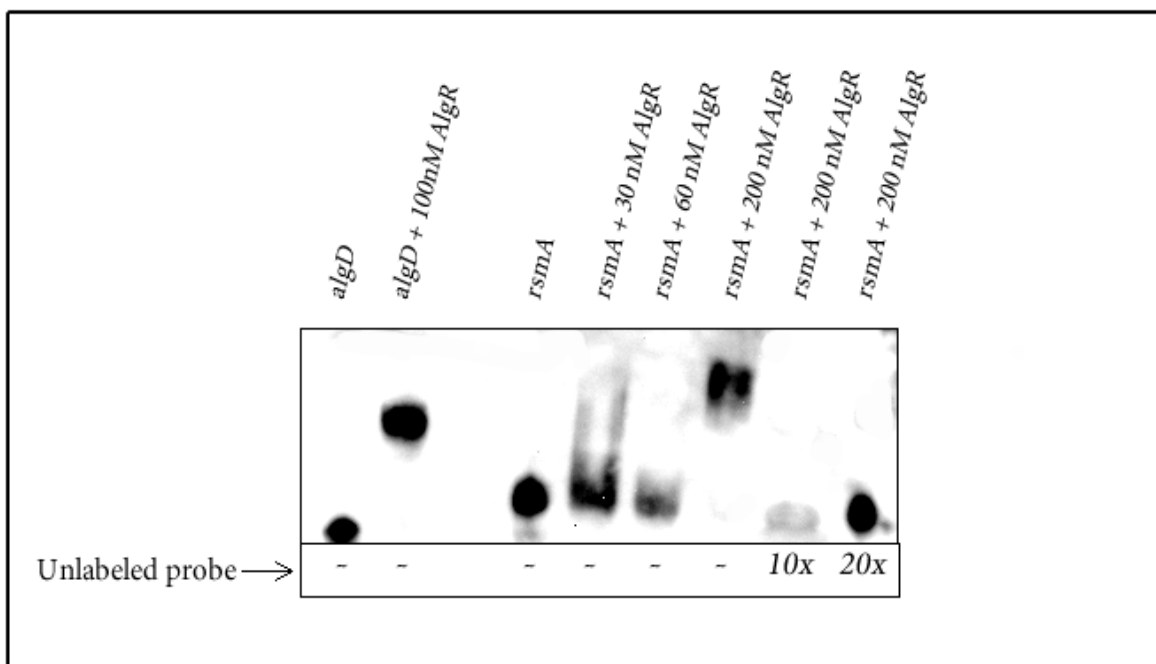
Figure 15: AlgR bound the algD probe at 100 nM and rsmA probe at 200 nM. Probe concentration was ~25 fmols.

The results above indicate the ability of the purified fraction to bind and shift *algD*

similar to that of other studies (44). Titration of the protein extract was conducted in binding

reactions with the *rsmA* probe, and complete binding occurred at a 200 nM concentration of

protein. This interaction was shown to be specific when excess amounts of unlabeled *rsmA*

probe were used to abrogate the shift, as depicted in the last two lanes of *Figure 12*. These

results indicate that the AlgR containing fraction correctly bound a gene known to be regulated

by AlgR, and specific binding also occurred to the *rsmA* probe.

CHAPTER 4

DISCUSSION

AlgR and *rsmA* Regulation

P. aeruginosa's ability to transition from acute to chronic infection within the cystic fibrosis lung is crucial for this pathogen's ability to cause severe mortality for these patients. *P. aeruginosa* uses complex and diverse genetic regulation to control gene expression for different virulence factors. While regulatory systems used during acute infection are becoming well understood, much remains unclear about regulation of chronic virulence factors and the genetic regulation involved in the switch to chronic infection. Characterizing pathways involved in pushing the cell into the mucoid phenotype can yield clues as to the signals processed by *P. aeruginosa* to induce expression of chronic virulence factors. In addition, interruption of these vital pathways may serve as a future drug target.

AlgR is a transcriptional regulatory protein required for alginate production, and is active in chronic strains (6, 7, 26, 64). RsmA is a post-transcriptional regulatory protein that encourages expression of acute associated virulence factors (32, 37, 51, 52). These two proteins serve crucial regulatory roles, and were previously thought to exist as part of two separate regulatory pathways. Recently, however, transcriptional analysis of *rsmA* has shown a novel interaction between these two global systems and has given clues as to how *P. aeruginosa* controls expression of virulence factors in the mucoid strains (32, 62).

A previous study by Intile, et al (32) demonstrated AlgR control of *rsmA* expression, but a mechanism was not determined. A primer extension of *rsmA* was conducted by Stacey, et al (62), and identified a possible AlgR binding site adjacent to an AlgU dependent promoter. Control of *P. aeruginosa* RsmA has previously been shown to occur by production of RsmY and

RsmZ, sRNAs that bind RsmA and thus modulate its ability to reach its mRNA regulatory targets (7). Control of *rsmA* at the transcriptional level, however, remains unclear. The AlgU/AlgR control of *rsmA* expression presented by this lab suggests that known regulatory pathways involved in alginate production tune *rsmA* expression. This illustrates a previously unknown interaction between two global systems that regulate virulence factor gene expression in *P. aeruginosa*. The goal of this study was to elaborate on the finding that AlgR controls *rsmA* expression and to determine the mechanism. Expression of *algR* was investigated for having an effect on *rsmA* transcription and protein levels. In addition, a purified extract containing AlgR was used in a gel shift to test its ability to bind the *rsmA* promoter.

 RsmA promoter activity *in vivo* was measured using a previously constructed *rsmA-lacZ* transcriptional fusion as a readout for *rsmA* expression. Results from *Figures 6, 7* and *8* showed that *mucA22rsmA-lacZ* expression is elevated compared to PAO1*rsmA-lacZ*, which agrees with the data from Intile, et al and Stacey, et al that shows *rsmA* transcription is elevated in a *mucA* mutant (32, 62). Deletion of *algR* in the *mucA22* background showed decreased *rsmA* expression. Western blotting of epitope tagged RsmA-HA was used to compare RsmA protein levels. Results of the Western blot analysis in *Figure 9* are suspect due to the lack of RsmA-HA increase in *mucA22* shown by other studies in this lab. Loading errors may explain the deviation between the Western blots and the transcriptional fusions. Though the *algR* mutants show less RsmA-HA than either wild-type, these results need further study before AlgR can be said to control RsmA protein levels.

 Additional methods could be used to measure *rsmA* expression in an *algR* mutant, such as directly measuring levels of mRNA in a Northern blot or quantitative real-time PCR to measure fold changes in *rsmA* expression in a *mucA22ΔalgR* strain compared to the wild-type mucoid

strain. Results from those studies could provide further evidence that an *rsmA* expression defect is AlgR dependent.

Since a loss of *rsmA* expression was seen in *mucA22ΔalgRrsmA-lacZ*, it was hypothesized that expression of *algR* would show an increase in *rsmA* activity in PAO1 *rsmA-lacZ* and PAO1RsmA-HA. Overexpressing *algR* in any background failed to increase *rsmA* transcription or protein levels, contrary to the results of Intile, et al (32). The *pAlgR* overexpression vector was able to restore twitching to an *algR* mutant (*Figure 4 and Table 5*), which suggests that *algR* was correctly being induced during the twitching assay. It is possible that induction differences occurred between the twitching assay, which was performed on solid media, compared with the β-galactosidase assay and Western blots, which were performed from broth cultures. Investigation into different concentrations and timing of arabinose induction with the strains used in this study could potentially show an AlgR-dependent increase in *rsmA* expression in future studies. If *algR* was correctly being induced in the β-galactosidase assay and Western blots, it is also possible that *algR* expression did not have an effect on *rsmA* in our strains. Overexpression of *algR* was unable to restore alginate production, an AlgR dependent phenotype (18). In addition, overexpression of *algR* has a deleterious effect on virulence (37). This suggests AlgR levels are tightly maintained in the cell and that large alterations in AlgR homoestasis are not favorable to *P. aeruginosa*. Intile, et al were able to see an increase in *rsmA* expression, but this occurs in PA103, a different strain than the wild-type used for this study (32). Additionally, the complementation construct used by Intile, et al. used both *algZ* and *algR*, which may suggest a role for AlgZ in controlling *rsmA*. In addition, the expression vector used in their study was plasmid based, compared to the chromosomal insertion used here. Overall, the *algR* expression in this study did not affect *rsmA* transcription or protein levels in a significant

manner, indicating that although *mucA22ΔalgR* possessed a noticeable RsmA defect, further

investigation must occur to implicate AlgR as the only reason for this decrease.

The second aim of this study was to define a mechanism for AlgR control of *rsmA* by

testing purified AlgR's ability to bind the *rsmA* promoter. A purified fraction containing AlgR

was used in an EMSA on a labeled section of the *rsmA* probe containing the possible AlgR

binding site. The purified extract contained proteins not corresponding in size to AlgR, and thus

it was determined that stringent controls should be performed before using the extract in a gel

shift with *rsmA*. In a previous study, AlgR was shown not to bind to *pscE/F* (52). In this study,

the AlgR containing extract did not bind a *pscE/F* probe at 100 nM (*Figure 14*), indicating that

non-specific binding of the extract was not occurring. In the future, the AlgR concentration used

in the *pscE/F* binding reaction should match that used in the reactions with *rsmA*. The AlgR

containing extract (100 nM) was also able to shift a section of the *algD* promoter previously

shown to be bound by AlgR (44). Since the extract bound in a manner expected from AlgR, gel

shifts were also performed on the *rsmA* promoter. Purified extract was titrated in a binding

reaction with the *rsmA* probe, and was shown to bind *rsmA* and completely shift the probe

(*Figure 15*). The results of these gel shifts show that the purified extract binds positive and

negative probes in a manner expected by AlgR, and that binding is occurring to the *rsmA* probe

at 200 nM. Further studies can identify the exact concentration of AlgR required for binding, as

well as the sequence bound by AlgR. In addition, AlgR antibody could be used in the *rsmA*

binding reaction to illustrate a super-shift, indicating the *rsmA* shift seen in this study was due to

AlgR, and not another protein present in the extract. PAGE-purification of AlgR in the future

would mean the concentration of protein used in the binding reaction reflects the concentration

of AlgR by eliminating the possibility of erroneous quantification from non-AlgR proteins

present in the purified lysate. Thrombin cleavage of the AlgR-GST fusion was completed for 12-16 hours, and lowering this treatment time may help lower the amount of background protein present in the purified eluate. In addition, stringent control of probe concentration using dot-blot analysis and a more specific titration by AlgR would yield more accurate results regarding binding concentration. To show the *in vivo* relevance of AlgR binding *rsmA* within the cell, site-directed mutagenesis was attempted at the AlgR binding site on the *rsmA-lacZ* transcriptional fusion and remains incomplete. Creating the mutagenized construct and performing β-galactosidase assays may show that an intact AlgR binding site is required for *rsmA* expression in the cell.

Intile, et al proposed that AlgR recalibrates *rsmA* expression to inhibit Type III Secretion, a known RsmA regulatory target (32). The data presented in this study show that *rsmA* expression in a *mucA22* mutant is defective without AlgR, and a mechanism may exist whereby AlgR directly binds and regulates the *rsmA* promoter. A concurrent study by this lab implicates AlgU in control of *rsmA* expression (62). A model representing AlgU and AlgR control of *rsmA* is depicted in *Figure 16*.
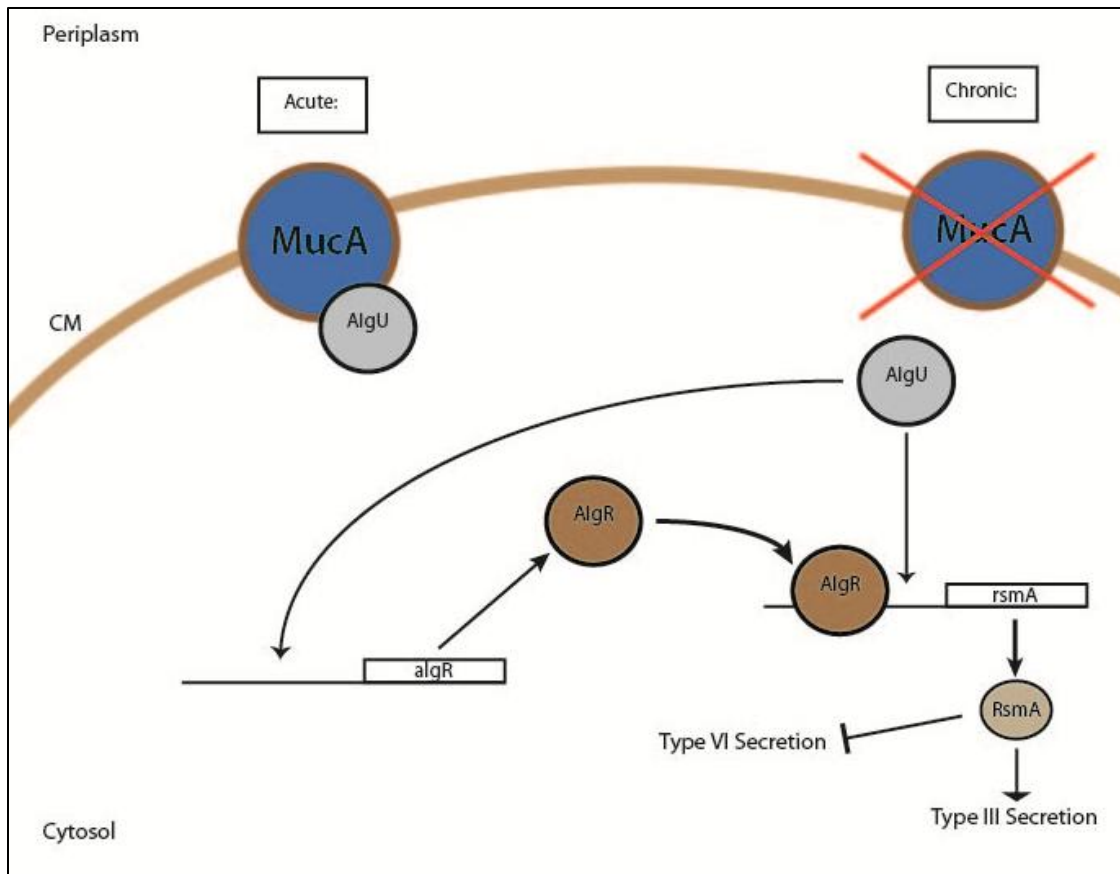
Figure 16: AlgU and AlgR control of rsmA expression in the mucoid background.

Prior to the studies by this lab, transcriptional regulation of *rsmA* in *P. aeruginosa* was unclear. Our findings suggest *P. aeruginosa* uses members of the alginate regulatory system to tune virulence factor gene expression, which may help the cell transition from acute to chronic infection. Future studies will attempt to elaborate upon the virulence implications of this interaction. RsmA has been shown to repress Type VI Secretion (6), another secretion system involved in *P. aeruginosa* virulence (46). Control of Type VI components was investigated as a downstream readout for AlgR control of *rsmA* (Appendix F), but further studies are needed to test the potential of this phenotype's relevance to AlgU/AlgR control of *rsmA*.

Continued understanding of the complex regulatory hierarchies involved in regulation of virulence factors by *P. aeruginosa* may identify crucial signals that push the cell towards the

debilitating chronic infection, and could potentially identify drug targets. In the absence of gene therapy treating cystic fibrosis, a treatment aimed at eliminating *P. aeruginosa* infection will have significant impact on the prognosis for CF patients.

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Appendix A: Media

Pseudomonas Isolation Agar (PIA):

In 2 L flask w/ stir bar:

940 ml ddH$_2$0
20 ml glycerol
25 mg Irgisan
20 g peptone
10 g K$_2$SO$_4$
1.4 g MgCl$_2$ hexahydrate
13.6 g agar

Mix and boil solution. Autoclave. Add antibiotics when warm to touch.

Antibiotics and Supplemental concentrations:

150 ug/ml gentamicin
80 ug/ml X-gal
Arabinose to 1% (replace 25 mL H$_2$0 with 25 mL of 20% arabinose stock)

Luria-Bertani (LB) Media:

In 2 L flask w/ stir bar:

950 ml ddH$_2$O
15g agar (if making plates)
10g NaCl
5g yeast extract
10g tryptone

Mix and autoclave. Add antibiotics when warm to touch.

Antibiotics and Supplemental concentrations:

15 μg/ml gentamicin
10 μg/ml tetracycline
100 μg/ml ampicillin
50 μg/ml kanamycin
1500 ug/ml trimethoprim
Arabinose to 1% (replace 25 mL H$_2$0 with 25 mL of 20% arabinose stock)

<u>LB no salt tet^{50} irg^{25}</u> :

950 ml ddH$_2$O
15g agar
5g yeast extract
10g tryptone
25 mg Irgasan

Mix and boil, then autoclave. Add 50 mg tetracycline when flask becomes warm to touch.

<u>Vogel-Bonner Minimal Media (VBMM):</u>

10X VBMM stock (500 ml):

400 ml ddH$_2$O
15g trisodium citrate
10g citric acid
50g K$_2$HPO$_4$
17.5g NaNH$_4$PO$_4$ x 6 H$_2$0
Adjust pH to 7 and autoclave

1X VBMM:

400 ml ddH$_2$O
7.5g Agar
autoclave and allow to cool to 50°C, then add:

50 ml 10X VBMM
500 µl 1M magnesium sulfate
50 µl 1M CaCl$_2$

For VBMM + Carb => When flask is warm to touch, add 300 ug/ml carbenicillin

<u>SOC Outgrowth Media:</u>

2% Peptone
0.5% Yeast
10 mM NaCl
2.5 mM KCl
10 mM MgCl$_2$
10 mM MgSO$_4$
20 mM Glucose

10X TBE
108g Tris base
55g boric acid
Dissolve into 900 ml ddH$_2$O and add 40 ml 0.5M EDTA (pH 8.0) and autoclave.

10X TE
100 ml 1M Tris-HCl (pH 7.5)
20 ml 0.5M EDTA (pH 8.0)
880 ml ddH$_2$O and autoclave

Z-buffer
16.1g Na$_2$HPO$_4$ x 7 H$_2$O
5.5g NaH$_2$PO$_4$ x 7 H$_2$O
0.75g KCl
0.246g MgSO$_4$ x 7 H$_2$O
Add 900 ml ddH$_2$O, adjust pH to 7 and autoclave

BME – Before assay, add 2.7 ul beta-mercaptoethanol / 1 ml Z buffer
ONPG – Just prior to assay, add 4 mg ONPG / 1 mL Z buffer and vortex until fully dissolved

1.5M Tris-HCl (pH 8.8)
27.23 g Tris base
80 ml ddH$_2$O
Adjust pH to 8.8 w/ conc. HCl
ddH$_2$O to 150 ml and autoclave

0.5M Tris-HCl (pH 6.8)
6.1 g Tris base
80 ml H$_2$O
Adjust pH to 6.8 w/ conc. HCl
ddH$_2$O to 100 ml and autoclave

10X Glycine Running Buffer
30.3 g Tris base
144.1 g glycine
10 g SDS
ddH$_2$O to 1 L and mix on stir plate

10X Towbin Buffer
30.3 g Tris base
144 g glycine
ddH$_2$O to 1 L and mix on stir plate

SDS-PAGE Transfer Buffer
10 ml 10X Towbin buffer
20 ml methanol
70 ml ddH$_2$O

10X Tris-buffered Saline
60.6 g Tris base
87.6 g NaCl
800 ml ddH$_2$O
Adjust pH to 7.6 w/ 1M HCl
Add ddH$_2$O to 1 L and autoclave

TBS-T:
Just before use, add 0.1% Tween-20 to 1X TBS

Western Blot Blocking Buffer
5 ml TBS-T
2.5 g Skim milk powder
ddH$_2$O to 50 ml

Western Blot Washing Buffer
5 ml TBS-T
45 ml ddH$_2$O

1˚ Antibody Solution
5 ml TBS-T
2.5 g Skim milk powder
ddH$_2$O to 50 ml
Thaw antibody on ice and add based on desired dilution (2.5 ul for 1:20,000)

2˚ Antibody Solution
5 ml TBS-T
2.5 g Skim milk powder
ddH$_2$O to 50 ml
Add anti-rabbit or anti-mouse based on desired dilution

Protein lysis buffer
10 ml 10X PBS (pH 7.4)
90 ml sddH$_2$O

Just before use add:
Lysozyme to desired concentration
5 mM DTT
PMSF or other protease inhibitor
Place on ice until use

10X Phosphate-buffered Saline (PBS)
80 g NaCl
2 g KCl
14.4 Na_2HPO_4
2.4 KH_2PO_4
Dissolve in 800 ml ddH_2O and adjust pH to 7.4
Add ddH_2O to 1 L and autoclave

4X SDS-PAGE loading buffer
2.2 ml 0.5M Tris HCl (pH 6.8)
2.2 ml glycerol
1.1 ml 20% SDS
250 ul 1% Bromophenol blue
250 ul β-mercaptoethanol
Aliquot 1 ml into tubes and store at -20˚C

5X EMSA Binding Buffer
For 10 ml:
0.5 ml 1M Tris HCl (pH 8.0)
3 ml 2.5M KCl
50 ul 0.5M EDTA (pH 8.0)
50 ul Triton X-100
6.5 ml glycerol
Add 1 mM DTT just prior to use

6X EMSA loading dye "Blue Juice"
25 mg Bromophenol blue
4 g sucrose
ddH_2O to 10 ml and filter sterilize
Aliquot 1 ml into tubes and store at -20˚C

4X Protein Storage Buffer
For 100 ml:
35 ml ddH_2O
2.4 g Tris base
2.98 g KCl
30 ml glycerol
Adjust pH to 7.4 and add ddH_2O to 100 ml
Filter sterilize and store at 4˚C

10x TNE
12.11 g Tris base
3.72 g EDTA
116.89 g NaCl

Dissolve above into 800 ml ddH_2O, pH to 7.4 w. HCl and fill to 1 L then autoclave.

Coomassie Staining Solution
For 100 mL 0.1% Solution:

Dissolve 0.1 g Coomassie Brilliant Blue into 100 mL containing the following:
50 ml Methanol
40 ml H_2O
10 ml Glacial acetic acid

Coomassie Destaining Solution:
For 1 L, combine:

500 ml ddH_2O
400 ml Methanol
100 ml Glacial acetic acid

Appendix C: Strains, Primers and Plasmids

Table 6: Strains used in the study

Strain	Description	Source
PAO1	Wild-type acute strain	Lab Stock
mucA22	Wild-type chronic strain	Lab Stock
PAO1Δ*algR*	*algR* mutant	This study
Δ*algRrsmAlacZ*	*ΔalgR; rsmAlacZ* in *attB* site	This study
mucA22rsmAlacZ	*mucA22; rsmAlacZ in attB site*	62
PAO1*rsmAlacZ*	*rsmAlacZ* in *attB* site	62
mucA22ΔalgRrsmAlacZ	*mucA22&ΔalgR; rsmAlacZ in attB site*	This study
PAO1RsmA-HA	HA by allelic exchange	62
*mucA*22RsmA-HA	HA by allelic exchange	62
Δ*algR*RsmA-HA	HA by allelic exchange	This study
*mucA22ΔalgR*RsmA-HA	HA by allelic exchange	This study
E. coli SM10	Sex pilus donor	Lab strain
E. coli BL21 (DE3)	Protein expression cells	New England Biolabs

Table 7: Primers and oligos used in study

Primer	Sequence
pHERDSF	ATCGCAACTCTCTACTGTTTCTC
pTJ1R	GTTTGGAACTAGATTTCACTTATCT
rsmASDMFcheck	GCCAAGGTTTCCATCGTCGG
rsmAGSR1	CACGCGAATATTTCAGGACAAC
lacZRforTF	CATCTGCTGCAAGGCGATTAAG
AlgRintF	GCAACTGGACTGGCAGGTGC
AlgRBXba1R	GCGCTCTAGAGCTCCAGCAGGTCCTCGGCC
pscFRBSF	GCTGGCGGAGTGTCGCCGGGAACTGGCCAGAGG
pscFRBSR	CCTCTGGCCAGTTCCCGGCGACACTCCGCCAGC
algDGS2F	CTTTCAGCCGCCATGCATTC
algDGS2R	GATGTTTTCTCTGCGAGGGAAG

Table 8: Plasmids used in study

Plasmid name	Description	Source
pTJ1algR	*algR* expression vector, tmp[r]	16
pGEX4T-2	GST-fusion vector, amp[r]	GE Healthcare
pGEX4T-2algR	*algR* in pGEX4T-2, amp[r]	This study
pRK2013	Tra+, Mob+, km[r]	Lab strain
pTNS3	Tra+, amp[r]	Lab strain

Table 9: 1% Agarose gel recipe

For 1% agarose:

45 ml sddH20
5 ml 10X TBE
0.5 g agarose

_____

Microwave 1 minute
When just warm to touch
add 2.7 ul EtBr

Table 10: SDS-PAGE recipe

4% Stacking Gel	15% Resolving Gel
500 ul 40% Bis/Bis-Acryl.	3 ml 40% Bis/Bis-Acryl.
1.25 ml 0.5 M Tris-HCl pH 6.8	2 ml 1.5 M Tris-HCl pH 8.8
50 ul 10% SDS	80 ul 10% SDS
3.1 ml ddH20	2.88 ml ddH20
5 ul TEMED	8 ul TEMED
25 ul fresh 10% APS	80 ul fresh 10% APS
Mix all components in order and pipette into cassette immediately.	Mix all components in order and pipette into cassette immediately.

Table 11: 6% TBE polyacrylamide gel recipe

6% TBE Polyacrylamide gel (10 ml) :

1.5 ml 40% Bis/Bis-Acryl.
1.0 ml 10X TBE
7.5 ml ddH20
8 ul TEMED
80 ul fresh 10% APS

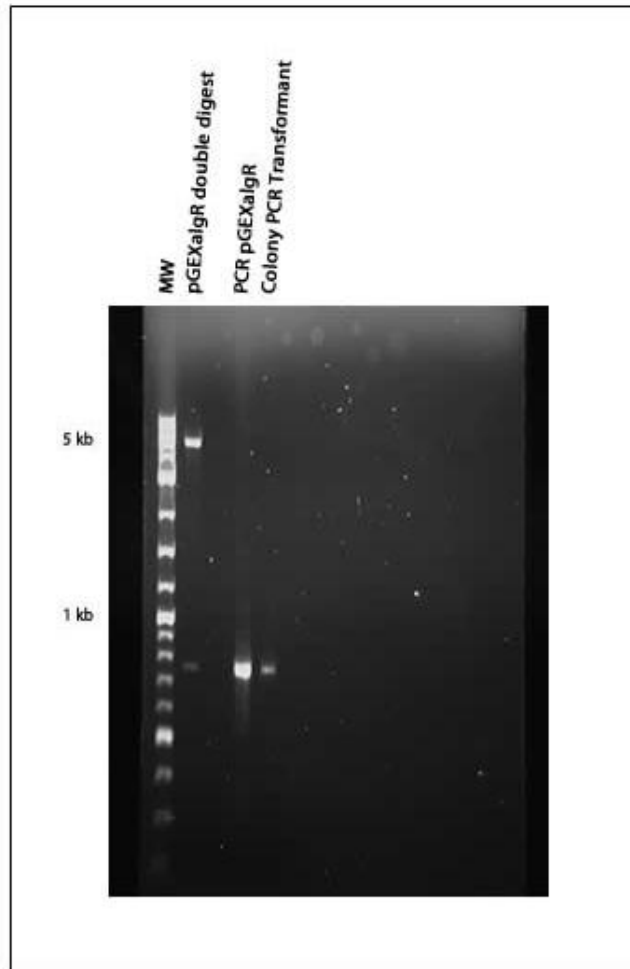Mix all components in order and pipette into cassette immediately.

Figure 17: Restriction digest and colony PCR confirm transformed BL21 has the algR expression vector.

BL21 cells transformed with pGEX*algR* were confirmed using restriction digest and colony PCR to show they possessed the overexpression construct. Sal1-HF and Not1-HF were used to drop out a ~750 bp fragment corresponding to the size of the cloned *algR* gene. PCR amplified a similar sized fragment using the purified plasmid prep generated previously in the lab as a positive control. Colony PCR of a single colony transformant generated a band of similar size, indicating expression cells had been successfully transformed with pGEX*algR*.

During the course of this study it became apparent that AlgR control of *rsmA* may be relevant to pathogenesis, since both *algR* and *rsmA* mutants are attenuated and each protein control a wide variety of virulence factors. A virulence associated phenotype was pursued as a readout for AlgR control of *rsmA*. *P. aeruginosa* uses the Type VI Secretion apparatus to counterattack antagonizing bacteria (31, 46). Although the *P. aeruginosa* Type VI has not been shown to attack eukaryotic cells, antibodies to Type VI components have been found in CF patient serum (31). Since RsmA negatively regulates Type VI Secretion (6), it was hypothesized that AlgR may modulate Type VI expression through RsmA. Antibody to HcP, a Type VI component, was obtained in order to use Western Blotting as a means to analyze Type VI expression in different mutant strains.
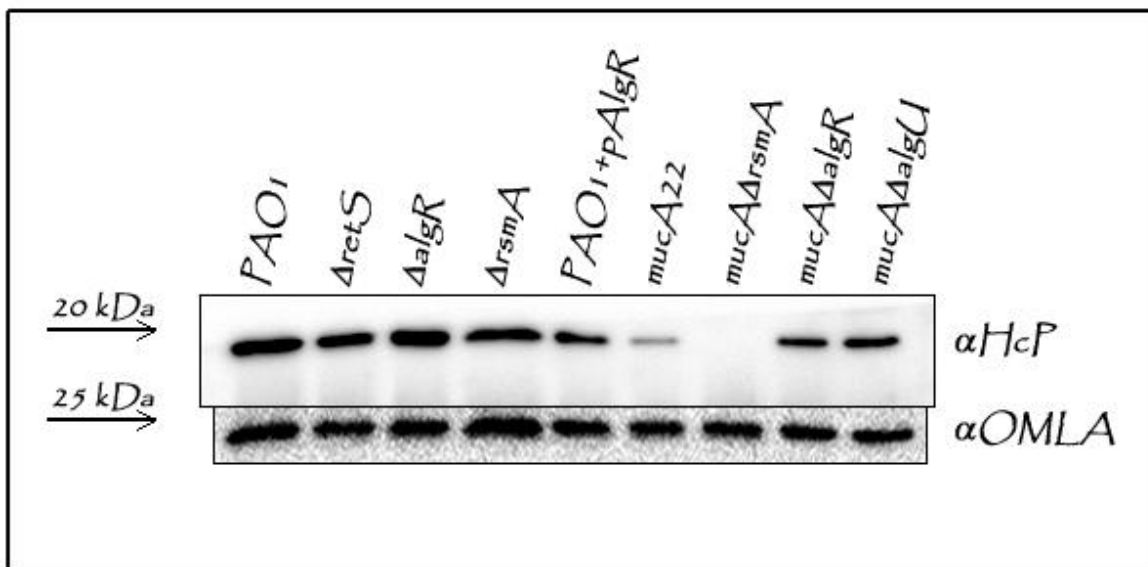

Figure 18: Differential expression of hcp occurs in various backgrounds. Cultures were grown in LB broth for 5 hours and 10 ug protein / lane was loaded onto a 15% SDS-PAGE and treated with antibody to either HcP (1:1000) or OMLA (1:20,000).

The strains shown in *Figure 16* were chosen to establish a preliminary reason for further investigation.

In the non-mucoid strains, no discernible difference is seen in HcP between wild-type and mutant strains. In the mucoid strains, HcP drops sharply. *RsmA, algR* and *algU* mutants show differential expression of Type VI. The *algR* and *algU* mutants show higher protein levels suggesting AlgR and AlgU are negative regulators of Type VI. The puzzling result was the decrease in HcP in the *rsmA* mutant. As RsmA is a negative regulator of Type VI, it would be expected that this mutant would have high levels of HcP. Further studies are needed to confirm these results and Type VI activity in different strains.

VITA

TYLER SPEAKS

Education: M.S. Biology, East Tennessee State University, Johnson City,
Tennessee 2015

B.S. Health Science conc. Microbiology, East Tennessee State
University, Johnson City, Tennessee 2013

Professional Experience: Graduate Research Assistant, East Tennessee State University,
Dept. Health Science, Johnson City, Tennessee, 2013-2015

Teaching Assistant, East Tennessee State University, Dept. of
Health Science, Johnson City, Tennessee, 2013-2015

Patient Transporter, Johnson City Medical Center, Johnson City,
Tennessee, 2012-2014

United States Army, Sergeant, 2008-2014

Honors and Awards: Quillen Scholarship, East Tennessee State University

Graduate Studies Research Grant

Dept. Health Science Summer Research Grant Award

Army Commendation Medal, Combat Action Badge, Afghanistan
Campaign Medal