12-2018

Virulence Regulation in Pseudomonas aeruginosa via the Alginate Regulators, AlgU and AlgR, the posttranscriptional regulator, RsmA, and the Two-component System, AlgZ/R

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Virulence Regulation in *Pseudomonas aeruginosa* via the Alginate Regulators, AlgU and AlgR, the posttranscriptional regulator, RsmA, and the Two-component System, AlgZ/R

A dissertation

presented to

the faculty of the Program of Biomedical Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

Biochemistry and Molecular Biology Concentration

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by

Sean Denver Stacey

December 2018

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Keywords: *Pseudomonas*, mucoid, post-transcriptional regulation, RsmA, two component system, type IV pili

ABSTRACT

Virulence Regulation in *Pseudomonas aeruginosa* via the Regulators, AlgU and AlgR, Posttranscriptional Regulator, RsmA, & Two-component System, AlgZ/R

by

Sean Denver Stacey

Pseudomonas aeruginosa is a Gram-negative bacillus able to colonize a wide variety of environments. In the human host, *P. aeruginosa* can establish an acute infection or persist and create a chronic infection. *P. aeruginosa* is able to establish a niche and persist in human hosts by using a wide array of virulence factors used for: movement, killing host cells, and evading immune cells and antibiotics. Understanding virulence factors and their regulation has proved to be an important means of combating the morbidity and mortality of *P. aeruginosa* as well as the ever-increasing threat of drug resistance. By targeting virulence factors or their regulators with antivirulence compounds, the bacterium is rendered defenseless and more readily cleared by the immune system. In this study, we examine three different contributors to virulence factor regulation. First, we examined the role of the extracellular sigma factor AlgU and its contribution to regulating a post-transcriptional RsmA. AlgU is most commonly active in chronic infecting strains that produce copious amounts of the virulence factor, alginate. We confirmed that not only was their more RsmA in this background, but that there was a previously unidentified promoter for *rsmA* regulated by AlgU. In concert with this study, we followed up by studying the effects of AlgR on this unknown promoter. AlgU and AlgR are known to work together, specifically on the alginate operon, and we hypothesized based off of bioinformatics data this was the case with RsmA. Second, due to increased RsmA in this chronic infection

strain, we set out to identify potential unknown virulence targets of RsmA. A previously unrevealed target, *pasP*, was shown to directly interact with RsmA. Third, in an acute infection model strain we identified a new regulatory loop involving the two-component system AlgZ/R. In a *pilW* strain deficient in the motility virulence factor type IV pili, we showed increased levels of AlgZ/R compared to wildtype, PAO1. The *pilW* strain produced less pyocyanin, rhamnolipid, and elastase and was attenuated in J774a.1 macrophages. Overall, these studies push the understanding of virulence factor regulation and open the door to potential therapeutic targets in treating *P. aeruginosa* infections.

DEDICATION

This manuscript is dedicated to the following,

My mother and father who have supported me throughout my academic career and have always encouraged me to achieve success with integrity and good intentions.

My friends who have supported me through tough times of this Ph.D. journey, who have told me to never give up, and have been a touchstone in my time of need.

To my fairy godmothers, Judy, Marianne, and Rhesa, thank you for helping me hone my scientific techniques and helping me grow to be a well-rounded scientist.

The ETSU Counseling Center can be accessed via https://www.etsu.edu/students/counseling/ or by calling (423) 439-3333 the Mental Health Help Line: (423) 439-4841. Obtaining a Ph.D. is not a cakewalk and it's certainly OK to ask for help from a counselor when under stress.

ACKNOWLEDGEMENTS

I would like to thank Dr. Chris Pritchett, my committee members: Dr. Lampson, Dr. Hurley, Dr. Rusiñol, and Dr. Agrawal for supporting me and helping me along the way.

Dr. Ranjan Chakraborty for stepping in as chair of my committee last minute. For helping me so much throughout my time as both a master's and Ph. D. student.

Dr. Hurley for helping immensely with editing my dissertation and guidance throughout my time as a Ph. D. student.

Dani Williams for helping with work, papers, and making too much media when the steam on campus went down.

The Department of Health Sciences for financial support for experiments travel to meetings, and encouraging me to succeed.

Emily Redd for helping with editing.

Dean Cecilia McIntosh for guidance and help.

Funding from the Research and Development Committee, ETSU (RDC12-012M and RDC13- 005M).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa

P. aeruginosa is most commonly known as an opportunistic pathogen. The Gram negative, bacillus *P. aeruginosa* can also colonize any tissue in the human host (Figure 1). In nature, *P. aeruginosa* can be found in a variety of environments from soil to water.

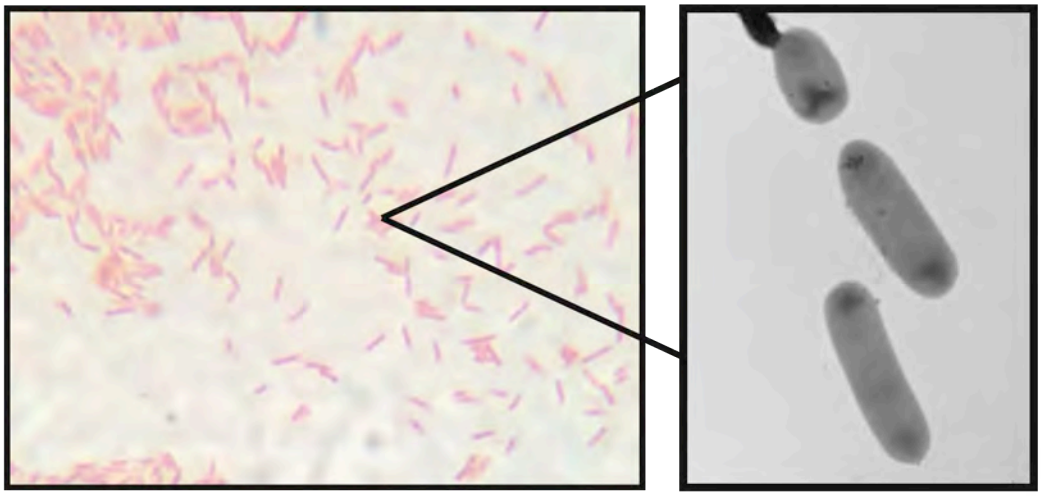

Figure 1.1 Gram Stain (left) and Transmission micrograph (right) of *Pseudomonas aeruginosa*

Besides humans, *P. aeruginosa* is a known pathogen to plants, insects, and other animals. As an opportunistic pathogen *P. aeruginosa* preys on hosts with compromised immune systems, which includes individuals with HIV/AIDS, on chemotherapeutic regimens, burns on a majority of their body, type II diabetes, or cystic fibrosis. *P. aeruginosa* causes infections in human hosts with optimal immune systems too. The bacterium is known to cause ear infections in swimmers as well as "hot tub rash," which is widespread folliculitis, due to a hot tub or pool not being properly treated. *Pseudomonas aeruginosa* has a long history as a hospital acquired infectious

agent and continues today to be a leader in morbidity and mortality from nosocomial infections. At the end of the 19th century, *Pseudomonas aeruginosa* née *Bacillus pyocyaneus* was isolated from the surgical wounds of two patients at a French hospital (Villavicencio 1998). The bacterium was characterized based off the blue appearance from the purulent discharge of the patient's wound and the rod-like shape under microscopic observation. In modern day hospitals ventilator associated pneumonia (VAP) due to *P. aeruginosa* has a 30% mortality rate (Gellatly and Hancock 2013). *P. aeruginosa* is also the leading cause of morbidity and mortality in patients with cystic fibrosis (CF) (Salunkhe et al. 2005). *P. aeruginosa* produces a resilient biofilm that aids in colonizing the endotracheal tube of ventilated patients or the CF lung. This biofilm is also important in establishing a niche in catheter associated urinary tract infections (CAUTI). As technology has advanced in treating microbial infections, so has the ability for *P. aeruginosa* to evade antibiotics and continue to infect hospital patients.

Pseudomonas Pathogenesis

The versatility of *P. aeruginosa* to thrive in a variety of environments and infect so many different hosts can be found in its ability to switch between planktonic and sessile states. As the bacterium scavenges for nutrients or a new niche *P. aeruginosa* will exhibit a number of structures used for locomotion, attachment, and obtaining foodstuffs (Moradali et al. 2017; Valentini et al. 2018). This *Pseudomonad* state is also believed to represent the phenotype of establishing an acute infection (Figure 1.2) (Luo et al. 2015; Valentini et al. 2018). The bacterium will use its flagellum and type IV pili (TFP) to adhere to a surface or host cell and proceed to lay down on the surface (Luo et al. 2015). From here, the bacterium will cease to express the structures most recognized by the host

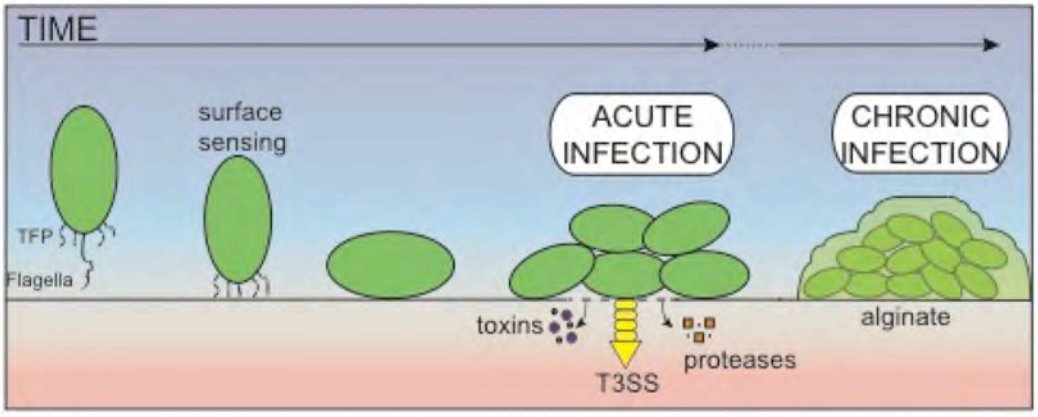

Figure 1.2. Generalization of *Pseudomonas aeruginosa* attachment, initiating and establishing an acute infection, and development of a chronic infection.

immune response and begin to reproduce and express various other weapons or virulence factors for uptake of nutrients (Gellatly and Hancock 2013; Valentini et al. 2018). Over time the bacteria will transition from the planktonic to sessile phenotype. The sessile state of the bacteria will produce compounds that will deter the inflammation response and immune cells to target *P. aeruginosa* (Gellatly and Hancock 2013; Valentini et al. 2018). As the chronic infection develops, the bacteria will express even fewer recognized targets of the immune system to curb the inflammation response (Gellatly and Hancock 2013; Valentini et al. 2018). In some cases, chronic *P. aeruginosa* infections have lasted for over 20 years (Gellatly and Hancock 2013). A number of researchers have mapped out some hierarchal pathways for both acute and chronic virulence factors, but there are still holes and question marks to be completed. By understanding the role virulence factors help in the two phenotypic states of *P. aeruginosa*, better ways to prevent acute infections or ameliorate chronic infections can be discovered.

Virulence Factors

P. aeruginosa has one of the most well stocked caches of virulence factors of any bacterium. *P. aeruginosa* can thrive in most environments and colonize any host tissue due to its wide array of weaponry. *P. aeruginosa* asserts dominance over other species of bacteria by

injecting toxins into competitors via the type VI secretion system, sequestering iron with siderophores, and secreting a force field of exopolysaccharide that protects from phagocytosis and antibiotics (Gellatly and Hancock 2013; Park et al. 2014; Valentini et al. 2018). *P. aeruginosa* has also developed a series of ways around antibiotics besides the exopolysaccharide alginate like: drug efflux pumps, modified surface receptors and beta-lactamases (Moradali et al. 2017). These enhancements have been either acquiring foreign plasmid DNA or mutations that enhance resistance (Moradali et al. 2017). However, understanding the role *P. aeruginosa* uses its virulence factors can lead to new ways to target and disarm this pathogen.

Secretion Systems

In Gram negative organisms there is no ATP energy available inside of the periplasmic space, so transport of virulence factors out of the cell the bacteria will use a variety of secretion systems (Costa et al. 2015; Green and Mecsas 2016). *P. aeruginosa* has multiple secretion systems, but lacks a type IV secretion system, which is commonly used by bacteria to inject DNA or DNA-protein complexes into host cells (Costa et al. 2015). *P. aeruginosa* uses multiple type I, type II, and type V secretion systems to secrete proteases, adhesins, and other enzymatic proteins (Filloux 2011). However, the most well studied secretion systems in *P. aeruginosa* are the type III and type VI.

The type III secretion system (T3SS) has been shown to have homology to the bacteria flagellar apparatus, but serves a completely different purpose in pathogenesis (Green and Mecsas 2016). Once *P. aeruginosa* contacts the host cell surface the T3SS is used to compromise the cell membrane. The needle-like apparatus pierces the membrane and will inject a series of toxins, some of which are extremely lethal (Filloux 2011; Gellatly and Hancock 2013). Multiple studies have shown the importance of the T3SS's importance in virulence of *P. aeruginosa* and

establishing an acute infection (Anderson et al. 2010; A.K. Jones et al. 2010; Filloux 2011; Schulmeyer and Yahr 2017).

The type VI secretion system (T6SS) is another needle-like appendage *P. aeruginosa* uses for injecting toxins. The T6SS is homologous to the T4-bacteriophage tail spike and used in a similar fashion (Green and Mecsas 2016). Uniquely, *P. aeruginosa* has three T6SS that have not been completely characterized fully, although it is believed one is for harming host cells (T6SS-2) while the first identified T6SS-1 is used to inject toxins into nearby bacteria (Filloux 2011 Jul 16; Costa et al. 2015; Green and Mecsas 2016). *P. aeruginosa* has one of the most lethal identified T6SS in *Eubacteria*, even dominating *Vibrio cholera* (Basler et al. 2013). The genetic loci for the T6SS also include antitoxins so that sister cells that may be fired upon do not lyse from injection of toxins (Filloux 2011). The T6SS-1 is also characterized as a chronic virulence factor (Valentini et al. 2018). *P. aeruginosa* uses secretion systems like the T6SS and T3SS to dominate its niche amongst other microbiota, pathogens, or host cells.

Secreted Virulence Factors

Besides using secretion systems, *P. aeruginosa* can use various membrane transporters or even outer membrane vesicles to release virulence factors into the extracellular milieu. Exopolysaccharides are used for a variety of functions to help *P. aeruginosa* persist in its environment. *P. aeruginosa* secretes three types of exopolysaccharide that contribute to biofilm formation: alginate, pel, and psl. Alginate is a combination of beta-D-mannuornic and alpha-L-guluronic acids that has a overall negative charge and the consistency of mucus(Moradali et al. 2017). Alginate not only shields the bacteria from antibiotics, but also causes frustrated phagocytosis because the pseudopods of the immune cell cannot wrap around the biofilm protected colony of cells (Lee et al. 2011; Moradali et al. 2017). Most commonly, *P. aeruginosa*

isolates from CF sputum undergo phenotypic shifts where the strains will produce copious amounts of alginate.

While alginate production is clearly seen on a plate, *P. aeruginosa* PAO1 still produces biofilms from the exopolysaccharides pel and psl (Figure 1.3). Pel has been

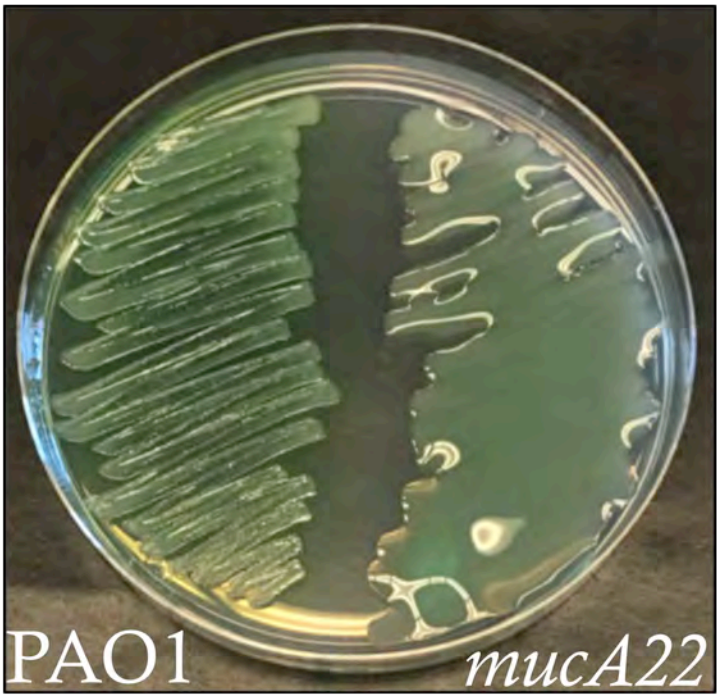

Figure 1.3 Nonmucoid (PAO1) and mucoid (*mucA22)* strains of *Pseudomonas aeruginosa* on *Pseudomonas* isolation agar.

characterized to have an overall positive charge has consists of linked N-acetylgalactosamine and N-acetylglucosamine (Moradali et al. 2017). Psl contains rhamnose, glucose, and mannose with an overall neutral charge (Moradali et al. 2017). Psl has also been shown to be regulated by the transcriptional regulator AmrZ, as well as the sigma factor RpoS and post-transcriptionally by RsmA (Irie et al. 2010; C.J. Jones et al. 2013). RpoS is considered the stress response sigma factor (Potvin et al. 2008). If *P. aeruginosa* is under attack by the innate immune response, biofilm production is a means of resisting the siege.

While most proteases are large and require secretion systems, there are other secreted virulence factors besides biofilm components that are secreted by diffusion or efflux pumps. Pyoveridine is a compound secreted by the efflux pump PvdRT/OmpQ system (Moradali et al. 2017). Pyoveridine acts as an iron chelator in order for *P. aeruginosa* to acquire exogenous iron from a host (Moradali et al. 2017; Little et al. 2018). Pyoveridine also reacts under ultraviolet light and can be used as a biomarker for *Pseudomonas* infections (Yin et al. 2014; Norbury et al. 2016). Hydrogen cyanide is a virulence factor produced by *P. aeruginosa* that is able to diffuse across its membrane and the membrane of host cells (Moradali et al. 2017). Hydrogen cyanide is a secreted weapon that compromises cellular respiration in host cells, limiting the ability for immune cells to attack (Gellatly and Hancock 2013; Moradali et al. 2017). RsmA has been shown to post-transcriptionally regulate hydrogen cyanide production in nonmucoid/acute infection strains, but in mucoid/chronic infection strains (Burrowes et al. 2006; Brencic and Lory 2009; Stacey et al. 2017). Secreted virulence factors are important for *P. aeruginosa* to establish and persist in a host or natural environment.

Motility Virulence

The most important virulence factors are those involved in motility. *P. aeruginosa* has a single polar flagellum, type IV pili, and produces surfactants called rhamnolipids for the ability to move across surfaces and swim in aqueous environments (Okkotsu et al. 2013; Kazmierczak et al. 2015; Moradali et al. 2017). The flagellum has been shown not only important in motility, but also has the ability to aid in surface attachment (Kazmierczak et al. 2015). The *Pseudomonas* flagellum is considered an acute virulence factor because it is solely expressed when the bacterium is searching to establish a niche in the environment (Kazmierczak et al. 2015; Valentini et al. 2018).

Touch is crucial for *P. aeruginosa* to take advantage of its environment and that is exactly what the type IV pili is used. Type IV pili are localized like the flagellum at the poles of the cell (Gellatly and Hancock 2013). The apparatus can be extended or retracted out of the cell and has an adhesin at the tip called PilY1 (Giltner et al. 2010; Kazmierczak et al. 2015). The stalk is comprised of a major pilin unit, PilA, and several minor pilin subunits, FimUPilVWXE (Giltner et al. 2010). Minor pilin mutants have been shown to have decreased virulence but the implications as to why have not been elucidated (Siryaporn et al. 2014; Luo et al. 2015; Marko et al. 2017). It is hypothesized that the type IV pili act not only as a mechanosensory system, but also relay expression of the sessile-phenotype upon touching a surface (Siryaporn et al. 2014; Kazmierczak et al. 2015; Luo et al. 2015; Marko et al. 2017). Marko et al. allude that there is influence of minor pilins on AlgZ, but to what effects have not been clarified (Marko et al. 2017).

Virulence Regulation

P. aeruginosa virulence is more complicated than just identifying virulence genes. Understanding the regulation of virulence factors proves to be just as important. Virulence factor regulation can in some cases consist of global networks activating , repressing or co-regulating targets. Virulence mechanisms can consist of cascades that go from a chemical signal sensed from outside the bacterium to the *P. aeruginosa* reacting, producing, and secreting a toxin. Luo et al. has shown that upon contact with a surface, the type IV pili will activate the diguanylate cyclase SadC to start producing cyclic di-GMP and in turn produce a biofilm (Luo et al. 2015). Knowledge of virulence regulation has potential for chemotherapeutic treatments to exploit *P. aeruginosa* and turn virulence weaponry on itself.

Sigma Factor

A sigma factor is the variable portion of the RNA polymerase holoenzyme to initiate transcription of a gene (Potvin et al. 2008). In *P. aeruginosa* there are a variety of sigma factors used for the expression of virulence factors, while most housekeeping genes and core metabolic processes are regulated by the main sigma 70, RpoD (Potvin et al. 2008). There are specific sigma factors in *P. aeruginosa* for expressing flagella, FliA, and nitrogen metabolism, RpoN (Potvin et al. 2008). Another type of alternative sigma factor is the extracytoplasmic function (ECF) sigma factors. These sigma factors will respond to extracellular signals or stresses and lead to the expression of protective targets (Potvin et al. 2008).

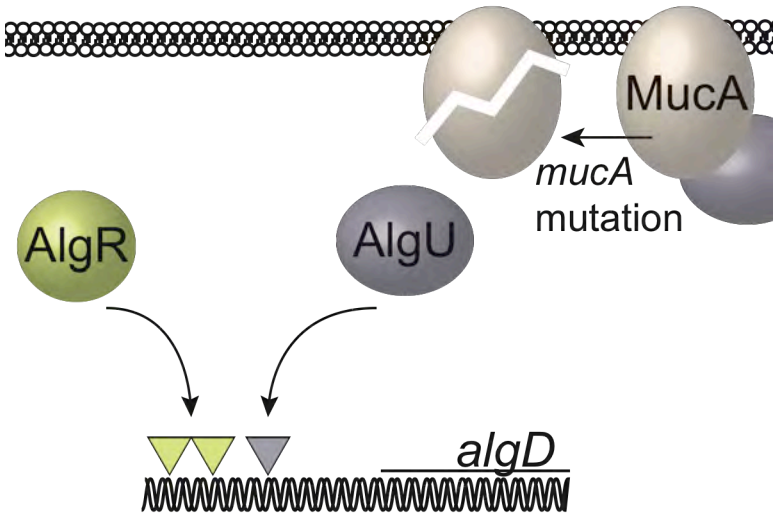

Figure 1.4 The alginate regulators, AlgU and AlgR, both converge on the *algD* promoter to activate alginate production in mucoid strains of *Pseudomonas aeruginosa* like the *mucA22* strain.

The most important ECF sigma factors involved with *P. aeruginosa* infections in the cystic fibrosis lung is AlgU. AlgU is normally sequestered by the anti-sigma factor, MucA, to the inner membrane (Potvin et al. 2008). If any oxidative damage causes stress to the cell membranes or denatures MucA, then AlgU is released to activate its regulon. A key component

of *P. aeruginosa* virulence and a chronic infection in the CF lung is the production of alginate (Hershberger et al. 1995; Gellatly and Hancock 2013). Clinical isolates from CF sputum very often contain mutations in *mucA* (Potvin et al. 2008; Gellatly and Hancock 2013). The *algD* operon is regulated by AlgU (Figure 1.4) (Hershberger et al. 1995). AlgU has been shown to also regulate AmrZ, which inhibits flagellum biosynthesis and conceals the bacterium from immune cells (Tart et al. 2006). Recently AlgU has been shown to regulate *rsmA*, in doing so, AlgU regulates hundreds of other targets via this post-transcriptional regulator (Stacey and Pritchett 2016).

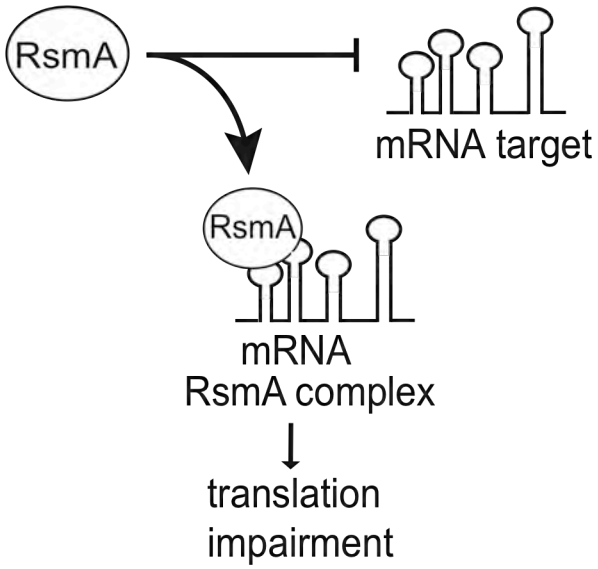

Figure 1.5 The post-transcriptional regulator RsmA inhibits translation in *Pseudomonas aeruginosa* by binding and blocking the ribosomal binding site of target messenger RNA.

Post-Transcriptional Regulation

Bacteria use post-transcriptional regulation as a means to control translation of mRNA. Depending on the strain of *P. aeruginosa* there is up to three post-transcriptional regulators of secondary metabolites, RsmA/F/E (Lapouge, Sineva, et al. 2007; Schulmeyer et al. 2016). These

regulators function by binding the ribosomal binding site (RBS) of the mRNA target (Figure 1.5). This site presents a binding motif usually consisting of AGGA on a stem-loop structure (Brencic and Lory 2009; Kulkarni et al. 2014). In *P. aeruginosa* the action of RsmA binding the mRNA represses the translation of the target, which is believed to be RsmA's sole function. However, the homolog of RsmA, CsrA, has been shown to interact with other proteins and positively influence mRNA targets(Wei et al. 2001; Mukherjee et al. 2011).

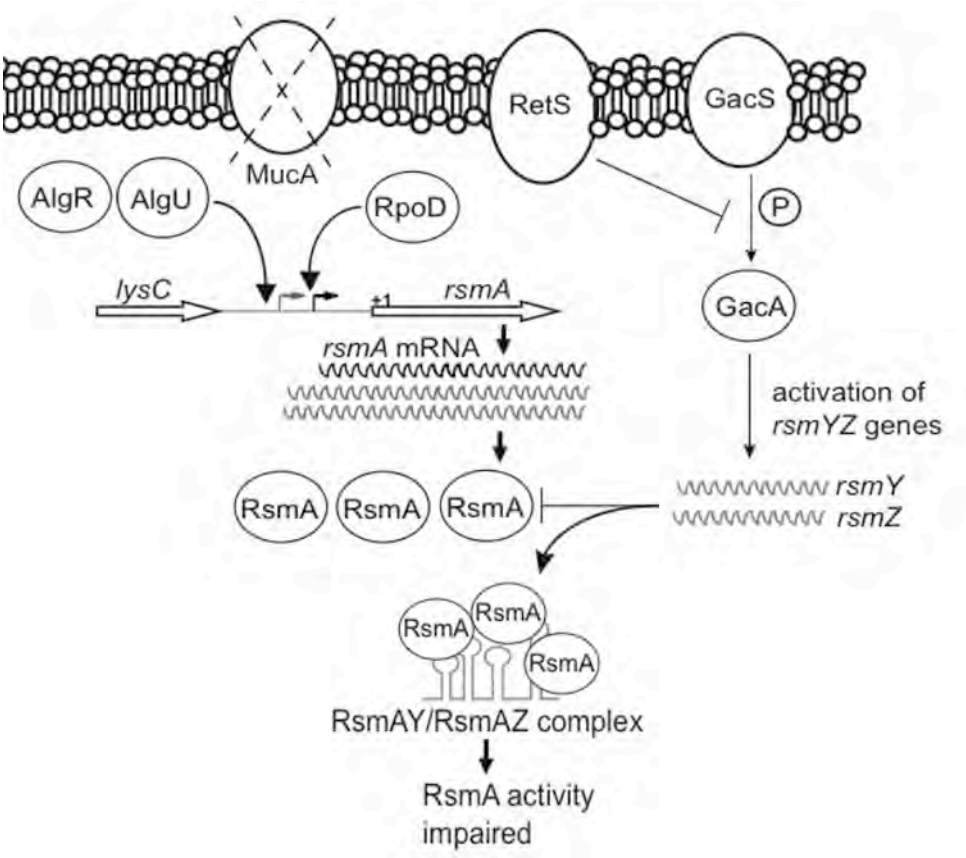

Figure 1.6 The Rsm/Gac hierarchy in *Pseudomonas aeruginosa.* The two-component system GacS/A activate the RsmA antagonists RsmY/Z. The GacS/A system is inhibited by the hybrid sensor kinase, RetS. RsmA is activated by two individual promoters. In strains with *mucA* mutations, the extracytoplasmic sigma factor, AlgU, increases expression of RsmA at its distal promoter.

RsmA is part of a larger network of virulence regulation called the Gac/Rsm system (Figure 1.6). In *P. aeruginosa* RsmA has two small noncoding RNA (ncRNA) antagonists,

RsmY and RsmZ (Janssen et al. 2018). These ncRNAs are regulated by the response regulator, GacA, which is part of the GacS/A two-component system (TCS) (Lapouge, Schubert, et al. 2007). GacS responds to a still unknown signal and phosphorylates GacA that in turn activates expression of the ncRNAs (Lapouge, Sineva, et al. 2007). An inhibitor of GacS autophosphorylation is the hybrid sensor kinase RetS. RetS, the regulator of exopolysaccharide and T3SS, will heterodimerize with GacS prohibiting the phosphorylation event (Vakulskas et al. 2015). Contrary to RetS is the lost adherence sensor, LadS, which will assist GacS in activating the ncRNAs (Vakulskas et al. 2015).

The Gac/Rsm components are all crucial for virulence as both mutations in *rsmA* or *gacA* lead to attenuation(Lapouge, Schubert, et al. 2007; Gellatly and Hancock 2013; Vakulskas et al. 2015; Valentini et al. 2018). The Gac/Rsm system has also been shown to play an important role in shift from planktonic to sessile states or acute infection to chronic (Valentini et al. 2018). Removing any portion of the Gac arm of the network will produce an unregulated RsmA strain that expresses T3SS, flagellum, and type IV pili, while removing *rsmA* or *retS* will cause increase in biofilm and T6SS expression (Valentini et al. 2018). As a result of either mutation the *P. aeruginosa* strain will be readily cleared by the host immune response.

Two Component Systems

P. aeruginosa has over 60 two component systems in its genome (Francis et al. 2017). A TCS consists of a sensor histidine kinase and a response regulator (Figure 1.7). These components are highly conserved and are usually arranged in an operon together (Galperin et al. 2016; Francis et al. 2017). The sensor will received an extracellular signal and autophosphorylate itself, this phosphate will then be transferred from the histidine residue to the

aspartate residue of the response regulator (Podgornaia and Laub 2013). Once phosphorylated, the response regulator will then activate the expression of its target genes.

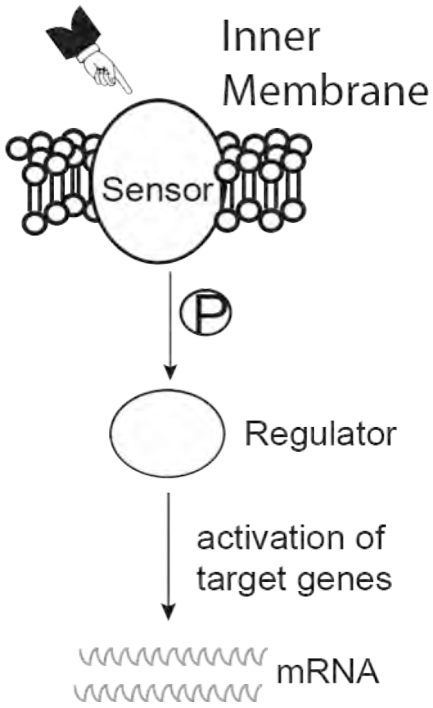

Figure 1.7 A classical interpretation of a two-component system in *Pseudomonas aeruginosa.* The sensor histidine kinase receives an external signal, which causes an autophosphorylation event from free ATP. This phosphate group is then transferred to the sensor's cognate response regulator at a conserved aspartate residue. The activated regulator causes the expression of its regulon.

The AlgZ/R two-component system has been shown to target multiple virulence genes in *P. aeruginosa*. The *algZ/R* loci are located in an operon and shown to be regulated by the virulence factor regulator, Vfr (Luo et al. 2015) (Figure 1.8). Upon phosphorylation, AlgR will activate expression of the minor pilin operon (Belete et al. 2008; Okkotsu et al. 2013). However, conflicting studies show AlgR influencing rhamnolipid production and the Rhl quorum sensing system, which also controls pyocyanin and elastase (Morici et al. 2007; Okkotsu et al. 2013; Little et al. 2018). AlgR when unphosphorylated can co-regulate with the ECF AlgU as seen in Figure 1.4 and 1.6 on the *algD* operon and *rsmA* (Salunkhe et al. 2005; Stacey et al. 2017). Most

recently Luo et al. and Marko et al. have shown that the AlgZ/R TCS plays a larger role in *P. aeruginosa* pathogenesis. Luo et al. demonstrated that the type IV pili regulated AlgZ/R has a feedback loop where by removing a minor pilin that expression of the minor pilin operon increased significantly (Luo et al. 2015). Marko et al. also demonstrated via bacterial adenylate

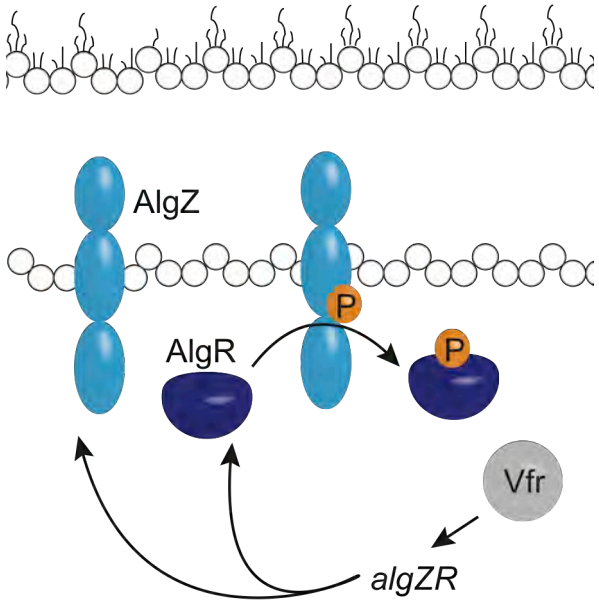

Figure 1.8 The AlgZ/R two-component system in *Pseudomonas aeruginosa.* Vfr regulated the *algZ/R* operon in *P. aeruginosa*. The signal that activates the phosphorylation cascade of AlgZ is still unknown. AlgZ phosphorylated AlgR at aspartate residue 54. AlgR can control the expression of targets dependent and independent of phosphorylation.

cyclase two hybrid system that AlgZ interacted with a portion of the minor pilins (Marko et al. 2017). The role of AlgZ/R is more complex than just regulating the minor pilin operon and may have greater implication in the mechanosensory network of the type IV pili.

Antivirulence Therapeutics

Treating *P. aeruginosa* infections with conventional antibiotics has become increasingly difficult due to the bacterium's ability to adapt and evolve like many other drug resistant

bacteria. A new means of eradicating *P. aeruginosa* infections is by targeting virulence factors with antivirulence chemotherapeutics. Multiple researchers are investigating ways to inhibit aspects of *P. aeruginosa's* ability to cause an infection by impairing different virulence factors. Novel targets include blocking adhesins from attaching to host cells, biofilm dispersal, and impairing the quorum sensing system, which all would lead to host clearance of *P. aeruginosa* (Fothergill et al. 2014). Understanding virulence factor regulation is crucial for developing antivirulence compounds. The regulation of the post-transcriptional regulator *rsmA* is characterized. Upon discovery of the alginate regulators AlgU and AlgR contributing to RsmA levels, the RsmA regulon is characterized in a mucoid strain, *mucA22.* Lastly, the role of AlgR on virulence in a minor pilin mutant, *pilW*, is characterized. Overall, through the understanding of how these regulators contribute to virulence, there is potential to utilize and target these virulence factors as an vulnerability to help clear *P. aeruginosa* acute and chronic infections.

Specific Aims

Aim1:

Analyze and characterize a potential second promoter upstream of the *rsmA* allele in *Pseudomonas aeruginosa*.

Aim 2:

Analyze and characterize the RsmA regulon in a *mucA22* strain, since there is more RsmA made in a *mucA22* strain of *Pseudomonas aeruginosa* due to the distal promoter.

Aim 3:

Analyze the expression and protein levels of the *algZ/R* operon in a *pilW* mutant and potential effects on virulence in *Pseudomonas aeruginosa*.

CHAPTER 2

PSEUDOMONAS AERUGINOSA ALGU CONTRIBUTES TO POSTTRANSCRIPTIONAL ACTIVITY BY INCREASING *RSMA* EXPRESSION IN A *MUCA22* STRAIN

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ABSTRACT

Pseudomonas aeruginosa thrives in multiple environments and is capable of causing life-threatening infections in immunocompromised patients. RsmA is a posttranscriptional regulator that controls virulence factor production and biofilm formation. In this study, we investigated the expression and activity of *rsmA* and the protein that it encodes, RsmA, in *P. aeruginosa mucA* mutant strains, which are common in chronic infections. We determined that AlgU regulates a previously unknown *rsmA* promoter in *P. aeruginosa*. Western blot analysis confirmed that AlgU controls *rsmA* expression in both a laboratory strain and a clinical isolate. RNase protection assays confirmed the presence of two *rsmA* transcripts and suggest that RpoS and AlgU regulate *rsmA* expression. Due to the increased amounts of RsmA in *mucA* mutant strains, a translational leader fusion of the RsmA target, *tssA1*, was constructed and tested in *mucA*, *algU*, *retS*, *gacA*, and *rsmA* mutant backgrounds to examine posttranscriptional activity. From these studies, we determined that RsmA is active in *mucA22* mutants, suggesting a role for RsmA in *mucA* mutant strains. Taken together, we have demonstrated that AlgU controls *rsmA* transcription and is responsible for RsmA activity in *mucA* mutant strains. We propose that RsmA is active in *P. aeruginosa mucA* mutant strains and that RsmA also plays a role in chronic infections.

IMPORTANCE

P. aeruginosa causes severe infections in immunocompromised patients. The posttranscriptional regulator RsmA is known to control virulence and biofilm formation. We identify a new *rsmA* promoter and determine that AlgU is important in the control of *rsmA* expression. Mutant *mucA* strains that are considered mucoid were used to confirm increased *rsmA* expression from the AlgU promoter. We demonstrate, for the first time, that there is RsmA activity in mucoid *P. aeruginosa* strains. Our work suggests that RsmA may play a role during chronic infections as well as acute infections.

INTRODUCTION

Pseudomonas aeruginosa produces a myriad of virulence factors and can cause both acute and chronic infections (1, 2). To survive and persist, *P. aeruginosa* must coordinate gene expression in response to changing environmental conditions. Global regulatory networks respond to changing environmental conditions to allow the physiological changes necessary for survival to be made. The *P. aeruginosa* genome encodes many global regulatory systems, including posttranscriptional regulators, such as RsmA. RsmA is important in regulating several virulence factors involved in acute and chronic infections (3).

Acute infections are characterized by motility and expression of the type III secretion system (4). RsmA positively regulates both motility and type III secretion system expression (4–6). A defining characteristic of chronic infections is biofilm formation, which includes the production of exopolysaccharides, such as alginate, and the type VI secretion system (T6SS) (7, 8). RsmA is a negative regulator of the exopolysaccharide gene *psl* and the first hemolysin-coregulated secretion island type VI secretion system (HSI-I T6SS) (5, 8, 9). A characteristic of some *P. aeruginosa* isolates causing chronic infections, such as those found in cystic fibrosis patients, is alginate overproduction, which is the result of the extracytoplasmic sigma factor AlgU (7, 10).

AlgU is normally sequestered at the cytoplasmic membrane by the anti-sigma factor MucA (7, 10). MucA prevents AlgU from binding and activating target promoters (7, 10). Once liberated from MucA, AlgU activates a variety of genes, including those important for biosynthesizing alginate (7, 10–13). Strains overproducing alginate are termed mucoid. *P. aeruginosa* strains that initially colonize cystic fibrosis patients frequently evolve into strains that overproduce alginate, often because of mutations in the *mucA* gene (7, 10). A previous study

found that a *mucA* mutant has increased *rsmA* expression (14). Because *mucA* mutations lead to hyperactive AlgU (7, 10), we hypothesized that AlgU might regulate *rsmA* transcription.

RsmA is a global posttranscriptional regulator that regulates several mRNAs involved in virulence (5, 6). RsmA positively regulates type III secretion and type IV pilus production, both of which are important virulence factors for acute infections (6, 14). In the case of biofilm formation, RsmA inhibits exopolysaccharide production (5, 9). Therefore, RsmA is important in determining what virulence factors are expressed by *P. aeruginosa*. RsmA activity is regulated by two small RNAs, RsmY and RsmZ, that bind RsmA and prevent RsmA from binding target mRNAs (15). RsmY and RsmZ sequester RsmA, allowing the production of exopolysaccharides and other RsmA target mRNAs (9, 16). Increased RsmY and RsmZ levels or decreased RsmA activity can allow bio- film formation to occur. The expression of *rsmY* and *rsmZ* is positively controlled by RsmA, GacA, and LadS and negatively con- trolled by RetS (16–18). RsmA binds to highly conserved GGA motifs. Usually, one of the GGA motifs is found in a stem-loop structure formed near the ribosome-binding site (19). Multiple copies of the same GGA motif are also found in the inhibitory small RNAs RsmY and RsmZ (20, 21).

Although RsmA is important for virulence and global gene expression (5, 22, 23), little is known regarding the genetic controls governing *rsmA* expression. Currently, only a single *rsmA* promoter region, which resembles a s^{70} (RpoD) promoter, has been identified in *P. aeruginosa* (24–26). A recent chromatin immunoprecipitation sequencing study showed that both RpoD and RpoS likely control *rsmA* expression (27). However, another study found similar RsmA levels in both an *rpoS* mutant and the wild- type strain (9). Early studies found that optimal *rsmA* expression requires GacA and that *rsmA* expression increases throughout growth (28), findings

which support a role for RpoS in controlling *rsmA* expression. These seemingly contradictory data have not yet been clarified.

Given the importance of RsmA in controlling *P. aeruginosa* virulence and biofilm formation, there is a critical need to under- stand *rsmA* expression. In this study, we determined the genetic elements controlling *rsmA* expression. We identified a new promoter region and determined that AlgU and RpoS contribute to *rsmA* expression. Importantly, we demonstrate that RsmA is active in *P. aeruginosa mucA* mutants. This work suggests that RsmA has a role in regulating gene expression in *mucA* mutants and that AlgU is an important activator of *rsmA* expression.

METHODS

Strains, plasmids, and media

The strains used in this study are presented in Table 2.1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics, as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB medium, or Vogel-Bonner minimal medium (VBMM) supplemented with the appropriate concentrations of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 mg/ml tetracycline, 15 mg/ml gentamicin, 100 mg/ml ampicillin, and 35 mg/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 mg/ml gentamicin, 50 mg/ml tetracycline, and 300 mg/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Genotype or Relevant properties	Reference or Source
E. coli strains		
NEB5α	*fhuA2 Δ(argF*-lacZ*) U169phoA, glnV44 Φ80Δ(*lacZ*)*M*15 gyrA96*	New England Biolabs
SM10	*Thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^R	(59)
pRK2013	Helper strain	(31)
pEX18Tc	Allelic exchange vector	(30)
pEX18Gm	Allelic exchange vector	(30)
Δ*rsmA* pEX18Tc	Allelic exchange for *rsmA* non-polar deletion	This study
Δ*algU* pEX18Tc	Allelic exchange for *algU* non-polar deletion	(60)
Δ*rpoS* pEX18Tc	Allelic exchange for *rpoS* non-polar deletion	This study
*rsmA*HA pEX18Gm	Allelic exchange for *rsmA* HA allele	This study
miniCTXlacZ	Transcriptional fusion vector	(37)
TF1-lacZ miniCTX	Transcriptional fusion	This study
TF3-lacZ miniCTX	Transcriptional fusion	This study
TF3USDM-lacZ miniCTX	Site-directed mutagenesis transcriptional fusion	This study
TF2-lacZ	Transcriptional fusion	This study
pTJ1	Integrating vector used for overexpression	(43)
algU pTJ1 (pAlgU)	*algU* complementation/overexpression	This study
P. aeruginosa strains		
PAO1	Wild type	(61)
Δ*alg*U	*algU* mutant	(60)
mucA22	*mucA22* mutation	(62)
mucA22 Δ*alg*U	*mucA22* strain, Δ*algU*	This study
2192		(45)
383		(45)
Δ*rsmA*	*rsmA* nonpolar deletion	This study
*mucA22*Δ*rsmA*	*rsmA* nonpolar deletion in *mucA22* strain	This study
PAO1 TF1-l*acZ*	*rsmA* transcriptional fusion strain	This study
Δ*algU* TF1-lacZ	*rsmA* transcriptional fusion strain	This study
mucA22 TF1-lacZ	*rsmA* transcriptional fusion strain	This study
mucA22, Δ*algU* TF1-*lacZ*	*rsmA* transcriptional fusion strain	This study
2192	*rsmA* transcriptional fusion strain	This study

TF1-lacZ		
383 TF1-lacZ	*rsmA* transcriptional fusion strain	This study
383HA	Nonmucoid clinical isolate with *rsmAHA* allele	This study
2192HA	Mucoid clinical isolate with *rsmAHA* allele	This study
2192HAΔ*algU*	2192 with *rsmAHA* allele and *algU* deleted	This study
PAO1 PE3-*lacZ*	*rsmA* transcriptional fusion strain	This study
Δ*algU* PE3-*lacZ*	*rsmA* transcriptional fusion strain	This study
mucA22 TF3-lacZ	*rsmA* transcriptional fusion strain	This study
mucA22, Δ*alg*U TF3-*lacZ*	*rsmA* transcriptional fusion strain	This study
PAO1 TF3USDM-*lacZ*	Mutant *rsmA* transcriptional fusion strain	This study
Δ*algU* TF3USDM-*lacZ*	Mutant *rsmA* transcriptional fusion strain	This study
mucA22 TF3USDM-*lacZ*	Mutant *rsmA* transcriptional fusion strain	This study
mucA22, Δ*alg*U TF3USDM-*lacZ*	Mutant *rsmA* transcriptional fusion strain	This study
PAO1 TF2-*lacZ*	*rsmA* transcriptional fusion	This study
mucA22 TF2-*lacZ*	*rsmA* transcriptional fusion	This study
Δ*algU* TF2-*lacZ*	rsmA transcriptional fusion	This study
mucA22 Δ*alg*U	rsmA transcriptional fusion	This study
PAO1HA	PAO1 with epitope tagged RsmA	This study
Δ*algU*HA	Δ*algU* with epitope tagged RsmA	This study
*mucA22*HA	*mucA22* mutant with epitope tagged RsmA	This study
mucA22 Δ*alg*UHA	*mucA22,* Δ*algU* with epitope tagged RsmA	This study
PAO1HA pTJ1	PAO1 *rsmAHA* allele and vector pTJ1 alone	This study
PAO1HA p*algU*	PAO1 *rsmAHA* allele and *algU* under pBAD promoter	This study

Strain construction

All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs [NEB], Ipswich, MA) and primers listed in Table 2.2. Crossover PCR (splicing by overhang extension PCR) (29) was used to construct deletion mutations and to clone sequences into the suicide vector pEX18Tc or pEX18Gm (30). All cloned

constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (31). Conjugations were performed overnight on LB plates at 37°C, and the conjugants were plated on the appropriate selective medium to obtain single-crossover mutants. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT medium–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR in Table 2.2 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of the proteins was accomplished using primers containing the HA tag at the 3= end of the gene, and HA was introduced as described above using the suicide vector pEX18Gm.

Table 2. Primers used in this study.

Primer Name	Sequence	Use
rsmARHindIII	GCGCAAGCTTCACGCGAATATTTCAGGACAAC	TF
rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGG	Mutant
rsmARXbaI	GCGCTCTAGAGCACGGTGATCCTGCAGACC	Mutant
rsmASOEF	GCGTGAGGAGAAAGGAATGCTGCATTAATTTTTATCTAATTTTCCCTTTGC	Mutant
rsmASOER	GCAAAGGGAAAATTAGATAAAAATTAATGCAGCATTCCTTTCTCCTCACGC	Mutant
algUKOF	GCGCGAGCTCGACAGCAACTCGATGTTCGGTC	Mutant
algUKOR	GCGCTCTAGAGTCGTACCAGGAAGCCAGCTG	Mutant
algUTJ1F2	GCGCCCATGGGAATGCTAACCCAGGAACAGGATCA	Complementation
algU HindIIIR	GCGCAAGCTTTCAGGCTTCTCGCAACAAAGGCTGCA	Complementation
algRTJ1F2	GCGCCCATGGGAATGAATGTCCTGATTGTCGATGAC	Overexpression
rpoS EcoRIF	GCGCGAATTCGATGGCACTCAAAAAAGAAGGGCC	Mutant

rpoS HindIIIR	GCGCAAGCTTTCACTGGAACAGCGCGTCACT	Mutant
rpoS intF	CGTCGCTGACCGGGAGTTCG	PCR check
rpoS intR	CATTTATCTACTTAGGCTCACACG	PCR check
rsmARXbaI	GCGCTCTAGAGCACGGTGATCCTGCAGACC	Mutant
rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGGG	Mutant
rsmASDMcheckF	GCCAAGGTTTCCATCGTCGG	PCR check
rsmAHAF	TACCCATACGATGTTCCAGATTACGCTTAATTTTTATCTAATTTTCCCTTTGC	HA allele
rsmAHAR	TTAAGCGTAATCTGGAACATCGTATGGGTAATGGTTTGGCTCTTGATCTTTCTC	HA allele
rsmASDMAlgUF	CAGAATTCTTCATTCCGGCGGGACTG	SDM
rsmASDMAlgUR	CCAAACGGTCAAAAACGACAAAAG	SDM
rsmAPE1F	GCGCGGTACCAAGGATCGCGCTCTTGATTTCTGCGGATCCGCCGCCATTTCTT	TF
rsmAPE3F	GCGCGGTACCGCTGACAGGCGAAAGGCG	TF/RPA
rsmAPE3R	GCGCGGATCCTCACCCAGTATTGACCAGTCC	TF
rsmAEcoRIR	GCGCGAATTCACGAGTCAGAATCAGCATTCCTTTC	TF
rsmAPE1	GGCGCGTTGACGCCGATGCGC	Primer extension
rsmAPE3	CACGCGAATATTTCAGGACAACAGTCTG	Primer extension
rsmAPE4	GCGCGATCCTTCACCCAGTATTGACCAGTCC	Primer extension
gacAF	GCGCGAGCTCGAGGCGACCTTGCGTCATTC	Mutant
gacAR	GCGCTCTAGACGGCGATCGCCGAAACCAG	Mutant
gacASOEF-2	GCGACGAGGTGCAGCGTGATTGTGAATTCCGCCAGCTAGATGAGCG	Mutant
gacASOER-2	CGCTCATCTAGCTGGCGGAATTCACAATCACGCTGCACCTCGTCGC	Mutant
gacAintF	CCAGGGTGCTTGCGCTTTAC	PCR check
gacAintR	CCACGTAGAGAAGCTTGGCC	PCR check
retSFSacI	GCGCGAGCTCGAACGTCGCCATCGACGTGC	Mutant
PA0082lacZCP	GCGCAGTACTCAGCACGGTGACGATCTCCC	TLF
tssA1HindIIICTXCP	GCGCAAGCTTAACCTTTCGAGTCATCCAATATTC	TLF
PA0082RminiCTX	GCGCGGATCCTCGAAAGGTTGGCAAAATTCTTGT	TXF
tacNotIF	GGCCGCTGTTGACAATTAATCATCGGCTCG	TLF vector

	TATAATGTGTG	
tacBamHIR	GATCCACACATTATACGAGCCGATGATTA ATTGTCAACAGC	TLF vector
retSRXbaI	GCGCTCTAGAGCAGGTCTGGCTATGCCGG	Mutant
retSSOEF	GACTTCGCCGTGGTACGGCTTTCCTGAGGG CAGCGACGTGCT	Mutant
retSSOER	AGCACGTCGCTGCCCTCAGGAAAGCCGTA CCACGGCGAAGTC	Mutant
retSintF	CAAGGTCGAGGCGACCTGGG	PCR check
retSintR	GAATAACCGCGTGCGGTTATCC	PCR check
lacZScaIF	GCTATGACCATGATTAGTACTGATTCACTG GCCGTC	TLF vector
lacZRXhoI	CCCCTCGAGCAGACATGGCCTGCCCGGTTA TTA	TLF vector
lacZRforTF	GATGTGCTGCAAGGCGATTAAG	SEQ
pHERDSF	ATCGCAACTCTCTACTGTTTCTC	SEQ
rsmASDMAlgRF	GAATTCGGCAGGAACTTTCATTCCGGC	RPA
rsmARPAR	TAATACGACTCACTATAGGGAGAGATAAA AATTAATGGTTTGGCTCTTG	RPA

Abbreviations: TF, transcriptional fusion, TLF, translational fusion, SEQ, sequencing primer; RPA, RNase protection assay

Transcriptional and translational leader fusion analysis

Upstream DNA fragments containing promoter regions were generated by using the primers listed in Table 2.2 in conjunction with Q5 polymerase (NEB, Ipswich, MA). PAO1 genomic DNA was used as the template. PCR products were cloned into pCR2.1 and then subcloned into miniCTX*lacZ* using the restriction enzymes HindIII/BamHI, HindIII/EcoRI, or KpnI/BamHI (New England BioLabs, Ipswich, MA). To construct *rsmA* transcriptional fusions, the primer pair rsmAHindIII/rsmAEcoRIR was used and the product was inserted into the SalI/EcoRI sites of miniCTX*lacZ* using T4 DNA ligase (NEB, Ipswich, MA). The transcriptional fusion PE3-*lacZ* was constructed using the primers rsmAPE3F and rsmAPE3R and cloning into the KpnI/EcoRI site of miniCTX*lacZ*. The transcriptional fusion TF2- *lacZ* was constructed

using primer pair rsmAPE1F/rsmAEcoRIR and cloned into miniCTX*lacZ*. The translational fusion vector CTX*lacZ*CP was constructed by amplifying the *lacZ* gene using the primers listed in Table 2.2 in order to delete the *lacZ* ribosome-binding site and the translational initiation codon. The *tssA1* leader fusion was constructed using the *lacUV5* promoter in the 5= primer (PA0082FUV5) and primer PA0082ScaIR to amplify the *tssA1* untranslated region on the basis of the data from the study of Brencic and Lory (5) and cloned into CTX*lacZ*CP. The fusion constructs were confirmed by sequencing and conjugated into *P. aeruginosa* strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with pFLP2 to remove vector sequences (30). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch plated onto VBMM supplemented with 300 g/ml carbenicillin and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 2.2). b-Galactosidase activity was deter- mined by incubating cell extracts with *o*-nitrophenyl-b-D-galactopyranoside (4 mg/ml) as described by Miller (32). A strain carrying the empty vector, miniCTX*lacZ*, was assayed, and the value for this background (28 Miller units) was subtracted from all transcriptional fusions. All mucoid strains were confirmed to be mucoid at the end of each experiment by plating on PIA plates and making sure that all colonies were mucoid. Experiments were performed in triplicate at least three times.

Site-directed mutagenesis

Phosphorylated primers rsmASDMAlgUF and rsmASDMAlgUR were used in an inverse PCR with the PE3-*lacZ* transcriptional fusions as the templates. The predicted AlgU-dependent promoter in the -35 consensus sequence was mutated from GAACTT to GAATTC. The

successful inverse PCR product was self-ligated, trans- formed into NEB5a cells (NEB, Ipswich, MA), and screened by plasmid digestion with EcoRI digestion and sequencing. The constructs obtained by site-directed mutagenesis were conjugated into *Pseudomonas* strains as described above.

Western blot analysis

P. aeruginosa strains were grown in LB broth at 37°C for various times. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 mg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:2,500 dilution of anti-HA monoclonal antibody (Thermo Fisher, Pittsburgh, PA), followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (33). Arbitrary units were determined by standardizing the Western blots to the Coomassie- stained SDS-polyacrylamide gel of each strain using total protein analysis as described previously (34, 35). Detection was performed using an ECL Plus kit (Thermo Fisher, Pittsburgh, PA) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). The blots and Coomassie-stained SDS-polyacrylamide gels were analyzed with ImageJ software. All Western blot analyses were repeated at least three times.

Primer extension

Total RNA (15 mg) from *P. aeruginosa* was isolated using a Pure-Link RNA minikit (Life Technologies, Grand Island, NY). Radiolabeled primers rsmAPE1 and rsmAPE3 (Table 2.2) were used in a reverse transcription reaction in a ThermoScript reverse transcription-

detected using autoradiography. Primer extension results were confirmed using two different primers a total of four times.

In vitro transcription and RPA

The *rsmA* probe for the RNase protection assay (RPA) was synthesized using a PCR product generated with primers that incorporated a T7 promoter sequence at the 5= end and a MAXIscript T7 kit (Life Technologies). The reverse primer containing the T7 promoter sequence contained a nonhomologous sequence to discriminate between the full-length probe and protected fragments. The primers rsmASDMAlgRF and rsmARPAR were used to produce the PCR product. The probes were labeled with UTP by the use of biotin-16-UTP (Life Technologies) at a ratio of 4:6 and were gel purified after *in vitro* transcription. Twenty micrograms of total RNA from each *P. aeruginosa* strain was precipitated with 800 pg of labeled probe and resuspended in hybridization buffer {80% formamide, 400 mM NaCl, 40 mM PIPES [piperazine-*N*,*N*=-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA}, heated at 95°C for 5 min, and incubated overnight at 42°C. Negative controls consisting of the probe incubated with 10 mg *Saccharomyces cerevisiae* yeast RNA were also included. Digestion of unhybridized RNA was performed using RNase A at 25 mg/ml and RNase T1 at 10 U/ml. A biotinylated RNA ladder (Kerafast) was also used to deter- mine the size of the protected probe fragments. The products of the reactions were run on a 5% acrylamide– 8 M urea gel, and the gels were transferred to a positively charged membrane (Hybond N+; Amersham Biosciences) using a semidry blotting system and 0.5M Tris-borate-EDTA buffer (Hoefer, Holliston, MA). Nucleic acid was UV cross-linked, and the probes were detected using a chemiluminescent nucleic acid detection module (Thermo Scientific). After washing, the blot was incubated for 5 min and developed using a FluoroChem M system (ProteinSimple). RNase protection assays were performed at least three

times.

Overexpression/complementation studies.

In order to overexpress *algU*, the coding sequence was amplified (Table 2.2) and cloned into the integrating vector pTJ1 for complementation or overexpression (36). Trimethoprim-resistant colonies were picked, cultured overnight, and diluted 1,000-fold on the following day. The presence of the gene of interest was confirmed using primers pTJ1R and either algUTJ1F2 or algRTJ1F2. Cultures requiring induction used 1% arabinose and were grown for 8 h in broth or overnight on agar plates.

Statistical analyses.

Statistical analyses were performed using Graph- Pad Prism (version 6.0) software (GraphPad Software, La Jolla, CA). The results of transcriptional and translational fusions and densitometry analysis were compared using a one-way analysis of variance (ANOVA) with Tukey's posttest. ImageJ software was used for Western blot analysis.

RESULTS

AlgU hyperactivity significantly contributes to *rsmA* expression in *mucA* mutant strains.

Previous studies using an *rsmA* translational fusion and/or Western blot analyses found that RsmA levels increase throughout the growth phases (5, 21, 28), which suggests a role of the stationary-phase sigma factor RpoS (37). Another study using a transcriptional fusion indicated that a *mucA* strain has increased *rsmA* expression (14). Because the alternative sigma factor AlgU is available in strains lacking functional MucA, AlgU has increased activity in this background, and we postulated that AlgU might play a role in *rsmA* expression. To further

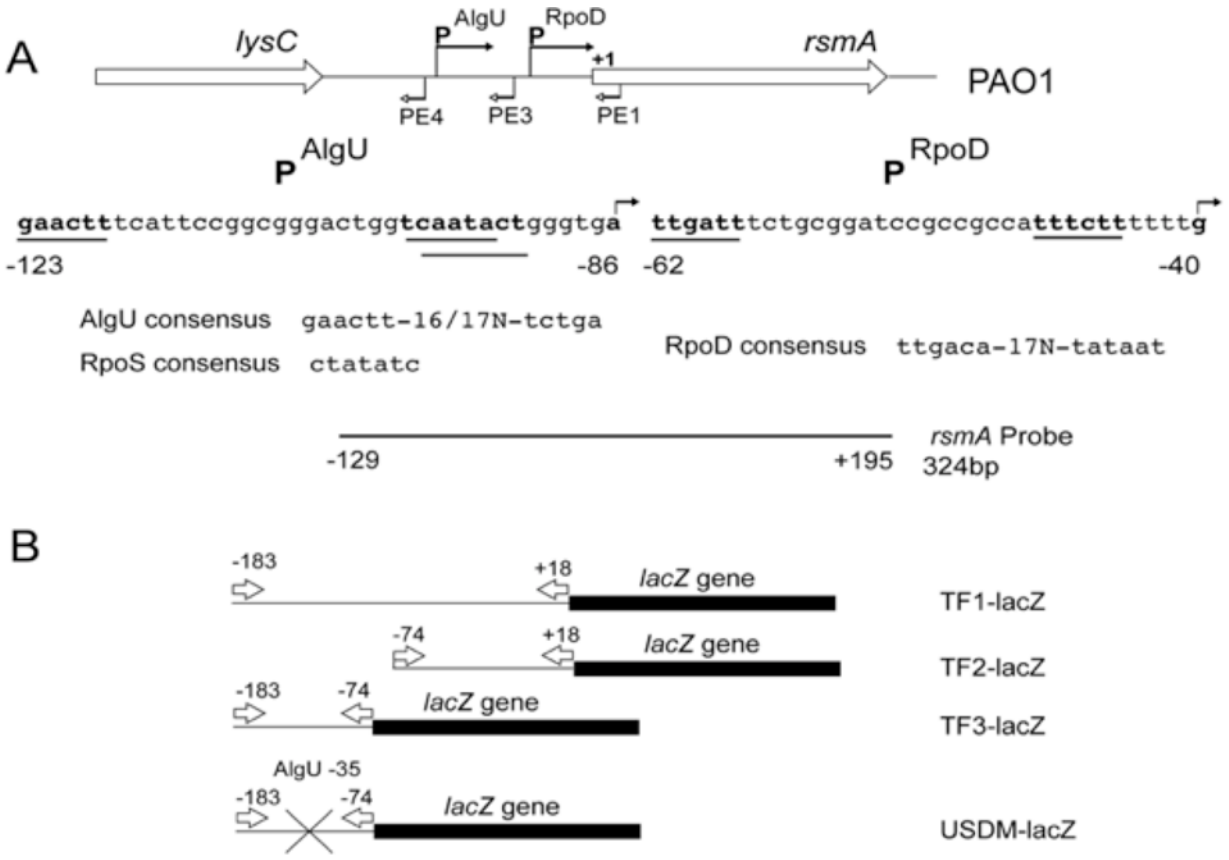

Figure 2.1 Depiction of the *rsmA* genomic region, the promoters controlling *rsmA* expression, and the reagents used in this study. (A) Schematic representation of the *rsmA* genomic region. The sequences below the schematic represent those of the two potential promoters. The bent arrows above the sequence indicate the transcriptional start sites identified by primer extension analysis. The primers used in the primer extension experiments are identified by bent arrows below the genomic schematic. The potential promoters are indicated by a line underneath the sequence and are in bold. Potential sigma factor consensus sequences are indicated below the sequence. The probe used for RNase protection assays is indicated below, and position numbers are relative to the *rsmA* translational start site. (B) The *rsmA-lacZ* transcriptional fusions constructed are indicated. Arrows indicate the primers used in the construction of the transcriptional fusions. The position numbers above the arrows are in relation to the *rsmA* translational start codon. The X indicates the site-directed mutagenesis of the putative AlgU -35 consensus sequence.

understand *rsmA* regulation at a transcriptional level, an *rsmA* transcriptional fusion, TF1-*lacZ*, was constructed by cloning the entire intergenic region between *rsmA* and *lysC* in the transcriptional fusion vector miniCTX*lacZ* (Fig. 2.1) (38).

Due to posttranscriptional *rsmA* autoregulation (39), it was important that only

transcription be monitored. The miniCTX*lacZ* vector allows insertion of single- copy *lacZ* fusion integrants and contains an RNase III processing site to ensure that transcription is uncoupled from posttranscriptional effects (38, 40). In wild-type strain PAO1, the TF1-*lacZ* fusion had b-galactosidase activity of 380 Miller units at 8 h (Fig. 2.2A). This result indicates that *rsmA* was expressed at low levels at this time point. The fusion was also tested at additional time points, including 16 h, and there was little change in TF1-*lacZ* activity (data not shown). In a Δ*algU* strain, the TF1-*lacZ* transcriptional fusion had no decrease in TF1-*lacZ* activity at 8 h (Fig. 2.2A). A Δ*rpoS* mutant was constructed and assayed for *rsmA* expression. There was no significant decrease in b-galactosidase activity at either 8 or 16 h (Fig. 2.2A). A Δ*algU* Δ*rpoS* strain was constructed, and the TF1-*lacZ* transcriptional fusion was assayed. No change in TF1-*lacZ* activity was seen in the double mutant. A Δ*algR* mutant had significantly increased *rsmA* expression at 8 h (Fig. 2.2A). A previous study indicated that AlgR activates *rsmA* expression (14), but the strain used, PA103, and the growth conditions differed from those used in our study. Our results suggest that *rsmA* is expressed at low levels in wild-type strain PAO1 and its mutant derivatives.

To further investigate the role of AlgU in controlling *rsmA* expression, the level of TF1-*lacZ* fusion expression was measured in a *mucA22* strain as well as the isogenic *mucA22* Δ*algU* nonmucoid derivative. As shown in Fig. 2.2A, a *mucA22* strain had a ~3- fold increase in *rsmA* expression compared to that in wild-type strain PAO1. In the *mucA22* Δ*algU* strain, *rsmA* transcriptional activity was reduced to the levels in wildtype strain PAO1 (Fig. 2.2A), confirming that AlgU does control *rsmA* expression. The analysis of the TF1-*lacZ* transcriptional fusion suggests that AlgU does play a role in regulating *rsmA* expression, but this effect is seen only when *mucA* is mutated and not able to sequester AlgU. In addition, RpoS does not regulate *rsmA*

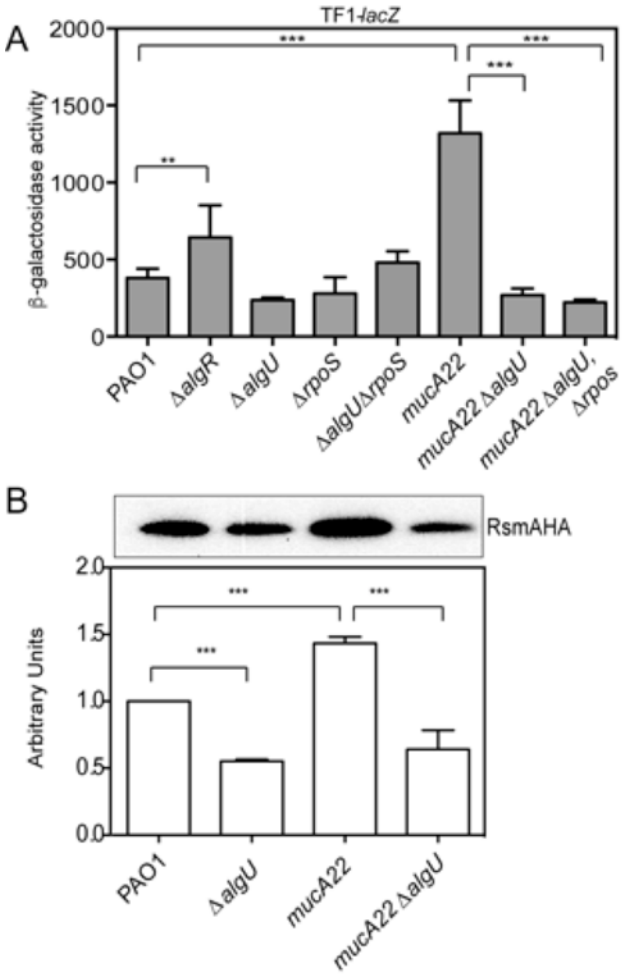

Figure 2.2 AlgU activates *rsmA* expression in *mucA* mutants. (A) Analysis of TF1- *lacZ* in mutant strains at 8 h of growth in LB broth. The TF1-*lacZ* transcriptional fusion was inserted into the *attB* site in the chromosomes of the indicated strains. Transcriptional fusion values are indicated as β-galactosidase activity. The values are the result of subtraction of the value for PAO1 containing the vector (miniCTX*lacZ*) alone (28 Miller units) from all test values. Fusion assays were all conducted at least three times. Determination of all significant differences from the results for the wild type was performed using a one-way analysis of variance and Tukey's posttest. **, $P < 0.01$; ***, $P < 0.001$. (B) (Top) Western analysis of RsmA in *algU* mutant strains. An *rsmA*-HA allele was constructed and introduced into various strains via homologous recombination, the strains were grown for 8 h, and whole-cell lysates were probed with anti-HA antibody. A single band was detected in all Western blots using the strains containing the *rsmA*-HA allele. A representative Western blot is shown. Western blot analysis was completed at least three times. The strains assayed are listed below the bottom panel. PAO1 without the *rsmA*-HA allele was run as a negative control (data not shown). (Bottom) Densitometry analysis was performed for each strain. Densitometry values, determined using ImageJ software, were standardized by dividing the intensity of the band in the Western blot by the intensity of the total protein in each lane stained with Coomassie. The result for PAO1 was set equal to 1, and the values for the other strains were normalized using the value for PAO1 as a reference. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

under these conditions.

To determine if RsmA protein levels mirrored the findings of *rsmA* expression analysis, we constructed a hemagglutinin (HA)- tagged *rsmA* allele that was introduced into strains by allelic exchange. As shown in Fig. 2.2B, the *mucA22* mutant had significantly higher RsmA levels than wild-type strain PAO1. Densitometry analysis indicated that there was a significant decrease in RsmA levels in the Δ*algU* mutant compared to those in wild-type strain PAO1 (Fig. 2.2B). The *mucA22* Δ*algU* mutant showed a drastic decrease in RsmA levels compared to those in the *mucA22* strain (Fig. 2.2B). The results of Western blot analysis suggested that AlgU is required for increased *rsmA* expression in *mucA* mutant backgrounds.

Identification of a second *rsmA* promoter

Because the sigma factor AlgU played a role in *rsmA* expression, the upstream region of *rsmA* was inspected for possible promoter sequences. A potential AlgU consensus sequence is located upstream of *rsmA* (Fig. 2.1A). To determine if this AlgU promoter is functional, we performed primer extension analysis. Total RNA was reverse transcribed using a radiolabeled primer (rsmAPE1) specific for a sequence located within the *rsmA* coding region, and extension products were detected on a denaturing polyacrylamide gel. Primer extension indicated that there were two transcriptional start sites (Fig. 2.3A), which suggests that *rsmA* has an additional promoter. The start site most distal to the coding region was identified as an A residue located 86 bp upstream of the predicted *rsmA* translational start codon. The first and closest transcriptional start site was a G residue located 40 bp upstream of the *rsmA* coding region. Primer extension analysis with an additional radiolabeled primer, rsmAPE3, produced a single extension product and confirmed the presence of the A residue identified to be a transcriptional start site 86 bp

upstream of *rsmA* (data not shown). The second transcriptional start site was independently identified four times. Primer extension analysis with a third primer, rsmAPE4, whose sequence overlapped the most distal transcriptional start site, failed to produce an extension product, suggesting that *rsmA* has only two active promoters under the conditions tested.

Sequences upstream of the identified transcriptional start sites were examined for potential promoters (Fig. 2.1A). Three previous studies identified using transcriptome sequencing (25, 26) and 5' rapid amplification of cDNA ends (24) a transcriptional start site that is 40 nucleotides upstream of the *rsmA* translational start codon. The sequence of the proximal promoter identified by our primer extension analysis resembled a sigma^{70} (RpoD) promoter sequence. Therefore, the proximal promoter that we identified was the same promoter previously identified, validating the findings of our primer extension analysis.

The second transcriptional start site has two possible promoter consensus regions upstream. A potential AlgU consensus sequence GAACTT in the -35 region (Fig. 2.1A) (11) and a potential RpoS -10 region are located upstream of this transcriptional start site. The potential RpoS consensus sequence CAATACT differs from the RpoS consensus sequence CTATACT by 1 nucleotide (41). *E. coli* RpoS promoters frequently have an extended structure, denoted by a G/T residue directly upstream of the conserved -10 region (42). The potential RpoS promoter upstream of *rsmA* also follows this extended structure (Fig. 2.1A). The potential RpoS -10 region overlaps the potential AlgU -10 region (Fig. 2.1A). On the basis of the results of primer extension and sequence analyses, *rsmA* has a second promoter that is controlled by AlgU and/or RpoS.

AlgU controls the distal *rsmA* promoter

Because two *rsmA* transcripts were detected by primer extension (Fig. 2.3A), this led us to test whether *rsmA* has two promoters. Two transcriptional fusions were constructed using the data generated by primer extension analysis (Fig. 2.1) to confirm the promoter activity of these regions. The activity of PAO1 carrying the vector alone (28 Miller units) was subtracted from the values obtained. A transcriptional fusion, TF2-*lacZ*, was constructed by deleting the putative distal promoter. TF2-*lacZ* has the 5' end at position -74 in relation to the *rsmA* translational start site and extends 18 nucleotides into the *rsmA* coding region (Fig. 2.1B). The transcriptional fusion TF3- *lacZ* was constructed using the intergenic region from *lysC* to the distal transcriptional start site at position +1 as the 3' end of the fusion and therefore contains only the putative distal promoter (Fig. 2.3B). When these fusions were analyzed in wild-type strain PAO1, both fusions produced b-galactosidase activity (Fig. 2.3B and C), but the level of expression was very low. The values for both the TF2-*lacZ* and TF3-*lacZ* fusions were much lower than the value for TF1-*lacZ*, suggesting that the entire region is necessary for optimal *rsmA* expression.

The TF2-*lacZ* transcriptional fusion was constructed and tested to determine the activity of the proximal promoter. The TF2-*lacZ* fusion did not have increased reporter activity in the *mucA22* strain (Fig. 2.4B). When *algU* was deleted in either background, no decrease in reporter activity was detected, indicating that this promoter was constitutively active at low levels in the strains analyzed (Fig. 2.4C).

To determine if AlgU affected the distal promoter that we identified, the TF3-*lacZ* fusion was analyzed in wild-type strain PAO1 and a Δ*algU* mutant. The activity of the TF3-*lacZ* fusion

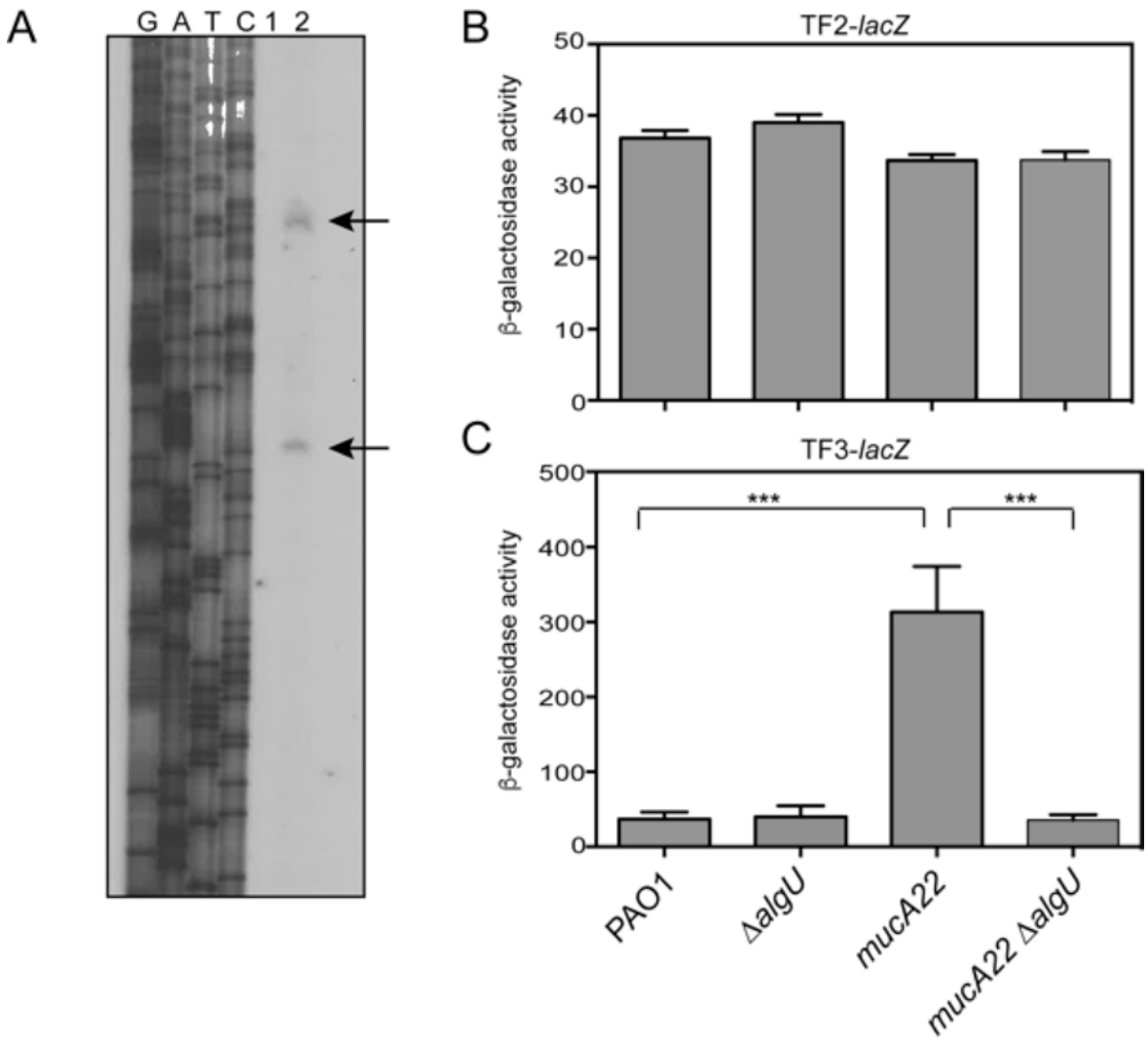

Figure 2.3 Identification of a second *rsmA* promoter region controlled by AlgU. (A) Primer extension analysis was performed to identify the transcriptional start sites of *rsmA*. Total RNA was reverse transcribed using a radiolabeled primer, rsmAPE1 (Table 2), and run alongside the products of the sequencing reactions on a denaturing polyacrylamide gel. Lanes G, A, T, and C, the sequencing ladder generated using the same primer used in the reverse transcription reaction; lane 1, a control lane with total RNA without reverse transcriptase; lane 2, reverse-transcribed PAO1 RNA. Arrows, extension products. (B) b -Galactosidase activity of chromosomal transcriptional fusion TF2-*lacZ* in the indicated strains tested in triplicate after incubation in LB broth for 8 h at 37°C as described in the legend to Fig. 2A. The value for PAO1 carrying the vector alone was subtracted from the value for each strain. (C) b -Galactosidase activity of chromosomal transcriptional fusions of TF3-*lacZ* in the indicated strains tested in triplicate after incubation in LB broth for 8 h at 37°C. The value for PAO1 carrying the vector alone was subtracted from the value for each strain. Transcriptional fusion assays were performed at least three times. All significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. ***, $P < 0.001$.

in PAO1 was low, and no difference in activity was seen when it was tested in a Δ*algU* mutant at 8 h. However, when the activity of the TF3-*lacZ* fusion was tested in a *mucA22* strain, there was an ~8-fold increase (Fig. 2.3C). The activity of the TF3-*lacZ* transcriptional fusion in a *mucA22* Δ*algU* strain was significantly reduced ~8- fold (Fig. 2.3C). These results show that the promoter contained in the TF3-*lacZ* fusion is AlgU dependent.

Overexpression and complementation of *algU* increase *rsmA* expression

For overexpression of *algU*, we used an arabinose- inducible promoter to control *algU* expression (43). As shown in Fig. 2.4A, there was a statistically significant increase in TF3-*lacZ* activity when *algU* was induced with arabinose in strain PAO1. The results suggest a role for AlgU in activating *rsmA* expression.

Additionally, the Δ*algU* mutant containing the TF3-*lacZ* transcriptional fusion was complemented with pAlgU. When 1% arabinose was used to induce *algU* expression, TF3-*lacZ* activity was also increased (Fig. 2.4B). Complementation and overexpression resulted in an increase in TF3-*lacZ* activity. From these results, we conclude that AlgU is necessary for increased *rsmA* expression.

To determine if RsmA protein levels matched those of the transcriptional fusions, we used the PAO1 *rsmA*-HA strain containing epitope-tagged *rsmA*. When *algU* expression was induced by the addition of arabinose, there was a significant increase in RsmA levels (Fig. 2.4C). On the basis of this result, it was determined that *algU* overexpression is able to increase *rsmA* transcription.

Site-directed mutagenesis of the putative AlgU-binding site in the distal promoter reduces *rsmA*

expression

Site-directed mutagenesis was performed on the TF3-*lacZ* transcriptional fusion to

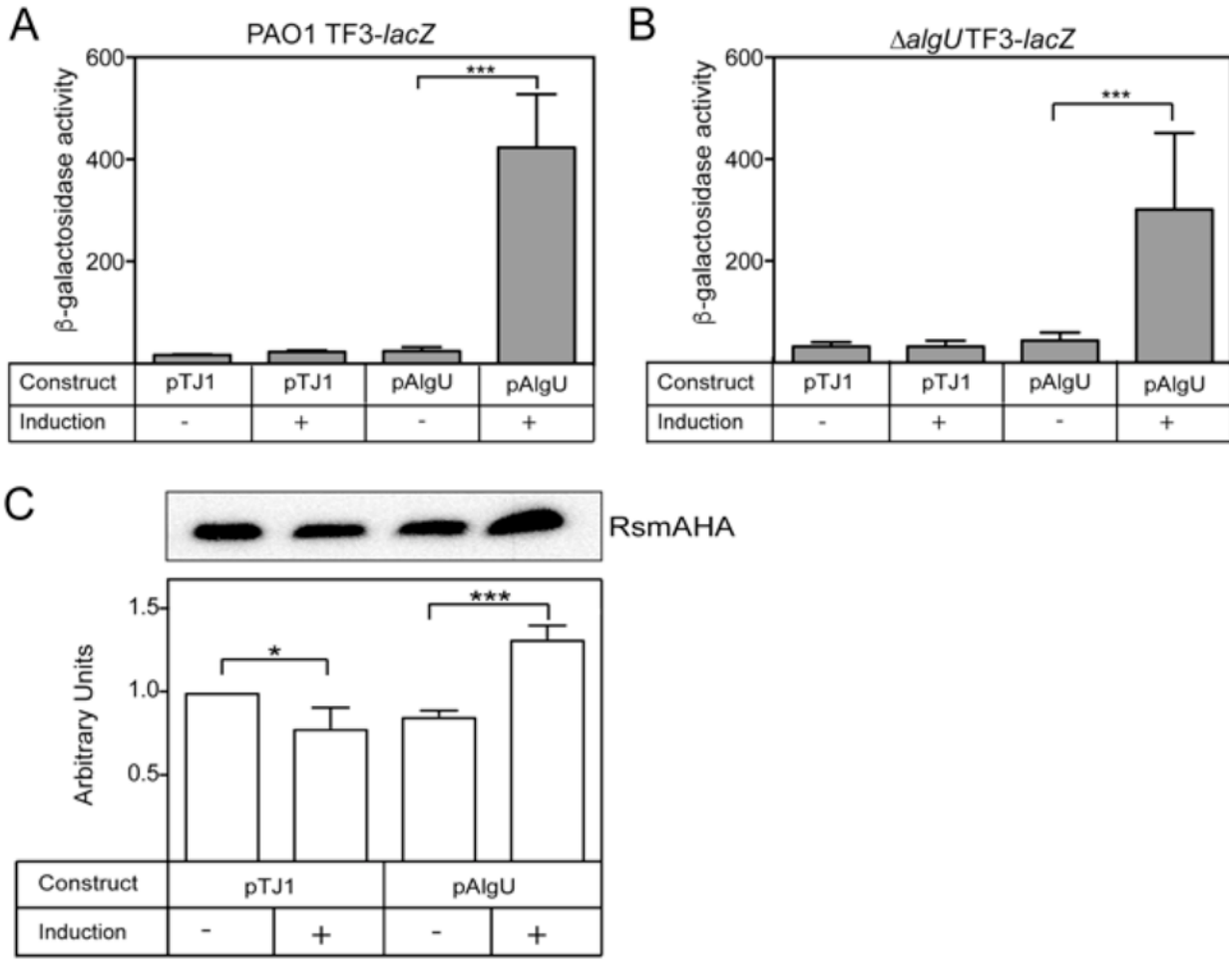

Figure 2.4 AlgU overexpression and complementation confirm the AlgU regulation of *rsmA*. (A and B) β–Galactosidase activity of the TF3-*lacZ* (A) and Δ*algU* TF3-*lacZ* (B) constructs. (A) The *algU* gene was cloned into the integrating vector pTJ1 and induced with 1% arabinose. Strains were grown in LB broth for 4 h and induced with arabinose for 4 h. PAO1 with the vector alone (pTJ1) was used as a negative control. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) Complementation of the Δ*algU* strain containing the transcriptional fusion TF3-*lacZ* was performed as described in the legend to panel A. (C) Overexpression of *algU* increased RsmA protein levels. PAO1-HA/pTJ1 was the PAO1 strain with the *rsmA*-HA allele and the vector alone. PAO1-HA/pAlgU contained the *rsmA*-HA allele and overexpressed *algU* when arabinose was added. ImageJ software was used for densitometry analysis, as described in the legend to Fig. 2B. (Top) A representative Western blot. Western blot analysis was performed at least three times. (Bottom) Densitometry results for the Western blots.

determine if the putative AlgU consensus sequence was required for increasing *rsmA* expression. The putative AlgU -35 sequence GAACTT was converted to GAATTC (underlined nucleotides indicate changes introduced) (Fig. 2.5). There was a ~3- fold decrease in the level of transcription in the *mucA22* strain with the mutagenized AlgU promoter (Fig. 2.5). The results of the site-directed mutagenesis experiment demonstrate the necessity of the conserved AlgU -35 sequence and strongly suggest that AlgU directly controls *rsmA* transcription by binding the distal *rsmA* promoter.

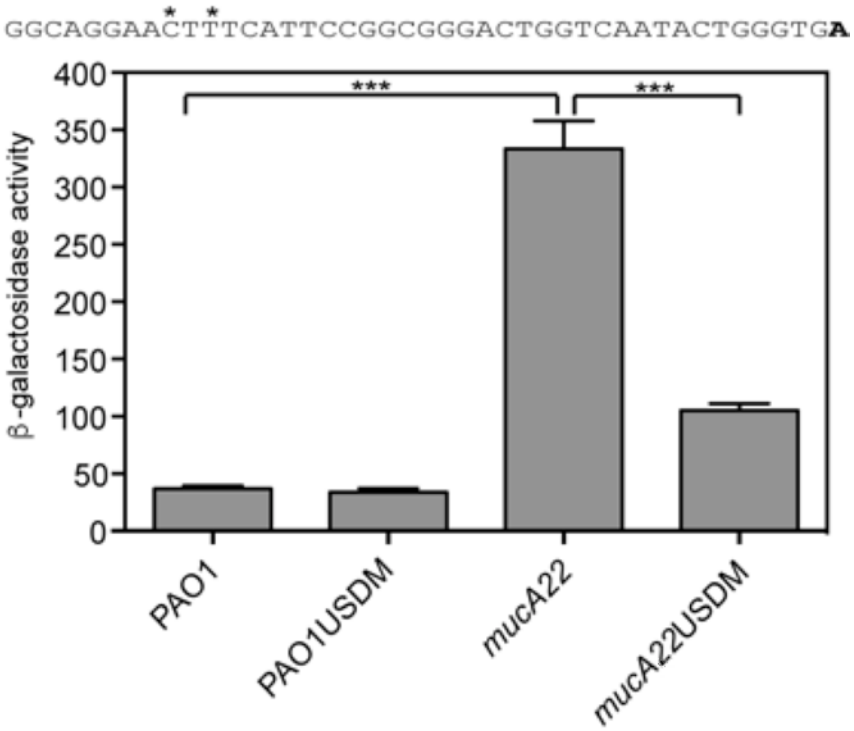

Figure 2.5 The potential AlgU-binding site is required for increased *rsmA* expression in a *mucA22* mutant. The TF3-*lacZ* transcriptional fusion was engineered with two mutations in the potential AlgU -35 consensus sequence (indicated by asterisks above the indicated nucleotides in the sequence above the graph). The transcriptional start site (the 3= end of the TF3-*lacZ* fusion) is indicated in bold. USDM indicates the fusion containing the mutation of the conserved AlgU -35 consensus sequence. β-Galactosidase activity was determined as described in Materials and Methods. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector (28 Miller units). A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

AlgU regulation of *rsmA* is conserved

To determine if AlgU control over *rsmA* is a conserved mechanism in other *P. aeruginosa* strains, we investigated the role of AlgU in the clinical strains 2192 and 383. These strains were isolated from a longitudinal study (44) and have been determined to be isogenic (45). Strain 2192 is mucoid and has a *mucA* mutation (46). We took a genetic approach and assayed our transcriptional fusions TF1-*lacZ* and TF3-*lacZ* to determine the role of AlgU in activating *rsmA* expression in these strains (Fig. 2.6). Strain 2192 had an ~3-fold increase in the amounts of both the TF1-*lacZ* fusion and the TF3-*lacZ* fusion compared to those in nonmucoid strain 383 (Fig. 2.6A and B). These results demonstrate that the AlgU-dependent promoter activity is increased in strain 2192 compared to that in its nonmucoid counterpart, strain 383. A 2192 Δ*algU* mutant was constructed, and assays for the expression of the TF1-*lacZ* and TF3-*lacZ* fusions were performed to confirm a role for AlgU in other *P. aeruginosa* strains. As shown in Fig. 2.6A and B, there was a statistically significant decrease in the levels of TF1- and TF3-*lacZ* expression, supporting the role of AlgU in regulating the distal promoter. To confirm the transcriptional fusion results, the levels of RsmA in the clinical strains were determined using the *rsmA*-HA allele. RsmA levels were increased in strain 2192 compared to those in the nonmucoid 383-parent strain (Fig. 2.6C), suggesting that AlgU activated *rsmA* expression in strain 2192. The 2192 Δ*algU* mutant had a significant decrease in the amount of RsmA compared to that in 2192 (Fig. 2.6C). On the basis of these results, it is likely that AlgU activates *rsmA* expression in multiple strains.

RpoS involvement in the control of *rsmA*

There are conflicting data regarding RpoS control of *rsmA* expression (9, 27). The potential RpoS-binding site upstream of *rsmA* implicated RpoS in the control of *rsmA* (Fig. 2.1A). However, the TF1-*lacZ* transcriptional fusion analysis did not demonstrate a decrease in

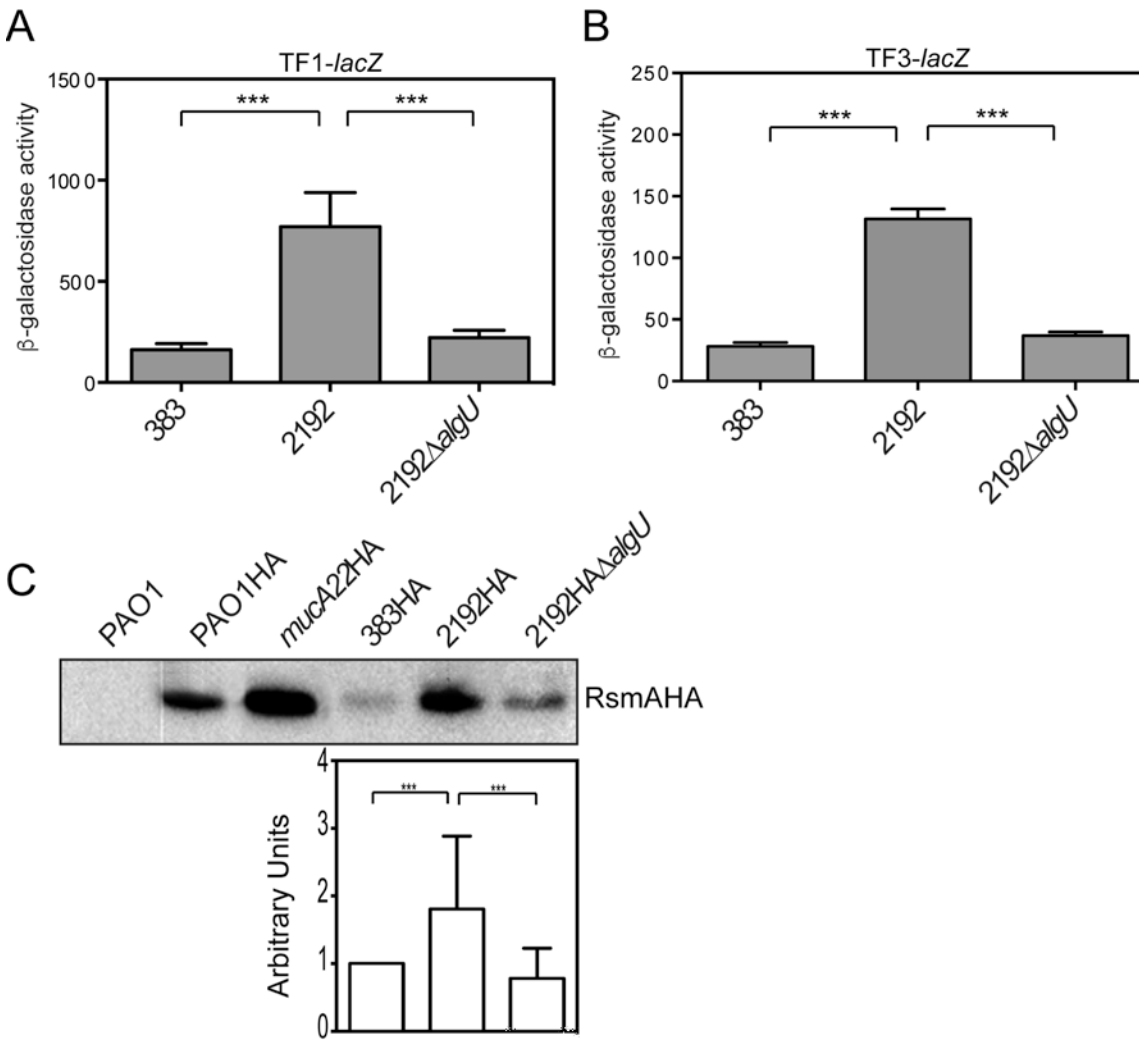

Figure 2.6 Clinical *mucA* mutant strains have increased *rsmA* expression. (A and B) β-Galactosidase activities of the TF1-*lacZ* (A) and TF3-*lacZ* (B) transcriptional fusions. (A) The β-galactosidase activity of the TF1-*lacZ* transcriptional fusion was assayed in clinical isolates 383 and 2192 and a 2192 Δ*algU* strain after 8 h, as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) The β-galactosidase activity of the TF3-*lacZ* transcriptional fusion was analyzed in strains 383, 2192, and 2192 Δ*algU* at 8 h, as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$ (C) RsmA levels are also increased in clinical *mucA* mutants. The *rsmA*-HA allele was exchanged for the wild-type *rsmA* allele in clinical isolates 383 and 2192. Whole-cell lysates were produced and probed with anti-HA antibodies. PAO1 was used as a negative control (data not shown). The results for PAO1-HA and *mucA22*-HA are shown for reference. (Top) A representative Western blot. (Bottom) Results of densitometry analysis of the blot performed using ImageJ software. Data are in arbitrary units and were produced from three Western blots. Densitometry analysis was performed as described in the legend to Fig. 2B.

rsmA

expression in a Δ*rpoS* mutant. Because sigma factor competition on the distal promoter could have influenced our ability to detect RpoS activity on the *rsmA* promoter (47), we tested a

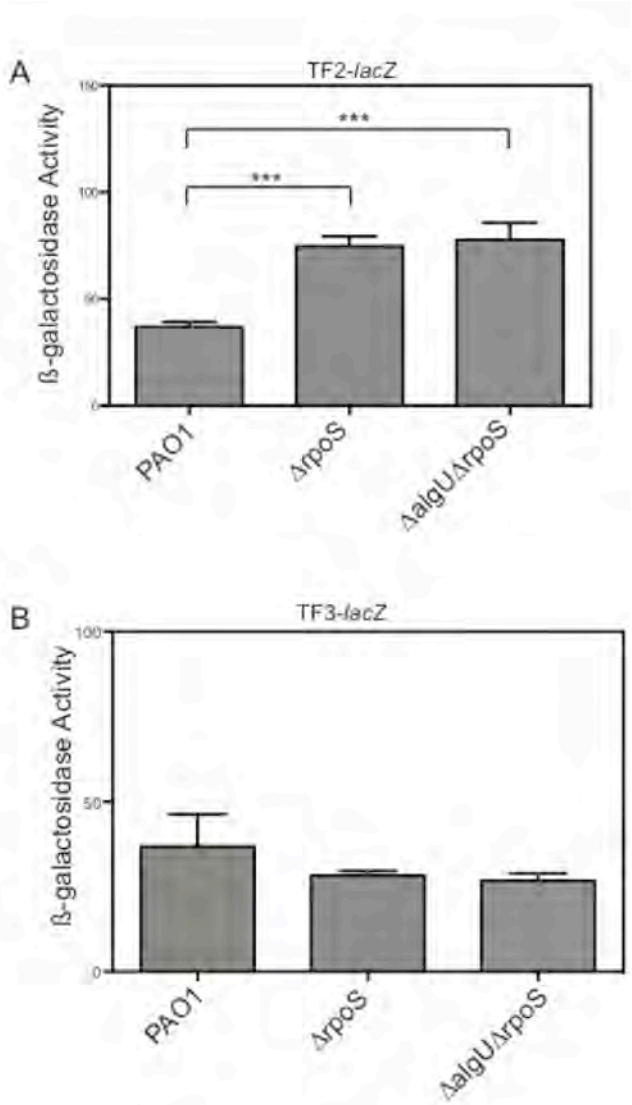

Figure 2.7 RpoS is not a significant regulator of *rsmA* expression. (A) β-Galactosidase activity of the TF2-*lacZ* transcriptional fusion in the denoted strains. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) β-Galactosidase activity of the TF3-*lacZ* transcriptional fusion in the denoted strains. β-Galactosidase activity was determined as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). Significant differences from the results for the wild type were determined using a one-way analysis of variance and Tukey's posttest. ***, $P < 0.001$.

double mutant, the Δ*algU* Δ*rpoS* mutant (Fig. 2.1A). No further decrease in TF1-*lacZ* activity in

the Δ*algU* Δ*rpoS* mutant was detected. To further define the role of RpoS in controlling *rsmA* expression, we tested the TF2- and TF3-*lacZ* fusions for activity in either Δ*rpoS* strains or Δ*algU* Δ*rpoS* strains (Fig. 2.7). Interestingly, both a Δ*rpoS* mutant and a Δ*algU* Δ*rpoS* mutant had significantly higher levels of activity when the TF2-*lacZ* promoter was used (Fig. 2.7A), suggesting that RpoS has a negative effect on transcription from the proximal *rsmA* promoter. No significant change was found when the TF3-*lacZ* transcriptional fusion was used in the Δ*rpoS* or Δ*algU* Δ*rpoS* mutant strains (Fig. 2.7B). From these results, the role of RpoS in regulating *rsmA* is inconclusive.

RpoS and AlgU are necessary for *rsmA* expression from the distal promoter

RNase protection assays (RPAs) were performed using an RNA probe specific for a region spanning the entire *rsmA* coding region and 129 bp upstream of the *rsmA* gene (Fig. 2.1A) to more directly analyze *rsmA* transcripts and better understand the role of AlgU and RpoS in controlling *rsmA* expression. The probe also includes non-template-encoded nucleotides to distinguish the full-length probe from protected fragments. As shown in Fig. 2.8, the RPAs confirmed the findings of our primer extension and transcriptional fusion analysis by detecting two *rsmA* transcripts in wild-type strain PAO1. The expected fragments were 281 bp and 235 bp, on the basis of the probe used. This matched what was seen in the RPAs (Fig. 2.8). Interestingly, the Δ*algU* mutant contained the same two transcripts as wild-type strain PAO1. When the Δ*rpoS* mutant was analyzed, both transcripts were detected, with the amount of the first transcript resulting from the amount of the proximal promoter being increased. A Δ*algU* Δ*rpoS* strain was also analyzed, and the larger transcript resulting from the distal promoter completely disappeared, demonstrating that both AlgU and RpoS contribute to transcription from the distal promoter (Fig. 2.8). The *mucA22* strain had both transcripts as well, with both transcripts having

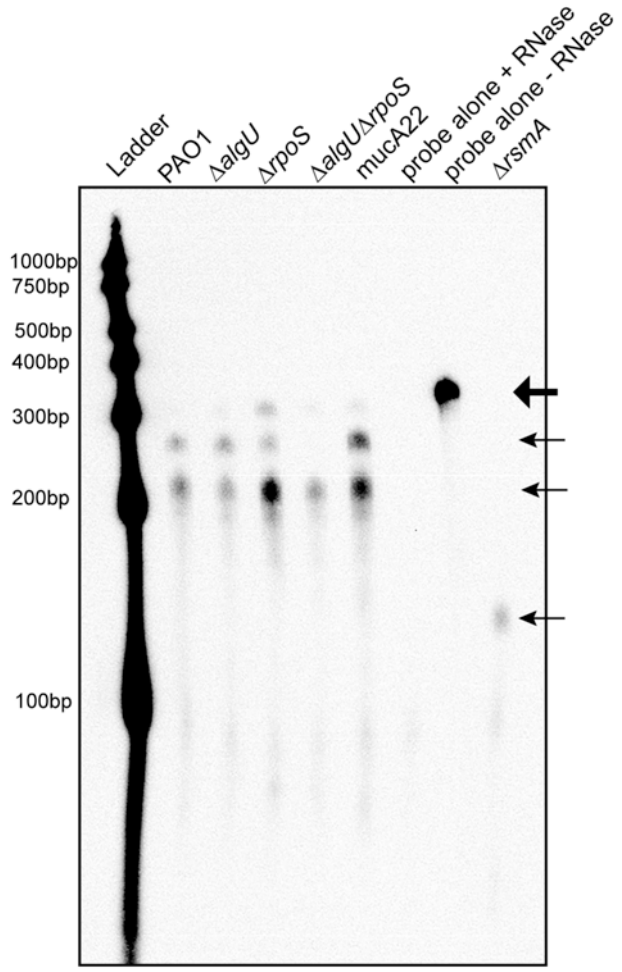

Figure 2.8 RPAs confirm the presence of two *rsmA* transcripts. RPAs of the indicated strains were performed after RNA was harvested from cultures grown in LB broth for 8 h at 37°C. Twenty micrograms of total RNA from the indicated strains was hybridized with 800 pg of biotinylated probe and subjected to RNase treatment. After RNase inactivation, RNA was precipitated from the samples and run on a 5% denaturing polyacrylamide gel, transferred to a nylon membrane, UV cross-linked, and developed using chemiluminescent detection. The undigested probe is indicated by the large arrow at the top. The protected *rsmA* probe is indicated by the three smaller arrows. Shown is a result representative of results from three RNase protection assays. Ladder, molecular size markers.

greater intensities. The highest band in the Δ*rpoS* and *mucA22* strains was likely undigested probe.

Three controls were included in the RPA. Two of the controls were the probe incubated

with yeast RNA; one of these was treated with RNase, and the other was not. As shown in Fig. 2.8, RNase treatment completely degraded the probe, which indicates that the probe did not bind nonspecifically. The intact probe can be seen at ~330 bp in the lane in Fig. 2.8 labeled "probe alone -RNase," which is consistent with the additional nucleotides that are non-template encoded. A Δ*rsmA* mutant was constructed and used as an additional control. In the Δ*rsmA* mutant, there was only one discernible protected probe fragment at ~150 bp. On the basis of how the Δ*rsmA* mutant was constructed, a protected fragment of 144 bp was expected. The results obtained with the Δ*rsmA* strain support the suggestion that there are only two *rsmA* transcripts, at least under the conditions used. Altogether, the results of the RPA suggested that there are two *rsmA* transcripts and that both AlgU and RpoS are involved in controlling the distal promoter region, suggesting overlapping promoters.

AlgU control of *rsmA* expression is responsible for RsmA activity in a *P. aeruginosa mucA22* strain

Because AlgU increased RsmA levels, we hypothesized that RsmA activity was also increased in a *mucA22* strain. We analyzed a variety of mutant strains, including strains that should alter the activity of RsmA on the basis of the current RsmA paradigm (5). RetS interferes with GacA phosphorylation, and mutation of *retS* should lead to increased production of the small RNAs RsmY and RsmZ (18, 48). Therefore, the level of RsmA activity should be reduced. In contrast, mutation of *gacA* should lead to a reduction in the amounts of the small RNAs RsmY and RsmZ (49). In this case, increased RsmA activity should be observed. Mutants with mutations in the *gacA* and *retS* genes were constructed in both the PAO1 and *mucA22* strain backgrounds.

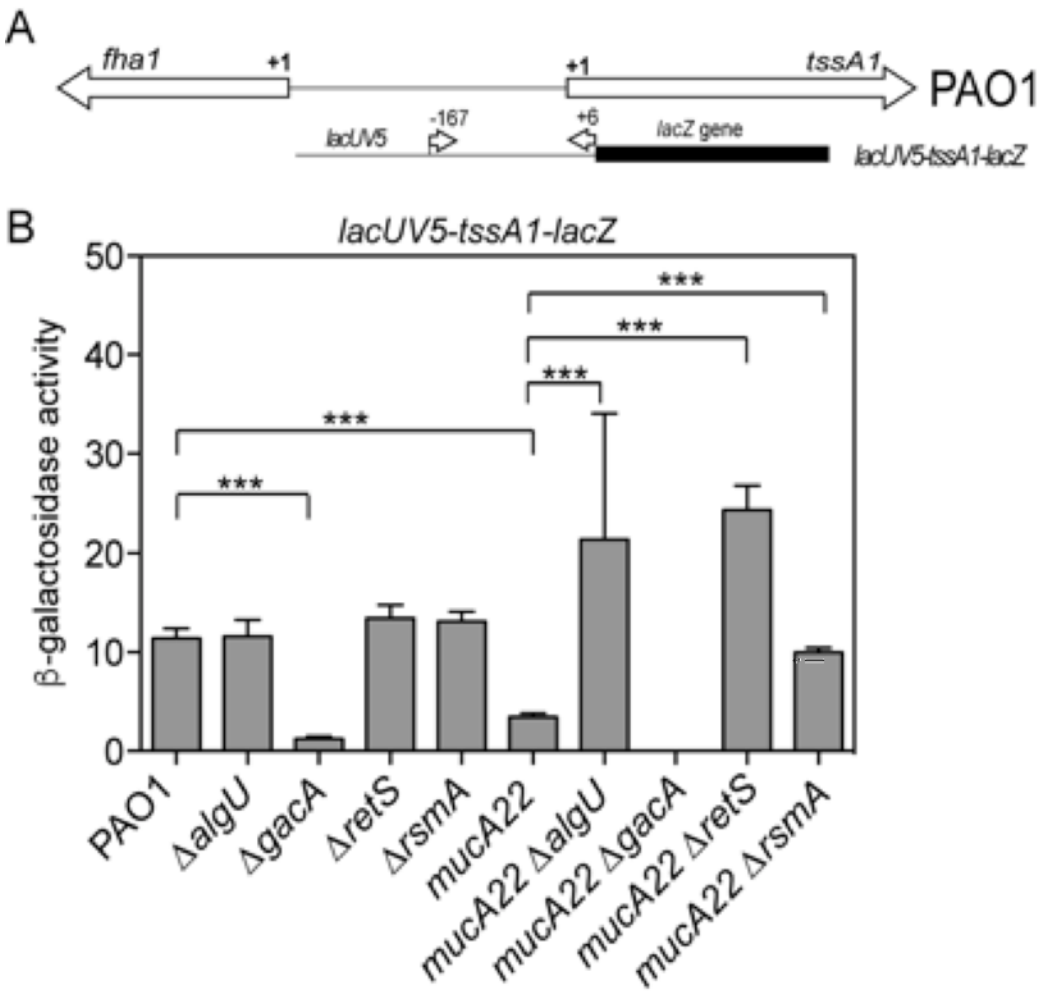

Figure 2.9 AlgU activation of *rsmA* leads to changes in *tssA1* expression. (A) Schematic of the genomic region of the RsmA-controlled genes *tssA1* and *fha1*. Below the genomic schematic is a depiction of the leader fusion that was constructed. The position numbers above the fusion construct indicate those of the primers used relative to the *tssA1* translational start codon. (B) Analysis of a TssA1 leader translational fusion driven by the *lacUV5* promoter in wild-type and mutant derivative strains. All assays were repeated at least three times in triplicate. Data were normalized to the mean for PAO1. PAO1 activity with the *lacUV5-tssA1-lacZ* fusion was 12 Miller units. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

RsmA has previously been shown to affect the production of the exopolysaccharide genes *pel* and *psl* (9, 16, 50). However, no studies have investigated the involvement of RsmA in alginate production. We hypothesized that if RsmA inhibits biofilm formation, then RsmA would inhibit alginate production. Interestingly, the *mucA22* Δ*gacA*, *mucA22* Δ*retS*, and *mucA22* Δ*rsmA* mutants still overproduced alginate (data not shown), and we conclude that RsmA does

not affect alginate production.

RsmA posttranscriptional activity was tested using a *tssA1* leader/translational fusion containing the *lacUV5* promoter and the *tssA1* untranslated sequence, based on the study of Brencic and Lory (5) (Fig. 2.9A). Due to the low level of activity of this reporter, the data are presented as a percentage of the wild-type activity, where the values were normalized to those for the PAO1 strain (12 Miller units). The *mucA22* strain had fusion activity decreased ~3-fold compared to that of PAO1 (Fig. 2.9B). There was no difference in activity between PAO1 and a Δ*algU* strain when the leader fusion was used (Fig. 2.9B). In the case of a *mucA22* Δ*algU* strain, there was a 5.5-fold increase in leader fusion activity compared to that in the *mucA22* strain (Fig. 2.9B). These results suggest that RsmA is active in the *mucA22* strain background and this is due to AlgU regulation of *rsmA*.

Further confirmation of RsmA activity in the *mucA22* strain was carried out using mutants with mutations in *gacA* and *retS*, as described above. While the differences in activity were not significant by statistical analysis, there was no activity from a *mucA22* Δ*gacA* strain, even after the reaction was allowed to proceed overnight. This result would be expected if RsmA activity were increased due to the absence of the small RNAs RsmY and RsmZ (49). As there was an increased amount of RsmA in the *mucA22* strain due to AlgU activation of the distal promoter, the difference between the *mucA22* strain and the *mucA22* Δ*gacA* strain should not be so different if our hypothesis is correct (Fig. 2.10). The leader fusion also had increased activity in both the *mucA22* Δ*rsmA* and the *mucA22* Δ*retS* strains compared to that in the *mucA22* strain (3-fold and 7-fold, respectively) (Fig. 2.9B). These results also indicate the RsmA activity in the *mucA22* strain background. The increased activity of the leader fusion indicates that the absence of RsmA or an abundance of RsmY and RsmZ due to the absence of RetS allows greater

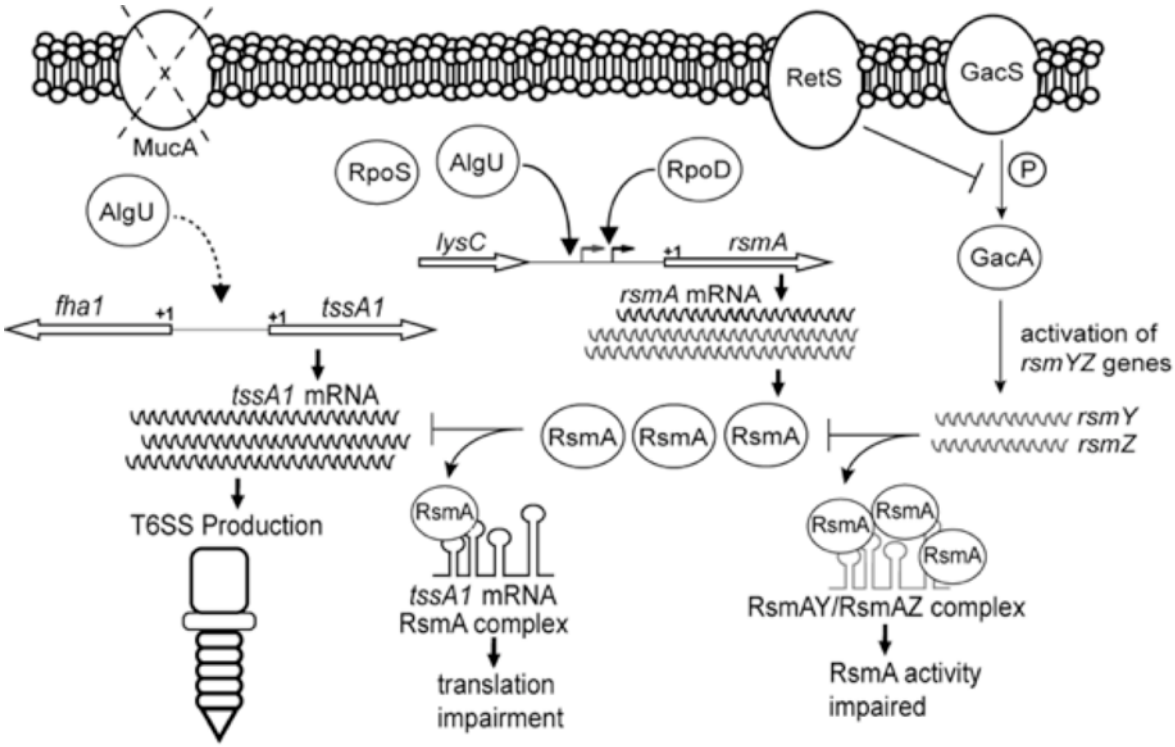

Figure 10. Model depicting the expression and activity of *rsmA* and the protein that it encodes, RsmA, in *mucA* mutant strains. Two transcripts of different sizes are produced. AlgU and RpoS control the longer transcript originating from the distal promoter. In the *mucA* strain background, AlgU increases the level of transcription from the distal promoter, but both transcripts are increased in amount either by transcription initiation at both promoters or by posttranscriptional processing of the longer message. Due to increased *rsmA* transcription, there is an increased amount of RsmA in *mucA* mutants. Transcriptional start sites are represented by bent arrows. The left and right bent arrows indicate the distal and proximal promoters, respectively. The +1 indicates the putative translational *rsmA* start site. RsmA levels increase, preventing translation of *tssA1* and therefore production of the HSI-I T6SS. The dashed curved arrow on the left side indicates that AlgU either directly or indirectly leads to the activation of the *tssA1* operon. RetS and GacA are depicted on the right to show how they are incorporated in the RsmA scenario. GacA, when phosphorylated by GacS, activates the expression of two small RNAs, RsmY and RsmZ, that bind and prevent RsmA from performing posttranscriptional regulation. RetS interferes with GacA phosphorylation by physically binding GacS and allows RsmA to regulate expression of mRNAs. The small RNAs RsmY and RsmZ bind and prevent RsmA activity.

expression from the leader fusion. Altogether, these data suggest that RsmA activity is present in a *mucA22* strain.

DISCUSSION

RsmA is considered an important posttranscriptional regulator involved in the positive regulation of acute virulence factors, such as the type III secretion system and motility (5, 14). Little is known of the transcriptional regulation of *rsmA*. Initially, we undertook studies to determine how *rsmA* was regulated in *P. aeruginosa*. We demonstrate that AlgU activates *rsmA* expression and increases RsmA levels in a *mucA22* mutant and a clinical isolate. Analysis of a leader fusion to assess the posttranscriptional activity of the RsmA target, *tssA1* (5), led us to discover RsmA activity in the *mucA22* strain. To our knowledge, this is the first demonstration of RsmA activity in a *mucA* mutant strain of *P. aeruginosa*. Our work demonstrates that increased *rsmA* expression due to AlgU activation leads to changes in the RsmA regulon. We hypothesize that RsmA plays an important role during *P. aeruginosa* chronic infection and that there are additional RsmA targets in *mucA* mutant strains.

One of the goals of this study was to determine how *rsmA* expression is regulated. Previous studies have raised questions about how *rsmA* is regulated in *P. aeruginosa* (9, 14, 24). In *E. coli*, five promoters control *csrA* (the orthologue of *rsmA*) expression (51). Only one *rsmA* promoter was previously identified in *P. aeruginosa* (24–26), and a previous study indicated that AlgR plays a role in regulating *rsmA* expression (14). Using primer extension, a new transcriptional start site was identified, indicating a second *rsmA* promoter (Fig. 2.2). Both transcriptional fusion analysis and RPA confirmed the presence of a second promoter (Fig. 2.3 and 2.8). Overexpression and Western blot analyses support a role of AlgU in controlling *rsmA* expression (Fig. 2.1E and 2.4). However, when MucA is functional, such as in PAO1, it is difficult to detect changes in *rsmA* expression. This explains the low levels of activity of the transcriptional reporters (Fig. 2.1A and 2.2). The role of RpoS in controlling *rsmA* expression is

somewhat less clear. While we were not able to detect RpoS regulation of *rsmA* by transcriptional fusion analysis (Fig. 2.7), the results of RPA suggested that RpoS does play a role (Fig. 2.8). Further studies are needed to determine the significance of RpoS in controlling *rsmA* expression. A previous study identified AlgR to be a regulator of *rsmA*, *rsmY*, and *rsmZ* (14). It is possible that AlgR and AlgU work together in regulating *rsmA* in *mucA* mutant strains. It is clear that *rsmA* regulation is complex, but this work solidifies the role of AlgU as an *rsmA* activator.

Our finding that AlgU controls *rsmA* expression is bolstered by the analysis of *rsmA* in the clinical strains 2192 (mucoid) and 383 (nonmucoid) (45). These data indicate higher levels of RsmA in the *mucA* mutant strain 2192 (Fig. 2.6). The findings of our regulation studies are consistent with a previous observation that a *mucA* mutant had increased levels of *rsmA* expression (14). Thus, our work implicates RsmA to be necessary for the persistence of *P. aeruginosa* during chronic infections, because *mucA* mutants are found at later time points during infection. This is consistent with the requirement of *Helicobacter pylori*, which requires CsrA (the RsmA orthologue) for persistence in the stomach (52). Whether RsmA participates in regulating virulence factors in *mucA* mutant strains is currently not known and is an unexplored area.

After finding that AlgU, an important sigma factor in chronic infections (7), regulates *rsmA* expression, we asked if the increased RsmA levels have activity in *mucA* mutant strains. Our results support a model (Fig. 2.10) where RsmA activity in the *mucA* mutant background is due to AlgU. First, using the *tssA1* leader fusion, the *mucA22* strain showed decreased activity, indicating in- creased posttranscriptional regulation compared to that in PAO1. Second, the *mucA22* Δ*algU* strain had increased leader fusion activity compared to that of the *mucA22* strain,

which supports our conclusion that increased *rsmA* expression leads to increased RsmA activity. This result also demonstrates the importance of AlgU control of *rsmA* expression. Third, the increase in the fusion activity in the *mucA22* Δ*rsmA* strain compared to that in the *mucA22* strain was greater than the increase in the Δ*rsmA* mutant of PAO1. Further support for RsmA activity in *mucA* mutant strains comes from the increased leader fusion activity of the *mucA22* Δ*rsmA* and *mucA22* Δ*retS* strains compared to that of the *mucA22* strain. An alternative explanation of our data is that some other form of posttranscriptional regulation occurs independently of RsmA. However, the *gacA* and *retS* mutants are known to regulate RsmA activity, even though *rsmA* is expressed in these strains (data not shown). We do not favor this alternative hypothesis, as previous studies have determined that GacA controls only *rsmY* and *rsmZ* expression (21, 49). Another posttranscriptional regulator, RsmF/RsmN, is also known to control *tssA1* expression (24, 53). This could possibly have affected the fusion results found in the present study as well and may explain the increased expression of the *tssA1* leader fusion in the *mucA*22 Δ*retS* strain. Regardless, the results still indicate increased posttranscriptional regulation of *tssA1* in a *mucA22* strain.

The results of a proteomic study of the isogenic clinical strains 383 and 2192 also support our findings. In this study, the mucoid strain 2192 has decreased amounts of HSI-I T6SS components, whereas the nonmucoid strain has increased HSI-I T6SS components (54). If the level of RsmA was increased due to AlgU in mucoid strain 2192, as presented here (Fig. 2.6), then the proteomic analysis suggests that RsmA is responsible for the decreased amounts of HSI-I T6SS components in mucoid strain 2192. We are currently examining RsmA activity in other clinical isolates.

However, a previous study did not find that RsmA posttranscriptionally regulates *tssA1* in

a *mucA* mutant (14). A possible reason for the discrepancy between the findings of that study and those of our study is that different strains and growth conditions were used. In addition, the previous study found that *rsmY* and *rsmZ* expression are increased in a PA103 *mucA* mutant (14). We analyzed *rsmY* and *rsmZ* expression using transcriptional fusions and also found the same result (data not shown). It is not known how RsmA functions if RsmY and RsmZ levels are increased, and this requires further investigation. One possibility is that the promoter fusions do not accurately monitor the levels of the small RNAs. We are directly measuring RsmY and RsmZ levels in a *mucA* mutant strain to determine if this is the case. If RsmY and RsmZ levels are increased and there is still RsmA activity, then we speculate the higher-affinity target mRNAs present compete with RsmY and RsmZ. Alternatively, other factors that appropriately direct RsmA to its targets, even in the presence of RsmY and RsmZ, exist.

The *P. aeruginosa mucA* mutant strains provide a snapshot of some of the bacteria found in the lungs of patients with cystic fibrosis during chronic infection. The use of *mucA* mutant strains may help provide an understanding of the factors required for persistence and adaptation in the lung environment. For instance, the overproduction of alginate by *mucA* mutant strains provides a competitive advantage by sequestering free radicals, preventing complement attack, and inhibiting phagocytosis (55). Our data suggest that RsmA also plays a role in chronic infections, such as those that occur in the lungs of cystic fibrosis patients. The RsmA regulon may have additional targets in *mucA* mutant backgrounds that have not yet been discovered. An understanding of RsmA in both acute and chronic infections may provide new ways to combat *P. aeruginosa*.

ACKNOWLEDGEMENTS

Thank you to the Department of Health Sciences at East Tennessee State University and

its Chair, Dr. Ranjan Chakraborty, for providing reagents, advice, and most importantly funds for this work. We thank Joseph and Julia Albright for providing equipment and supplies for these studies. We thank Joanna Goldberg (Emory University) for plasmid construct pTJ1. We thank Laraine Powers and David Hurley (ETSU) for critical review of the manuscript. We thank Eric Jones for expert technical assistance.

FUNDING INFORMATION

This work, including the efforts of Chris L. Pritchett, was funded by Research and Development Committee, ETSU (RDC12-012M and RDC13- 005M).

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CHAPTER 3

THE *PSEUDOMONAS AERUGINOSA* TWO-COMPONENT REGULATOR ALGR DIRECTLY ACTIVATES *RSMA* EXPRESSION IN A PHOSPHORYLATION-INDEPENDENT MANNER

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Keywords: *P. aeruginosa*, RsmA, AlgR, mucoid, *Pseudomonas aeruginosa*, cystic fibrosis, *mucA*, two-component regulatory systems

ABSTRACT

Pseudomonas aeruginosa is an important pathogen of the immunocompromised, causing both acute and chronic infections. In cystic fibrosis (CF) patients, *P. aeruginosa* causes chronic disease. The impressive sensory network of *P. aeruginosa* allows the bacterium to sense and respond to a variety of stimuli found in di- verse environments. Transcriptional regulators, including alternative sigma factors and response regulators, integrate signals changing gene expression, allowing *P. aeruginosa* to cause infection. The two-component transcriptional regulator AlgR is important in *P. aeruginosa* pathogenesis in both acute and chronic infections. In chronic infections, AlgR and the alternative sigma factor AlgU activate the genes responsible for alginate production. Previous work demonstrated that AlgU controls *rsmA* expression. RsmA is a posttranscriptional regulator that is antagonized by two small RNAs, RsmY and RsmZ. In this work, we demonstrate that AlgR directly activates *rsmA* expression from the same promoter as AlgU. In addition, phosphorylation was not necessary for AlgR activation of *rsmA* using *algR* and *algZ* mutant strains. AlgU and AlgR appear to affect the antagonizing small RNAs *rsmY* and *rsmZ* indirectly. RsmA was active in a *mucA22* mutant strain using leader fusions of two RsmA targets, *tssA1* and *hcnA*. AlgU and AlgR were necessary for posttranscriptional regulation of *tssA1* and *hcnA*. Altogether, our work demonstrates that the alginate regulators AlgU and AlgR are important in the control of the RsmA posttranscriptional regulatory system. These findings suggest that RsmA plays an unknown role in mucoid strains due to AlgU and AlgR activities.

IMPORTANCE

P. aeruginosa infections are difficult to treat and frequently cause significant mortality in CF patients. Understanding the mechanisms of persistence is important. Our work has demonstrated that the alginate regulatory system also significantly impacts the posttranscriptional regulator system RsmA/Y/Z. We demonstrate that AlgR directly activates *rsmA* expression, and this impacts the RsmA regulon. This leads to the possibility that the RsmA/Y/Z system plays a role in helping *P. aeruginosa* persist during chronic infection. In addition, this furthers our understanding of the reach of the alginate regulators AlgU and AlgR.

INTRODUCTION

The opportunistic pathogen *Pseudomonas aeruginosa* possesses multiple virulence factors for causing disease. This allows *P. aeruginosa* to cause both acute and chronic infections. Acute infecting strains are characterized by the presence of type IV pili (T4P), flagella, and a type III secretion system (T3SS) (1–3). In contrast, chronic infecting strains diversify (4, 5) and frequently do not express T3SS, T4P, or flagella (6, 7). Chronic infecting strains often form biofilms composed of exopolysaccharides, such as alginate, and signal a decline in lung function in CF patients (8–11). Alginate biosynthesis requires activation of the alternative sigma factor AlgU (12, 13) and the two-component transcriptional regulator AlgR (12, 14, 15).

Two-component signal transduction systems are important in regulating the bacterial response to environmental conditions. AlgR controls both acute and chronic virulence genes (16–18). In the case of acute infections, AlgZ phosphorylates AlgR and activates the *fimU* operon encoding minor pilins important in pilus biogenesis (19–21). The production of alginate, indicative of chronic infection, does not require AlgR phosphorylation (22). This fact has been demonstrated by deletion of the histidine sensor kinase gene *algZ* (23) and by the expression of an *algR* mutant allele encoding an asparagine in place of the conserved aspartate (22).

In chronic *P. aeruginosa* infections, the bacteria frequently acquire spontaneous *mucA* mutations that free the alternative sigma factor AlgU (12, 14, 24–26). AlgU and AlgR regulate the alginate biosynthesis genes producing mucoid colonies (12). In addition, AlgR decreases the expression of acute virulence factors, such as the T3SS in chronic infections when *mucA* mutations occur (7, 27). Therefore, AlgR has multiple roles depending on the type of infection and the phosphorylation state of AlgR: phosphorylated in acute infections activating the *fimU*

operon and unphosphorylated in chronic infections activating alginate biosynthesis.

Previous work implicated the AlgZ/R system in the control of *rsmA* expression (28), but a mechanism for AlgR activation of *rsmA* was not investigated. RsmA is considered a global regulator that controls the expression of many *P. aeruginosa* genes by binding to mRNAs (29). Two noncoding RNAs, RsmY and RsmZ, counteract RsmA (29, 30). In this study, we sought to determine how AlgR activates *rsmA* and further investigate the role of RsmA in *P. aeruginosa* strains containing an *mucA* mutation. We further examined *rsmY* and *rsmZ* expression in *algR* mutant strains.

We demonstrate that AlgR directly activates *rsmA* expression in a phosphorylation-independent manner and that both AlgR and AlgU are important in activating *rsmA* expression in mucoid strains. The activity of RsmA is not thought to be significant in chronic infecting strains, such as *mucA* mutants. However, we also provide evidence that RsmA is active in mucoid *P. aeruginosa*. Altogether, our work shows that the two-component regulator AlgR can affect gene expression through RsmA, providing another mechanism for how AlgR impacts virulence gene regulation. We postulate that AlgR and RsmA likely function as a rheostat, as opposed to a switch, and suggest that RsmA plays a role in chronic infections.

MATERIALS AND METHODS

Strains, plasmids, and media.

The strains used in this study are presented in Table 1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics as appropriate.

Pseudomonas aeruginosa strains were grown on *Pseudomonas* isolation agar (PIA), LB, or Vogel-Bonner minimal medium (64) and supplemented with the appropriate concentration of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 μg/ml tetracycline, 15 μg/ml gentamicin, 100 μg/ml ampicillin, and 35 μg/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 μg/ml gentamicin, 50 μg/ml tetracycline, and 300 μg/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	**Genotype or relevant properties**[a]	**Reference or source**
E. coli strains		
NEB5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA ø80*Δ(*lacZ*)M15 *gyrA96*	New England BioLabs
pRK2013	Helper strain	67
Plasmids		
pEX18Tc	Allelic exchange vector	66
pEX18Gm	Allelic exchange vector	66
pEX18Tc Δ*rsmA*	Allelic exchange for *rsmA* nonpolar deletion	31
pEX18Tc Δ*algR*	Allelic exchange for *algR* nonpolar deletion	31
algZHSDM/pEX18Gm	Allelic exchange for making *algZ* mutant	This study
*rsmA*HA/pEX18Gm	Allelic exchange for *rsmA* HA allele	31
pGEX4T-3	GST fusion vector	GE Healthcare

pGEX4T-3 *algR*	GST fusion vector AlgR purification	This study
miniCTX*lacZ*	Transcriptional fusion vector	73
rsmATF1-*lacZ*	Transcriptional fusion	31
rsmATF3-*lacZ*	Transcriptional fusion	31
rsmATF2-*lacZ*	Transcriptional fusion	31
rsmYTF1-*lacZ*	Transcriptional fusion	This study
rsmYTF2-*lacZ*	Transcriptional fusion	This study
rsmYTF3-*lacZ*	Transcriptional fusion	This study
rsmZTF1-*lacZ*	Transcriptional fusion	This study
rsmZTF2-*lacZ*	Transcriptional fusion	This study
*lacUV5*CTXCP	Transcriptional fusion	This study
lacUV5 tssA-lacZ	Leader fusion vector	This study
lacUV5 hcnA-lacZ	*tssA1* leader fusion	This study
P. aeruginosa strains		
PAO1	Wild type	74
Δ*algR* mutant	*algR* mutant	75
D54N mutant	*algR* with asparagine instead of aspartate at residue 54 *algR*	This study
D54E mutant	*algR* with glutamate instead of aspartate at residue 54	This study
algZ mutant	*algZ* mutant	This study
mucA22 mutant	mucA22 mutant	76
mucA22 Δ*algR* mutant	*mucA22* Δ*algR* mutant	This study
*mucA22*D54N mutant	*mucA22* mutant strain with *algR* with asparagine instead of aspartate at residue 54	This study
*mucA22*D54E mutant	*mucA22* mutant strain with *algR* with glutamate instead of aspartate at residue 54	This study
mucA22 algZ mutant	*mucA22* mutant strain, *algZ* mutation	This study

Δ*rsmA* mutant	*rsmA* nonpolar deletion	31
mucA22 Δ*rsmA* mutant	*rsmA* nonpolar deletion in *mucA22* strain	31
Δ*rsmY* mutant	*rsmY* nonpolar deletion mutant	This study
Δ*rsmZ* mutant	*rsmZ* nonpolar deletion mutant	This study
PAO1 rsmATF1-l*acZ*	*rsmA* transcriptional fusion strain	31
Δ*algR* TF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	31
D54N rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
D54E rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
*mucA22*D54N rsmATF1-*lacZ*	*rsmA* transcriptional fusion strain	This study
mutant *mucA22*D54E rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
mucA22 algZ mutant	*rsmA* transcriptional fusion strain	This study
mucA22 TF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	*31*
mucA22 Δ*algR* rsmATF1-*lacZ* mutant	*rsmA* transcriptional fusion strain	*31*
PAO1 rsmATF3-*lacZ*	*rsmA* transcriptional fusion strain	31
Δ*algR* rsmATF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	31
mucA22 TF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	*31*
mucA22 Δ*algR* rsmATF3-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
PAO1 rsmATF2-*lacZ*	*rsmA* transcriptional fusion strain	This study
Δ*algR* rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study

mucA22 rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
mucA22 Δ*algR* rsmATF2-*lacZ* mutant	*rsmA* transcriptional fusion strain	This study
PAO1 rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
Δ*algR* rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
algZ rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 Δ*algR* rsmYTF1-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 algZ mutant	*rsmY* transcriptional fusion strain	This study
PAO1 rsmYTF2-*lacZ*	*rsmY* transcriptional fusion strain	This study
Δ*algR* rsmYTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
mucA22 Δ*algR rsm*YTF2-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study

PAO1 rsmYTF3-*lacZ*	*rsmY* transcriptional fusion strain	This study
mucA22 rsmYTF3-*lacZ* mutant	*rsmY* transcriptional fusion strain	This study
PAO1 rsmZTF1-*lacZ*	*rsmZ* transcriptional fusion strain	This study
Δ*algR* rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
algZ rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 Δ*algR* rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
mucA22 algZ rsmZTF1-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
PAO1 rsmZTF2-*lacZ*	*rsmZ* transcriptional fusion strain	This study
mucA22 rsmZTF2-*lacZ* mutant	*rsmZ* transcriptional fusion strain	This study
PAO1HA	PAO1 with epitope-tagged RsmA	31
Δ*algR*HA mutant	Δ*algR* mutant with epitope-tagged RsmA	This study

*mucA22*HA mutant	*mucA22* mutant with epitope-tagged RsmA	*31*
mucA22 Δ*algR*HA mutant	*mucA22* Δ*algR* mutant with epitope-tagged RsmA	This study
PAO1 *lacUV5 hcnA-lacZ* mutant	PAO1 with *hcnA* leader fusion	This study
mucA22 lacUV5 hcnA-lacZ	*mucA22* mutant with *hcnA* leader fusion	This study
mucA22 Δ*rsmA lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*rsmA* mutant with *hcnA* leader fusion	This study
mucA22 Δ*algU lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algU* mutant with *hcnA* leader fusion	This study
mucA22 Δ*algR lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algR* mutant with *hcnA* leader fusion	This study
PAO1 *lacUV5 tssA1-lacZ*	PAO1 with *tssA1* leader fusion	This study
mucA22 lacUV5 tssA1-lacZ mutant	*mucA22* mutant with *tssA1* leader fusion	This study
mucA22 Δ*rsmA lacUV5 tssA1-lacZ* mutant	*mucA22* Δ*rsmA* mutant with *tssA1* leader fusion	This study
mucA22 Δ*algU lacUV5 tssA1-lacZ* mutant	*mucA22* Δ*algU* mutant with *tssA1* leader fusion	This study
mucA22 Δ*algR lacUV5 hcnA-lacZ* mutant	*mucA22* Δ*algR* mutant with *tssA1* leader fusion	This study

Mutant construction

All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs). Crossover PCR (SOE'ing) (65) was used to construct deletion mutations and to clone into the suicide vector pEX18Tc or pEX18Gm (66). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (67). Conjugations were performed overnight on LB plates at 30°C, and conjugations were plated for single-crossover mutants on the appropriate selective media. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR shown in Table 3.2 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of proteins was accomplished using primers containing the HA tag at the 3' end of the gene and introduced as described above using the suicide vector pEX18Gm.

Table 2. Primers used in this study.

Primer Name	Sequence	Use
algRTJ1F2	GCGCCCATGGGAATGAATGTCCTGATTGTCGATGAC	Overexpression
algRTJ1R	GCGCAAGCTTTCAGAGCTGATGCATCAGACGC	Overexpression
algRintF	GCAACTGGACTGGCAGGTGC	Mutant
algRintR	CGCGACTGGTCATCGGCAG	Mutant
algRBamHIR	GCGCGGATCCGTCAGAGCTGATGCATCAGACG	Mutant
algZHindIIIF	GCGCAAGCTTCTCTCGCTGCAACAAGAAACGG	Mutant
algZSDMcheckF	CAGCTGGGCGGAGAACTGAC	Mutant
algRD54XbaIF	AACATCCGCATGCCCGGTCTAGACGGC	Mutant

algRD54NR	CAGCAGGACGATATCGGGCTTGA	Mutant
algRD54EF	GTCCTTCTAGAGATCCGCATGC	Mutant
algRD54ER	GATATCGGGCTTGAGGCTGTCG	Mutant
algZHSDMF	GAATTCCTGTTCAACAGCCTGAACAG	Mutant
algZHSDMR	CGGGCGAATCCGCGCCTGCA	Mutant
rsmARXbaI	GCGCTCTAGAGCACGGTGATCCTGCAGACC	Mutant
rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGG G	Mutant
rsmASDMcheckF	GCCAAGGTTTCCATCGTCGG	PCR check
rsmAHAF	TACCCATACGATGTTCCAGATTACGCTTA ATTTTTATCTAATTTTCCCTTTGC	HA allele
rsmAHAR	TTAAGCGTAATCTGGAACATCGTATGGGT AATGGTTTGGCTCTTGATCTTTCTC	HA allele
rsmASDMAlgUF	CAGAATTCTTCATTCCGGCGGGACTG	SDM
rsmASDMAlgUR	CCAAACGGTCAAAAACGACAAAAG	SDM
rsmYRPART72	ATGCTAATACGACTCACTATAGGGAGACTG ACATCCGTGCTACGCCAC	RPA
rsmYRPAF	CTTGGACGTCAGGACATTGCGCAGGAAG	Northern
rsmYT7RPAR	TAATACGACTCACTATAGGGAGACAATAAA AAACCCCGCCTTTTGGG	Northern
rsmZRPAT72	ATGCTAATACGACTCACTATAGGGAGAGTAT TACCCCGCCCACTCTTCA	RPA
rsmZRPAF	GGGCCCCACTCCTGCGTACAGGGAACAC	Northern
rsmZRPART7	TAATACGACTCACTATAGGGAGACAAAAAA AAGGGGCGGGGTATTAC	Northern
rsmYTFFEcoRI	GCGCGAATTCGTGTTGCCGTCGGTCCGGC	TF
rsmYTFRBamHI	GCGCGGATCCTTCCTGCGCAATGTCCTGACG	TF
rsmYTFFUAS	GCGCGCGGCCGCCAGCGTGTAAGCCAAGGC TTAC	TF/RPA
rsmYshortEcoRIF	GCGCGAATTCAAACGGCGGTGGTTTTGGCTG	TF
dnrTFRBamHI	GCGCGGATCCCCATGCTGGGAAGGCTCGC	TF
rsmZKpnIF	GCGCGGTACCCGTCTGGCGCAGAAGGGCG	Mutant
rsmZXbaIR	GCGCTCTAGAGAGCGAAACCGCCAACATCC	Mutant
rsmZSOEF	GTTTTCTGGCAGGTTGCGGGATTTGCCTGCC GTTTTACTCGTC	Mutant
rsmZSOER	GACGAGTAAAACGGCAGGCAAATCCCGCAA CCTGCCAGAAAAC	Mutant
rsmZIntF	GCGCGCGGCCGCACCGATCCGTGCGAGCTG	Mutant
rsmZintR	CATGCAGGAATTCATCGAGCTG	Mutant
rsmYKOF	CGCGGAGCTCCTGTTCACTCGAAGCACTCCA G	Mutant
rsmYKOR	CGCGTCTAGATTCGCCAACTCCGCTATTTCG	Mutant
rsmYSOEF	GCGTAATCTTCAAACCGTCAGGATCCTGCGG	Mutant

	CCCGAGGAAAACCGCGTCG	
rsmYSOER	CGACGCGGTTTTCCTCGGGCCGCAGGATCCT GACGGTTTGAAGATTACGC	Mutant
rsmYintF	CCTGGAGCTGGACGGGAG	Mutant
rsmYintR	GGAATTCAGGAAGGTGTCCC	Mutant
tssA1annealF	GATCCTTCGATGATAGGGAGATCGTCACCGT GCTG	Leader fusion
tssA1annealR	CAGCACGGTGACGATCTCCCTATCATCGAAG	Leader fusion
5SrRNAI	TTGGACAGGATGGGGTTGGA	Northern
5SrRNA2T7	TAATACGACTCACTATAGGGA GACGATTGTGTGTTGTAAGG	Northern
lacZScaIF	GCTATGACCATGATTAGTACTGATTCACTGG CCGTC	TLF vector
lacZRXhoI	CCCCTCGAGCAGACATGGCCTGCCCGGTTAT TA	TLF vector
lacZRforTF	GATGTGCTGCAAGGCGATTAAG	SEQ
pHERDSF	ATCGCAACTCTCTACTGTTTCTC	SEQ
rsmASDMAlgRF	GAATTCGGCAGGAACTTTCATTCCGGC	RPA
rsmARPAR	TAATACGACTCACTATAGGGAGAGATAAAA ATTAATGGTTTGGCTCTTG	RPA
rsmARSDMF	CCTTTTGTCGTTTTTGAGAATTCGGCAGGAA CTTTCATTC	Gel Shift
rsmARSDMR	GAATGAAAGTTCCTGCCGAATTCTCAAAAAC GACAAAAGG	Gel Shift
algDBS1F	CGCCTCCTGGCGCTACCGTTCGTCCCTCCGA CACCCCTGCTGCGTCGC	Gel Shift
algDBS1R	GCGACGCAGCAGGGGTGTCGGAGGGACGAA CGGTAGCGCCAGGAGGCG	Gel Shift
rsmARBS1F	GCGCCTTTTGTCGTTTTTGACCGTTTGGCAG GAACTTTCATTCCG	Gel Shift
rsmARBS1R	CGGAATGAAAGTTCCTGCCAAACGGTCAAA AACGACAAAAGGCGC	Gel Shift
rsmYRBS1F	CGGCTGGGTGTTGCCGTCGGTCCGGCGAGCG GAACTATTACAGCG	Gel Shift
rsmYRBS1R	CGCTGTAATAGTTCCGCTCGCCGGACCGACG GCAACACCCAGCCG	Gel Shift
pscFRBSF	GCTGGCGGAGTGTCGCCGGGAACTGGCCAG AGG	Gel Shift

pscFRBSR	CCTCTGGCCAGTTCCCGGCGACACTCCGCCAGC	Gel Shift
rsmYEMSAR2	GGGCGGGGTTTTGCAGACCT	Gel Shift
rsmZEMSAR2	GTATTACCCCGCCCACTCTTCA	Gel Shift
rsmAPE1F	GCGCGGTACCAAGGATCGCGCTCTTGATTTCTGCGGATCCGCCGCCATTTCTT	Gel Shift
rsmAPE3F	GCGCGGTACCGCTGACAGGCGAAAGGCG	Gel Shift
rsmAPE3R	GCGCGGATCCTCACCCAGTATTGACCAGTCC	Gel Shift
rsmAEcoRIR	GCGCGAATTCACGAGTCAGAATCAGCATTCCTTTC	Gel shift
rsmAR1SDMannealF	CCGCGGAATTCGTCGTTTTTGACCGTTTGGCAGGAACTTTC	Gel Shift
rsmAR1SDMannealR	GAAAGTTCCTGCCAAACGGTCAAAAACGACGAATTCCGCGG	Gel Shift
rsmAR2SDMannealF	CCGCGGAATTCGTCGTTTTTGAGAATTCGGCAGGAACTTTC	Gel Shift
rsmAR2SDMannealR	GAAAGTTCCTGCCGAATTCTCAAAAACGACGAATTCCGCGG	Gel Shift
rsmAGSWTannealF	CCGCGCCTTTTGTCGTTTTTGACCGTTTGGCAGGAACTTTC	Gel Shift
rsmAGSWTannealR	GAAAGTTCCTGCCAAACGGTCAAAAACGACAAAAGGCGCGG	Gel Shift

Abbreviations: TF, transcriptional fusion, TLF, translational fusion, LF, leader fusion, SEQ, sequencing primer; RPA, RNase protection assay

algR mutant construction

The wild-type *algR* genomic region was amplified using Q5 (NEB) and primers algRXbaIR and algRHindIIIF and cloned into pEX18Tc. Site-directed mutagenesis was performed using the algRD54XbaIF/algRD54NR primers for the D54N allele or the algRD54EF/algRD54ER primer pair for the D54E mutation. The *algZ* mutant was constructed using the algZHSDMF/algZHSDMR primers. The primers were phosphorylated and used in site-directed mutagenesis, in accordance with the manufacturer's instructions, using Q5 (NEB). Constructs were analyzed by restriction enzyme analysis and sequencing. Mutant strains were constructed using homologous recombination, as described above, and were checked using PCR and the algRintF/algRintR primer pair and digestion with the appropriate restriction enzyme.

Additional PCR amplicons were sequenced to confirm the mutation in each strain using the same primers. Further confirmation of mutants was done using phenotypic assays.

Transcriptional and translational leader fusion analysis

Upstream DNA fragments containing promoter regions were generated by using primers listed in Table 1 in conjunction with Q5 polymerase (New England BioLabs). PAO1 genomic DNA was used as the template. PCR products were cloned into pMiniT (NEB) and then subcloned into miniCTX*lacZ* using the restriction enzymes HindIII and BamHI, HindIII and EcoRI, or KpnI and BamHI (NEB). The *rsmA* transcriptional fusions have been previously described (31). To construct *rsmY* and *rsmZ* transcriptional fusions, the rsmYTFFEcoRI/rsmYTFR and rsmZTFF/rsmZTFR primer pairs, respectively, were used. PCR products were purified, cut with restriction enzymes, and inserted into the EcoRI and BamHI sites of miniCTX*lacZ* using T4 DNA ligase (NEB). The leader fusion vector was constructed by annealing the lacUV5NotI/lacUV5BamHI primer pair together and cloning into the NotI/BamHI site of CTXCP (31). Translational/leader fusions were constructed using the tssA1annealF/R and hcnAannealF/R primer pairs (Table 1) and were cloned into the ScaI/BamHI site of the leader fusion vector. Fusion constructs were confirmed by sequencing and conjugated into *P. aeruginosa* strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with pFLP2 to remove vector sequences (66). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch-plated onto VBMM CB300 and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 1). b -Galactosidase activity was determined by incubating cell extracts with

o-nitrophenyl- b -D-galactopyranoside (ONPG) (4 mg/ml), as described by Miller (68). A strain carrying the empty vector miniCTX*lacZ* was also conjugated into PAO1 and assayed, and this background (28 Miller units) was subtracted from all transcriptional fusions. The translational/leader fusion backbone CTXCPlacUV5 had no background activity. All mucoid strains were confirmed mucoid at the end of each experiment by plating on PIA plates to ensure all colonies were mucoid. Three biological replicates were reproduced for all assays.

AlgR purification

The *algR* gene was PCR amplified using Q5 (NEB) and PAO1 chromosomal DNA using oligonucleotides algRSal1F and algRNot1R (Table 1). A 754-bp SalI/NotI fragment was cloned into pGEX-4T-3 (Novagen) to be expressed as a glutathione *S*-transferase–AlgR (GST-AlgR) fusion protein. The resulting plasmid (pGEX-4TAlgR) was transformed into *E. coli* BL21(DE3) (NEB) cells and incubated overnight. The colonies from this transformation were collected, inoculated into LB supplemented with 100 μg/ml ampicillin, and grown to an optical density at 600 nm (OD_{600}) of 0.6; 0.2 mM isopropyl-b-D- galactopyranoside (IPTG) was added to induce AlgR expression for 4 h at 15°C. The cells were collected by centrifugation (6,740 x *g*, 15 min), washed once in 20 ml of phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS containing protease inhibitors (Thermo Fisher). AlgR was purified from this supernatant using the GST spin purification kit (Thermo Fisher). After binding of the fusion protein and washing the column, AlgR was cleaved away from GST with 10 U of thrombin/column overnight at 22°C. Purified protein was dialyzed using a Slide-A-Lyzer (Thermo Fisher) and storage buffer (20% glycerol, 20 mM Tris [pH 7.5], 5 mM $MgCl_2$, and 1 mM dithiothreitol [DTT]) overnight at 22°C. The identity of the purified protein was determined by mass spectrometry. The purity of

AlgR was visually determined in a Coomassie-stained 7.5% electrophoresis gel (SDS-PAGE).

EMSAs

Purified AlgR was used in electrophoretic mobility shift assays (EMSAs) using either PCR amplicons or annealed primers using the LightShift kit, in accordance with the manufacturer's instructions (Thermo Fisher Scientific). For *rsmA*, the rsmAPE3F/rsmAPE3R, rsmAPE1F/rsmAEcoRIR, or rsmAPE3F/ rsmAEcoRIR primer pair was used to produce PCR amplicons using *Taq* polymerase (NEB). Amplicons were gel extracted and biotinylated using the 3' biotinylation kit (Thermo Fisher Scientific). Annealed primers were heated to 95°C for 5 min and cooled to room temperature. The annealed primers were biotinylated and incubated with purified AlgR. Purified AlgR was incubated at increasing concentrations to determine the suitable concentrations to be used. The nonspecific competitor poly(dI-dC) at 25 ng/µl was used for all gel shift reactions. DNA and protein were electrophoresed through 5% or 10% native polyacrylamide gels, transferred to a nylon membrane using a semidry apparatus (Hoefer), UV cross- linked, developed using the chemiluminescence detection kit (Thermo Fisher Scientific), and visualized using a charge-coupled-device (CCD) camera (ProteinSimple). The gel shift assays were repeated at least two times.

Western blot analysis

P. aeruginosa strains were grown in LB at 37°C for 8 h. The bacteria were collected by centrifugation, resuspended in sterile PBS, and lysed by sonication using a Branson sonifier. Total protein concentrations were determined by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 to 10 µg) were separated by 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using

a 1:2,500 dilution of anti-HA monoclonal antibody in blocking buffer (Thermo Fisher, Pittsburgh, PA), followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (69). Densitometry of Coomassie-stained SDS-PAGE gels was used to standardize the Western blots using total protein analysis, as described previously (70, 71). Detection was performed using the ECL Plus kit (Thermo Fisher) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). The blots and Coomassie-stained SDS-PAGE were analyzed using ImageJ analysis. All Western blotting was repeated at least four times.

Primer extension

Total RNA (15 µg) from *P. aeruginosa* was isolated using the PureLink RNA minikit (Life Technologies, Grand Island, NY). Radiolabeled primers rsmYPE1 and rsmZPE1 (see Table 1) were used in a reverse transcription reaction using ThermoScript (Life Technologies). A temperature of 42°C was used for the extension. The same primer used for primer extension was also used in a sequencing reaction with the *rsmY* or *rsmZ* upstream sequence in pGEM-T (Promega, Madison, WI) using the Sequenase 7-deaza-deoxy-GTP (7-deaza-dGTP) kit (Affymetrix, Cleveland, OH). Both primer extensions and sequencing reactions were run on a 6% acrylamide-8 M urea gel. The gel was dried and extension products detected using autoradiography.

In vitro transcription and RPA

The *rsmA*, *rsmY*, and *rsmZ* probes for RNase protection assay (RPA) were synthesized using a PCR product generated with primers that incorporated a T7 promoter sequence at the 5= end and using the MAXIscript T7 kit (Life Technologies) (Table 1). The reverse primer containing the T7 promoter sequence contained a nonhomologous sequence to discriminate

between the full- length probe and protected fragments. The primers rsmASDMAlgRF and rsmARPAR were used to produce the PCR product for *rsmA*. The rsmYRPAT&R/rsmYRPAF primer pair for *rsmY* or rsmZRPAT7RPAF/ rsmZRPAF primer pair for *rsmZ* was used to produce probes for the appropriate target. Probes were labeled by biotin-11-UTP (Life Technologies) at a ratio of 4:6 with UTP and were gel purified after *in vitro* transcription. Five micrograms of total RNA from each *P. aeruginosa* strain was precipitated with 800 pg of labeled probe and resuspended in hybridization buffer [80% formamide, 400 mM NaCl, 40 mM piperazine-*N*,*N*=-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA], heated 95°C for 5 min, and incubated overnight at 42°C. Negative controls using the probe incubated with 10 µg of yeast RNA were also included. Digestion of unhybridized RNA was performed using RNase A at 25 mg/ml and RNase T1 at 10 U/ml. A biotinylated RNA ladder (Kerafast) was also used to determine the size of protected probe fragments. The reactions were run on a 5% acrylamide-8 M urea gel for *rsmA* and a 10% acrylamide-8 M urea gel for *rsmY* and *rsmZ*. RNA was transferred to a positively charged membrane (Hybond N+; Amersham Biosciences) using a semidry blotting system and 0.5X Tris-borate-EDTA (TBE; Hoefer, Holliston, MA). Nucleic acid was UV cross-linked and probes detected using the chemiluminescent nucleic acid detection module (Thermo Scientific). After washing, the blot was incubated for 5 min and developed using a FluorChem M (ProteinSimple). RNase protection assays were performed at least three times.

Northern blot analysis

Total RNA was extracted from strains using the PureLink RNA minikit (Thermo Fisher). RNA was quantitated using a NanoDrop (Thermo Fisher). Two to 5 µg of total RNA was electrophoresed through a 10% acrylamide-8 M urea gel using formaldehyde-loading buffer. After electrophoresis, RNA was transferred using a semidry blotter (Hoefer, Holliston, MA) at

300 mAmp for 45 min and the membranes cross-linked using UV light (Bio-Rad). Probes produced by *in vitro* transcription were biotin labeled and hybridized to the membrane using ULTRAhyb buffer (Thermo Fisher) at 65°C overnight. The primers used to make probes for *rsmY* and *rsmZ* are listed in Table 2. For *rsmY*, the rsmYRPAF/rsmYT7RPAR primer pair was used. For *rsmZ*, the rsmZRPAF/rsmZRPART7 primer pair was used. Membranes were washed with two 5-min low-stringency washes at room temperature and 2 high-stringency washes for 15 min at 65°C. Blots were developed using the chemiluminescence detection kit (Thermo Fisher), in accordance with the manufacturer's instructions. For normalization, 5S RNA probes (Table 2) made by *in vitro* transcription were used using primers 5SrRNAI and 5SrRNA2T7. Northern blotting was repeated more than three times.

Statistical analyses

Statistical analyses were performed using Graph- Pad Prism (version 6.0) software (GraphPad Software, La Jolla, CA). The results of transcriptional and translational fusions and densitometry analysis were compared using a one-way analysis of variance (ANOVA) with Tukey's posttest. ImageJ software was used for Western blot analysis.

RESULTS

mucA mutant strains require AlgR for increased *rsmA* expression

Previous studies suggested that AlgU and AlgR control *rsmA* expression (28, 31). However, whether both AlgZ and AlgR were involved in the control of *rsmA* expression was not tested. We constructed and assayed an *rsmA* transcriptional fusion (Fig. 3.1A) that contains both *rsmA* promoters (rsmATF1-*lacZ*). The rsmATF1-*lacZ* fusion was analyzed in the wild-type

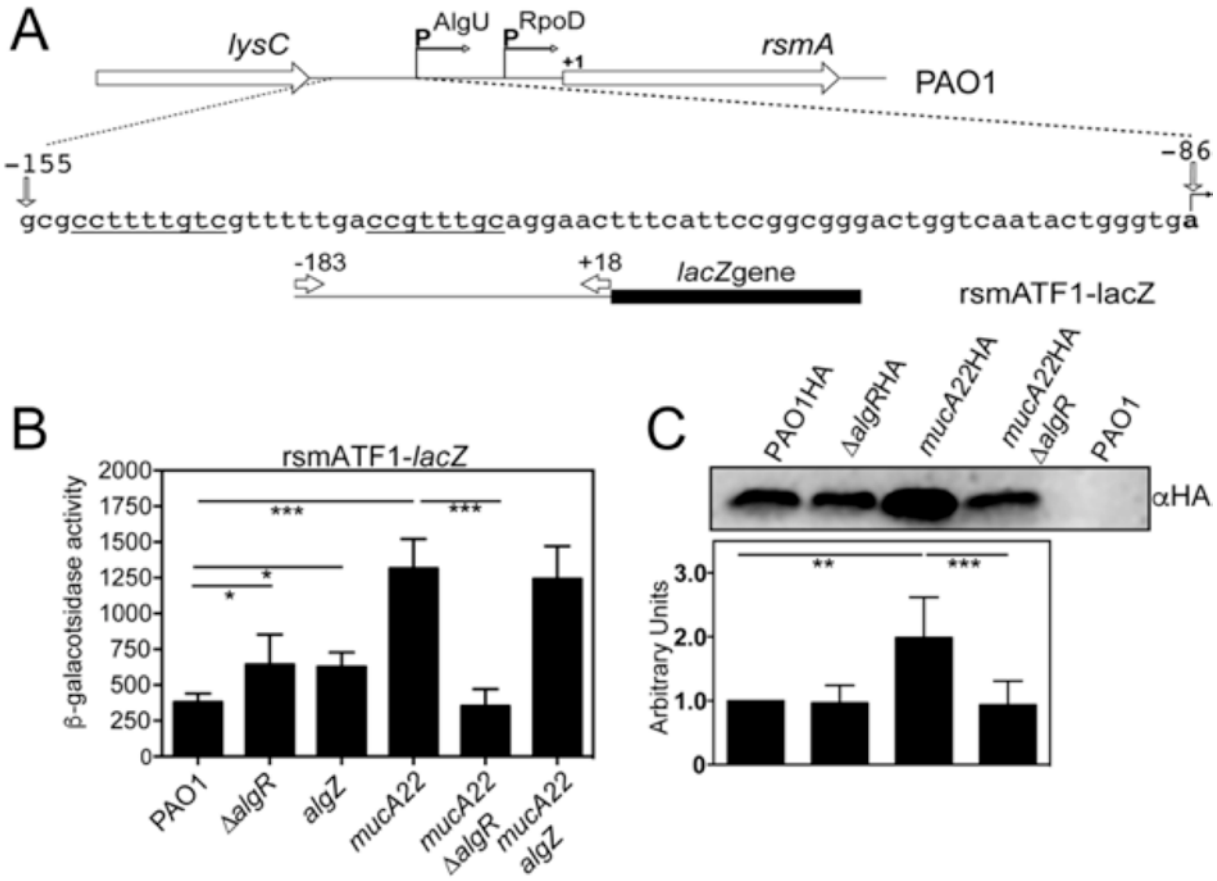


Figure 3.1 AlgR, but not AlgZ, is necessary for increased *rsmA* expression in mucoid strains. (A) Schematic of *rsmA* genomic region. The sequence below the genomic schematic indicates the AlgU-dependent promoter region. Underlined sequences are potential AlgR-binding sites. The bent arrow and bold nucleotide indicate the transcriptional start sites. The *rsmA* transcriptional fusion rsmATF1-*lacZ* used in this study is listed below. Arrows indicate the primers used and the numbers indicate the primer locations relative to the translational start site. (B) The transcriptional fusion rsmATF1-*lacZ* (Fig. 1A) was assayed in the indicated strains after growth for 8 h in LB broth. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.01 (*) and <0.0001 (***). (C) Western blot analysis of the indicated strains containing an HA-tagged *rsmA* allele. PAO1 without the *rsmAHA* allele was run as a negative control. Densitometry analysis was performed using a duplicate gel and staining for total protein using Coomassie blue, and all strains were normalized to PAO1 containing the *rsmAHA* allele. Western blotting was performed four times, and densitometry analysis is indicated below the Western blot. A representative Western blot is shown above the densitometry. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. **, $P < 0.001$; ***, $P < 0.0001$.

strain *P. aeruginosa* PAO1, Δ*algR* and *algZ* mutants, and in the corresponding *mucA22* mutants.

The *algZ* mutant has a mutation in the conserved histidine residue, which prevents AlgZ-

mediated phosphorylation of AlgR (19, 32) but does not disrupt the internal *algR* promoter (33). As shown in Fig. 1B, there was a slight increase in rsmATF1-*lacZ* activity in a Δ*algR* mutant. When the rsmATF1-*lacZ* fusion was assayed in an *algZ* mutant strain, there was also a slight increase in reporter activity (Fig. 3.1B). The modest increase in reporter activity suggests that AlgZ and AlgR have a minor role in *rsmA* regulation in strain PAO1.

As previous studies also indicated a role for AlgU in regulating *rsmA* expression, the rsmATF1-*lacZ* transcriptional fusion was assayed in a *mucA22* strain, where AlgU is most active. Reporter activity was increased ~3-fold in a *mucA22* strain, as previously described (31) (Fig. 3.1B). However, in the *mucA22* Δ*algR* double mutant, there was a drastic decrease (~3-fold) in rsmATF1-*lacZ* activity (Fig. 3.1B). The *mucA22 algZ* mutant strain had rsmATF1-*lacZ* activity almost identical to that of the *mucA22* mutant strain (Fig. 3.1B). These data implicate AlgR but not AlgZ in *rsmA* regulation in a *mucA22* mutant strain and suggest that AlgR phosphorylation is not necessary for *rsmA* activation.

To confirm that AlgR affected *rsmA* expression, an epitope-tagged *rsmA* allele was introduced into the wild-type strain (PAO1), *mucA22* mutant, and respective *algR* mutant strains and analyzed by Western blot analysis. As shown in Fig. 3.1C, a slight decrease in RsmA levels in the Δ*algR* mutant strain was detected compared to the wild-type strain PAO1. As reported previously (31), a *mucA22* mutant strain had drastically increased RsmA levels (Fig. 3.1C). The *mucA22* Δ*algR* mutant strain had significantly decreased RsmA compared with the *mucA22* mutant strain (Fig. 3.1C). The Western blot analysis confirmed the transcriptional fusion analysis and supported a significant role for AlgR activating *rsmA* in the mucoid *mucA22* mutant strain but not in the nonmucoid strain PAO1.

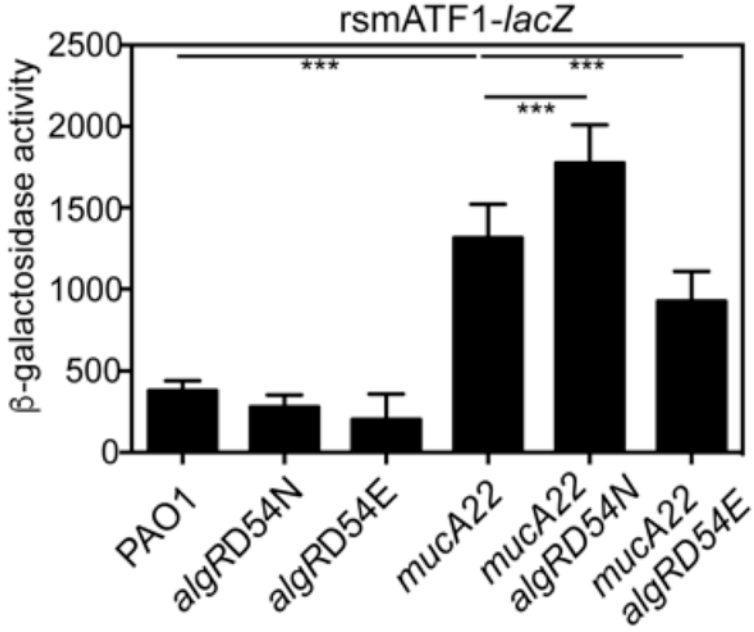


Figure 3.2 AlgR phosphorylation is not required for *rsmA* activation. Mutants containing either a mutated aspartate to asparagine (D54N) or aspartate to glutamate (D54E) were constructed in both the wild-type background PAO1 and the *mucA22* mutant background. The *rsmA* transcriptional fusion rsmATF1-*lacZ* was introduced into the strains indicated, resulting in single-copy chromosomal transcriptional fusions. The indicated strains were grown for 8 h in LB broth and assayed for b-galactosidase activity. Values indicate the actual b - galactosidase activity minus the vector control (~28 Miller units). Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001.

AlgR phosphorylation is not required for activation of *rsmA* expression

To further investigate the role of AlgR phosphorylation in the regulation of *rsmA*, *algR* site-directed mutants were constructed that mimic either the unphosphorylated (D54N) or phosphorylated form of AlgR (D54E). The *algR*D54N and *algR*D54E mutant alleles were also constructed in the *mucA22* mutant background to confirm a phosphorylation- independent mode of AlgR activation of *rsmA*. Both the *mucA22 algR*D54N and the *mucA22 algR*D54E mutant strains were mucoid (data not shown), supporting the notion that phosphorylation is not necessary for alginate production and that the mutant AlgR proteins produced in these strains were still functional. The *mucA22*D54N mutant had increased *rsmA* reporter activity compared to a *mucA22* mutant strain (Fig. 3.2). The *mucA22 algR*D54E strain had decreased rsmATF1-

lacZ activity compared to the *mucA22* mutant (Fig. 3.2). Because the *mucA22 algR*D54N and the *mucA22 algZ* mutant strains had elevated and similar levels, respectively, of rsmATF1-*lacZ* activity (Fig. 3.2 and 3.1B, respectively) compared to the *mucA22* mutant strain, this suggests that phosphorylation of AlgR is not required for *rsmA* activation.

AlgR regulates the distal *rsmA* promoter

Previous work determined that *rsmA* has two promoters (31). An RNase protection assay was performed using a probe spanning the upstream region of *rsmA* (Fig. 3.3A) to determine which *rsmA* message AlgR affected. As we previously reported (31), two *rsmA* messages were seen in both the wild-type strain PAO1 and the *mucA22* mutant strain, with the longer transcript increased in the *mucA22* mutant strain (Fig. 3.3B). There was little difference in the *rsmA* transcripts between PAO1 and the Δ*algR* mutant (Fig. 3.3B). In contrast, there was a substantial decrease in the longer *rsmA* transcript in the *mucA22* Δ*algR* mutant strain compared to the *mucA22* parent strain (Fig. 3.3B). The specificity of the *rsmA* probe was confirmed using the probe incubated with yeast tRNA (-RNase) and using a Δ*rsmA* mutant (Fig. 3.3B). These results suggest that AlgR is most important for *rsmA* control in the *mucA22* background and that AlgR controls the distal *rsmA* promoter that is also under control by AlgU.

To confirm that AlgR regulates the distal *rsmA* promoter, transcriptional fusions containing individual *rsmA* promoters were used (Fig. 3.3A; see also Fig. 3.S1A in the supplemental material). The transcriptional fusion rsmATF2-*lacZ* contains only the proximal *rsmA* promoter (Fig. 3.S1A). The transcriptional fusion rsmATF3-*lacZ* (Fig. 3.3A) contains only the distal promoter that has been shown to be controlled by AlgU (31). The deletion of *algR* had no effect in the rsmATF2-*lacZ* transcriptional fusion in the wild-type or a *mucA22* background

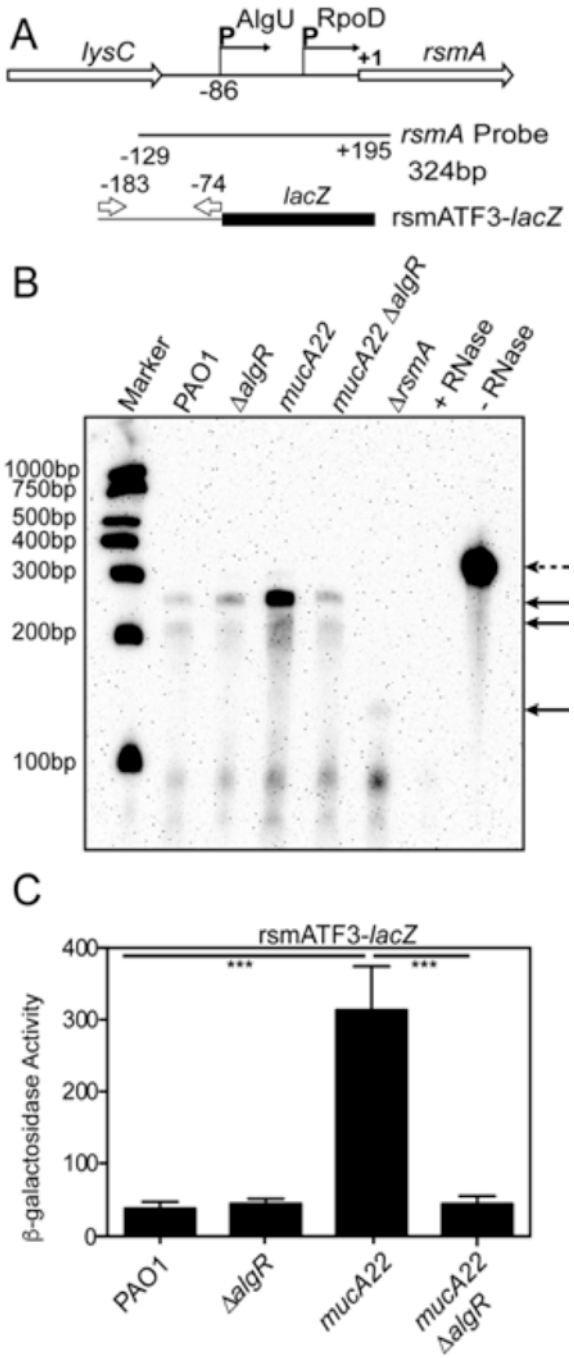


Figure 3.3 AlgR controls the distal AlgU-dependent *rsmA* promoter. (A) Schematic of the RNase protection assay probe used. Numbers indicate distances from the *rsmA* translational start site. The transcriptional fusion rsmATF3-*lacZ* is indicated at the bottom. Arrows indicate the primers used and their location in reference to the *rsmA* translational start site. (B) A representative RNase protection assay performed on the indicated strains after 8 h of growth in LB broth. Total RNA was isolated from each strain and hybridized to 800 pg of biotinylated *rsmA* probe. After treatment with RNase A/RNase T1, protected probe fragments were detected after electrophoresis through a denaturing polyacrylamide gel and transfer to a nylon membrane. Protected probe fragments are indicated by solid arrows. The broken arrow indicates a full-length probe. Biotinylated molecular size markers are indicated to the left. RNase protection assays were performed three times. (C) The *rsmA* transcriptional fusion rsmATF3-*lacZ* was introduced into the indicated strains to generate strains containing single-copy chromosomal fusions. The strains were grown for 8 h in LB broth and assayed for b -galactosidase activity minus the vector control. Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. Fusion analysis was performed in triplicate three times.

(Fig. 3.S1B). The transcriptional fusion rsmATF3-*lacZ* had increased reporter activity in a *mucA22* mutant strain compared to that in PAO1 (Fig. 3.3C). Compared to PAO1, no change in rsmTF3-*lacZ* activity was seen in a Δ*algR* mutant strain (Fig. 3.3C). However, *algR* deletion in a *mucA22* mutant strain (*mucA*22 Δ*algR*) resulted in a significant decrease in rsmATF3-*lacZ* activity (Fig. 3.3C), supporting the RNase protection assay. Taking these results together, we conclude that AlgR activates the distal *rsmA* promoter, and this further suggests that AlgU and AlgR both are required to activate *rsmA* transcription from the distal promoter.

AlgR directly binds the distal *rsmA* promoter

We hypothesized that AlgR directly activated the distal *rsmA* promoter. In support of this idea, two potential AlgR-binding sites are located upstream of *rsmA*. One of the potential AlgR-binding sites is 2 nucleotides away from the AlgU -35 consensus promoter (Fig. 3.4A). Purified AlgR was tested in gel shift studies with biotinylated *rsmA* promoter fragments (Fig. 3.4A). A biotinylated *pscF* gene, a component of the T3SS (34, 35), was used as a negative control and did not shift when incubated with purified AlgR (Fig. 3.4B to D). A PCR fragment representing the entire *rsmA* upstream region containing both promoters was dramatically shifted using purified AlgR (Fig. 3.4B), suggesting that AlgR directly binds to at least one of the AlgR-binding sites upstream of *rsmA*. To further define the region upstream of *rsmA* that was bound by AlgR, PCR amplicons that corresponded to the transcriptional fusion constructs containing individual *rsmA* promoters (Fig. 3.4A) were biotinylated and tested via gel shift analysis. A biotinylated probe containing the proximal promoter (TF2) and upstream sequence to the more distal transcriptional start site did not shift when incubated with purified AlgR (Fig. 3.4B). This result suggested that AlgR does not bind the proximal *rsmA* promoter. As shown in Fig. 3.4B,

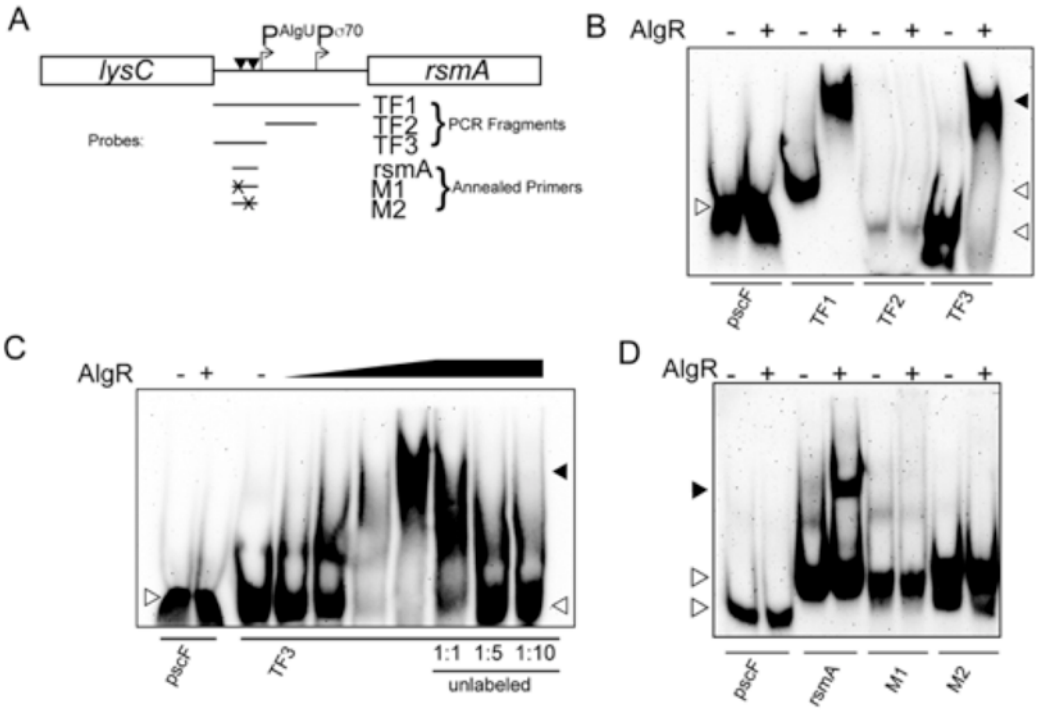


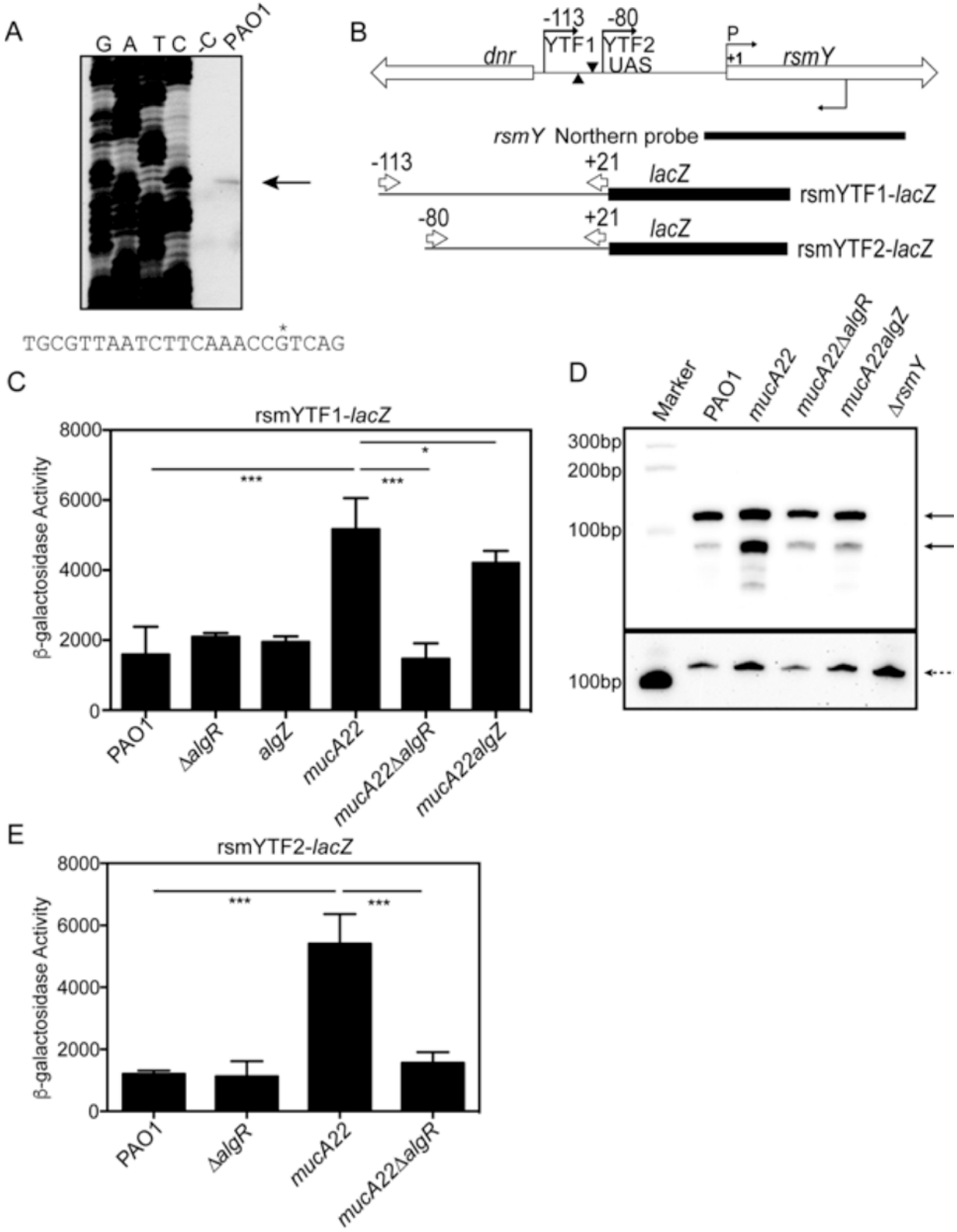
Figure 3.4 AlgR directly binds the AlgU-dependent *rsmA* promoter and requires both AlgR-binding sites. Purified AlgR was incubated with either biotinylated PCR products or annealed primers. (A). Schematic of the *rsmA* genomic region. Below are fragments or annealed primers and the approximate location upstream of *rsmA*. Promoters are indicated above as bent arrows and are denoted by P^{AlgU} or P^{RpoD}. The arrowheads indicate the approximate location of the putative AlgR-binding sites. (B) Analysis of biotinylated PCR fragments in gel shift studies. The PCR fragment TF1 represents the entire *rsmA* upstream region (see panel A). Fragments TF2 and TF3 correspond to the separated *rsmA* promoters (see Fig. 1). TF2 corresponds to the proximal *rsmA* promoter, and TF3 corresponds to the distal AlgU-dependent promoter. A minus sign indicates probe alone. A plus sign indicates AlgR concentration of 2.5 μM. *pscF* was used as a negative control. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. (C) Titration and competition assay using purified AlgR and the TF3 fragment corresponding to the distal *rsmA* promoter. The *pscF* fragment was used as a negative control. TF3 was incubated with different concentrations of AlgR (indicated by the graded triangle; 0.05, 0.25, 1.25, and 2.5 μM). TF3 was also used in a competition assay using unlabeled TF3 as a competitive inhibitor. Ratios below indicate the ratio of labeled to unlabeled probe. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. A minus sign indicates probe alone. (D) Two AlgR-binding sites are upstream of the distal *rsmA* promoter. Annealed primers containing either wild-type or two different mutated AlgR-binding sites were tested in gel shift experiments. The *pscF* fragment was used as a negative control. The *rsmA* lanes indicate the probe using annealed primers with both AlgR-binding sites intact. M1 indicates the same as *rsmA*, except that the furthest upstream AlgR-binding site was mutated. M2 indicates the same as *rsmA*, except that the further downstream AlgR-binding site is mutated. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. Minus signs indicate probe alone. Plus signs indicate an AlgR concentration of 2.5 μM.

AlgR bound the distal promoter sequence fragment, TF3, containing the AlgU-dependent promoter and potential AlgR-binding sites. The TF3 fragment containing the AlgU promoter was tested in a competition assay using increasing concentrations of AlgR and unlabeled probe. A 1:1 ratio of labeled to unlabeled probe was capable of decreasing the shift of the TF3 probe (Fig. 3.4C). Competition with the unlabeled specific probe in a 1:5 or 1:10 ratio was able to compete for AlgR binding, as indicated by severe reduction of the probe shift (Fig. 3.4C). These data suggest that AlgR specifically binds the AlgU- dependent promoter region and strongly suggests that AlgR binds at least one of the AlgR consensus sequences located in this promoter region.

AlgR has previously been shown to bind an 11-bp consensus sequence, with nucleotides 2 to 10 being the most conserved (36). To determine if one, or both, of the putative AlgR-binding sites were required for AlgR binding, we used annealed primers upstream of the distal promoter in gel shift analyses. Approximately 30-bp primers were annealed and biotinylated that contained the predicted AlgR-binding sites. AlgR was able to shift the annealed biotinylated primers containing the two predicted AlgR-binding sites (Fig. 3.4D). The first putative AlgR-binding site, CCTTTTGTC, was mutated, and this mutation resulted in a loss of AlgR binding (Fig. 3.4D), suggesting that the first AlgR-binding site is required for AlgR binding to the *rsmA* promoter. The second putative AlgR-binding site, CCGTTTGGC, is located 7 bp downstream and directly adjacent to the AlgU -35 consensus sequence. When the second AlgR-binding site was mutated, AlgR was no longer able to bind to the annealed primers (Fig. 3.4D). These data suggest that AlgR binds both of the consensus sequences in order to activate *rsmA* expression.

AlgR regulates *rsmY* expression indirectly

A previous study indicated that *rsmY* and *rsmZ* expression was also increased in a *mucA*


A
G A T C C PAO1
TGCGTTAATCTTCAAACCGTCAG
B
-113
-80
YTF1
YTF2
UAS
P
+1
dnr
rsmY
rsmY Northern probe
-113
+21
lacZ
rsmYTF1-lacZ
-80
+21
lacZ
rsmYTF2-lacZ
C
rsmYTF1-lacZ
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8000
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PAO1
ΔalgR
algZ
mucA22
mucA22ΔalgR
mucA22algZ
D
Marker
PAO1
mucA22
mucA22ΔalgR
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rsmYTF2-lacZ
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8000
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PAO1
ΔalgR
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mucA22ΔalgR

Figure 3.5 AlgR indirectly increases *rsmY* expression in a *mucA22* mutant strain. (A) Primer extension analysis performed on total RNA from strain PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the *rsmY* promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the *rsmY* genomic region. The UAS is indicated between the numbers from the transcriptional start site. The bent arrows above indicate the primers used for the transcriptional fusions, and the numbers correspond to the distance from the transcriptional start site. P and the bent arrow indicate the start site of transcription. The arrowheads represent potential AlgR-binding sites. The bent arrow below indicates the primer used in the primer extension experiment. Indicated below is the probe generated by *in vitro* transcription for Northern analysis. Transcriptional fusions are indicated below. Numbers above the arrows indicate the distance from the transcriptional start site. (C) Transcriptional fusion rsmYTF1-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for b -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.001 (**) and <0.0001 (***). (D) Northern blot analysis of RsmY in the indicated strains after growth for 8 h in LB broth. The Δ*rsmY* mutant was used as a control. The top half is a membrane probed with the RsmY probe, and the bottom portion is a second independent blot of the loading control probed with a 5S rRNA probe. Northern blotting was performed at least three times. (E) Transcriptional fusion rsmYTF2-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for b -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. All transcriptional fusion analyses were performed at least three times.

mutant background and required AlgR (28). The transcriptional start site of *rsmY* was determined in *Pseudomonas fluorescens* using 5' rapid amplification of cDNA ends (RACE), and this information was used to deduce the transcriptional start site in *P. aeruginosa* (30, 37). To confirm the *P. aeruginosa rsmY* transcriptional start site, we performed primer extension. As indicated in Fig. 3.5A, a single extension product was obtained after reverse transcription of total RNA that confirmed the *rsmY* transcriptional start site assigned using *P. fluorescens*.

A transcriptional fusion, rsmYTF1-*lacZ* (Fig. 3.5B), was constructed and assayed in *algR* and *algZ* mutants in both the PAO1 (wild-type) and the *mucA22* background. When a Δ*algR* or *algZ* mutant was tested for *rsmY* reporter activity, there was no decrease compared to PAO1 (Fig. 3.5C). There was a ~3-fold increase in rsmYTF1-*lacZ* activity in a *mucA22* mutant strain

compared to PAO1 (Fig. 3.5C). When tested in a *mucA22* Δ*algR* mutant strain, the increased activity in the *mucA22* mutant strain was reduced to PAO1 levels (Fig. 3.5C). In the case of the *mucA22 algZ* mutant strain, there was a slight but statistically significant decrease in rsmYTF1-*lacZ* activity (Fig. 3.5C). Overall, the fusion results suggest that AlgR affects *rsmY* expression, but only in the *mucA22* background.

To confirm that the *rsmY* levels decreased, Northern blotting was performed on the same strains. Two bands hybridized to the *rsmY* probe, as has been seen previously (Fig. 3.5D) (30, 38). Little difference was seen between a Δ*algR* mutant strain and the wild-type PAO1 (data not shown). However, an increase in both hybridizing RNAs was seen in a *mucA22* mutant strain (Fig. 3.5D). The *mucA22* Δ*algR* and *mucA22 algZ* mutant strains had decreased hybridizing fragments (Fig. 3.5D). The loading control used (5S rRNA) was consistently uneven upon repeated attempts, but the Northern blotting does confirm the transcriptional fusion analysis. A second probe using *proC* was also used and gave similar results. A Δ*rsmY* strain had no hybridizing RNA, confirming the specificity of the *rsmY* probe. Overall, these results support AlgR control of *rsmY* expression in the *mucA22* strain.

Both *rsmY* and *rsmZ* contain upstream activating sequences (UAS) that GacA con- trols (39). A potential AlgR-binding site is located 23 nucleotides upstream of the UAS for *rsmY*. An additional AlgR-binding site is present further upstream on the opposite strand (Fig. 3.5B). Two additional transcriptional fusions, rsmYTF2-*lacZ* and rsmYTF3-*lacZ* (Fig. 3.5B and 3.S2A), were constructed to determine the sequences required for AlgR control. The rsmYTF2-*lacZ* transcriptional fusion deletes the AlgR-binding sites but retains the UAS sequence (Fig. 3.5B). When assayed in the *algR* mutant strains in both PAO1 and *mucA22* backgrounds, a difference was only seen in the *mucA22* background (Fig. 3.5E). This result suggests that the potential

AlgR-binding sites are not necessary for increased *rsmY* reporter activity.

An additional *rsmY* reporter, rsmYTF3-*lacZ*, was constructed that does not contain the UAS sequence. This fusion had very low activity in both PAO1 and *mucA22* (Fig. 3.S2B). This result suggests that the UAS is required for increased *rsmY* reporter activity in a *mucA22* mutant strain as well as PAO1. Overall, the data suggest that AlgR and AlgU indirectly affect *rsmY* expression.

AlgR regulates *rsmZ* expression indirectly

The regulation of *rsmZ* is more complex than *rsmY* and includes regulators other than GacA (38, 40, 41). Primer extension analysis confirmed the location of the transcriptional start site in *P. aeruginosa* that was predicted from *P. fluorescens* (Fig. 3.6A) (42, 43). A transcriptional fusion, rsmZTF1-*lacZ*, was assayed in the *algZ* and *algR* mutants (Fig. 3.6B). There was no difference in the *rsmZ* reporter in the Δ*algR* or *algZ* mutant compared to PAO1 (Fig. 3.6C). There was a 5-fold increase in rsmZTF1-*lacZ* activity in the *mucA22* mutant strain compared to PAO1 (Fig. 3.6C). In a *mucA22* Δ*algR* mutant strain, there was a significant decrease in rsmZTF1-*lacZ* activity. In a *mucA22 algZ* mutant strain, there was a significant but slight decrease in rsmZTF1-*lacZ* activity compared to the *mucA22* mutant (Fig. 3.6C). In all, these results suggest that AlgR plays a major role and AlgZ plays only a minor role, if any, in terms of *rsmZ* expression in the *mucA* mutant background.

To confirm the transcriptional fusion results, we again wanted to monitor the actual RNA levels. Using Northern blot analysis, a trend was seen similar to that in the transcriptional fusions. As shown in Fig. 3.6D, there were two bands detected, with the larger fragment being the most intense. Previous studies utilizing Northern blotting suggested that the smaller

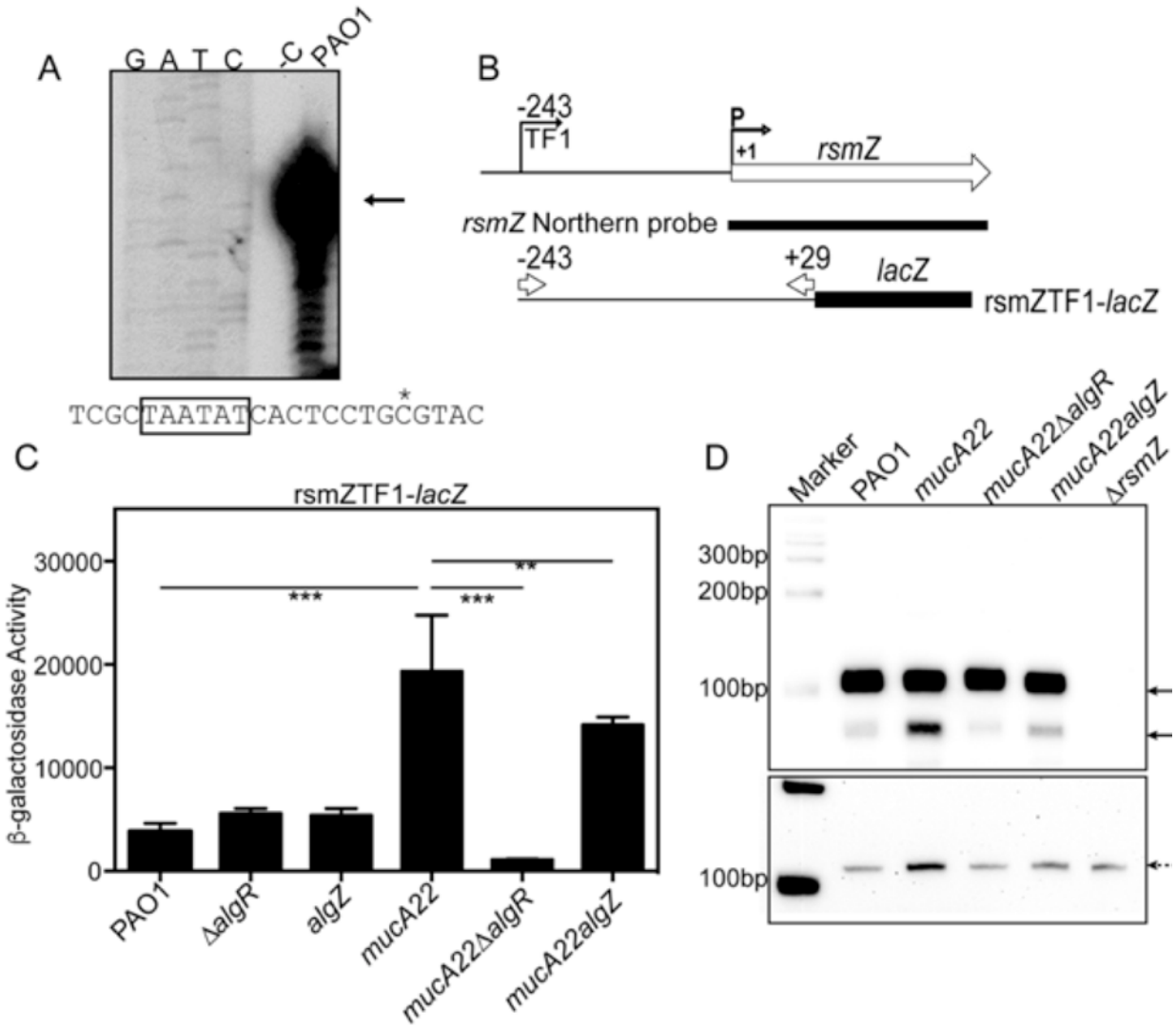

Figure 3.6 AlgR increases *rsmZ* expression in a *mucA22* mutant strain indirectly. (A) Primer extension analysis using total RNA isolated from PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the *rsmZ* promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the *rsmZ* genomic region. The bent arrow on top indicates the primer used for transcriptional fusion construction. The bent arrow indicated by a "P" indicates the transcriptional start site. The bent arrow below indicates the primer used in primer extension. (C) Transcriptional fusion ZTF1-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for b - galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.001 (**) and <0.0001 (***). Transcriptional fusion analysis was performed in triplicate three times. (D) Northern analysis of the indicated strains grown for 8 h in LB broth and total RNA probed with and *rsmZ* probe. A Δ*rsmZ* mutant strain was used to denote the specificity of the probe. A separate blot of the same strains was probed using a 5S rRNA probe (shown below). Northern blotting was performed three times.

hybridizing fragment represents the small RNA lacking the stem-loop found at the 3' end of the small RNA (30). Therefore, the smaller hybridizing bands may represent the small RNAs lacking a transcriptional terminator. A Δ*rsmZ* mutant was used as a negative control. The *mucA22* mutant strain had increased RsmZ compared to PAO1 (Fig. 3.6D). The *mucA22* Δ*algR* mutant strain had the most drastic decrease in RsmZ levels, and there was a slight decrease in RsmZ levels in the *mucA22 algZ* mutant (Fig. 3.6D). Altogether, these results suggest that AlgR plays a role in increasing the RsmZ small RNA in the *mucA22* background.

We hypothesized that like *rsmY* expression, AlgR indirectly affects *rsmZ* expression. An additional transcriptional fusion, rsmZTF2-*lacZ* (Fig. 3.S3A), was constructed and assayed in the wild-type strain PAO1 and a *mucA22* mutant strain, which lacks the UAS sequence. Both strains had drastically decreased reporter activity (Fig. 3.S3B). Interest- ingly, the *mucA22* mutant strain had significantly decreased activity compared to PAO1 (Fig. 3.S3B). Further support for an indirect mechanism of AlgR activation was obtained using gel shift analysis of a PCR amplicon of the *rsmZ* upstream region (Fig. 3.S3A). Purified AlgR was not able to shift the *rsmZ* upstream region tested (Fig. 3.S3C), suggesting AlgR does not directly activate *rsmZ* expression. These results suggest that the UAS is required for increased *rsmZ* expression, and we conclude that AlgR affects *rsmZ* expression indirectly.

AlgR is necessary for RsmA activity in a *mucA* mutant background

To ascertain whether AlgR control of *rsmA* was significant in a biological context, the direct RsmA targets *hcnA* and *tssA1* were used to assess RsmA activity (29). A new integrating vector was constructed that contains the *lacUV5* promoter and lacks a ribosome-binding site or a start codon for *lacZ*. When tested in the wild-type strain PAO1, there was no activity of the

vector alone.

The *hcnA* gene encodes part of the hydrogen cyanide synthase enzyme that is implicated in virulence (44, 45). RsmA was shown to previously negatively affect a leader fusion using the *tac* promoter and the ribosome-binding site region of *hcnA* (45). An *hcnA* leader fusion was constructed by annealing 33 nucleotides, including one of the predicted RsmA-binding sites, the ribosome-binding site, and the translational start codon of *hcnA*. As shown in Fig. 3.7A, the *lacUV5 hcnA-lacZ* fusion had activity in strain PAO1. When assayed in a *mucA22* mutant strain, there was an ~2.5-fold decrease in reporter expression (Fig. 3.7A), consistent with increased RsmA levels in the *mucA22* mutant strain. If *rsmA* expression requires AlgR and AlgU, mutating *algR* and *algU* should result in increased fusion activity in the *mucA22* background due to decreased *rsmA* expression. Both the *mucA22* Δ*algR* and the *mucA22* Δ*algU* mutant strains had at least a 3-fold increase in reporter expression compared to the *mucA22* strain (Fig. 3.7A). The *mucA22* Δ*rsmA* mutant strain was assayed and had a 3.6-fold increase in reporter activity compared to the *mucA22* mutant (Fig. 3.7A). Overall, these results are consistent with the conclusion that RsmA is active in the *mucA22* strain and that AlgU and AlgR are important for the decreased posttranscriptional regulation of *hcnA*.

Another RsmA target, *tssA1*, was also analyzed. The *tssA1* gene encodes a portion of the T6SS and is directly regulated by RsmA (29, 46). Previous studies have used a *lacUV5 tssA1* construct containing 227 bp of *tssA1* upstream sequence that had extremely low activity (28, 29, 31). However, this fusion was found to contain additional transcriptional controls that resulted in low activity (data not shown). To more carefully assess RsmA activity using *tssA1*, we constructed a new leader fusion by annealing 31 nucleotides, including the predicted RsmA-binding site, the ribosome-binding site, and the translational start codon of *tssA1*. The *lacUV5*

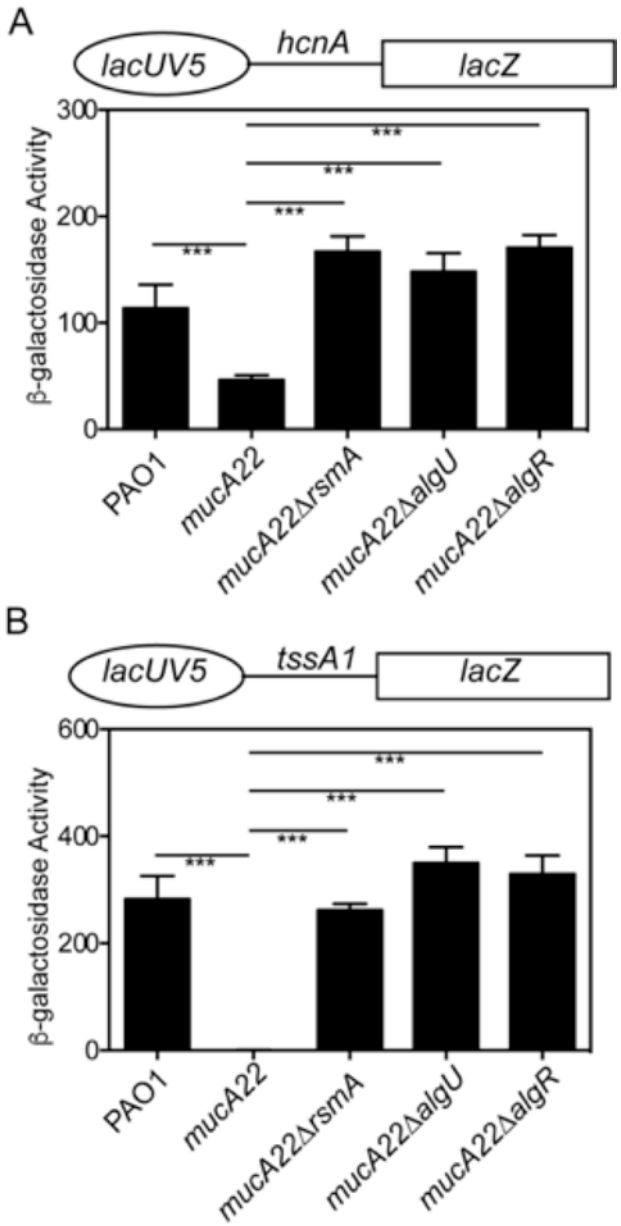

Figure 3.7 RsmA is active in a *mucA22* mutant strain. (A) Leader/translational fusion *lacUV5 hcnA-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for b -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. (B) Leader/ translational fusion *lacUV5 tssA1-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for b -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. All leader/translational fusions were assayed in triplicate three times.

tssA1-lacZ fusion had approximately 200 times the activity as the previously constructed *lacUV5 tssA1-lacZ* fusion containing 227 bp of upstream sequence in the wild-type strain PAO1 (Fig. 3.S4). There was no activity when this fusion was assayed in a *mucA22* mutant strain (Fig. 3.7B and 3.S4). The *lacUV5 tssA1-lacZ* fusion confirmed our previous result (31), demonstrating increased activity in the *mucA22* Δ*algU* mutant strain, validating the use of this construct. When a *mucA22* Δ*algR* mutant was tested, the fusion was also increased from the *mucA22* mutant strain to levels similar to those of the *mucA22* Δ*algU* mutant strain (Fig. 3.7B). A *mucA22* Δ*rsmA* mutant strain also had statistically significant activity compared to that of the *mucA22* mutant (Fig. 3.7B). These results demonstrate that AlgR and AlgU are necessary for posttranscriptional regulation of *tssA1*. From these data, we conclude that AlgU and AlgR are both required for increased *rsmA* expression in the *mucA* mutant background and that this leads to posttranscriptional regulation on two known RsmA targets, most likely through RsmA.

DISCUSSION

AlgR is an important two-component regulator having roles in both acute and chronic infections. In the case of acute infections, AlgR activates the *fimU* operon, enabling the production of T4P (19, 21, 47). In chronic infections, AlgR, AlgU, and other transcriptional regulators activate the production of alginate (22, 48–50). These and other virulence factors in *P. aeruginosa* are often considered mutually exclusive, de- pending on whether there is an acute or chronic infection (51–53). This work demonstrates that another effect of a *mucA* mutation is that AlgR activity is directed at increasing the posttranscriptional regulator RsmA. AlgR activation of *rsmA* may help explain how AlgR participates in the mutually exclusive production of some

virulence factors depending on acute or chronic infection.

The initial description of AlgR as a regulator of *rsmA* (28) did not evaluate the contribution of AlgR phosphorylation by AlgZ or a mechanism for AlgR activation of *rsmA*. Because AlgR is part of a two-component system that is important for both acute and chronic infections (19, 32), it was necessary to address the mechanism for AlgR activation of *rsmA* in order to create a framework to understand how AlgR regulation of *rsmA* might impact virulence gene expression. AlgR binds a consensus sequence CCGTTCGTC (21, 48, 49), and phosphorylation is thought to enable AlgR to bind potential binding sites that deviate from this consensus, such as the sites found in the *fimU* promoter (32, 47). However, the *rsmA* promoter deviates from the AlgR-binding consensus, and AlgR phosphorylation was not required for *rsmA* expression. This argues against phosphorylation as the sole mechanism for AlgR binding less well-conserved consensus sequences. The studies using the *mucA22* mutant strain, the *algZ* mutant, and the D54N mutant are consistent with the conclusion that AlgR phosphorylation is not required for *rsmA* activation. As AlgR phosphorylation is not required for alginate expression (22), our results are consistent with the conclusion that AlgR activates *rsmA* expression in mucoid strains.

The mechanism for AlgR activation of *rsmA* is by directly binding upstream of the AlgU-dependent promoter, further supporting our model (Fig. 8). Gel shift studies determined that two AlgR-binding sites are required for *in vitro* binding. The location of the AlgR-binding sites, the *in vitro* gel shift analysis, transcriptional fusions, and RNase protection assay all support AlgR binding the distal promoter region of *rsmA* and are in accord with the activation of the AlgU-dependent promoter.

Our data suggest that AlgR plays a more important role in regulating *rsmA* expression in *mucA*

mutant strains, such as those found in CF patients. If AlgU and AlgR activate *rsmA* expression, as predicted by our model (Fig. 8), this would explain why AlgR did not appear to play a significant role in regulating *rsmA* in the wild-type PAO1 strain (Fig. 1B and C, 2, and 3). From this work, we conclude that AlgR and AlgU play a greater role in *rsmA* activation in *mucA* mutant strains. However, it is possible that *in vivo* conditions differ and that nonmucoid wild-type strains also have increased regulation of *rsmA* under other conditions.

The *mucA* mutation leads to AlgU and AlgR activation of *rsmA*, suggesting that RsmA

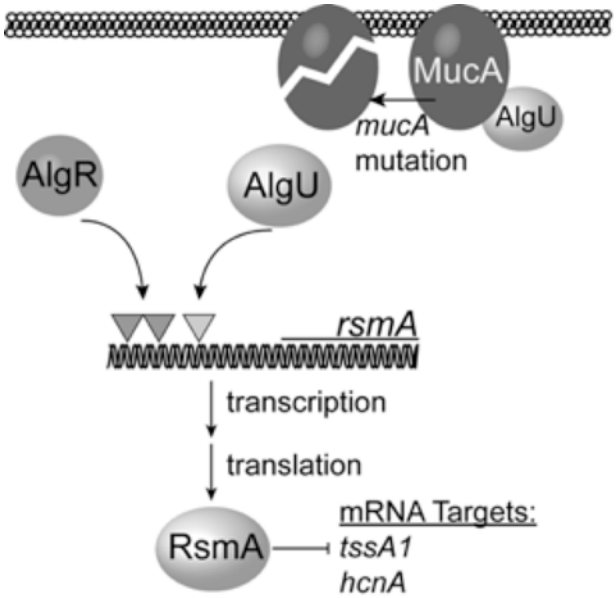

Figure 3.8 Model for AlgR activation of *rsmA* and RsmA activity in mucoid strains. In chronic infections, *mucA* mutants arise, leading to increased AlgU activity. In the case of *rsmA*, AlgR and AlgU activate the distal *rsmA* promoter. The dark-shaded triangles indicate the AlgR-binding sites located upstream of *rsmA*. The light-shaded triangle indicates the AlgU-dependent promoter. The model predicts that increased expression of *rsmA* leads to increased RsmA activity, as indicated by the negative regulation of the RsmA targets, *hcnA* and *tssA1*.

may regulate specific targets in *mucA* mutant strains. RsmA is a posttranscriptional regulator that binds mRNAs, in many cases, at or near the ribosome-binding site of targets (54). It is likely that

RsmA has unknown targets in a *mucA* mutant background due to transcriptional differences between nonmucoid and mucoid strains (55–59). Therefore, RsmA will bind only to targets that are present at a given time, supporting RsmA working as a rheostat. An alternative explanation is that our conditions of growth in the laboratory environment do not represent the conditions in the CF lung. While this is quite obvious, it is known that the mutation in *mucA* leads to alginate production *in vivo* (11, 60, 61). In addition, nonmucoid *P. aeruginosa* also has active AlgU and AlgR during infection because of alginate secretion by nonmucoid *P. aeruginosa* from CF patients (62, 63). Therefore, it is highly probable that AlgU and AlgR activity *in vivo* would increase RsmA levels during infection. What role RsmA plays due to AlgU and AlgR regulation *in vivo* is not known.

The significance of AlgU and AlgR control of *rsmA* is demonstrated by the analyses of RsmA targets. The posttranscriptional activity of the RsmA targets, *tssA1* and *hcnA*, was greater in the *mucA22* background than in the wild-type PAO1 strain (Fig. 7). We also observed a lack of posttranscriptional activity on RsmA targets when *algU* or *algR* was inactivated in a *mucA22* mutant strain (Fig. 7), due to the decreased expression of *rsmA*. Therefore, we conclude that RsmA is active in an *mucA22* mutant strain and that RsmA activity requires AlgU and AlgR to increase *rsmA* gene expression in this back- ground. The correlation between increased AlgR activity and increased RsmA activity in *mucA* mutants also supports these systems acting as a rheostat to fine-tune gene expression, as opposed to an on/off switch. When AlgU and AlgR increase RsmA levels, the indirect effects of these two regulators may be due to RsmA. Further work is necessary to pursue this exciting discovery that could help explain the exclusive expression of particular virulence genes in a given background.

The role of AlgU and AlgR on the RsmA-antagonizing small RNAs *rsmY* and *rsmZ* is

likely indirect. While our Northern blot data were not strong, they did support our transcriptional fusion analysis. Our transcriptional fusions mirrored what was seen in a previous study (28), supporting the idea of AlgR indirectly controlling *rsmY* and *rsmZ* expression. We hypothesize that AlgU and AlgR activities may coincide with factors that lead to increased GacA phosphorylation, resulting in the increased expression of *rsmY* and *rsmZ*. Further work is necessary to establish the mechanism for increased expression of the small RNAs in a *mucA* background.

Another question raised by our study is how RsmA remains active when the antagonizing small RNAs are increased. One possibility is that RsmA preferentially binds its targets in the *mucA* mutant strains better than the antagonizing small RNAs. This might also result from other regulators, either protein or other small RNAs that are currently unknown and affect the ability of RsmA to interact with the antagonizing small RNAs RsmY and RsmZ.

Altogether, our work demonstrates that AlgR is required for increased *rsmA* expression in *mucA* mutant strains. Phosphorylation of AlgR was not required for *rsmA* activation. The increased RsmA levels in a *mucA22* mutant strain result in increased RsmA activity, even though the antagonizing small RNAs are also increased. What roles RsmA plays and how RsmA functions in chronic infecting strains are not known. To better understand the role of RsmA, we are currently investigating new possible RsmA targets in AlgU-active strains. A further understanding of the RsmA regulon in *mucA* mutants may provide additional insight into how *P. aeruginosa* becomes such a successful CF pathogen and has implications for the important role of RsmA in all types of *P. aeruginosa* infections.

ACKNOWLEDGEMENTS

Thank you to the Department of Health Sciences at East Tennessee State University and its Chair, Dr. Ranjan Chakraborty, for providing reagents, advice, and most importantly funds for this work. We thank Joseph and Julia Albright for providing equipment and supplies for these studies. We thank Joanna Goldberg (Emory University) for plasmid construct pTJ1. We thank Laraine Powers and David Hurley (ETSU) for critical review of the manuscript. We thank Eric Jones for expert technical assistance.

FUNDING INFORMATION

This work, including the efforts of Chris L. Pritchett, was funded by Re- search and Development Committee, ETSU (RDC12-012M and RDC13- 005M).

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SUPPLEMENTAL FIGURES

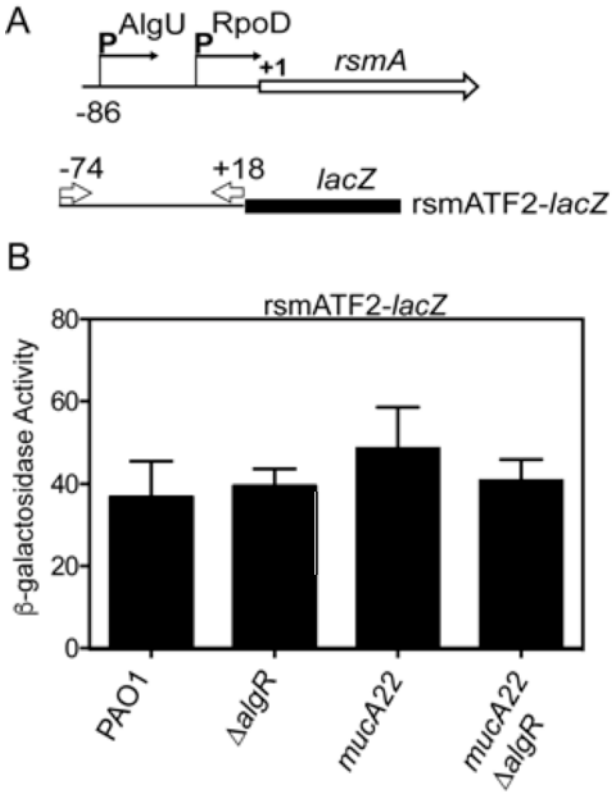

Figure 3. S1 *algR* deletion has no effect on the proximal *rsmA* promoter. (A) Schematic of the *rsmA* genomic region and the transcriptional fusion rsmATF2- *lacZ*. Numbers indicate distance from the *rsmA* translational start site. The translational start site is indicated by a +1. The AlgU and RpoD promoters are indicated by bent arrows. The transcriptional fusion rsmATF2-*lacZ* is indicated directly below and the arrows indicate the primers and the numbers their location relative to the *rsmA* translational start site. (B) The *rsmA* transcriptional fusion rsmATF2-*lacZ* was introduced into the indicated strains to generate strains containing single-copy, chromosomal fusions. The strains were grown for 8 hours in LB broth and assayed for β-galactosidase activity minus the vector control. Differences from the wild-type strain PAO1, *mucA22,* or Δ*algR* mutants, were determined using a one-way analysis of variance and a Tukey's post-test. Transcriptional fusions were assayed in triplicate three times.

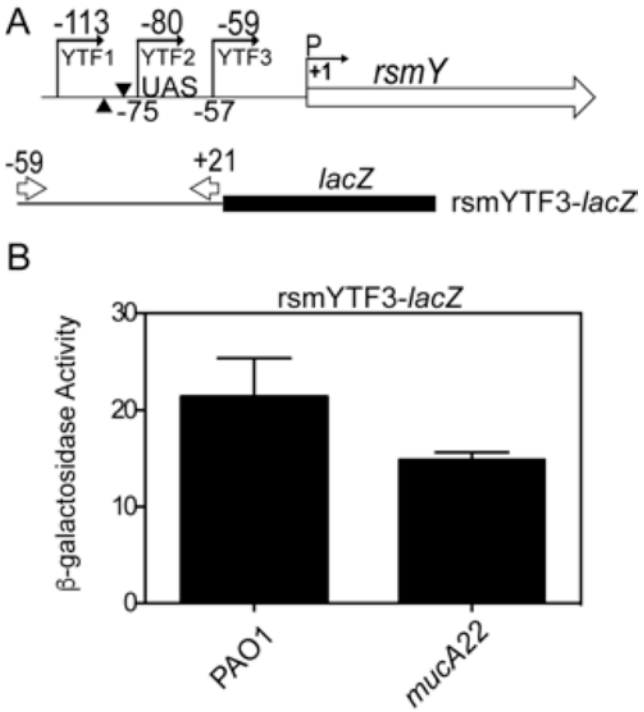

Figure 3. S2. The GacA binding site is necessary for increased *rsmY* expression. (A) Schematic of the *rsmY* genomic region and the transcriptional fusion rsmYTF3-*lacZ*. Numbers indicate distance from the *rsmY* transcriptional start site. The transcriptional start site is indicated by a bent arrow and +1. The UAS controlled by GacA is indicated with the distances from the transcriptional start site indicated below. The transcriptional fusion rsmYTF3-*lacZ* is indicated directly below and the arrows indicate the primers and the numbers their location relative to the *rsmY* transcriptional start site. (B) Transcriptional fusion rsmYTF3-*lacZ* was introduced into the indicated strains in single-copy, grown for 8 hours in LB broth and assayed for β-galactosidase activity. Significant differences from the wild type were performed using a one-way analysis of variance and a Tukey's post-test. Transcriptional fusions were assayed in triplicate three times.

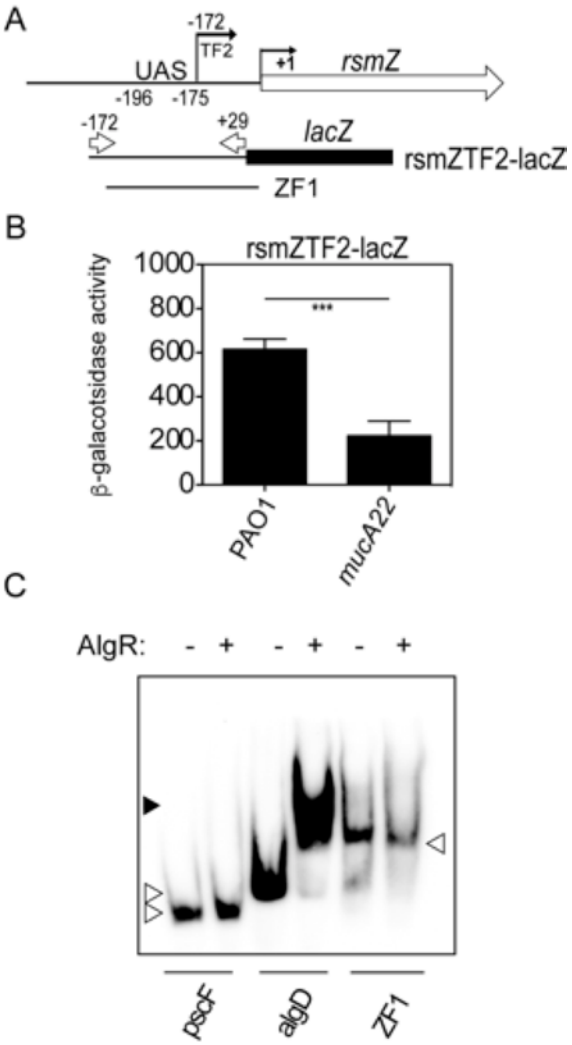

Figure 3. S3. AlgR does not directly affect *rsmZ* expression. (A) Schematic of the *rsmZ* genomic region and the transcriptional fusion rsmZTF2-*lacZ* and the PCR product used for gel shift analysis. Numbers indicate distance from the *rsmZ* transcriptional start site. The transcriptional start site is indicated by a bent arrow and +1. The UAS controlled by GacA is indicated with the distances from the transcriptional start indicated below. The transcriptional fusion rsmZTF2-*lacZ* is indicated directly below and the arrows indicate the primers and the numbers their location relative to the *rsmZ* transcriptional start site. (B) Analysis of rsmZTF2-*lacZ* in PAO1 and a *mucA22* strain. The indicated strains were grown for 8 hours and assayed for β-galactosidase activity. Assays were repeated three times. Significant differences from the wild type were performed using a one-way analysis of variance and a Tukey's post-test. Asterisks indicate *P* values of 0.001 (**) to < 0.0001 (***). (C) Gel shift analysis of the *rsmZ* upstream region with purified AlgR. Open arrowheads indicate probes alone. Blackened arrowheads indicate shifted probe. *pscF* is a negative control. *algD* is a positive control. ZF1 is the *rsmZ* upstream region. The – symbol means a probe with no added AlgR. The + symbol indicates 2.5μM of AlgR added with the probe.

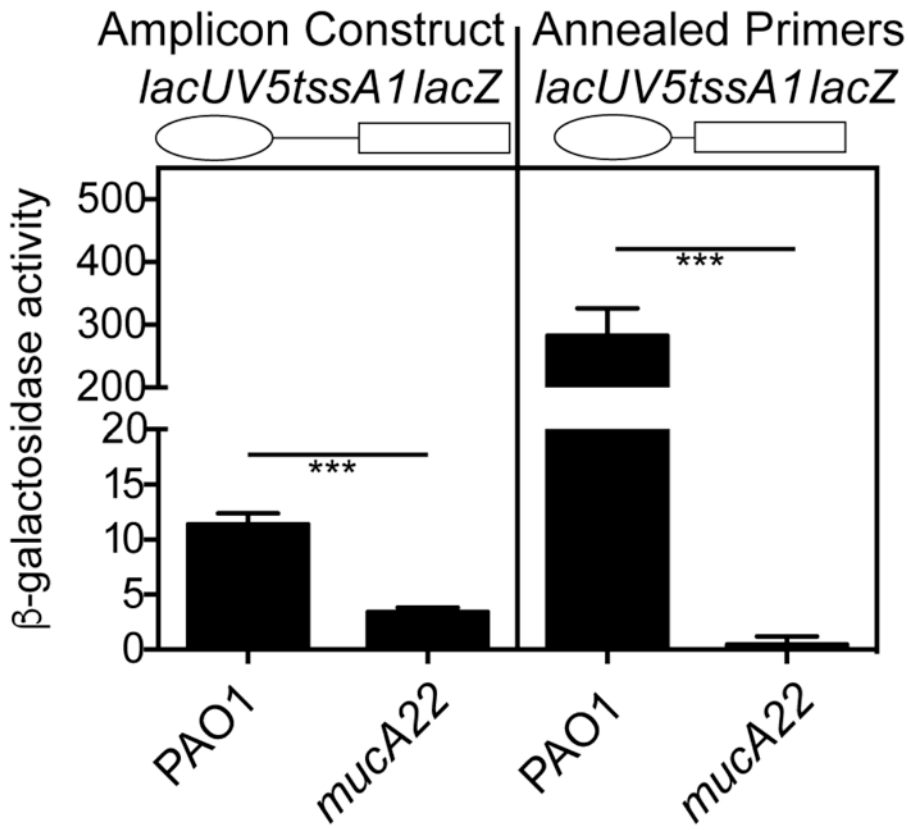

Figure 3. S4. Comparison of *tssA1* leader/translational fusions. The previously used leader/translational fusion was based on a 5' RACE study by Brencic and Lory (1) and includes 227bp of upstream sequence of *tssA1* that was previously used to investigate posttranscriptional regulation of *tssA1* (1-3). On the left is the original *lacUV5tssA1-lacZ* fusion assayed in PAO1 and *mucA22* at 8 hours. On the right is the new *lacUV5tssA1-lacZ* leader/translational fusion using annealed primers of 31bp cloned into the same vector and assayed at 8 hours in the indicated strains. Assays were repeated three times. Significant differences from the wild type were performed using a one-way analysis of variance and a Tukey's post-test. Asterisks indicate *P* values of 0.001 (**) to < 0.0001 (***).

CHAPTER 4

ANALYSIS OF THE RSMA REGULON AND IDENTIFICATION OF A PREVIOUSLY UNKNOWN RSMA TARGET IN A *MUCA22* STRAIN OF *PSEUDOMONAS AERUGINOSA*.

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Keywords: mucoid, Cystic fibrosis, AlgU, RsmA, PasP, RNA EMSA

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen able to colonize any host tissue. When Cystic fibrosis (CF) patients are infected with *P. aeruginosa,* the bacteria can develop a niche in the CF lung and establish a chronic infection. A majority of CF chronic infection isolates contain a mutation in *mucA* that encodes an anti-σ factor that sequesters the σ factor AlgU. AlgU regulates the chronic virulence factor alginate. Strains that overproduce alginate are termed mucoid. We have shown that AlgU also regulates the gene for the posttranscriptional regulator *rsmA*. Based on our previous data demonstrating that the alginate regulators AlgU and AlgR activate *rsmA* expression, we hypothesize *rsmA* has increased expression due to AlgU and AlgR in mucoid strains of *P. aeruginosa* and this in turn affects the RsmA regulon. First, we wanted to characterize observed phenotypic variations in the *mucA22* Δ*rsmA* strain and study any effects on virulence. Due to increased RsmA levels in mucoid strains and blatant variation in phenotypes between *mucA22* and *mucA22 rsmA*, we expected to identify novel RsmA targets in the mucoid background. Therefore the *mucA22* and *mucA22*Δ*rsmA* proteomes were analyzed using 2D-gel electrophoresis and isobaric tagging by relative and absolute quantification (iTRAQ). The *mucA22*Δ*rsmA* strain showed increases in previously unidentified targets: PA3866, AlgP, PasP, AhpC, and OprF. We constructed a *pasP* leader fusion with a constitutively active *lacUV5* promoter and compared PAO1, *mucA22*, and the *mucA22* Δ*rsmA* mutant strains to confirm the iTRAQ data. Next, we demonstrated a direct interaction of RsmA with *pasP.* These data show that *rsmA* has increased expression in *mucA* mutant strains and identified new RsmA targets. We also showed a significant increase in *rsmA* expression in several mucoid clinical isolates compared to nonmucoid strains. Overall, RsmA regulation is important not only in nonmucoid strains like PAO1, but also mucoid strains of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacillus capable of growing in a wide variety of environments. While *P. aeruginosa* is capable of causing an infection in any human host, the bacterium is more widely known as an opportunistic pathogen, causing afflictions in individuals with a compromised immune system (1) (2). *P. aeruginosa* is a major contributor to morbidity and mortality in individuals with major burns, type II diabetes, HIV/AIDS, and cystic fibrosis(CF) (3-6). In the case with CF, *P. aeruginosa* establishes a niche in the lung that can develop into a chronic infection (7). *P. aeruginosa* chronic infection isolates often present a variant phenotype from acute infection isolates and express less inflammatory virulence factors, like flagella, allowing them to persist in the CF lung (8).

P. aeruginosa in the CF lung is targeted by neutrophils releasing reactive oxygen and nitrogen species (7). This attack causes osmotic stress on the bacteria and affects the integrity of membrane bound proteins. The anti-sigma factor, MucA, is an inner membrane bound protein that sequesters the extracytoplasmic sigma factor, AlgU (9). The release of AlgU allows for a phenotypic shift where *P. aeruginosa* begins to produce copious amounts of the exopolysaccharide alginate. *P. aeruginosa* strains that overproduce alginate are termed mucoid. This thick protective biofilm allows for *P. aeruginosa* to evade phagocytosis, secreted reactive species, and antibiotics (7, 9, 10). The AlgU regulon has been shown to contribute to the expression of virulence factors like proteases *lasB* and the metalloproteinase *aprA* (11). Our previous work has also shown AlgU contributes to posttranscriptional regulation of virulence factors by regulating the expression of *rsmA* (12).

RsmA regulates hundreds of targets by binding the ribosomal binding site of a messenger RNA (mRNA) and inhibiting translation (13). RsmA is antagonized by two small noncoding

RNA called RsmY and RsmZ (14). RsmA is also parth of the Gac/Rsm hierarchal system. The two component system GacA/S activate *rsmY/Z* expression (15). GacS, the sensor, can also dimerize with another sensor, RetS, which will inhibit the phosphorylation of GacA and therefore not make the RsmA antagonists (15-17). The RsmA homolog, CsrA, has been more characterized and shown to not only inhibit translation, but also positively influence the translation of some targets (18). CsrA has also been shown to form protein-protein interactions (19, 20). In *P. aeruginosa* RsmA has been shown to inhibit the production of virulence factors like the Type VI secretion system and hydrogen cyanide synthase while positively influencing other virulence factors like type IV pili (13). Originally, the function of RsmA was to regulate as a switch from acute to chronic infection virulence factors (21). Most studies of RsmA targets via microarray were investigated in acute/nonmucoid strains of *P. aeruginosa* like PAO1 or PAK.

However, our work investigating AlgU controlling *rsmA* expression showed that more RsmA was present in a chronic/mucoid strain where AlgU is not sequestered by MucA (12). Not only was there more RsmA present in the *mucA22* strain, but we also showed this abundance in RsmA was inhibiting the translation of the type six secretion component, *tssA1,* and hydrogen cyanide synthase, *hcnA* (12). Due to bacteria tightly controlling the regulation of their various products, these data suggested an importance to the abundance of RsmA in mucoid chronic infection strains. We also hypothesized from these results that there might be more RsmA targets or different targets in mucoid strains.

In this study, we continue our understanding of RsmA in a mucoid chronic infection model strain. We characterize a mucoid Δ*rsmA* strain in terms of virulence factor production and also the effects on virulence of this mutation. Since RsmA is known to regulate hundreds of

targets post-transcriptionally and previous works examined the transcriptome of *P. aeruginosa*, we decided to compare the proteome of the mucoid *mucA22* strain and its *rsmA* mutant. We hypothesized proteomic analysis would produce a better representation of RsmA targets in the mucoid background. Lastly, we demonstrated a direct interaction with RsmA and a previously unknown target, *pasP,* a small protease. Overall, this work furthers the understanding of the RsmA regulon in mucoid strains of *P. aeruginosa.*

METHODS

Strains, plasmids, and media

The strains used in this study are presented in Table 4.1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics, as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB medium, or Vogel-Bonner minimal medium (VBMM) supplemented with the appropriate concentrations of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 μg/ml tetracycline, 15 μg/ml gentamicin, 100 μg/ml ampicillin, and 35 μg/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 μg/ml gentamicin, 50 μg/ml tetracycline, and 300 μg/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

TABLE 4.1

Strain or Plasmid	Genotype or Relevant properties	Reference or Source
E. coli strains		
NEB5α	*fhuA2 Δ(argF*-lacZ*) U169phoA, glnV44*	New England

	*Φ80Δ(*lacZ)M*15 gyrA96*	Biolabs
pRK2013	Helper strain	21d
*rsmA*HA pEX18Gm	Allelic exchange for *rsmA* HA allele	12
TF3-lacZ miniCTX	Transcriptional fusion	12
hcp1-lacZ	Transcriptional fusion	This study
lacUV5-pasp-lacZ	Leader fusion	This study
P. aeruginosa strains		
PAO1	Wild type	50
mucA22	*mucA22* mutation	51
FRD1	Mucoid clinical isolate	52
PAO568	Mucoid clinical isolate	53
8830	Mucoid clinical isolate	54
383	Nonmucoid clinical isolate	55
2192	Mucoid clinical isolate	55
Δ*lasR* Δ*rhlR*	Attenuated control strain	56
mucA22 Δ*retS*	Mucoid *retS* mutant	12
Δ*rsmA*	*rsmA* nonpolar deletion	12
*mucA22*Δ*rsmA*	*rsmA* nonpolar deletion in *mucA22* strain	12
PAO1 TF3-*lacZ*	*rsmA* transcriptional fusion strain	12
mucA22 TF3-lacZ	*rsmA* transcriptional fusion strain	12
2192 TF3-*lacZ*	*rsmA* transcriptional fusion strain	12
383 TF3-*lacZ*	*rsmA* transcriptional fusion strain	12
FRD1 TF3-*lacZ*	*rsmA* transcriptional fusion strain	This Study
PAO568 TF3-*lacZ*	*rsmA* transcriptional fusion strain	This Study
8830 TF3-*lacZ*	*rsmA* transcriptional fusion strain	This Study
PAO1 hcp1-lacZ	*hcp1* transcriptional fusion strain	This Study
mucA22 hcp1-lacZ	*hcp1* transcriptional fusion strain	This Study
*mucA22*Δ*rsmA* hcp1-lacZ	*hcp1* transcriptional fusion strain	This Study
PAO1 lacUV5-pasp-lacZ	PAO1 with *pasP* leader fusion	This Study
mucA22 lacUV5-pasp-lacZ	*mucA22* with *pasP* leader fusion	This Study
*mucA22*Δ*rsmA* lacUV5-pasp-lacZ	*mucA22*Δ*rsmA* with *pasP* leader fusion	This Study
PAO1HA	PAO1 with epitope tagged RsmA	This study
*mucA22*HA	*mucA22* mutant with epitope tagged RsmA	This study

Strain construction

All PCR products were amplified with DNA from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs [NEB], Ipswich, MA) and primers listed in Table 4.2. Crossover PCR (splicing by overlapping extension PCR) (21b) was used to construct deletion mutations and to clone sequences into the suicide vector pEX18Tc or pEX18Gm (21c). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (21d). Conjugations were performed overnight on LB plates at 37°C, and the conjugants were plated on the appropriate selective medium to obtain single-crossover mutants. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT medium–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR in Table 4.2 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of the proteins was accomplished using primers containing the HA tag at the 3' end of the gene, and HA was introduced as described above using the suicide vector pEX18Gm.

TABLE 4.2

Primer	Sequence	Use
Hcp1-lacZF	GCGCGGATCCACACCGACTTCGCCGCCTTC	Transcriptional fusion
Hcp1-lacZR	GCGCAAGCTTCTTTCCTCCCGTGTGGCTC	Transcriptional fusion
lacUV5pasPF	GATCCGGACGTTAAGGAGAATGTCCAT	Leader fusion
lacUV5paspR	AATTCATGGACATTCTCCTTAACGTCC	Leader fusion
Paspt7emsaF	TGTAATACGACTCACTATAGGGACGTTAAGGAGAATGTCCAT	EMSA
Paspt7emsaR	ATGGACATTCTCCTTAACGTCCCTATAGTGAGTCGTATTACA	EMSA
rsmyemsaF	TGTAATACGACTCACTATAGGGGTCAGGACATTGCGCAGGAAG	EMSA

rsmyemsaR	AAAACCCCGCCTTTTGGGCGG	EMSA

Phenotypic Assays

Various virulence products were assayed in the *P. aeruginosa* strains in this study. All strains were grown in LB broth at 37ºC for 16 hours. Biofilm production was assayed by using Congo Red plates and a 96-well plate biofilm quantification (22, 23). Strains were spotted onto agar plates in 10 µl drops and incubated at 37ºC overnight. For quantification, strains were resuspended in M63 media per O'Toole and quantified using a spectrophotometer. Skim milk agar (Difco) was used to observe any differences of casein digestion (24). Strains were spotted onto the agar in 10 µl drops and incubated overnight at 37ºC. Pyocyanin was assayed by centrifuging the culture and collecting the supernatant. Then 2 mL of chloroform was used to extract any pyocyanin produced in the culture by vortexing vigorously and allowing to set for 10 minutes. The chloroform layer was removed and 1 mL of 0.1 N HCl was added and vigorously vortexed and allowed to set for 10 minutes. 0.1 N HCl was used to blank the spectrophotometer at OD_{562nm}. Values were normalized to PAO1.

C. elegans Assay

Caenorhabditis elegans was maintained on NGM agar plates with OP50 *E. coli* (25). Once plates were fully saturated with *C. elegans* eggs, eggs were harvested and worms were synced via bleaching (26). The following day worms were put on NGM media with OP50 and allowed to grow to L1 stage. *P. aeruginosa* strains were incubated at 37ºC on fast killing media on 20 cm plates(27). *C. elegans* were harvested and cleaned of *E. coli* and 30-50 worms were plated on *P. aeruginosa* strains (27). *C. elegans* viability was recorded every 12 hours for 2.5

days. Experiments were performed in triplicate two times.

Macrophage Survival Assays

P. aeruginosa strains were incubated with ATCC J774a.1 macrophages to assess macrophage survivability. Macrophages were grown in Corning cellgro DMEM with L-Glutamine, 4.5g/L Glucose, and 10% fetal bovine serum and 100 I.U./ml penicillin 100 µg/mL streptomycin. Cells were maintained in a 37ºC incubator with 5% CO_2 and humidity. Cells were counted and plated in 12-well plates at 1x10^6 cells per well in media without pen/strep. Overnight LB cultures of *P. aeruginosa* strains were diluted to 1% and allowed to grow for 2 hours at 37ºC at 250 rpm. Cultures were then centrifuged and washed with 1X PBS. Strains were then aliquot to an OD_{600nm} of 0.1 and incubated with macrophages up to 6 hours. The inoculum of bacteria was also measured in CFU/mL. Macrophages were collected at 2, 4, and 6 hour time points and 1 mL of culture was mixed with 100 µL of 0.4% trypan blue to measure viability. Experiments were performed three times in triplicate (n=9).

Western blot analysis

P. aeruginosa strains were grown in LB broth at 37°C for various times. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 µg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:20,000 dilution of anti-HA monoclonal antibody (Thermo Fisher, Pittsburgh, PA), followed by a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody or a 1:20,000 dilution of anti-Hcp1 polyclonal antibody followed by a 1:20,000 dilution

of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (27b). The membrane was stripped per Abcam mild stripping protocol and then probed with RpoA primary antibody (1:20,00 dilution) followed by 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody. Detection was performed using an ECL Plus kit (Thermo Fisher, Pittsburgh, PA) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). All Western blot analyses were repeated at least three times.

Two dimensional gel electrophoresis

Strains were started as overnight cultures and the following day diluted to a fresh LB broth at 1% and grown for 8 hours at 37ºC at 250 rpm. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication with 10 µL of lysozyme 20 mg/ml added. Samples were centrifuged for 10 minutes at 10,000 x g. Samples were quantified using a BCA assay. Four hundred micrograms of each sample was then acetone precipitated with ice-cold acetone and left at -20ºC overnight. Samples were then centrifuged at 14K x g for 15 minutes at 4ºC, then washed with 80% cold acetone and centrifuged at 14K x g for 15 minutes at 4ºC twice. Pellet was dried in a speed vac centrifuge after aspirating off 80% acetone. Using the Invitrogen ZOOM 2D kit, 1X IEF rehydration buffer was mixed per 400 µg (309 µl ZOOM 2D Protein Solubilizer 1.1, Ampholytes 6.4 µl, 7 µl 0.1% bromophenol blue, 31.6 µl deionized water, 6 µl 1M DTT). 1X IEF rehydration buffer was added to each pellet. Samples were incubated on over end rotator for 1 hr at room temp. Centrifuged samples at 12K x g for 10 minutes at 4ºC. Each Novex ZOOM strip was rehydrated with 155 µl of rehydration buffer and incubate cassette for at least 2 hours on rocker at room temp. Strips were then run following the IPG Runner program protocol B (200 V for 20 min, 450 V for 15 min, 750 V for 15 min, 2000 V for 65 min). ZOOM trays were then reduced and

alkylated before running second dimension. Strips were added to a Novex 4-20% Tris-glycine ZOOM gel and run for 165 minutes at 125V. Gels were fixed and Coomassie Blue stained and then analyzed with Dymension Software. Significant spots were in-gel trypsin digested and cleaned up to run through a Thermofisher LTQ XL mass spectrometer for protein identification. Experiments were performed in duplicate twice.

In-gel trypsin digestion

Gel pieces were added to a microcentrifuge tube. To shrink the pieces, 50 µl of acetonitrile was added and incubated for 15 minutes at RT. Gel pieces were then dried using a speed vac centrifuge. To digest, 75 µl of a 10 ng/µl stock of trypsin was added to cover the gel pieces and incubated at 37ºC overnight. The solution was removed and added to a new microcentrifuge tube. Elution buffer (0.1% formic acid, 50% acetonitrile, 49.9% water) was then added to cover the gel piece and incubated for 15 minutes then added to the new microcentrifuge tube. Using Zip Tips (µ-C18) purification to prepare the sample for mass spectrometry, samples were run through the tip column and then eluted off with elution buffer. The sample was pelleted and dried using speed vac centrifugation. Samples were resuspended in 8 µl of mass spec buffer (0.1% formic acid in 100 µl of acetonitrile and 400 µl HPLC water). Samples were then sent through the LTQ XL mass spectrometer and analyzed using Proteome Discover 3.1 software to determine protein identified in sample and identifying only high confidence peptides.

iTRAQ

Strains were started as overnight cultures and the following day diluted to a fresh LB broth at 1% and grown for 8 hours at 37ºC at 250 rpm. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication with 10

µL of lysozyme 20 mg/ml added. Samples were centrifuged for 10 minutes at 10,000 x g. Samples were quantified using a BCA assay. One hundred and ten micrograms of each sample was then acetone precipitated with ice-cold acetone and left at -20ºC overnight. Samples were then centrifuged at 14K x g for 15 minutes at 4ºC, then washed with 80% cold acetone and centrifuged at 14K x g for 15 minutes at 4ºC twice. The Sciex iTRAQ labeling protocol was followed for labeling peptides. After labeling trypsin digested peptides, SCX spin tips were used to create a gradient to identify lower frequency peptides in the labeled samples. Samples were fractionated over 20 concentrations of increasing ammonium formate (15 to 500 mM) and decreasing amount of 10% acetonitrile (985 µl to 500 µl). Fractions were then speed vac centrifuged and resuspended in 8 µl of mass spec buffer. iTRAQ Samples were analyzed using Bioworks 3.3 software with peptide confidence threshold set to 10^{-3} for only high confidence peptides that also had iTRAQ label quantification. The p-value of comparison between labels was performed using Student's t-test. Experiments were performed in duplicate twice.

Transcriptional and translational leader fusion analysis

Upstream DNA fragments containing promoter regions were generated by using the primers listed in Table 4.2 in conjunction with Q5 polymerase (NEB, Ipswich, MA). PAO1 genomic DNA was used as the template. PCR products were cloned into pCR2.1 and then subcloned into miniCTX*lacZ* using the restriction enzymes HindIII/BamHI, HindIII/EcoRI, or KpnI/BamHI (New England BioLabs, Ipswich, MA). The translational fusion vector CTX*lacZ*CP was constructed by amplifying the *lacZ* gene using the primers listed in Table 4.2 in order to delete the *lacZ* ribosome-binding site and the translational initiation codon. The fusion constructs were confirmed by sequencing and conjugated into *P. aeruginosa* strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with

pFLP2 to remove vector sequences (21c). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch plated onto VBMM supplemented with 300 µg/ml carbenicillin and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 4.2). β-Galactosidase activity was deter- mined by incubating cell extracts with *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) as described by Miller (27c). All mucoid strains were confirmed to be mucoid at the end of each experiment by plating on PIA plates and making sure that all colonies were mucoid. Experiments were performed in triplicate at least three times.

RsmA purification

The *rsmA* gene was PCR amplified using Q5 (NEB) and PAO1 chromosomal DNA using oligonucleotides rsmASal1F and rsmANot1R (Table 4.2). A 186-bp SalI/NotI fragment was cloned into pGEX-4T-3 (Novagen) to be expressed as a glutathione *S*-transferase–RsmA (GST-RsmA) fusion protein. The resulting plasmid (pGEX-4T-RsmA) was transformed into *E. coli* BL21(DE3) (NEB) cells and spread on LB Amp^{100} agar. The colonies from this transformation were PCR confirmed and struck for isolation. The confirmed isolate was inoculated into LB Amp^{100} at 37ºC at 250 rpm overnight. A 1% dilution was added to 250 mL of LB Amp^{100} and grown to an OD_{600nm} of 0.6 at 37ºC at 250 rpm; 0.2 mM isopropyl-β-D- galactopyranoside (IPTG) was added to induce RsmA expression for 6 h at the same conditions. The cells were collected by centrifugation (7,000 *g*, 10 min) and frozen overnight. Pellets were thawed and lysed using BPER with 4 µl of lysozyme 20 mg/mL and 2 µL of DNase I per mL of BPER along

with a 1X concentration of protease inhibitors. RsmA was purified from this supernatant using the GST spin purification kit (Thermo Fisher). After binding of the fusion protein and washing the column, RsmA was cleaved away from GST with 10 U of thrombin/column overnight at 22°C on an end over rocker. Purified protein was dialyzed using a Slide-A-Lyzer (Thermo Fisher) and storage buffer (20% glycerol, 20 mM Tris [pH 7.5], 5 mM $MgCl_2$, and 1 mM dithiothreitol [DTT]) for 2 hours at room temperature twice and overnight at 4°C. The identity of the purified protein was determined by mass spectrometry. The purity of RsmA was visually determined in a Coomassie-stained 15% electrophoresis gel (SDS-PAGE).

RNA EMSA

Annealed oligomers or PCR amplicons with T7 promoters on the forward primer were used to construct RNA probes for electromobility shift assays with RsmA (Table 4.2). Oligomers were either annealed or amplified using *Taq* polymerase from NEB and gel purified using an NEB Monarch gel extraction kit. *In vitro* transcription was performed using the NEB HiScribe T7 High Yield RNA synthesis kit. Upon completion, the Pierce RNA 3' End Biotinylation kit was used to generate probes. The kit control was also biotinylated as a control for this step and the EMSA as it has an RsmA binding motif (AGGA). Purified RsmA was used in this study. The Pierce RNA EMSA kit was used for the detection of protein-RNA interactions. Reactions were run on a 15% native PAGE and transferred to a nylon membrane at 35 mA for 35 minutes. The membrane was then UV cross linked for 60 seconds. The membrane was then treated using the Thermofisher LightShift Chemiluminescent detection kit. Membranes were developed using chemiluminescence detection on a ProteinSimple developer.

Statistical analyses.

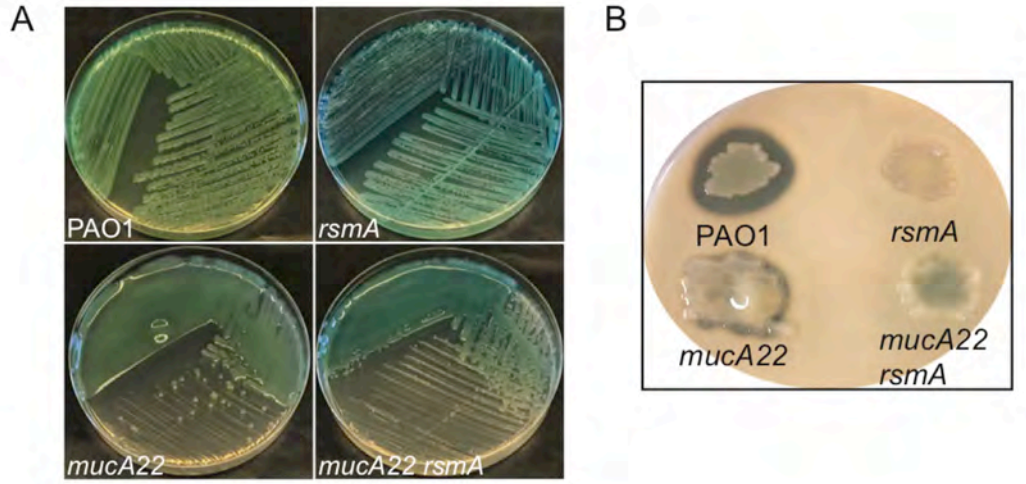
Statistical analyses were performed using Microsoft Excel software (Microsoft, Redmond, WA).

RESULTS

mucA22 Δ*rsmA* has variant phenotypes

In *P. aeruginosa* RsmA has been shown to regulate hundreds of targets through posttranscriptional regulation (13, 28). Our lab has previously shown that in a *mucA22* strain, AlgU and AlgR increase the expression of *rsmA* (12, 29). The *rsmA* mutation did not affect the ability for alginate to be produced and both *rsmA* and *mucA22 rsmA* both appeared bluish on PIA

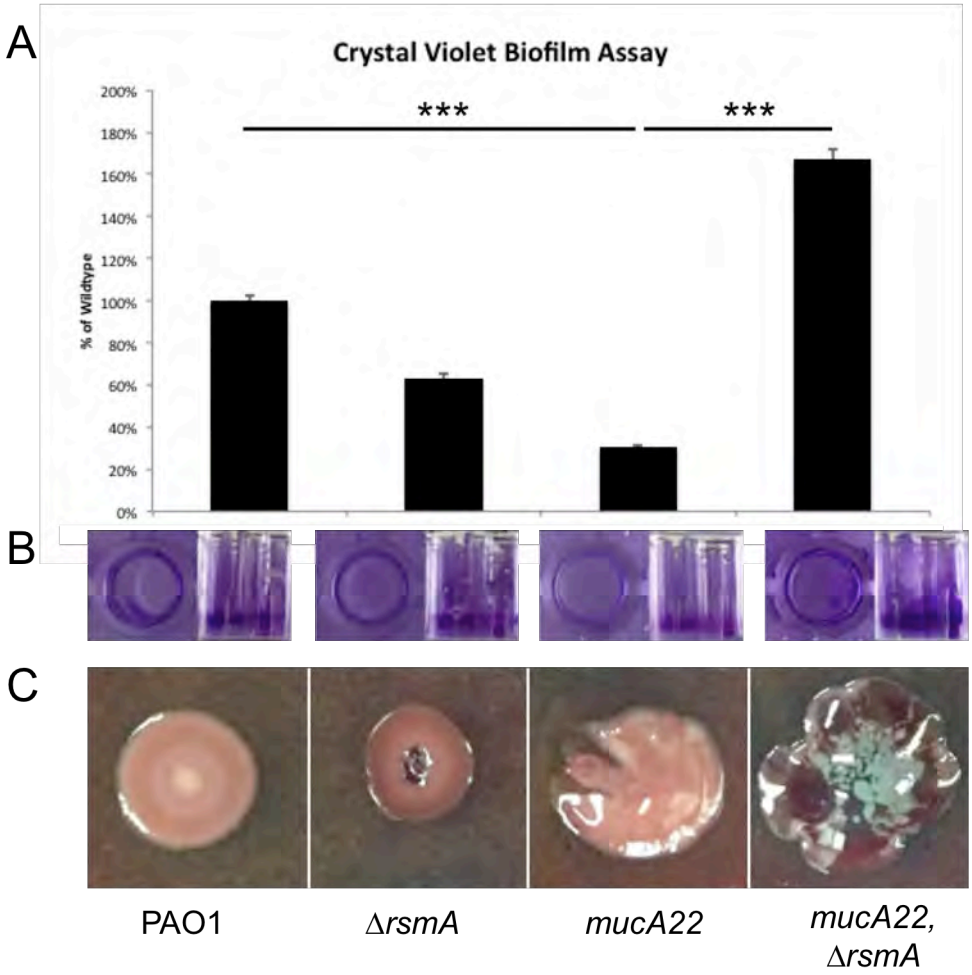
Figure 4.1 Appearance of *rsmA* mutants appear similar on PIA and skim milk agar.



P. aeruginosa strains on PIA (A) or skim milk agar (B). PIA is standard growth media for *Pseudomonas* strains and blue pigmentation is typical of *rsmA* mutants. Skim milk plates are used to observe the ability of a strain to digest casein protein.

(Figure 4.1a). To investigate other simple phenotypic assays, the *rsmA* mutants were also

Figure 4.2 *mucA22 rsmA* produces more biofilm.



A) A 96-well microtiter plate biofilm assay was performed to quantify amounts of biofilm between *P. aeruginosa* strains. All strains were normalized to PAO1 or percent of wildtype. ***represents a $p < 0.01$. B) Photos of below and the side of wells with *P. aeruginosa* strains stained with crystal violet. C) Colonies of *P. aeruginosa* strains grown on LB supplemented with Congo Red and Bromophenol Blue. Experiments were performed in triplicate (n=9).

screened on skim milk agar (Figure 4.1b) and analyzed for biofilm production (Figure 4.2). Skim milk agar looks specifically at the ability of the bacteria to digest the milk protein, casein (24). The parent strains were able to generate clear zones of digested casein much more readily than either *rsmA* mutant.

Biofilm production was quantified using a 96-well plate and visualized using crystal violet and Congo Red agar (22, 30). *rsmA* mutants produce more of the secondary

messenger cyclic di-GMP (31). This compound indirectly contributes to biofilm formation. The transcriptional regulator, AmrZ, also contributes to biofilm formation and is regulated by AlgU (32, 33). In the *mucA22 rsmA* strain both AlgU is active and RsmA does not repress cyclic di-GMP production. The *rsmA* strain showed a slight decreased in the biofilm assay, but appeared slightly darker than PAO1 on the Congo Red plate. This result has been previously reported where *rsmA* has impaired binding on a plate (28). *mucA22* showed an even more significant decrease in biofilm production. *P. aeruginosa* produces three known exopolysaccharides: alginate, pel, and psl (32, 34, 35). The *mucA22* strain has an abundance of alginate and RsmA, which can repress the ability for biofilm formation (36). The *mucA22 rsmA* strain showed the most increase in biofilm production in the microtiter plate assay and was also starkly different on the Congo Red plate. Congo Red is bound by the biofilm compounds of *P. aeruginosa* and causes the visual difference (22). The difference between *rsmA* mutants suggests RsmA has potential for regulation of other targets in the *mucA22* background compared to the nonmucoid PAO1 strain.

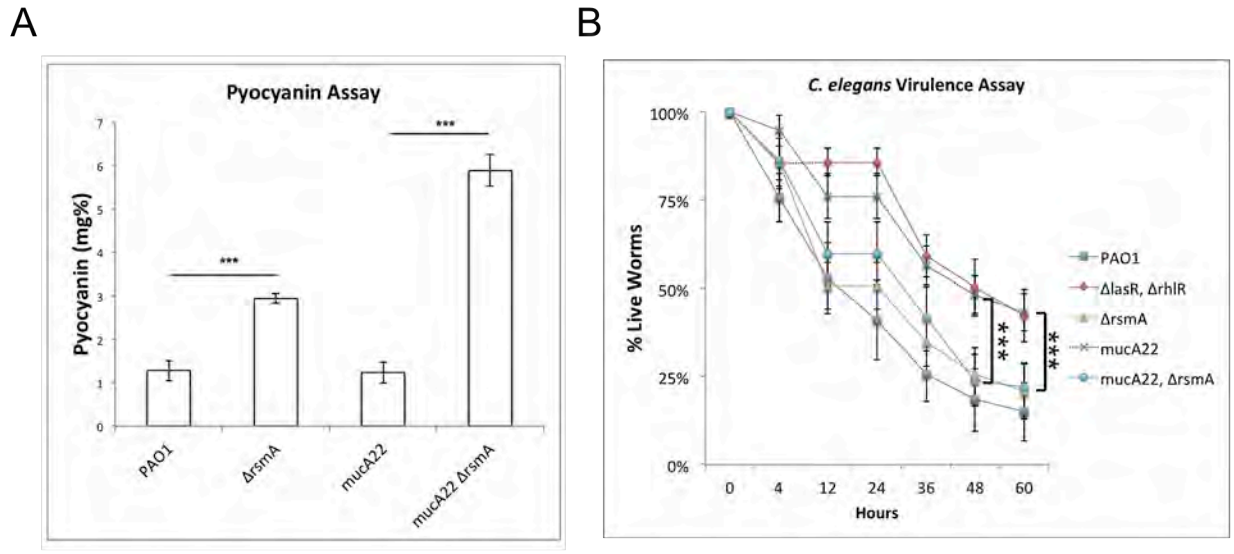
<u>*C. elegans* more susceptible to *mucA22* Δ*rsmA* pyocyanin levels</u>

RsmA has been shown to regulate pyocyanin production through anthranilate levels increasing in an *rsmA* mutant (28). Pyocyanin is a blue pigment produced by *P. aeruginosa* that blocks the effects of reactive oxygen species from host immune cells and creates an observable blue appearance during skin infections (2, 37). *Pseudomonas* on PIA media will appear bluer if there is increased pyocyanin production. Both *rsmA* mutants appeared blue on PIA, but pyocyanin can be quantified as well (Figure 4.3a). The parent strains, PAO1 and *mucA22,* produced no difference in pyocyanin levels. The nonmucoid *rsmA* mutant showed the expected increase has reported previously (28). Similarly to the biofilm assay, the *mucA22 rsmA* strain

produced a four-fold increase in pyocyanin compared to *mucA22.* This significant increase showed further importance of studying the effects of RsmA in the mucoid background.

To analyze the effects of increased pyocyanin on virulence, a fast killing assay with *C. elegans* can be used to screen for viability(27). In Figure 4.3b the *rsmA* were more lethal to the

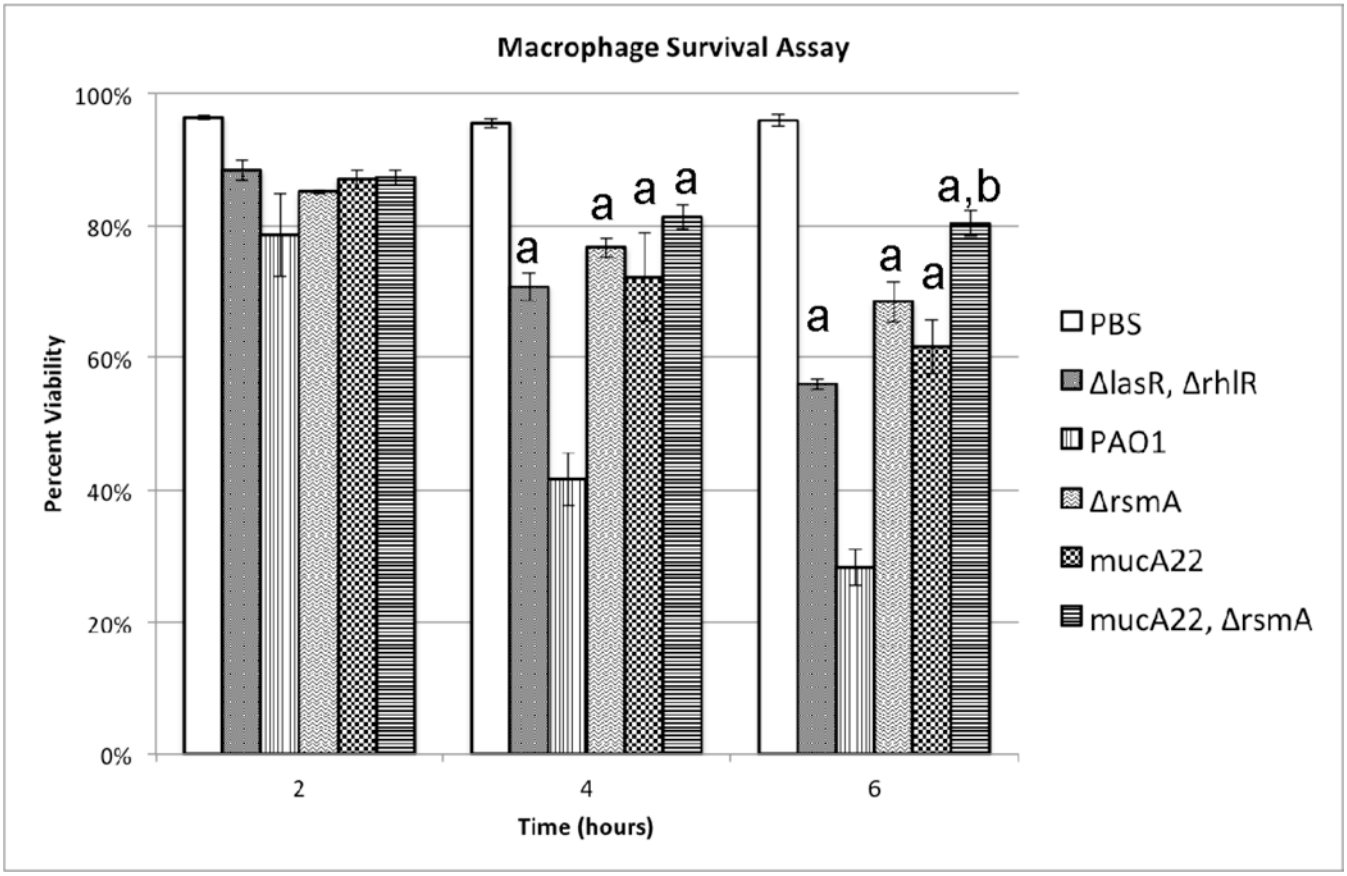
Figure 4.3 *mucA22 rsmA* mutant produces more pyocyanin and is more lethal in a fast-killing assay than *mucA22*.



A) Pyocyanin assay was used to quantify production of pyocyanin by *P. aeruginosa* strains. Experiments were performed in triplicate (n=9). B) Fast-killing assays were used to confirm production of pyocyanin. *C. elegans* is more susceptible to pyocyanin with this media. *P. aeruginosa* strains were incubated with *C. elegans* for 2.5 days. A *lasR rhlR* strain produces little if no pyocyanin and was used as a control. Experiments were performed in triplicate (n=6). *** represents a $p < 0.01$.

C. elegans due to increased pyocyanin production. A *lasR rhlR* mutant was included due to this strain being attenuated and also deficient in pyocyanin production (38). There was no difference between the lethality of PAO1 with *rsmA*, however the four-fold increase in pyocyanin production was significant after two days between *mucA22* and *mucA22 rsmA*. The fast killing assay with *C. elegans* showed increased lethality in the *mucA22 rsmA* strain. Pyocyanin is an important factor in this assay and the *mucA22 rsmA* strain produces four times as much compared to the attenuated *mucA22* strain.

Figure 4.4 *mucA22* Δ*rsmA* kills fewer J774a.1 macrophages than PAO1 and *mucA22* after 6 hour incubation.



J774a.1 macrophages were incubated with PBS or *P. aeruginosa* strains for up to 6 hours in 12-well plates. Macrophages were scraped and strained for viability with 0.4% Trypan blue strain. Experiments were performed in triplicate (n=9). a represents $p < 0.01$ compared to PAO1. b represents $p < 0.01$ compared to *mucA22*.

Macrophages have increased survivability with *mucA22* Δ*rsmA*

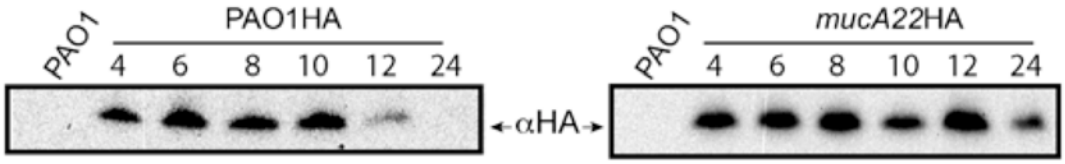
To investigate the effects of *mucA22 rsmA* virulence further on a level more relevant to higher organisms, *P. aeruginosa* strains were assayed with J774a.1 macrophages (Figure 4.4). A *lasR rhlR* strain was used as a negative control since this strain is attenuated for multiple virulence factors(39). *mucA22, rsmA, mucA22 rsmA* were all attenuated compared to PAO1. Even more interesting was the significant increase in macrophage viability in the *mucA22 rsmA* compared to *mucA22.* Since RsmA regulates multiple virulence factors and indirectly promotes

acute virulence factors an *rsmA* mutant has been shown to be attenuated with epithelial cell infection, which explains the increase in survivability with macrophages(15). Pyocyanin production has also been demonstrated to affect macrophage phagocytosis but due to incubation time and the cell culture media used, pyocyanin production does not seem to effect this assay (37). Overall, the role of RsmA in the mucoid background effects virulence factor regulation and pathogenesis, as we show herein that the *mucA22* Δ*rsmA* is less lethal to J774a.1 macrophages.

Proteomics approach reveals new targets of RsmA

RsmA targets had been studied in the nonmucoid background previously using PAO1 and PAK via a transcriptomics approach (13, 28). RsmA is expressed throughout the *P. aeruginosa* growth phases and most expression of RsmA in nonmucoid strains occurs around 9 hours (13, 40). A time course western blot was performed to identify the optimally expressed hour for investigating RsmA in the mucoid strain, *mucA22* (Figure 4.5). While RsmA is present even at 24 hours in *mucA22*, *P. aeruginosa* cultures were collected at 8 hours to be consistent with nonmucoid studies.

Figure 4.5 RsmA levels are increased in *mucA22* across all time points compared to PAO1.

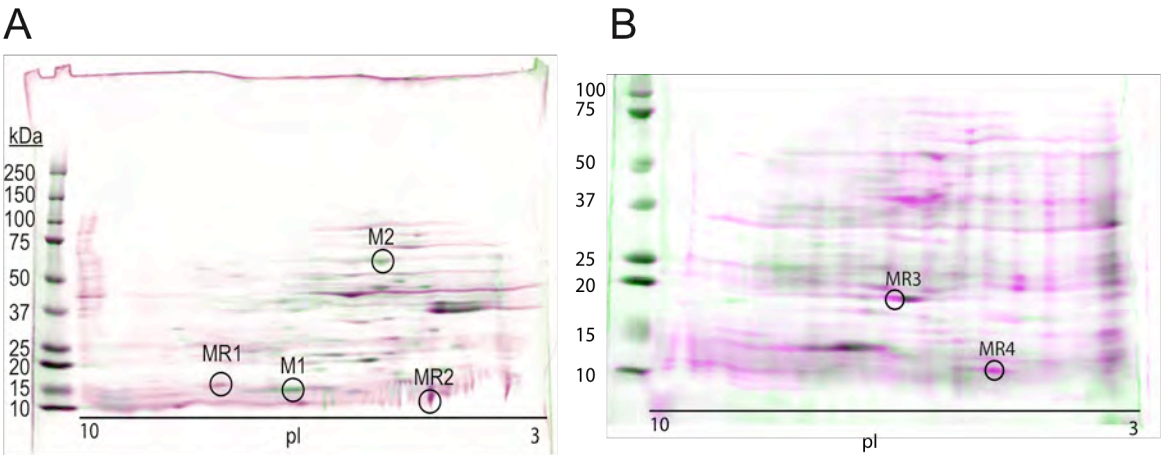


Western blot analysis of PAO1 and *mucA22* strains at 4, 6, 8, 10, 12, and 24 hours. Blots were probed for RsmA-HA with anti-HA monoclonal antibodies. A PAO1 strain lacking an HA-epitope was used as a negative control. Experiments were performed twice.

Since RsmA regulates targets by inhibiting translation of mRNA, a better representation of RsmA targets would be through a proteomics approach versus transcriptomics (15). In Figure 4.6a and b 2-dimensional gels were initially used to investigate the proteomes between *mucA22*

and *mucA22 rsmA*. In this proteomic approach, a known target of RsmA, Hcp1, was shown to be increased in the *mucA22 rsmA* background(13). In order to streamline the proteomic approach, the *P. aeruginosa* strains were analyzed though iTRAQ (isobaric tagging relative and absolute quantification). *mucA22 rsmA* was compared to *mucA22* and an abbreviated list of proteins can be observed in Table 4.3. In the iTRAQ screen the *mucA22 rsmA* strain showed increases in known RsmA targets, *hcp1* and *phnA* (28, 41). The small protease PasP was increased in not only the 2D gel but also 2-fold in the iTRAQ. PasP had

Figure 4.6 2D-gel analysis reveals new RsmA targets.



C

Match	PA Number	Protein	Function
M1	PA4922	Azurin	Azurin precursor
M2	PA4385	GroEL	60 kDa Chaperone
MR1	PA0085	Hcp1	T6SS component
MR2	PA4271/PA1804	RplL/HupB	50S Ribosomal protein/DNA binding Protein
MR3	PA0423	PasP	Protease
MR4	PA3940	PA3940	Probable DNA binding Protein

A) and B) 2D gels of *mucA22* (green) and *mucA22 rsmA* (pink) overlaid using Dymension software. Molecular weight markers are designated on the left side of the gels. Isoelectric points are designated on the bottom of the gels. Increases in the *mucA22* background (M) or in the *mucA22* Δ*rsmA* background (MR) were picked and in-gel trypsin digested. C) Table identifying the proteins via mass spectrometry analysis present in the selected spots.

not shown up in any of the transcriptome studies of RsmA, and it has been implemented in

Table 4.3. iTRAQ analysis between *mucA22* and *mucA22 rsmA*

Protein	Function	114	115	116	117	Average
		mucA22	*mucA22 rsmA*	*mucA22*	*mucA22 rsmA*	
OprF	Outermembrane protein F	1	3.44	1	2.13	2.785
PA5178	Uncharacterized protein	1	2.7	1	2.54	2.62
NusA	Transcription termination protein	1	0.71	1	3.66	2.185
Hcp1	T6SS component	1	2.3	1	1.98	2.14
PA5339	hypothetical conserved deaminase	1	2.79	1	1.32	2.055
PasP	small protease	1	1.59	1	2.43	2.01
GroEL	60 kDa Chaperonin	1	2.26	1	1.35	1.805
PhnA	Pyocyanin precursor	1	1.55	1	1.98	1.765
TufA	Elongation Factor Tu	1	2.41	1	1.05	1.73
PA4495	hypothetical protein	1	2.01	1	1.36	1.685
MvaT	Transcriptional regulator	1	1.18	1	2.18	1.68
RpsA	30S Ribosomyl Protein S1	1	1.68	1	1.56	1.62
PpiB	Peptidylprolyl cis trans isomerase	1	2.1	1	1.03	1.565
AlgD	GDP-mannose-6 dehydrogenase	1	1.1	1	1.91	1.505
Tig	Trigger Factor	1	1.79	1	1.21	1.5
AtpD	ATP synthase subunit b	1	1.45	1	1.45	1.45
AlgP	transcriptional regulator	1	1.37	1	1.52	1.445
HtpG	Chaperone protein	1	1.49	1	1.33	1.41
FusA	Elongation factor G1	1	0.89	1	1.91	1.4
Azu	Azurin	1	1.56	1	1.2	1.38
RplI	50S Rb protein L9	1	2.04	1	0.64	1.34
RplY	50S Rb protein L25	1	1.26	1	1.41	1.335
PA3940	Probable DNA binding Protein	1	1.36	1	1.17	1.265
RpsN	30S Ribosomyl Protein S14	1	0.86	1	1.52	1.19
AtpA	ATP synthase subunit a	1	1.18	1	1.07	1.125
InfC	Translation Initiation Factor	1	1.05	1	1.16	1.105
RplV	50S Rb protein L22	1	1.28	1	0.88	1.08
Icd	isocitrate dehydrogenase	1	1.13	1	0.97	1.05
SucC	Succinyl CoA ligase	1	1.29	1	0.77	1.03
AdhA	alcohol dehydrogenase	1	0.82	1	1.21	1.015
GyrB	DNA gyrase B subunit	1	1.09	1	0.92	1.005
AcpP1	acyl carrier protein 1	1	1.21	1	0.77	0.99
HupB	DNA binding protein Hu beta	1	0.75	1	1.01	0.88
ArcC	Carbamate Kinase	1	0.84	1	0.9	0.87
PA1579	hypothetical lipid binding	1	0.6	1	0.92	0.76
GreA	Transcription Elongation Factor	1	0.88	1	0.5	0.69
LptF	lipotoxin F	1	0.46	1	0.57	0.515
PhaF	Polyhydroxyalkanoate synthesis protein	1	0.73	1	0.17	0.45
OprI	Major Outermembrane Lipoprotein	1	0.2	1	0.33	0.265

Abbreviated data of iTRAQ results. Green shows a > 1.5 increased protein abundance in *mucA22 rsmA* compared to *mucA22*. Red shows a ≤ 0.76 decreased protein abundance in *mucA22 rsmA* compared to *mucA22*. Bold lines outline previously identified targets, or targets identified in 2D-gel analysis. Experiments were performed in biological duplicate for each iTRAQ label. Thermofisher Bioworks software was used for determining peptide confidence and iTRAQ label amounts. No proteins under a 10^{-3} peptide confidence were included meaning peptides had 99.95% confidence of correct identification.

affecting *P. aeruginosa* virulence (42, 43). Interestingly, these proteomic experiments are more

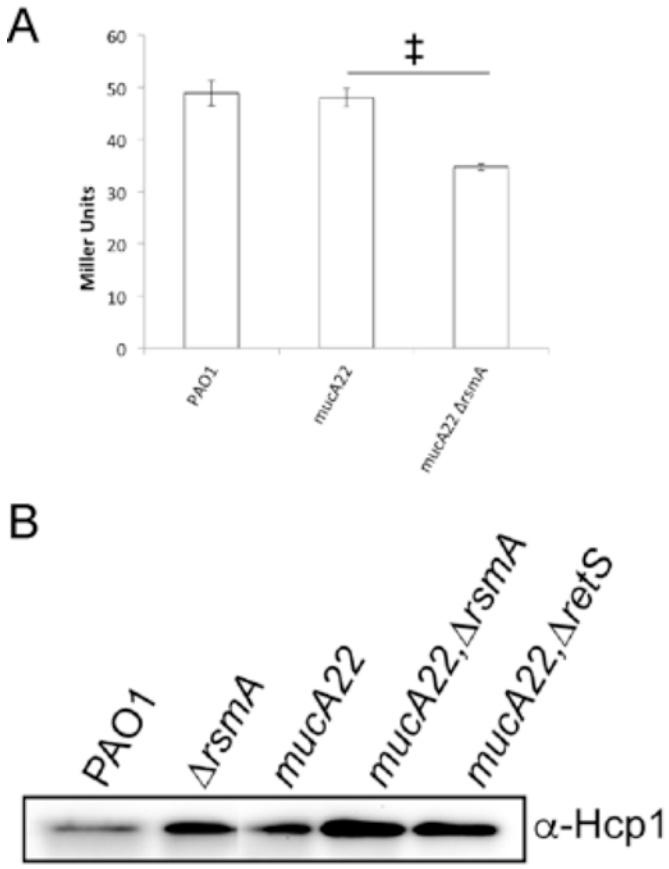
insight into RsmA's regulon in the mucoid background.

RsmA targets *hcp1* and *pasP* in *mucA22*

iTRAQ, just like an RNA-seq or microarray, has to be confirmed because effects can be indirect results of a mutation. In the case of RsmA, this is especially necessary since it regulates hundreds of targets. Since RsmA had been known to target *hcp1* already, there was no need to prove a direct interaction (41). The effects of *hcp1* in the mucoid background had not been investigated so transcription and western blot analysis was employed to confirm the 2D gel and iTRAQ results. In Figure 4.7a *hcp1* expression was no different between PAO1 and *mucA22*, however expression was decreased in *mucA22 rsmA*. The decreased expression levels of *hcp1* in an Δ*rsmA* mutant was not expected as nonmucoid Δ*rsmA* mutant is known to have increased levels of Hcp1. Western blot analysis was employed to look at whether RsmA was in fact having a role in Hcp1 translation in the mucoid strain. A *mucA22 retS* strain was included as a control since RetS has been shown to negatively affect the levels of type VI secretion system (T6SS) protein (44). The *mucA22 retS* strain showed more Hcp1 compared to *mucA22* as expected. Both *rsmA* mutants showed increased Hcp1 levels compared to the parent strains. The T6SS is used to fend off other foreign bacteria, but not thought to be expressed in mucoid strains. A previous proteomic comparison between co-cultured nonmucoid and mucoid strains, 383 and 2192, showed 2192 had less Hcp1 (45). Because RsmA is in higher abundance in *mucA22*, RsmA inhibits the translation of the T6SS in the mucoid background.

Since *pasP* has never been shown to be a target of RsmA, confirmation of the 2D gel and iTRAQ had to also include a direct interaction. The posttranscriptional effect on *pasP* was first investigated using a *lacUV5* leader fusion for its intergenic region (Figure 4.8a). The fusion was

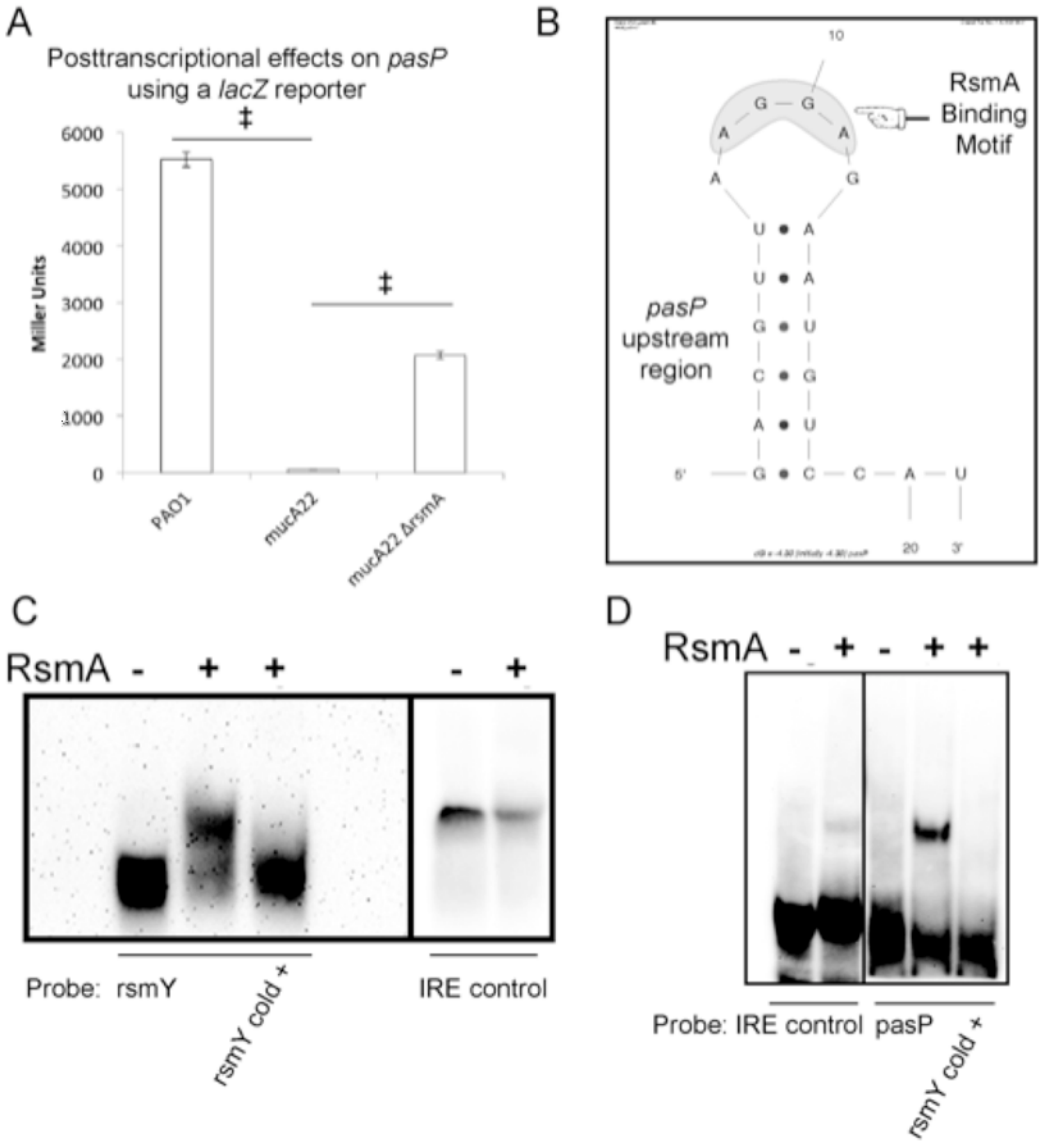
Figure 4.7. RsmA represses T6SS component Hcp1 in *mucA22.*



A) Strains were assayed for *hcp1* expression using a transcriptional fusion with *lacZ* reporter. P. *aeruginosa* strains were grown in LB broth overnight and then a 1% inoculum was grown for 8 hours. β-galactosidase assay was performed to measure expression using a LacZ reporter per Miller. Experiments were performed in three technical triplicates with three biological replicates (n=9). Student's t-test showed no significant difference between PAO1 and *mucA22*, however statistical analysis showed a decrease with $p < 0.001$ in *mucA22 rsmA* compared to *mucA22* represented by ‡. B) Western blot analysis with antibodies to Hcp1 in *P. aeruginosa* strains. *P. aeruginosa* strains were grown in LB broth overnight and then a 1% inoculum was grown for 8 hours. Nonmucoid and mucoid *rsmA* mutants had increased Hcp1 levels compared to their respective parent strains. A *retS* mutation, known to suppress T6SS production, is similar to a *rsmA* mutation and was used as a control in this analysis. ImageJ software was used to analyze densitometry.

very active in PAO1, but in *mucA22* where there is an abundance of RsmA, the fusion decreased 100-fold. When the *rsmA* allele was removed in *mucA22*, reporter activity went up 50- fold. RsmA is known to target an AGGA motif that is usually presented in the loop of a stem-loop structure (13). Using m-fold predictive software, Figure 4.8b shows the intergenic region of

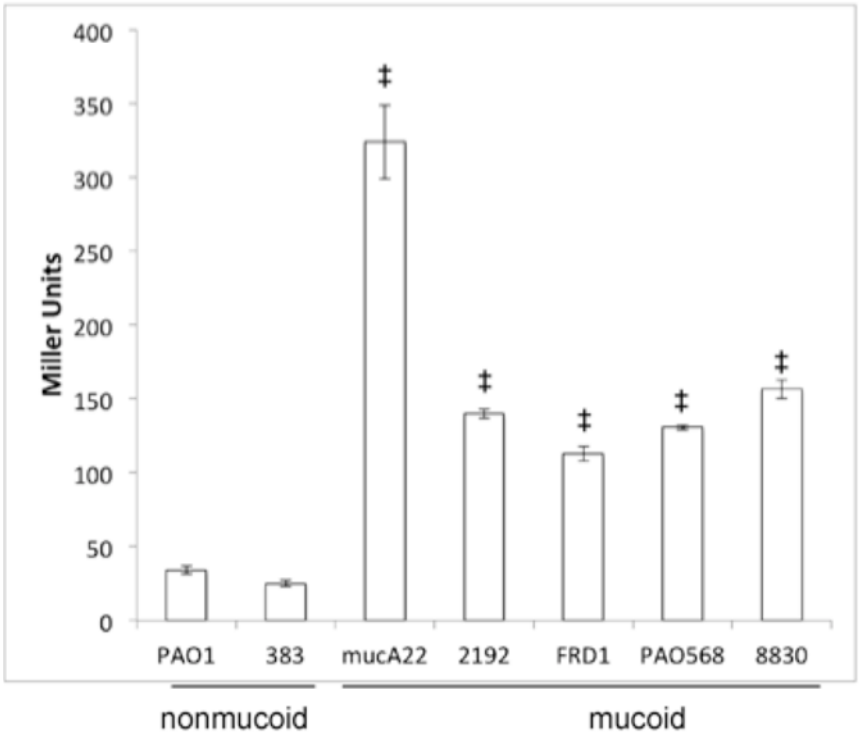
Figure 4.8. RsmA interacts directly with *pasP* in *mucA22.*



A) Constitutively active fusion with *lacZ* reporter constructed with *lacUV5* promoter and intergenic upstream region of *pasP* to quantify posttranscriptional activity of RsmA. . *P. aeruginosa* strains were grown in LB broth overnight and then a 1% inoculum was grown for 8 hours. β-galactosidase assay was performed to measure expression using a LacZ reporter per Miller. Experiments were performed in three technical triplicates with three biological replicates (n=9). Student's t-test was performed to compare PAO1 and *mucA22* as well as *mucA22* and *mucA22 rsmA*. Both showed statistical significance with $p < 0.001$ demonstrated by ‡. B) Predicted secondary RNA structure of the *pasP* upstream intergenic region between *PA0422* and *pasP*. The RsmA binding motif "AGGA" is highlighted at the top of the stem loop structure. C) and D) RNA electrophoretic mobility shift assay (EMSA) with purified RsmA to illustrate direct interaction between RNA of rsmY (C) or pasP (D) and RsmA. Twice as much unbiotinylated (cold) rsmY RNA was used to demonstrate specificity and outcompete the biotinylated probe. IRE (iron responsive element) RNA provided in the Pierce 3' OH RNA biotinylation kit has no RsmA binding motif and was used as a negative control.

PA0422 and *pasP*, which contains the AGGA motif in a stem-loop. To confirm a direct interaction with *pasP,* RsmA was purified and first tested with its ncRNA antagonist RsmY (Figure 4.8c). RsmA was then incubated with a biotinylated *pasP* probe to prove if *pasP* was a previously unknown target of RsmA (Figure 4.8D). RsmA could also be competed away with unlabeled RsmY transcript in both experiments confirming specificity of RsmA for the probe, RsmY or PasP (Figure 4.8c and d).

Figure 4.9. Mucoid strains of *P. aeruginosa* express more *rsmA* through *rsmA*'s distal promoter.



Strains of *P. aeruginosa* were assayed for *rsmA* expression using a transcriptional fusion (TF3-*lacZ*) that is regulated by AlgU and AlgR. *P. aeruginosa* strains were grown in LB broth overnight and then a 1% inoculum was grown for 8 hours. β-galactosidase assay was performed to measure expression using a LacZ reporter per Miller. Experiments were performed in three technical triplicates with three biological replicates (n=9). Student's t-test was performed between PAO1 and 383, which showed no significant difference. Student's t-test was performed between nonmucoid PAO1 and mucoid strains where ‡ represents $p < 0.001$.

Mucoid clinical isolates have increased *rsmA* expression

P. aeruginosa commonly undergoes phenotypic variation when exposed to the hostile response of a host's immune system (46). Isolates from cystic fibrosis patients commonly

convert to the mucoid phenotype. In our previous work, we investigated *rsmA* expression in a nonmucoid isolate 383 and a mucoid isolate 2192 that were co-cultured from the same cystic fibrosis patient (12, 55). The mucoid 2192 strain had increased RsmA levels via western blot analysis (12). Because our iTRAQ results showed new potential targets, more mucoid clinical isolates were investigated for *rsmA* expression using the TF3-*lacZ* fusion which relies on the AlgU promoter (12) (Figure 4.9). *mucA22* has the most increased *rsmA* expression all of the mucoid clinical isolates have at least 3-fold increased expression compared to the nonmucoid strains, PAO1 and 383. The increase of *rsmA* levels in mucoid strains lends credence to the importance of understanding the RsmA regulon in the mucoid background.

DISCUSSION

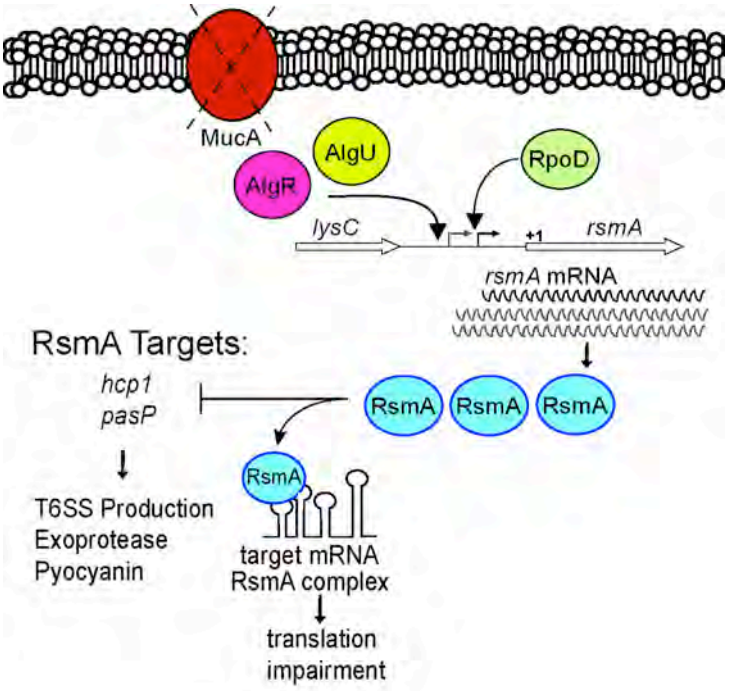
P. aeruginosa can cause many life threatening infections from acute to chronic. In the case of cystic fibrosis, *P. aeruginosa* will establish an infection in the lung where it can evade the host response and antimicrobials to persist and develop into a chronic infection (47, 48). Some clinical isolates from cystic fibrosis patients produce the phenotype termed mucoid, which is commonly indicative of a chronic infecting strain. The mucoid phenotype is produced by the extracytoplasmic sigma factor AlgU which regulates the alginate operon along with other transcription factors like AlgR(9, 49). Our previous work showed that AlgU and AlgR also contribute to *rsmA* expression and that there is increased RsmA levels in mucoid strains (12, 29). Most of the work on the RsmA regulon has been done in nonmucoid strains investigating the transcriptome (13, 28). However when compared to the nonmucoid *rsmA* mutant, the mucoid *mucA22 rsmA* varies phenotypically. The *mucA22 rsmA* strain produced more casein digestion, more biofilm, and more pyocyanin than the *rsmA* strain. We hypothesized that these results

would lead to increased virulence of the *mucA22 rsmA* strain. However, the *mucA22 rsmA* strain was attenuated compared to *mucA22* and *rsmA* with J774a.1 macrophages due to an unknown mechanism.

These stark differences between *rsmA* mutants in virulence factor production and attenuation with macrophages led us to hypothesize that the RsmA regulon in mucoid strains would be different that previously reported targets from transcriptome analysis. Our proteomic approach demonstrated a better representation of posttranscriptional regulation by RsmA in comparison to past transcriptomics analyses. If RsmA was not present to inhibit the translation of its targets, the proteins would appear in abundance. We initiated this screen with 2D-gel electrophoresis and identified previously unknown targets as well as known targets. In order to screen the potential hundreds of targets of RsmA, we streamlined this approach with iTRAQ. Using iTRAQ we confirmed already known targets of RsmA like Hcp1 and PhnA but also previously unknown targets (Figure 4.10). Unfortunately with all global experiments, results can be indirect. Direct interactions of RsmA and iTRAQ targets must be confirmed. However, the virulence factors that are repressed in the *mucA22 rsmA* have potential to be positively regulated RsmA targets just like CsrA is known to do with flagellum synthesis proteins in *E. coli* (18).

One new target we were able to confirm a direct interaction with RsmA is the *P. aeruginosa* small protease, PasP. PasP has been shown to aid in *P. aeruginosa* virulence in a rabbit corneal infection model (42, 43). Purified PasP was shown to also digest type I and IV collagens in these studies (42, 43). PasP has also been shown to be important in the infection process of a cystic fibrosis patient (48). We were able to show a direct interaction between biotinylated *in vitro* transcribed probes for RsmY and PasP with purified RsmA. By competing the RsmA away with cold *in vitro* transcribed RsmY, RsmA was shown to have specificity for

Figure 4.10. Model of RsmA in mucoid strains.



In mucoid strains of *P. aeruginosa* AlgR and AlgU co-regulated the distal promoter of *rsmA*. This activity leads to increased expression of *rsmA* in the mucoid background. The mucoid *P. aeruginosa* RsmA regulon is similar and different to the targets of RsmA in nonmucoid *P. aeruginosa* strains, PAO1 and PAK. RsmA targets T6SS components by blocking the ribosomal binding site and inhibiting translation, but also represses previously unknown targets like *pasP*. RsmA affects pyocyanin in both nonmucoid and mucoid backgrounds, however a complicated pathway produces pyocyanin and direct targets have yet to be elucidated.

the *pasP* message. Further work on the role PasP plays on the infection of *P. aeruginosa* is important to elucidate the purpose of RsmA in regulating this target so heavily in mucoid strains. Overall, studying the RsmA regulon in mucoid strains can help explain the role RsmA plays in the infection process especially with cystic fibrosis. Also, by knowing what targets will and will not be expressed direct antivirulence compounds in concert with antimicrobials can lower the morbidity and mortality in cystic fibrosis patients with *P. aeruginosa* infections.

ACKNOWLEDGEMENTS

The Department of Health Sciences for always supporting our lab's research and making available resources and equipment. Marianne Brannon, Ph.D. and Bill Stone, Ph.D. for the immense help with proteomics analysis and experiment design. The Filloux lab for generous donation of Hcp-1 antibodies. We are grateful for the donation of from the Kruppa lab at ETSU of *C. elegans* and training us to work with *C. elegans*.

FUNDING INFORMATION

This work, including the efforts of Chris L. Pritchett, was funded by Research and Development Committee, ETSU (RDC12-012M and RDC13- 005M).

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CHAPTER 5

PSEUDOMONAS AERUGINOSA MINOR PILIN PROTEIN, PILW, NEGATIVELY REGULATES THE ALGZ/R TWO-COMPONENT SYSTEM

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Keywords: Type IV Pili, Two-component system, PilW, AlgZ, AlgR, virulence

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen and causes the most complications in hosts with compromised immune systems like diabetes, HIV/AIDS, and cystic fibrosis. *P. aeruginosa* wreaks havoc with an armamentarium of virulence factors able to evade immune defenses and thrive in these hosts. *P. aeruginosa* regulates multiple virulence factors via two component systems (TCS). TCS are composed of a sensor histidine kinase that receives an external signal, autophosphorylates, and then phosphorylates its response regulator, which activates or represses target genes. One TCS, AlgZ/R, activates the expression of the minor pilin operon (*fimUpilVWXY1Y2E*) important in type IV pili (TFP). Previous work has shown that some minor pilins can cause increased expression of the minor pilin operon via a feedback loop, but the mechanism is unclear. *algZ* and *algR* transcriptional fusions were analyzed in a *pilW* mutant strain of PAO1 to see if activity was affected due to the increase of minor pilin expression. A 10-fold increase in *algZ* expression was seen in the *pilW* mutant. A previous work performed a transposon mutagenesis screen to identify the regulator of this increase in *algZ* expression. In this study, we show that the increase in minor pilin expression in a *pilW* mutant is due to increased *algZR* expression and that phosphorylated AlgR is required for this increase. These results were confirmed with qPCR and western blot analysis. These effects were also examined in a *pilW,* phospho-deficient *algR* mutant (*pilW algRD54A*) that restored *algZR* expression to wildtype levels. The biological effects of increased phosphorylated AlgR levels were assayed with phenotypic assays for pyocyanin, elastin, and rhamnolipids, all showing significant decreases in a *pilW* strain, but restoring levels to wildtype in *pilW algRD54A*. Lastly, due to decreases in these virulence factors in *pilW*, the survivability of J774a.1 and RAW264.7 macrophages were assayed in the presence of the *pilW* and *pilW algRD54A* strains. Overall the

overexpression of *algZR* in a *pilW* mutant leads to decreased virulence and demonstrates an Achilles' heel for *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting any host tissue. *P. aeruginosa* mostly causes acute infections in individuals with HIV/AIDS, with burns over a majority of their body, and with ventilator assisted respiration (1, 2). These acute infections can lead to septicemia and eventually death (3, 4). *P. aeruginosa* compromises its host and evades the host immune system with the help of an armamentarium of virulence factors (1, 5). The bacterium contains a diverse network of sensors and regulators controlling the expression of virulence factors as to efficiently establish a niche and fully reap the host's nutrients.

One simple mechanism that *P. aeruginosa* uses to sense environmental signals is a two-component system (TCS) (6). TCSs are composed of a membrane bound sensor histidine kinase that autophosphorylates upon binding its signal and then transfers this phosphate to a specific response regulator. While many pathogens use TCSs, there are more identified TCSs in *P. aeruginosa* than any other studied pathogen (7). These TCSs contribute to *P. aeruginosa* pathogenesis in multiple ways including: biofilm development, attachment, antibiotic resistance, suppressing posttranscriptional regulation, cyclic di-GMP production, and motility (5).

While some TCSs can have specific control over a single virulence mechanism, there are TCSs that contribute to regulation of multiple virulence factors. The TCS AlgZ/R has been shown to regulate rhamnolipids, pyocyanin, small noncoding RNAs, and type IV pili (TFP) (8, 9). This system consists of the histidine kinase, AlgZ, and the response regulator, AlgR. AlgZ and AlgR are located in an operon together, like most TCSs (6). AlgZ is bound to the inner membrane and the environmental signal it receives has still not been elucidated. The histidine residue at 175 transfers the phosphate to AlgR's aspartate at residue 54. AlgR contains the same

motifs of most response regulators including the helix-turn-helix DNA binding domain (10). Interestingly, AlgR can activate its targets in the phosphorylated form, like the TFP, or in a phosphorylation independent manner, like *rsmA* (8, 11).

AlgR contributes to the production of TFP by regulating the expression of the minor pilin operon (*fimUpilVWXY1Y2E)*. These minor pilins are incorporated into the TFP stalk along with the major pilin, PilA, and allow for *P. aeruginosa* to attach to surfaces and also perform motility in the form of twitching (12). The TFP structure has two ATPase motors at its base for extension and retraction of the stalk, allowing the bacterium to move across a surface (13, 14). Previous work has shed light on the fact that the TFP not only contribute to adhesion and motility, but that the tip of the TFP stalk, PilY1, contributes to expression of other virulence factors by acting as a mechano-sensory apparatus, proving to be important in initiating an infection (14). Luo et al. also showed that by removing the minor pilin operon, it caused a significant increase in minor pilin expression (14). However, the actual mechanism by which the TFP works on regulating other virulence factors has not been elucidated.

In this study, we expand upon the master's thesis of Danielle A. Williams where she performed a transposon mutagenesis with a *pilW* mutant containing an *algZ-lacZ* transcriptional fusion (30). Her master's work identified that in this background *algZ* expression was elevated, but was significantly decreased when *algR* was interrupted by the transposon (30). We hypothesized due to AlgR regulating minor pilin in a phosphorylation dependent manner and by removing a minor pilin allele like *pilW* caused a significant increase in minor pilin expression that not only was this due to increased levels of phosphorylated AlgR, but also these levels of AlgR were causing a significant increase in *algZ* expression. In order to confirm this hypothesis a *pilW* phospho-deficient strain, *pilW algRD54A,* was used. We assessed the *pilW* strain for

AlgZ and AlgR levels, production of virulence factors regulated by AlgR, and whether or not the increase in *algZ/R* expression had an effect on virulence. Overall, our work shows that targeting the TFP can lead to manipulating the AlgZ/R TCS to make *P. aeruginosa* less virulent and in combination with antibiotics, more readily cleared by the immune system.

METHODS

Strains, plasmids, and media

The strains used in this study are presented in Table 5.1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics, as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB medium, or Vogel-Bonner minimal medium (VBMM) supplemented with the appropriate concentrations of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 μg/ml tetracycline, 15 μg/ml gentamicin, 100 μg/ml ampicillin, and 35 μg/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 μg/ml gentamicin, 50 μg/ml tetracycline, and 300 μg/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

Table 5.1

Strain or Plasmid	Genotype or Relevant properties	Reference or Source
E. coli strains		
NEB5α	*fhuA2 Δ(argF*-lacZ*) U169phoA, glnV44 Φ80Δ(*lacZ*)*M*15 gyrA96*	New England Biolabs
pRK2013	Helper strain	16
BTH101	F^-, *cya-99, araD139, galE15, galK16, rpsL1 (*Str^r*),*	Euromedex

	hsdR2, mcrA1, mcrB1	
*algZ*HA pEX18Gm	Allelic exchange for *algZ* HA allele	30
algZ-lacZ miniCTX	Transcriptional fusion	30
pAlgRD54E	Overexpression of AlgRD54E	This study
P. aeruginosa strains		
PAO1	Wild type	32
algR	*algR* mutant	33
algZ	*algZ* mutant	30
algZHSDM	*algZ* mutant with histidine 175 replaced with glutamate	11
pilA	*pilA* mutant	30
lasR rhlR	Quorum sensing deficient strain	29
pilW	*pilW* mutant	31
pilY1	*pilY1* mutant	30
vfr pilW	Double mutant	30
pilW algR	Double mutant	30
pilW algRD54A	Double mutant	30
pilW algZHSDM	Double mutant	30
PAO1HA	PAO1 with epitope tagged AlgZ	This study
*pilW*HA	*mucA22* mutant with epitope tagged AlgZ	This study

Strain construction

All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs [NEB], Ipswich, MA) and primers listed in Table 5.2. Crossover PCR (splicing by overhang extension PCR) (14b) was used to construct deletion mutations and to clone sequences into the suicide vector pEX18Tc or pEX18Gm (15). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (16). Conjugations were performed overnight on LB plates at 37°C, and the conjugants were plated on the appropriate selective medium to obtain single-crossover mutants. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT medium–10% sucrose plates. Mutations were confirmed

Table 5.2		
Primer	Sequence	Use
algZqPCRF	GACCTGTCCGACCTGTTCCGG	qPCR
algZqpcrR	CCAGCAGCGGCTGCAAGGTC	qPCR
algRqPCRF	GCGGCAGCGGTCCGCGAG	qPCR
algRqpCRR	CTTGTGGTCGGCAATGAAG	qPCR
uvrDqpcrR	GGCGTCCCTGGTTGTT	housekeeper
uvrDqpcrF	GCAGTTCAGCGACGAC	housekeeper
mreBqpcrR	CGCCGCAACTACGGCAG	housekeeper
mreBqpcrF	GGTTGCGGCCGCGGA	housekeeper
algZTSFF	GCGCGGATCCGTTCATGTGCACGTCTTCCAGG	Transcriptional fusion
algZTSFR	GCGCAAGCTTCGACAGAGTTTCCGCAAGG	Transcriptional fusion
AlgRR	GCGCGGTACCCAGAGCTGATGCATCAGACGC	BACTH
AlgRF	GCGCGGATCCGAATGTCCTGATTGTCGATGACG	BACTH
AlgZF	GCGCGGATCCGCCTATCCGATTCAAGCCCGG	BACTH
AlgZR	GCGCGGTACCCAGGCTTCCTGCATGAGTCGC	BACTH
AlgZTruncR	GCGCGGTACCGCGCGTGCCGCAGATACAG	BACTH

using PCR with primers containing the suffix intF and intR in Table 5.2 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of the proteins was accomplished using primers containing the HA tag at the 3' end of the gene, and HA was introduced as described above using the suicide vector pEX18Gm.

Site-directed mutagenesis

Phosphorylated primers algRD54AF/algRD54EF and algRD54AR/algRD54ER were used in an inverse PCR with the *algR* allele in pEX18Gm as the templates. The aspartate at residue 54 of the *algR* allele was mutated into either an alanine with an NheI restriction site or a glutamate with an XbaI site. The successful inverse PCR product was self-ligated, transformed into NEB5α cells (NEB, Ipswich, MA), and screened by plasmid digestion with respective restriction enzyme digestion and sequencing. The constructs obtained by site-directed mutagenesis were conjugated into *Pseudomonas* strains as described above.

BACTH

Bacterial adenylate cyclase two-hybrid system from Euromedex was used to test for protein-protein interactions (17). The *algR, algZ,* and a truncated form of *algZ* were amplified from PAO1 with primers listed in Table 5.2. These genes were cloned into the BACTH vectors. For *algR* and the truncated C-terminus *algZ* either N-terminus or C-terminus fusions were used. For the full-length *algZ* allele, only the C-terminus fusion was used. These vectors were transformed into NEB5α cells and selected for on their respective antibiotic LB media, Amp100 or Kan50. After colony PCR confirmation, the strain was struck for isolation, frozen down and set up for a plasmid precipitation using NEB Monarch plasmid prep kits. These plasmids were than digested with the respective restriction enzymes and confirmed on a 1% agarose gel. BTH101 cells were then transformed with the appropriate control vectors: empty Kan and Amp vectors for negative control, leucine zipper positive controls, and the alleles to be investigated. Cells were spread on MacConkey agar (Difco) with 1% Maltose, Amp100, and Kan50 or LB/IPTG/XGal, Amp100, and Kan50 per Euromedex protocol. Colonies were PCR confirmed for inserts before assaying. Assaying consisted of measuring β-galactosidase activity per Miller.

qPCR

P. aeruginosa strains were grown in LB broth at 37ºC overnight and then a 1% dilution of the overnight culture was grown for 6 hours. The bacteria were collect by centrifugation following the ZymoResearch Direct-zol RNA kit protocol. Upon elution from the column, RNA was treated with DNase-I from NEB for 30 minutes at 37ºC. RNA was then precipitated with 200 proof EtOH and frozen overnight. The following day, the samples were centrifuged at 15k x g for 10 minutes at 4ºC. The EtOH was aspirated off, 1 mL of 70% EtOH was added , and

centrifugation repeated. EtOH was again aspirated off and samples were left to air dry. Pellets were resuspended in 40 µl of Nuclease free water and quantified using a Thermofisher NanoDrop. To generate cDNA, 5 ng of RNA was used with 2.5 ng random hexamers/µL (New England Biolabs, Ipswitch, MA), 0.5 mM dNTPS, and followed Invitrogen superscript IV reverse transcriptase protocol. A no RT control was generated. For qPCR NEB Luna was used with a BioRad CFX96 Real-Time PCR detection system to quantify amplicons. Primers listed in Table 5.2 were designed for inside the coding region of the target. Two house-keeping genes, UvrD and RpoS, were used as controls along with no RT wells and wells for dilution of a PCR amplicon of the gene of interest. Data analysis was performed using the CFX Manager software. Experiments were repeated four times (n=12).

Western blot analysis

P. aeruginosa strains were grown in LB broth at 37°C overnight and then a 1% dilution of the overnight culture was grown for 6 hours. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 µg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:20,000 dilution of anti-HA monoclonal antibody (Thermo Fisher, Pittsburgh, PA), followed by a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (18)or 1:20,000 dilution of AlgR antibody followed by 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody. The membrane was stripped per Abcam mild stripping protocol and then probed with RpoA primary antibody (1:20,00 dilution) followed by 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin

antibody. Detection was performed using an ECL Plus kit (Thermo Fisher, Pittsburgh, PA) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). All Western blot analyses were repeated at least twice.

Phenotypic Assays

Various virulence products were assayed in the *P. aeruginosa* strains in this study. All strains were grown in LB broth at 37ºC for 16 hours. Pyocyanin was assayed by centrifuging the culture and collecting the supernatant. Then 2 mL of chloroform was used to extract any pyocyanin produced in the culture by vortexing vigorously and allowing to set for 10 minutes. The chloroform layer was removed and 1 mL of 0.1 N HCl was added and vigorously vortexed and allowed to set for 10 minutes. 0.1 N HCl was used to blank the spectrophotometer at OD_{562nm}. Values were normalized to PAO1. Elastase was assayed using Elastin-Congo Red (ECR) from Sigma Aldrich. Strains were centrifuged and 100 µL taken from the supernatant. This was mixed with 900 µL of ECR solution (100 mM Tris, 1 mM $CaCl_2$, pH 7.5, 20 mg/mL) and incubated at 37ºC for 4 hours at 250 rpm. The negative control used to blank the spectrophotometer was 1 mL of ECR solution with no strain supernatant. Values were normalized to PAO1. Rhamnolipid production was assayed using the orcinol method listed in Morici et al. (19). All experiments were performed in triplicate (n=9).

Macrophage Survival Assays

P. aeruginosa strains were incubated with ATCC J774a.1 or Raw 264.7 macrophages to assess macrophage survivability. Macrophages were grown in Corning cellgro DMEM with L-Glutamine, 4.5g/L Glucose, and 10% fetal bovine serum and 100 I.U./ml penicillin 100 µg/mL streptomycin. Cells were maintained in a 37ºC incubator with 5% CO_2 and humidity. Cells were

counted and plated in 12-well plates at 1x10^6 cells per well in media without pen/strep. Overnight LB cultures of *P. aeruginosa* strains were diluted to 1% and allowed to grow for 2 hours at 37ºC at 250 rpm. Cultures were then centrifuged and washed with 1X PBS. Strains were then aliquot to an OD_{600nm} of 0.1 and incubated with macrophages up to 6 hours. The inoculum of bacteria was also measured in CFU/mL. Macrophages were collected at 2, 4, and 6 hour time points and 1 mL of culture was mixed with 100 µL of 0.4% trypan blue to measure viability. Experiments were performed in triplicate (n=9).

Overexpression/complementation studies.

In order to overexpress *algD54E*, the coding sequence was amplified (Table 5.2) and cloned into the integrating vector pTJ1 for complementation or overexpression(20). Trimethoprim-resistant colonies were picked and patched on trimethoprim-irgasan media and 0.1% arabinose PIA. The presence of the gene of interest was confirmed using primers pHERDSF and algRTJ1R. Cultures requiring induction used 0.1% arabinose and were grown for overnight in broth or on agar plates.

β-galactosidase Assay

β-Galactosidase activity was determined by incubating cell extracts with *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) as described by Miller (20b). Experiments were performed in triplicate (n=9).

Statistical analyses.

Statistical analyses (Student's t-test) were performed using Microsoft Excel software (Microsoft, Redmond, WA).

RESULTS

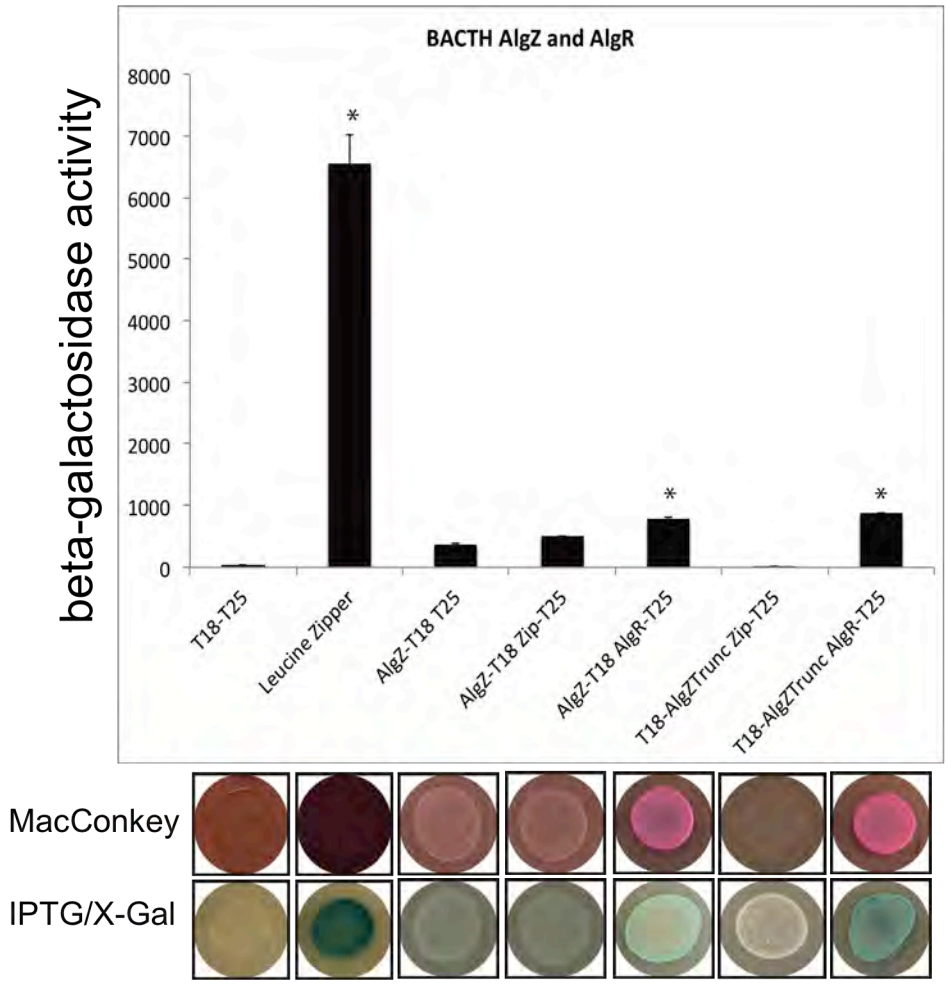
AlgZ interacts with AlgR and AlgR dimerizes

The response regulator AlgR has been thoroughly studied and also implicated to affect virulence in *P. aeruginosa* (4, 10). While AlgR and its cognate sensor histidine kinase have been shown to be in an operon together, they have never been shown to actually interact (21). It is important to demonstrate this interaction to complete a portion of *P. aeruginosa* knowledge. With TCSs the sensor will share a phosphate with the response regulator at specific amino acid residues, however they also have protein-protein interaction domains. In the case of AlgZ and AlgR the C-terminus region of AlgZ is proposed to interact with AlgR. By using a BACTH system, AlgZ and AlgR were shown to interact (Figure 5.1). In this analysis, appropriate controls consist of empty vectors (T18 or T25), which produce pieces of adenylate cyclase, but do not interact. The positive controls consisted of a leucine zipper (Zip) that is known to dimerize and allow adenylate cyclase to make cyclic AMP (cAMP). Positive results consist of 700 to 7000 miller units with a β-galactosidase assay and colorimetric indication by pH indicator phenol red on MacConkey agar or cleavage of X-gal on LB/IPTG/Xgal agar.

Since the AlgZ protein is incorporated into the inner membrane of the bacteria, the fusion was placed on the C-terminus, so that the adenylate cyclase portion on AlgR had greater potential to interact. An empty vector and leucine zipper-containing vector were co-transformed with AlgZ carrying vector to test for any aberrant interactions. While the full-length AlgZ and AlgR interaction did show positive results, some reporter activity was observed with our negative controls. One possible explanation for these results is the fact that the structure of AlgZ has not been elucidated and this could lead to arbitrary interactions. To combat this result, a

truncated form of AlgZ was cloned into the BACTH vector, which just included the C-terminus

Figure 5.1 AlgZ and AlgR interact.



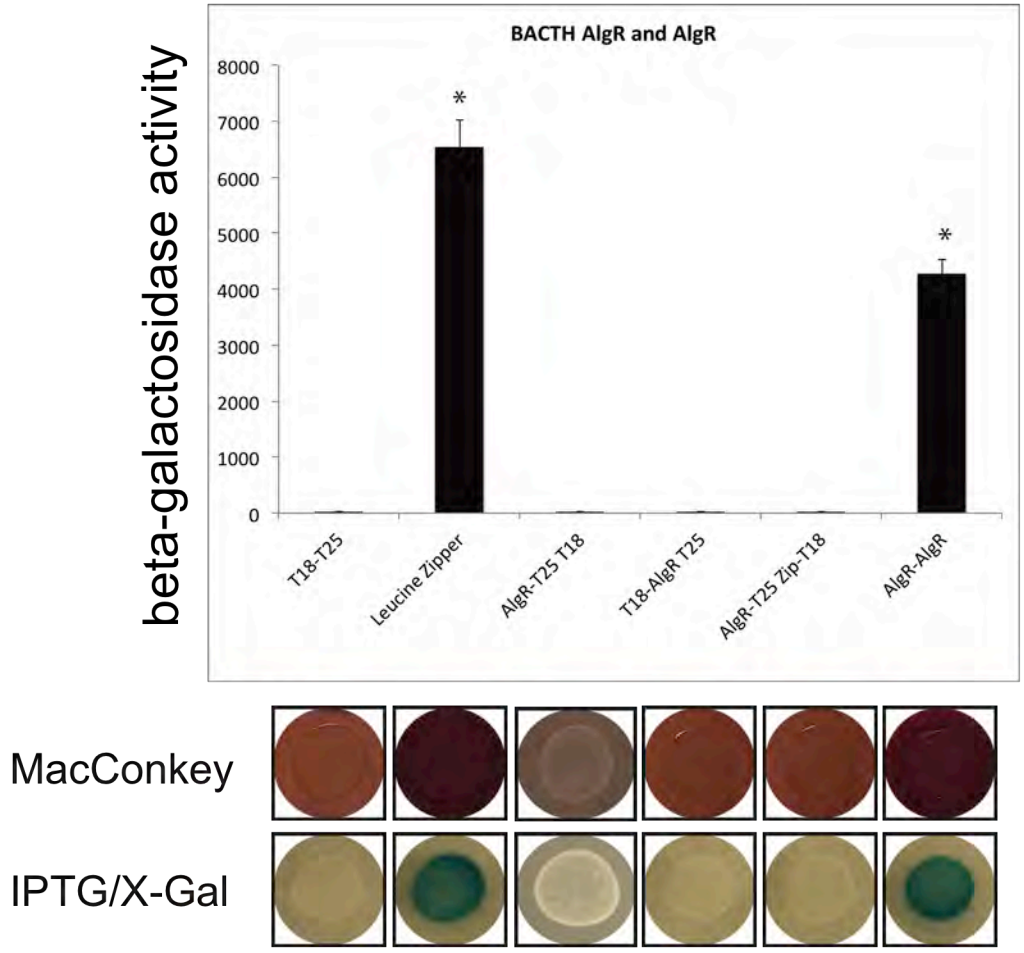
BACTH assay for AlgZ or truncated AlgZ interacting with AlgR using T18 or T25 portions of adenylate cyclase. β-galactosidase assay, MacConkey agar supplemented with 1% maltose, and LB agar supplemented with IPTG/X-Gal were screened for interaction between AlgZ, AlgZ C-terminus, and AlgR. * signifies beta-galactosidase activity units between 700 – 7000, the significance threshold for the BACTH. . β-galactosidase assay was repeated in triplicate (n=9).

region (AlgZTrunc). The truncated form of AlgZ did not interact with the leucine zipper, however the same reporter activity was observed with the truncated form of AlgZ and AlgR-T25.

Response regulators are commonly known to dimerize to activate their genetic targets. AlgR has been shown to activate the minor pilin operon and the posttranscriptional regulator

rsmA with two binding sites (8, 11). This consistent motif led us to also analyze the ability for

Figure 5.2 AlgR dimerizes.



BACTH assay for AlgR-AlgR interaction using T18 or T25 portions of adenylate cyclase. β-galactosidase assay, MacConkey agar supplemented with 1% maltose, and LB agar supplemented with IPTG/X-Gal were screened for interaction between AlgR and AlgR (T18-AlgR, AlgR-T25). * signifies beta-galactosidase activity units between 700 – 7000, the significance threshold for the BACTH. β-galactosidase assay was repeated in triplicate (n=9).

AlgR to dimerize using the BACTH system. The same controls were repeated in this experiment except with AlgR. No activity with the empty vectors or the leucine zipper was reported with AlgR (Figure 5.2). However, when both vectors containing AlgR were transformed, around 4000 units of β-galactosidase activity was observed as well as increased colorimetric reporter.

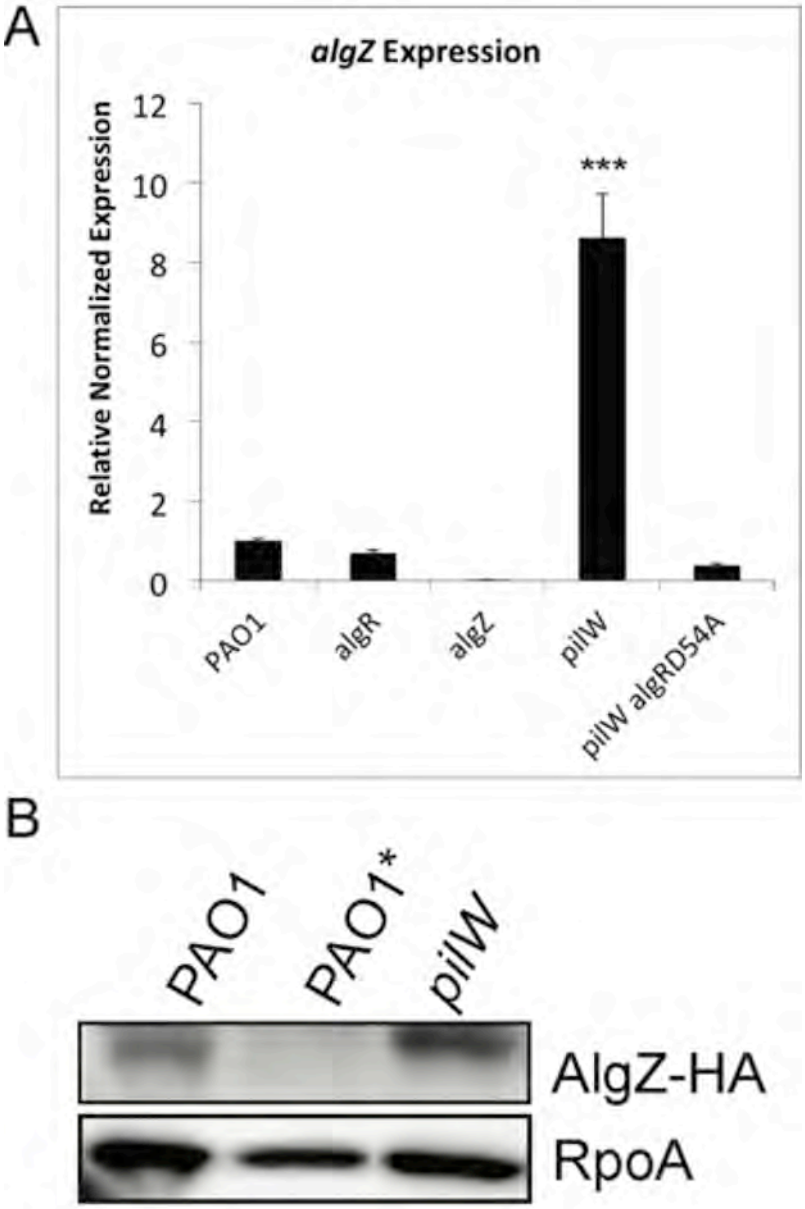
Confirming the interactions of AlgZ with AlgR and AlgR dimerizing demonstrates the AlgZ/R TCS truly interacts.

AlgZ levels are increased in *pilW* strain

When studying the effects of a *pilY1* mutation, Luo et al. showed that the increase in minor pilin expression was due to AlgR and not the known regulator of *algZ,* Vfr (14). However, this group never investigated the effects on *algZ* expression; considering if AlgR needs to be phosphorylated to activate the minor pilin operon then there might be an increase in *algZ* to cause more phosphorylation. Williams identified that not only was there increased *algZ* expression in a *pilW* strain but that this was dependent on AlgR (30). Since Williams only investigated these effects with *lacZ* reporter fusions, *algZ* levels were examined with qPCR and western blot (Figure 5.3a). Williams reported expression of *algZ* was maximal at 6 hours with an *algZ-lacZ* transcriptional fusion (30). An *algZ* strain was used as a negative control in our experiment. The *pilW* mutant had a 8-fold increase in *algZ* expression compared to the PAO1 wildtype strain. Interestingly, the phospho-deficient AlgR *pilW* strain showed *algZ* expression levels less than PAO1. These results further asserted that there was a phosphorylated AlgR dependent phenomenon causing the increase in *algZ* expression.

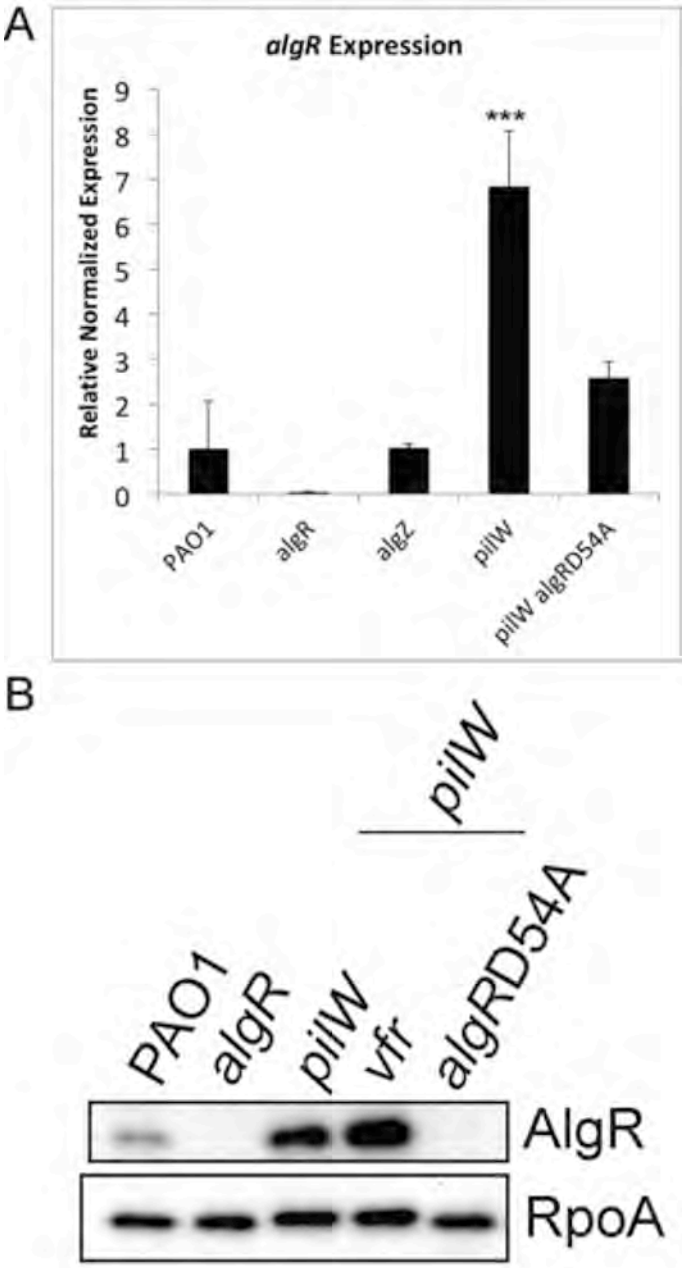
AlgZ levels between PAO1 and *pilW* were studied using a HA-epitope tagged *algZ* allele (Figure 5.3b). These strains were also grown in LB and collected after 6 hours to mimic the qPCR experiments. Western blot analysis also showed a significant increase in AlgZ levels in the *pilW* background. The lower levels of AlgZ protein versus *algZ* transcript might be due to posttranscriptional regulation or the fact that cell lysates were assayed instead of membrane fractions also.

Figure 5.3 AlgZ levels are increased in a *pilW* mutant.



A) qPCR analysis of *algZ* expression. *pilW* has a significant increase while the phospho-deficient *pilW* was less than wildtype. Values were normalized to housekeeper genes (*uvrD* and *mreB*) and compared to PAO1. *** signifies $p < 0.01$. Experiments performed in triplicate (n=12). B) Western blot analysis of AlgZ in PAO1HA and *pilWHA* from 6 hour cultures using anti-HA monoclonal antibodies. PAO1* is lacking the HA epitope. RpoA was used as a loading control.

Figure 5.4 AlgR levels are increased in a *pilW* mutant.



A) qPCR analysis of *algR* expression. *pilW* has a significant increase while the phospho-deficient *pilW* was less than wildtype. Experiments were performed in triplicate at least three times. Values were normalized to housekeeper genes (*uvrD* and *mreB*) and compared to PAO1. *** signifies $p < 0.01$. Experiments performed in triplicate (n=12). B) Western blot analysis of AlgR in PAO1, *algR, pilW,pilW vfr,* and *pilW algRD54A* from 6 hour cultures using anti-AlgR serum. RpoA was used as a loading control.

AlgR levels are increased in *pilW* strain

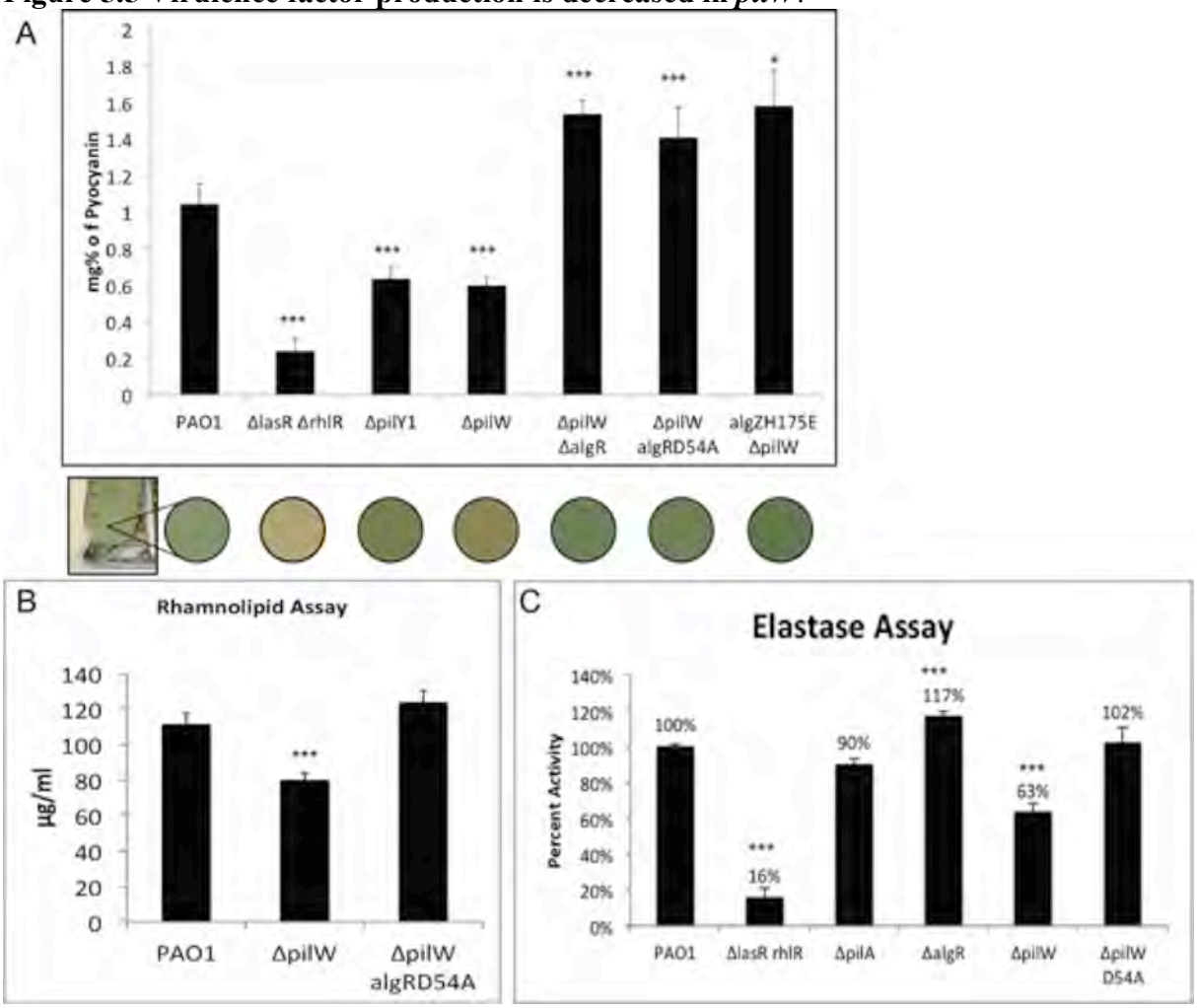
Since the AlgZ/R TCS is genetically organized in an operon, *algR* expression and protein levels were investigated. Depending on which regulators are active, *algR* can be expressed as a message with *algZ* or the extracellular sigma factor AlgU can activate expression of *algR* with a promoter in the *algZ* coding region (14, 22). Williams studied the effects of the AlgU dependent promoter in *pilW* and showed no increase in *algR* expression compared to PAO1. The AlgZ levels were increased in the *pilW* strain as well as Williams confirming the Luo et al. data that minor pilin expression also significantly increased, which is an effect of phosphorylated AlgR. To see if *algR* mRNA was increased, *algR* expression was also investigated via qPCR (Figure 5.4a). This time the *algR* mutant was the negative control and the *algZ* mutant showed no difference in expression compared to PAO1. The *pilW* strain showed an increase in expression levels similar to the *algZ* expression (Figure 5.3a). Just like *algZ* expression, the phospho-deficient AlgR *pilW* strain showed wildtype levels. This datum showed more evidence of *algZ/R* co-transcription due to phosphorylated AlgR, but were AlgR protein levels also decreased? Western blot analysis showed significant increases in *pilW* and in the *pilW vfr* in AlgR levels (Figure 5.4b). Luo et al. reported that while significant, the *pilX vfr* mutant still had increased levels of minor pilin expression compared to wildtype (14). Williams also reported this same result with a *pilW vfr* mutant and transcriptional fusion analysis. However, the phospho-deficient AlgR *pilW* strain showed little AlgR protein present, further arguing the importance of phosphorylated AlgR on activating the expression of the *algZ/R* operon.

pilW has decreased AlgR-regulated virulence factor production

AlgR has been show to regulate multiple virulence factors including pyoverdine, rhamnolipids, and TFP in a phosphorylation dependent manner (8, 9). Since the *pilW* strain

showed increases in the AlgZ/R TCS, then were the same increases in virulence targets of AlgR present in this background? Pyocyanin is known to be regulated by AlgR in a phosphorylation

Figure 5.5 Virulence factor production is decreased in *pilW*.



A) Pyocyanin assay from overnight LB cultures. Circles underneath the strain represent color taken from image of culture tube. B) Rhamnolipid assay from overnight LB cultures. C) Elastase assay using Elastin-Congo Red (ECR), and values were normalized to PAO1. Overnight LB cultures were pelleted and 100 µL of supernatant was incubated with ECR for four hours. * represents $p < 0.05$ *** represents $p < 0.01$. All experiments were repeated in triplicate (n=9).

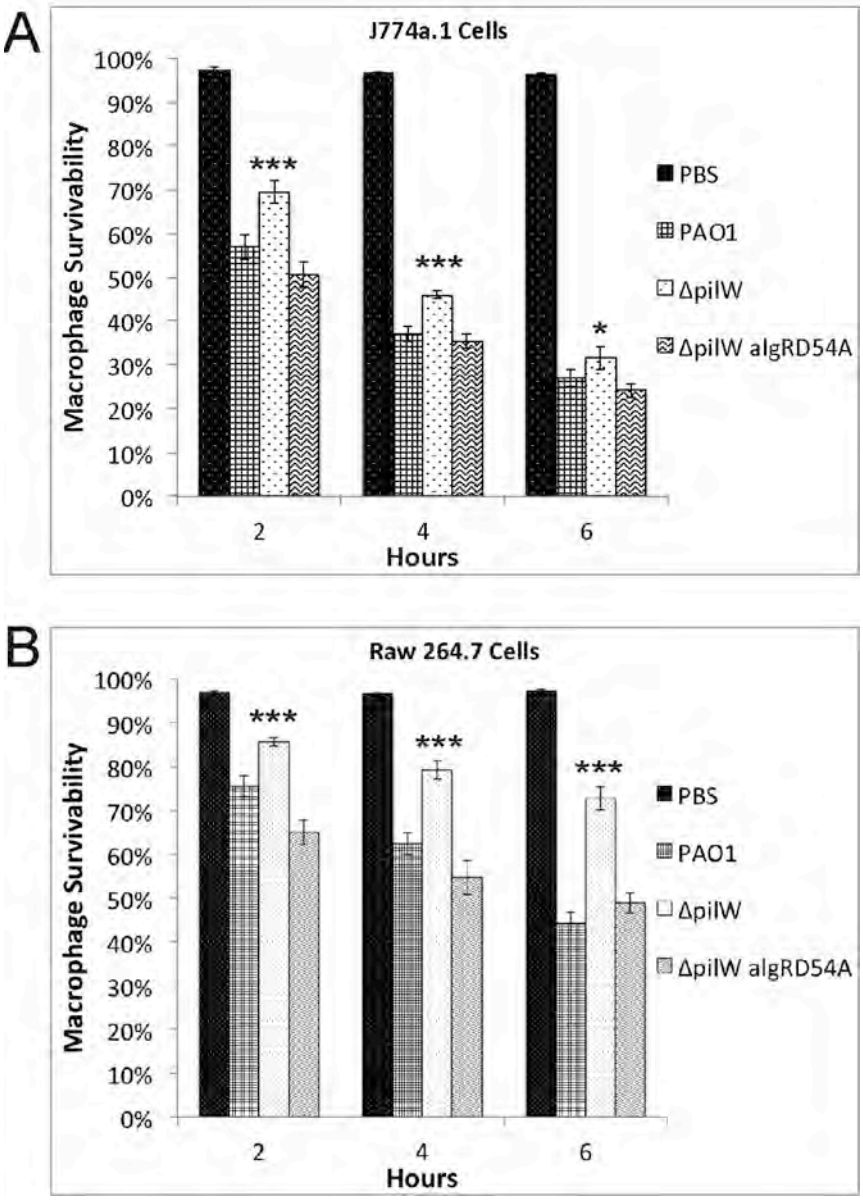
independent manner (9). Little et al. showed that in a phospho-deficient AlgR strain, pyocyanin production was increased while in the phosphomimetic strain, pyocyanin levels were much lower than wildtype (9). Pyocyanin production was assessed in minor pilin mutants (*pilY1* and *pilW*) as well as strains lacking the ability to phosphorylate AlgR in a *pilW* (*algR, algRD54A,*

algZH175E) (Figure 5.5a). A *lasR rhlR* strain was used for a negative control as these regulators are known to contribute to pyocyanin production (23). Bohn et al. and Siryaporn et al. have both characterized the effects of *pilY1* mutations and how they mimic *lasR* mutations. Both minor pilin mutants showed decreases in pyocyanin production compared to wildtype (24, 25). However, loss of the *algR* allele in the *pilW* mutant or inability for this mutant to phosphorylate AlgR showed significant increases in pyocyanin levels. Next, rhamnolipid production was assayed in *pilW* and *pilW algRD54A* (Figure 5.5b). A similar pattern of decrease in *pilW* and increase in the phospho-deficient strain was observed. The literature in this case is conflicting. Morici et al. states that AlgR represses the Rhl quorum sensing (QS) system and *rhlA* expression where an *algR* mutant has increased rhamnolipid production, while Okkotsu et al. demonstrate the opposite and AlgR actually activates *rhlA*. Both also show AlgR binding upstream. The results in Figure 5.5b were performed in a broth instead of collected from a biofilm on a plate and may explain the lack in distinct significance. Also, the western blot analysis from Figure 5.4b shows more AlgR present in *pilW* and less in *pilW algRD54A*, which supports the argument from Morici et al. that AlgR would be repressing rhamnolipid production. Lastly, the quorum sensing target, elastase, was quantified. Due to the effects of decreases in QS targets because of increased AlgR, an elastase assay was used to study the effects *pilW* had on elastase activity (Figure 5.5c). Since elastase is a direct target of the Las and Rhl QSs, the *lasR rhlR* mutant again was used as a negative control. Again, the same pattern of decrease in *pilW* and increase in *pilW algRD54A* was observed. Further arguing the work of Morici et al. that increased AlgR levels are repressing the QS response. These data also argue that minor pilin mutants can affect the QS response via AlgR's ability to influence the Rhl quorum system.

Increased macrophage survivability in *pilW* strain

To find out if the decreases in virulence factors had any effect on virulence in the *pilW* mutant, macrophage survival was assayed (Figure 5.6). Strains were incubated up to 6 hours

Figure 5.6 Macrophages have increased survivability with *pilW*.



A) J774a.1 and B) Raw 264.7 macrophages were incubated with PAO1, *pilW,* and *pilW algRD54A* for viability with 0.4% Trypan blue stain at 2, 4, and 6 hours. * represents $p < 0.05$ and *** represents $p < 0.01$. All experiments were performed in triplicate (n=9).

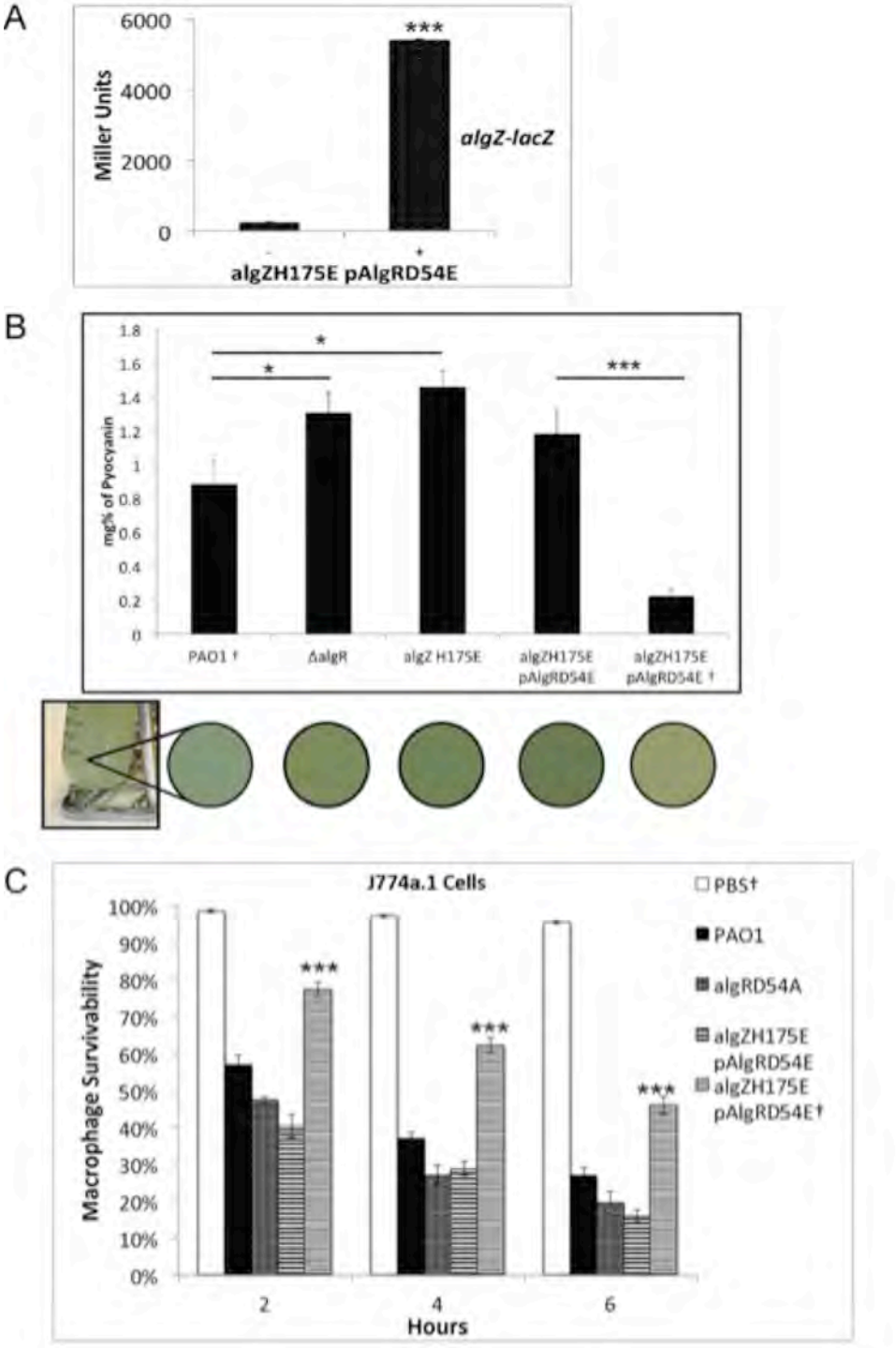
with either J774a.1 (Figure 5.6a) or Raw 264.7 cells (Figure 5.6b). PBS inoculated wells were used as a negative control in both experiments. Both cell lines had increased survivability with

the *pilW* strain. There was also no significant difference between PAO1 and the *pilW algRD54A* strains. Siryaporn et al. demonstrated that both *pilY1* and *pilW* mutants were attenuated in killing the amoeba *Dictyostelium discoideum* (25). These results were more visible in the incubation with the Raw 264.7 macrophages. The major difference between J774a.1 and Raw 264.7 cells is the production of interleukin-1β (IL-1β) by J774a.1. This overproduction of IL-1β with the *pilW* strain may be causing the lesser significance as compared to the Raw 264.7 cells. However, due to overall decreased virulence products produced in the *pilW* mutant, the *pilW* mutant is attenuated when incubated with macrophages. But when AlgR is unable to be phosphorylated in the *pilW* background, virulence factor production is equal to wildtype if not increased and this effect leads to macrophage death.

Overexpression of phosphomimetic AlgR mimics *pilW* strain

The *pilW* strain not only has increased levels of AlgR but that these are increased levels of phosphorylated AlgR due to increases in AlgZ also. The *pilW* strain also had decreased virulence factors and was attenuated with two lines of macrophages. To further drive home the fact that this is due to increased levels of phosphorylated AlgR, an inducible phosphomimetic AlgR (pAlgRD54E) was constructed and put it in a strain that could not phosphorylate AlgR (*algZH175E*). The pAlgRD54E construct was induced in all scenarios with 0.1% arabinose. This strain was first analyzed with the *algZ-lacZ* transcriptional fusion to confirm the increased *algZ* expression in the *pilW* was phosphorylated AlgR dependent (Figure 5.7a). Upon induction, the expression of *algZ* increased by ~100 fold. The *algZH175E* strain cannot phosphorylate AlgR due to the mutated histidine. Due to the increase of *algZ* expression, pyocyanin was quantified in uninduced and induced strains (Figure 5.7b). PAO1 was also incubated with 0.1% arabinose to show no effects of the added compound. *algR* and *algZH175E* strains were also

Figure 5.7 Overexpression of phosphomimetic AlgR mimics *pilW* mutant and shows decreased virulence.



A) β-galactosidase assay of *algZ* expression of uninduced and induced AlgRD54E in *algZH175E* strain that cannot phosphorylate AlgR. B) Pyocyanin assay of uninduced and induced AlgRD54E. PAO1† represents wildtype with 0.1% arabinose. C) Macrophage survivability assay with J774a.1. PBS† represents macrophages inoculated with PBS and 0.1% arabinose. All experiments were performed in triplicate (n=9). * represents $p < 0.05$ and *** represents $p < 0.01$.

used as controls in the comparison to the *algZH175E* pAlgRD54E strain. The induced *algZH175E* pAlgRD54E strain had levels of pyocyanin similar to the *lasR rhlR* strain. Lastly, this strain was assayed for macrophage survivability to see if increased levels of AlgRD54E had a decrease in virulence like *pilW* (Figure 5.7c). An *algRD54A* strain was also assayed to show a similarity with the phospho-deficient strain *algZH175E* when pAlgRD54E was not induced. The induced pAlgRD54E strain had significantly increased levels of macrophage survivability. At the 6-hour time point, around 50% of macrophages survived which again mimics the *lasR rhlR* mutant strain (data not shown). The overexpression of the phosphomimetic AlgR construct further suggests that the same activation of *algZR* expression and decrease in virulence occurs in the *pilW* mutant due to the increase in phosphorylated AlgR via autoregulation.

DISCUSSION

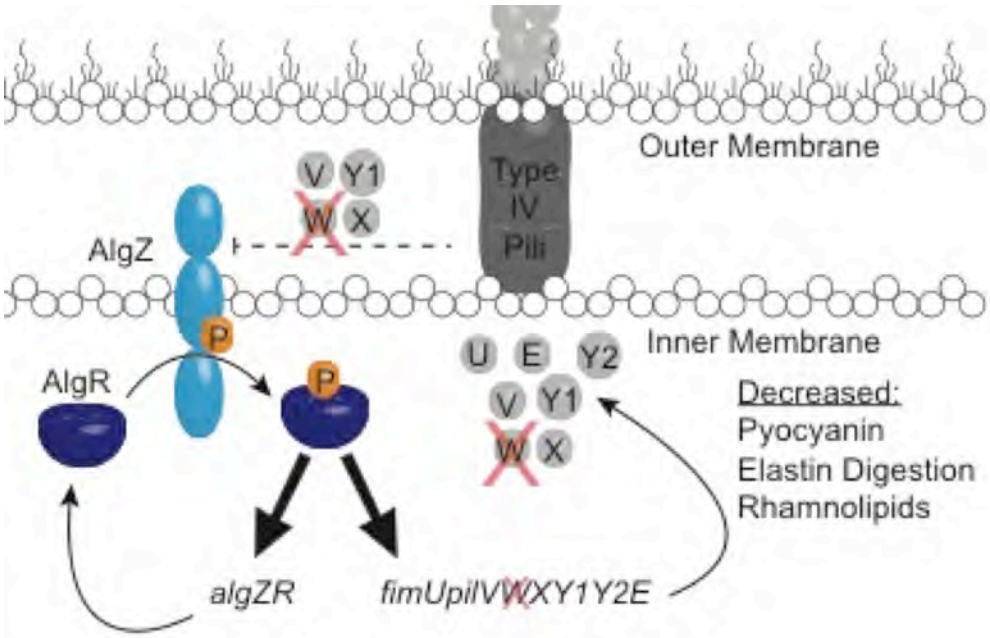
The response regulator AlgR is used by *P. aeruginosa* to control the expression of multiple virulence factors and aid in establishing an infection and persisting in a compromised host a (8-10, 26). While genetically arranged in an operon with *algZ,* no evidence to this date has demonstrated that AlgZ is the sensor histidine kinase to AlgR except for bioinformatic prediction. We demonstrated using a BACTH system that AlgZ and AlgR do in fact interact. While more information can be determined from studying the 3D structure with X-ray crystallography of AlgZ and AlgR, overall this interaction proves a wide-held belief with this TCS in *P. aeruginosa.* AlgR has also been proposed to have DNA binding sites that require two AlgRs (8, 11). In activating these AlgR targets, AlgR would dimerize and bind the DNA consensus sequence. By using the BACTH system we demonstrated that AlgR is capable of interacting with itself. This datum affirms that like other response regulators, AlgR can, at least,

dimerize (27). In the case with AlgZ and AlgR, a phosphotransfer assay would be more pertinent to show that actual exchange between these members of this TCS. However, AlgR has the ability to autophosphorylate and could prove to be difficult to visualize unless studied in a time dependent manner(8, 13).

Confirming the interaction with AlgZ and AlgR is important for understanding the increase in *algZR* expression in a *pilW* mutant. Williams demonstrated that not only did a *pilW* mutant have increased minor pilin expression confirming the Luo et al. work, but there was also increased *algZ* expression. Autoregulation is common amongst TCSs that are arranged in an operon (6, 23). We confirmed that there was increased *algZR* message and also increased levels of AlgR protein in *pilW*. When AlgR was mutated to a phospho-deficient *algRD54A* these levels dropped. Lastly, because AlgR regulates the expression of the minor pilin operon in a phosphorylation dependent manner and by overexpressing the AlgR phosphomimetic, pAlgRD54E, showed the same increase in *algZ* expression; we theorize that the *pilW* mutant has no way of turning off the *algZR* autoregulation mechanism. Marko et al. demonstrated using the BACTH system that four minor pilin proteins, PilVWXY1, form a complex in the innermembrane/periplasmic space and interact with AlgZ(28). This interaction would be a mechanism for AlgZ to "turn off" the phosphorylation of AlgR and decrease expression of minor pilins. However, when one of these prominent minor pilins is missing, the off-switch is nonfunctional and the AlgZ/R autoregulation mechanism (Figure 5.8) increases *algZ* expression and minor pilin expression. This mechanism would also explain the effects on the Rhl quorum sensing system virulence factors all being decreased. Since the *pilW* strain is also producing more AlgR and Morici et al. showed that increased levels of AlgR repress this QS mechanism, naturally pyocyanin, rhamnolipid, and elastase production would also decrease (19).

Many groups have shown that *pilY1* mutations or *pilW* mutations lead to attenuation with amoebae and *C. elegans* (25, 28). *in vivo* studies and global knowledge experiments, like

Figure 5.8 Model of *pilW* mutation and the effects on *algZR* expression.



We show with this work herein that minor pillin mutants, in this case *pilW*, that the AlgZ/R TCS is no longer regulated in *P. aeruginosa*. *algZ/R* expression increases greatly, therefore increasing minor pilin expression. The effects of this increase also influence other phosphorylated AlgR dependent targets besides TFP and decreases virulence when incubated with J774a.1 macrophages.

transcriptomics or proteomics, would allow for better understanding and the coordination between the AlgZ/R TCS, TFP, and the Rhl QS network in *P. aeruginosa* virulence. To this point, we showed that a *pilW* mutant and a strain overexpressing phosphomimetic AlgR were attenuated with macrophages. These results lead the TFP of *P. aeruginosa* to be targeted as an Achilles's heel to compromise the bacterium's virulence capabilities. Since the TFP are crucial for twitching motility and attaching to surfaces; by targeting this apparatus, a pilicide would not only render the bacterium unable to attach and twitch, but also decrease the production of virulence factors. This effect in concert with an additional antimicrobial could allow for clearance of an infection, preventing the development of septicemia in acute infections or

persister cells in chronic infections.

ACKNOWLEDGEMENTS

The Department of Health Sciences for always supporting our lab's research and making available resources and equipment. Danielle A. Williams with initiating this project with her master's work and brainstorming the layout of the paper with me.

FUNDING INFORMATION

This work, including the efforts of Chris L. Pritchett, was funded by Research and Development Committee, ETSU (RDC12-012M and RDC13- 005M).

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CHAPTER 6

CONCLUSIONS

P. aeruginosa is a versatile opportunistic pathogen capable of infecting any tissue in the human body (Winstanley et al. 2016; Moradali et al. 2017). *P. aeruginosa* has always posed the most threat to immunocompromised individuals with HIV, type II diabetes, or cystic fibrosis (Bahemia et al. 2015; Winstanley et al. 2016; Stefani et al. 2017). *P. aeruginosa* is the leading cause of morbidity and mortality in people with cystic fibrosis (Salunkhe et al. 2005). However, over time *P. aeruginosa* infections are becoming more difficult to treat as the bacteria become more resistant to antibiotics, specifically carbapenem-resistant strains (Woodworth et al. 2018). New tactics for treating *P. aeruginosa* acute and chronic infections are necessary for increasing the quality of life for people susceptible to this pathogen.

Because *P. aeruginosa* has become resistant to multiple antibiotics and adapts to newly developed antibiotics, researchers have started looking for alternative means to clear bacterial infections. In the case with uropathogenic *E. coli* (UPEC) the Hultgren lab has investigated crippling the pathogen by targeting the Fim pathway (Chorell et al. 2012). This group has generated a compound called a pilicide that outcompetes the affinity for UPEC to bind host and instead inactivates the attachment mechanism clearing the pathogen from the bladder (Chorell et al. 2012; Greene et al. 2014). Similar work is occurring with *P. aeruginosa*. Multiple groups are investigating virulence targets of *P. aeruginosa* like: inhibiting the quorum sensing system, disrupting biofilms, and interfering with two component systems and adhesins (Fothergill et al. 2014). Antivirulence studies require understanding of genetic regulation and regulation of virulence factors in order to compromise the infectious pathogen. This work investigates the multifactorial components of virulence factor regulation in both acute and chronic infections of

P. aeruginosa. The regulation of the post-transcriptional regulator, *rsmA*, by two known regulators of virulence AlgU and AlgR, the RsmA regulon of virulence targets in the mucoid background, and the role of minor pilins of the type IV pili in regulating the *algZ/R* two-component system.

RsmA has mainly been studied in nonmucoid strains of *P. aeruginosa* like PAO1 and PAK (Burrowes et al. 2006; Brencic and Lory 2009). RsmA is studied in this background because RsmA has always been assumed to regulate virulence factors associated with establishing infections and repressing virulence factors known to be expressed in a chronic infection (Lapouge, Schubert, et al. 2007; Valentini et al. 2018). With a *P. aeruginosa* infection in the cystic fibrosis lung, the bacteria face osmotic stress from reactive oxygen and nitrogen species (ROS and RNS) from the host immune system (Palmer et al. 2015). This immune cell assault causes the release of the extracytoplasmic sigma factor, AlgU, which then activates the expression of its regulon(Schnider-Keel et al. 2001). The most well known AlgU directed target is the alginate operon that produces a copious amount of exopolysaccharide (Hershberger et al. 1995). The alginate biofilm can protect *P. aeruginosa* from ROS and RNS, phagocytosis, and antibiotics (Firoved et al. 2002). The overproduction of alginate allows for *P. aeruginosa* to persist in the cystic fibrosis lung and develop into a chronic infection. The most common phenotype isolated from cystic fibrosis chronic infections are alginate overproducers of *P. aeruginosa* (Boucher et al. 2000; Winstanley et al. 2016).

Interestingly we identified a predicted AlgU consensus sequence upstream of the previously identified RpoD promoter for *rsmA* (Stacey and Pritchett 2016). This work went against the paradigm that RsmA only had a role in regulating acute virulence factors and RsmA activity was repressed by the small RNA antagonists RsmY and RsmZ in later phases of growth

(Brencic and Lory 2009). We showed that there was more RsmA present in a mucoid mutant of PAO1, *mucA22* and the mucoid cystic fibrosis isolate, 2192. The increase in RsmA was a result of AlgU activity in these mucoid strains. AlgU's role was confirmed via site directed mutation of the -35 consensus sequence and deletion of the *algU* allele. While there was no direct interaction demonstrated between AlgU and this promoter, an electromobility shift assay to show protein-DNA interaction or interferometry between AlgU and RpoS to identify which sigma factor has a greater affinity of the distal *rsmA* promoter. However, this discovery proved that RsmA has a role in mucoid strains and still contributes to virulence factor regulation in chronic infection strains.

In mucoid strains, AlgU is known to work in concert with the response regulator AlgR. Together they are known as the alginate regulators and contribute to expression of the *algD* operon that produces alginate (Salunkhe et al. 2005). Our next study investigated if this co-regulation occurred on the distal *rsmA* promoter. Bioinformatics showed two possible AlgR binding sites upstream of the AlgU binding sites. An *algR* mutant *mucA22* showed decreases in RsmA levels and increases in RsmA targets *hcnA* and *tssA1*. AlgR was shown to directly bind this distal *rsmA* promoter and both AlgR binding sites were required, a common feature of AlgR (Morici et al. 2007; Okkotsu et al. 2013). AlgR also binds the *rsmA* promoter independent of phosphorylation, further arguing this is a mucoid strain phenomenon. To reiterate not only is there more RsmA in mucoid strains but the increase is due to the alginate regulators, AlgU and AlgR. These works demonstrated a new dimension to RsmA in *P. aeruginosa* virulence regulation and had importance in relation to pathogenesis in cystic fibrosis where the alginate regulators give way to chronic infections.

The RsmA regulon has been studied in PAO1 and PAK by using global transcriptome experiments (Burrowes et al. 2006; Brencic and Lory 2009). These strains are nonmucoid and are used as acute infection model strains. These studies found that RsmA positively influences motility genes for flagellum synthesis and type IV pili, alginate and biofilm production, and the type III secretion system (Burrowes et al. 2006; Brencic and Lory 2009). These virulence factors are used by *P. aeruginosa* for establishing an infection and creating a niche inside the host. RsmA was also found to repress the type VI secretion system, a multidrug efflux pump system, and genes involved in iron regulation(Burrowes et al. 2006; Brencic and Lory 2009). These virulence factors are commonly found expressed in chronic infecting strains of *P. aeruginosa,* where the bacteria have to dominate other pathogens and acquire the valuable nutrient iron. It is easy to see why RsmA would be associated with initiating a *P. aeruginosa* infection and be repressed in the chronic infection phase of growth.

However, due to the fact that RsmA is in abundance in mucoid strains, the RsmA regulon is more complex than believed. Our proteomic approach of studying the RsmA regulon was more indicative of whether or not translation had occurred versus the transcriptome approach. The transcriptome approach will give better results to repressed targets, which will show increases in fold change, while targets positively influenced by RsmA are potentially indirect. In our iTRAQ study, we identified similar targets that were repressed by RsmA like *hcp1*. However, some of our targets also differed in previously reported results. AlgD , the protein regulated by AlgU and AlgR which generates alginate, was increased in the *mucA22 rsmA* strain while the *rsmA* mutant was reported to show a -3 fold change compared to wildtype (Burrowes et al. 2006). Lastly, our study identified previously unknown targets. We were able to confirm a

direct interaction with the *P. aeruginosa* small protease, PasP. We can now argue that not only

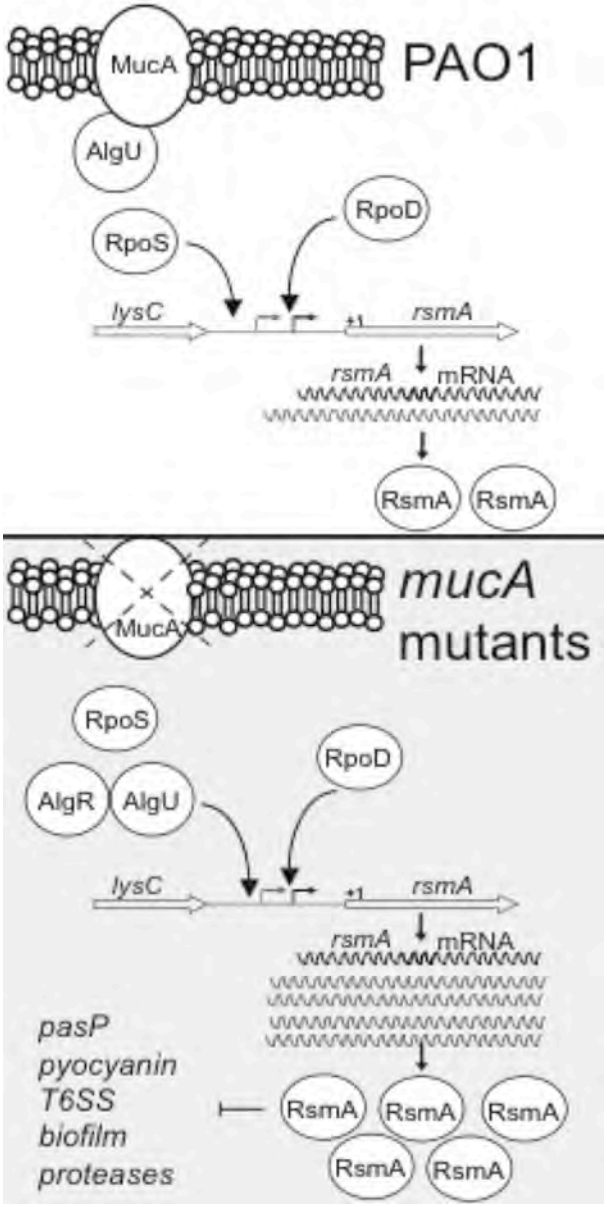

Figure 6.1 Schematic of RsmA production in nonmucoid (PAO1) and mucoid (*mucA22)* strains of *Pseudomonas aeruginosa.* In mucoid strains, the alginate regulators, AlgU and AlgR, activate the distal promoter of *rsmA*, which causes an increase in RsmA levels in this background. In the mucoid background, there is a previously unidentified target, the *P. aeruginosa* small protease, *pasP*.

is RsmA important in the *P. aeruginosa* chronic infection, but it regulates multiple virulence factors not previously known (Figure 6.1). This work has opened a door for further

understanding of the RsmA regulon and its role in infecting the cystic fibrosis lung, which has potential to lead to antivirulence drug targets.

Like CsrA in *E. coli*, RsmA has potential to positively interact with some of the products that were repressed in the *mucA22 rsmA* strain (Wei et al. 2001). Using m-fold predictive software, alignments of known targets and these RsmA "positive targets" could show the possibility of the AGGA stem-loop motif. These positive targets that present the RsmA binding motif would then be tested with purified RsmA. *P. aeruginosa* RsmA has never been shown to interact with other proteins too, unlike CsrA in *Campylobacter jejuni* and *Bacillus subtillis* (Mukherjee et al. 2011; Dugar et al. 2016). The interaction between CsrA and mRNA positively influences motility, which RsmA is known to contribute to also. To identify possible protein-protein interactions of RsmA a simple bait-prey experiment with the bacterial adenylate cyclase two-hybrid system could be performed. The most important experiment to be performed to understand RsmA in mucoid strains is *in vivo* experiments. Because RsmA influencing virulence factor regulation in mucoid strains is still relatively new, the *mucA22 rsmA* strain should be screened in a higher organism than *C. elegans* or macrophages. If the *mucA22 rsmA* strain were attenuated in a respiratory infection in a mouse, rat, or cystic fibrosis mouse, then an antimicrobial compound targeting RsmA would be of the utmost importance in clearing *P. aeruginosa* infections.

Another important vulnerability in *P. aeruginosa* is the type IV pili. Pili are commonly used for attachment, motility, mechanosensory mechanisms(Giltner et al. 2010; Luo et al. 2015). More recently they have been used as antivirulence targets (Greene et al. 2014; Novotny et al. 2017). This work examines the effects of *P. aeruginosa* with deficient type IV pili. If a single minor pilin protein is missing, the whole type IV pili is malformed and twitching and sensing are

no longer efficient (Giltner et al. 2010). It has also been reported that if one of the four

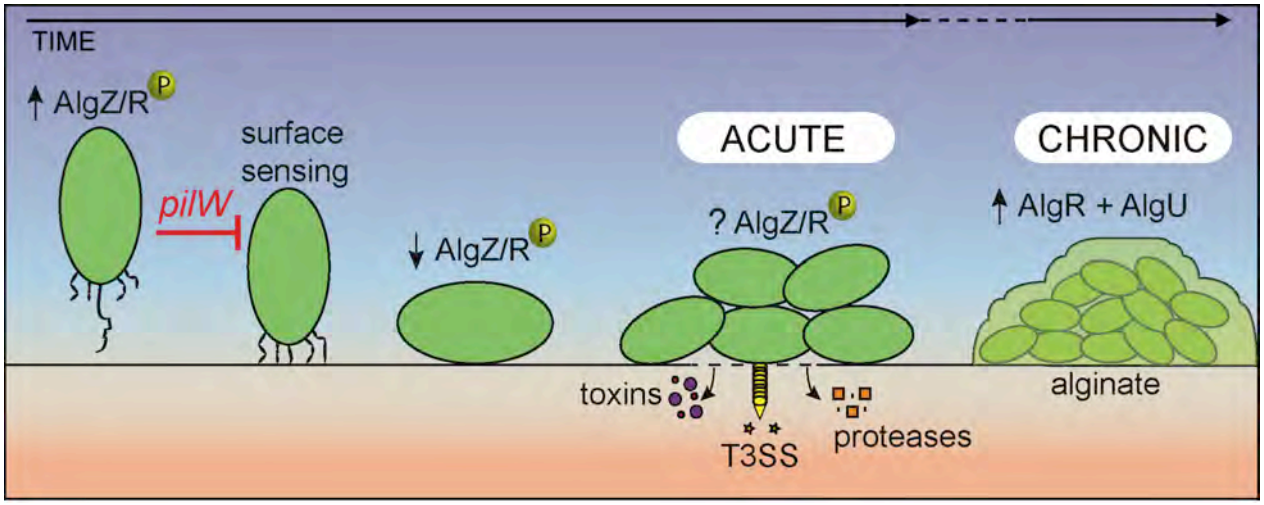

Figure 6.2 The role of the response regulator, AlgR, in colonizing and establishing an infection in *Pseudomonas aeruginosa*. Phosphorylated AlgR is extremely important in virulence factor production. By deleting the minor pilin protein, PilW, this process is interrupted due to the lack of surface sensing capabilities in the type IV pili.

important minor pilins, PilVWXY1, are missing, then this complex cannot interact with AlgZ (Marko et al. 2017). In a *pilW* mutant we showed increase AlgZ/R levels, and decreased virulence factor production. More work is still required to show a direct interaction of AlgR with the *algZ* promoter region and confirm the autoregulation effect. But there is major potential for a pilicide compound to disrupt the of the type IV pili and send the AlgZ/R system into overdrive, decrease virulence, and limit the ability for *P. aeruginosa* to establish an infection (Figure 6.2).

AlgR has been studied *ad nauseam* but there are still global experiments yet to be performed. While CHIP-SEQ has been performed with AlgR to identify targets as well as microarrays, no one has elucidated the difference between the AlgR regulon dependent and independent of phosphorylation(Lizewski et al. 2004; Kong et al. 2015). Many researchers have used phosphodeficient and phosphomimetic forms of AlgR, AlgRD54A or N and AlgRD54E, however, we know these strains present phenotypically correct, but western blot analysis shows lower levels of AlgR present (data not shown). Using iTRAQ with a *pilW* mutant strain, which

we have shown has more AlgZ/R, would be a better strain to identify AlgR targets dependent of phosphorylation. Understanding the phospho-dependent targets of AlgR could lead to more virulence targets to combine with a minor pilin target and generate a multivalent vaccine. Therefore never allowing the *P. aeruginosa* strain to initiate an infection in the human host.

Overall, this work was to investigate virulence regulation in *P. aeruginosa* by a variety of factors. Genetic regulation is multifactorial in terms of sigma factors, response regulators, post-transcriptional regulation, and external sensing systems. By understanding regulation of virulence factors in *P. aeruginosa* are regulated and visualizing these complex networks, researchers can provide the individuals susceptible to this opportunistic pathogen a little more hope with advanced treatments and preventative measures and less morbidity and mortality.

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Cover Photo on Journal of Bacteriology Vol. 199 Issue 19

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Sean Stacey and Christopher Pritchett, The MpsR/T Operon Influences Biofilm Development and Motility. Poster presentation at Appalachian Student Research Forum April 2014.

Sean Stacey and Christopher Pritchett, A Putative Membrane Transporter Increases *rsmA* Expression in *Pseudomonas aeruginosa*. Poster presentation with additional data at the American Society of Microbiology General Meeting May 2013.

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