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Genetic Regulation of Mucoidy in *Pseudomonas aeruginosa*

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Genetic Regulation of Mucoidy in *Pseudomonas aeruginosa*

DISSERTATION

Submitted to the Graduate College of Marshall University

**In Partial Fulfillment of the Requirements for
The Degree of Doctor of Philosophy
In Biomedical Sciences**

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This work is dedicated to the loving memory of my grandmother, Dorothy Juanita Jennings. Thank you for teaching me how to enjoy life with both tenacity and grace; a lesson I hope to pass on to your great-granddaughter.

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Dissertation Abstract

Cystic fibrosis is a genetic disorder that results from mutations in the CF transmembrane conductance regulator gene. These mutations cause a disruption in the chloride transport in mucosal tissues causing the accumulation of dehydrated mucus, and a decrease in the mucocilliary removal of environmental pathogens within the lungs. Additionally, the accumulation of dehydrated mucus within the lungs provides a hospitable environment for various bacteria, including the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* uses the overproduction of a surface polysaccharide called alginate to form a biofilm to evade the host's immunological defenses. The overproduction of alginate, often referred to as mucoidy, is a virulence factor that is responsible for chronic *P. aeruginosa* infections, as well as an increased resistance to antibiotics and phagocytosis by the host defense cells. Chronic *P. aeruginosa* infections are the leading cause of morbidity and mortality in CF patients, and the detection of mucoid isolates is a proven predictor of a decline in the patient's health. The transition from the non-mucoid phenotype, found in environmental isolates, to the mucoid phenotype found within the CF lung is typically due to "loss-of-function" mutations in the transmembrane anti-sigma factor MucA. However, *P. aeruginosa* can overproduce alginate independent of mutations in *mucA*, through the regulated proteolysis of MucA. A series of proteases, beginning with AlgW, can degrade MucA, and release the alternative sigma factor AlgU to drive transcription of the alginate biosynthetic operon. It is generally accepted that the regulated proteolysis of MucA is a mechanism used by early colonizing strains prior to the selection for MucA mutations. Therefore, understanding this mechanism employed by those early colonizing strains may prove beneficial in preventing the establishment of chronic *P.*

aeruginosa respiratory infection. In this dissertation, I identify and characterize two novel regulators of alginate overproduction in *P. aeruginosa* strains possessing a wild-type MucA. Using the model strain PAO579, I determined that mutations that result in the truncation of the type-IV pilin precursor protein, PilA, can induce alginate overproduction through activation of the AlgW resulting in an increased rate of proteolysis of MucA. Additionally, I identify that expression of the genetic locus PA1494, referred to as mucoid inhibitor A (*muiA*), can suppress mucoidy in *P. aeruginosa* strains with a wild-type MucA. Collectively, these findings provide needed insight into the regulation of mucoidy in those early colonizing strains, as well as identifies potential therapeutic targets for the prevention of chronic *P. aeruginosa* infections in the CF lung.

CHAPTER 1: Introduction and Literature Review

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder predominantly associated with individuals of European descent. CF afflicts approximately 30,000 individuals in the United States and 70,000 worldwide, with about 1,000 new cases being diagnosed each year. CF is the result of mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), located on the long arm of chromosome 7 at locus q31.2. Currently, >1,500 mutations in *CFTR* have been identified that cause CF, with the most common being a deletion of a single codon for phenylalanine at amino acid position 508 ($\Delta F508$). The $\Delta F508$ mutation is found in over 90% of all CF cases in the United States, and approximately 70% worldwide (8).

CFTR is categorized as an ATP-binding cassette membrane transporter, and is responsible for the export of chloride ions (Cl^-) across epithelial membranes. Disruption in the *CFTR* gene causes a decrease in export of chloride ions to the surface of epithelial cells. Chloride ions are required to attract water molecules to sufficiently hydrate the epithelial cell surface. Following the loss of chloride export and hydration of the epithelial cells, a thick, dehydrated mucus accumulates. This accumulated mucus affects several organ systems including the lungs, liver, pancreas and gastrointestinal tract. Symptoms associated with CF include salty-tasting sweat, poor growth and weight gain, constipation, frequent chest infections, coughing, shortness of breath and infertility.

Of particular importance, with regards to the overall morbidity and mortality of CF patients, is the accumulation of mucus within the lungs. This accumulation causes a

decrease in mucociliary removal of microbial pathogens, and provides a hospitable environment for their cultivation (6, 15, 73). Indeed, microbial infection is the leading cause of mortality in individuals with CF (35). However, the spectrum of microbial pathogens that are associated with CF pulmonary infections is limited. Those pathogens that are associated with CF pulmonary infections are primarily bacterial and include *Burkholderia cepacia*, *Haemophilus influenza*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Initial colonization by bacterial pathogens occurs early in life (<1 year), with 50-60% caused by *S. aureus*. However, starting in adolescents, *P. aeruginosa* surpasses *S. aureus* to become the leading cause of morbidity and mortality among bacterial pathogens associated with CF (35). Increased susceptibility of CF patients to bacterial infections is likely due to a direct interaction of microbes with the mutated CFTR protein (61, 62). However, Parker *et al.* suggest that mutations in CFTR provide an opportunity for microbial cultivation because of a decrease in immune surveillance due to disruptions in type-I interferon signaling (57). Additionally, mice possessing CFTR mutations were unable to properly to kill phagocytosed bacteria due to poor lysosomal acidification (25). Therefore, the function of CFTR mutation with regards to the increased susceptibility in CF lungs is still not completely understood.

Historically, individuals with CF did not live past infancy. However, due to the implementation of second and third generation antibiotics, as well as improved therapeutics, CF patients are able to live well into adulthood, with the average lifespan being 37.5 years of age (Cystic Fibrosis Foundation). Ultimately, increased mortality rates in all CF patients is due to the “overwhelming sequelae of repeated pulmonary exacerbations arising from persistent *P. aeruginosa* colonization” (35).

***Pseudomonas aeruginosa* and Biofilms**

Pseudomonas aeruginosa is a motile, Gram-negative bacterium that is found throughout the environment. *P. aeruginosa* is also classified as an opportunistic pathogen and has been linked to severe infections in immuno-compromised individuals, such as burn victims (40), those afflicted with HIV/AIDS (2, 78), and pediatric patients



Figure 1. Mucoid phenotype in *P. aeruginosa*. Shown on the left side of the illustration is a mucoid *P. aeruginosa* strain (PAO579), and on the right is the nonmucoid progenitor strain PAO1. The bacteria are cultured on plates containing *Pseudomonas* isolation agar (PIA) and were incubated at 37°C for 24 hours and then at room temperature for 24 hours.

undergoing chemotherapy (11, 81). *P. aeruginosa* has also been shown to successfully colonize abiotic surfaces of equipment and vehicles used in space exploration (12). Moreover, *P. aeruginosa* is the leading cause of hospital-acquired pneumonia and bacteremia by a Gram-negative pathogen (26, 39).

P. aeruginosa is particularly adept at establishing chronic infections in individuals with CF through the formation of a biofilm. The term “biofilm” was first introduced in 1977, and is used to define a collection of bacteria adhered to a surface surrounded by

a self-produced exopolysaccharide matrix (37, 64). Biofilm formation confers a selective advantage upon the bacteria. Specifically with *P. aeruginosa* biofilms, there is an increased resistance to antimicrobial therapies (17, 18, 47, 59, 79, 80), increased protection against opsonization (49, 52, 63), and decreased engulfment by amoeba and macrophages (10, 36, 45, 46, 54). Current estimates are that the vast majority of all pathogenic *P. aeruginosa* infections, and 80% of all microbial infections, are due to biofilm formation.

A biofilm is both functionally and morphologically different from a single free-floating, or planktonic, bacterium. Moreover, the formation of a bacterial biofilm is often compared to the developmental processes associated with multi-cellular organisms. A biofilm develops in four distinct stages: attachment, development of microcolonies, maturation, and dispersal (18, 74, 82). Initially, planktonic cells use flagella, type-IV pili and other outer membrane adhesion proteins to adhere to biotic or abiotic surfaces (55). Following initial adherence, the bacteria transition into microcolonies through the production of an extracellular matrix consisting primarily of an extracellular polymeric substance (EPS), or exopolysaccharide. In *P. aeruginosa*, the exopolysaccharides Psl and Pel are involved in this initial stage of biofilm development (34, 41). The regulation of Psl and Pel is often facilitated through cell-density dependent signaling or quorum sensing. Two quorum-sensing systems, *las* and *rhl*, are used in *P. aeruginosa* (58).

During the maturation stage, the biofilm increases in population and volume and begins to take on a three dimensional structure. In *P. aeruginosa*, this is due to an overproduction of an exopolysaccharide called alginate (30, 35). The terminal stage of biofilm development, dispersal, is marked by the evacuation of the bacteria following a

return to the planktonic state (18, 74). This transition the planktonic state is often in response to environmental signals such as changes in pH, the concentrations of carbon and oxygen, the presence of nitric oxide, the presence of heavy metals, as well as the presence of various quorum-signaling molecules (4, 5, 38, 43, 75). Interestingly, the use of alginate in this maturation stage of biofilm development is generally not found outside of the CF lung, and is indicative of a chronic infection (35). Moreover, alginate is not used by nonmucoid, environmental isolates of *P. aeruginosa* in the formation of biofilms (89).

Alginate Production and Regulation

In the CF lung, the transition of *P. aeruginosa* between initial attachment and the development of mature biofilm is marked by the overproduction of the exopolysaccharide alginate. Alginate is a biopolymer consisting of two monomers of β -D-mannuronate acid and its epimer α -L-guluronate. Overproduction of alginate is often referred to as the mucoid phenotype, or simply mucoidy. The emergence of mucoid isolates from CF patient sputa is indicative of a chronic infection, and is pathognomic for both CF and *P. aeruginosa* (24, 35) . Additionally, the conversion from the nonmucoid to the mucoid phenotype is considered a prognostic marker for the poor overall health of the CF patient (35).

Table 1. Summary of the alginate biosynthetic operon

Gene Locus	Gene Name	MW (kDa)	Predicted Localization	Product Name	References
PA3540	<i>algD</i>	47.6	Cytoplasm	GDP-mannose 6-dehydrogenase	(22, 23)
PA3541	<i>alg8</i>	56.5	Inner Membrane	glycosyltransferase	(48, 56, 70)
PA3542	<i>alg44</i>	41.8	Inner Membrane	c-di-GMP binding-activation/membrane fusion protein	(48, 53, 71)
PA3543	<i>algK</i>	52.5	Periplasm	periplasmic multi-protein complex	(44)
PA3544	<i>algE</i>	54.4	Outer Membrane	outer membrane protein (porin)	(16, 68, 85)
PA3545	<i>algG</i>	59.8	Periplasm	alginate C5-epimerase	(14, 28)
PA3546	<i>algX</i>	52.6	Periplasm	alginate o-acetyltransferase	(72, 84)
PA3547	<i>algL</i>	40.8	Periplasm	poly(β -D-mannuronate) lyase	(1, 42)
PA3548	<i>algI</i>	58.7	Inner Membrane	alginate o-acetyltransferase	(29, 32, 33)
PA3549	<i>algJ</i>	43.1	Inner Membrane	alginate o-acetyltransferase	(29, 32, 33)
PA3550	<i>algF</i>	22.8	Periplasm	alginate o-acetyltransferase	(29, 31, 33)
PA3551	<i>algA</i>	53.1	Cytoplasm	phosphomannose isomerase/guanosine 5'-D-mannose pyrophosphorylase	(21, 60)

The overproduction of alginate in *P. aeruginosa* is the result of increased expression of the alginate biosynthetic operon (22). The alginate biosynthetic operon is located between genome positions 3,962,825 and 3,979,476 in the reference strain PAO1, and consists of 12 genes responsible for the manufacture, assembly and export of the alginate polymer across the periplasmic space to the extracellular matrix (Table 1). The regulation of the entire alginate biosynthetic operon occurs at a single promoter site upstream of the *algD* gene (P_{algD}). Transcription at P_{algD} is primarily controlled by the

alternative sigma factor AlgU (also known as AlgT, σ^{22} , σ^E) (50, 88).

Increased expression of AlgU also modulates the two-

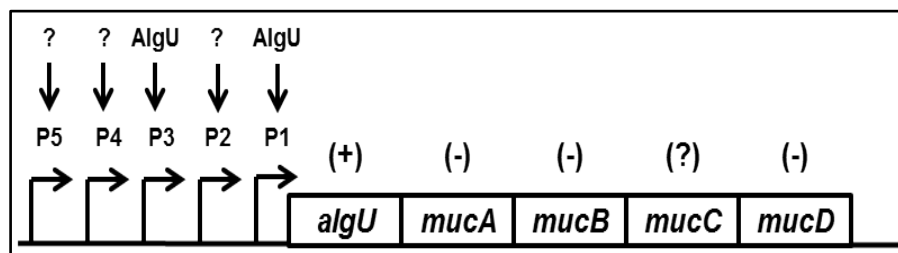


Figure 2. Regulation of the *algU-mucA-mucB-mucC-mucD* operon. The regulation of the *algUmucABCD* operon occurs at five promoter sites (P1-P5) upstream of *algU*. Expression of *algU* and its subsequent negative regulators, *mucA*, *mucB* and *mucD*, is auto-regulated at the P1 and P3 promoter sites. (+) and (-) identify the associated gene as a positive or negative regulators of *algU*, respectively. (?) signifies either unknown function or transcriptional regulator.

component regulators, AlgB and AlgR, as well as the transcription factor AmrZ (67). Subsequently, the increased expression of AlgB, AlgR, and AmrZ, which directly bind to different sites upstream of *algD*, also causes an increase in activity at P_{algD} and mucoidy (7, 67, 76, 88). Therefore, the initiation of the alginate biosynthetic operons hinges upon the regulation of AlgU.

The *algU* gene (PA0762) is found at the 5' end of an operon containing the negative regulators *mucA* (PA0763), *mucB* (PA0764) and *mucD* (PA0766) (Figure 2). AlgU is localized in the cytoplasm and is a stress-related alternative sigma factor, which belongs to a superfamily of sigma factors known to control extra-cytoplasmic function

(ECF). MucA is the cognate anti-sigma factor for AlgU, and is associated at its C-terminus with the periplasmic protein MucB (51). MucB is thought to improve the stability of MucA by protecting its periplasmic portion from proteolytic degradation. The *mucC* gene codes for a 15.9 kDa protein of unknown function. The *algU-mucA-mucB-mucC-mucD* operon is regulated transcriptionally by 5 promoters (P1-P5) upstream of *algU*. P1 and P5 are constitutively active in nonmucoid *P. aeruginosa* strains with a wild-type MucA, while P1 and P3 are AlgU-dependent and responsive to external stressors (77). MucD is a homolog of DegP in *E. coli*, and is classified as a periplasmic chaperone-protease. Inactivation of MucD causes mucoidy in *P. aeruginosa* strain PAO1 (20, 66).

The positive regulation of alginate overproduction is primarily attributed to AlgU and its cognate anti-sigma factor MucA. MucA is a transmembrane protein that spans the inner membrane, with the C-terminus and N-terminus localized in the periplasm and cytoplasm, respectively. In a nonmucoid wild-type *P. aeruginosa* strain, AlgU is sequestered by MucA to the cytoplasmic leaflet of the inner membrane. This prevents AlgU from moving freely about the cytoplasm and initiating transcription of the alginate biosynthetic operon at the *algD* promoter site, as well as the promoter sites for the additional modulators of alginate biosynthesis, *algB*, *algR* and *amrZ*.

Currently there are two mechanisms identified by which AlgU is released from MucA. The first is through the abrogation of MucA. In the majority of clinical CF isolates, selective pressure causes the emergence of mucoid isolates through the selection of MucA mutants (3, 9). This is commonly due to frameshift or insertion/deletion (INDEL)

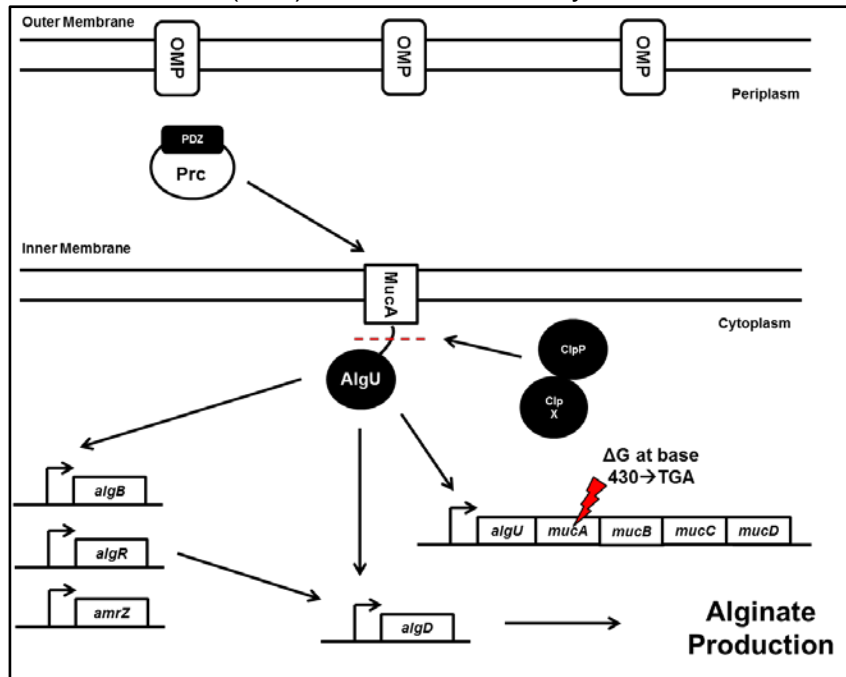


Figure 3. Mutations in MucA cause mucoidy in *Pseudomonas aeruginosa*. In the nonmucoid PAO1 strain, the alternative sigma factor AlgU is sequestered to the inner membrane. The emergence of mucoid isolates in the CF lung is typically due to mutations in that either abrogate or truncate the gene for the transmembrane anti-sigma factor MucA. The loss of function for MucA causes the release of AlgU and an increase in expression of the alginate biosynthetic operon at the algD promoter site. AlgU also modulates expression of the *algB*, *algR* and *amrZ*. Previously it has been shown that the proteases Prc and ClpXP are required for mucoidy in the truncated MucA strains *mucA22* and *mucA25*, respectively.

mutations that result in a complete loss of function or diminished function due to the translation of truncated MucA protein. The truncated protein has decreased overall stability when compared to the wild-type, full length MucA

(65). Interestingly, Reiling *et al.* reported that the periplasmic protease Prc is required for mucoidy in the truncated MucA

mutant, *mucA22*, but not in strains possessing a wild-type MucA (69). Prc has both a PDZ and a tail-specific protease domain; however the substrate specificity is not fully understood. Additionally, the cytoplasmic protease complex ClpXP is required for mucoidy in the truncated MucA mutant, *mucA25* (65). Hypothetically, the site-2

protease MucP (YaeL) could degrade the intramembrane portion of the truncated MucA; however no data confirming this hypothesis is currently available (19).

The second mechanism for the release of AlgU involves the regulated intramembrane proteolysis (RIP) of wild-type MucA. More specifically, MucA is systematically degraded by a series of proteases beginning in the periplasm with AlgW,

continuing with the site-2 intramembrane protease MucP, and completed with the cytoplasmic protease complex ClpXP (Figure 4). AlgW, a serine protease and a functional homologue of DegS in *E. coli*,

contains an N-terminal anchor, a PDZ domain and a protease domain (27, 66, 86). Activation

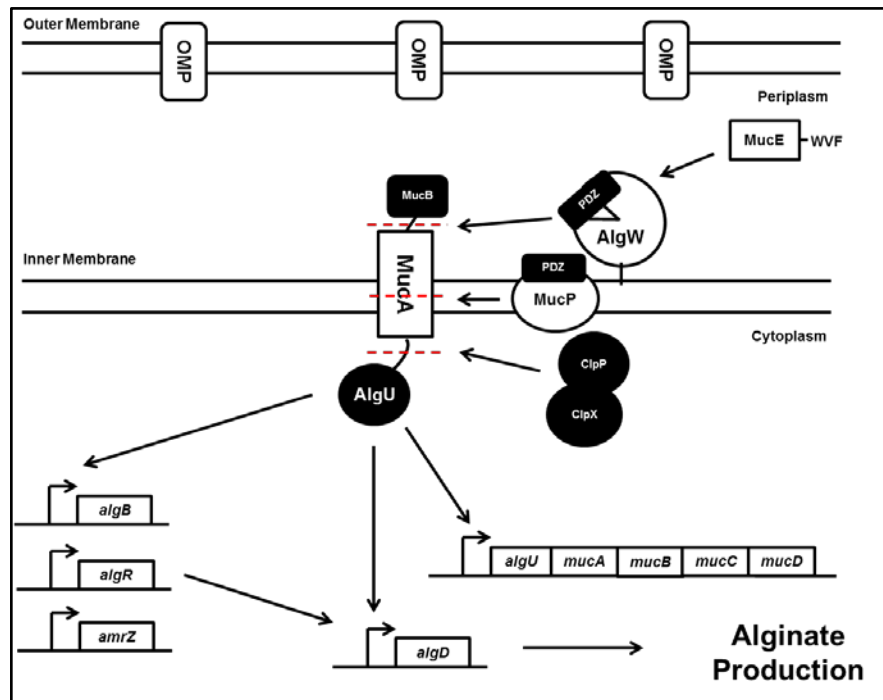


Figure 4. Activation of mucoidy through regulated intramembrane proteolysis (RIP). When activated by the presence of stress agents (not shown) or the accumulation of envelop proteins, the serine protease AlgW can positively regulate AlgU by cleaving the periplasmic portion of MuA. This is followed by proteolytic cleavage of the intramembrane portion of MucA by the site-2 metalloprotease MucP. The protease complex ClpXP degrades the cytoplasmic portion of MucA. AlgU is now free to drive transcription of the alginate biosynthetic operon at the *algD* promoter, as well as the *algB*, *algR* and *amrZ*.

of AlgW results in the cleavage of MucA at a major cut site between the alanine residue at position 136 and the glycine residue at position 137 (13). Activation of AlgW occurs in response to the presence of external stress agents, such as D-cycloserine (86). Additionally, the overexpression of the small periplasmic protein MucE (PA4033)

induces mucoidy via AlgW in the nonmucoid strain PAO1 (66). This activation of AlgW is due to an interaction between the C-terminal motif of tryptophan-valine-phenylalanine (WVF) of MucE and the PDZ domain of AlgW (13, 66, 83). MucP is also required for activation of mucoidy in both the presence of D-cycloserine and the overexpression of MucE (66, 87). MucP possesses two PDZ domains and a zinc metallo-proteolytic domain consisting of a motif of histidine-glutamic acid-X-X-histidine (HExxH), which is required for alginate overproduction (20). It has also been proposed that the cytoplasmic portion of MucA is degraded by the ClpXP complex following cleavage by MucP (65). Following the complete degradation of the wild-type MucA, AlgU is free to drive transcription of the alginate biosynthetic operon at the *PalgD* promoter site, as well as modulate transcription of *algB*, *algR* and *amrZ*.

Dissertation Introduction

Colonization of the CF lung by *P. aeruginosa* via infiltration of nonmucoid environmental strains with a wild-type MucA, and natural selection for mutations in MucA that provide protection from the host's defenses through biofilm formation using the overproduction of alginate is the current dogma for the evolution of chronic *P.aeruginosa* infections in CF patients. Although, the majority of mucoid CF isolates possess mutations in MucA, regulated proteolysis of MucA is proposed as the mechanism for biofilm formation used by those early colonizing strains. **Therefore, the goal of my study was to identify novel regulators of alginate overproduction in *P. aeruginosa* strains with a wild-type *mucA*.** To achieve this goal, I used a prototypic mucoid strain PAO579 as the model system (shown in Figure 1). PAO579 possesses a wild-type *mucA* and is mucoid via an unclassified mutation referred to as *muc23*. Additional details regarding the origin of PAO579 are described in Chapters 2 and 3. I used whole genome sequencing to identify the *muc23* mutation. The parameters and results are provided in Chapter 2. In Chapter 3, I describe the use these sequencing results, as well as various molecular and microbiological techniques, to determine that mucoidy in PAO579 is due to three tandem mutations in the gene locus PA4525. These three tandem mutations result in a truncation in the type-IV pilin precursor protein, PilA, which activates the regulated proteolysis of MucA via AlgW. In Chapter 4, I describe the use of complementation and *in vitro* transposon mutagenesis of a whole genome cosmid library, derived from the reference strain PAO1, to identify novel inhibitors of alginate production in PAO579. I identified gene locus PA1494 that can inhibit alginate overproduction after localization of the gene product to the periplasm. As set forth in

Chapter 5, I explore the relationship between the expression of gene locus PA1494 and the periplasmic chaperone/protease MucD. These chapters identify and detail both the positive and negative regulatory pathways involved in mucoidy in the *P. aeruginosa* strain PAO579. The summation of these chapters, as well as the proposal of new questions and ideas, is found in Chapter 6. Collectively, the research described in this dissertation identifies novel mechanisms for the genetic regulation of alginate production in *Pseudomonas aeruginosa*. More importantly, it provides insights that may aid in the suppression of biofilm formation in the CF lung.

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**CHAPTER 2: Draft genome sequence for *Pseudomonas aeruginosa* strain
PAO579, a mucoid derivative of PAO381**

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Chapter 2 Overview and Rationale

In this study, I used the model strain PAO579 to identify novel regulators of mucoidy in *P. aeruginosa* strains possessing a wild-type MucA. PAO579 is a mucoid mutant derived from the non-mucoid progenitor strain PAO1. The genetic mutation(s) responsible for mucoidy in PAO579 is unknown and is referred to as *muc-23*. In order to identify potential candidates for the *muc-23* mutation, I used next-generation sequencing technology to sequence the entire genome of PAO579, and compared it to the previously published PAO1 reference genome. These results are presented and discussed in this chapter.

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that establishes a chronic infection in individuals afflicted with cystic fibrosis. Here we announce the draft genome of *P. aeruginosa* strain PAO579, a mucoid derivative of strain PAO381.

Pseudomonas aeruginosa is a ubiquitous, opportunistic pathogen and the leading cause of mortality in individuals afflicted with cystic fibrosis. *P. aeruginosa* uses the overproduction of an exopolysaccharide, called alginate, to form biofilms. Biofilm

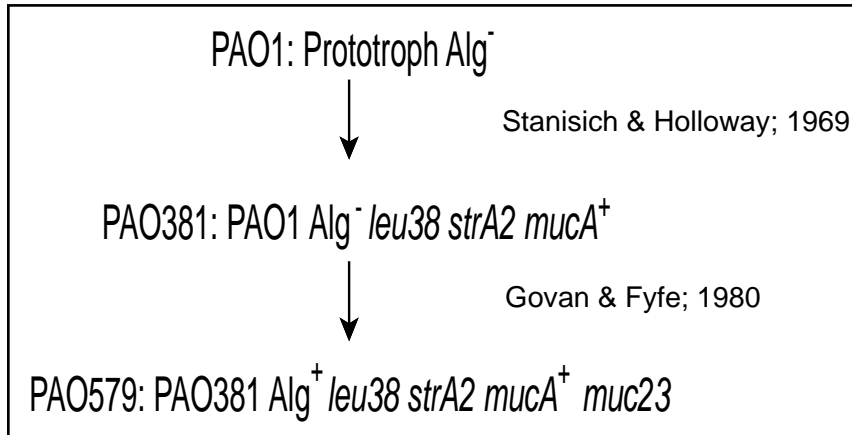


Figure 1. Origin of *P. aeruginosa* strain PAO579. PAO579 was derived from a series of mutagenic experiments beginning with bacteriophage-mediate mutagenesis of PAO1 yielding PAO381. Next, mucoid mutants were isolated following exposure of PAO381 to sub-lethal concentrations of carbenicillin. Alg⁻=nonmucoid; Alg⁺=mucoid; *leu38*=leucine auxotrophic; *strA2*=streptomycin resistant; *mucA*⁺=wild-type *mucA*; *muc23*=unclassified mutation(s).

formation is responsible for the development of chronic infections, as well as increased resistance to antibiotic treatment (2), and reduced phagocytosis by macrophages (4). *P. aeruginosa* strain PAO579 was first isolated as mucoid variant of

PAO381, a derivative of the non-mucoid strain PAO1 (3, 5). Both PAO579 and PAO381 have a wild-type *mucA* (*mucA*⁺), and both strains are leucine auxotrophic (*leu38*) and streptomycin resistant strains (*strA2*).

Genomic DNA from *P. aeruginosa* strain PAO579 was isolated using phenol/chloroform extraction and ethanol precipitation, and paired-end sequencing libraries were generated using vendor protocols (Illumina, San Diego, CA) and genome sequencing was performed on an Illumina GAIIx with a total of 54,496,482 60-bp paired-end reads resulting in 3,269,788,920 bp for ~500x coverage. Data was generated and assembled using Illumina Pipeline version SCS 2.8.0 paired with OLB 1.8.0, and aligned and annotated according to the *P. aeruginosa* strain PAO1 reference

genome (GenBank accession no. NC_2516.2) using NovoAlign version 2.07.10. All specifics regarding aligner algorithms can be obtained from novocraft.com. Further analysis of the genome was performed using Samtools version 01.16a for the generation of pileup after sorting and removing duplicate reads, and then applying analysis pipeline software developed by CoFactor Genomics (St. Louis, MO, USA). The genome was annotated and prepared for submission using NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; <http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>) server at NCBI.

Based upon our analysis, single nucleotide polymorphisms (SNPs) and INDELS were observed between the sequenced reads and the PAO1 reference genome and were tabulated for each genomic position, as well as the total coverage of bases observed at that location. As a result, 16 non-synonymous and 15 synonymous single nucleotide polymorphisms (SNPs) were identified using two criteria: more than 4X coverage and greater than 60% frequency. This data is shown in Table 1. Of particular interest is the substitution of thymine for adenine at genome position 4980548 in the DegS-like MucA protease gene, *algW* (PA4446). This alteration results in a change in the primary structure of AlgW, more specifically a substitution of isoleucine for phenylalanine at amino acid position 239. This predicted substitution is thought to effect the proteolytic activity of AlgW (1). Also of note, we observed mutations in *leuA* (PA3792), 2-isopropylmalate synthase, and *rpsL* (PA4268), 30S ribosomal protein S12. These mutations are contiguously part of the PAO381 lineage, and are responsible for leucine auxotrophy and streptomycin resistance, respectively.

Nucleotide sequence accession number. The draft genome sequence of *P. aeruginosa* strain PAO579 has been deposited in GenBank under accession number AFLOF00000000.

Table 1. Complete sequencing results for PAO579							
SNP	Genome Position	Change	SNP Position (Gene Size)	ORF	Gene Name	Gene Product	Protein
1	5036891	A→C	-183(1602)	PA4500		Probable binding protein component of ABC transporter	
2	4771865	T→C	263(372)	PA4268	<i>rpsL</i>	30S ribosomal protein S12	K88R+
3	183697	T→G	930(939)	PA0159		Probable LysR transcriptional regulator	C310W
4	4251149	G→A	322(1779)	PA3792	<i>leuA</i>	2-isopropylmalate synthase	E108K+
5	6115455	T→G	858(1254)	PA5434	<i>mtr</i>	Tryptophan permease	K266N
6	4980548	A→T	715(1170)	PA4446	<i>algW</i>	DegS-like serine protease	I239F
7	4924552	C→G	532(837)	PA4394	<i>yggB</i>	Conserved hypothetical protein	V178L+
8	4924553	G→C	531(837)	PA4394	<i>yggB</i>	Conserved hypothetical protein	
9	6098781	G→C	1758(3018)	PA5418	<i>soxA</i>	Sarcosine oxidase α subunit	
10	1871272	T→C	157(342)	PA1728		Hypothetical protein	S53P
11	4212201	A→G	1907(2529)	PA3760	<i>nagF</i>	N-acetyl-D-Glucosamine phosphotransferase system transporter	H636R
12	1589438	G→C	101(1107)	PA1459	<i>cheB</i>	Probable methyl-transferase	G34A
13	6079222	A→G	1179(1962)	PA5399	<i>dgcB</i>	Dimethylglycine catabolism	

14	5743462	G→C	1292(1680)	PA5100	<i>hutU</i>	Urocanase	T431S+
15	5743461	C→G	1293(1680)	PA5100	<i>hutU</i>	Urocanase	
16	4869855	T→G	474(771)	PA4341		Probable IclR transcriptional regulator	E158D+
17	4344266	A→G	570(1296)	PA3877	<i>nark1</i>	Nitrite extrusion protein 1	
18	721611	C→T	(1200)+55	PA0668	<i>tyrZ</i>	tyrosyl-tRNA synthetase 2	
19	2239547	T→G	-280(408)	PA2046		Hypothetical protein	
20	413850	T→C	-196(291)	PA0369		Hypothetical protein	
21	4334140	G→C	207(990)	PA3870	<i>moaA1</i>	Molybdopterin biosynthetic protein A1	
22	4448855	C→G	(1500)+38	PA3970	<i>amn</i>	AMP nucleosidase	
23	4448856	G→C	(1500)+37	PA3970	<i>amn</i>	AMP nucleosidase	
24	1603577	T→G	-94(759)	PA1477	<i>ccmC</i>	Heme exporter protein CcmC	
25	5069207	G→A	325(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop
26	5069206	T→C	326(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop
27	5069205	C→T	327(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop
28	1440625	A→G	1920(1971)	PA1327		Probable protease	
29	169284	G→C	-77(546)	PA0149		Probable σ^{70} factor, ECF	

30	2768847	C→G	9958(16884)	PA2462		Hypothetical protein	A3320P
31	2813321	A→G	128(1419)	PA2495	<i>oprN</i>	Multidrug efflux outer membrane protein	D43G

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CHAPTER 3: Truncation of type IV pilin induces mucoidy in *Pseudomonas aeruginosa* strain PAO579

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Chapter 3 Overview and Rationale

In chapter 2, I identified 31 mutations unique to *P. aeruginosa* strain PAO579, as compared to the previously published PAO1 reference genome. In order to determine the specific mutation(s) responsible for mucoidy in PAO579, I used functional analyses and PCR sequencing to narrow the search to two potential candidate loci. Using standard cloning and molecular techniques, I determined that three tandem mutations in the *pilA* gene (*pilA108*) are responsible for the induction of mucoidy by activating the serine protease AlgW to degrade MucA, thereby releasing the AlgU to drive transcription of the alginate biosynthetic operon. Therefore, the three tandem mutations in the *pilA* gene are the *muc-23* mutation. These results are presented and discussed in this chapter.

Abstract

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that uses the overproduction of alginate, a surface polysaccharide, to form biofilms. Overproduction of alginate, also known as mucoidy, affords the bacterium protection from the host's defenses and facilitates the establishment of chronic lung infections in individuals with cystic fibrosis. Expression of the alginate biosynthetic operon is primarily controlled by the alternative sigma factor AlgU (AlgT/ σ^{22}). In a non-mucoid strain, AlgU is sequestered by MucA, a transmembrane anti-sigma factor, to the cytoplasmic membrane. AlgU can be released from MucA by regulated intramembrane proteolysis by proteases AlgW and MucP, causing the conversion to mucoidy. *P. aeruginosa* strain PAO579, a derivative of the non-mucoid strain PAO1, is mucoid due to an unidentified mutation (*muc-23*). Using whole genome sequencing, we identified 16 non-synonymous and 15 synonymous single nucleotide polymorphisms. We then identified three tandem single point mutations in the *pilA* gene (PA4525), as the cause of mucoidy in PAO579. These tandem mutations generate a premature stop codon resulting in a truncated version of PilA (PilA¹⁰⁸), with a C-terminal motif of phenylalanine-threonine-phenylalanine (FTF). Deletion of *pilA*¹⁰⁸ confirmed it was required for mucoidy. Additionally, *algW* and *algU* were also required for mucoidy of PAO579. Western blot analysis indicated that MucA was less stable in PAO579 than non-mucoid PAO1 or PAO381. The mucoid phenotype and high P_{algU} and P_{algD} promoter activities of PAO579 require *pilA*¹⁰⁸, *algW*, *algU* and *rpoN*. We also observed that the alternative sigma factor RpoN regulates expression of *algW* and *pilA* in PAO579. Together, these results suggest that truncation in PilA can induce mucoidy through an AlgW/AlgU-

dependent pathway.

Introduction

Cystic fibrosis (CF) is a genetic disorder that results from mutations in the CF transmembrane conductance regulator gene (32). These mutations cause a disruption in chloride transport of mucosal tissues resulting in an accumulation of dehydrated mucus. This accumulation of mucus within the lungs prevents the removal of infectious agents by interfering with the mucociliary escalator (6). This provides a hospitable environment for the adherence and cultivation of microbial pathogens (2, 34). As a result, individuals afflicted with CF are highly susceptible to various bacterial infections including *P. aeruginosa* (13). This bacterium is a Gram-negative, opportunistic pathogen that uses the overproduction of alginate, a surface polysaccharide, to form biofilms. The overproduction of alginate, also known as mucoidy, is responsible for the establishment of chronic infections, as well as an increased resistance to antibiotics (13) and phagocytosis by macrophages (21) in CF patients. Chronic lung infections with *P. aeruginosa* cause an increase in morbidity and mortality in individuals afflicted with CF (22), and this transition from the non-mucoid to the mucoid phenotype is a proven predictor of an overall decline in the patient's health (16).

Typically, constitutively mucoid strains arise in the lungs of CF patients due to mutations in the *mucA* gene which encodes the inner membrane-spanning anti-sigma factor (4, 23). MucA is a negative regulator of alginate overproduction due to sequestering AlgU (AlgT, σ^E , σ^{22}), the primary sigma factor responsible for activation of the alginate biosynthetic operon at the *algD* promoter (40). Alternatively, the conversion to mucoidy can occur when MucA is degraded by regulated intramembrane proteolysis

which will release AlgU (31). Proteolytic degradation is initiated through cleavage of the C-terminus of MucA between the alanine and glycine at position 136 by the serine protease AlgW (5), anchored in the periplasmic leaflet of the inner membrane, and followed by the transmembrane protease MucP (YaeL) and the cytoplasmic proteases ClpX and ClpP (5, 7, 30, 31). The activation of AlgW, and subsequent proteolysis of MucA, is thought to be in response to extracellular stress, as well as the accumulation of misfolded envelope proteins (31, 39). We previously found that induction of a small envelope protein called MucE causes mucoidy (31). MucE has an AlgW activation signal with a C-terminal motif of tryptophan-valine-phenylalanine (WVF) (31). The MucE peptide is also a potent ligand that activates AlgW resulting in the degradation of the periplasmic fragment of MucA (5).

P. aeruginosa strain PAO579 was first generated in the 1970s through the isolation of mucoid variants of PAO381 (14), a non-mucoid derivative of the progenitor strain PAO1, following exposure to a sub-lethal concentration of carbenicillin. PAO579 is highly mucoid due to unclassified mutation(s) that is referred to as *muc-23* (14). Mucoidy in PAO579 depends on the alternative sigma factor RpoN (σ^{54}) (3). In this study, we used whole genome sequencing to identify mutation(s) that cause the mucoidy of PAO579. We found three tandem mutations in *pilA* that are responsible for the mucoid phenotype in this strain. Moreover, the mucoid phenotype of strain PAO579 is dependent upon AlgW, as well as AlgU and RpoN. Our data suggests truncation of pilin induces mucoidy in *P. aeruginosa* strain PAO579.

Experimental Procedures

Sequence analysis of PAO579. Methods and parameters used in the sequencing of *P. aeruginosa* strain PAO579 were previously described (37).

Bacterial strains and growth conditions. Bacterial strains used in this study are indicated in Table 1. *P. aeruginosa* and *E. coli* strains were grown at 37°C in Lennox broth (LB), on LB agar, or *Pseudomonas* Isolation Agar (PIA). When indicated, the media was supplemented with carbenicillin, gentamycin, tetracycline, kanamycin and/or arabinose.

Construction of mutant strains. In-frame deletion of target genes *algU* (PA0762) and *algW* (PA4446) in PAO579 was performed through PCR amplification of the upstream and downstream regions (500 to 1000 base pairs) flanking the target gene. Using crossover PCR, these upstream and downstream regions were fused and ligated into pEX100T-NotI. A two-step allelic exchange procedure was used by first screening the possible deletions mutants for carbenicillin resistance and sucrose sensitivity, then screening for sucrose resistance and carbenicillin sensitivity. For construction of the PAO579 *rpoN*::Tc^R strain, *rpoN* (PA4462) was amplified through PCR, cloned into the pCR[®]4-TOPO[®] Vector (Invitrogen) and transformed into *E. coli* DH5 α . *In vitro* transposon mutagenesis was performed on the pCR[®]4-TOPO[®]-*rpoN* vector using the EZ::TN <KAN-2> insertion kit (Epicentre Biotechnologies). The mutant library was recovered and triparentally conjugated en masse into PAO579. Mutants were selected on PIA containing tetracycline and screened for the non-mucoid phenotype.

Table 1. Bacterial strains and plasmids used in this study.

Strain, Plasmid	Genotype, phenotype, description	Reference
E. coli		
TOP10	DH5α derivative	Invitrogen
P. aeruginosa		
PAO1	<i>algU</i> ⁺ <i>mucA</i> ⁺ ; non-mucoid	P. Phibbs*
PAO381	<i>algU</i> ⁺ <i>mucA</i> ⁺ ; non-mucoid, derived from PAO1	J. Govan**
PAO579	<i>algU</i> ⁺ <i>mucA</i> ⁺ <i>muc-23</i> ; mucoid, derived from PAO381	J. Govan**
PAO579Δ <i>algU</i>	<i>mucA</i> ⁺ <i>muc-23</i> , In-frame deletion of <i>algU</i> (PA0762); non-mucoid	This study
PAO579Δ <i>algW</i>	<i>algU</i> ⁺ <i>mucA</i> ⁺ <i>muc-23</i> , In-frame deletion of <i>algW</i> (PA4446); non-mucoid	This study
PAO579 <i>pilA</i> :: <i>aacC1</i>	<i>algU</i> ⁺ <i>mucA</i> ⁺ <i>muc-23</i> , <i>pilA</i> ::Gm ^R (PA4525) encoding a Type-IVa pilin precursor; non-mucoid	This study
PAO579 <i>rpoN</i> ::Tc ^R	<i>algU</i> ⁺ <i>mucA</i> ⁺ <i>muc-23</i> , <i>rpoN</i> :: Tc ^R (PA4462) of the sigma factor RpoN (σ54); non-mucoid	This study
Plasmids		
pCR4-TOPO	3.9-kb, Ap ^R , Km ^R ; TA cloning vector	Invitrogen
pRK2013	Km ^R , <i>Tra Mob ColE1</i>	(12)
pHERD20T	pUCP20T P _{lac} replaced by 1.3-kb AflIII-EcoRI fragment of <i>araC</i> -P _{BAD} cassette	(29)
pHERD20T- <i>algW</i>	<i>algW</i> (PA4446) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>algW</i> ^{I239F}	<i>algW</i> (PA4446) from PAO579 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>pilA</i>	<i>pilA</i> (PA4525) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>pilA</i> ¹⁰⁸	<i>pilA</i> (PA4525) from PAO579 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>pilA</i> -HA	C-terminally tagged <i>pilA</i> -HA ending with the PKGCDN motif cloned in pHERD20T; EcoR1/HindIII	This study

pHERD20T- <i>pilA</i> ¹⁰⁸ -HA	C-terminally tagged <i>pilA</i> -HA ending with the DITFTF motif cloned in pHERD20T; EcoR1/HindIII	This study
pHERD20T- <i>oprF</i>	<i>oprF</i> (PA1777) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>oprF</i> -FTF	<i>oprF</i> (PA1777) from PAO1 with FTF-motif fused to the C-terminal; EcoRI/HindIII	This study
pHERD20T-HA- <i>mucA</i>	N-terminally tagged HA- <i>mucA</i> in pHERD20T; EcoRI/HindIII	(9)
pUCP20T-P _{BAD} - <i>rpoN</i>	<i>araC</i> -P _{BAD} - <i>rpoN</i> fusion in pUCP20; XbaI/HindIII	(9)
miniCTX- <i>lacZ</i>	Gene delivery system used to fuse target genes to <i>lacZ</i> and integrate onto the chromosome at the CTX phage <i>att</i> site in <i>P. aeruginosa</i> , Tc ^R	(17)
miniCTX-P _{algD} - <i>lacZ</i>	Complete P _{algD} promoter (1,525 bp upstream of ATG) HindIII/BamHI in miniCTX- <i>lacZ</i>	(9)
miniCTX-P _{algU} - <i>lacZ</i>	Complete P _{algU} promoter (541 bp upstream of ATG) EcoRI/HindIII in miniCTX- <i>lacZ</i>	(9)
pEX100T-NotI	Pseudomonas suicide vector with NotI restriction site fuse into SmaI of pEX100T, <i>sacB</i> , <i>oriT</i> , CbR	(9)
pEX100T-Δ <i>algW</i>	1.4-kb fragment flanking the <i>algW</i> (PA4446) gene ligated into pEX100T-NotI with in-frame deletion of <i>algW</i>	(31)
pEX100T-Δ <i>algU</i>	2.5-kb fragment flanking the <i>algU</i> (PA0762) gene ligated into pEX100T-NotI with in-frame deletion of <i>algU</i> with 24 bp remaining.	(9)
pCR4- <i>pilA</i> ::Gm ^R	1941 bp fragment contained 966 bp upstream of ATG and 975 bp downstream of TAA with a MluI Gm ^R cassette (750 bp) inserted 9 bp before ATG of an in-frame deleted <i>pilA</i> ligated into pCR4-TOPO	This study
pLP170	8.3-kb, <i>lacZ</i> , Ap ^R , multiple cloning site	(28)
pLP170-P _{algU}	Complete P _{algU} promoter (541 bp upstream of ATG) fused with <i>lacZ</i> in pLP170 BamHI/HindIII	This study
pLP170-P _{algD}	Complete P _{algD} promoter (989 bp upstream of ATG) fused with <i>lacZ</i> in pLP170 BamHI/HindIII	This study
pLP170-P _{pilA}	Complete P _{pilA} promoter (500 bp upstream of ATG) fused with <i>lacZ</i> in pLP170 BamHI/HindIII	This study
pLP170-P _{algW}	Complete P _{algW} promoter (1,000 bp upstream of ATG) fused with <i>lacZ</i> in pLP170 BamHI/HindIII	This study

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The *PAO579pilA::aacC1* strain was constructed using crossover PCR of 1000 bp upstream and downstream fragments of *pilA* (PA4525) containing an internal *MluI* restriction site. This crossover PCR product was cloned into the pCR[®]4-TOPO[®] vector and digested using *MluI*. A cassette containing a gentamycin resistance marker was digested with *MluI* and ligated into the pCR[®]4-TOPO[®]-*pilA* construct. Finally, the pCR[®]4-TOPO[®]-*pilA* construct was triparentally conjugated and a two-step allelic exchange procedure was used by first screening for gentamycin resistance and carbenicillin resistance, then gentamycin resistance and carbenicillin sensitivity. All strains were amplified by PCR and sequenced to confirm proper insertion or deletion of the target genes.

Plasmid construction and complementation. The plasmids used in this study are indicated in Table 1. Standard recombinant DNA cloning techniques were used in the construction of all plasmids used in this study (33). Briefly, oligonucleotide primers were designed based on PAO1 sequence information and synthesized by Eurofin MWG Operon. Primer sequence information is available in Table 1. PCR amplifications were done using EasyStart[™] Micro 50 PCR Mix-in-a-Tube (Molecular BioProducts) and *Taq* DNA Polymerase (New England BioLabs). The pCR[®]4-TOPO[®] Vector (Invitrogen, Inc.) was used as an intermediary before ligation into the target vector. All plasmids were purified using QIAprep[®] Spin Miniprep Kit (Qiagen Sciences). All plasmid constructs were sequenced to confirm they contained no mutations. Plasmids were transformed into *E. coli* DH5 α for all intermediate cloning steps. Completed plasmids were triparentally conjugated into target *P. aeruginosa* strains using pRK2013 as a helper strain(12).

Alginate assay. Alginate was measured using the previously published carbazole reaction (19). Bacterial strains were streaked onto triplicate PIA plates, and incubated at 37° C for 24 hrs. The bacterial cells were scraped into 10 mL of PBS and the OD₆₀₀ was recorded. The amount of uronic acid was measured and compared to an alginate standard curve made with D-mannuronic acid lactone (Sigma-Aldrich) in the range 0 to 100 µg/mL. The reported values represent an average of three independent experiments with standard deviation.

β-galactosidase activity assay. *Pseudomonas* strains carrying the plasmid pLP170 (empty vector) or pLP170 containing P_{algD}, P_{algU}, P_{algW} or P_{pilA} or PAO1 miniCTX-P_{algU}-*lacZ* and miniCTX-P_{algD}-*lacZ* with pHERD20T, pHERD20T-*pilA* or pHERD20T-*pilA*¹⁰⁸ were cultured at 37°C on three PIA plates supplemented with carbenecillin or carbenicillin and tetracycline. Bacterial cells were harvested, resuspended in PBS and the OD₆₀₀ was recorded. The cells were permeabilized using toluene, and β-galactosidase activity was measured with results calculated and reported in Miller Units (25). One Miller Unit equivalent to $1000 \times (A_{420}/1.75 \times A_{550} / OD_{600} \text{ mL}^{-1} \text{ min}^{-1})$. The reported values represent an average of three independent experiments with standard deviation. Student's *t* test was performed to determine statistical significance.

Protein analyses. Bacterial strains were grown at 37°C on PIA or LB media supplemented with the appropriate antibiotics. Cells were harvest and whole cell

lysates were prepared using the ProteaPrep Cell Lysis Kit (Protea Biosciences) and the total protein content was quantified using D_c Assay (Bio-Rad). Using a HA Tag immunoprecipitation kit (Pierce[®]), HA-tagged proteins were isolated by combining cell lysates with anti-HA agarose beads, incubating overnight at 4°C, washing with a TBS-Tween solution, and eluting the proteins from the anti-HA agarose beads. The protein samples (25 µg) were boiled for 10 min in Tricine Sample Buffer (Bio-Rad) and electrophoresed on a 16.5% Tris-Tricine gel (Bio-Rad). Samples were then electroblotted onto a Hybond™-P PVDF transfer membrane (GE Healthcare). The membrane was blocked using 3% skim milk/PBS. Mouse monoclonal antibody for the alpha subunit of RNA polymerase (Neoclone) and rat monoclonal antibody for hemagglutinin (Roche Diagnostics) were used as primary antibodies. Anti-pilin serum was gifted from the Lory laboratory (University of California, San Francisco, CA). Horseradish peroxidase-labelled goat anti-mouse IgG, goat anti-rabbit or goat anti-rat IgG were used as secondary antibodies. Primary and secondary antibodies were diluted in 3% skim milk/PBS to 1:5000 and 1:10,000, respectively. Western blot results were imaged using ECL Advance Western Blotting Detection Kit (Amersham; GE Healthcare) and UVP BioImaging Systems. When necessary, blots were stripped using 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol for 10 minutes at 40°C.

Epifluorescent microscopy. *Pseudomonas* strains containing pHERD20T-*pilA*-HA-PKGCDN (WT), pHERD20T-*pilA*-HA-DITFTF or pHERD20T were cultured on PIA plates supplemented with 300 µg/mL carbenicillin and arabinose and incubated at 37°C. For the detection of extracellular PilA, cells were stained according to the procedure

provided with the Anti-HA-Fluorescein, High Affinity (3F10) antibody (Roche Diagnostics). Briefly, cells were harvested, washed and resuspended in incubation buffer. Alternatively, for the detection of intracellular PiiA, the cells were permeabilized using toluene. Anti-HA-Fluorescein antibody was added to the cells, which were then incubated on ice for 30 minutes, washed with incubation buffer. Images were initially collected and recorded using an Olympus EX51 microscope with an Olympus DP70 digital camera, and subsequently with a Leica SP5 TCSII equipped with a Coherent Chameleon multiphoton VisionII laser. Images were further analyzed using ImageJ 1.45s software.

Results

PAO579 has polymorphisms in *algW* and *pilA*.

Using PAO1 as a reference genome, we performed next-generation sequencing to determine the mutation(s) (*muc-23*) responsible for mucoidy in PAO579 (ALOF00000000) (37). We identified 16 nonsynonymous and 15 synonymous single nucleotide polymorphisms (SNPs) by using two criteria: more than 4X coverage and greater than 60% frequency. (previously shown in Table 1 of Chapter 2). Consistent with previous phenotypic observations (14, 36), we detected mutations at loci *rpsL* (PA4268) and *leuA* (PA3792), both corresponding to previous genetic and phenotypic markers in the parent strain PAO381 (Table 2). Further PCR sequencing revealed that only two genes, *algW* (PA4446) and *pilA* (PA4525) contained SNPs in PAO579 when compared to strain PAO381. Our results showed a substitution of an adenine for a thymine at nucleotide 715 of the coding region of *algW* (PA4446), resulting in the exchange of phenylalanine for isoleucine at amino acid 239 (I239F) in AlgW (Table 2). We designated this mutation as *algW*^{I239F}. We also observed three tandem nucleotide substitutions (C→T³²⁵, A→G³²⁶, G→A³²⁷) in *pilA* (PA4525) that predicted a premature stop codon (TGA) (Table 2). The *pilA* gene encodes the protein precursor that constitutes the type-IVa pilin. Further analysis of these tandem mutations at nucleotides 352-327 confirmed a truncation in PilA from 149 to 108 amino acids (Table 2 and Figure 4). We designated this mutation as *pilA*¹⁰⁸. We hypothesized that one, or both of these mutations could be responsible for mucoidy in PAO579.

Table 2. Summary of sequencing results for PAO579							
SNP	Genome Position	Change	SNP Position (Gene Size)	ORF	Gene Name	Gene Product	Protein
1	4771865	T→C	263(372)	PA4268	<i>rpsL</i>	30S ribosomal protein S12	K88R+
2	4251149	G→A	322(1779)	PA3792	<i>leuA</i>	2-isopropylmalate synthase	E108K+
3	4980548	A→T	715(1170)	PA4446	<i>algW</i>	DegS-like serine protease	I239F
	5069207	G→A	325(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop
4	5069206	T→C	326(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop
	5069205	C→T	327(450)	PA4525	<i>pilA</i>	Type IV fimbrial precursor	Stop

***algW* and *algU* are required for alginate overproduction in strain PAO579.**

AlgW is the first in a cascade of proteases responsible for the degradation of MucA, (5, 38, 39). To determine if *algW* is required for mucoidy in PAO579, we deleted *algW* and observed a decrease in alginate production and a conversion to the non-mucoid phenotype (Figure 1). Next, we cloned *algW* and *algW*^{I239F} into the shuttle vector pHERD20T containing the arabinose-inducible P_{BAD} promoter (29). The expression of *algW* *in trans* restored mucoidy to PAO579Δ*algW* (Figure 1). Similarly, the expression *algW*^{I239} *in trans* restored mucoidy in PAO579, however we did not observe a significant difference in the amount of alginate produced (Figure 1). More importantly, *algW*^{I239F} did not induce mucoidy in PAO1Δ*algW* (Figure 1). These data support AlgW being required for mucoidy in PAO579, however the I239F mutation is not responsible for inducing mucoidy in PAO579.

While *algU* is reportedly not required for alginate overproduction in the strain PAO579 (3), our data showing that AlgW is required for mucoidy, suggests that MucA degradation, and subsequently, the release of AlgU, is occurring in PAO579. If this is correct, expression of MucA would result in a loss of mucoidy. To test this hypothesis, *mucA* was expressed from both pHERD20T and the low-copy number Ptac vector (15), and indeed loss of mucoidy was observed (data not shown). Additionally, deletion of *algU* from PAO579 resulted in a loss of mucoidy (Figure 1). Expression of *algU* *in trans* in PAO579Δ*algU* restored mucoidy (data not shown). These data suggest that AlgU is required for alginate production in PAO579.

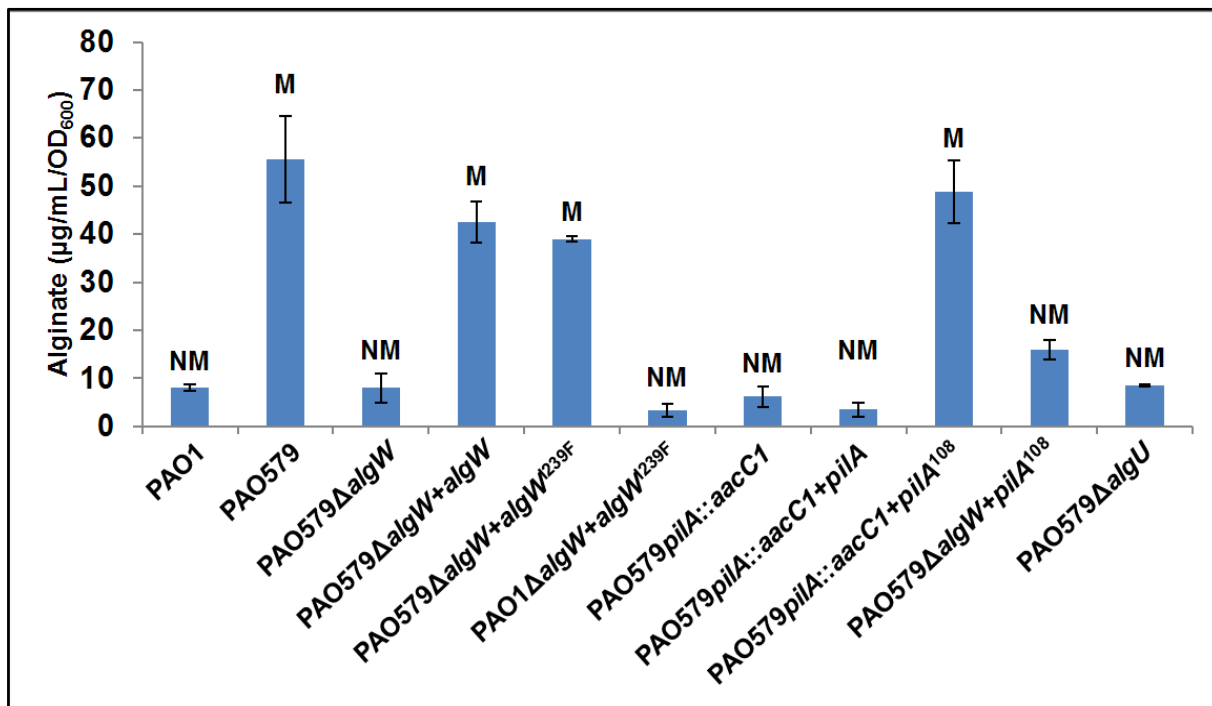


Figure 1. Alginate produced by *P. aeruginosa* strains PAO1, PAO381 and PAO579 mutants. All strains were grown on PIA plates for 24h at 37°C then for 24h at room temperature. The alginate was collected and measured using the carbazole assay. The values are reported as mean ± standard deviation of three independent experiments. M, Mucoid; NM, Nonmucoid

Expression of *pilA*¹⁰⁸ induces mucoidy in PAO579.

Since *algW*^{I239F} is not responsible for the induction of mucoidy, we next examined the role of *pilA*¹⁰⁸ in the regulation of alginate overproduction in PAO579. Sequence analysis, indicates that *pilA*¹⁰⁸ encodes for ~11 kDa protein. Western blot

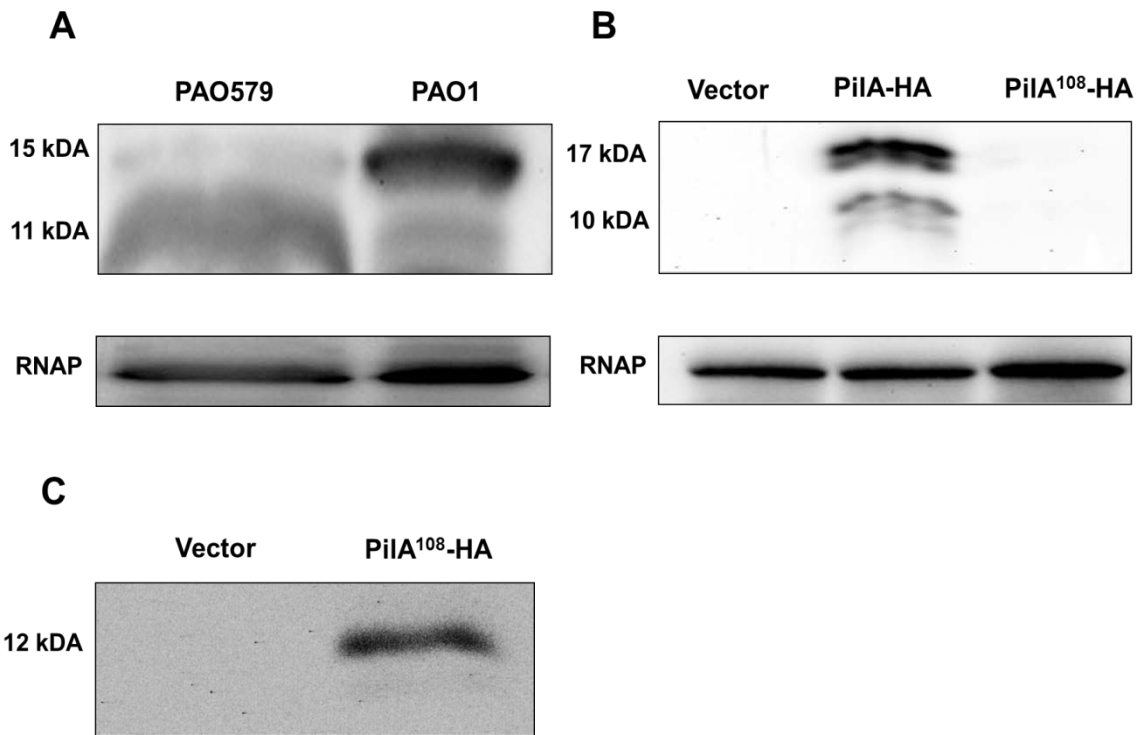


Figure 2. Western blot analysis of PilA108 Panel A) PAO579 and PAO1 were grown at 37°C on PIA. Cellular protein was harvested and was subjected to SDS-PAGE electrophoreses, membrane transfer, and probed with anti-Pilin polyclonal antibody. Panel B) PAO1 cells containing pHERD20T (Vector), pHERD20T-*pilA*-HA and pHERD20T-*pilA*¹⁰⁸-HA were grown at 37°C on PIA plates supplemented with carbenicillin and 0.1% arabinose. Cellular protein was harvested was subjected to SDS-PAGE electrophoreses, membrane transfer, and probed with anti-HA monoclonal antibody. Panel C) PAO1 pHERD20T and pHERD20T-*pilA*¹⁰⁸-HA was cultured on PIA plates supplemented with carbenicillin and 0.1% arabinose. Cellular protein was harvested then purified using high affinity anti-HA immunoprecipitation and analyzed using SDS-PAGE electrophoresis, membrane transfer, and probed with anti-HA monoclonal antibody.

analysis using anti-PilA polyclonal antibody revealed a lack of the full length pilin protein in PAO579 (Figure 2A). Additionally, HA-tagged PilA108 was only detected with Western blot analysis after immunopurification (Figure 2B and Figure 2C). Similarly, HA-tagged PIIA108 was not detected on the cell surface of PAO1 using epifluorescent confocal microscopy (Figure 3).

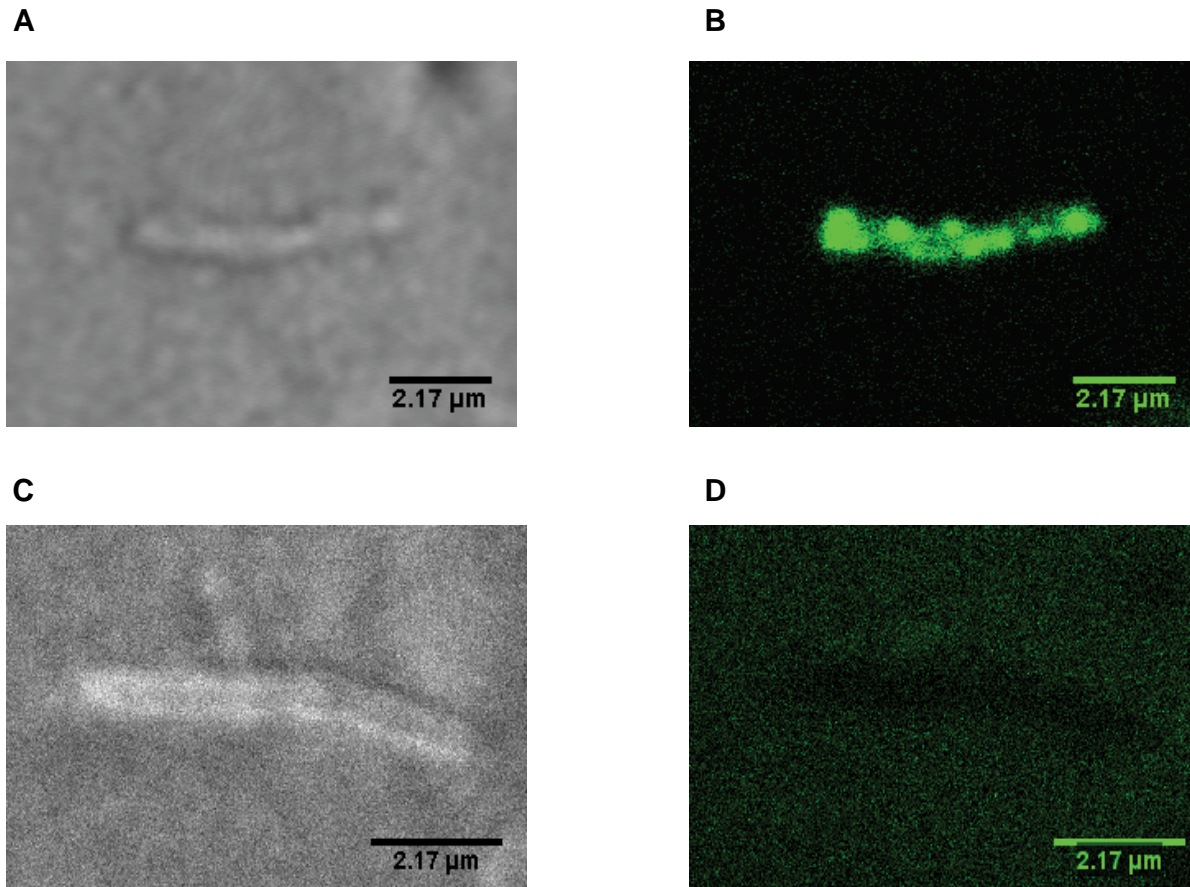


Figure 3. Localization of PilA108. Epifluorescent confocal microscopic images showing the localization of PilA-HA (WT) and HA-tagged PilA108 in PAO1: A) confocal and B) epifluorescent image of PAO1 pHERD20T-*pilA*-HA; C) confocal and D) epifluorescent image of PAO1 pHERD20T-*pilA*¹⁰⁸-HA.

The sequence of C-terminal of PilA¹⁰⁸ consists of a 3 amino acid motif of phenylalanine-threonine-phenylalanine (FTF) (Figure 3A and Figure 3B). Previously, we reported that the C-terminal motif tryptophan-valine-phenylalanine (WVF) found on the

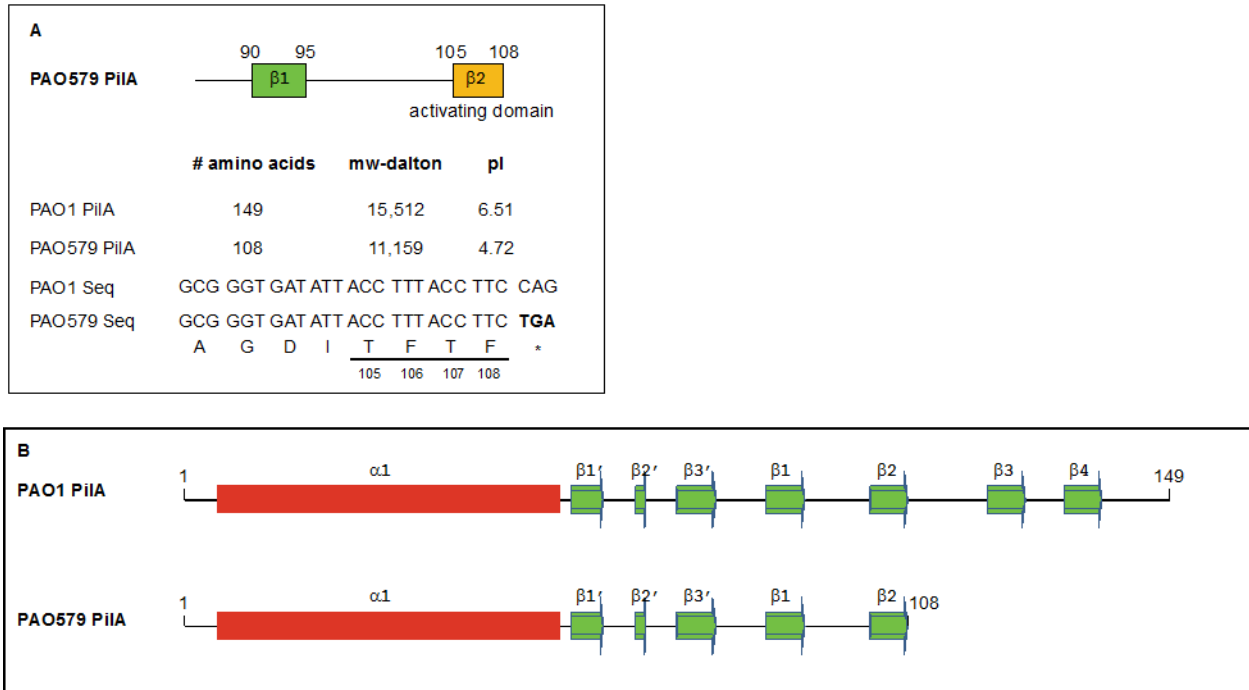


Figure 4. Diagram showing the predicted structure for PAO1 PilA and PAO579 PilA. A) Base pair substitutions in the *pilA* gene in PAO579 result in a premature STOP codon and a truncation in the PilA protein from 149 amino acid residues and molecular weight of 15.5 to 108 amino acid protein with a molecular weight of 11.2 kDa. PAO579 PilA has an activating domain ($\beta 2$) that is responsible for the positive regulation of alginate production which consists of a phenylalanine-threonine-phenylalanine (FTF) motif at its C-terminal. B) The truncation of PAO579 PilA at amino acid 108 also results in the loss of the $\beta 3$ and $\beta 4$ antiparallel sheets, as well as revealing the activating domain in the $\beta 2$ sheet.

small periplasmic protein MucE can induce mucoidy through the activation of AlgW (31). Based on this information, we hypothesized that the truncated *pilA*¹⁰⁸ could induce mucoidy through AlgW. We tested this hypothesis by first inactivating *pilA* in PAO579 through the insertion of a gentamycin cassette (PAO579*pilA*::*aacC1*). We observed a decrease in alginate production and a conversion to the non-mucoid phenotype in PAO579*pilA*::*aacC1* (Figure 1). Next, we complemented these experiments by cloning the wild-type *pilA* and *pilA*¹⁰⁸ into pHERD20T containing the arabinose-inducible P_{BAD}

promoter and expressed them *in trans*. Expression of *pilA*¹⁰⁸ increased alginate production inducing mucoidy in PAO579*pilA::aacC1* (Figure 1), while expression of expression of *pilA* wild-type did not (Figure 1). Similar results were also observed in PAO1 (data not shown). In addition, the expression of *pilA*¹⁰⁸ did not confer mucoidy in PAO579Δ*algW*, suggesting that PilA¹⁰⁸ acts through AlgW. To confirm whether the FTF-motif found in PilA¹⁰⁸ can induce mucoidy via AlgW, we cloned the major outer membrane porin precursor *oprF* (PA1777) and *oprF* with the addition of the FTF motif to its C-terminal (*oprF*-FTF) into pHERD20T. Next, we conjugated this construct, as well as pHERD20T-*pilA* and pHERD20T-*pilA*¹⁰⁸ into PAO1 and PAO1Δ*algW*. After incubating in the presence of 0.1% arabinose, expression of *oprF*-FTF and *pilA*¹⁰⁸ increased alginate production and conferred mucoidy in PAO1 (Table 3). Expression of *oprF* did not induce mucoidy in PAO1, which is consistent with our previously published results (29). Expression of *pilA* did not induce mucoidy in PAO1. As expected, we did not observe any phenotypic change when *pilA*, *pilA*¹⁰⁸, *oprF*, *oprF*-FTF were expressed in PAO1Δ*algW*. These results suggest that the FTF-motif found at the C-terminal of PilA¹⁰⁸ can activate mucoidy through AlgW.

Table 3. Complementation analyses of <i>pilA</i>, <i>pilA</i>¹⁰⁸, <i>oprF</i> and <i>oprF</i>-FTF					
<i>Pseudomonas</i> strains	Vector Control	<i>pilA</i>	<i>pilA</i> ¹⁰⁸	<i>oprF</i>	<i>oprF</i> -FTF
PAO1	NM(3.6±0.4)	NM(11.7±1.6)	NM(52.7±7.1)	NM(6.3±5.8)	NM(40.8±6.8)
PAO1Δ <i>algW</i>	NM(6.7±3.2)	NM(4.4±4.4)	NM(5.2±3.3)	NM(4.7±1.9)	NM(7.4±1.2)

NM, non-mucoid; M, mucoid; pHERD20T was used in this study. All strains were grown on PIA supplemented with 300 µg/mL carbenicillin and 0.1% arabinose and incubated at 37°C for 24h. The alginate measurements for three independent experiments are represented as (Mean µg of alginate/mL/OD₆₀₀±Standard Deviation).

***pilA*¹⁰⁸ and *algW* are required for proteolytic degradation of MucA.**

Since expression of *pilA*¹⁰⁸ required *algW* to confer mucoidy in PAO579, we hypothesized that the activation of alginate production was due to increased MucA degradation. In order to test this hypothesis, we measured the degradation of MucA by

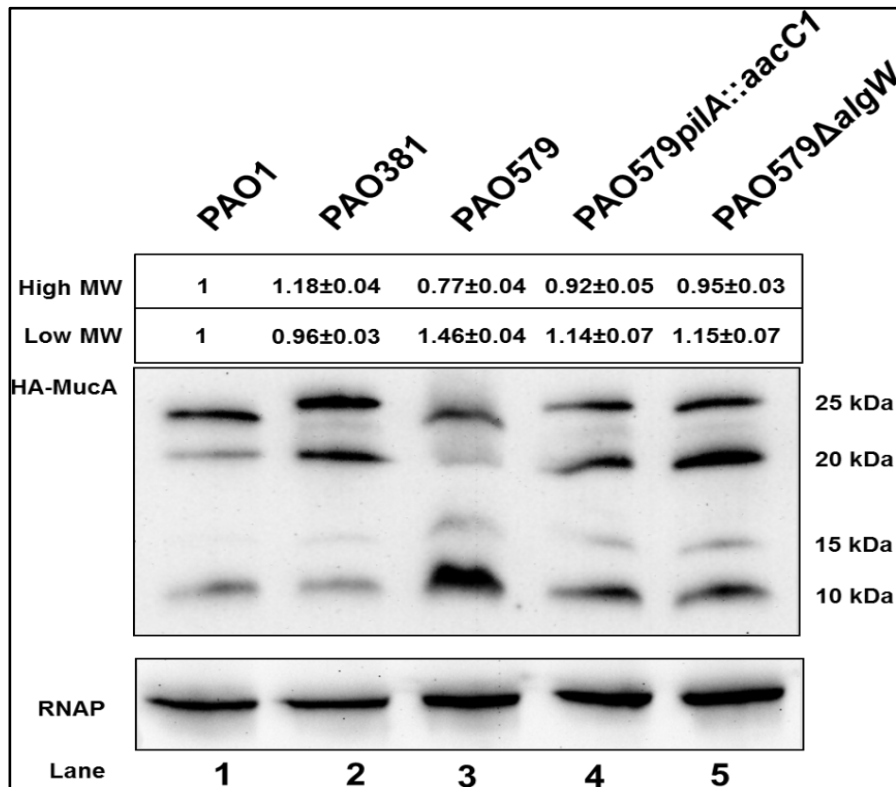


Figure 5. Western blot analysis of N-terminally tagged HA-MucA in PAO1, PAO381, PAO579, PAO579*pilA::aacC1* and PAO579Δ*algW*. Shown are representative panels of three independent experiments. All strains were grown on PIA plates supplemented with carbenicillin and 0.1% arabinose for 24h at 37°C then for 24h at room temperature. Cell lysates were prepared and 25 μg of total protein was loaded for each sample for SDS-PAGE electrophoresis. Following transfer, the membrane was immunoblotted with primary rat anti-HA and secondary horseradish peroxidase-labelled goat anti-rat IgG. Protein levels were categorized as High MW (>20 kDa) or Low MW (<20kDa), normalized to PAO1 pHERD20T-HA-*mucA*, and presented as relative means ± standard deviations.

expressing an N-terminally HA-tagged MucA (9) via the *P*_{BAD} arabinose-inducible promoter (pHERD20T-HA-*mucA*) in PAO1, PAO381, PAO579, PAO579*pilA::aacC1* and PAO579Δ*algW*. All strains were cultured on PIA plates supplemented with carbenicillin

and 0.1% arabinose. Western blot analysis of PAO1 and PAO381 showed similar levels of full length HA-MucA, although we detected greater accumulation of protein at 20 kDa and 10 kDa in PAO381 (Figure 4, Lane 1 and 2). We detected a decrease in full length HA-MucA and an increase in lower molecular weight products (~10 kDa) in PAO579 when compared to all other test strains (Figure 4, Lane 3). We also detected similar amounts of full length HA-MucA in PAO579*pilA::aacC1* and PAO579 Δ *algW* as PAO381 (Figure 4, Lane 4 and 5). These results suggest that there is an increase in MucA degradation in PAO579 when compared to its progenitor strains PAO1 and PAO381. Additionally, *pilA*¹⁰⁸ and *algW* are required for increased MucA degradation in PAO579.

Increased transcriptional activity at the P_{algD} and P_{algU} promoters in PAO579 requires *pilA*¹⁰⁸, *algW*, *algU* and *rpoN*.

Based on our Western blot analyses of MucA, we hypothesized that deletion of *pilA*¹⁰⁸, *algW* and *algU* would result in a decrease in transcriptional activity for the alginate biosynthetic operon. To test this, we measured promoter activity by fusing the entire P_{algD} promoter to *lacZ* in the plasmid pLP170 (28), and performed a Miller assay (25). We observed a significant increase in P_{algD} activity in PAO579 as compared to its progenitor strains PAO1 and PAO381 (Figure 5). We also observed a significant decrease in P_{algD} activity in the *pilA*¹⁰⁸, *algW* and *algU* mutants in PAO579 (Figure 5). Since the expression of the AlgU gene is auto-regulated, it is possible to indirectly measure the release of AlgU following MucA degradation using a β -galactosidase promoter fusion assay. Similar to our analysis of the *algD* promoter, we used the plasmid pLP170 to fuse the entire *algU* promoter region to *lacZ*, and performed a Miller

assay. Similar to our *algD* promoter analysis, we observed a significant increase in P_{algU} activity in PAO579 compared to PAO1 and PAO381, and a significant decrease in P_{algU} activity in the *pilA*¹⁰⁸, *algW* and *algU* mutants (Figure 5).

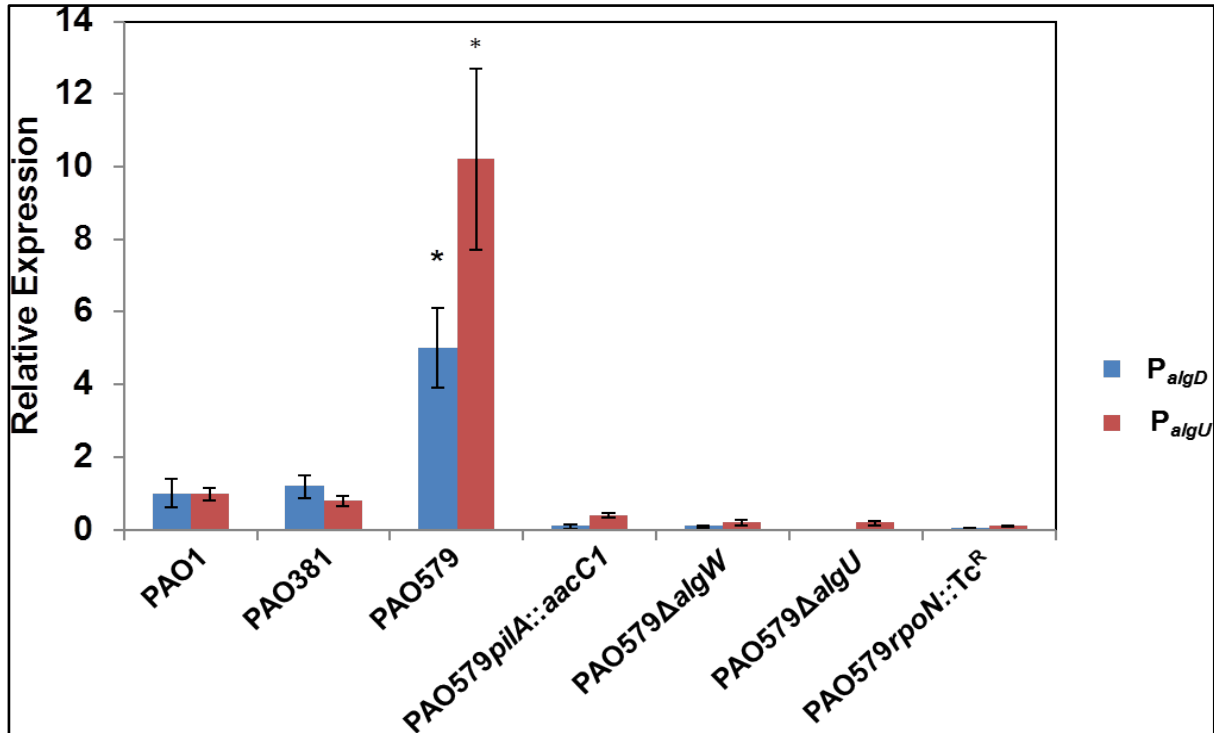


Figure 6. The β -galactosidase activity of the P_{algD} and P_{algU} promoter fusions was measured using the pLP170- P_{algD} -*lacZ* and pLP170- P_{algU} -*lacZ* reporter constructs. Each strain was on incubated at 37° C on PIA plates supplemented with 300 ug/mL of carbenicillin. The values for the mean \pm standard deviation are shown as relative expression, and are representative of three independent experiments. Asterisks indicate statistical significance (* P <0.05).

Additionally, we measured the effect of wild-type *pilA* and *pilA*¹⁰⁸ expression on merodiploid strains carrying P_{algD} and P_{algU} fused with the *lacZ* reporter gene (9) in the presence of the shuttle vector pHERD20T, pHERD20T-*pilA* and pHERD20T-*pilA*¹⁰⁸. Induction of *pilA*¹⁰⁸ with 0.1% arabinose, caused a significant increase in P_{algD} activity as compared to the vector control and wild-type *pilA* (Figure 6). There was no significant difference in P_{algD} activity between the vector control and *pilA* wild-type

(Figure 6). A similar trend was observed when measuring the P_{algU} promoter activity (Figure 6).

The alternative sigma factor RpoN was reported to be required for alginate production in PAO579 (3). Consistent with these findings, inactivation of *rpoN* in PAO579 (PAO579*rpoN*::Tc^R) resulted in a significant decreases in activity at the *algD*

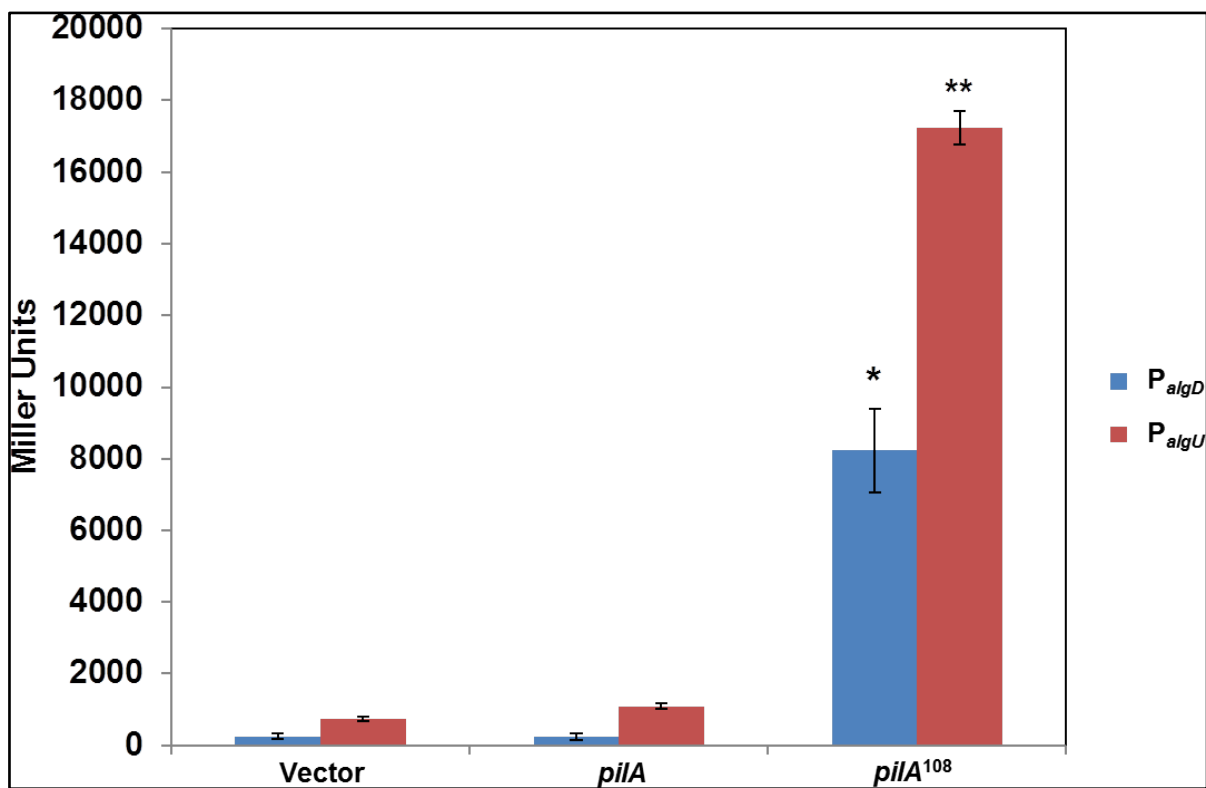


Figure 7. The β -galactosidase activity of the *algD* promoter and *algU* promoters with *pilA* and *pilA*¹⁰⁸ expressed *in trans*. The β -galactosidase activity was measured using the miniCTX- P_{algD} -*lacZ* and miniCTX- P_{algU} -*lacZ* reporter constructs integrated to *att* site in PAO1 and pHERD20T, pHERD20T-*pilA* or pHERD20T-*pilA*¹⁰⁸ were conjugated using the helper plasmid pRK2013. Each strain was incubated at 37° C on PIA plates supplemented with tetracycline, carbenicillin and 0.1% arabinose. The values for the mean and standard deviation are shown as relative expression, and are representative of three independent experiments. Asterisks indicate statistical significance (* P <0.01; ** P <0.0005).

and *algU* promoters when compared to PAO579 (Figure 5). Interestingly, overexpression of *rpoN* using pHERD20T failed to induce mucoidy in PAO579*pilA*::*aacC1* and PAO579 Δ *algU*, suggesting that RpoN regulates mucoidy in PAO579 upstream of PilA and AlgU. We performed Western blot analysis to measure

the level of RpoN in PAO1, PAO579 and PAO381 and found the level of RpoN was comparable in these three strains (data not shown). RpoN regulates global gene expression of many motility genes in non-mucoid strains of *P. aeruginosa* (10). Likewise, RpoN is responsible for transcription of *pilA* through the PilS/PilR two-component regulatory system (18). Deletion of *rpoN* from a mucoid strain resulted in dysregulation of ~20% of the genome (8). RpoN may be involved in the expression of

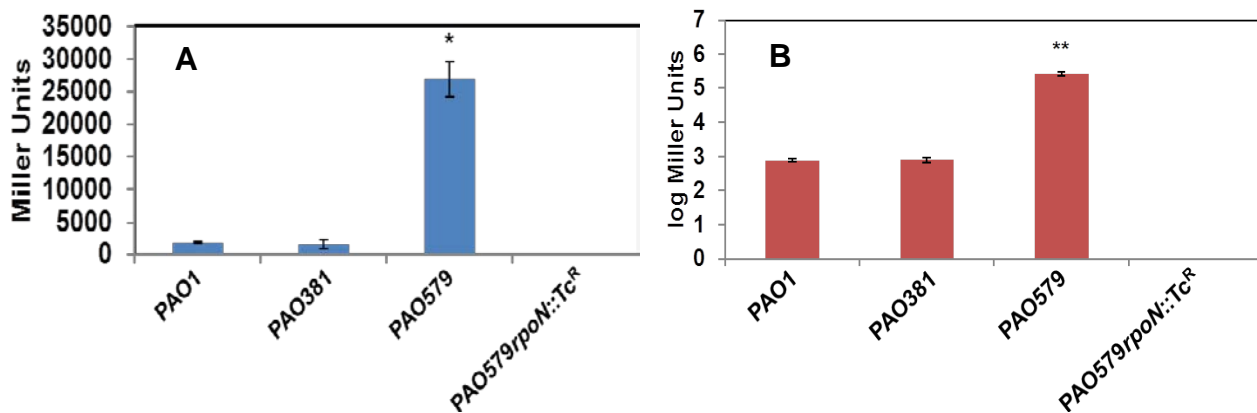


Figure 8. The β -galactosidase activity of the *algW* promoter (A) and *pilA* promoter (B) was measured using the pLP170- P_{algW} -*lacZ* and pLP170- P_{pilA} -*lacZ* reporter constructs. Each strain was incubated at 37°C on PIA plates supplemented with 300 μ g/mL of carbenicillin. The values for the mean and standard deviation are representative of three independent experiments. The measurement for P_{pilA} activity is presented in log Miller Units. Asterisks indicate statistical significance (* $P < 0.005$; ** $P < 0.0005$).

algW (8). Expression of *pilA*¹⁰⁸ in PAO579rpoN::Tc^R did not restore mucoidy, indicating *rpoN* may have multiple roles in alginate overproduction in strain PAO579. We hypothesized that the inability of *pilA*¹⁰⁸ to confer mucoidy in PAO579rpoN::Tc^R could be due to RpoN's role in driving transcription at both the *pilA* and *algW* promoters. We tested this hypothesis by measuring the level of promoter activities of P_{pilA} and P_{algW} . Promoter activity of P_{pilA} and P_{algW} between strains PAO1 and PAO381 was similar; however, we observed a significant increase in promoter activity in PAO579 at both sites (Figure 7A and Figure 7B). The level of promoter activity for both P_{pilA} and P_{algW} fell below the threshold for detection in PAO579rpoN::Tc^R (Figure 7A and Figure 7B). These

results are consistent with previous reports, stating that RpoN drives transcription of *pilA* and *algW* in PAO579. Collectively, our results suggest a pathway where RpoN regulates mucoidy in PAO579 upstream of *pilA*¹⁰⁸, *algW* and *algU*.

Discussion

Generally speaking, there are two types of mucoid isolates found in CF sputum samples: those with mutations mapped within the *mucABCD* cluster (1, 4, 35), and those with undefined mutations mapped outside of the *mucABCD* cluster. While *mucA* mutants are associated with chronic infections, it is not clear what mucoid-related genotypes are present in those early colonizing strains that precede chronic infection. In this study, we used whole genome sequence analysis to identify the unknown positive regulator(s) of alginate production in *P. aeruginosa* strain PAO579 (*muc-23*), an isogenic derivative of PAO1. We identified three tandem point mutations in the *pilA* gene resulting in a premature stop codon. These alterations cause a truncation in the major subunit of type-IVa pilin at amino acid 108. This truncated version of PilA reveals a C-terminal primary amino acid sequence of FTF that functions as a signal to activate alginate overproduction through the proteolytic degradation of MucA. The transcriptional activity at the *algD* and *algU* promoters was increased in PAO579, while inactivation of *algW*, *algU*, *rpoN* and the truncated *pilA* caused a significant decrease in activity at these promoters. The sigma factor RpoN regulated transcription at both the *pilA* and *algW* promoters in PAO579.

Initially we identified a non-synonymous mutation in *algW* of PAO579 (*algW*^{Δ239F}). However, this mutation did not have an impact on AlgW activity (Figure 1). Deletion of *algW* in PAO579 did result in a loss of mucoidy, however expression of *algW* and *algW*^{Δ239F} *in trans* from the *P_{BAD}* promoter did not result in a significant difference in alginate overproduction (Figure 1). The amino acid substitution occurs in a non-conserved region of the L2 loop (5) and this may explain why we did not observe any

significant difference in the amount of alginate produced. Collectively, these results indicate that *algW*^{I239F} is not the positive regulator involved in activating alginate overproduction. However, the requirement of AlgW for mucoidy does implicate the

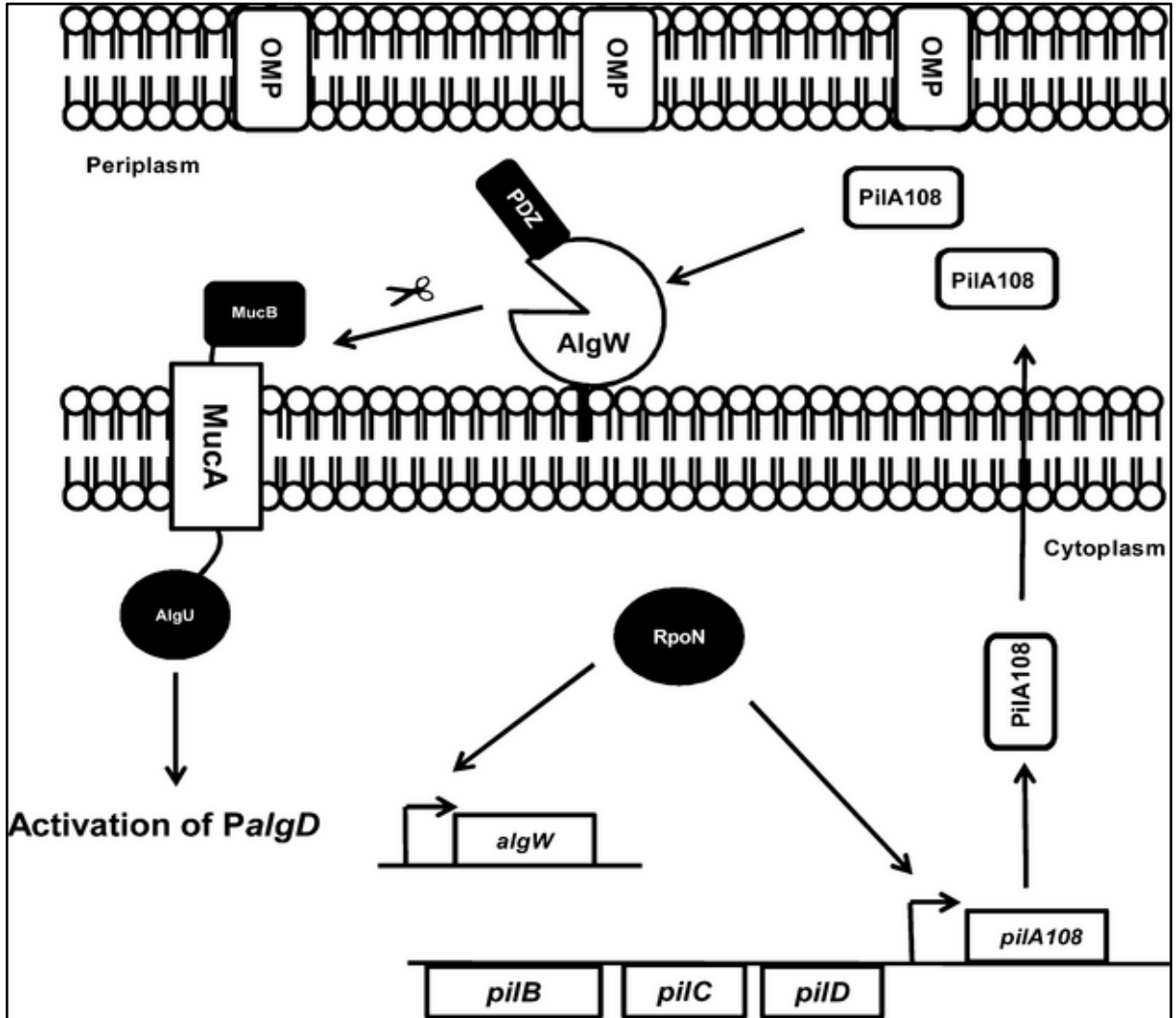


Figure 9. Schematic diagram summarizing the induction of alginate production and mucoid conversion by PiiA108 in *P. aeruginosa* strain PAO579. The sigma factor RpoN is required for transcription of *pilA*¹⁰⁸ and *algW*. PiiA¹⁰⁸ is transported to the periplasm where it activates the periplasmic protease AlgW which proteolytically degrades the anti-sigma factor MucA releasing the sequestered sigma factor AlgU. AlgU drives transcription of the alginate biosynthetic operon via the *algD* promoter.

release of AlgU due to proteolytic degradation of MucA. Western blot analysis of the HA-MucA confirmed an increase in lower molecular weight products in PAO579 as

compared to PAO1, PAO381, PAO579*pilA::aacC1* and PAO579 Δ *algW* (Figure 5), indicating increased MucA degradation in PAO579.

We observed that the deletion of *algU* resulted in a loss of mucoidy in PAO579 (Figure 1). In Boucher *et. al.*, RpoN was shown to be involved in driving transcription at P_{algD} (3). In this same study *algU* was inactivated by an insertion of a tetracycline resistance cassette and observed to not be essential for the mucoid phenotype of PAO579 (3). In our study, since AlgW was required for the mucoid phenotype of PAO579, degradation of MucA, and transcriptional activity at the P_{algD} promoter (Figure 1, Figure 4 and Figure 5), we hypothesized that AlgU was most likely required for the mucoid phenotype of PAO579. We then in-frame deleted *algU* from PAO579 and observed this strain to be non-mucoid (Figure 1). We were also able to complement this mutated strain by expressing *algU* *in trans* and observed a return to the mucoid phenotype (data not shown). The essential difference between our present work and the Boucher *et. al.* study is the complete deletion of *algU* from PAO579 in our study. Although our data argues that *algU* is required, it also confirms that RpoN is required for mucoidy in PAO579 (Figure 6 and 8). However, overexpression of RpoN in PAO579*pilA::aacC1* and PAO579 Δ *algU* did not confer mucoidy. Additionally, we observed that *rpoN* may be regulating alginate production upstream of AlgU through controlling expression of *algW* and *pilA* (Figure 8). Collectively these data suggest a pathway where RpoN acts upstream of *pilA*¹⁰⁸ and *algU* in regulating mucoidy in PAO579, as illustrated in Figure 9. RpoN drives transcription of *algW* and *pilA*¹⁰⁸; PilA108 then activates AlgW to begin proteolytic degradation of MucA. Upon release,

which three tandem point mutations may occur is quite low. Although the C-termini of pilin displays a high diversity, those found in CF isolates tend to cluster together into one phylogenic group (20). Through BLAST searches, we identified 6 CF isolates containing an internal FTF motif (Figure 10). It is known that mucoid mutants are selected for in the CF lung. Our study suggests that mutations can arise in envelope proteins, such as *pilA*, and induce alginate overproduction. Since regulated proteolysis is controlled by the AlgW protease and envelope proteins, we wonder if a treatment strategy targeting these proteins could block alginate overproduction and allow for better clearance of chronic *P. aeruginosa* infections.

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Conflict of Interest

The authors do not have a conflict of interest to declare.

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CHAPTER 4: Identification and characterization of a novel inhibitor of alginate overproduction in *Pseudomonas aeruginosa*.

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Running title: PA1494 inhibits alginate overproduction in *P. aeruginosa*

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Chapter 4 Overview and Rationale

In chapter 3, I determined that a series of single nucleotide polymorphism in the *pilA* gene result in the overproduction of alginate in PAO579. However, inhibiting mucoidy and biofilm formation is of particular importance regarding the prevention of chronic respiratory infections in individuals afflicted with cystic fibrosis.

In this chapter, I change the trajectory of our study to target novel genetic loci that can suppress alginate overproduction in *P. aeruginosa* strains possessing a wild-type MucA. To do so, I used whole-genome complementation coupled with *in vitro* transposon mutagenesis to identify an uncharacterized locus which suppresses mucoidy in the model strain PAO579. I also explore its efficacy and specificity in suppressing mucoidy in a variety of *P. aeruginosa* strains.

Abstract

In this study, we used whole genome complementation of a PAO1 cosmid library, coupled with *in vitro* transposon mutagenesis, to identify a gene locus (PA1494) encoding a novel inhibitor of alginate overproduction in *Pseudomonas aeruginosa* strains possessing a wild-type *mucA*.

Keywords: *Pseudomonas aeruginosa*, *alginate*, *biofilm*, inhibitor, PA1494

Introduction

Cystic fibrosis (CF) is autosomal recessive disorder that afflicts approximately 70,000 individuals throughout the world. CF can have serious effects on the pulmonary, gastrointestinal and cardiovascular organ systems. This is due to the accumulation of mucus at the surface of epithelial cells. In the lungs, the accumulated mucus causes a greater susceptibility to bacterial infection, with the most common pathogen being Gram-negative bacterium *Pseudomonas aeruginosa* (9). This bacterium establishes a chronic infection in individuals afflicted with CF, through the formation of a mucoid biofilm, which is facilitated by the overproduction of an exopolysaccharide called alginate (9).

Alginate overproduction is achieved through increased transcription of the alginate biosynthetic operon at the *algD* promoter (7). Regulation of alginate overproduction primarily involves the alternative sigma factor AlgU (AlgT, σ^{22}) and its cognate anti-sigma factor, MucA (18, 19). Typically, in environmental isolates of *P. aeruginosa*, AlgU is sequestered by MucA to the inner membrane (20, 27). However, with a loss of MucA, AlgU is free to regulate transcription at the *algD* promoter (18, 32). Previous reports have determined the role of AlgW, a DegS-like serine protease, as the first in a series of proteases to degrade MucA (2, 25, 31). Transcription of the alginate operon can also be dependent on the AlgK-AlgX-MucD multiprotein complex (11). Overexpression of MucE (PA4033) induces mucoidy through the regulated proteolysis of MucA (25). Additionally, we found that the truncation of the type IV precursor protein *pilA* can also induce mucoid through the regulated proteolysis of MucA in strain

PAO579 (29). Importantly, *P. aeruginosa* isolates initially colonizing the CF lung are typically nonmucoid, environmental strains.

In this study, we used our knowledge of pathways for overproduction of alginate in the *P. aeruginosa* strain PAO579 to identify additional regulators of alginate synthesis. Preventing the overproduction of alginate (26), and more specifically inhibiting the regulated proteolytic degradation of MucA (4), is an attractive strategy to prevent the establishment of chronic *P. aeruginosa* infections. To investigate this possibility, we employed whole-genome complementation coupled with *in vitro* transposon mutagenesis to identify new genetic loci that can inhibit alginate overproduction in *P. aeruginosa* strains with a wild-type *mucA*.

Materials and Methods

Bacterial strains and growth conditions. All pertinent information regarding bacterial strains and plasmids used in this study are provided in Table 1. *P. aeruginosa* and *E. coli* strains were grown at 37°C in Lennox broth (LB), on LB agar, or *Pseudomonas* Isolation Agar (PIA). When indicated, the media was supplemented with 300 µg/mL carbenicillin, 200 µg/mL gentamycin, 200 µg/mL tetracycline and L-arabinose for *P. aeruginosa* strains, and 100 µg/mL carbenicillin, 15 µg/mL gentamycin, 10 µg/mL tetracycline and 40 µg/mL kanamycin for *E. coli* strains.

MTP complementation and *in vitro* mutagenesis. A PAO1-derived minimal tiling pathway (MTP) genomic cosmid library was conjugated into PAO579 using the helper plasmid pRK2013, and screened for the ability to complement mucoidy. MTP cosmids that complemented mucoidy were isolated using Qiagen Maxi Prep Kit (Qiagen). *In vitro* transposon-mediated mutagenesis of the complementing cosmid was performed using EZ::TN <KAN-2> insertion kit (Epicentre). To construct a mutant library, transposon-inserted cosmids were transformed into *E. coli* DH5α and cultured on LB plates with kanamycin. The mutant library was conjugated en masse into PAO579 and cultured on PIA with tetracycline overnight at 37°C. Cosmids were screened for loss of complementation (*i.e.*; return to mucoidy).

Plasmid construction and complementation. Standard recombinant DNA cloning techniques were used in the construction of all plasmids used in this study (28). Briefly, oligonucleotide primers were designed based on PAO1 sequence information and

synthesized by Eurofin MWG Operon. PCR amplifications were done using EasyStart™ Micro 50 PCR Mix-in-a-Tube (Molecular BioProducts) and *Taq* DNA Polymerase (New England BioLabs) was used for PCR amplification. The pCR®4-TOPO® Vector (Invitrogen, Inc.) was used as an intermediary before ligation into the target vector. Plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen Sciences). All cloning constructs were sequenced to confirm the absence of mutations. Completed plasmids were tri-parentally conjugated into target *P. aeruginosa* strains using *E. coli* containing the helper plasmid pRK2013 (8).

β-galactosidase activity assay. PAO1 or PAO579 carrying the chromosomal integration vector miniCTX- P_{algD} -*lacZ* and either pHERD20T, pHERD20T-*muiA* or pHERD20T-*muiA*ΔN22 were cultured at 37°C on three PIA plates supplemented with 300 μg/mL carbenicillin and 0.1% arabinose. Bacterial cells were harvested, resuspended in PBS and the OD₆₀₀ was recorded. The cells were permeabilized using toluene, and β-galactosidase activity was measured with results calculated and reported in Miller Units (21). One Miller Unit equivalent to $1000 \times (A_{420} / -1.75 \times A_{550} / OD_{600} \text{ mL}^{-1} \text{ min}^{-1})$. The reported values represent an average of three independent experiments with standard deviation. Student's *t* test was performed to determine statistical significance.

Table 1. Bacterial strains and plasmids used in this study.

Strain, Plasmid	Genotype, phenotype, description	Reference
E. coli		
TOP10	DH5α derivative	Invitrogen
P. aeruginosa		
PAO1	<i>algU⁺ mucA⁺</i> ; nonmucoid	P. Phibbs*
PAO579	<i>algU⁺ mucA⁺ muc-23</i> ; mucoid	J. Govan**
PAO1-VE2	<i>algU⁺ mucA⁺</i> , P _{GM⁻} <i>mucE</i> (PA4033); mucoid	(25)
PAO1-VE19	<i>algU⁺ mucA⁺</i> , <i>mucD::aacC1</i> (PA0766); mucoid	(25)
C4700m	<i>algU⁺ mucA⁺</i> ; clinical isolate; mucoid	D. Speert***
C7447m	<i>algU⁺ mucA⁺</i> ; clinical isolate; mucoid	D. Speert***
Plasmids		
pCR4-TOPO	3.9-kb, Ap ^R , Km ^R ; TA cloning vector	Invitrogen
pRK2013	Km ^R , <i>Tra Mob ColE1</i>	(8)
pHERD20T	pUCP20T P _{lac} replaced by 1.3-kb AfIII-EcoRI fragment of <i>araC</i> -P _{BAD} cassette	(23)
pHERD20T- <i>muiA</i>	<i>muiA</i> (PA1494) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>muiA</i> -HA	<i>muiA</i> (PA1494) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD20T- <i>muiA</i> ΔN22	<i>muiA</i> (PA1494) from PAO1 in pHERD20T with a deletion of the N-terminal signal sequence; EcoRI/HindIII	This study
miniCTX- <i>lacZ</i>	Gene delivery system used to fuse target genes to <i>lacZ</i> and integrate onto the chromosome at the CTX phage <i>att</i> site in <i>P. aeruginosa</i> , Tc ^R	(12)
miniCTX-P _{algD} - <i>lacZ</i>	Complete P _{algD} promoter (1,525 bp upstream of ATG) HindIII/BamHI in miniCTX- <i>lacZ</i>	(6)
pEX100T-NotI	<i>Pseudomonas</i> suicide vector with NotI restriction site fuse into SmaI of pEX100T, <i>sacB</i> , <i>oriT</i> , <i>CbR</i>	(6)
pEX100T-Δ <i>muiA</i>	1.0-kb fragment flanking the <i>muiA</i> (PA1494) gene ligated into pEX100T-NotI with in-frame deletion of <i>muiA</i>	(25)

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Cell fractionation and protein analyses. *P. aeruginosa* strain PAO579 containing the pHERD20T-*muiA*-HA was cultured at 37°C on PIA supplemented 300 µg/mL of carbenicillin and 0.1% arabinose. Cells were collected and whole cell lysates were prepared using the ProteaPrep Cell Lysis Kit (Protea Biosciences). Periplasmic proteins were harvested using the Epicentre Periplasting Kit (Epicentre). Outer membrane proteins were collected and isolated using 2% sarkosyl with 2 mM PMSF, sonication and ultracentrifugation as previously published (5). The total protein content for each fraction was quantified using D_c Assay (Bio-Rad). Protein samples (25 µg) were boiled for 10 min in Tricine Sample Buffer (Bio-Rad) and electrophoresed on a 12.5% Tris-Glycine gel (Bio-Rad). Samples were then electro-blotted onto a nitrocellulose transfer membrane (GE Healthcare). The membrane was blocked using 3% skim milk/PBS. Rat monoclonal antibody for hemagglutinin (Roche Diagnostics) was used as primary antibody. Horseradish peroxidase-labeled goat anti-rat IgG was used as secondary antibody. Primary and secondary antibodies were diluted in 3% skim milk/PBS to 1:5000 and 1:10,000, respectively. Western blot results were imaged using ECL Advance Western Blotting Detection Kit (Amersham; GE Healthcare) and UVP BioImaging Systems.

Alginate assay. Alginate was measured using the previously published carbazole reaction (16). Bacterial strains were streaked in triplicate on PIA supplemented with antibiotics and/or L-arabinose when appropriate, and incubated at 37° C for 24 hrs. The bacterial cells were collected and the OD₆₀₀ was recorded. The amount of uronic acid was measured and compared to an alginate standard curve made with D-mannuronic

acid lactone (Sigma-Aldrich) in the range 0 to 100 $\mu\text{g/mL}$. The reported values represent an average of three independent experiments with standard deviation.

Results and Discussion

PA1494 encodes a novel inhibitor of alginate overproduction.

Alginate overproduction in *P. aeruginosa* strain PAO579 (*muc-23*), a derivative of PAO1, is caused by the release of AlgU from MucA by regulated intramembrane proteolysis (29, 30). This regulated intramembrane proteolysis is initiated by the

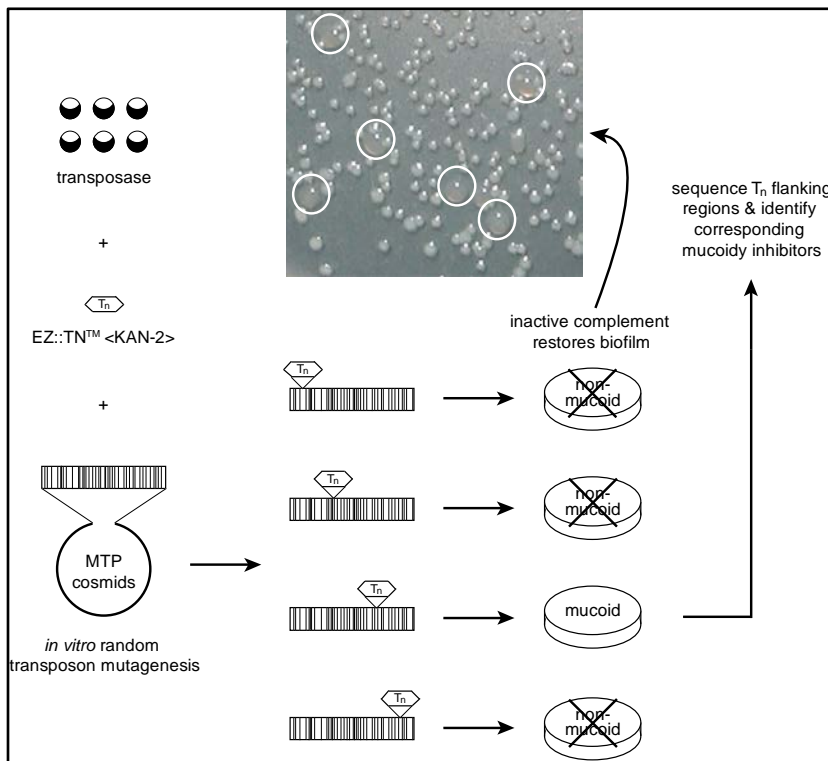


Figure 1. Identification of PA1494 as a novel inhibitor of alginate overproduction. MTP87 cosmid was subjected to *in vitro* transposon mutagenesis to generate random gene knockouts. Shown in the inset are PAO579 (*muc-23*) exconjugants carrying cosmid MTP87 randomly mutagenized with an EZ::TN transposon (Epicentre), selected on a PIA plate supplemented with the appropriate antibiotic, and incubated at 37°C for 48h.

(data not shown). MTP87 covers a region of 22,757 bp from the genome of PAO1 (start: 1,618,021; end: 1,640,777). To identify the exact gene within this cosmid responsible for the multi-copy suppression of alginate overproduction, MTP87 was subjected to random transposon-mediated *in vitro* mutagenesis, and the mutated cosmids were then

activation of the protease AlgW by a truncated type-IV pili (PilA108) (29). To identify novel inhibitors of alginate overproduction in *P. aeruginosa* strains with a wild-type *mucA*, a PAO1-derived, minimal tiling path (MTP) genomic cosmid library (13) was conjugated into PAO579 (10). The cosmid MTP87 completely suppressed alginate overproduction in PAO579

conjugated en masse into PAO579 and screened for alginate overproduction (Figure 1). We observed the presence of alginate overproducing clones indicating a transposon-mediated inactivation of a specific inhibitory gene within cosmid MTP87. PCR and

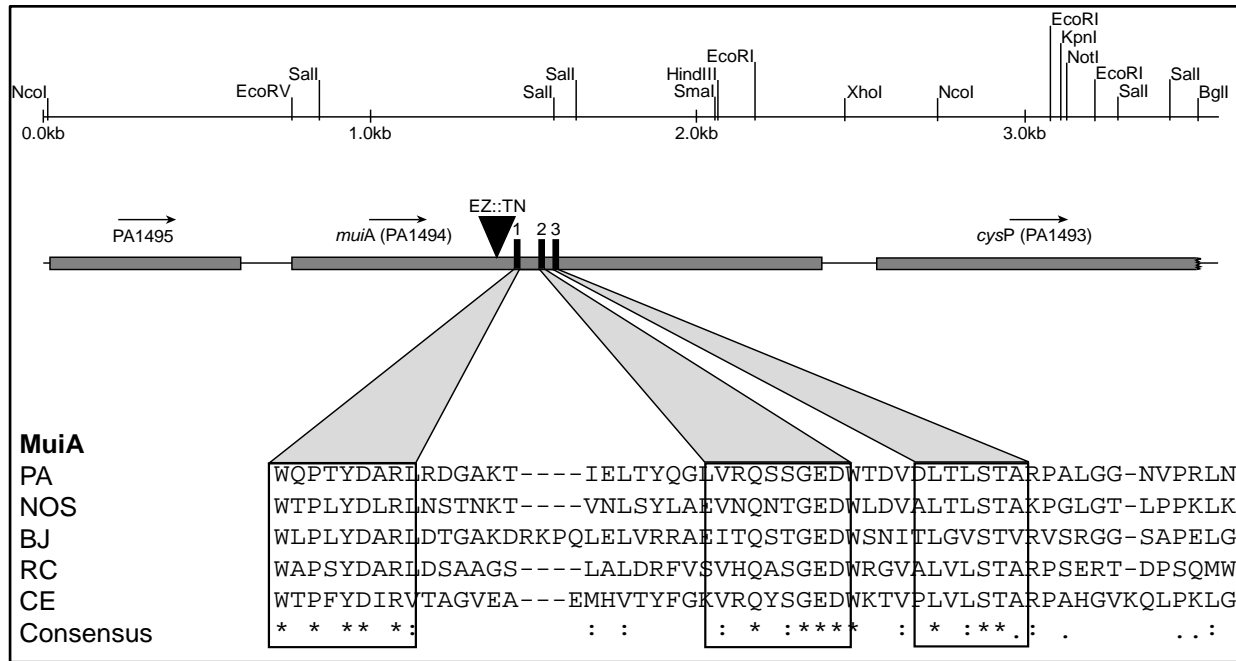


Figure 2. Characterization of PA1494. Restriction map, gene organization and Tn insertion in the *muiA* gene. Homology of MuiA with its orthologs. Shown are the most homologous regions (1, 2 and 3): *R. capsulatus* (RC; ORF1654; 534 aa), *B. japonicum* (BJ; CAC38742; 560 aa), *Nostoc sp.* (NOS; NP_484904; 545 aa), and *C. elegans* (CE; NP_500427; 556 aa). A single Tn insertion occurred before regions 1, 2 and 3.

sequence analysis of the mutagenized MTP87 confirmed a single transposon insertion in open reading frame PA1494. This gene is up-regulated when *P. aeruginosa* is exposed to azithromycin (15, 22) and hydrogen peroxide (3). However, since PA1494 belongs to a class of unclassified/hypothetical genes, and its exact function is unknown, we refer to PA1494 as mucoidy inhibitor gene A, or *muiA*.

The *muiA* gene is predicted to encode a polypeptide of 551 amino acids with a predicted molecular mass of 61 kDa and an isoelectric point (pI) of 5.5. Located immediately downstream is the *E. coli* periplasmic sulfate-binding ortholog gene (*cysP*: PA1493). The *muiA* gene uses GTG as a start codon with a typical type-I signal

sequence encoding 22 amino acids (NH₂-MNRLAASPLLFAGLFASAPLLA-COOH) (17), and previous proteomic analysis detected MuiA in the periplasm of PAO1 (14). Through BLAST analysis, we determined that MuiA is highly conserved amongst other *P. aeruginosa* strains; however no orthologs were identified in *E. coli* species, or other Pseudomonads. MuiA orthologs were found in other organisms including *Rhodobacter capsulatus*, *Bradyrhizobium japonicum*, *Nostoc sp.*, and *Caenorhabditis elegans* (Figure 2).

These orthologs are all of similar size ranging from 530 to 560 amino acids in length, and are classified as conserved

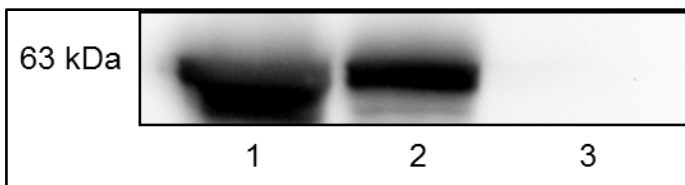


Figure 3. Western blot analysis and localization of MuiA. PAO579 pHERD20T-*muiA*-HA was cultured on PIA supplemented with carbenicillin and 0.1% arabinose. Shown is a representative panels from three independent experiments in which 25 µg total protein for whole cell lysate (Lane 1), periplasmic (Lane 2) and outer membrane (Lane 3) extracts were loaded on a 12.5% Tris-Glycine gel, transferred to a nitrocellulose membrane, and probed with anti-HA antibody.

hypothetical proteins. An internal region of MuiA (232-274aa) displayed 3 highly conserved regions. In addition, the transposon insertion in MTP87 was located 15 bps in front of these conserved domains (Figure 2). Additionally, using cell fractionation and Western blot analysis, we confirmed the results presented in the previous study by Imperi *et al.* (14) by detecting the presence of MuiA in the periplasm (Figure 3).

Expression of *muiA* suppresses alginate overproduction.

In order to confirm whether *muiA* is responsible for suppressing alginate overproduction, we used standard molecular techniques (28) to clone *muiA* into the shuttle vector pHERD20T that contains the P_{BAD} arabinose inducible promoter (23). PAO1 pHERD20T (vector control), PAO579 pHERD20T and PAO579 pHERD20T-*muiA* were cultured on PIA supplemented with carbenicillin and 0.1% arabinose, and the

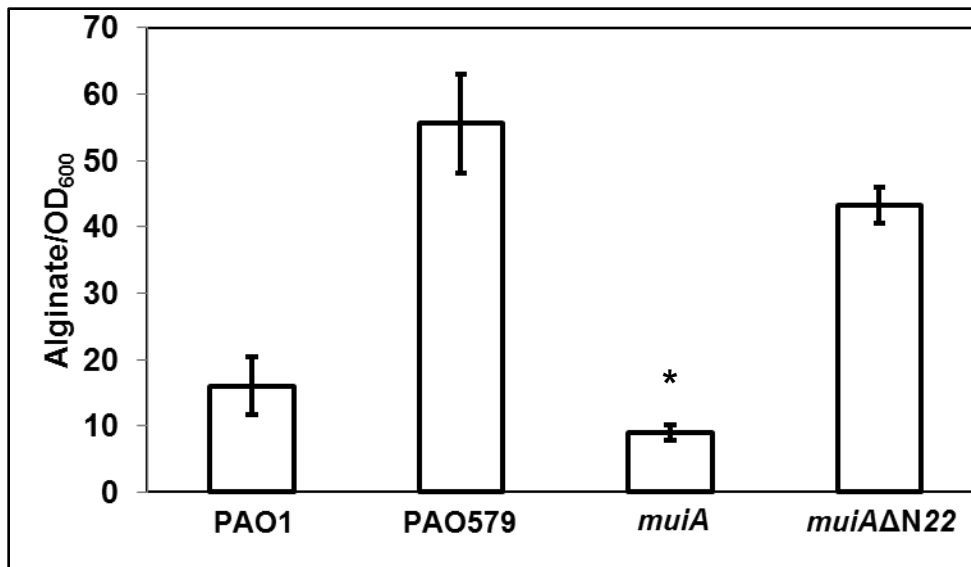


Figure 4. MuiA suppresses alginate overproduction. PAO1 pHERD20T, PAO579 pHERD20T (vector control), PAO579 pHERD20T-*muiA* (wild-type *muiA*) and PAO579 pHERD20T-*muiA*ΔN22 (deletion of N terminal signal sequence) were grown on PIA plates supplemented with carbenicillin and 0.1% arabinose for 24h at 37°C then for 24h at room temperature. The alginate was collected and measured using the standard carbazole assay. The unit of measurement used is μg of alginate/mL/OD₆₀₀, and the values are representative of three independent experiments. Statistical significance was determined using the Student's t-test in comparison with PAO579 (*P<0.05).

amount of alginate was measured using the standard carbazole assay (16). When compared to the PAO1 and the vector control, there was a decrease in alginate overproduction when *muiA* was expressed *in trans* (Figure 4). Additionally, we observed that pHERD20T-*muiA* can suppress mucoidy even in the absence of arabinose on PIA, suggesting that the basal expression from pHERD20T-*muiA* was sufficient for the suppression. Removal of the N-terminal signal sequence (pHERD20T-

*muiA*ΔN22) abrogated MuiA's ability to suppress alginate overproduction in PAO579 (Figure 4). An in-frame deletion of *muiA* in strain PAO1 did not result in alginate overproduction, suggesting that MuiA does not play a central role in alginate regulation (data not shown). Thus, MuiA likely suppresses alginate overproduction after localization to the periplasm, and also functions as a multi-copy suppressor for alginate overproduction in PAO579.

Expression of *muiA* decreases P_{algD} transcriptional activity.

Alginate overproduction in PAO579 has been reported to be due to increased transcriptional activity at the P_{algD} promoter site of the alginate biosynthetic operon (1, 29). To test the effect of expression of *muiA* on P_{algD} activity, we used PAO1 and PAO579 merodiploid strains (generated via miniCTX- P_{algD} -*lacZ*) that carry a chromosomal copy of the *algD* promoter fused with a reporter gene, *lacZ*. Next, we conjugated pHERD20T (vector control) pHERD20T-*muiA* and pHERD20T-*muiA*ΔN22 into the PAO1 or PAO579 miniCTX- P_{algD} -*lacZ*, and cultured them on PIA plates supplemented with carbenicillin, tetracycline and 0.1% arabinose. We measured the transcriptional activity of the P_{algD} promoter using the Miller Assay (21). As expected, the level of transcriptional activity in PAO579 pHERD20T was significantly higher than that in PAO1 (Figure 5). The activity at P_{algD} decreased when pHERD20T-*muiA* was expressed *in trans* in PAO579 (Figure 5). Additionally, expression of pHERD20T-*muiA*ΔN22 did not result in a decrease in P_{algD} activity in PAO579 (Figure 5). Based on these results, we conclude that expression of *muiA* suppresses transcriptional activity at the alginate biosynthetic operon at the *algD* promoter.

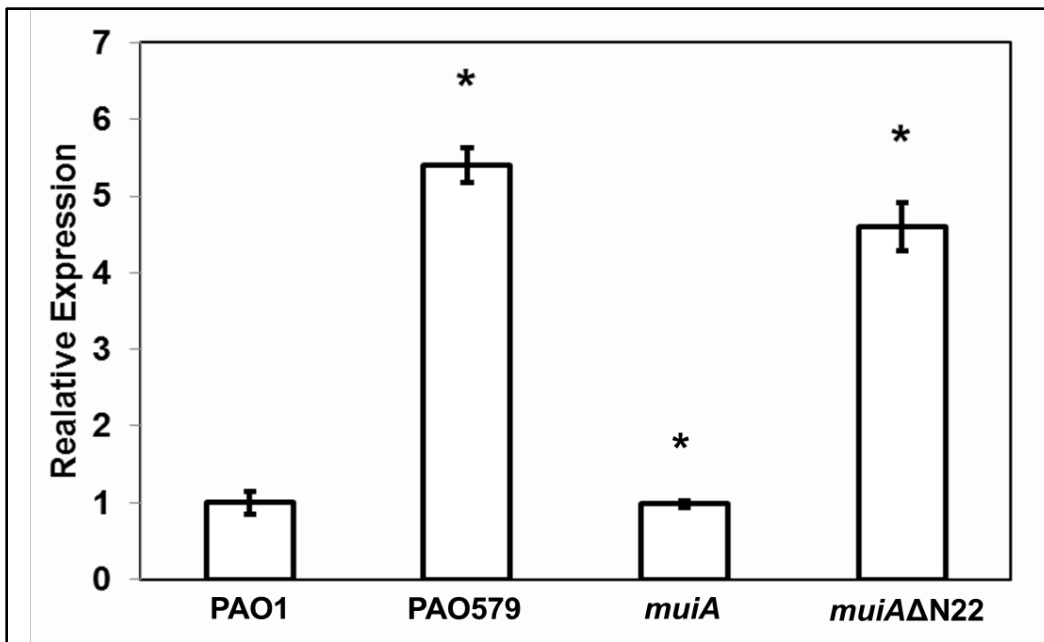


Figure 5. MuiA suppresses transcription activity at the P_{algD} promoter. The β -galactosidase activity of the *algD* promoter was measured using PAO1 and PAO579 miniCTX- P_{algD} -lacZ with pHERD20T, pHERD20T-*muiA* or pHERD20T-*muiA*ΔN22. All strains were incubated at 37° C on PIA plates supplemented with tetracycline, carbenicillin and 0.1% arabinose. The values for the mean and standard deviation Miller Units (One Miller Unit=1000 X $(A_{420}/1.75 \times A_{550} / OD_{600} \text{ mL}^{-1} \text{ min}^{-1})$) are shown as relative expression as compared to PAO1, and are representative of three independent experiments. Statistical significance was determined using the Student's t-test in comparison to PAO579 (* $P < 0.05$).

Expression of *muiA* suppresses alginate overproduction only in strains with a wild-type MucA.

To determine the overall robustness, and also elucidate the possible mechanism by which MuiA suppresses alginate overproduction, we conjugated pHERD20T-*muiA* into a variety of laboratory and clinical strains. Expression of *muiA in trans* suppressed alginate overproduction in PAO1-VE2 (Table 2). PAO1-VE2 is a derivative of PAO1, and overproduces alginate due to activation of AlgW by MucE, a small envelope protein (2, 25). When *muiA* was expression *in trans* it suppressed alginate overproduction in clinical strains possessing a wild-type MucA (C7447m and C4700m), but *muiA* was unable to suppress alginate overproduction in the PAO1-derived, *mucA25* strain, PAO581 (Table 2). PAO581 carries a truncated MucA25 protein which lacks the

transmembrane domain of the wild-type MucA, suggesting that MucA25 is likely localized in the cytoplasm (24). Additionally, expression of *muiA* did not suppress alginate overproduction in the strain PDO300 (Table 2). PDO300 carries a truncated MucA22 protein. Interestingly, expression of *muiA* did not complement mucoidy in the PAO1-VE19 (Table 2). This strain is mucoid due the inactivation of the serine protease MucD (PA0766). The relationship between MuiA and MucD is explored further in Chapter 5. Importantly none of the strains examined in this study, aside from PAO579, have three tandem mutations resulting in a truncation of type IV pili. Collectively, these data suggest that MuiA's ability to suppress alginate overproduction is not unique to PAO579; however it is only effective at suppressing alginate overproduction in strains with a wild-type MucA.

Table 2. MuiA-mediated suppression of alginate overproduction in laboratory and clinical strains			
Strains	Genotype	Vector Control ^a	muiA ^b
Laboratory Strains			
PAO1	<i>mucA</i> ⁺	NM(16.0±4.4)*	NM(6.8±1.3)
PAO579	<i>mucA</i> ⁺ ; <i>muc23</i>	M(56.6±7.4)*	NM(9.0±1.2)*
PAO581	<i>mucA25</i>	M(102.2±12.5)	M(111.6±9.3)
PAO1-VE2	<i>mucA</i> ⁺ ; P _{Gm} - <i>mucE</i>	M(76.3±8.3)	NM(12.2±3.4)
PAO1-VE19	<i>mucA</i> ⁺ ; <i>mucD</i> :: <i>aacC1</i>	M(32.6±6.8)	M(40.1±4.3)
PDO300	PAO1 <i>mucA22</i>	M(81.8±9.5)	M(76.0±9.6)
CF Clinical Strains			
C4700m	<i>mucA</i> ⁺	M(66.7±20.1)	NM(17.2±2.5)
C7447m	<i>mucA</i> ⁺	M(71.8±3.3)	NM(18.2±1.8)

All strains were conjugated with pHERD20Ta or pHERD20T-muiAb, using *E. coli* pRK2013 and cultured on PIA supplemented with 300 µg/mL of carbenicillin and 0.1% arabinose and incubated at 37°C for 24h, then at room temperature for 24h. The alginate measurements for three independent experiments are represented as (Mean±Standard Deviation). *-as presented in Figure 4.

Summary

In summary, we coupled whole genome complementation of a PAO1 cosmid library and *in vitro* transposon mutagenesis to identify a genetic loci, PA1494 (*muiA*), as a novel inhibitor of alginate overproduction in *P. aeruginosa* strains with a wild-type

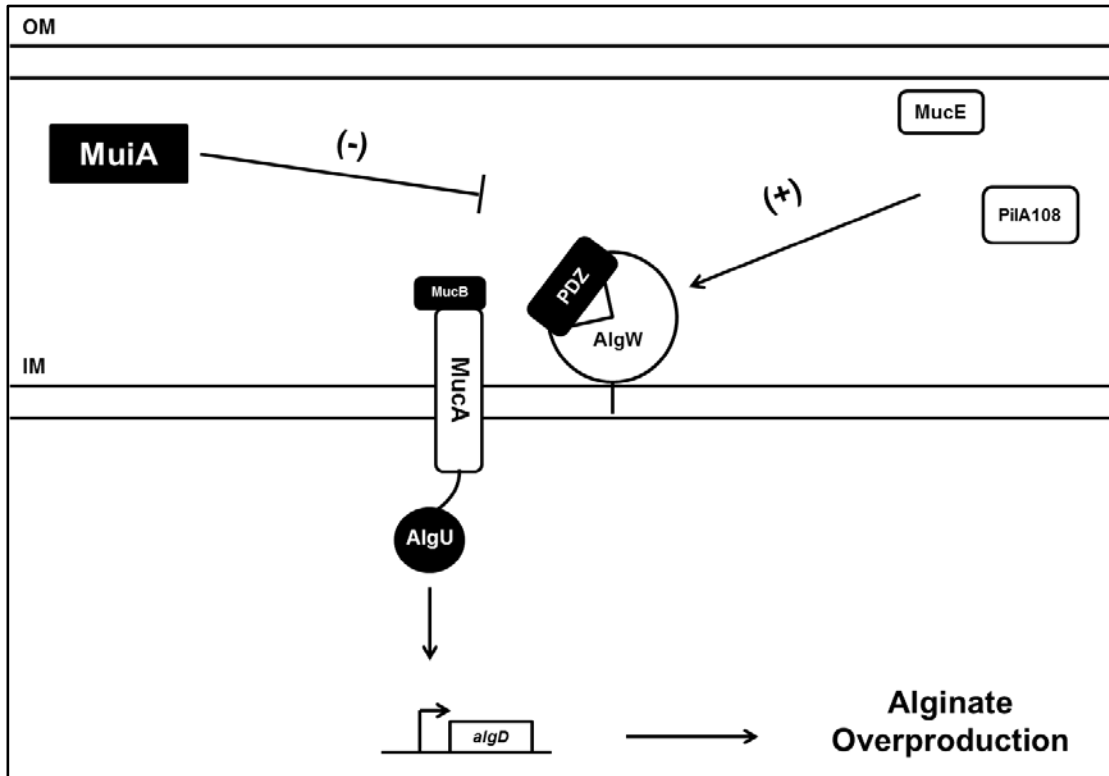


Figure 6. Schematic diagram illustrating the suppression of alginate overproduction by MuiA. The sigma factor AlgU drives transcription of the alginate biosynthetic operon at the P_{algD} promoter following regulated intramembrane proteolysis of MucA in *P. aeruginosa* strains PAO579 and PAO1-VE2 (2, 25, 29). Expression of MuiA suppresses alginate overproduction in these strains following localization to the periplasm.

mucA. Also, expression of *muiA* *in trans* resulted in a decrease in alginate production, as well as transcriptional activity at the P_{algD} promoter. As outlined in Figure 6, we propose that expression of *muiA* suppresses alginate overproduction via the periplasm in *P. aeruginosa* strains PAO579, PAO1-VE2, and clinical strains with a wild-type MucA.

Nucleotide sequence accession numbers. The GenBank accession number for *P. aeruginosa* strain PAO579 mucoidy inhibitor A (*muiA*) gene is AF226874.

Competing interests

HDY is the co-founder of Progenesis Technologies, LLC.

Authors' Contributions

T.R.W. designed and performed experiments, analyzed data and wrote the manuscript.

Y.Y. and H.D.Y. analyzed data and helped revise the manuscript.

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CHAPTER 5: Expression of PA1494 (*muiA*) affects MucD and suppresses proteolytic activity in periplasm of *Pseudomonas aeruginosa* strain PAO579

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Chapter 5 Overview and Rationale

In chapter 4, I discovered that expression of gene locus PA1494 (*muiA*) can only inhibit mucoidy in *P. aeruginosa* strains possessing a wild-type MucA. Additionally, I observed that MuiA required the periplasmic chaperone protease MucD to suppress mucoidy. Based on these results, I wanted to determine the molecular mechanism by which MuiA suppresses mucoidy, as well as MucD's role in this regulation.

In this chapter, I used Western blot and protease analyses to examine the relationship between MuiA and MucD. Additionally, I compared the proteomic profiles of two different mucoid strains expressing MuiA *in trans* to identify potential effector proteins.

Abstract

In Chapter 4, we reported that the expression of the gene locus PA1494 (*muiA*) did not suppress alginate overproduction in the PAO1-VE19, a mucoid mutant of PAO1 containing a transposon-inactivated *mucD*. The protein encoded by *mucD* is a periplasmic serine protease classified as a negative regulator of AlgU and alginate production. Inactivation of MucD bypasses the requirement of AlgW for alginate overproduction. In this chapter, I present my work that further explored the relationship between the newly identified alginate regulator, MuiA, and the periplasmic protease MucD. Western blot analysis of MucD showed an increase in low molecular weight products in PAO579 when compared to PAO1, and expression of *muiA in trans* reduced the amount of these low molecular weight products to the level found in PAO1. Additionally, we observed a low amount of low molecular weight products of MucD in PAO579 Δ *algU*. Zymogram protease analysis showed an increase in periplasmic proteolytic activity in PAO579 as compared to PAO1 at the molecular weight of ~50 kDa. Moreover, we observed a decrease in periplasmic proteolytic activity at a molecular weight of ~50 kDa when *muiA* was expressed *in trans*. Using iTRAQ analysis, we observed the down-regulation of three proteins common between PAO579 and PAO1-VE2 when *muiA* was expressed *in trans*. These data indicate a relationship between the expression of MuiA, MucD and periplasmic protease activity, as well as identifies three potential downstream effectors of MuiA: elongation factor-Tu (PA4277), PasP (PA0423) and trigger factor (*tig*, PA1800).

Introduction

The overproduction of alginate, or mucoidy, is a virulence factor commonly associated with the development of a biofilm, and the establishment of a chronic *P. aeruginosa* infection in individuals with cystic fibrosis (9). There is considerable research identifying many of the key proteins involved in the regulation of alginate production. Most notable are the proteins AlgU, AlgW, and MucA. The production of alginate is primarily controlled by the sigma factor known as AlgU, whose activity is modulated by its cognate anti-sigma factor MucA (15, 16, 20). AlgW is a periplasmic protease localized in the inner leaflet of the cytoplasmic membrane, and acts on the C-terminus of MucA. The accumulation of periplasmic or outer membrane proteins, as well as environmental stressors, can activate AlgW to initiate proteolytic cleavage of MucA, thereby releasing AlgU to up-regulate the expression of the alginate biosynthetic operon (2, 19, 23). In Chapters 3 and 4, we presented evidence that the accumulation of type-IV pili induces mucoidy through an AlgW-dependent pathway in the model strain PAO579 (22). Additionally, expression of the gene locus PA1494, referred to as *muiA*, suppressed mucoidy in laboratory and clinical strains of *P. aeruginosa* possessing a wild-type MucA.

The suppression of mucoidy by the expression of *muiA in trans* required the periplasmic serine protease MucD (Chapter 4, Table 2). MucD is a homolog of the periplasmic serine protease DegP found in *E. coli*, and negatively regulates alginate production (1). Similar to the role of DegP in *E. coli*, expression of MucD can remove misfolded or accumulated periplasmic proteins that may activate alginate production, such as the small envelope protein MucE (19). Boucher *et al.* and Qiu *et al.* also

observed that inactivation of MucD in PAO1 resulted in a conversion to mucoidy (1, 19). Interestingly, inactivation of MucD resulted in the degradation of MucA independent of the periplasmic protease AlgW, and is dependent on the site-2 protease MucP (6). The cytoplasmic chaperone protease complex ClpXP, in conjunction with Trigger Factor (*tig*), is required for the induction of mucoidy by degrading the cytoplasmic portion of MucA (18).

In this study, we used Western blot of analysis to explore the relationship between expression of MuiA and MucD. Expression of *muiA* from arabinose-inducible vector pHERD20T in PAO579 caused a decrease in the amount of lower molecular weight products of MucD. Similarly, a decrease in the amount of lower molecular weight products of MucD was observed in PAO579 Δ *algU*. Based on these observations, we examined the proteolytic activity in the periplasm of PAO579 using zymogram protease gel analysis. We observed an increase in protease activity in PAO579 at a molecular weight of 50 kDa in PAO579 compared to PAO1. Expression of *muiA in trans* resulted in a decrease in 50 kDa proteolytic activity to below detectable levels. Additionally, a comparative proteomic analysis of PAO579 and PAO1-VE2 with and without expression of *muiA in trans* identified EF-Tu (PA4277), Trigger Factor (PA1800) and the extracellular protease PasP (PA0423) as potential effectors of MuiA.

Materials and Methods

Bacterial strains and growth conditions. Information regarding the bacterial strains and plasmids used in this study are provided in Table 1. *P. aeruginosa* strains were grown at 37°C on *Pseudomonas* Isolation Agar (PIA). When indicated, the media was supplemented with 300 µg/mL carbenicillin and 0.1% L-arabinose.

Western blot analysis of MucD. Bacterial strains were grown overnight at 37°C on PIA plates supplemented with carbenicillin and L-arabinose. Cells were harvested and whole cell lysates were prepared using the ProteaPrep Cell Lysis Kit (Protea Biosciences), and the total protein content was quantified using D_c Assay (Bio-Rad). Protein samples (50 µg) were boiled for 10 min in Tris-Glycine Sample Buffer (Bio-Rad) and electrophoresed on a 12.5% Tris-Glycine polyacrylamide gel. Samples were then transferred to a nitrocellulose membrane and blocked using 3% skim milk/PBS. Anti-MucD monoclonal antibody was used as the primary antibody, and horseradish peroxidase-labelled goat anti-mouse IgG was used as the secondary antibody. Primary and secondary antibodies were diluted in 3% skim milk/PBS to 1:10,000 and 1:10,000, respectively. Western blot results were imaged using ECL Advance Western Blotting Detection Kit (Amersham; GE Healthcare) and UVP BioImaging Systems. Blots were stripped using 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol for 10 minutes at 40°C, and re-probed using anti-RNAPα and horseradish peroxidase-labelled goat anti-mouse IgG.

Table 1. Bacterial strains and plasmids used in this study.

Strain, Plasmid	Genotype, phenotype, description	Reference
E. coli		
TOP10	DH5 α derivative	Invitrogen
P. aeruginosa		
PAO1	<i>algU</i> ⁺ <i>mucA</i> ⁺ ; nonmucoid	P. Phibbs*
PAO579	<i>algU</i> ⁺ <i>mucA</i> ⁺ <i>pilA108(muc23)</i> ; mucoid	J. Govan**
PAO1-VE2	<i>algU</i> ⁺ <i>mucA</i> ⁺ , P _{GM} ⁻ <i>mucE</i> (PA4033); mucoid	(19)
PAO1-VE19	<i>algU</i> ⁺ <i>mucA</i> ⁺ , <i>mucD::aacC1</i> (PA0766); mucoid	(19)
Plasmids		
pCR4-TOPO	3.9-kb, Ap ^R , Km ^R ; TA cloning vector	Invitrogen
pRK2013	Km ^R , <i>Tra Mob ColE1</i>	(7)
pHERD20T	pUCP20T P _{lac} replaced by 1.3-kb AflIII-EcoRI fragment of <i>araC</i> -P _{BAD} cassette	(17)
pHERD26T	pUCP26T P _{lac} replaced by 1.3-kb AflIII-EcoRI fragment of <i>araC</i> -P _{BAD} cassette	(17)
pHERD20T- <i>muiA</i>	<i>muiA</i> (PA1494) from PAO1 in pHERD20T; EcoRI/HindIII	This study
pHERD26T- <i>muiA</i>	<i>muiA</i> (PA1494) from PAO1 in pHERD26T; EcoRI/HindIII	This study

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Protein isolation and zymogram protease analysis. *P. aeruginosa* strains PAO1 pHERD20T, PAO579 pHERD20T, PAO579 Δ *algU* and PAO579 pHERD20T-*muiA* were cultured at 37°C on PIA plates supplemented 300 μ g/mL of carbenicillin and 0.1% arabinose. Cells were collected and the periplasmic proteins were harvested using the Epicentre Periplasting Kit (Epicentre). Electrophoresis was performed as previously described, but with 12.5% polyacrylamide gel containing zymogen and casein (Bio-Rad). Following electrophoresis, gels were incubated in renaturing solution (2.5% Triton X-100) for 30 minutes at room temperature, then at 37°C for overnight in development solution (50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, pH 7.5). Finally, gels were stained with Coomassie Blue and then destained. Completed gels were imaged using an UVP BioImaging System.

iTRAQ MALDI-TOF/TOF proteome analysis. The comparative isobaric tags and relative and absolute quantitation (iTRAQ) analyses of PAO579:PAO579+*muiA* and PAO1-VE2:PAO1-VE2+*muiA* were performed as previously described (5). Briefly, *P. aeruginosa* strains PAO579 pHERD26T, PAO579 pHERD26T-*muiA*, PAO1-VE2 pHERD26T and PAO1-VE2 pHERD26T-*muiA* were streaked on PIA plates supplemented with tetracycline and 0.1% L-arabinose and incubated overnight at 37°C. Cells were harvested and whole cell lysates were isolated using the ProteaPrep Cell Lysis Kit (Protea Biosciences) and total protein content was quantified using the D_c Assay (Bio-Rad). Samples were labeled with isobaric tags for relative and absolute quantification, and were spotted using an ABI Tempo liquid chromatography-matrix assisted laser desorption ionization (LC-MALDI) instrument with a Merck Chromolith

CapRod monolith column. Samples were then further analyzed using an ABI 4800 MALDI-time of flight (TOF/TOF) using ABI ProteinPilot and Paragon software programs. These data are reported as an average ratio either greater or less than 1, indicating up- or down-regulation, respectively. The number of peptides identified with at least 95% confidence level is also represented, as well as a P-value indicating the certainty that the average ratio differs from 1. The smaller the P-value, the more likely any differential expression is significant.

Results and Discussion

MuiA suppresses the accumulation of low molecular weight products of MucD.

Previously, we found that expression of MuiA failed to suppress mucoidy in the *mucD* mutant, indicating a possible relationship between MuiA and the periplasmic chaperone/protease MucD. To explore this relationship further, we PCR amplified *muiA* and fused it into the P_{BAD}-arabinose-inducible shuttle vector pHERD20T (pHERD20T-*muiA*) (17). The completed pHERD20T-*muiA* construct was then conjugated into PAO579 and the recipient bacteria cultured on PIA plates supplemented with 300 µg/mL carbenicillin and 0.1% L-arabinose, and incubated for 24 hrs at 37°C. Similarly, we conjugated pHERD20T into PAO1, PAO579 and PAO579Δ*algU*, to use as a vector control. Western blot analysis probing for MucD showed a distinct banding pattern

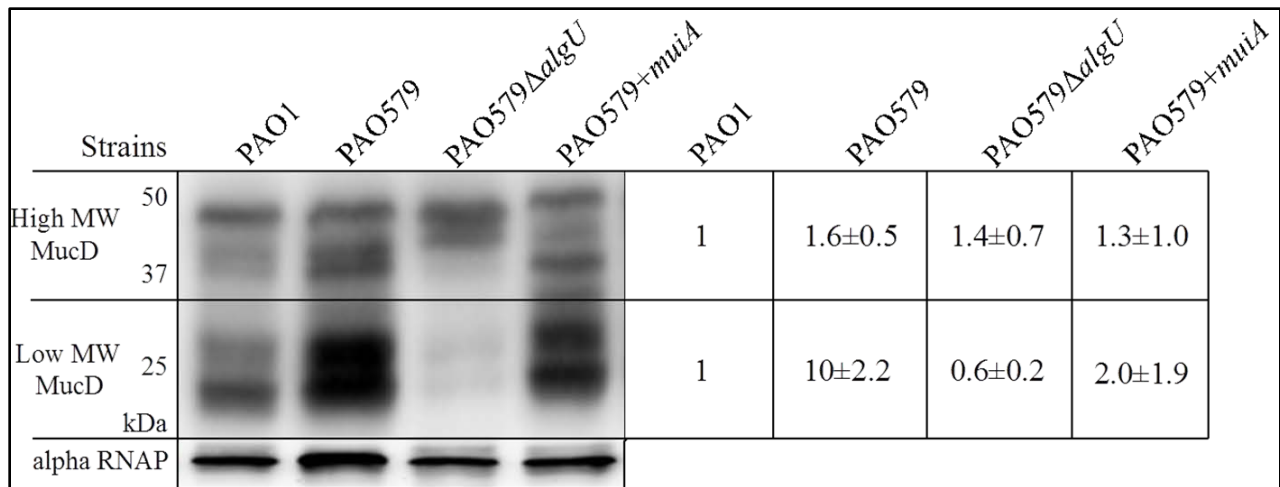


Figure 1. Western blot analysis of MucD. Shown is a representative panels from three independent experiments in which 50 µg of whole cell lysate was loaded on a 12.5% Tris-Glycine polyacrylamide gel, electro-transferred, and probed with anti-MucD monoclonal antibody.

consistent amongst all test strains (Figure 1). In PAO1 pHERD20T, we observed full length MucD at ~50 kDa, and subsequent bands at ~37 kDa, ~30 kDa and ~20 kDa (Figure 1). These bands observed between ~50 to ~37 kDa are identified as high molecular weight (High MW), while banding between molecular weights ~30 and ~20

kDa are identified as low molecular weight (Low MW). Interestingly, we observed an accumulation of Low MW products in PAO579. Previously, it has been shown that the MucD homologue in *E. coli*, DegP, is auto-catalytic (12). Additionally, expression of *mucD* is operonic (*algUmucABCD*), and the regulation occurs at a series of promoters, two of which are AlgU-dependent (20). We previously showed a significant increase in transcriptional activity at the AlgU-dependent promoters in PAO579 (22). Therefore, the accumulation of Low MW products could be due to a combination of increased transcription of the *algUmucABCD* operon along with MucD's ability for auto-proteolytic activity. Supporting this hypothesis, we observed a decrease in the Low MW products in the PAO579 Δ *algU* (Figure 1). *MucD* has been reported to have an independent promoter in the region corresponding to *mucC*, although the sigma factor that drives this promoter remains unknown (24). In the PAO579 Δ *algU* strain, the amount of MucD is reduced when AlgU is not present, suggesting that AlgU is responsible for the production of MucD. We also observed that expression of MuiA decreased the accumulation of Low MW products, again possibly a result of decreased transcriptional activity at the AlgU-dependent promoter sites of *algUmucABCD* operon.

MuiA suppresses proteolytic activity at ~50 kDa.

Since MuiA expression suppresses the function of MucD, we wanted to determine if this activity corresponded to a decrease in periplasmic proteolytic activity. Previously, we discovered that the regulation of mucoidy in PAO579 requires the systematic degradation of MucA, initiated by the periplasmic protease AlgW, possibly activated by a truncated type IV pilin (22). Since the expression of MuiA only suppresses alginate overproduction in *P. aeruginosa* strains that utilize the degradation

of the wild-type MucA by AlgW, we would expect to see a decrease in proteolytic activity for AlgW. Therefore, expression of MuiA should result in a decrease in proteolytic activity in the periplasm of PAO579. In order to test this hypothesis, we extracted the total protein from the periplasms of PAO1 pHERD20T, PAO579 pHERD20T,

PAO579 Δ *algW* and

PAO579 pHERD20T-*muiA*.

Equal amounts of protein

were electrophoretically

separated on 12.5% poly-

acrylamide gels containing

the common proteolytic

substrates zymogen and

casein. Interestingly, we

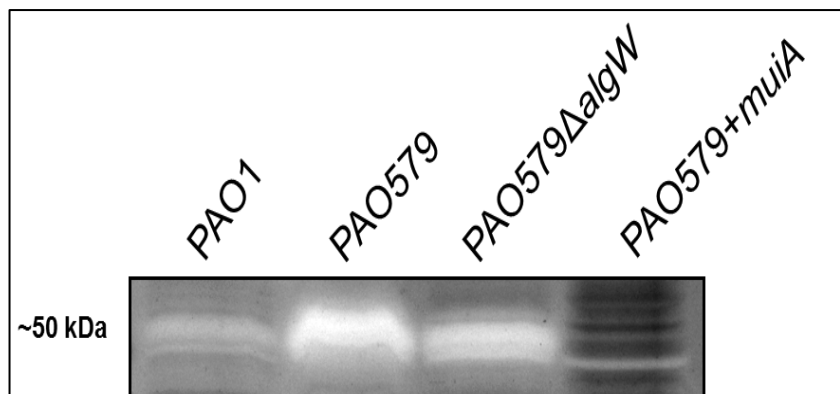


Figure 2. Zymogram analysis of protease activity. Analysis of protease activity of PAO1 pHERD20T, PAO579 pHERD20T (vector control), PAO579 Δ *algW* pHERD20T and PAO579 pHERD20T-*muiA* in which 25 μ g of total protein extracted from the periplasm was loaded on a 12.5% polyacrylamide gel containing zymogen and casein, renatured, and stained with R250 SuperBlue Coomassie Stain.

did not detect any proteolytic activity at ~41 kDa, the predicted molecular weight of

AlgW, in any of our test strains (data not shown). This may be due, in part, to low

amounts of AlgW found within the cell. However, we did detect an increase in

proteolytic activity for a ~50 kDa protein (Figure 2) in PAO579 as compared to PAO1.

Additionally, we observed a decreased proteolytic activity of the ~50 kDa protein in

PAO579 Δ *algW* (Figure 2). As illustrated in Figure 1, we observed an increase in

expression and activity of the 50 kDa periplasmic protein MucD in PAO579 as

compared to PAO1. The increase in MucD activity may correlate with the increased

proteolytic activity observed at ~50 kDa (Figure 2), however further analysis will be

required to identify the specific protease responsible for this activity. Interestingly, we

also observed a complete loss of proteolytic activity at ~50 kDa when *muiA* is expressed *in trans* (Figure 2). Collectively, these data suggest the expression of *muiA in trans* causes a decrease in proteolytic activity of the protein located at 50 kDa.

Comparative iTRAQ analyses of PAO1-VE2 and PAO579.

To further explore the effect of MuiA on protein profiles of mucoid *P. aeruginosa* strains with a wild-type MucA, we used the iTRAQ technology to performed comparative proteomic analysis of PAO1-VE2 and PAO579 following expression of *muiA in trans*. Both PAO1-VE2 and PAO579 are mucoid due to the increased degradation of the wild type MucA by activated AlgW. Both can be suppressed by *muiA in trans*. Simply pHERD26T (vector control) and pHERD26T-*muiA* plasmids were conjugated into PAO1-VE2 and PAO579 and cultured on PIA plates supplemented with 200 µg/mL tetracycline, and expression of *muiA* was induced by the addition of 0.1% arabinose. Cultures were incubated at 37°C for 24 hrs, cells were collected, and total protein was extracted. From the proteomic analysis of PAO1-VE2 pHERD26T compared to PAO1-VE2 pHERD26T-*muiA*, we identified 211 distinct peptides, at a 95% confidence value, that correspond to 35 proteins (Table 2). As expected, the most detected protein was MuiA (PA1494), due to its overexpression from the multi-copy plasmid, pHERD26T-*muiA*. Interestingly, we observed an overall down-regulation in most proteins, with the exception of increases in the outer membrane porin precursor OprO, the conserved hypothetical protein at gene locus PA4739, and the alginate regulatory protein, AlgP (Table 2).

Comparative analysis of PAO579 pHERD26T and PAO579 pHERD26T-*muiA* identified 95 unique peptides, at a 95% confidence value, that correspond to 12 proteins

(Table 3). Similar to the analysis of PAO1-VE2 pHERD26T vs. PAO1-VE2 pHERD26T-*muiA*, the most detected protein was MuiA (average ratio of 6.09). Overall, we observed a down-regulation in the remaining proteins. However, only 3 of the remaining 10 proteins detected in the comparative analysis of PAO579 pHERD26T vs. PAO579 pHERD26T-*muiA*, were also detected in the analysis of PAO1-VE2 pHERD26T vs. PAO1-VE2 pHERD26T-*muiA*: PA4277 (Elongation Factor-Tu), PA0423 (PasP), PA1800 (*tig*, Trigger Factor).

Translational Elongation Factor-Tu binds to amino-acylated tRNA, following GTP-mediated activation, and assists in transporting it to the A site of the ribosome. After the dephosphorylation of GTP to GDP, EF-Tu is removed from the tRNA and repeats the cycle. EF-Tu is encoded in duplicate on the gene *tufA* and *tufB*, and is the most abundant protein found within the bacterial cell (11, 13). Typically, the amount of EF-Tu found within the bacterial cell is equimolar with that of tRNA (8, 13). We observed a 1.88- and 1.27-fold down-regulation of EF-Tu in PAO1-VE2 and PAO579 when *muiA* was expressed *in trans* (Table 2 and Table 3). This may be due to the decreased translation of all the biosynthetic operons including the alginate biosynthetic operon, although this remains to be determined.

Additionally, we observed a down-regulation in PA1800 in both PAO1-VE2 and PAO579 when *muiA* was expressed *in trans*. The gene loci *tig* (PA1800), also known as Trigger Factor, encodes for a 48.6 kDa cytoplasmic chaperone involved in translocation and prevention of improper molecular interactions with nascent polypeptides (4, 21). In 2008, Qiu *et al.* reported that *tig*, as well as the genes encoding the cytoplasmic chaperone/ serine protease complex ClpXP, were required for mucoidy

in the truncated *mucA* strain PAO581 (18). ClpXP was also reported by Qiu *et al* to be responsible for the degradation of the cytoplasmic portion of a truncated MucA (MucA-25), and was also required for mucoidy in *P aeruginosa* strains possessing a wild-type MucA (18). Thus, the down-regulation of Trigger Factor could be in response to the overall decrease in the total activity of the proteolytic cascade responsible for the stabilization of cytoplasmic MucA resulting the loss of mucoidy.

Induction of mucoidy in PAO1-VE2 and PAO579 requires AlgW to initiate the regulated intramembrane proteolysis of MucA. However, overproduction of alginate in *mucD* mutant (PAO1-VE19) requires the site-2 protease MucP, but not AlgW (6). Therefore, through the genetic analysis of mucoidy, we found that there are two types of signals that can activate protease-mediated alginate overproduction: those that activate AlgW such as *mucE*, and those that activate MucP through the loss of MucD. Our data in chapter 4 shows that the expression of MuiA is unable to suppress mucoidy in the truncated MucA strain PAO581, as well as the *mucD* mutant PAO1-VE19. As reported in chapter 4, suppression of mucoidy by MuiA requires an intact N-terminal signal sequence for proper translocation to the periplasm. These data suggest that MuiA is only capable of suppressing mucoidy in the AlgW-dependent pathway.

Lastly, we observed a down-regulation in PAO423, or PasP, PAO1-VE2 and PAO579 when *muiA* was expressed *in trans*. PasP is 20.8 kDa protein that possesses a type I export signal and is secreted to the extracellular matrix (3, 10). Marquart *et al.* identified PasP to be an extracellular protease that causes erosion of the corneal epithelial tissue (14). Zymogram proteolytic analysis of purified recombinant PasP showed distinct protease activity at 50 kDa (14). This may explain the decrease in

proteolytic activity observed at 50 kDa when MuiA is expressed *in trans* (Figure 2). Stated more simply, expression of MuiA down-regulates PasP possibly resulting in a decrease in proteolytic activity at 50 kDa. However, identification of PasP as the 50 kDa protease seen in our experiments remains to be done.

Summary

In this chapter, we explored the possible mechanism by which MuiA suppresses mucoidy in *P. aeruginosa* strains with a wild-type MucA. We observed that expression of MuiA caused a decrease in the accumulation of low molecular weight protein products in the chaperone/protease MucD. We also observed that expression of MuiA cause a decrease in proteolytic activity at ~50 kDa. Finally, comparative iTRAQ analyses of PAO1-VE2 and PAO579 identified three shared downstream effectors of MuiA.

Table 2. iTRAQ proteomic analysis of *muiA* expression in PAO1-VE2.

Gene Locus	Name and Functional Description^a	Localization^b	Average Ratio^c	Peptides (95%)	P-Value
PA1494	Conserved hypothetical protein, MuiA	P	5.50	43	2.19E-10
PA4277	Elongation Factor-Tu	C	0.53	28	2.34E-05
PA3280	Pyrophosphate-specific outer membrane porin OprO precursor	O	1.57	13	0.024
PA1342	Probable binding protein component of ABC transporter	P	0.66	12	0.049
PA5556	ATP synthase alpha chain	C	0.70	11	0.037
PA4739	Conserved hypothetical protein	P	1.84	14	0.032
PA0423	PasP	E	0.72	11	0.004
PA3529	Probable peroxidase	C	0.77	10	0.039
PA2300	Chitinase	E	0.74	10	0.019
PA4236	Catalase	P	0.68	9	0.001
PA4266	Elongation factor G	P	0.64	6	0.013
PA4922	Azurin precursor	C	0.57	8	0.016
PA3162	30S ribosomal protein S1	C	0.44	5	0.013
PA1589	Succinyl-CoA synthetase alpha chain	C	0.56	6	0.010
PA3309	Conserved hypothetical protein	C	0.42	6	0.004
PA4431	Probable iron-sulfur protein	C	0.51	4	0.003
PA5489	Thiol:disulfide interchange protein DsbA	P	0.67	4	0.025
PA3692	Probable outer membrane protein precursor, LptF	O	0.35	4	0.030
PA4495	Hypothetical protein	P or O	0.59	3	0.005

PA4453	Conserved hypothetical protein	P or O	0.64	3	0.047
PA4935	30S ribosomal protein S6	C	0.41	3	0.025
PA0548	Transketolase DsbB	C	0.55	3	0.027
PA5046	Malic enzyme	C	0.50	4	0.026
PA2743	Translation initiation factor IF-3	C	0.74	2	0.019
PA5173	Carbamate kinase	C	0.47	2	0.030
PA0865	4-hydroxyphenylpyruvate dioxygenase	C	0.49	2	0.018
PA1579	Hypothetical protein	P or O	0.63	2	0.032
PA1800	<i>tig</i>, Trigger factor	C	0.39	2	0.017
PA2001	Acetyl-CoA	C	0.64	1	0.037
PA5253	Alginate regulatory protein, AlgP	C	1.78	5	0.018
PA5161	dTDP-D-glucose 4,6-dehydratase	C	0.58	1	0.026
PA3480	Probable deoxycytidine triphosphate deaminase	C	0.52	1	0.019

a, PseudoCAP Function Class (www.pseudomonas.com); b, C=cytoplasm; E=extracellular, O=outer membrane, P=periplasm, U=unknown; c, >1=up-regulated, <1=down-regulated

Table 3. iTRAQ proteomic analysis of *muiA* expression in PAO579.

Gene Locus	Name and Functional Description ^a	Localization ^b	Average Ratio ^c	Peptides (95%)	P-Value
PA1494	Conserved hypothetical protein, MuiA	P	6.09	26	1.65E-14
PA4277	Elongation Factor-Tu	C	1.27	24	2.77E-06
PA4385	GroEL protein	C	2.24	13	1.48E-07
PA1092	Flagellin type B	E	1.47	10	0.0076
PA0139	Alkyl hydroperoxide reductase subunit C	C	2.04	5	0.040
PA0423	PasP	E	0.50	4	0.029
PA3250	Hypothetical protein	C	1.39	4	0.011
PA4386	GroES protein	C or P	0.49	3	0.025
PA1800	<i>tig</i>, Trigger factor	C	0.83	3	0.0039
PA5554	ATP synthase beta chain	C	0.80	2	0.031
PA4847	Biotin carboxyl carrier protein	C	0.70	2	0.033
PA3480	DNA-directed RNA polymerase alpha chain	C	0.72	1	0.031

a, PseudoCAP Function Class (www.pseudomonas.com); b, C=cytoplasm; E=extracellular, O=outer membrane, P=periplasm, U=unknown; c, >1=up-regulated, <1=down-regulate

CHAPTER 5 REFERENCES

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CHAPTER 6: General Discussion and Conclusions

Pseudomonas aeruginosa is an opportunistic pathogen that is primarily associated with severe respiratory infections in individuals with the autosomal recessive disorder cystic fibrosis. The ability of *P. aeruginosa* to readily form a biofilm through the overproduction of alginate allows for greater adhesion to the lung epithelial cells and impairs mucociliary clearance (4). Moreover, the overproduction of alginate assists in the avoiding the host's immunological response, and confers increased resistance to the antimicrobial and chemotherapeutic agents (7, 11). The regulation of alginate production is primarily controlled by the alternative sigma factor AlgU and its cognate transmembrane anti-sigma factor MucA.

In non-mucoid environmental strains, AlgU is sequestered to the inner membrane by the wild-type MucA. However, upon contact with the environment found in the cystic fibrosis lung, *P. aeruginosa* quickly transitions from the non-mucoid phenotype to the alginate overproducing, or mucoid phenotype. This transition results from two regulatory mechanisms: First, response to internal or external stressors causes the release of AlgU through the regulated proteolytic degradation of MucA; Second is the selection for those strains that possess a "loss of function" mutation in MucA. It is generally accepted that those early colonizing strains utilize the proteolytic degradation of MucA for induction of alginate overproduction prior to the selection for mutations in MucA. Moreover, this transition from the non-mucoid to the mucoid phenotype is emblematic of a decline in the overall health in individuals afflicted with cystic fibrosis (9). Therefore, it is beneficial to understand the regulatory mechanisms responsible for the conversion to mucoidy in those *P. aeruginosa* strains with a wild-type MucA, in order

to prevent a chronic respiratory infection. The overall goal of this dissertation was to identify and characterize novel regulators of alginate overproduction in those *P. aeruginosa* strains carrying a wild-type MucA. In this chapter, I will review and discuss the research detailed in this dissertation and suggest future studies.

In Chapter 2, we used next generation sequencing to identify those mutations that are unique to the *P. aeruginosa* strain PAO579. Based on our comparative genomic analysis, we identified 31 mutations that were present in PAO579 but were not present in PAO1 (Chapter 2, Table 1). Of these 31 mutations, 16 were classified as non-synonymous, while the remaining 15 were classified as synonymous mutations. As expected, we identified mutations in the *leuA* and *rpsL* genes, corresponding to the leucine-axotrophic and streptomycin-resistant phenotypes observed in PAO579. Interestingly, we identified a single nucleotide polymorphism resulting in a non-synonymous mutation in the DegS-like serine protease AlgW. AlgW has been shown to confer mucoidy in *P. aeruginosa* by proteolytically degrading the transmembrane anti-AlgU factor, MucA (3, 15). Also of importance, we identified three sequential mutations in *pilA*, a gene encoding the precursor for type-IV pilin. Therefore, next-generation sequencing is an excellent tool for comparative genomic analyses and the identification of classical mutations

In Chapter 3, I determined which mutations, identified through the sequencing analysis detailed in Chapter 2, were ultimately responsible for conferring mucoidy in PAO579. Using standard molecular techniques and complementation analyses, I determined that the sequential mutations present in the *pilA* gene were responsible for the induction of mucoidy in PAO579. Further analyses determined that these sequential

mutations result in a truncation in the PilA protein, revealing a C-terminal amino acid motif of phenylalanine-threonine-phenylalanine (FTF), which causes a release of the alternative sigma factor AlgU following the proteolytic degradation of MucA by AlgW. Previously, Boucher *et al.* reported that the sigma factor RpoN was required for mucoidy in PAO579, while the alternative sigma factor AlgU was not required for mucoidy (2). Based on their analysis, they suggested that the use of RpoN in place of AlgU for the induction of mucoidy was due to sigma factor antagonism (2). My results suggest otherwise. I did observe that RpoN is required for mucoidy in PAO579, however my data suggests that this is due to RpoN's role in regulating transcription of AlgW and the truncated PilA. Therefore, my results indicated that RpoN acts upstream of *algD*. Further supporting this model, I observed that the deletion of AlgU resulted in loss of mucoidy, confirming AlgU's role in regulating mucoidy in PAO579. I also observed that the truncated PilA protein did not localize to the cell surface, due to decreased stability.

Taken together, the results from chapters 2 and 3 clearly outline a pathway in which truncated pilin can confer mucoidy in *P. aeruginosa* strains possessing a wild-type MucA. However, these results pose many interesting questions. First, will exposure to subclinical doses of carbenicillin consistently select for mucoid colonies containing these same mutations? This could be determined by repeating the previous experiments conducted by Govan and Fyfe (8), with the addition of PCR and sequence analysis targeting the particular genetic loci identified in this study. Second, what is the clinical relevance of the sequential mutations causing a truncation of type-IV pilin? Simply stated, are these mutations found in clinical isolates? Based on my preliminary analysis, I was unable to detect the three sequential mutations in *pilA* found in PAO579

in clinical strains possessing a wild-type MucA. However, I was only able to examine a small sample size of early colonizing clinical isolates possessing a wild-type MucA. Expanding this analysis to included hundreds of clinical isolates could help determine the clinical relevance of this mutation. Finally, does the truncation of PilA have a synergistic effect regarding evasion from the host's immune system? I determined that the truncated PilA found in PAO579 does not localize to the cell surface due to an overall lack of stability. PilA is a known immunogenic target for macrophages and neutrophils (12, 16). Simultaneously, the truncation of PilA activates alginate overproduction, providing additional protection from the host's immunological response (11). Additional experiments analyzing immunological markers should be performed to determine whether this synergistic relationship is present. Specifically, western blot analyses targeting interleukin-8 in CF- and non-CF cell lines cultured in the presence of PAO579 could best characterize this relationship. Together, these future studies will identify the prevalence and relevance that mutations resulting in the truncation of type IV pili have with respect to the formation of a mucoid biofilm, and the development of a chronic respiratory infection.

In Chapter 4, I shifted the focus of my study to identifying possible genetic mechanisms to prevent the formation of a mucoid biofilm. To do so, I expanded upon the initial observations of a previous graduate student, Dr. Nathan Head. In this study, he used whole genome complementation analysis using a PAO1-derived cosmid library, coupled with *in vitro* transposon-mediated mutagenesis, to identify possible inhibitors of alginate overproduction. These experiments were performed using PAO579 as the model strain; however these results were preliminary due to a lack of data determining

the precise mechanism by which PAO579 conferred mucoidy. As a result of my continuance of this study, I identified the expression of gene locus PA1494 can inhibit mucoidy in *P. aeruginosa* strain PAO579. Moreover, expression of PA1494, also referred to as mucoid inhibitor A (*muiA*), suppressed mucoidy in strains possessing a wild-type MucA. This was not observed in mucoid *P. aeruginosa* strains with MucA mutations. MuiA's ability to suppress alginate overproduction required correct localization to the periplasm. This finding supports previous reports identifying MuiA as a periplasmic protein (10). Interestingly, characterization of MuiA based on sequence analysis identified no homologs in other *Pseudomonads* or *E. coli*. While this study identifies MuiA as a potential target for the suppression of mucoid biofilm formation, many questions remain regarding the role of MuiA within the cell. Primarily, what function does MuiA play in the physiology of *P. aeruginosa*? To answer this question, X-ray crystallography analysis of the protein could be performed to determine the three-dimensional structure of MuiA, as well as identify any potential active sites or binding domains. Additionally, identifying those proteins which interact with MuiA within the periplasm could be particularly useful information in determining MuiA's function. Since the expression of MuiA was unable to suppress mucoidy in the absence of the periplasmic serine protease MucD, then, generally speaking, the mechanisms that activate protease-mediated alginate overproduction can be classified into two types. The first mechanism requires the activation of AlgW to initiate the degradation of MucA (3, 5, 15, 17), while the second occurs following the inactivation of MucD, and subsequent activation of the site-2 protease, MucP (6). Despite this analysis, additional experiments to identify and characterize proteins that specifically interact with MuiA

should be performed. Techniques such as the co-immunoprecipitation of MuiA, as well as bacterial of yeast two-hybridization, should be performed, with priority given to proteins found within the periplasm. Comparison of the results garnered from these methods could provide potential candidates for further analysis.

Experiments further examining the relationship between MuiA and MucD are presented in chapter 5. MucD is a periplasmic serine protease that has considerable homology with the protease DegP found in *E. coli* (1). Moreover, MucD plays a role in both the suppression and induction of mucoidy in *P. aeruginosa* (1, 6, 19, 20). Western blot analysis probing for MucD revealed an accumulation of lower molecular weight bands in the mucoid strain PAO579, when compared to the non-mucoid progenitor strain PAO1. However I did not observe the level of accumulation of lower molecular weight bands when the alternative sigma factor AlgU was deleted in PAO579. Also, expression of *muiA in trans* reduced the amount of lower molecular weight bands in PAO579 to levels comparable to PAO1. Collectively, these data suggest that there is an increase in expression of *mucD* in PAO579 as compared to PAO1. Moreover, we observed a decrease in the accumulation of lower molecular weight products in PAO579 Δ *algU*, suggesting that the increased expression of *mucD* in PAO579 is likely due to auto-regulation of the *algUmucABCD* operon by AlgU. However, these data do not identify a clear association between MuiA and MucD outside of MuiA's ability to suppress the release of AlgU. Based on these results, I wanted to determine if the expression of *muiA* affected proteolytic activity in PAO579. I observed increased proteolytic activity at ~50 kDa. Moreover, we also observed a decrease in proteolytic activity at ~50 kDa when MuiA is expressed *in trans*. These data possibly corresponds

to the proposed increased expression of MucD in PAO579, suggested in the previous western blot analysis, however the specific protease needs to be determined using either tandem mass spectroscopy or *de novo* peptide sequencing. In future studies, the possible relationship between of MuiA and MucD could be succinctly determined using *in vitro* protein-protein interaction assays, or through bacterial two-hybridization.

The comparative iTRAQ proteomic analyses to identify changes in the protein profiles of mucoid strains PAO579 and VE2, due to the expression of *muiA in trans* is presented in chapter 5. Based on these analyses, we identified three common proteins effectors down-regulated in both VE2 and PAO579 when *muiA* is expressed *in trans*: Elongation Factor-Tu (EF-Tu), Trigger Factor and the extracellular protease PasP. The down-regulation in EF-Tu could be in response to the decrease in expression of the alginate biosynthetic operon; however this remains to be determined. Previously, Qiu *et al.* identified Trigger Factor as being required for the proteolytic degradation of the cytoplasmic portion of MucA (14). Therefore, the down-regulation of Trigger Factor may be in response to the decrease in regulated intramembrane proteolysis of MucA. An interesting protein down-regulated by expression of *muiA* is PasP. This extracellular protease is associated with corneal pathogenicity, and causes proteolytic degradation at ~50 kDa (13). These findings correlate with the decrease in proteolytic activity at ~50 kDa observed in the prior zymogram protease analysis of PAO579. Tandem mass spectroscopy is needed to determine whether the protein responsible for the increased protease activity at ~50 kDa is PasP.

Summary and Conclusions

The goal of this thesis was to *identify novel regulators of alginate overproduction, also known as mucoidy, in P. aeruginosa strains possessing a wild-type MucA*. The work in Chapters 2 and 3 identified the mutations resulting in the truncation of type-IV pili and demonstrated that this mutated protein can induce mucoidy via the regulated intramembrane proteolysis of wild-type MucA in the model strain PAO579. Chapters 4 and 5, describe the identification and characterization of a novel inhibitor of alginate overproduction in *P. aeruginosa* strains possessing a wild-type MucA. An illustration summarizing the findings presented in this dissertation can be found on page 114 (Figure 1). However, many questions remain regarding the prevalence and clinical relevance of truncated type-IV pili-mediated alginate overproduction, and the mechanism by which MuiA suppresses mucoidy in *P. aeruginosa*.

There has been a considerable amount research focusing on the role of the alternative sigma factor AlgU and its cognate anti-sigma factor MucA in the regulation of alginate overproduction in *P. aeruginosa*. Previous studies have shown the importance of *mucA* mutations in establishing a chronic infection within the cystic fibrosis lung through the formation of mucoid biofilm. However, there are few studies that identify the genetic regulatory components involved in induction of mucoidy prior to selection for MucA mutants. In this study, I identified and characterized two novel regulators that control alginate overproduction in strains possessing a wild-type MucA. Understanding the mechanism by which these regulators control alginate overproduction will give us a better understanding of the progression from the non-mucoid to the mucoid phenotype in early colonizing strains of *P. aeruginosa*. More importantly, the identification of novel

inhibitors of alginate overproduction may provide a solution for preventing the establishment of chronic respiratory infection in individuals afflicted with cystic fibrosis.

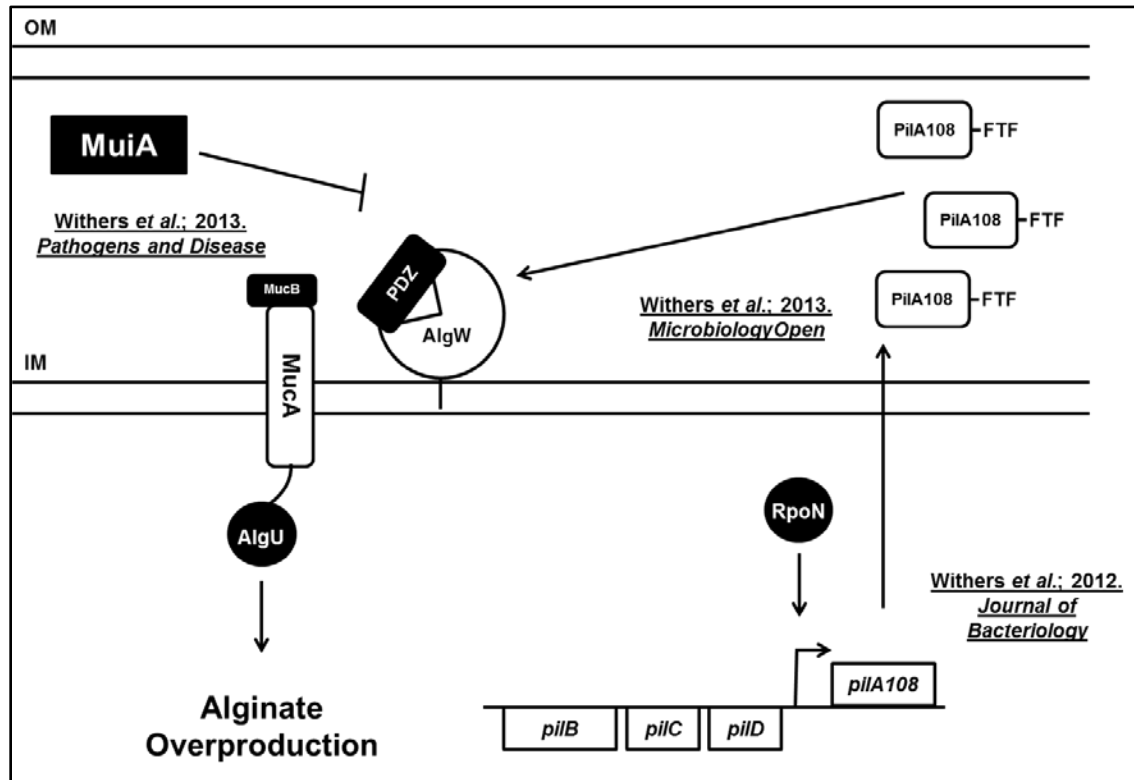


Figure 1. Illustration summarizing the research contained in this dissertation. In PAO579, three tandem mutations coding a premature stop codon in *pilA* results in the truncation of the pilin protein to 108 amino acids (PilA108) (18). The sigma factor RpoN drives for transcription of *pilA*¹⁰⁸ (*muc-23*) and *algW* (not shown). PilA108 possesses a C-terminal motif of phenylalanine-threonine-phenylalanine (FTF) which activates the periplasmic protease AlgW after translocation to the periplasm (17). Upon activation, AlgW initiates the proteolytic degradation of MucA, thereby releasing AlgU. The release of AlgU drives transcription at the alginate biosynthetic operon. Expression of MuiA suppresses alginate overproduction following localization to the periplasm. **IM**, inner membrane; **OM**, outer membrane.

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Appendix: Internal Review Board Letter of Exemption



Office of Research Integrity

May 10, 2013

Thomas Ryan Withers
1329 Neel Street
Huntington, WV 25701

Dear Mr. Withers:

This letter is in response to the submitted dissertation abstract titled "Genetic Regulation of Mucoidity in *Pseudomonas aeruginosa*." After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

A handwritten signature in blue ink that reads 'Bruce F. Day'.

Bruce F. Day, ThD, CIP
Director

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