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Regulation of Alginate Production of Pseudomonas Aeruginosa Pseudomonas aeruginosa

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REGULATION OF ALGINATE PRODUCTION OF *Pseudomonas aeruginosa*

DISSERTATION

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By

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ABSTRACT

Pseudomonas aeruginosa (*P.a*.) is a major threat to the health and well being of a person living with cystic fibrosis (CF). *P.a.* is capable of conversion to mucoidy or alginate overproducing phenotype. Mucoidy facilitates the chronic infection of *P.a.* infection in the CF lung. Mutations in the anti-sigma factor *mucA,* result in activation of the sigma factor AlgU which promotes expression of alginate through the alginate biosynthetic operon. However, *P.a*. can produce alginate independent of *mucA* mutation via a mechanism known as regulated proteolysis. Proteases AlgW and MucP can degrade MucA to liberate and activate AlgU. In this dissertation, two pathways to alginate production will be characterized through investigation of two negative regulators of alginate production: KinB and MucD. KinB is a histidine kinase which controls alginate production through two transcription factors: AlgB and RpoN along with the MucA protease AlgW. In the absence of KinB, the degradation rate of MucA is elevated which results in alginate production. MucD is a periplasmic protease that is presumed to degrade protein signals in the periplasm. It is hypothesized that if MucD does not degrade certain protein signals then they will accumulate and activate alginate production. Here, it is presented that in the absence of MucD, the intramembrane protease MucP activates degradation of MucA. To further characterize MucD, the roles of the PDZ domains in suppression of alginate production were probed. PDZ domains are conserved protein domains that function in protein to protein interaction. The PDZ domains of MucD were found to be dispensable for suppression of alginate production but required for the stability of MucD. When regulated proteolysis is activated, AlgU promotes expression of many genes. One such gene is LptF or Lipotoxin F. Here it is shown that in the absence of *kinB*, LptF is highly expressed. LptF was shown to be an outer membrane protein, but it is not involved in production of alginate. However, LptF mediates adhesion to lung epithelia and resistance to reactive oxygen species. *P.a.* is capable of activating AlgU through MucA proteolysis by several mechanisms that are characterized in this dissertation. Through elucidation of these pathways, we will be more prepared to combat alginate production and *P.a.* infection of the CF lung.

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Pseudomonas aeruginosa (*P.a.*) is a gram negative bacterium regarded as both an environmental organism and an opportunistic pathogen. HIV, burn or other patients with compromised immune systems are susceptible to infection by *P.a.* In particular, humans with the genetic disease cystic fibrosis (CF) are highly susceptible to lung infections. Due to the intrinsic antibiotic resistance of *P.a.,* these infections cannot be effectively controlled and ultimately lead to the decreased lifespan of CF patients.

P.a. is easily recognized by its characteristic blue-green pigment due to production of two virulence factors known as pyocyanin and pyoverdin. *P.a.* also has a characteristic smell of grapes. *P.a.* has one of the largest bacterial genomes. Within the *P.a* genome, numerous secreted virulence factors are coded such as elastase, proteases, phospholipase C, enzymes for hydrogen cyanide production and exotoxins. Each of these secreted virulence factors has been recognized to be involved in *P.a.* pathogenesis in CF. Also, *P.a.* can communicate through quorum-sensing via small molecules and coordinate gene expression of virulence factors. To persist in the CF lung, *P.a.* overproduces and secretes an exopolysaccaride known as alginate (Fig. 1). High alginate production provides the organism with a protective coating, resulting in a phenotype referred to as mucoidy. The morbidity and mortality of a CF individual is directly correlated with the time at which mucoid *P.a.* emerges. Therefore, knowledge of how *P.a.* regulates alginate production is of critical importance. Even though the initial mechanism of alginate production due to mutation of a *P.a.* gene known as *mucA* was elucidated in 1993,

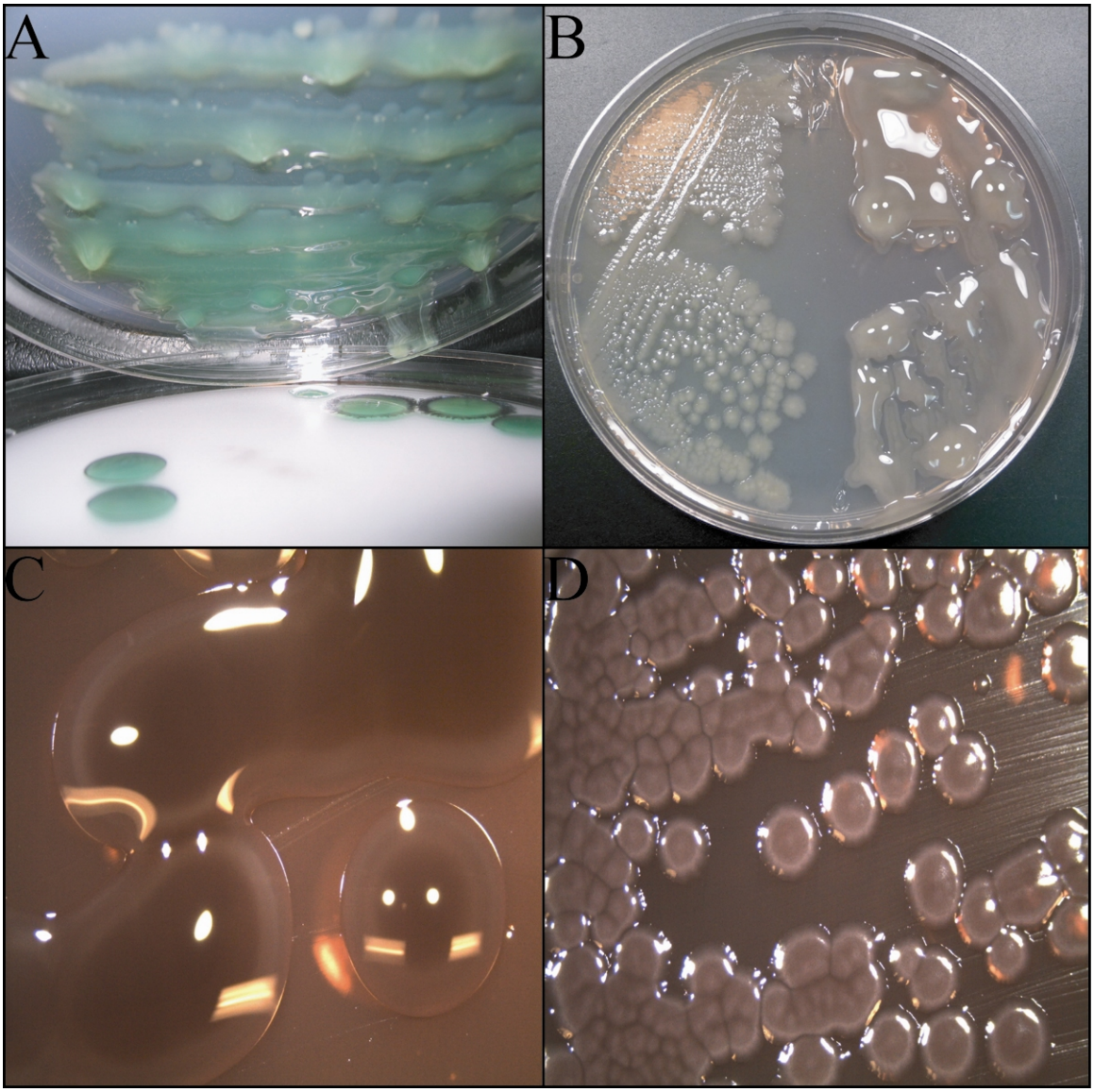


Figure 1. Alginate overproduction and mucoid phenotype. Panel A shows a *Pseudomonas* isolation agar (PIA) plate with a mucoid *mucA* mutant cultured for 48 hrs at 37° C. Of note droplets of alginate and cells have fallen from the agar on to the lid of the plate due to the hyper alginate production. Panel B indicates a mucoid strain PAO579 with both nonmucoid (left) and mucoid phenotype (right) due to expression of an alginate regulatory gene. In Panel C mucoid strain VE2 is shown on PIA supplemented with Congo red for staining the exopolysaccaride. In Panel D alginate production in VE2 has been abolished by the inactivation of *algB*, a transcriptional regulator of the alginate biosynthetic operon.

recent data have shown *P.a.* is capable of alginate production independently of classical *mucA* mutations (30). Furthermore, data suggests alginate production occurs in the lung before *mucA* mutations are selected (6). Collectively, these data indicate alginate production can be modulated by *P.a.* *The hypothesis of this dissertation states that P.a. utilizes signal transduction pathways which activate regulated proteolysis of MucA to regulate and control the activity of AlgU, the master regulator of alginate production.* The goal of this work was to use genetic, molecular and biochemical techniques to describe how alginate production by *P.a.* is modulated through transcriptional and post-translational modifications.

Cystic Fibrosis

CF is the most common inherited lethal disorder in the Caucasian population. The reason why CF individuals are susceptible to chronic *P.a.* lung infection is due to the compromised lung functions caused by recessive mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). Individuals homozygous for mutant CFTR have severe defects in chloride ion transport. When the concentration of these ions are not properly modulated then abnormalities in fluid secretion occurs and dehydrated mucus buildup results. The major organs affected by CF mutations include sweat glands, exocrine glands, pancreas, and the intestines. Eventually 95% of all CF patients die from *P.a.* lung infections due to defective mucociliary clearance.

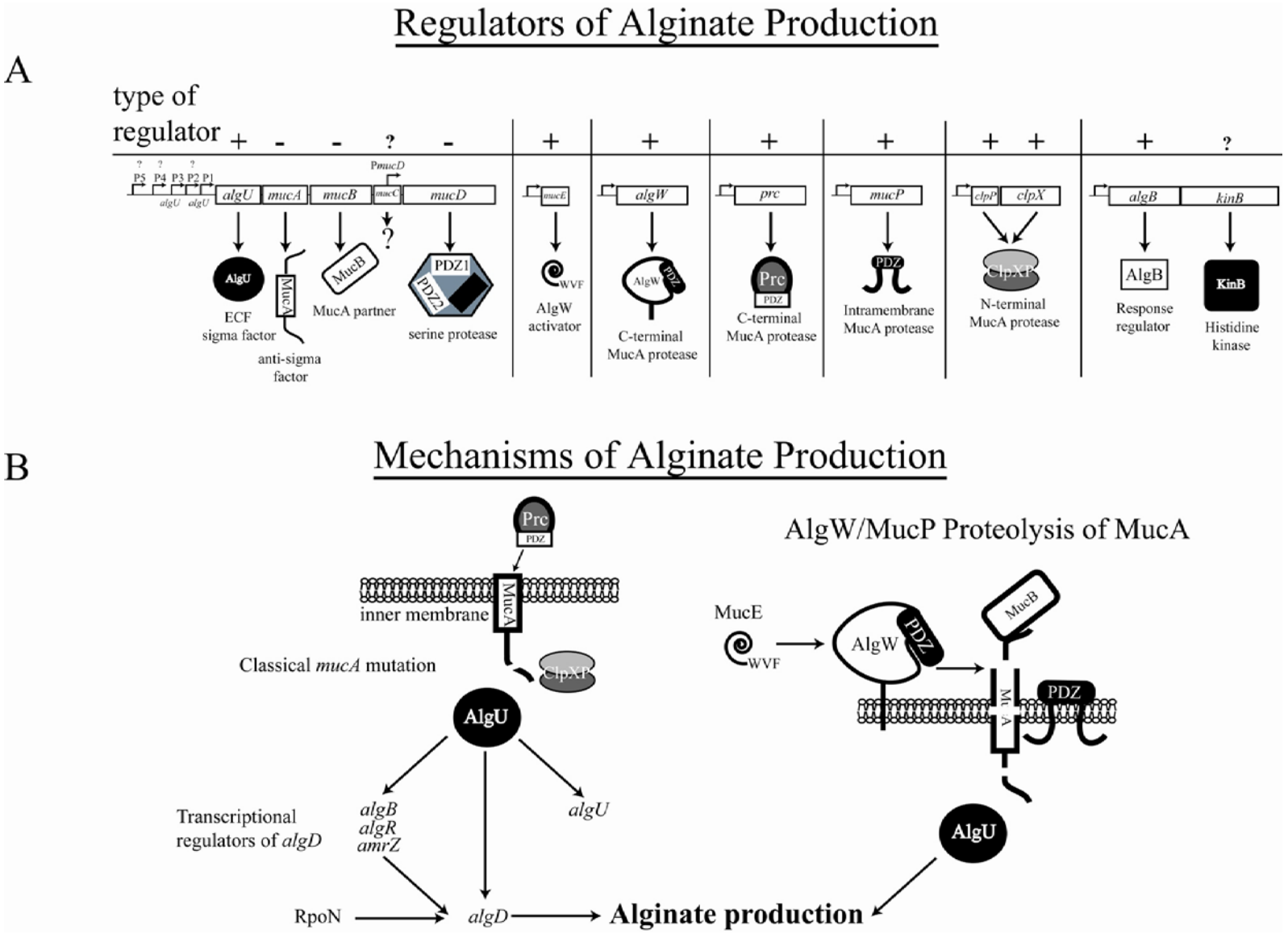
CF was appreciated as a disease in the 1930-1940's, and before antibiotic therapies were available most CF individuals did not survive beyond infancy. Today, most CF individuals survive into adulthood mainly due to improved antibiotic and nutritional therapy. However, chronic lung infection by *P.a.* dictates the life expectancy

of those with CF. The characteristic thick mucosal secretions of the CF lung trap microorganisms, such as *P.a.*, which facilitates establishment of infection (11). Emergence of alginate overproducing or mucoid *P.a.* signifies chronic infection and a poor prognosis for the patient. Furthermore, rigorous antibiotic treatment is ineffective and once chronic infection has been established, these infections will ultimately lead to the death of the CF patient (19). Therefore, elucidation of the molecular modulation of alginate production by *P.a.* is of paramount importance to the control or eradication of *P.a.* infection of the CF lung.

LITERATURE REVIEW

Pseudomonas aeruginosa and mucoidy

In 1927, mucoid strains were first isolated from a patient with an abscess in the gallbladder (35). However, the correlation of mucoid *P.a.* strains and chronic pulmonary disease was not described until the 1960's (19). As lung disease progresses, the emergence of alginate producing colonies is considered the onset of chronic infection (11, 19). The visual phenotype of alginate producing colonies is referred to as mucoid (Fig.1C). High transcription of the first gene of the alginate biosynthetic operon, *algD* (Fig. 2), leads to mucoidy (10). The *algD* biosynthetic operon codes for the enzymes and machinery for manufacture and secretion of alginate. The first mechanism elucidated for conversion to mucoidy was through mutation of the *mucA* gene (25). MucA is the anti-sigma factor


Regulators of Alginate Production
A
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algU
mucA
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algB
kinB
AlgU
ECF sigma factor
MucA
anti-sigma factor
MucB
MucA partner
PDZ1
PDZ2
serine protease
WVF
AlgW activator
AlgW
C-terminal MucA protease
Prc
PDZ
C-terminal MucA protease
Intramembrane MucA protease
ClpXP
N-terminal MucA protease
AlgB
Response regulator
KinB
Histidine kinase
B
Mechanisms of Alginate Production
inner membrane
Classical mucA mutation
Transcriptional regulators of algD
algB
algR
amrZ
algU
RpoN
algD
Alginate production
AlgW/MucP Proteolysis of MucA
MucE
WVF

Figure 2 Regulators and mechanisms of alginate production. Alginate production is controlled by an array of operons and proteins. Panel A indicates the genes that code for alginate regulatory proteins. Above each genetic element, the genes are classified as positive and negative based on their effect on AlgU activity. The *algU* promoters control an operon of *algU, mucA, mucB, mucC* and *mucD.* Promoters 1 and 3 are dependent upon AlgU. Therefore, AlgU regulates its own expression. MucA is the direct negative regulator of AlgU. If the *mucA* gene is mutated then AlgU activity is not repressed and causes alginate overproduction as shown in panel B. MucB is another principal negative regulator of AlgU. MucB binds the C-terminus of MucA and protects it from proteolytic cleavage. *mucC* has not been demonstrated to code for a protein, however a promoter is located in the *mucC* open reading frame that drives *mucD* expression. MucD is the third known negative regulator of alginate production. MucD is a serine protease that likely performs quality control in the periplasm. *mucE* codes for a small periplasmic protein that has been shown to activate proteolysis of MucA by the serine protease AlgW. When activated, AlgW can degrade the C-terminus of MucA. Another periplasmic protease known as Prc, or tail specific protease, has been suggested to facilitate proteolysis of truncated MucA proteins such as those that result from mutated *mucA* genes. Once AlgW degrades the C-terminal of MucA, then MucP has been suggested to facilitate degradation of MucA at its transmembrane domain. AlgW, Prc, and MucP are the three known periplasmic proteases that act upon MucA (B). After MucA has been cleaved by the periplasmic proteases then ClpXP facilitates degradation of the remainder of MucA. Once all MucA has been degraded, then AlgU is active and can drive transcription at its target promoters. AlgU has been shown to drive transcription of several transcriptional regulators of the *algD* alginate biosynthetic operon. AlgB, AlgR, and AmrZ have been shown to regulate expression of *algD*. The alginate biosynetic operon codes for all the machinery and enzymes necessary for production and secretion of alginate. AlgB is coded in an operon with a histidine kinase known as KinB. KinB has been previously shown to be not required for alginate production in *mucA* mutants. Alginate production is controlled by AlgU-MucA interactions. When AlgU is not repressed by MucA then alginate production occurs.

that sequesters the alternative sigma factor AlgU (also called AlgT) (Fig. 2) (19, 31). Increased transcription directed by AlgU at the *algD* promoter (P_{algD}) activates alginate biosynthesis (24) (42).

AlgU is the gateway to alginate production and MucA is the primary negative regulator (Fig. 2). Analysis of transcription has revealed that *algU* is expressed from multiple promoters (33), two of which being AlgU-dependent (12, 33) (Fig. 2). Therefore, mutations in *mucA* lead to derepression of AlgU-MucA, interactions which then causes up-regulated levels of AlgU due to the fact two of the P_{algU} promoters are AlgU-dependent. The periplasmic protease, known as Prc, has been shown to be required for mucoidy of *mucA* mutant strains (32). Prc is thought to degrade mutant forms of *mucA* which will facilitate AlgU activation in a *mucA* mutant strain (Fig. 2). Loss of repression of AlgU causes increased transcription of AlgU-dependent promoters such as the *algD* promoter (25). Even though AlgU directs transcription at P_{algD}, several other factors also participate in transcriptional activation as well. Significant research has focused on the alginate regulators that enhance the transcriptional activity at P_{algD}. The two component response regulators, AlgB and AlgR, along with the DNA binding protein, AmrZ, promote AlgU-dependent P_{algD} activity (Fig. 2) (3, 41). Another transcriptional activator, AlgR, binds to the far upstream region of *algD* to activate the alginate overproduction (26). Also at $P_{algD,}$ the alternative sigma factor RpoN has dual roles as both a positive and a negative regulator (5). Conversion to mucoidy by *mucA* mutations in *P.a.* has been well characterized. However the mechanisms of alginate production in wild type *mucA* strains are not fully understood.

The strains that initially colonize the CF lung most resemble environmental strains due to their nonmucoid phenotype (19). One study has shown 97.5% of children with CF had evidence of *P.a.* infection by age 3 (7). The majority of these strains from early infection displayed non-mucoid phenotype and were antibiotic susceptible. In a CF mouse model, it has been shown that *algD* is required for respiratory colonization (9). Taken together these data suggest, even though early colonizing *P.a.* are non-mucoid, alginate production may be critical in establishing these infections. Recently, nonmucoid *P.a.* has been shown to express alginate in both murine lungs and in CF patient sputum samples (6). The study also found that alginate genes were increased in anaerobic conditions. CF sputum contains enough nitrate to support growth of *P.a.* anaerobically (28). Collectively evidence from the literature indicates conditions in the CF lung are favorable for alginate production by *P.a.* and that alginate production provides a selective advantage for infection.

Only limited research has shed light on alginate production by wild type *mucA* strains. In wild type *mucA* strains, MucB cooperates with MucA-AlgU sequestering presumably by protecting the periplasmic portion of MucA from degradation by proteases and thus stabilizing MucA-AlgU interaction (Fig. 2) (34). Derepression of AlgU-MucA interactions has recently been the focus of *P.a.* alginate regulation research. Cell wall synthesis inhibitory antibiotics such as D-cycloserine have been shown to up-regulate the alginate biosynthetic operon (38). Also our group has shown that overexpression of the small periplasmic protein, MucE, causes mucoidy mediated by the protease AlgW (30). *E. coli* possesses a homologue to AlgW known as DegS. It has been shown that in *E. coli* the interaction of the anti-sigma factor RseA and σ^E can be

derepressed by DegS and RseP in response to high expression of mis-folded periplasmic and outer membrane proteins (2, 30). The DegS-like AlgW has been shown to be required for mucoidy when MucE is overexpressed (30) or for activation of P_{algD} in the presence of D-cycloserine (38). This conserved signal transduction pathway through DegS in *E. coli* or AlgW in *P.a.* is referred to as regulated intramembrane proteolysis (RIP) (Fig. 2B) (1). In *E. coli,* post proteolysis by DegS and RseP, released σ^E still has residual RseA peptide that must be degraded before σ^E can interact with RNA polymerase for transcription. The N-terminus of RseA is then bound by ClpP (17) and finally degraded by the ClpXP protease complex (8). *P.a.* has three *clp* family genes (*clpP*, *clpX*, *clpP2*) which are required for mucoidy in a *mucA*-25 mutant (Fig. 2B) (29). Together, these recent findings show that *P.a.* harbors the machinery to activate alginate production via regulated proteolysis of the anti-sigma factor MucA.

AlgB-KinB two-component signal transduction system.

Bacteria can control signal transduction through two-component systems. Two-component systems are comprised of a transcription factor known as a response regulator and a histidine kinase. Classically, when the response regulator is phosphorylated by the sensor (histidine kinase) then transcription at target promoters will be activated (Fig. 3). Before regulated proteolysis was described as a mechanism of alginate regulation, a transcriptional regulator known as AlgB was shown to be required for alginate production in the classical *mucA* mutant strains (42). AlgB is an response regulator homologous to *E. coli* nitrogen fixation regulator NtrC (41) (18). The primary role of AlgB elucidated thus far has been to affect the transcriptional activation of P_{algD} (Fig. 2) (21). The cognate sensor kinase of AlgB is (23) an inner membrane protein known as KinB (23). KinB is

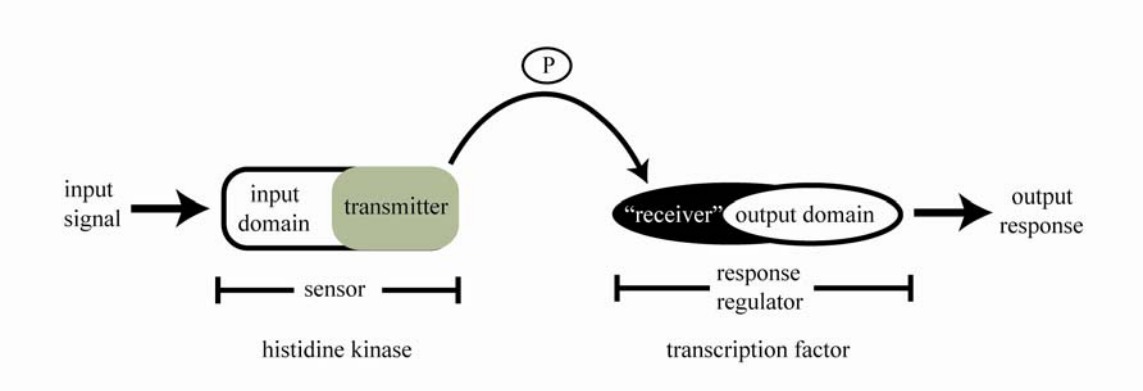

Figure 3. Two-component system dogma. Bacteria utilize two-component systems for signal transduction. A two-component system is comprised of a histidine kinase (sensor) and a transcription factor (response regulator). When input signals activate the histidine kinase then autophosphorylation occurs followed by transfer of the phosphate to the transcription factor. Typically phosphorylation of the transcription factor causes activation of gene expression at target promoters.

capable of autophosphorylation and transfer of the phosphate to AlgB (23). Interestingly, phosphorylation of AlgB is not required for alginate biosynthesis in a clinical mucoid isolate that carries *mucA22* mutation (22). It has been shown AlgB can bind upstream of the P_{algD} (21). Bioinformatic data shows AlgB has an interacting domain for the alternative σ^{54} (RpoN). RpoN is required for mucoidy in the prototype strain that carries an undefined mutation, *muc-23* (5). However *rpoN* is not required for alginate synthesis in the *mucA* mutant strains with the *mucA22* allele (5) or the *mucA2* allele (26, 27). AlgB and RpoN have been demonstrated to have independent functions in alginate synthesis and regulation of transcriptional activation at P_{algD}. AlgB has been characterized as a positive regulator (18), however RpoN has been shown to be either a positive or a negative regulator (5). The AlgB-KinB two-component system has been studied;

however it is clear that it does not follow the established dogma. In this dissertation, the role of KinB in alginate production will be probed in Chapter 3.

Negative regulation of alginate production by MucD.

Another negative regulator of AlgU is MucD. MucD is homologous to heat shock protein HtrA (also known as DegP or Do) of *E. coli* which is a protease that degrades unfolded proteins in the periplasm (20) and also functions as a chaperone (36). MucD is a serine protease and has been characterized as a negative regulator of alginate production (4). MucD was shown to have a role in modulating heat shock and reactive oxygen intermediates (4) and has also been implicated in pathogenesis (43). It has been proposed that MucD may facilitate folding and chaperoning of virulence factors such as toxins (43). When MucD was classified as a negative regulator, no clear mechanism was evident showing how MucD would regulate alginate production. In Chapter 4 of this dissertation, the roles of MucD are investigated and a novel pathway of regulated proteolysis of MucA is proposed.

Virulence factors co-expressed with alginate overproduction

Several studies have shown that when AlgU is activated a global change in gene expression occurs (13-16, 21, 37, 39, 40). Interestingly, it seems AlgU controls many other genes in addition to alginate genes. Microarray analysis has revealed that AlgU controls a whole class of lipoproteins (13-16). These lipoproteins were shown to cause inflammation in epithelial cells and were designated as lipotoxins (14, 15). Interestingly, most of the lipotoxins are membrane associated lipoproteins, however one lipotoxin known as Lipotoxin F or LptF is a predicted outer membrane protein (15). LptF shares a high amount of homology with the major outer membrane porin OprF of *P.a.* In Chapter

5, LptF is further characterized and is implicated in resistance to oxidative stress and adhesion.

DISSERTATION INTRODUCTION

From the literature that was detailed above, it is clear alginate production and regulation of AlgU in *P.a.* have been extensively investigated. While several mechanisms of alginate production have been described, it is still not clear how *P.a.* senses its environment and produces alginate. This dissertation details a series of studies aimed at addressing both long-standing questions about regulation of alginate production as well as descriptions of novel pathways. Molecular biology is built upon the design and construction of novel tools. Since regulatory genes are tightly regulated there is a need for expression systems that can be controlled to test hypotheses regarding gene expression. Therefore, Chapter 2 of this dissertation details the construction and validation of the pHERD series of vectors. These vectors allow for controlled expression from the arabinose-inducible PBAD promoter. These vectors were essential for the research detailed in all of the remaining chapters. In Chapter 3, characterization of inactivation of the histidine kinase KinB is detailed. As previously described, KinB was originally characterized as nonessential for alginate production, however in this work KinB is described as a negative regulator of alginate production and a novel pathway of alginate regulation is proposed. MucD is yet another negative regulator of alginate production; and in Chapter 4, a MucP pathway by which MucD regulates alginate production is described. Chapters 3 and 4 both show how AlgU is regulated by post-translation modifications to MucA, and from this data it is also clear that multiple pathways to activation of AlgU exist. Once AlgU is activated due to derepression of

MucA, then many genes are co-expressed with the alginate genes (13-16, 21, 37, 39, 40). In Chapter 5, a novel outer membrane protein known as lipotoxin F was identified up-regulated and implicated in resistance to oxidative stress and adhesion. Collectively, these Chapters of this dissertation describe two novel pathways of alginate production as well as an important co-expressed factor. However, these data propose more novel questions that will be discussed in Chapter 6. Furthermore, this dissertation indicates that regulation of alginate production is complex and continues to require investigation in order to understand how this virulence factor is regulated in hopes we can someday combat *P.a.* colonization especially in CF more effectively.

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CHAPTER 2: A series of P_{BAD}-based shuttle vectors for functional analysis of toxic and highly-regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria

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Keywords: P_{BAD} promoter, recombinant protein, shuttle vectors, *Pseudomonas, Burkholderia*

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ABSTRACT

We report the construction of a series of *Escherichia-Pseudomonas* broad-host-range expression vectors, utilizing the P_{BAD} promoter and the *araC* regulator for routine cloning, conditional expression, and analysis of tightly-controlled and/or toxic genes in pseudomonads.

Gene cloning, disruption, deletion, complementation analysis, and allelic exchange are central to prokaryotic molecular genetics. In *P. aeruginosa*, Schweizer and colleagues developed the pUCP family of general-purpose cloning/expression vectors (25, 30), based on the well-characterized pUC18/19 vectors (32) and the cryptic mini-plasmid pRO1614 (19). However other promoters are also in routine use such as the *tac* (4, 6), T7 (29), and *araBAD* promoter -based (8, 11) vectors for regulated expression in *Escherichia coli* and many other bacterial species (e.g., (2, 18, 26)). In *E. coli* AraC, the product of the constitutively expressed *araC* represses the *araBAD* promoter (P_{BAD}), and the expression of a cloned gene is induced by the addition of L-arabinose. *Pseudomonas* researchers have utilized the inducible properties of the *araC* regulator and the P_{BAD} promoter cassette for the controlled gene expression by integrating the *araC*-P_{BAD}-specific transcription fusion into the chromosome using a suicide vector or an integration-proficient vector (1, 3, 13, 17, 31). In the present study, we modified the four existing *Escherichia-Pseudomonas* shuttle vectors pUCP20T/26/28T/30T by replacing the *lac*-promoter with the *araC*-P_{BAD} cassette to achieve highly-controlled and inducible expression in the pseudomonads and other bacteria, e.g. *Burkholderia* spp.

Construction and features of pHERD vectors. Functional genetic analysis requires vectors capable of conditional expression. The P_{BAD} promoter has been used for gene expression extensively in *E. coli* and some in *P.aeruginosa* and *Burkholderia spp* (12, 28). We first constructed three shuttle vectors, pHERD20T/28T/30T (Fig. 1), based on the *Escherichia-Pseudomonas* shuttle vectors pUCP20T, pUCP28T, and pUCP30T (30), and the commercial expression vector, pBAD/Thio-TOPO® (Invitrogen). The 368 bp fragment of the pUCP vectors spanning two restriction sites *Afl*II and *Eco*RI was replaced with the *araC*-P_{BAD}

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Genotype, phenotype and primer sequences [a]	Source
P. aeruginosa		
PAO1	Alg^{wt} Prototroph	P. Phibbs
PAO1VE2Δ*algW*	Alg^{+}, PAO1 *mucE*$^{+oe}$ (*himar1*Gm^{R}::P_{GM}::*mucE*) Δ*algW*	(21)
P. fluorescens		
Pf-5	Alg^{wt}, Prototroph	ATCC
E. coli		
DH5α	F^{-}, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^{-}, mk^{+}), *phoA*, *supE*44, λ^{-}, *thi*-1, *gyrA*96, *relA*1	Lab strain
B. pseudomallei		
Bp50	Δ(*amrRAB-oprA*) derivative of wild-type strain 1026b	(5)
Plasmids		
pUCP20T	*Escherichia-Pseudomonas* shuttle vector, P_{lac}, Ap^{R}, ori_{pBR322}, $ori_{pRO1600}$, *oriT*	(30)
pUCP30T	*Escherichia-Pseudomonas* shuttle vector, P_{lac}, Gm^{R}, ori_{pBR322}, $ori_{pRO1600}$, *oriT*	(30)
pUCP28T	*Escherichia-Pseudomonas* shuttle vector, P_{lac}, Tp^{R}, ori_{pBR322}, $ori_{pRO1600}$, *oriT*	(30)
pUCP26T	*Escherichia-Pseudomonas* shuttle vector, P_{lac}, Tet^{R}, ori_{pBR322}, $ori_{pRO1600}$	(30)
pBAD/Thio-TOPO®	4,454 bp, *araC*-P_{BAD}, ori_{pUC}, Ap^{R}	Invitrogen
pHERD20T	pUCP20T P_{lac} replaced by 1.3 kb *Afl*II-*Eco*RI fragment of *araC*-P_{BAD} cassette	This study
pHERD30T	pUCP30T P_{lac} replaced by 1.3 kb *Afl*II-*Eco*RI fragment of *araC*-P_{BAD} cassette	This study
pHERD26T	pUCP26 P_{lac} replaced by 2.4 kb *Adh*I-*Eco*RI fragment of *araC*-P_{BAD} cassette and *oriT*	This study
pHERD28T	pUCP28T P_{lac} replaced by 1.3 kb *Afl*II-*Eco*RI fragment of the *araC*-P_{BAD} cassette	This study
pHERD20T-*mucE*	*mucE* in pHERD20T *Eco*RI/*Hin*dIII	This study
pHERD28T-*mucE*	*mucE* in pHERD28T *Eco*RI/*Hin*dIII	This study
pHERD30T-*mucE*	*mucE* in pHERD30T *Eco*RI/*Hin*dIII	This study

pHERD20T-*algU*	*algU* in pHERD20T *Eco*RI/*Hin*dIII	This study
pHERD20T-*oprF*	*oprF* (*PA1777*) in pHERD20T *Eco*RI/*Hin*dIII	This study
pHERD20T-*oprF*-WVF	*oprF* allele encoding OprF ending with the WVF motif cloned in pHERD20T *Eco*RI/*Hin*dIII	This study
Primers		
pBAD-F	AGTATACCTTAAGGAATCCCCAAATTATGACAACTTGACGGCTACATCAT	This study
pBAD-R	AGGATCCCCGGGTACCGAGCTCGAATTCTTATCAGATCCCATGGGTATGTATA	This study
pHERD-SF	ATCGCAACTCTCTACTGTTTCT	This study
pHERD-SR	TGCAAGGCGATTAAGTTGGGT	This study
algU-F	AGAATTCGATGCTAACCCAGGAACAGGA	This study
algU-R	CAAGCTTTCAGGCTTCTCGCAACAAAGGCTGCA	This study
algW-F	AGAATTCGATGCCCAAGGCCCTGCGTTTCCT	This study
algW-R	TGCCAAGCTTTCACTCGCCGCCGTCCTGTTT	This study
mucE-F	AGAATTCGATGGGTTTCCGGCCAGTTA	This study
mucE-R	GAAGCTTCAAAACACCCAGCGCAACTCGTC	This study
oprF-F	AGAATTCGATGAAACTGAAGAACACCTTA	This study
oprF-R	CAAGCTTTTACTTGGCTTCAGCTTCTACTTCGGCT	This study
oprF-WVF-R	AAGCTTAAAACACCCAGCGCTTGGCTTCAGCTTCTACTTCGGCT	This study
lacZ-RT-For2	GTCGTGACTGGGAAAACC	This study
lacZ-RT-Rev2	GCCTCTTCGCTATTACGC	This study
Bp23S_F	GTAGACCCGAAACCAGGTGA	This study
Bp23S_R	CACCCCTATCCACAGCTCAT	This study

[a] Alg^{wt}, wild-type non-mucoid phenotype. Alg^{-}, non-mucoid phenotype. Alg^{+}, mucoid phenotype. Primers used for cloning purposes carried a built-in restriction sites (underlined) with F denoting forward and R reverse primers, respectively.

fragment (1.3 kb), produced via PCR using pBAD/Thio-TOPO® as the template and primers pBAD-F and pBAD-R (Table 1). The PCR product was purified and directly digested with *Afl*II and *Eco*RI, and the two fragments were ligated into the pUCP vectors, creating pHERD20T (Figure 1; Cb^R, 5,087bp), pHERD28T (Tp^R, 4,993bp), and pHERD30T (Gm^R, 5,216bp). The *Eco*RI/*Afl*II regions of these vectors were sequenced to confirm that no mutations were introduced during the cloning process. We next transferred the 2.4 kb *Adh*I-*Eco*RI fragment from pHERD20T to pUCP26, generating pHERD26T (Tet^R, 6,166 bp) which includes the *araC*-P_{BAD} cassette and the *oriT* sequence. The GenBank accession numbers for the nucleotide sequences of the *Escherichia-Pseudomonas* shuttle vectors, pHERD20T, 26T, 28T 30T are EU603324, EU603327, EU603325, EU603326, respectively.

The pHERD vectors have the features of the pUCP vector family, including the pBR322 origin, four different antibiotic resistance markers, the *oriT* region for conjugation-mediated plasmid transfer (24), ori_{1600}, and the *rep* gene encoding the replication-controlling protein (25, 30). However, the main advantage for cloning into the pHERD vectors is low expression occurs from the P_{BAD} promoter when it is not induced. α-complementation is inducible for blue-white screening which facilitates identification of recombinants on an X-gal-containing plate supplemented with arabinose (0.01%). The P_{BAD} promoter responds in a dose dependent manner (Fig. 2). Two sequencing and PCR primers were designed that anneal to regions on both sides of the MCS, pHERD-SF 78 bp upstream of *Eco*RI and pHERD-SR 49 up downstream of *Hin*dIII. If a gene is cloned in-frame into the *Eco*RI site, a fusion protein with an additional seven NH_2-terminal amino acids (MGSDKNS) derived from thioredoxin of the pBAD-TOPO/Thio will result. Thioredoxin acts as a translation leader to facilitate high-level expression and, in some cases, increase solubility in *E. coli* (9) These amino acids at the

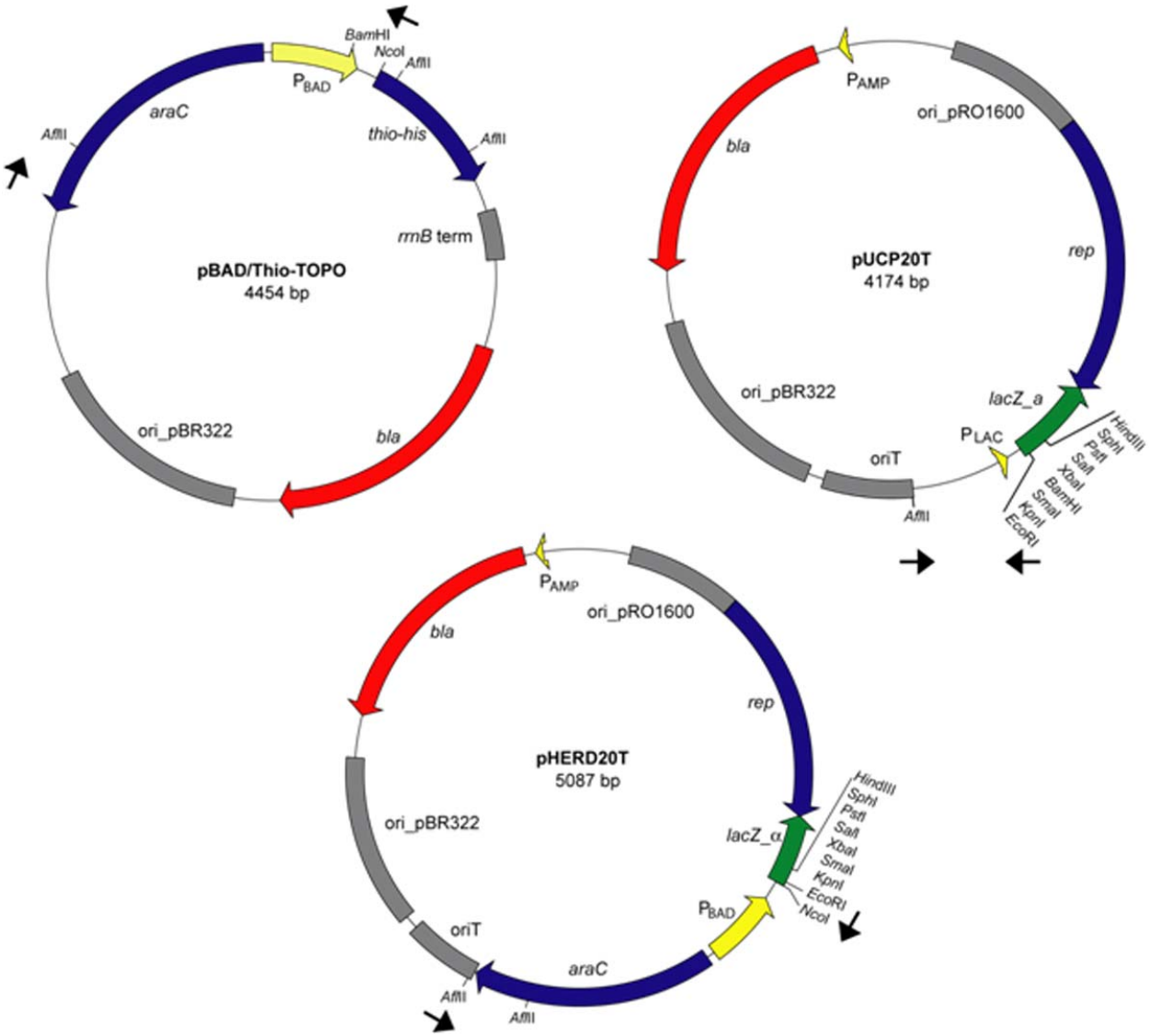

Figure 1. **Construction of an *Escherichia-Pseudomonas* shuttle vector, pHERD20T, an arabinose-inducible expression vector.** pHERD20T is a pUCP20T-based, conjugatable vector with pBR322 and pRO1600 derived replicons which support replication in *E. coli*, and *P. aeruginosa* and other bacteria, respectively. The P_{BAD} promoter was derived from the expression vector, pBAD/Thio-TOPO (Invitrogen). The P_{lac} promoter in pUCP20T was replaced by the P_{BAD} promoter containing segment with an *Eco*RI-*Afl*II fragment generated via PCR containg the *araC* gene and P_{BAD}. Black arrows indicate the region transferred from pBAD/Thio-TOPO into pUCP20T. pHERD20T contains a multiple cloning site within *lacZ*α encoding the β-galactosidase α peptide.

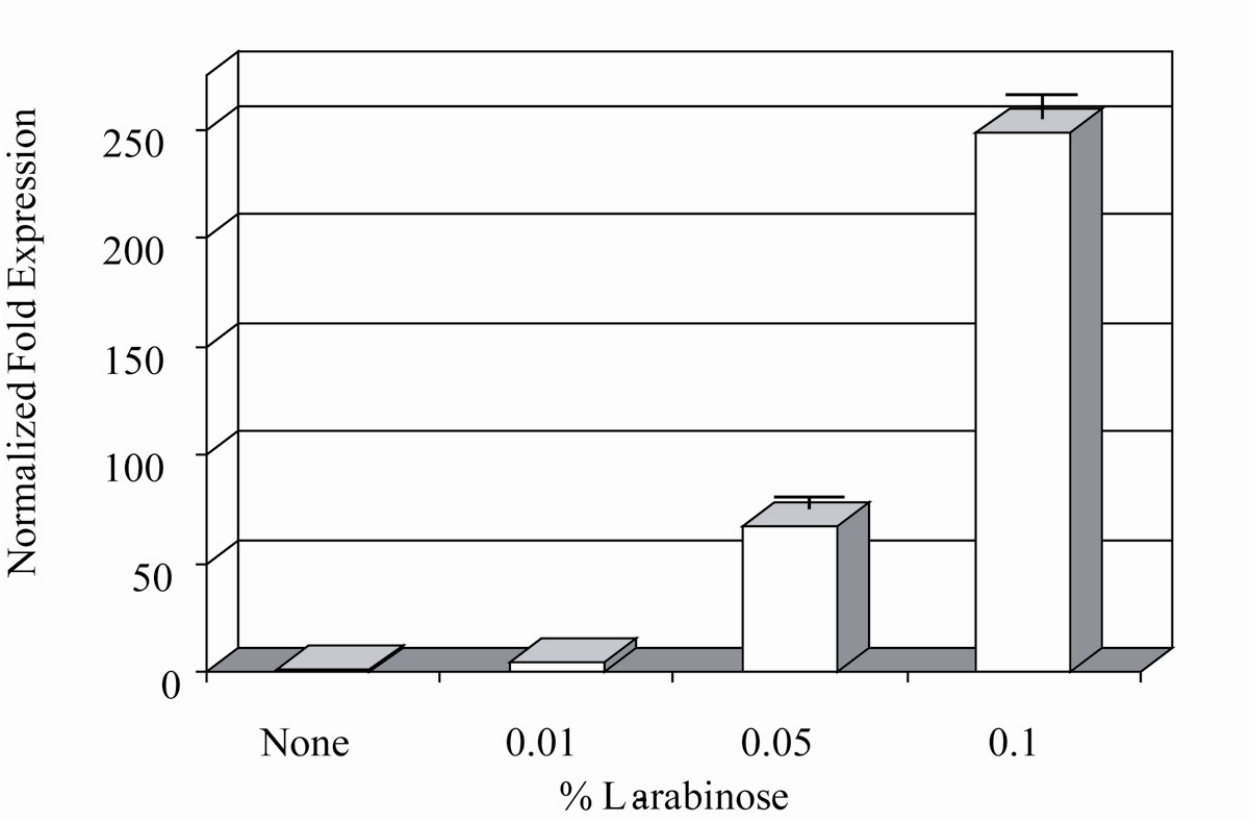

Figure 2. Arabinose-regulated *lacZα* expression in *B. pseudomallei*. RNA was extracted from log phase *B. pseudomallei* Bp50 and *P. aeruginosa* PAO1 cells harboring pHERD30T that either had no arabinose added (None) or were induced for 2 h by addition of the indicated amounts of L-arabinose. Quantitative real-time PCR was performed using *lacZα* specific primers. Data were normalized using the 23S rRNA gene as the housekeeping control.

NH_2-terminus of the target protein may also serve as an epitope tag for protein analysis. pHERD vectors can be readily transferred from *E. coli* into *Pseudomonas* species and other bacteria via triparental conjugation (7) or by electroporation. It has been shown that the progenitor plasmid pRO1614 could replicate in a series of bacterial species, including *P. aeruginosa*, *P. putida*, *P. fluorescens*, *Klebsiella pneumoniae* (19) and *Burkholderia* spp. (5, 27). Therefore, the pHERD vectors are most likely functional in these bacteria. Another feature of the P_{BAD} promoter is catabolite repression of expression in the presence of glucose in the growth medium, which reduces intracellular cAMP concentrations in *E. coli* cells, preventing transcriptional activation of many genes by the cAMP-binding protein (8).

We have observed pHERD vectors can be used for the high-fidelity cloning of toxic and essential genes due to the low level of the P_{BAD} transcription in the absence of L-arabinose (10). Initial attempts to clone the *P. aeruginosa* alternative sigma factor *algU* into pUCP20T were not successful. All *algU* alleles cloned were not functional and sequence analysis showed only mutant *algU* alleles were cloned into pUCP20T. This was consistent with the previous observations that *algU/T* can not be cloned into the common expression vectors (16, 22). However, the *algU* gene was readily cloned into pHERD20T. Upon expression of *algU* from P_{BAD} on pHERD20T, we observed dose-dependent alginate production in *P.aeruginosa* strain PAO1 in response to arabinose in the growth media (Figure 3).

Validation of pHERD20T in *P. aeruginosa* by modulating alginate production. Overexpression of the small peptide encoded by *mucE*, activates AlgW inducing alginate production in *P. aeruginosa* PAO1 and PA14 (20). Overexpression of *mucE* caused mucoidy in *P.aeruginosa* and PAO1 and *P.fluoresences* Pf-5 (Table 2). The C-terminal WVF signal coded by *mucE* is required for activation of AlgW. The outermembrane protein OprF does not

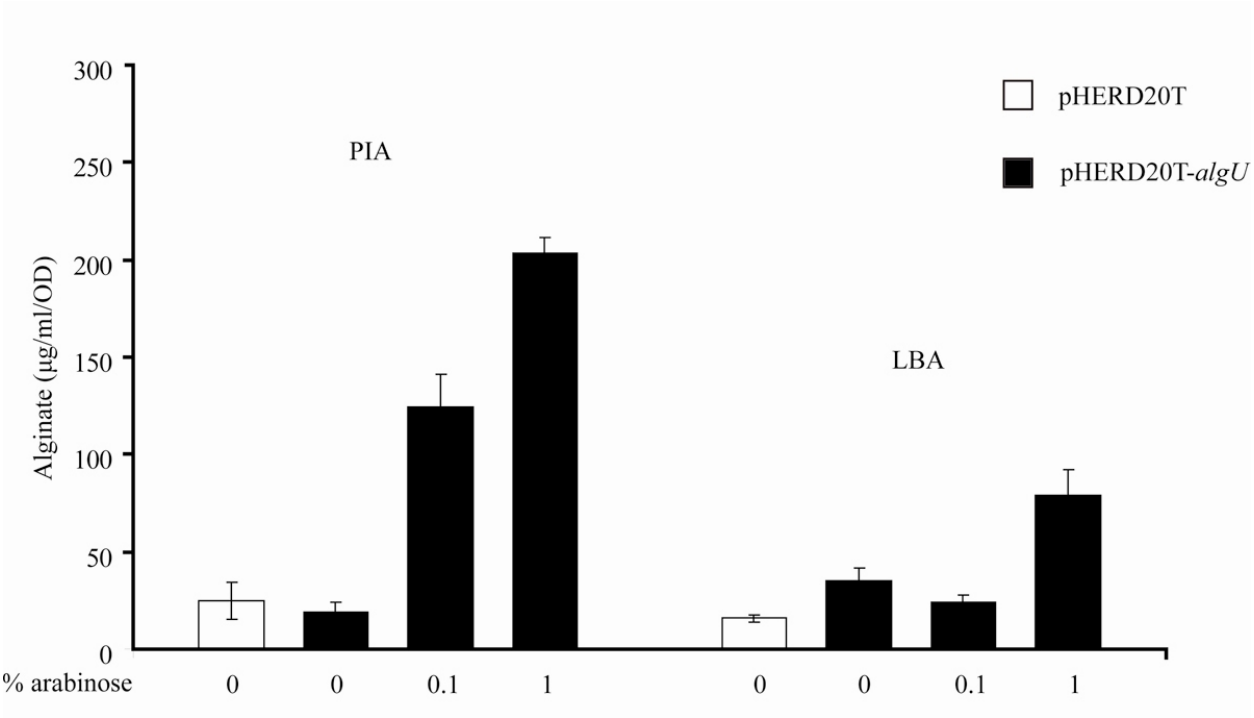

Figure 3. Arabinose-dependent induction of alginate production in *P. aeruginosa* PAO1 carrying pHERD20T-*algU*. PAO1 with pHERD20T-*algU* was grown at 37˚C for 24 h on PIA and LB plates supplemented with carbenicillin and 0%, 0.1% and 1.0% of arabinose, respectively. The empty pHERD20T vector was used as the control (open box). Bars indicate means with standard error. OD, optical density.

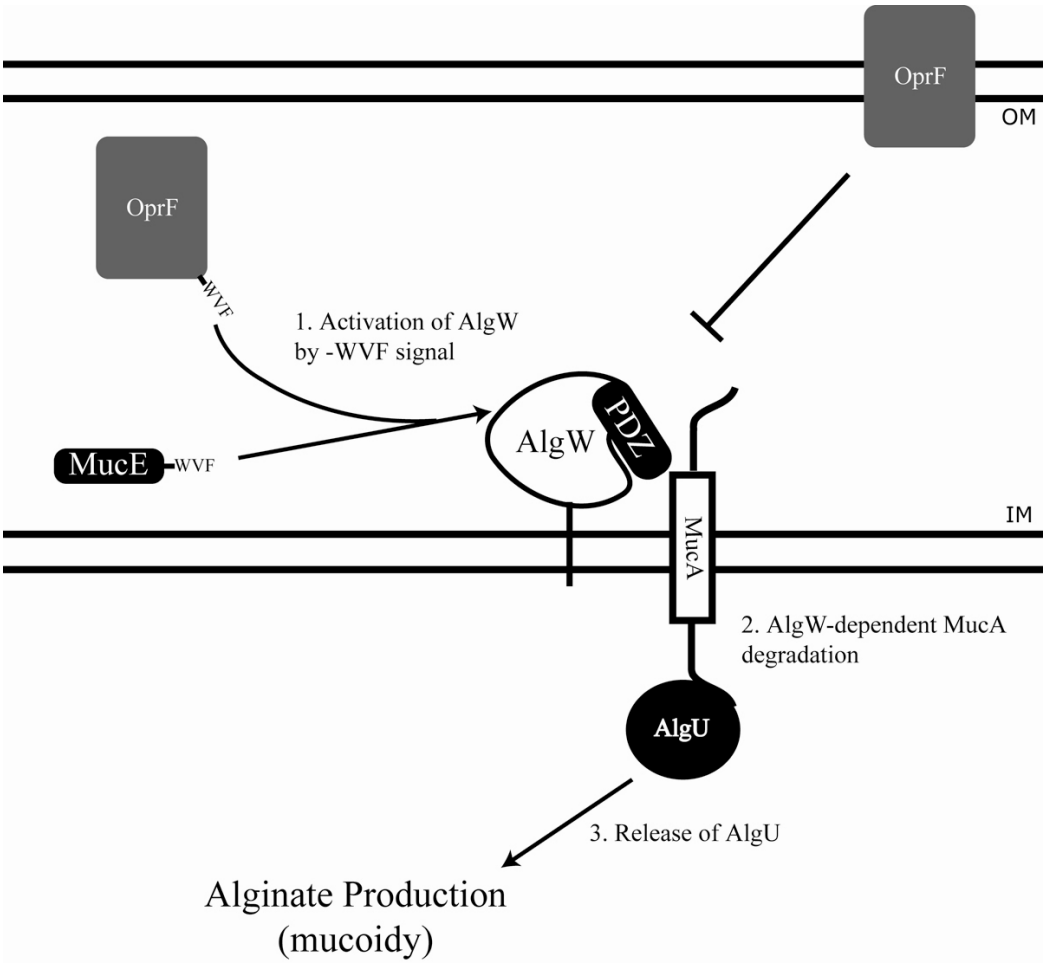

Figure 4. Regulated Alginate Production in *P. aeruginosa*. Regulation of alginate production in *P.aeruginosa* involves many genes coding for products of many different functions. Mucoidy or alginate production is directed by the alternative σ^{22}, AlgU (14). MucA is the cognate anti-sigma factor that negatively regulates AlgU activity by sequestering AlgU to the inner membrane (23). Sequestering of AlgU by MucA can be relieved by either mutation of *mucA* (15) or by proteolytic degradation of MucA by the intramembrane protease AlgW (21). Derepression of MucA causes AlgU activation and alginate production**.**

Table 2. Modulation of mucoidy in *P. aeruginosa* and *P. fluorescens* by pHERD20T-borne alginate regulators. M indicates mucoid and NM indicates nonmucoid phenotypes.

Strain (genotype)	Colony morphology	Plasmid	Colony morphology with plasmid				
			1% gluc	0% ara	0.1% ara	1% ara	2.5% ara
P.aeruginosa							
PAO1	NM	pHERD20T-*algU*	NM	NM	M	M	NM
		pHERD30T-*mucE*	NM	NM	M	M	M
		pHERD20T-*oprF*		NM	NM		
		pHERD20T-*oprF*-WVF		NM	M		
PAO1VE2Δ*algW* (P_{Gm}-*mucE* Δ*algW)*	NM	pHERD20T-*algW*	NM	M	M	NM	NM
P.fluorescens							
Pf-5	NM	pHERD30T-*mucE*		NM	M		

activate alginate production (Figure 4), however addition of the MucE WVF signal motif to the C-terminal of OprF did cause alginate production (Table 2). Some genes are not highly expressed and therefore expression in trans for complementation needs to be conditional. Expression of *algW* from P_{BAD} can complement an *algW* mutant back to alginate due to titratable expression (Table 2).

In summary, we constructed a series of small *Escherichia-Pseudomonas* shuttle vectors with the *E. coli araC* and P_{BAD} promoter for highly-regulated expression of cloned genes in *Pseudomonas* species and other bacteria and demonstrated their utility by modulation of alginate production. These results demonstrate that pHERD vectors are useful tools for bacterial physiological research and gene function studies in the pseudomonads, as well as other bacteria, including medically significant *Burkholderia* spp.

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CHAPTER 3: *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis

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Running title: Negative regulation of alginate biosynthesis by KinB

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ABSTRACT

Mucoidy or overproduction of the exopolysaccharide known as alginate in *Pseudomonas aeruginosa* is a poor prognosticator for lung infections in cystic fibrosis. Mutation of the anti-σ factor MucA is a well-accepted mechanism for mucoid conversion. However, certain clinical mucoid strains of *P. aeruginosa* have a wild-type (wt) *mucA*. Here, we describe a loss-of-function mutation in *kinB* that causes overproduction of alginate in the wt *mucA* strain PAO1. KinB is the cognate histidine kinase for the transcriptional activator AlgB. Increased alginate production due to inactivation of *kinB* was correlated with the high expression at the alginate-related promoters P_{algU} and P_{algD}. Deletion of alternative σ factor RpoN (σ^{54}) or the response regulator AlgB in *kinB* mutants decreases alginate production to wt nonmucoid levels. Mucoidy was restored in the *kinB algB* double mutant by expression of wt AlgB or phosphorylation defective AlgB.D59N, indicating that phosphorylation of AlgB was not required for alginate overproduction when *kinB* was inactivated. The inactivation of the DegS-like protease *algW* in the *kinB* mutant caused loss of alginate production and an accumulation of the hemagglutinin (HA)-tagged MucA. Furthermore, we observed the *kinB* mutation increased the rate of HA-MucA degradation. Our results also indicate AlgW-mediated MucA degradation requires *algB* and *rpoN* in the *kinB* mutant. Collectively, these studies indicate KinB is a negative regulator of alginate production in wt *mucA* strain PAO1.

INTRODUCTION

Cystic fibrosis (CF) patients are predisposed to bacterial respiratory infections due to the mucus buildup in their airways (17). Mutation of the chloride ion transporter called CFTR creates a hospitable environment for the opportunistic pathogen *Pseudomonas aeruginosa* (27). The emergence of mucoid or alginate-overproducing strains marks the beginning of chronic infection by *P. aeruginosa* (13). The presence of mucoid strains causes significant deterioration of lung function (40). Mucoid strains produce alginate by increasing transcription of the *algD* promoter of the alginate biosynthetic operon (Fig. 1) (11). The first molecular mechanism for the conversion to mucoidy elucidated was mutation of the *mucA* gene (32). MucA is the anti-σ factor that sequesters the alternative sigma factor AlgU (also called AlgT or σ^{22}) (Fig. 1) (33, 46). When MucA is not functional due to mutation, increased transcription directed by AlgU at the *algD* promoter (P_{algD}) activates alginate biosynthesis (Fig. 1) (57).

Activation of alginate production by AlgU is controlled at transcriptional and post-translational levels (Fig. 1). Transcription of *algU* occurs from multiple promoters two of which are AlgU-dependent (12, 45) therefore AlgU autoregulates its expression. Alginate production is also negatively controlled by MucB and MucD which are coded downstream of *algU* and *mucA*. MucB cooperates with MucA-AlgU sequestering presumably by protecting the periplasmic portion of MucA from degradation thus stabilizing MucA-AlgU interaction (46). Inactivation of *mucB* in wt *mucA* strain causes elevated alginate production (31). MucD is homologous to DegP of *E. coli* which degrades unfolded proteins in the periplasm (22) and also functions as a chaperone (49). In *P. aeruginosa, mucD* inactivation causes alginate overproduction and sensitivity to H_20_2 and heat (6).

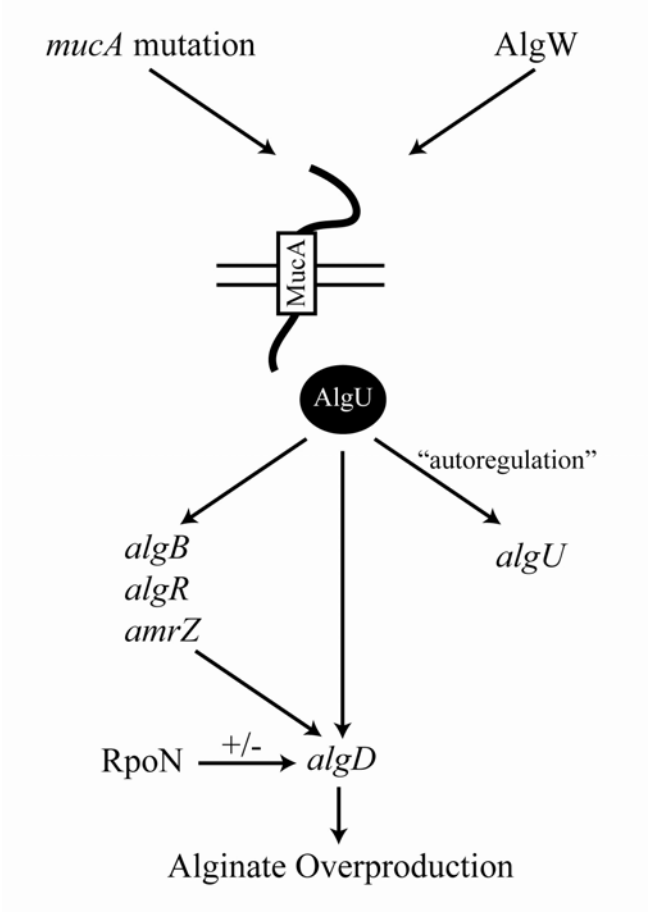

Figure 1. MucA-AlgU is the central regulatory pathway controlling the expression of mucoid phenotype in *P. aeruginosa*. In *mucA* mutants, AlgU is not repressed (32) and activates transcription of downstream promoters. The *algU* gene is transcribed by 5 promoters two of which (P1 and P3) are dependent on AlgU (46). AlgU activates transcription of *algB*, *algR*, and *amrZ* whose gene products participate in transcriptional activation of P_{algD} (4, 38, 56). In a *muc-23* mucoid mutant RpoN has also been shown to bind to P_{algD} and activate or repress transcription during certain environmental conditions (7). AlgU also activates transcription of P_{algD} (57). Activation of transcription at P_{algD} results in alginate overproduction and mucoid phenotype. MucA is the anti-σ-factor that sequesters AlgU(T) (46). The predicted protease AlgW can cleave MucA which results in derepression of AlgU. Overexpression of the periplasmic peptide, MucE, results in mucoidy due to activation of AlgW (43) which leads to degradation of MucA. Cell wall inhibitors such as D-cycloserine have been shown to up-regulate AlgW-dependent transcription at P_{algD} (54).

There is a high level of conservation between *E. coli* σ^E-RseA and *P. aeruginosa* AlgU-MucA. Activation of σ^E occurs after sequential proteolytic cleavage of the anti-σ factor, RseA, first by activated DegS and finally by RseP proteases (3). DegS is a serine protease that is activated in response to unfolded proteins via a conserved C-terminal sequence (51, 52). This conserved signal transduction pathway is referred to as regulated intramembrane proteolysis (1). In *P. aeruginosa*, AlgU is associated with the inner membrane and MucA in wild type, non-mucoid strains (44). Recently, the *P. aeruginosa* DegS homologue, AlgW, has been shown to activate alginate production through regulated proteolysis of MucA in response to increased expression of *mucE* (Fig. 1)(43). Also, the cell wall inhibitor, D-cycloserine can activate the AlgU-stress response in *P. aeruginosa* dependent upon AlgW (Fig. 1) (54).

When MucA does not repress AlgU, transcriptional activation at P_{algD} and alginate overproduction occurs. Significant research has focused on the multitude of regulators that bind and or regulate transcriptional activity at P_{algD}. Most P_{algD} transcriptional regulators are AlgU-dependent such as AlgR, AmrZ, and AlgB (Fig. 1). The response regulator AlgR, binds multiple sites within P_{algD} and is required for P_{algD} expression (21, 38). Additionally, the alginate and motility regulator Z (AmrZ) also promotes activity at P_{algD} (5, 50). The NtrC-family response regulator AlgB has recently been shown to bind at P_{algD} and cause transcriptional activation (26). Beyond AlgU and the AlgU-dependent transcription factors, a second alternative sigma factor, RpoN, has been suggested to have dual roles as both a positive and a negative regulator at P_{algD} (Fig. 1) (7).

The P_{algD} transcriptional regulator, AlgB (15, 26, 56) is a response regulator of a two-component signal transduction system. Typically two-component signal transduction systems are comprised of a response regulator and a sensor kinase. Upon phosphorylation of the

response regulator by the sensor kinase, the response regulator binds specific DNA sequences near a promoter and modulates transcription. The *E. coli* homologue of AlgB, known as NtrC, activates phosphorylation-dependent transcription at target promoters with the σ^{54}-holoenzyme (24). σ^{54} (RpoN) is required for mucoidy in a *P. aeruginosa* prototype strain (*muc23*) (7), but *rpoN* is not required for alginate synthesis in several different *mucA* mutant strains (7, 37, 38). AlgB is a NtrC-family response regulator that mediates alginate biosynthesis in *mucA* mutants (16). The primary role of AlgB that has been elucidated thus far has been transcriptional activation of P_{algD} (26). KinB is the cognate sensor kinase of AlgB (29) and furthermore KinB is capable of autophosphorylation and transfer of phosphate to AlgB (29). Interestingly, phosphorylation of AlgB is not required for P_{algD} activation (28). Unlike *algB*, *kinB* is not required for alginate production in a *mucA22* mutant (28).

Previous extensive research has focused on regulation of alginate production in *mucA* mutant strains. However, recent data shows *algD* expression can occur independent of *mucA* mutations by regulated proteolysis of MucA (43, 54). Studies have shown expression of *algD* is increased in anaerobic conditions (9, 19) which may occur in the CF lung (39). Given the data that *P. aeruginosa* can produce alginate irrespective of *mucA* mutation, we sought to further characterize mucoidy in wild type *mucA* strain PAO1. In this report, we present data that shows inactivation of *kinB* in nonmucoid *P. aeruginosa* strain PAO1 results in alginate overproduction that requires the predicted protease AlgW. We observed that *algB* and *rpoN* are also required in *kinB* mutants for alginate production and high P_{algU} and P_{algD} expression. We also show evidence of regulated MucA degradation in *P. aeruginosa*. A novel role for AlgB and RpoN is proposed in signal transduction of regulated proteolysis to release AlgU from sequestering by MucA in the

kinB mutant background. Our results support a model that KinB negatively regulates the AlgU-signal transduction pathway in *P. aeruginosa* strain PAO1.

MATERIALS AND METHODS

Bacterial strains, plasmids, transposons, growth conditions and oligonucleotides. Bacterial strains, plasmids, and transposon used in this study are indicated in Table 1. *P. aeruginosa* strains were grown at 37° C in Lennox broth (LB), on LB agar or *Pseudomonas* isolation agar plates (PIA; Difco, Sparks, MD). PIA plates were prepared containing 20 ml of glycerol per liter as recommended by the manufacturer. When necessary, PIA media was supplemented with carbenicillin, tetracycline, or gentamicin at a concentration of 300μg/ml. The sequences of the primers used in this study are available upon request.

Transposon mutagenesis. The mariner transposon-containing plasmid pFAC (53) was introduced into PAO1 by biparental conjugations. The locations of the transposon insertion of the mucoid mutants were determined by inverse PCR (42, 43). The chromosomal DNA of these strains were digested with *Sal*I and ligated to generate circular closed DNA molecules (Fast-Link DNA ligation kit; Epicentre, Madison, WI). The ligated DNA was then used as the template for inverse PCR using primers (Gm3OUT and Gm5OUT) as previously described (42) that anneal to the gentamicin resistance gene (Gm^r). The resulting amplicons were sequenced by Marshall University Genomics Core Facility.

Mutant strain construction. For in-frame deletion of specific genes (*algU*, *algB, algB-kinB*, *algW, kinB, rpoN*), the upstream and downstream sequence fragments (500-1,000bp) flanking the target gene were PCR amplified and fused by using the cross-over PCR method. The PCR products with the in-frame deletion of target gene were digested and ligated into pEX100T-*Not*I

vector. A two-step allelic exchange procedure was employed with the pEX100T constructs for in-frame deletion. The single cross-over merodiploid exconjugants were selected based on carbenicillin resistance and for sensitivity on PIA supplemented with 10% (w/v) sucrose (*sacB*). After incubation of the merodiploids in LB broth at 37°C, the double cross-over recombinants were isolated on PIA 10% sucrose. The in-frame deletion of the target gene was confirmed by antibiotics-resistance assays and then PCR amplification of the flanking region of target gene with multiple sets of primers, and amplicon sequencing.

Plasmid construction and complementation analyses. Alleles were cloned into the shuttle vector pHERD20T (41) for complementation with gene expression driven by P_{BAD} arabinose-inducible promoter. For *lacZ* reporter analysis, *algU* and *algD* promoters were fused with *lacZ* in miniCTX-*lacZ* (20) (Fig. 3A and 3C respectively). All plasmid constructs containing PCR products were sequenced and confirmed that no mutations occurred.

Alginate assay. *P. aeruginosa* strains were grown at 37˚C on PIA plates supplemented with carbenicillin and 0.1% w/v arabinose for 24hr. Bacterial growth was removed from plates with PBS and suspended in 50ml of PBS per plate. The optical density at OD_{600} of bacterial suspension in PBS was measured and adjusted. Cell suspensions containing bacterial alginates were used for assay of the amounts of the uronic acid using a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich, St. Louis, MO) in the range of 0-100μg/ml as described (23).

β-galactosidase activity assay. The promoter fusion constructs miniCTX-P_{algU}-*lacZ* and miniCTX-P_{algD}-*lacZ* were integrated onto the *P. aeruginosa* chromosome at the CTX phage *att* site (20). β-galactosidase activity assay was based on the method as originally described by Miller (36) with the following modification. The cells were grown on PIA plates in triplicate for

24hr at 37° C and harvested in PBS. The β-galactosidase activity was assayed after toluene permeabilization of the cells. The reported values represent the average in triplicate of three independent experiments. The values displayed are normalized to PAO1 pHERD20T for each respective promoter fusion.

Western blot analysis. Cell lysates were prepared with Ready-Preps (Epicentre, Madison, WI) by manufacturer's protocol. Cell lysates were quantified by D_C assay (BIO-RAD, Hercules, CA). 40μg of protein was boiled in SDS-loading buffer. The samples were electrophoresed on 12% polyacrylamide gels or 15% ProteaGel (Protea Morgantown, WV) SDS-PAGE polyacrylamide and then electroblotted (Trans-Blott Cell, BIO-RAD, Hercules, CA) on to 0.45μm nitrocellulose. The membrane was blocked with 3% non-fat dry milk in PBS (pH 7.4). Primary antibodies were diluted 1:1000 in 3% non-fat dry milk in PBS. The membranes were probed with mouse monoclonal antibodies against AlgU (46), RpoN (Neoclone, Madison, WI), alpha RNA subunit polymerase subunit (Neoclone), rabbit polyclonal antibody against AlgB (28) or rat monoclonal antibody against HA (Roche, Mannheim, Germany) overnight at 4°C with shaking. HRP-labeled goat anti-mouse IgG or HRP-labeled anti-rabbit IgG were diluted 1:5000 in 3% non-fat dry milk in PBS and used as the secondary antibodies. Advanced ECL or ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ) was used for detecting HRP-labeled goat anti-mouse IgG or anti-rabbit IgG (Roche) by the manufacturer's procedure. The signals were detected with an EC3 Imaging System (UVP, Upland, CA) by capturing with a BioChemi HR camera. For re-probing, membranes were stripped with 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol for 15 min at 50°C and then washed in PBS.

HA-MucA steady state and kinetic concentration Western blots analysis. To assay the HA-MucA degradation profile after 48 hours of growth at 37° C, HA-MucA was expressed in *trans*

in mucoid and nonmucoid strains from pHERD20T with 0.1% arabinose on PIA carbenicillin plates. Cells were scraped from the plate suspended in PBS pH 7.4 and pelleted by centrifugation. Proteins were isolated and prepared as described for Western blot analysis.

To observe the rate of degradation of HA-MucA in PAO1 and PAO1*kinB::aacC1* we analyzed cell lysates over a time course. We utilized the conditional expression of HA-MucA from pHERD20T to compare the rate of degradation. Cultures of PAO1 and PAO1*kinB::aacC1* were incubated overnight at 37° C in LB carbenicillin 100 μg/ml. The cultures were OD matched and equal number cells were inoculated into 500ml of LB carbenicillin (100 μg/ml) supplemented with 1% w/v arabinose to induce expression of HA-*mucA*. At OD_{600} of 0.2 the cells were harvested by centrifugation at 7000 x g for 10min. The cells were then resuspended in 500ml of M9 broth with 0.4% glucose supplemented with 100 μg/ml carbenicillin. Samples were taken at ten min intervals by harvesting 50ml of culture at 4000 x g at 4° for 10min. Pellets were immediately stored at -80° until proteins were prepared with Ready-Preps (Epicentre, Madison, WI) by manufacturer's protocol and submitted to Western blot analysis with anti-HA and anti-alpha RNA polymerase subunit antibodies.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Phenotype, genotype, and description [a]	Source
P. aeruginosa		
PAO1	Alg$^-$ Prototroph	P. Phibbs
PAO1Δ*algB*	Alg$^-$, PAO1 in-frame deletion of *algB* (PA5483)	This study
PAO1Δ*algU*	Alg$^-$, PAO1 in-frame deletion of *algU* (PA0762)	This study
PAO1Δ*kinB*	Alg$^+$, PAO1 in-frame deletion of *kinB* (PA5484)	This study
PAO1Δ*rpoN*	Alg$^-$, PAO1 in-frame deletion of *rpoN* (PA4462)	This study
PAO1*kinB::aacC1*	Alg$^+$, PAO1 *kinB*:: Gm^r	This study
PAO1*kinB::aacC1*Δ*algU*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *algU* (PA0762)	This study
PAO1Δ*algB*Δ*kinB*	Alg$^-$, PAO1 in-frame deletion of *algB* (PA5483) and *kinB* (PA5484)	This study
PAO1*kinB::aacC1*Δ*algW*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *algW* (PA4446)	This study
PAO1*kinB::aacC1*Δ*rpoN*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *rpoN* (PA4462)	This study
E. coli		
DH5α	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Lab strain
TOP10	DH5α derivative	Invitrogen
SM10/λpir	*thi recA thr leu tonA lacY supE RP4-2-Tc: Mu 1::pir* Km^r	Lab strain
Plasmids		
pRK2013	Km^r *Tra Mob ColE1*	(14)
pFAC	Mini-*himar1* mariner transpson in *Pseudomonas* suicide plasmid; Ap^r Gm^r	(53)
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^r Km^r	Invitrogen

pUS56	*algB45* in pTrcHisA *Bam*H1-*Eco*RI	(28)
pHERD20T	pUCP20T P_{lac} replaced by 1.3 kb *Afl*II-*Eco*RI fragment of *araC*-P_{BAD} cassette	(41)
pHERD20T-*algU*	*algU* (PA0762) from PAO1 in pHERD20T *Eco*RI/*Hind*III	This study
pHERD20T-*algB*	*algB* from PAO1in pHERD20T *Eco*RI/*Hind*III	This study
pHERD20T-*algB45*	*algB45* from pUS56 in pHERD20T *Eco*RI/*Hind*III	This study
pHERD20T-*algW*	*algW* (PA4446) from PAO1in pHERD20T *Eco*RI/*Hind*III	This study
pHERD20T-MPDZ*algW*	*algW* with partial PDZ domain in pHERD20T *Eco*RI/*Hin*dIII	This study
pHERD20T-ΔPDZ*algW*	*algW* with complete deletion of PDZ domain in pHERD20T *Eco*RI/*Hin*dIII	This study
pHERD20T-*kinB*	*kinB* from PAO1 in pHERD20T *Kpn*I/*Hin*dIII	This study
pHERD20T-HA-*mucA*	N-terminal tagged HA-*mucA* in pHERD20T *Eco*RI/*Hin*dIII	This study
pUCP20T-P_{BAD}-*rpoN*	*araC*- P_{BAD}-*rpoN* fusion in pUCP20 *Xba*I/*Hind*III	This study
miniCTX-*lacZ*	Gene delivery vector for inserting genes at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^r	(20)
miniCTX-P_{algU}-*lacZ*	Complete P_{algU} promoter (541 bp upstream of ATG *Eco*RI/*Hind*III fused with *lacZ* for integration at the CTX phage *att* site in *P. aeruginosa*	This study
miniCTX-P_{algD}-*lacZ*	Complete P_{algD} promoter (1,525 bp upstream of ATG) *Hind*III/*Bam*H1 fused with *lacZ* for integration at the CTX phage *att* site in *P. aeruginosa*	This study
pEX100T	*Pseudomonas* suicide vector, *sacB*, *oriT*, Cb^r	(47)
pEX100T-*Not*I	*Pseudomonas* suicide vector with *Not*I restriction site fused into *Sma*I of pEX100T, *sacB*, *oriT*, Cb^r	This study
pEX100T-Δ*algU*	A 2.5-kb fragment flanking *algU* gene fused with pEX100T-*Not*I with in-	This study

	frame deletion of *algU* with only 24 bp left coding for 8 amino acids of *algU*.	
pEX100T-Δ*algB*	A 1.5-kb fragment flanking *algB* gene fused with pEX100T-*Not*I with in-frame deletion of *algB*	This study
pEX100T-Δ*algB*Δ*kinB*	A 1.5-kb fragment flanking *algB* gene fused with pEX100T-*Not*I with in-frame deletion of *algB* and *kinB*	This study
pEX100T-Δ*algW*	A 1.4-kb fragment flanking *algW* gene fused with pEX100T-*Not*I with in-frame deletion of *algW*	(43)
pEX100T-Δ*kinB*	A 2.5-kb fragment flanking *kinB* gene fused with pEX100T-*Not*I with in-frame deletion of *kinB*	This study
pEX100T-Δ*rpoN*	A 1.9-kb fragment flanking *rpoN* gene fused with pEX100T-*Not*I with in-frame deletion of *rpoN*	This study

[a] Alg^-, non-mucoid phenotype and Alg^+, mucoid phenotype

RESULTS

Inactivation of *kinB* in *P. aeruginosa* strain PAO1 results in alginate overproduction. To discover novel negative regulators of alginate biosynthesis, the standard genetic strain PAO1 was subjected to mariner transposon mutagenesis (53). Stable mucoid gentamicin-resistant mutants were isolated. Mucoid mutants were verified for single transposon insertions by Southern hybridization (data not shown) and the pFAC transposon insertions were mapped by inverse PCR and sequencing as previously described (42, 43). Numerous mucoid mutants were identified with insertions into the well characterized negative regulators, *mucA*, *mucB*, and *mucD*. Interestingly, an insertion into *kinB* converted PAO1 to mucoid phenotype (GenBank accession for the *kinB* insertion in PAO1: FJ209363) (Fig. 2A). To show mucoidy due to *kinB* inactivation was not caused by polar effects on nearby genes, we constructed an in-frame deletion of *kin*B in PAO1. Alginate overproduction results when *kinB* is deleted (Fig. 2A). However, PAO1Δ*kinB* produced less alginate at 62 ± 4 μg/ml/OD_{600} vs. 103 ± 10 of PAO1*kinB::aacC1* (Fig 2A). The mucoid phenotypes of PAO1*kinB::aacC1* and PAO1Δ*kinB* were complemented by conditional expression of *kinB* (Fig. 2A). Expression of *kinB* in *trans* in PAO1*kinB::aacC1* and PAO1Δ*kinB* decreased alginate production to wt PAO1 levels as expected (Fig. 2A). Furthermore, sequencing analysis confirmed the *mucA* of PAO1*kinB::aacC1* did not harbor mutations (GenBank accession: FJ209362). Thus, inactivation or deletion of *kinB* in wt *mucA* background causes alginate overproduction. This suggests that KinB is a negative regulator of alginate in *P. aeruginosa* strain PAO1.

Alginate production in *kinB* mutants requires *algB* and *rpoN*. Alginate overproduction in *mucA* mutants, requires AlgB, an NtrC type of transcriptional activator (56). The *algB* gene is located immediately upstream of *kinB* in the genome. The *kinB* gene encodes the cognate kinase

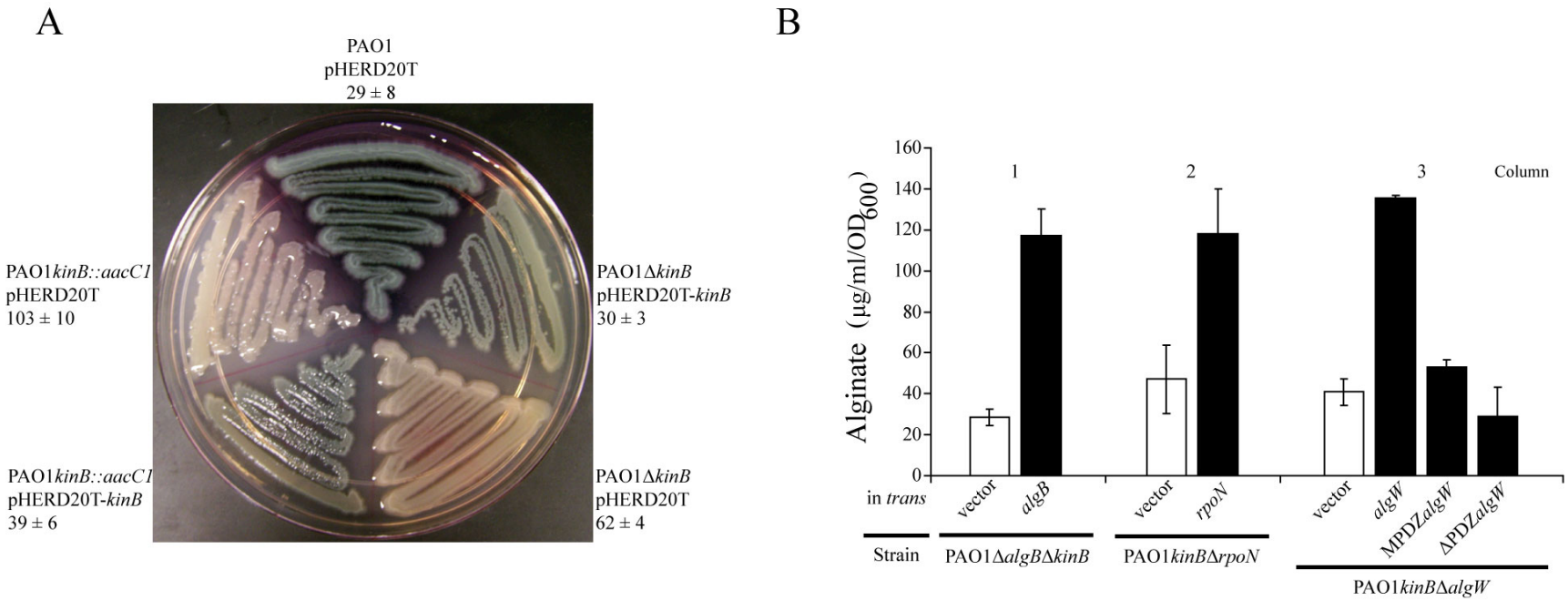

Figure 2. Mutation of *kinB* in PAO1 results in mucoid phenotype dependent upon *algB, rpoN,* and *algW*. 2A. Colony morphologies of *P. aeruginosa* PAO1, and mucoid *kinB* mutants with or without *kinB* expressed in *trans*. For complementation, *kinB* was expressed from the P_{BAD} promoter of pHERD20T. Strains were grown on a PIA carbenicillin plate supplemented with 0.1% arabinose at 37° C for 24hr and room temperature for 24hr. Alginate production was assayed by the carbazole assay (23) after 24hr at 37° C. The amount of alginate is indicated as μg/ml/OD_{600}. Values are expressed as mean ± SD from three independent experiments. 2B. *kinB* mutants require *algB, rpoN,* and *algW* for alginate overproduction. Each mutant strain was assayed for alginate production with vector control (pHERD20T) or with the gene indicated in *trans* expressed from the P_{BAD} promoter of pHERD20T. The strains were grown for 24hr at 37°C on PIA supplemented with carbenicillin and 0.1% arabinose.

that has been shown to phosphorylate AlgB (29). Deletion of both *algB* and *kinB* together, results in wt nonmucoid alginate production (Fig. 2B column 1). Alginate production was restored in the PAO1Δ*algB*Δ*kinB* double mutant by expression of *algB* in *trans* (Fig. 2B column 1). Since *rpoN* has been shown to be required for alginate production in a mucoid strain with an undefined *muc23* mutation (7), we examined if *rpoN* is required in PAO1*kinB::aacC1*. Deletion of *rpoN* from PAO1*kinB::aacC1* resulted in loss of mucoidy and could be complemented with *rpoN* expressed in *trans* (Figure 2B column 2).

Alginate production in *kinB* mutants requires *algW*. Since the *mucA* gene is not mutated in PAO1*kinB::aacC1*, one possible explanation for the mucoid phenotype is that MucA is being degraded. AlgW has been shown to be required for activation of the alginate biosynthetic operon by D-cycloserine (54) and AlgW mediates regulated proteolysis of MucA during overexpression of *mucE* (43). We next tested whether mucoidy due to loss of *kinB* is dependent upon AlgW-regulated proteolysis. Deletion of *algW* from PAO1*kinB::aacC1* resulted in nonmucoid phenotype and lowered alginate production (Fig. 2B column 3). Expression of *algW* in *trans* restored alginate production (Fig. 2B column 3). The PDZ domain of AlgW is required for MucE-mediated signal transduction (43). Therefore, to show PAO1*kinB::aacC1* utilizes activated AlgW for derepression of MucA, we introduced an *algW* allele with the PDZ domain truncated and an *algW* allele with the PDZ domain completely deleted. When these mutant *algW* alleles were expressed in *trans* in the double mutant PAO1*kinB::aacC1*Δ*algW* alginate overproduction was not restored (Figure 2B column 3). These data suggest activation of AlgW is required for alginate overproduction in PAO1*kinB::aacC1*.

In the absence of *kinB*, phosphorylation of AlgB at D59 is not required for alginate production. KinB has been shown to effectively phosphorylate AlgB *in vitro* (29). However

AlgB derivatives such as AlgB.D59N, which cannot be phosphorylated by KinB, still promote alginate production in *mucA* mutants (28). The *algB45* allele encodes AlgB.D59N where the phosphorylation site (D59) has been mutated to asparagine (N) (28) We presumed that AlgB was not phosphorylated in the absence of the cognate histidine kinase KinB. To confirm phosphorylation of AlgB at position 59 was not required for alginate production in the absence of KinB, we cloned the *algB45* allele into pHERD20T for conditional expression. The *algB45* gene was PCR amplified from pUS56 (28) and directionally cloned. The construct was sequenced to observe the expected D59N mutation and to ensure no other mutations resulted. Expression of *algB45* from the P_{BAD} promoter in the presence of arabinose complemented the PAO581*algB::aacC1* (*mucA25 algB*::Gm^r) mutant (Table 2) (42)which is consistent with the previous finding that *algB45* allele can still promote alginate production in a *mucA22* mutant (28). Since the construct was functional, we introduced *algB45* into PAO1Δ*algB*Δ*kinB*. Alginate overproduction occurred when *algB45* was expressed in PAO1Δ*algB*Δ*kinB* (Table 2). This data suggests that in the absence of KinB, phosphorylation of AlgB at position 59 was not required for mucoidy. Interestingly when we overexpressed *algB* or *algB45* in wt PAO1 and PAO1Δ*algB* we did not observe an increase in alginate production even when cultured on 1% arabinose (data not shown). It seems deletion of *kinB* affects alginate production independent of the phosphorylation status of AlgB. Similar to *mucA* mutants, phosphorylation of AlgB is not required for alginate overproduction in the *kinB* mutant with the wt *mucA* background.

P_{algU} and P_{algD} activity in *kinB* null mutants are dependent on *algU*, *algB*, *rpoN* and *algW*. To examine the effect of the *kinB* mutation on the alginate-related promoters, P_{algU} and P_{algD}, we integrated a single copy of the entire *algU* or *algD* promoter region (Fig. 3A and 3C respectively) fused with *lacZ* onto the chromosome of PAO1 and PAO1*kinB::aacC1* as well as *kinB/algU,*

Table 2. Complementation of alginate production by *algB* mutants with wt *algB* and phosphorylation defective *algB45*

Strain (Genotype)	Plasmid	Arabinose[a]	Phenotype[b]	Alginate ($\mu g/ml/OD_{600}$)
PAO581*algB*::*aacCC1* (*mucA25 algB*::Gm^r)	pHERD20T-*algB*	0%	NM	49.0 ± 7.3
		1%	M	285.7 ± 12.9
	pHERD20T-*algB45*	0%	NM	51.9 ± 3.0
		1%	M	228.2 ± 21.0
PAO1Δ*algB*Δ*kinB*	pHERD20T-*algB*	0%	NM	64.7 ± 10.9
		1%	M	215.7 ± 13.5
	pHERD20T-*algB45*	0%	NM	49.5 ± 2.9
		1%	M	263.8 ± 2.5

[a] Strains were cultured for 24 hr at 37 °C on Pseudomonas Isolation Agar (PIA) supplemented with carbenicillin and arabinose concentration indicated (w/v).
[b] NM and M indicate nonmucoid and mucoid phenotype respectively.

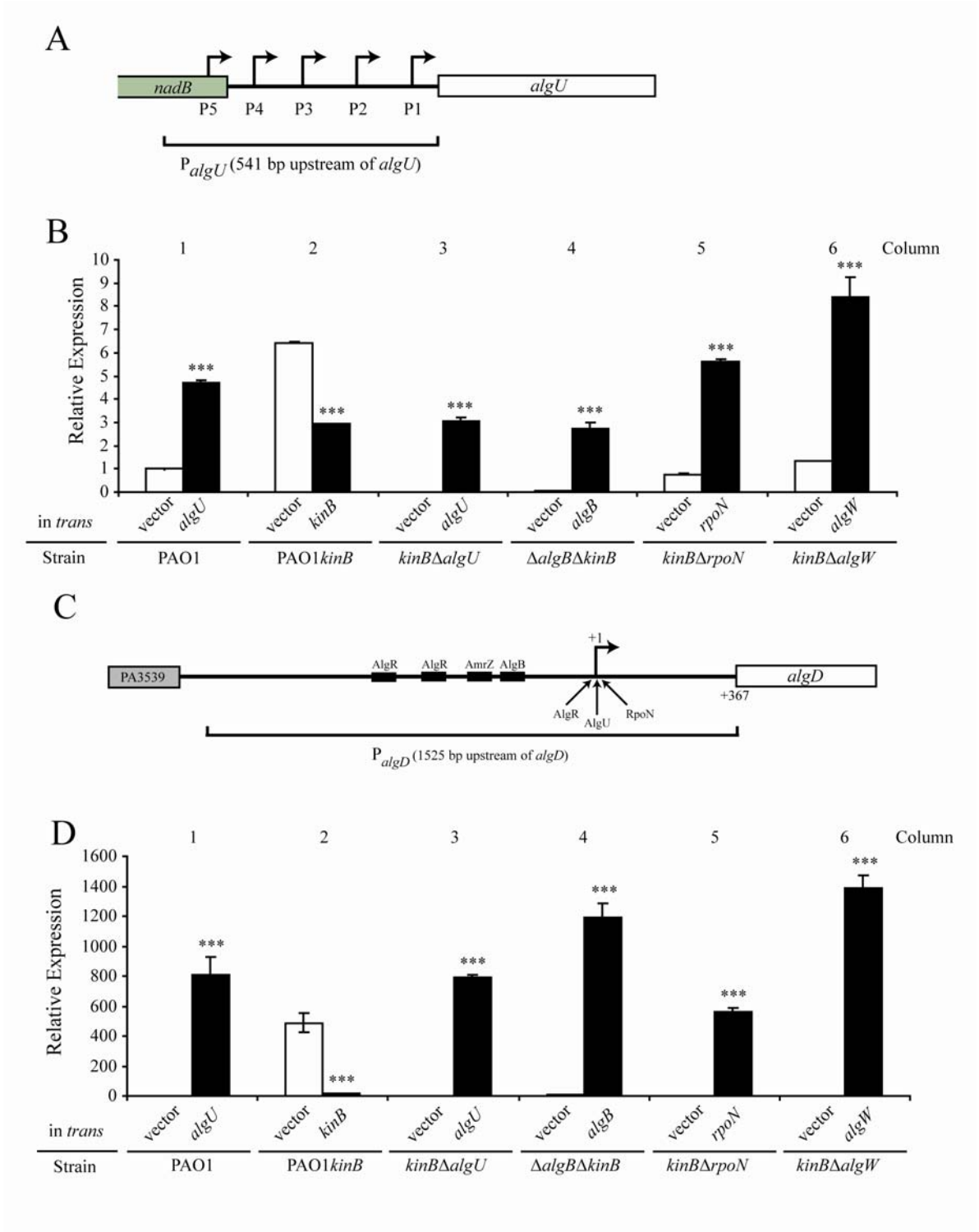
A
nadB
algU
P5
P4
P3
P2
P1
P algU (541 bp upstream of algU)
B
Column
Relative Expression
in trans
Strain
vector
algU
kinB
algB
rpoN
algW
PAO1
PAO1kinB
kinBΔalgU
ΔalgBΔkinB
kinBΔrpoN
kinBΔalgW
C
PA3539
AlgR
AmrZ
AlgB
+1
algD
+367
RpoN
AlgU
P algD (1525 bp upstream of algD)
D
Column
Relative Expression
in trans
Strain

Figure 3. Loss of *kinB* causes upregulation of both P_{algU} and P_{algD}. β-galactosidase activity from P_{algU}-*lacZ* and P_{algD}-*lacZ* reporters on the chromosome of PAO1, PAO1*kinB::aacC1*, and PAO1*kinB::aacC1* isogenic mutants. P_{algU}-*lacZ* and P_{algD}-*lacZ* reporter constructs were integrated into the chromosome of the strains indicated. Genes indicated were expressed in *trans* from the P_{BAD} promoter of pHERD20T. β-galactosidase activities were determined after 24hr growth on PIA with 0.1% arabinose. Values were normalized to PAO1 pHERD20T (empty vector) reporter expression and indicated as mean ± SD from three independent experiments. A student's *t* test was performed for comparison of activity of the strain with vector only or with complementing gene in *trans*. Asterisks indicate significant differences (*** $p<0.0001$). Strain PAO1*kinB::aacC1* is indicated as PAO1*kinB*. Note expression of *algU* in PAO1 is a positive control for the analysis due to the AlgU-dependent nature of both P_{algU} and P_{algD}. 3A. A schematic of the entire P_{algU} promoter region with the relative positions of the five promoters that were utilized for the *lacZ* promoter fusion. 3B. The P_{algU} activity in PAO1, PAO1 *kinB::aacC1* and strains isogenic to PAO1 *kinB::aacC1*. High P_{algU} activity in PAO1 *kinB::aacC1* and PAO1Δ*kinB* mutants requires *algU, algB, rpoN*, and *algW*. Note *kinB* expression significantly lowers P_{algU} activity. 3C. A schematic of the entire P_{algD} promoter region that was for the *lacZ* promoter fusion. The relative binding sites of the P_{algD} transcriptional activators are indicated. 3D. The P_{algD} activity in PAO1, PAO1 *kinB::aacC1* and strains isogenic to PAO1 *kinB::aacC1*. High P_{algD} activity in PAO1 *kinB::aacC1* and PAO1Δ*kinB* requires *algU, algB, rpoN*, and *algW*. Note *kinB* expression significantly lowers P_{algD} activity.

kinB/algB, kinB/rpoN, and kinB/algW double mutants. The effect of each deletion or inactivated gene on the expression of the promoter fusions in the PAO1 and *kinB* backgrounds were assessed by complementation. The β-galactosidase activity was measured with vector alone (pHERD20T) and compared to when the mutation was complemented with expression of the gene from the P_{BAD} promoter of pHERD20T (41)in the presence of 0.1% arabinose. As a control for these experiments, P_{algU} and P_{algD} expression were measured when *algU* was overexpressed (Fig. 3B column 1 and 3D column 1 respectively).

Previously studies have shown only small changes in P_{algU} expression are required for mucoidy (33). Inactivation of *kinB* in PAO1*kinB::aacC1* caused significantly increased P_{algU} expression compared to parent strain PAO1 (Fig. 3B column 2). The high P_{algU} expression of PAO1*kinB::aacC1* can be reduced with *kinB* expressed in *trans* (Fig. 3B column 2). Deletion of *algU* eliminated detectible P_{algU} expression in PAO1*kinB::aacC1* (Fig. 3B column 3). Since *algB* was observed to be required for alginate production in *kinB* mutants, we next examined if *algB* was required for high levels of expression of P_{algU}. The high level of P_{algU} expression in the absence of *kinB* requires *algB* (Fig. 3B column 4). AlgB has been established as a transcriptional activator at P_{algD} in *mucA22* mutant FRD-1 (56). Here we show a possible new role for AlgB above P_{algD}. We also observed that *rpoN* has a role influencing high expression of P_{algU} (Fig. 3B column 5) that can be restored with *rpoN* expressed in *trans*. This information shows a possible role of *rpoN* outside of characterized interactions at P_{algD} (7). As expected, P_{algU} expression is also influenced by the serine protease AlgW in PAO1*kinB::aacC1* (Fig. 3B column 6). However, the level of expression of P_{algU} with *algW* in *trans* exceeded the P_{algU} expression level in PAO1*kinB::aacC1*. A possible explanation for this is *algW* expression from the arabinose

promoter in the presence of 0.1% on a multi-copy vector, may exceed endogenous expression levels of *algW in vivo*.

The P_{algD} expression was measured with the same strategy utilized for P_{algU}. Unlike P_{algU} activity, P_{algD} activity was minimally detectable in PAO1 (Fig. 3D column 1). The elevated level of P_{algD} expression in PAO1*kinB::aacC1* is significantly reduced when *kinB* was expressed in *trans* (Fig. 3D column 2). The elevated level of P_{algD} in *kinB* mutants requires *algU, algB, rpoN*, and *algW* which correlates with the observations of P_{algU} expression. The *kinB* mutants with deletions of *algU, algB, rpoN*, and *algW* had minimally detectable of P_{algD} (Fig. 3D columns 3-6). When *algU*, *algB*, *algW* and *rpoN* were expressed in *trans* to complement their respective gene deletions in *kinB* mutants, elevated P_{algD} expression was returned. Collectively these promoter fusions in the PAO1 and *kinB* backgrounds show *algU*, *algB*, *algW*, and *rpoN* influence the P_{algU} and P_{algD} activity which correlates with alginate production (Fig. 2B).

AlgU and AlgB expression are increased in PAO1*kinB::aacC1*. Next we measured the expression of AlgU and AlgB in the whole cell lysates of PAO1*kinB::aacC1* (Fig. 4A). To control for cross-reactivity of anti-AlgU and anti-AlgB, total lysates of PAO1Δ*algU* and PAO1Δ*algB* were blotted and very low cross-reactivity was noted (Fig. 4A lanes 1 and 5 respectively). Western blot analysis revealed that AlgU was up-regulated 2.6 ± 0.8 fold in PAO1*kinB::aacC1* compared to PAO1 (Fig. 4A lanes 2 and 3). AlgB expression was also increased in PAO1*kinB::aacC1* which is consistent with a previous observation that *algB* transcription requires *algT/U* (57). Interestingly, AlgB was detected in PAO1Δ*algU* cell lysate which suggests AlgB expression may also be controlled by another σ factor in addition to AlgU (Fig. 4A lane 1).

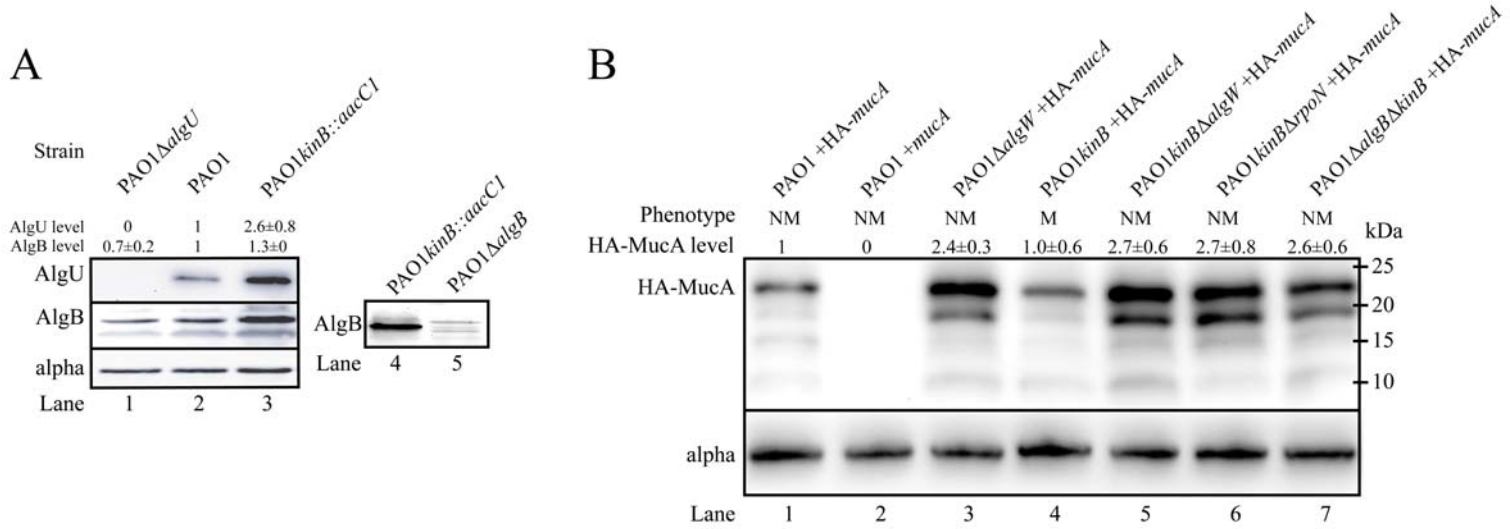

Figure 4. PAO1 *kinB::aacC1* exhibits elevated levels of AlgB and AlgU. HA-MucA degradation in PAO1*kinB::aacC1* requires *algW*, *algB* and *rpoN*. Shown are representative panels of blots from three independent experiments with 40μg of total lysate. 4A. Western blot of total cell lysate of PAO1*kinB::aacC1* shows elevated levels of AlgB and AlgU. Western blots of cell lysates were prepared from cells after 24 hr growth on PIA. The membranes were probed with anti-AlgU, anti-AlgB, and anti-alpha subunit of RNAP antibodies (loading control). Levels of each protein were adjusted for loading and then normalized to PAO1 levels and expressed as mean ± SD. Note that deletion of *algU* did not abolish AlgB expression. 4B. Western blot analysis of N-terminal HA-tagged MucA in PAO1 and PAO1 *kinB::aacC1* isogenic backgrounds. Cell lysates were prepared from 48 hr growth on PIA carbenicillin plates supplemented with 0.1% arabinose. The membranes were immunoblotted with Rat anti-HA diluted 1:1000 (Roche). Lane 2 PAO1 pHERD20T-*mucA* is a negative control for background and cross-reactivity. Lanes 1, 3-7 express HA-*mucA* in *trans* from pHERD20T. Levels of each protein were adjusted for loading and then normalized to PAO1 pHERD20T-HA-*mucA* levels and expressed as mean ± SD. Positions of apparent molecular masses are depicted. NM and M indicate nonmucoid and mucoid phenotypes respectively.

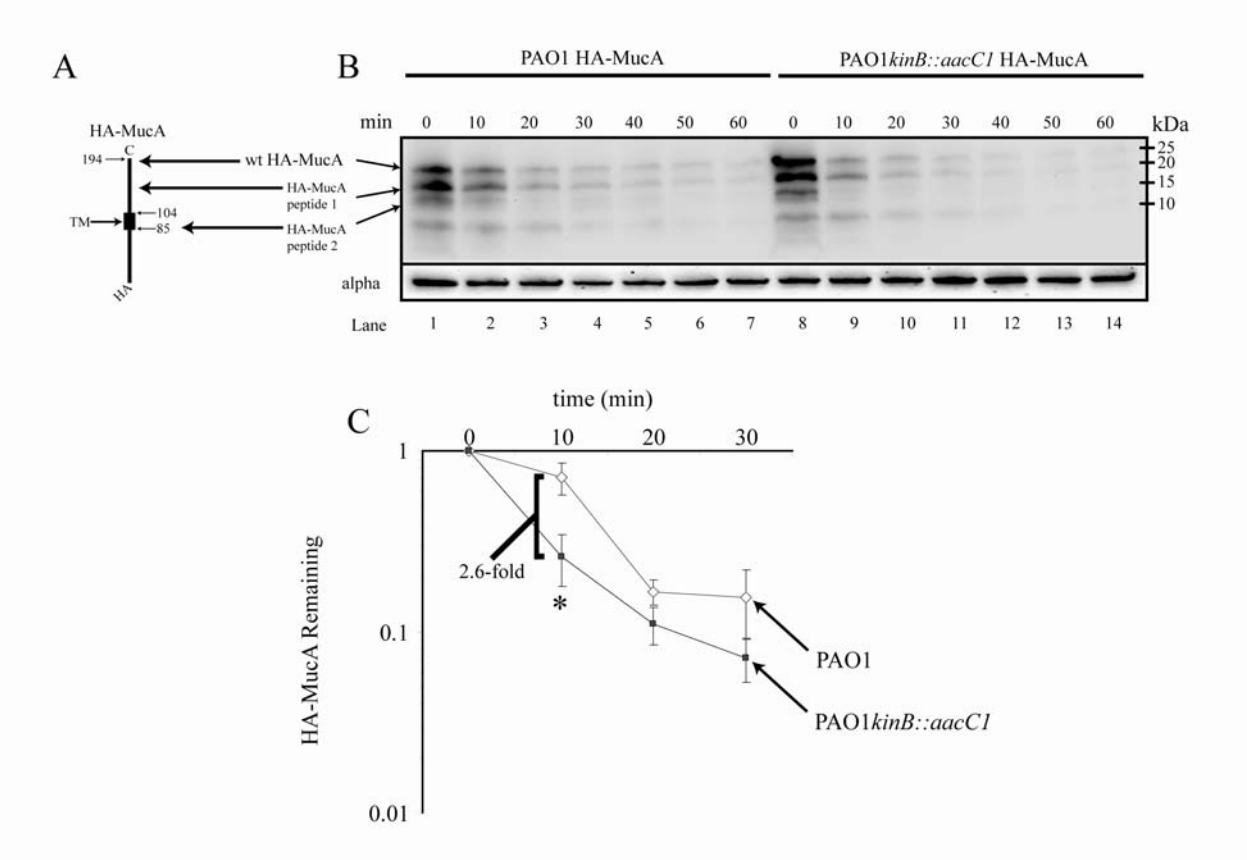

Figure 5. Kinetic comparison of HA-MucA degradation in PAO1 and PAO1*kinB::aacC1*. 5A. Schematic diagram of HA-MucA. Indicated are the N-terminal HA tag, the transmembrane domain, and the relative cleavage sites resulting in the truncated HA-MucA peptides observed by Western blotting. 5B. Western blotting analysis of a time course of HA-MucA degradation. PAO1 and PAO1*kinB::aacC1* expressing HA-*mucA* from pHERD20T were grown at 37 °C with shaking in LB supplemented with carbenicillin and arabinose till OD_{600} 0.2. Cells were harvested and resuspended in M9 broth supplemented with 0.4% glucose. Glucose enhances repression of the P_{BAD} promoter. During the time course the OD_{600} was monitored and remained stable throughout. Equal numbers of cells were extracted at 10 min intervals and harvested at 4°C and pellets were frozen at -80°C till cell lysates were prepared. Shown is a representative panel of blots with 40µg of total lysate transferred and blotted with anti-HA from three independent experiments. Positions of apparent molecular masses are indicated. 5C. Quantitative measurement of wt HA-MucA in PAO1 and PAO1 *kinB::aacC1* during t = 0 min to t = 30 min. Levels of each protein were adjusted for loading and then normalized to PAO1 pHERD20T-HA-*mucA* or PAO1 *kinB::aacC1* pHERD20T-HA-*mucA* levels and expressed as mean ± SD from three independent experiment (* indicates $p<0.01$).

MucA proteolytic degradation facilitates alginate overproduction in PAO1*kinB::aacC1*. Since *mucA* is wt in PAO1*kinB::aacC1,* MucA repression of AlgU must be relieved for activation of AlgU and alginate production. Based on the fact that alginate overproduction by PAO1*kinB::aacC1* requires AlgW, our hypothesis is alginate production in the *kinB* mutant occurs by regulated proteolysis of MucA. To test this model we needed to observe MucA degradation. N-terminal haemagglutinin (HA)-tagged MucA expressed from pHERD20T-HA-*mucA* under induction of arabinose into nonmucoid and mucoid PAO1-deriviative strains. Wild type *mucA* without HA was expressed in *trans* as the negative control. Western blotting of PAO1 without HA-tagged *mucA* showed no background or cross-reactivity with other proteins (Fig. 4B lane 2). In PAO1, full length HA-MucA exists as well as other truncated degradation products (Fig. 4B lane 1). HA-MucA degradation in PAO1 is consistent with degradation of RseA in *E. coli* which occurs in the absence of stress signals (2). Also PIA contains triclosan which has been shown to activate P_{algD} activity (54) suggesting regulated proteolysis occurs in the presence of cell wall inhibitory antibiotics. In PAO1Δ*algW,* full length HA-MucA is 2.4 ± 0.3 fold increased relative to PAO1 HA-MucA level (Fig. 4B lanes 1 and 3). This implies HA-MucA is not as rapidly degraded in PAO1Δ*algW* compared to PAO1. However, PAO1Δ*algW* also exhibits a truncated HA-MucA with an apparent molecular mass of 19 kDa (Fig. 4B lane 3). The absence of this band in PAO1 suggests deletion of *algW* inhibited efficient proteolysis of HA-MucA resulting in accumulation of two major fragments of HA-MucA. Mucoid PAO1*kinB::aacC1* lacks the secondary truncated peptide of HA-MucA (Fig. 4B, lane 4) and the concentration of HA-MucA full length is lower than in PAO1*kinB::aacC1*Δ*algW* (Fig. 4B lane 5). Interestingly, smaller degradation intermediates ~15 kDa of HA-MucA are still observed when *algW* is deleted in PAO1 and PAO1*kinB::aacC1* (Fig. 4B lanes 3 and 5). These data

suggest that regulated proteolysis of MucA may occur independent of AlgW as has been shown when PAO1*algW*::Tc^r converts to mucoidy in the presence of the reactive oxygen-producing paraquat (6). The presence of the truncated HA-MucA is apparent upon deletion of *algW*, *algB*, and *rpoN* in PAO1 *kinB* mutants (Fig. 4B lanes 5, 6 and 7). Taken together these observations suggest that *algB*, *algW* and *rpoN* influence HA-MucA degradation and derepression of AlgU in PAO1*kinB::aacC1*.

In order to better measure the differences in HA-MucA degradation between PAO1 and PAO1*kinB::aacC1* a time course was conducted to show *in vivo* depletion of HA-MucA in PAO1 and PAO1*kinB::aacC1*. To quantify the degradation of HA-MucA, cells were grown in LB broth containing arabinose to express HA-MucA and then transferred to M9 minimal medium supplemented with 0.4% glucose lacking arabinose. The OD_{600} remained stable during the time course. Therefore, depletion of HA-MucA was due to *in vivo* proteolytic degradation and not division of the cells. Samples were taken every 10 min for the cell lysis and Western blotting. We also performed the time course with 30 min intervals but found degradation of HA-MucA even in PAO1 was rapid indicating shorter time points needed to be taken (data not shown). The HA-MucA levels were assayed by Western blotting of 40 μg of total cell lysate (Fig. 5B). The rate of HA-MucA degradation in PAO1*kinB::aacC1* is 2.6-fold greater than that of PAO1 from 0 to 10 min (Fig. 5C). However, after 10 min the amounts of HA-MucA levels off and are not significantly different between PAO1 and PAO1*kinB::aacC1* (Fig. 5C). Assuming the mobility of HA-MucA peptide 1 is not aberrant, it appears there is a cleavage site between the transmembrane domain and the C-terminus of MucA (Fig. 5A). The smaller major truncated MucA peptide recognized with a HA epitope (HA-MucA peptide 2) is likely created by cleavage near the transmembrane domain. This study indicates HA-MucA degradation is rapid

even in PAO1, however inactivation of *kinB* causes an increased rate of degradation. The increased HA-MucA degradation likely contributes to the mucoid phenotype of *kinB* mutants.

DISCUSSION

We discovered mutation of *kinB* in PAO1 results in overproduction of alginate (Fig. 2A). Alginate regulation in *mucA* mutant strains was the first characterized mode of conversion to mucoidy and is the best elucidated (32). However, recently studies have shown regulated proteolysis mediated by AlgW is a mechanism for alginate production in *P. aeruginosa* (43, 54). Here we have presented data that inactivation of *kinB* causes mucoidy and is dependent upon *algB*, *algW*, and *rpoN* (Fig. 2B). We also observed through complementation analysis that phosphorylation of AlgB at the confirmed phosphorylation site is not required for alginate production in the *kinB* mutant. Our data suggests the *kinB* mutation increases the rate of degradation of MucA by regulated proteolysis which causes the mucoid phenotype of *kinB* mutants.

KinB is the cognate kinase of the alginate regulator AlgB (29), and alginate biosynthesis occurs independent of phosphorylation of AlgB (28). However the role of *kinB* in alginate production has only been examined in mucoid *mucA*22 mutant strains such as FRD-1 (28). In *mucA* mutants, the requirement for regulated proteolysis to activate AlgU would likely be bypassed due to the *mucA* mutation. We observed that in *kinB* mutants, *algB* and *rpoN* are both required for alginate production (Fig. 2B) and increased P_{algU} and P_{algD} promoter activity (Fig. 3B and 3D, respectively). Previously both *rpoN* (7) and *algB* (26) have been shown to affect transcription at P_{algD}. Conversely, our data shows that these regulators, AlgB and RpoN, also affect P_{algU} transcription. Only relatively small changes in P_{algU} expression are required for

mucoidy (33) however, PAO1*kinB::aacC1* exhibits significantly elevated expression of both P_{algU} and P_{algD} (Fig. 3B and 3D respectively). We also noted deletion of *algU* from PAO1*kinB::aacC1* resulted in complete loss of detectable P_{algU}-*lacZ* activity as measured by β-galactosidase assay (Fig. 3B column 3). This has also been observed when *algU* is deleted from PAO1 (data not shown). Two of the *algU* promoters are AlgU-dependent (12, 45), however it is not clear which σ factors the other promoters depend upon. Therefore it is possible that *in vivo* AlgU contributes the bulk of transcriptional activation of the AlgU promoters that is detectable by our reporter assay, but further analysis is required to fully understand the *algU* promoters.

Based on our data, we propose two alternative models for activation of alginate production through regulated proteolysis in *kinB* mutants (Fig. 6). In both models, regulated proteolysis of MucA occurs by AlgW but the cause of the increased concentration of activating signals differs. The first model suggests mutation of *kinB* affected expression of a protease or chaperone responsible for removal of misfolded proteins (Fig. 6). Mutation of an aminopeptidase, *phpA*, has been shown to cause increased P_{algD} activity and mucoidy (55). The second model proposed is that *algB* and *rpoN* directly control expression of peptide signals in the absence of *kinB* that activate AlgW and therefore increase proteolytic degradation of MucA (Fig. 6). Deletion of *algB* and *rpoN* in *kinB* mutants caused an accumulation of the major HA-MucA truncation product that was also observed in when *algW* was deleted in PAO1 or PAO1*kinB::aacC1* (Fig. 4B). We have also observed *algB* and *rpoN* are not required for *algW* expression (data not shown) which suggested loss of *algB* or *rpoN* may affect the proteolytic activity of AlgW. From this information we hypothesize that *algB* and *rpoN* may be required for expression of signals that activate AlgW and regulated proteolysis. Our data suggests increased

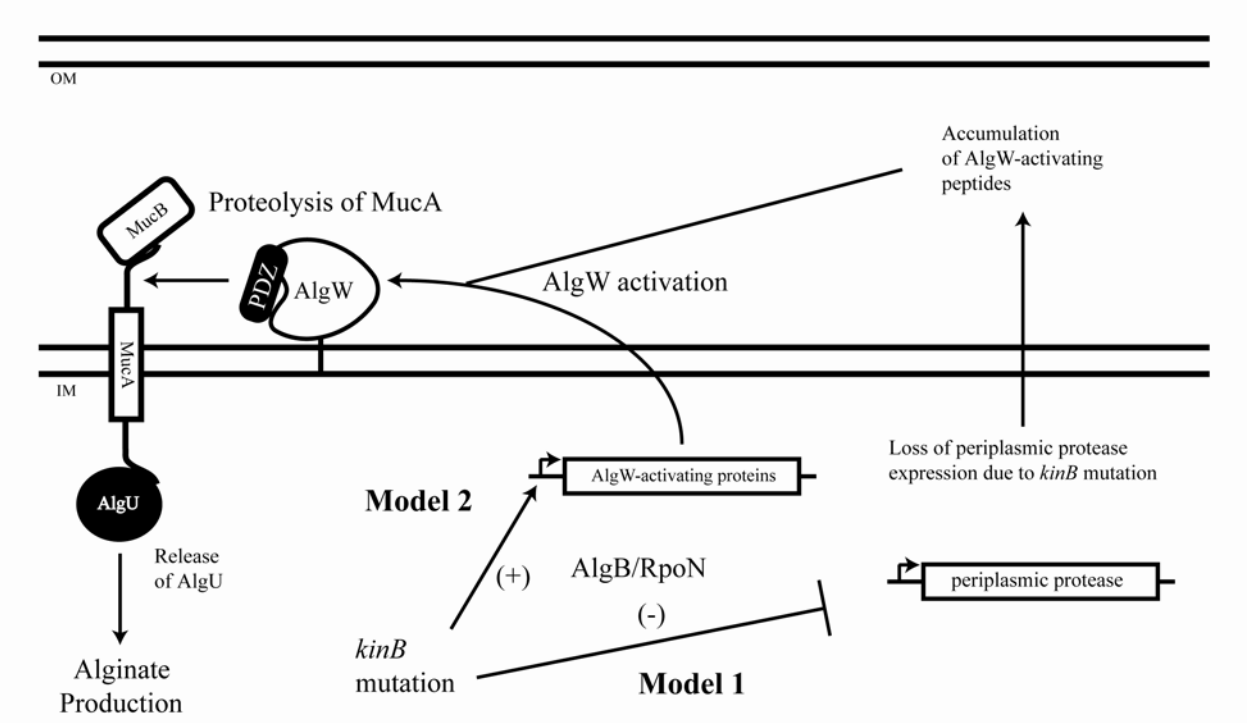

Figure 6. Proposed models of negative regulation of alginate production by KinB in *P. aeruginosa*. Mutation of *kinB* in wt *mucA* strain PAO1 caused alginate overproduction. Alginate overproduction of k*inB* mutants requires *algB* and *rpoN*. We propose in model 1, mutation of *kinB* causes loss of expression of a periplasmic protease(s) which leads to accumulation of AlgW-activating factors. In model 2, we propose *algB* and *rpoN* control expression of factors which can activate the AlgW protease to release repression of MucA by proteolytic degradation and activate AlgU. In either case, derepression of MucA by regulated proteolysis causes AlgU activation which facilitates *algU* expression resulting in mucoidy by up-regulating the alginate biosynthetic operon. OM and IM indicate outermembrane and innermembrane respectively.

regulated proteolysis occurs in *kinB* mutants of PAO1 (Fig. 4B, 5B, 5C). In *E. coli*, many outer-membrane and periplasmic proteins have been shown to activate DegS protease activity through interaction with the PDZ domain (18). Interestingly in *E. coli,* inactivation of the two-component histidine kinase EnvZ causes upregulation of the porin OmpC (48). Porins such as OmpC can activate regulated proteolysis (18). Analysis of the *P. aeruginosa* genome shows no significant homologs to the DegS-activating peptides such as OmpC of *E. coli.* This is conceivable because *P. aeruginosa* and *E. coli* reside in different habitats, therefore it is likely activation of AlgU and σ^E require different types of signals. However, proteins with probable activating sequences are coded throughout the *P.aeruginosa* genome (43). Thus, *P. aeruginosa* likely has novel proteins that could potentially activate AlgW degradation of MucA. It is possible that RpoN, in tandem with response regulators such as AlgB, control numerous genes of various functions which may be involved in signal transduction of the AlgU stress response.

AlgB and or RpoN could drive both *algU* and *algD* transcription. This is an alternative hypothesis to the models already described. Both AlgB and RpoN have been shown to bind at P_{algD} and are required for *algD* expression (7, 26). It has been suggested that AlgB may interact with other σ factors than RpoN (26). We have attempted to show AlgB binding with P_{algU} using gel shift assay however interaction has not been observed (data not shown). Recent studies have employed special conditions to detect AlgB DNA binding at P_{algD} (26.). Since exhaustive studies have not been performed, we cannot dismiss the possibility that AlgB and or RpoN may initiate transcription at P_{algU}. Based on our data, both the P_{algU} and the P_{algD} promoters are highly upregulated in *kinB* mutants (Fig. 3B and 3D). Therefore it is possible in the absence of *kinB*, AlgB could activate transcription of the both the P_{algU} and P_{algD} promoters.

Do *P. aeruginosa* CF isolates have *kinB* mutations? Most clinical observations have focused on surveying *mucA*, *mucB*, and *mucD* (8, 10, 32). Therefore, large scale surveys have not been performed looking for *kinB* mutants. However, one recently sequenced epidemic CF isolate, C3719, does have a mutation that truncates the KinB protein to 526 aa instead of the wt 595 aa PAO1 KinB (http://www.broad.mit.edu). Therefore a CF isolate has been shown to have a *kinB* mutation but C3719 is apparently nonmucoid (34). This suggests either the mutation is not completely detrimental to KinB regulation or C3719 may have additional suppressor mutations in either known or novel alginate regulators. PAO579 is another strain that requires *rpoN* for mucoidy (7), however the mucoid phenotype cannot be suppressed by complementation with *kinB* (data not shown). We are currently surveying for wt *mucA* CF isolates for *kinB* mutations. Many two-component signal systems can be activated by environmental conditions. The PhoP-PhoQ (30) and PmrA-PmrB (35) systems of *P. aeruginosa* are activated by low Mg^{2+} whereas the conserved PhoB-PhoR system is activated by low phosphate (25). Therefore, elucidation of the environmental signals that relieve the negative regulation of KinB on alginate overproduction will be as interesting as finding *kinB* mutant CF isolates.

In this report we have characterized KinB as a negative regulator of alginate production and proposed novel regulation of AlgW-dependent MucA derepression that is mediated by AlgB and RpoN. These data are a step closer towards understanding the molecular events leading to alginate production which preclude the classically described *mucA* mutations in *P. aeruginosa*. It will be interesting to further elucidate the unknown genes that may be under the negative control of the sensor kinase KinB and determine the environmental stimulus that affects KinB regulation in *P. aeruginosa*

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CHAPTER 4: Characterization of the serine protease MucD PDZ domains of *Pseudomonas aeruginosa* reveals autocleavage and MucP-dependent MucA proteolysis

Running title: MucP-dependent mucoidy in *P. aeruginosa*.

Keywords: Alginate, AlgU/T, MucD, AlgW, MucP, regulated intramembrane proteolysis

ABSTRACT

P. aeruginosa alginate overproduction or mucoidy is a marker for chronic respiratory infection in cystic fibrosis. AlgU(T), an extracytoplasmic function (ECF) σ factor, is the master regulator of alginate production. In nonmucoid cells, most AlgU is repressed by anti-sigma factor MucA. When *mucA* is inactivated, or MucA is proteolytically degraded, AlgU will be released to activate alginate overproduction. MucD (DegP/HtrA family serine protease) is also a negative regulator of alginate production and is presumed to control the protein quality of the envelope. Fluctuations in the envelope can initiate pathways of stress responses such as alginate overproduction. Here we report that the PDZ domains of MucD were dispensable for suppression of alginate production. However, MucD without both PDZ domains was unstable and rapidly degraded *in vivo,* suggesting the PDZ domains have roles in the regulation of autocleavage. Next, we probed how the loss of MucD quality control affected proteolysis of MucA activating alginate production. Inactivation of *mucD* resulted in proteolysis of HA tagged-MucA mediated by the intramembrane RseP-like protease MucP, but not the DegS-like protease AlgW. Overexpression of *algW* decreased alginate production in the *mucD* mutant, indicating AlgW can also function as a negative alginate regulator. Analysis of mucoidy-associated gene expression further supports the model indicating that the activation of AlgU in the absence of MucD was via MucP but not AlgW. Finally, we observed MucD can recognize divergent signals that activate AlgW-dependent and -independent (MucP) proteolysis of MucA, however, KinB-mediated signals were not regulated by MucD. Collectively, these data indicate the PDZ domains of MucD are not necessary in alginate regulation, but function in the control of autocleavage. Furthermore, when MucD quality control was absent, alginate production occurs through derepression of MucA dependent upon MucP.

INTRODUCTION

Alginate production by *P.aeruginosa* is a critical virulence phenotype that directly contributes to the morbidity and mortality of individuals with cystic fibrosis (CF) (15). Alginate overproduction, phenotypically termed mucoidy, facilitates survival of *P. aeruginosa* within the viscous mucosa found in the CF lung (24). Expression of the alginate biosynthetic operon results in production and secretion of alginate (9). Activation of transcription of the alginate biosynthetic operon at the P_{algD} promoter is dependent upon the ECF sigma factor AlgU(T) (42). AlgU is under negative regulation of the anti-sigma factor MucA (27). MucA sequesters AlgU to the inner membrane (28, 33, 35). Mutations in *mucA* result in a loss of AlgU repression (27).

Downstream of *mucA*, two more negative regulators are encoded by the genes *mucB* (26) and *mucD* (3). MucB is a periplasmic protein (35) that protects the C-terminal of MucA from proteolytic degradation (4). MucD is a member of the HtrA/DegP family of endoserine proteases (3). Expression of MucD can suppress P_{algD} expression (3). Inactivation of *mucD* or mutation of the proteolytic domain of *mucD* both result in alginate production (3, 40, 43). Recently, it has also been shown that inactivation of MucD decreases stability of C-terminal tagged MucA (41). MucD expression is both AlgU-dependent and independent with one promoter initiated from within the *mucC* ORF (40). Some of *mucD* expression occurs via the upstream AlgU-dependent P3 and P1 promoters of *algU* however some *mucD* expression occurs via a promoter in *mucC* Bioinformatic analysis shows MucD is the homolog of *E. coli* DegP (HtrA) (Fig. 1), which has three functionally-distinct domains, the protease domain (residues 100-258) and two PDZ domains, PDZ1 (residues 260-349) and PDZ2 (residues 380-460). PDZ domains are conserved protein-protien interacting domains. In *E. coli* DegP, PDZ2 domain has

roles in oligomerization (18) and PDZ1 is required for substrate recognition (17). The role of PDZ domains in suppression of alginate production in *P. aeruginosa* has not been investigated.

In addition to MucD, another serine protease, AlgW, has been shown to play roles in alginate production. AlgW was first identified for its ability to suppress mucoidy in *mucA* mutants (3). AlgW is homologous to *E. coli* DegS, which activates derepression of the anti-sigma factor RseA in response to the accumulation of envelope proteins with specific C-terminal residues (1). Recently, AlgW has been shown to positively regulate alginate production when *mucE* is overexpressed or in the presence of certain cell wall inhibitory antibiotics (31, 39). Through *in vitro* experiments, MucE peptide has been shown to bind directly to AlgW and activate cleavage of the periplasmic portion of MucA (4). AlgW has also been shown to activate degradation of MucA when the histidine kinase KinB is inactivated (7).

A recent model suggests that AlgW is activated in the absence of MucD (41) in a manner similar to the dogma that has been described in *E. coli* with degradation of RseA first by DegS and then by YaeL/RseP (MucP homologue) (1). However, AlgW has also been observed to be not required for alginate production in the absence of MucD (31). In a *mucD* mutant strain, the intramembrane protease MucP is essential for alginate production (31). Interestingly, alginate production can occur independent of AlgW in the presence of paraquat, a redox cycling compound that induces intracellular levels of superoxide (3). This supports the notion that MucA can be proteolytically degraded independent of AlgW.

In this study we sought to define which domains of MucD are critical for negative regulation of alginate production and to elucidate the activation pathway of AlgU in the absence of MucD. In this study, we that report the PDZ domains of MucD have a minimal role in suppression of alginate production but the PDZ domains are critical for *in vivo* stability of MucD.

Our data also suggests that MucD undergoes autocleavage in a similar manner to DegP. Here we also suggest a model by which MucD regulates protein signals that cause the proteolysis of MucA (Fig. 1A). When quality control of periplasmic proteins is lost due to inactivation of *mucD*, MucA degradation occurs which is dependent upon MucP but not AlgW. Also, MucD can suppress signals that activate AlgW-dependent or -independent MucA proteolysis. Our data indicate MucD negatively regulates alginate production by repressing the signals that activate MucA degradation through intramembrane proteolysis.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids and oligonucleotides. Bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* strains were grown at 37°C in Lennox broth (LB; Difco, Sparks, MD), on LB agar or *Pseudomonas* isolation agar plates (PIA; Difco, Sparks, MD). Whenever necessary, the PIA plates were supplemented with carbenicillin, tetracycline, or gentamicin at a concentration of 300 μg/ml. *E. coli* strains were grown in LB or on LB agar and when necessary supplemented with carbenicillin at a concentration of 100 μg/ml. Transfer of plasmids from *E. coli* to *P. aeruginosa* strains was performed by pRK2013 (11) mediated triparental conjuation. The sequences of the oligonucleotides used in this study are indicated in Table 1.

Plasmid construction and complementation analyses. PCR amplicons with restriction endonuclease tails were cloned into pCR4-TOPO and then digested for ligation into pHERD20T (30). All plasmid constructs containing PCR products were sequenced to confirm that no mutations occurred. Amplicon sequencing was performed by the Marshall University Genomics Core Facility.

Alginate assay. *P. aeruginosa* strains were grown in triplicate at 37˚C on PIA supplemented with carbenicillin for selection of the complementation vector and arabinose as indicated. Alginate was measured as previously described (7).

Western blot analysis. Cell lysates were prepared with ProteaPrep Bacterial cell lysis (Protea Morgantown, WV) by the manufacturer's protocol. Cell lysates were quantified by D_C assay (BIO-RAD, Hercules, CA). 50 μg of protein was boiled in SDS-loading buffer and loaded on each gel. The samples were electrophoresed on 15% ProteaGel (Protea Morgantown, WV) polyacrylamide gels (SDS-PAGE) and then electroblotted (Trans-Blott Cell, BIO-RAD, Hercules, CA) onto 0.2μm or 0.45μm nitrocellulose. The membranes were blocked with 3% non-fat dry milk in PBS (pH 7.4). Primary antibodies were diluted 1:1,000 - 1:4,000 in 3% non-fat dry milk in PBS. The membranes were probed with all or some of these antibodies: mouse monoclonal antibodies against AlgU (35), AlgB (25), alpha subunit of RNA polymerase subunit (Neoclone), or rat monoclonal antibody against HA (Roche, Mannheim, Germany) overnight at 4°C with shaking. HRP-labeled goat anti-mouse IgG or HRP-labeled anti-rabbit IgG were diluted 1:5,000 - 1:20,000 in 3% non-fat dry milk in PBS and used as the secondary antibodies. Advanced ECL or ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ) was used for detecting HRP-labeled goat anti-mouse IgG or anti-rabbit IgG (Roche) by the manufacturer's procedure. The signals were detected with an EC3 Imaging System (UVP, Upland, CA) by capturing with a BioChemi HR camera. For re-probing, membranes were stripped with 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol for 15 min at 50°C and then washed in PBS and reanalyzed.

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

Bacterial strains	Genotype, phenotype or sequence [a b]	Source
P. aeruginosa		
PAO1	Prototroph, NM	P. Phibbs
PAO1*mucD*	PAO1 *mucD*::Gm^r, M	(31)
PAO1*mucD / algW*	PAO1 *mucD*::Gm^r *algW*::Tc^r, M	(31)
PAO1*mucD / mucP*	PAO1 *mucD*::Gm^r *mucP*::Tc^r, NM	(31)
PAO1*kinB*	PAO1 *kinB*::Gm^r, M	(7)
PAO1 (P_{Gm}::*mucE*)	PAO1 with chromosomal fusion of P_{Gm}-*aacC1*-*mucE*, M	(31)
E. coli		
DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Lab strain
TOP10	DH5α derivative	Invitrogen
BL21(DE3)pLysS	F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	(37)

Oligonucelotides		
ecoR1-mucD-F	AGAATTCGATGCATACCCTAAAACGCTGTAT	This study
hindIII-HA-mucD-R	TGCCAAGCTTTTAAGCGTAATCTGGAACATCGTA TGGGTATTCGGCCAGCTTGAAGGTAATGAAGCT	This study
hindIII- HA-ΔPDZ2-R	TGCCAAGCTTTTAAGCGTAATCTGGAACATCGTA TGGGTACTCGTCGTCGTCCGGAAGGCTGCCT	This study
hindIII- HA vΔPDZ1 ΔPDZ2-R	TGCCAAGCTTTTAAGCGTAATCTGGAACATCGTA TGGGTAGCGACTGACCTTGCCGGCTTTCTTCA	This study
Plasmids		
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^r Km^r	Invitrogen
pRK2013	Km^r *Tra Mob ColE1*	(11)
pUCP20T	*Escherichia-Pseudomonas* shuttle vector, P_{lac}, Ap^r, *ori*pBR322, *ori*pRO1600, *oriT*	(38)
pUCP20T-*mucP*	*mucP* (PA3649) from PAO1 in pUCP20T *BamH1*/*Hind*III	(31)
pHERD20T	pUCP20T P_{lac} replaced by 1.3 kb *Afl*II-*Eco*RI fragment of *araC*-P_{BAD} cassette	(30)
pHERD20T-*mucD*-HA	C-terminal HA-tagged *mucD* (PA0766) from PAO1 in pHERD20T *EcoRI*/*Hind*III	This study

pHERD20T-*mucD*-ΔPDZ2-HA	C-terminal HA-tagged *mucD* (PA0766) without PDZ2 domain in pHERD20T *EcoRI*/*Hind*III	This study
pHERD20T-*mucD*-ΔPDZ1-ΔPDZ2-HA	C-terminal HA-tagged *mucD* (PA0766) without PDZ2 or PDZ1 domains in pHERD20T *EcoRI*/*Hind*III	This study
pHERD20T-*algW*	*algW* (PA4446) from PAO1 in pHERD20T *Eco*RI/*Hind*III	(7)
pHERD20T-HA-*mucA*	N-terminal tagged HA-*mucA* in pHERD20T *Eco*RI/*Hind*III	(7)

[a] NM, non-mucoid phenotype; M, mucoid phenotype.

[b] Underline indicates the restriction site utilized for the cloning

Release of periplasmic proteins by chloroform shock. To observe periplasmic protein profiles of *P. aeruginosa* strains, an assay based on the method as described by Ames et al (2) was used with the following modifications. Cells were cultured on PIA supplemented with carbenicillin and 0.1% arabinose for 24 hrs at 37°C. The cells were scraped off the agar plates into 6 ml of PBS (pH 7.4) for harvesting by centrifugation. The supernatant fluid was decanted and the resulting pellet was vortexed to suspend in residual buffer. 40µl of chloroform (Fisher Scientific) was added to the cell suspension. After 15 min incubation at room temperature, 100 µl of tris buffered saline (pH 7.6) was added to the cell suspension and mixed before the cells were pelleted. The supernatant contained the periplasmic proteins of the cells that were released by chloroform shock. The protein preparations were quantified by D_C assay (BIO-RAD) and separated on SDS-PAGE. Periplasmic protein profiles were visualized by silver staining (Bio-Rad Silver Stain Plus) by the manufacturer's protocol.

Isolation of RNA and RT-PCR. *P.aeruginosa* strains were cultured on PIA for 12 hrs at 37°C. The cells were scraped from the PIA plates and resuspended in PBS. RNA was isolated using Qiagen RNeasy spin columns (Qiagen, Valencia, CA) processed by the manufacturer's protocol. To remove potential contaiminating genomic DNA, the eluted RNA was treated with DNase (Qiagen). The DNase treated RNA was cleaned up by adding the solution to another RNeasy column with subsequent wash steps followed by elution. The DNA-free RNA was quantified and aliquoted for analysis to prevent freeze-thaw degradation. 100ng of RNA from each strain was submitted to one-step RT-PCR (Qiagen) with gene specific primers (*algU*, *algD*, *lptF*, 16S rDNA). The PCR amplification protocol utilized was the standard protocol suggested by the manufacturer with an annealing temperature of 58°C. Three independent experiments were performed along with no RT controls (data not shown). RT-PCR amplicons were analyzed by

1% argarose gel electrophoresis with ethidium bromide for visualization. The amplicons were detected with an EC3 Imaging System (UVP, Upland, CA) by capturing with a BioChemi HR camera.

RESULTS

Construction and expression of HA-tagged wild type and truncated MucD. To investigate the roles of the MucD PDZ domains in the suppression of alginate production, we cloned C-terminal HA-tagged *mucD* genes (Fig. 1B). Wild type *mucD, mucD*ΔPDZ1-ΔPDZ2 and *mucD*ΔPDZ2 were fused into the pHERD20T vector (30) for conditional expression from the PBAD promoter. The constructs were sequenced and expressed in *E. coli* strain BL21. Western blot analysis shows the size of each of the MucD-HA proteins (Fig. 2A). The proteins migrated to their predicted sizes on SDS-PAGE. However, two peptides were detected when PDZ2 domain was deleted from MucD (Fig. 2 lane 3). Since the MucDΔPDZ2-HA is C-terminal tagged, the truncated protein would have resulted from N-terminal cleavage. Furthermore, the concentration of MucDΔPDZ1-PDZ2-HA was notably lower than that of the MucD-HA or MucDΔPDZ2-HA when expressed in *E. coli* (Fig. 2A lanes 2-4).

Effect on alginate production due to expression of HA-tagged MucD in PAO1*mucD* mutant. With *mucD* inactivated, alginate production was elevated in PAO1 (Fig. 2B lane 1) as

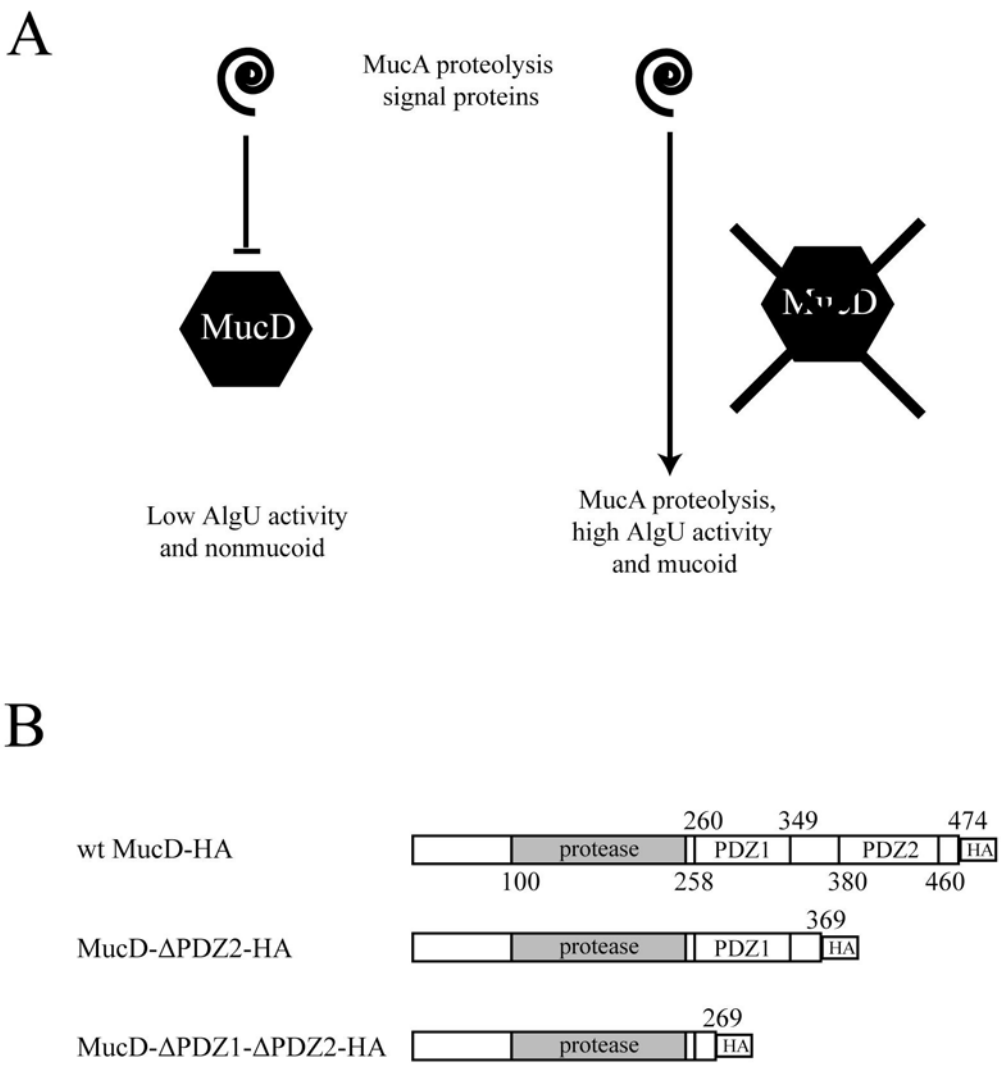


Figure 1. Hypothetical model of MucD regulation of alginate production and the HA-tagged *mucD* constructs utilized for this study. In the absence of *mucD*, alginate production occurs (3). This hypothetical model proposes that MucD regulates proteins which activate proteolysis of the AlgU anti-sigma factor, MucA. A. In the presence of MucD, such as in lab strain PAO1, low AlgU activity and nonmucoid phenotype are observed. However, in the absence of MucD, peptides which activate regulated proteolysis accumulate which results in high AlgU activity and mucoidy. B. MucD contains three domains: a serine protease domain and two PDZ domains (PDZ1 and PDZ2). The amino acid positions of the serine protease, PDZ1 and PDZ2 domains, and HA epitope tags are indicated.

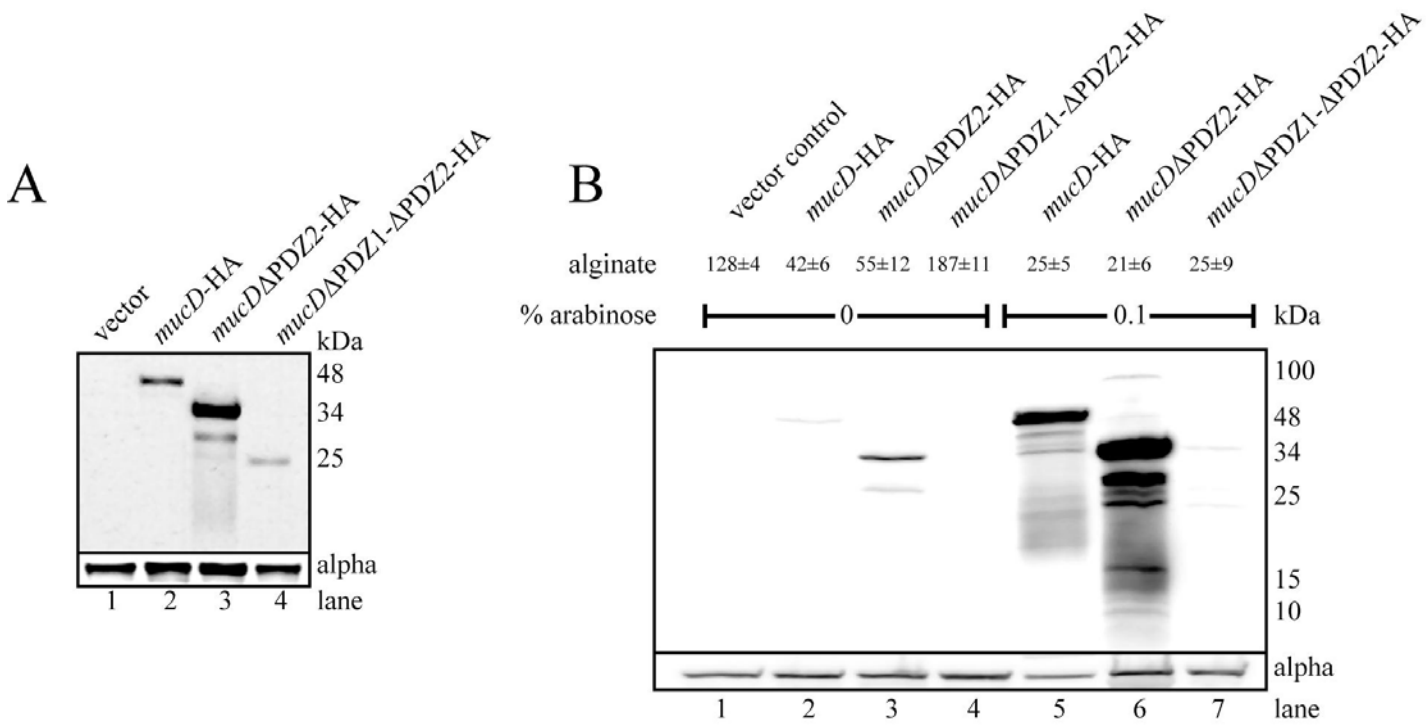


Figure 2. Western blot analysis of C-terminal HA-tagged MucD in BL21 and PAO1*mucD* strains. Shown are a representative panels (from three experiments) with 50 μg of total protein blotted and probed with Rat anti-HA and anti-Alpha subunit RNA polymerase (loading control) from three independent experiments. Positions of apparent molecular masses are indicated. A. Cell lysates were prepared from *E. coli* BL21 strains harboring C-terminal HA-tagged *mucD* constructs. The constructs used are indicated on the above the blot. Cells with the *mucD* constructs were grown in LB supplemented with carbenicillin. B. Cell lysates were prepared from *P. aeruginosa* strain PAO1*mucD* harboring C-terminal HA-tagged *mucD* constructs. The percent arabinose used to drive expression of the HA-tagged MucD constructs is indicated along with the amount of alginate produced by each strain (indicated as μg/ml/OD_{600}). Of note the bands in lane 7 are not due to carryover from nearby lanes (data not shown).

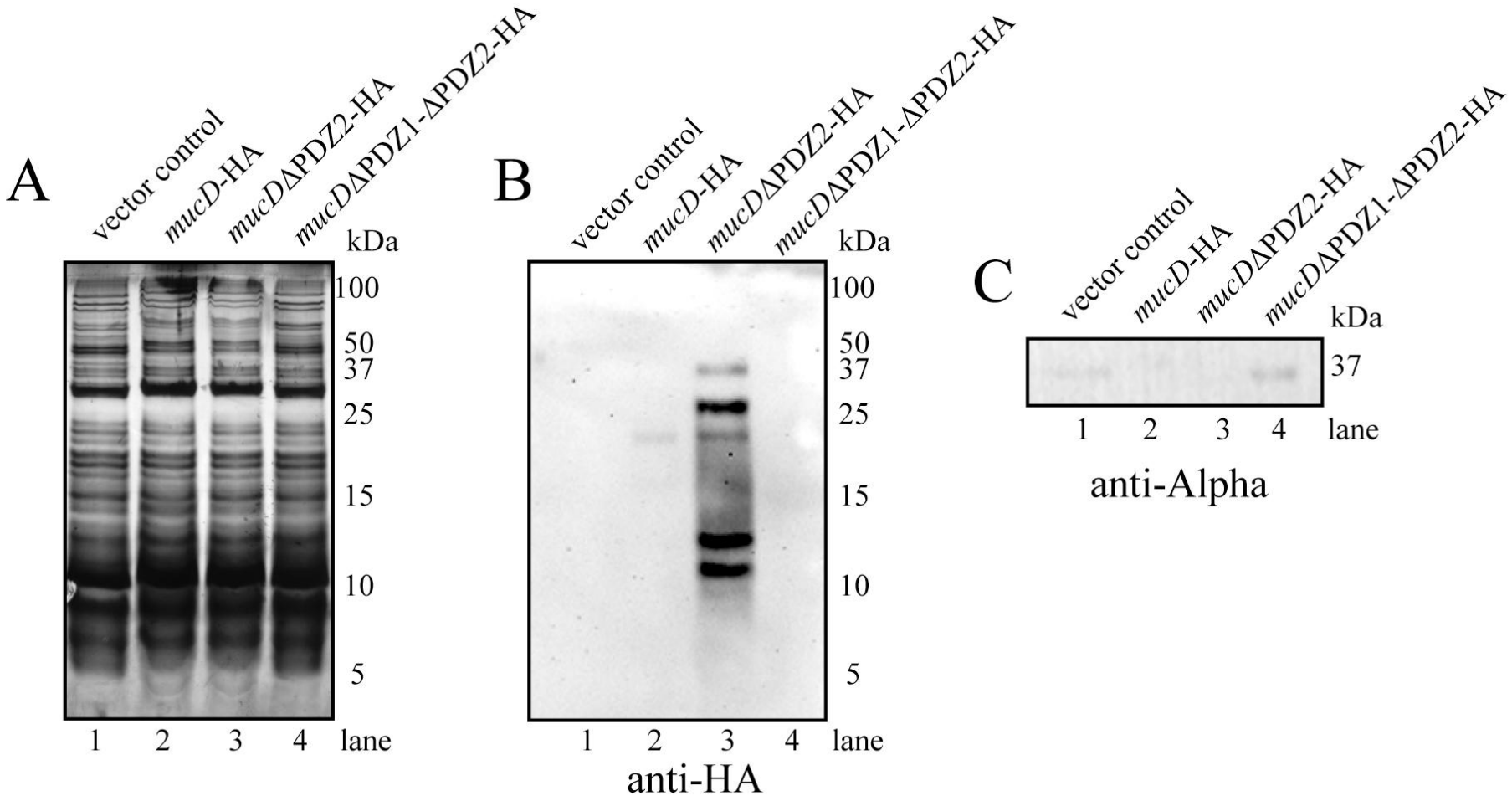
previously reported (3) . Under basal expression (in the absence of arabinose) of MucD-HA, alginate production decreased to nonmucoid levels (Fig. 2B lanes 1 and 2). Interestingly, when the PDZ2 domain was absent, a similar suppression of alginate production was observed (Fig. 2B lanes 1-3). Since PDZ2 in *E.coli* DegP is critical for oligomer formation (17), this data suggests that monomers of MucD without the PDZ2 domain may be capable of processing the signals which would activate alginate production. Of note, a second band of lower molecular mass was detected in both *E. coli* and *P. aeruginosa* (Fig 2A lane 3 and Fig. 2B lane 3). When MucD lacking both PDZ domains was expressed under basal conditions in the *mucD* mutant, alginate production persisted (Fig. 2B lane 4). Interestingly, at basal expression the MucDΔPDZ1-ΔPDZ2-HA peptide was not detected (Fig. 2B lane 4). This suggests that deletion of both PDZ domains resulted in a highly unstable protein (Fig. 2B lane 4).

When MucD-HA was expressed at an elevated level (0.1% arabinose), alginate production decreased more than that observed with basal expression (Fig. 2B lanes 2 and 5). Lower molecular mass HA-tagged peptides were observed when MucD-HA expression was elevated (Fig. 2B lane 5). These truncations would occur at the N-terminus because MucD-HA was C-terminal tagged. Recently DegP/HtrA has been shown to autocleave as a mechanism to eliminate excess DegP/HtrA once stress conditions cease (20). It is likely that due to the elevated expression of MucD-HA, we were able to visualize these autocleavage peptide products of MucD. When PDZ2 was absent from MucD, even more truncation products at higher concentrations were detected (Fig. 2B lane 6). Interestingly a HA peptide near 100 kDa was detected which has higher molecular mass than what was predicted for MucDΔPDZ2-HA (Fig. 2B lane 6). DegP is capable of forming trimers with the protease domains interacting to each other and PDZ1 and PDZ2 deletion mutants of DegP have been shown to elute as trimers (19). It

is possible deletion of PDZ2 causes a structural change resulting in tight association of the MucDΔPDZ2-HA monomers into a trimer structure that could not be dissociated by denaturing conditions of SDS-PAGE. Alternatively, it is also possible the MucDΔPDZ2-HA is linked with a larger unknown molecule.

Under basal expression, MucDΔPDZ1-ΔPDZ2-HA could not suppress alginate production (Fig. 2B lane 4). However, under elevated expression (0.1% arabinose) MucDΔPDZ1-ΔPDZ2-HA suppressed alginate production to the same level as wild type MucD-HA (Fig. 2B lanes 5 and 7), indicating that protease domain alone of MucD was sufficient for suppression of alginate production. Interestingly, several bands of higher molecular mass were observed when MucDΔPDZ1-ΔPDZ2-HA was expressed under arabinose induction (Fig. 2B lane 7). Since these bands appeared to correspond to the major products in lane 6, we performed a separate experiment where only the MucDΔPDZ1-ΔPDZ2-HA total proteins were separated by SDS-PAGE alongside molecular mass markers. HA-peptides corresponding to the products as in Fig. 2B lane 7 were detected which concluded carryover from nearby wells did not occur (data not shown). By measuring alginate and detecting expression of truncated MucD via the HA epitope tag, it is clear that both PDZ domains are dispensable for the control of alginate production, but are important for the *in vivo* stability of MucD.

Release of periplasmic proteins suggests deletion of PDZ2 causes loss of oligomerization and deletion of both PDZ domains decreases stability of the MucD peptide. DegP is a periplasmic protein; however, the cellular localization of MucD had not previously been confirmed. DegP has recently been shown to be tightly associated with lipid membranes as bowl-shaped oligomeric structures (36). We sought to detect HA-tagged MucD in periplasmic fractions produced by chloroform shock (2). We reasoned that chloroform shock may allow the


A
vector control
mucD-HA
mucDΔPDZ2-HA
mucDΔPDZ1-ΔPDZ2-HA
kDa
100
50
37
25
15
10
5
1 2 3 4 lane
B
vector control
mucD-HA
mucDΔPDZ2-HA
mucDΔPDZ1-ΔPDZ2-HA
kDa
100
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anti-HA
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vector control
mucD-HA
mucDΔPDZ2-HA
mucDΔPDZ1-ΔPDZ2-HA
kDa
37
1 2 3 4 lane
anti-Alpha

Figure 3. Western blot analysis of released periplasmic proteins of PAO1*mucD* harboring C-terminal HA-tagged MucD constructs. Periplasmic proteins were released by chloroform shock (2) with slight modifications (see *Materials* and *Methods*). The strains were cultured for 24 hrs on PIA supplemented with carbenicillin and 0.1% arabinose to express the constructs from the PBAD promoter of pHERD20T.

A. Silver stained SDS-PAGE analysis shows the profile of periplasmic proteins (50 μg) released by chloroform shock of PAO1*mucD* harboring the plasmids indicated.

B. On the same SDS-PAGE gel that was submitted to silver staining, a duplicate set of proteins (50 μg) were transferred to nitrocellulose and blotted with anti-HA. The positions of the apparent molecular masses are indicated. Of note a light band of approximately 20 kDa is present in lane 2. Shown is a representative of three independent experiments.

C. The preparations are enriched for periplasmic proteins, the same membrane that was probed with anti-HA was stripped and reprobed with anti-Alpha (for the alpha subunit of RNA polymerase). Of note the signal visualized was achieved with long exposure with high sensitivity chemiluminescent substrate (Amersham ECL™ Advance).

release of MucD and its derivatives from the envelope. Periplasmic fractions were prepared and duplicate sets of proteins were separated by SDS-PAGE. One set of samples was silver stained to show proteins released and the other set was Western blotted to visualize HA-tagged MucD. The periplasmic fraction profile of each of the strains with truncated MucD constructs were similar (Fig. 3A). However, we did not detect a monomer of MucD-HA in the Western blot of the periplasmic fraction (Fig. 3B lane 2). It is possible the large MucD oligomer could not be released to the extracellular milieu under the conditions of chloroform shock. Of note, a peptide of approximately 20 kDa could be visualized (Fig. 3B lane 2). When PDZ2 was absent from MucD-HA, several distinct HA-peptides were detected (Fig. 3B lane 3). This suggests that MucDΔPDZ2-HA can be released from the periplasm by chloroform shock and without PDZ2 the large oligomer formation would not occur. When both PDZ domains were absent, no HA peptides could be detected in the periplasmic fraction (Fig. 3B lane 4). This shows that loss of both PDZ domains results in a peptide that can be released by chloroform shock. To show the periplasmic fractions were enriched for periplasmic proteins with minimal cytoplasmic proteins, we blotted for alpha subunit of RNA polymerase subunit (Fig. 3C). Only a small amount of alpha RNA polymerase was detected (Fig. 3C) which suggests periplasmic fractions released by chloroform shock had minimal contamination with cytoplasmic proteins.

Inactivation of MucD causes instability of MucA dependent upon MucP. In the absence of MucD, alginate production occurs (3); therefore we speculated that inactivation of *mucD* causes loss of quality control in the envelope. Quality control imbalance in the envelope activates intramembrane proteases that act upon MucA resulting in release of AlgU. AlgW and MucP are two intramembrane proteases that have been shown to have a role in degradation of MucA (31, 39). In *E. coli*, degradation of RseA occurs first by DegS and then by YaeL/RseP (MucP

homologue) (1). In *P. aeruginosa*, when AlgW is activated by MucE, AlgW degrades the periplasmic C-terminal tail of MucA (4). We previously reported that PAO1 *mucD / algW* was mucoid (31) (Fig. 4C) and that PAO1 *mucD* / *mucP* was nonmucoid (Fig. 4C). To observe proteolysis of MucA in these strains, we utilized conditional expression of N-terminal HA-tagged MucA driven from the PBAD promoter of pHERD20T (7). The PBAD promoter has low (basal) expression even in the absence of arabinose (30). The HA-MucA degradation profiles were first observed with basal expression (Fig. 4A). Unlike MucD-HA peptides described above, these peptides detected are derivatives of N-terminal HA-tagged MucA due to C-terminal truncations. Inactivation of *mucD* caused decreased full length HA-MucA and truncated HA-MucA Peptide 1 (Fig. 4A lanes 2 and 3), which suggested MucA undergoes degradation in the absence of MucD, as expected. A similar level of HA-MucA and Peptide 1 were observed in both PAO1 *mucD* and PAO1 *mucD/algW* mutants (Fig. 4A lanes 3 and 4). This data suggested that MucA degradation in the absence of MucD quality control is not dependent solely upon AlgW. In the PAO1 *mucD / mucP* double mutant, there was an accumulation of a 10 kDa HA-MucA peptide (Peptide 3), suggesting incomplete proteolysis occurs when *mucP* is inactivated (Fig. 4A lane 5). Peptide 3 is likely lacking the periplasmic domain of HA-MucA due to C-terminal truncations. If MucP does not cleave at the MucA transmembrane domain, complete proteolysis of MucA is blocked.

To observe if differences in HA-MucA degradation exist between PAO1*mucD* and PAO1*mucD/algW*, HA-MucA was overexpressed (Fig. 4B). Under these conditions, four major HA-tagged peptides were detected in PAO1 (Fig. 4B lane 2). PAO1 *mucD* and PAO1*mucD/algW* HA-MucA degradation profiles were distinctive when HA-MucA was

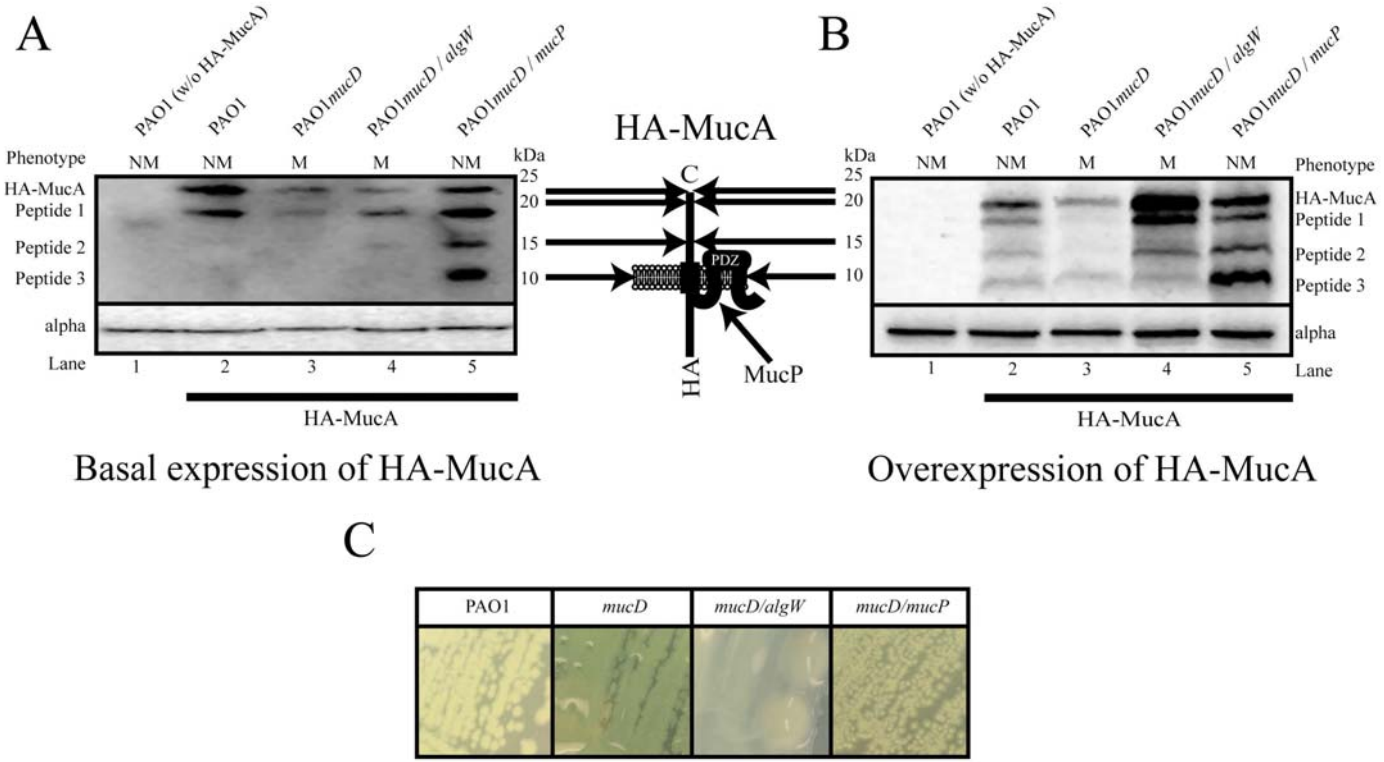

Figure 4. Degradation of HA-tagged MucA indicates MucD regulates MucP-dependent proteolysis of MucA. Western blotting analysis of HA-MucA in different *P. aeruginosa* strains. Cell lysates were prepared after 24 hrs of growth at 37 °C on PIA supplemented with carbenicillin. The relative positions of the peptides visualized are aligned with the predicted regions of HA-MucA. As a control for cross-reactivity, total protein samples lacking HA-tagged peptides were analyzed and indicated the HA antibody was specific for the HA-tagged peptides (Fig. 4A lane 1 or Fig. 4B lane 1). A. The profile of HA-MucA when it is expressed from the PBAD promoter of pHERD20T without arabinose. The highly regulated PBAD promoter allows basal expression in the absence of arabinose (30). Degradation of HA-MucA occurs in PAO1*mucD* and PAO1*mucD/algW*, however inactivation of *mucP* causes incomplete proteolysis of HA-MucA. B. The profile of HA-MucA when it is overexpressed from the PBAD promoter of pHERD20T with 0.1% arabinose. Inactivation of *algW* in PAO1*mucD* causes accumulation of full length HA-MucA; however, this strain is mucoid. When *mucP* was inactivated, peptide 3 was accumulated, which corresponds to approximately the transmembrane region which MucP probably recognizes. C. The visual phenotypes of PAO1, *mucD, mucD/algW* and *mucD*/*mucP* are shown when cultured on PIA for 24hrs at 37°C.

overexpressed (Fig. 4B lanes 3 and 4) indicating proteolysis of MucA was affected by loss of AlgW. Interestingly C-terminal truncation of HA-MucA occurred independent of AlgW (Fig. 4AB lane 4). Since in the absence of MucD quality control alginate production is not dependent upon *algW* (31), these data suggest that AlgW acts upon MucA in the absence of MucD; however in the absence of AlgW, MucP-dependent MucA proteolysis controls alginate production.

Negative regulation of alginate production by AlgW. We next used the PAO1 *mucD/algW* double mutant to investigate the relationship between AlgW and MucD. Interestingly the visual phenotype of PAO1*mucD/algW* was stable and different than the PAO1 *mucD* parent strain (Fig. 4C). This suggested AlgW may somewhat inhibit alginate production in the absence of MucD. When *algW* was overexpressed in PAO1*mucD*/*algW* mutant, alginate production was suppressed (Table 2). This indicated that AlgW may have a role in negatively regulating alginate production under certain conditions. This is consistent with the role of AlgW that was originally identified to suppress alginate production in *mucA* mutants (3). To further test the hypothesis of negative regulation of AlgW, we measured alginate production in PAO1*mucD* with overexpression of *algW in trans* (Table 2). Overexpression of *algW* in PAO1 *mucD* resulted in nonmucoidy and lower alginate production (Table 2). These data suggest that while AlgW may positively regulate alginate production, it can also act as a negative regulator.

AlgU accumulates when either *mucP* or *algW* are inactivated in PAO1 *mucD*. When MucA is proteolytically degraded, AlgU is free to activate transcription at target promoters such as the auto-regulated *algU* promoters (10) as well as the *algD* biosynthetic operon (42). The mucoid phenotypes of PAO1*mucD* and PAO1*mucD/algW* would predict an increase in free AlgU concentration. To test this, Western blot analysis was utilized to check AlgU concentration in

Table 2. Complementation of genes affecting alginate production when *mucD* is inactivated.

Strain [a]	Plasmid	Phenotype[b]	Alginate (μg/ml/OD_{600})
PAO1*mucD* / *algW*	pHERD20T	M	141±3
	pHERD20T-*algW*	NM	58±2
PAO1*mucD*	pHERD20T-*algW*	NM	65±8
PAO1*mucD* / *mucP*	pUCP20T	NM	27±3
	pUCP20T-*mucP*	M	80±18

[a] Strains were cultured for 24 hrs at 37 °C on Pseudomonas Isolation Agar (PIA) supplemented with carbenicillin and 0.1% arabinose (w/v).
[b] NM and M indicate nonmucoid and mucoid phenotype respectively.

PAO1*mucD* and the isogenic derivatives. PAO1 and PAO1*mucD* displayed similar levels of AlgU (Fig. 5A lanes 1 and 2). However, inactivation of *mucP* and *algW* in PAO1*mucD* caused elevated levels of AlgU over their parent strain (Fig. 5A lanes 3 and 4). The PAO1*mucD*/*algW* strain was expected to have increased AlgU because it is mucoid. However, since *mucD/mucP* was nonmucoid, this data was unexpected. The HA-MucA profile of the *mucD/mucP* double mutant suggested incomplete degradation (Fig. 4AB lane 5). It is likely the 10 kDa HA-MucA is a C-terminal truncated MucA (Peptide 3) protein still capable of sequestering AlgU.

AlgU activity as measured by AlgB expression is low in *mucD/mucP* but upregulated in *mucD/algW* double mutants. Experiments above suggested concentration of AlgU does not necessarily reflect the activity of AlgU, since PAO1 *mucD/mucP* is nonmucoid (Table 2 and (Fig. 4C)) but has a high amount of AlgU (Fig. 5A lane 3). To ascertain the activity of AlgU in the *mucD*, *mucD/algW,* and *mucD/mucP* mutants, we examined the expression of the AlgU-dependent alginate response regulator AlgB (42). AlgB is upregulated in mucoid strains with mutations in AlgU negative regulators such as *mucA* and *kinB* mutants (7, 14). Therefore, expression of AlgB is a better indicator of AlgU activity than AlgU expression alone. We observed inactivation of *mucD* caused a slight increase in AlgB expression (Fig. 5A lanes 1 and 2). However, low AlgB expression was detected in nonmucoid PAO1*mucD/mucP* (Fig. 5A lane 3). Furthermore, AlgB expression did not require *algW* in the absence of MucD (Fig. 5 lane 4), which is consistent with mucoidy of PAO1*mucD/algW* (Fig. 4C). These data suggest AlgU activity in the absence of MucD requires MucP but not AlgW.

AlgU activity assayed by expression of *algU, algD,* and *lptF* shows MucP is required for activation of AlgU. To further determine the activity status of AlgU in each of the strains, we performed semi-quantitative RT-PCR analysis of *algU* dependent genes *algD, algU,* and *lptF*.

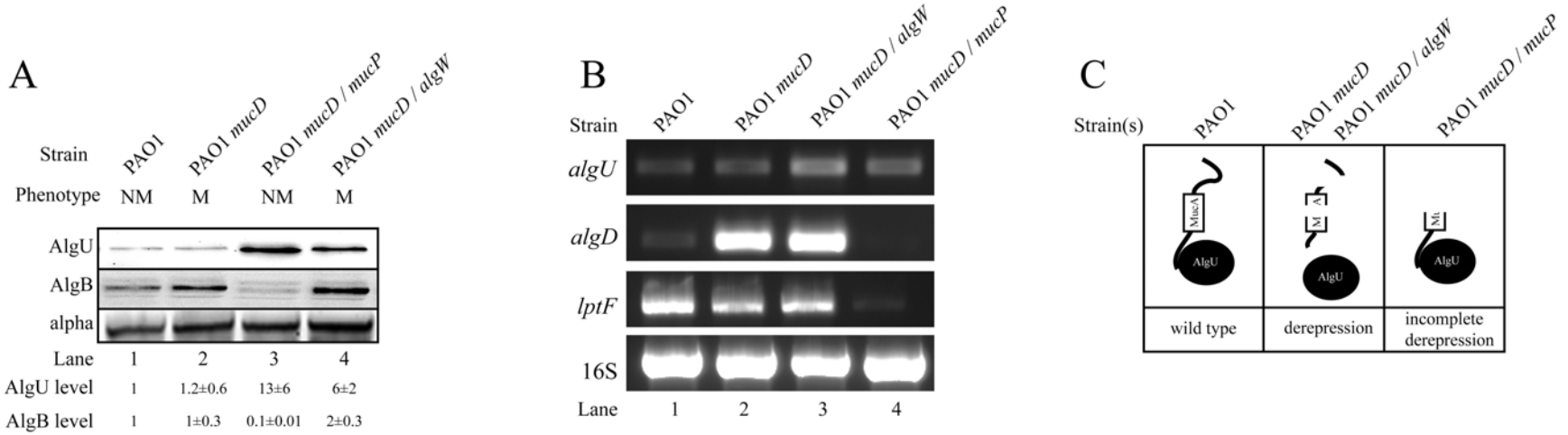

Figure 5. AlgW is not required for activation of AlgU when MucD is absent; however, MucP is required.

A. Western blot analysis reveals inactivation of *algW* and *mucP* increases AlgU concentration, Cell lysates from strains PAO1, PAO1*mucD, mucD/mucP, mucD/algW* were probed with anti-AlgU, anti-AlgB and anti-alpha RNAP antibodies. Shown is a representative panel of blots of 50μg of total protein from three independent experiments. The relative levels of AlgU and AlgB are indicated.

B. In the absence of MucD, AlgU activation requires MucP. Agarose gel analysis of semi-quantitative RT-PCR was performed on the strains indicated with *algU, algD, lptF,* and 16S rDNA gene specific primers. RNA was isolated from strains cultured on PIA for 12 hrs. Expression of *algD* (42) and *lptF* (Lipotoxin F) (6) are dependent upon activation of AlgU. Two of the five *algU* promoters are dependent upon *algU* and the other three are unknown (34). In the *mucD/mucP* double mutant, expression of *algD* and *lptF* is decreased. Mucoid strains PAO1*mucD* and PAO1*mucD/algW* both have high expression of *algD.*

C. In the absence of MucD*,* derepression of MucA or activation of AlgU requires MucP protease and not AlgW. Based on data presented here when *mucD* is inactivated then increased activation of AlgU occurs. MucP is required to release MucA from AlgU; however, AlgW is not required for MucA degradation in the absence of MucD.

There was a small amount of *algD* expression in nonmucoid PAO1; however, high expression of *algD* was observed in the mucoid PAO1*mucD* and PAO1*mucD/algW* strains (Fig. 5 lanes 1-3). Furthermore, *algU* expression was elevated in PAO1*mucD/algW* (Fig. 5B lane 3) consistent with mucoidy (Fig. 4C) and increased *algD* transcription in this mutant. Interestingly, *algU* expression was not blocked in the PAO1 *mucD/mucP* mutant (Fig. 5B lane 4). This can be explained by the fact that only two of the *algU* promoters are AlgU-dependent (P1 and P3) while it is unknown which σ factor(s) drive expression from the other *algU* promoters (P2, P4, P5) (34). The *algD* expression was detected in PAO1*mucD/mucP* at a level even lower than PAO1 (Fig. 4B lanes 1 and 4), consistent with nonmucoidy in PAO1*mucD/mucP* mutant. To further support the notion that *mucP* is required for AlgU activation, we measured *lptF* expression. The *lptF* gene encodes lipotoxin F which is an AlgU-dependent lipoprotein (6, 12, 13, 39). The *lptF* gene was observed to be expressed in PAO1, PAO1*mucD,* and PAO1*mucD/algW* (Fig. 5 lanes 1-3). However, inactivation of *mucP* also decreased *lptF* expression dramatically (Fig. 5 lane 4). In the PAO1*mucD/mucP* double mutant, AlgU is not active because it is expected to still be sequestered by MucA.

MucD suppresses alginate production by mediating signals that activate both AlgW-dependent and -independent pathways. Since AlgW is not required for regulated proteolysis of MucA in the absence of MucD, it can be suggested that MucP may be directly activated by signals mediated by MucD. We have also previously reported that expression of MucD can suppress MucE-AlgW mediated mucoid phenotype (31). Here we show that when MucD-HA was expressed from pHERD20T, overexpression (1% arabinose) was required for suppression of the MucE-mucoid phenotype (Table 3). Recently, we have reported that inactivation of sensor kinase *kinB* causes AlgW-dependent MucA proteolysis (7). We hypothesized that MucD could

Table 3. Suppression of alginate production of AlgW-mediated mucoid strains by MucD

Strain [a]	Plasmid	Alginate Production [b] (Phenotype [c])	
		0% [d]	1%
PAO1 (P_{Gm}::*mucE*)	pHERD20T-*mucD*-HA	188±6 (M)	65±16 (NM)
PAO1*kinB*	pHERD20T-*mucD*-HA	155±16 (M)	147±20 (M)

[a] Strains were cultured for 24 hr at 37 °C on Pseudomonas Isolation Agar (PIA) supplemented with carbenicillin and arabinose concentration indicated (w/v).
[b] Alginate production is indicated as μg/ml/OD_{600}
[c] NM and M indicate nonmucoid and mucoid phenotype respectively.
[d] The percent of arabinose utilized to express *mucD*-HA from the PBAD promoter of pHERD20T.

suppress this alginate production pathway as well. However, alginate production was not suppressed by MucD overexpression in the absence of *kinB* (Table 3). Collectively our data indicate that MucD can mediate signals that activate AlgW-independent proteolysis, MucE-mediated AlgW proteolysis, but MucD does not mediate the unknown signals that activate AlgW proteolysis of MucA in PAO1*kinB*.

DISCUSSION

Nonmucoid *P. aeruginosa* strains with wild type *mucA* initially colonize the CF lung. Lab strains with inactivated *mucD* have been shown to convert to mucoidy (3, 26), and CF isolates have also been reported with *mucD* mutations (5). Since MucD mediates alginate production, virulence, as well as other factors (43), it is important to better understand the multiple facets of this dynamic protein in *P. aeruginosa*. We hypothesized that without MucD alginate production occurs because the protein signals activate regulated proteolysis of MucA. We also tested the roles of the PDZ domains in modulation of alginate production.

Previous reports have shown that the *mucD217* allele encoding a defective protease domain could suppress mucoidy when overexpressed (40). These data suggest that MucD likely performs both proteolytic and chaperone functions in *P. aeruginosa* homologous to the roles of DegP in *E. coli* (21). It is clear that the protease domain of MucD is critical for the control of alginate production (40). However, since PDZ domains of DegP have roles in the formation of "giant" oligomers like molecular "death stars" (18, 22, 23), we would not have predicted the PDZ domains of MucD are dispensable for suppression of alginate production. Interestingly, CF isolates with mutations in PDZ1 and PDZ2 are still nonmucoid; however, a *mucD* null mutant (CF172 stop codon residue 41) is mucoid (5).

Recent reports have shown that DegP undergoes autocleavage to eliminate excess DegP (20). Here we observed the same phenomenom *in vivo;* however, in the absence of PDZ2 or both PDZ2 and PDZ1, MucD becomes unstable (Fig. 2B and Fig. 3B). In fact, loss of both PDZ domains have resulted in a highly unstable peptide (Fig 2B lanes 4 and 7). This is an interesting observation may add to the *E. coli* DegP/HtrA autocleavage model. When DegP hydrolyzes protein substrates and relaxes back into a hexameric cage (6-mer), then autocleavage occurs (20). With our *in vivo* analysis of MucD-HA peptides, we have observed that increased autocleavage occurs more when both PDZ domains are missing or when MucD molecules are in an elevated concentration (Fig. 2 and 3). Therefore, our data suggest that the PDZ domains may play a negative role in the autocleavage events to eliminate excess MucD.

In the absence of MucD alginate production occurs. We observed that the PDZ domains do not seem to be involved in the regulation of alginate production. Our hypothesis was that without MucD, regulated proteolysis of MucA occurs do to the accumulation of proteins that MucD would have recognized for degradation or re-folding. Our data suggests that MucA degradation occurs independent of AlgW but dependent upon MucP. Recently another study demonstrated that AlgW has a role in a PAO1 *mucD* mutant (41). Using C-terminal tagged MucA, inactivation of AlgW caused an accumulation of MucA in a PAO1*mucD* mutant (41). In our report, during overexpression of HA-MucA, accumulation of full length peptide was also observed in PAO1*mucD/algW* (Fig. 4B lane 4). Since the HA-MucA degradation profiles differ under basal and overexpression, our data also suggests AlgW could act upon MucA. However, AlgU-dependent genes are expressed in mucoid PAO1*mucD/algW* (Fig. 5), indicating that activation of AlgU could occur in the absence of AlgW. Furthermore we show that MucD

mediates AlgW-signals such as MucE as well as signals that don't require AlgW for MucA proteolysis. In addition it appears KinB-mediated signals are not modulated by MucD (Table 3).

AlgW and MucD can be aligned and show high homology (3). Originally it was shown that AlgW expression could decrease alginate production (3). Therefore it could be possible that AlgW acts as a protease on not just MucA. Since AlgW can suppress alginate production in *mucA* mutants (3), it is possible AlgW suppresses signals which could activate MucP. Some mutant MucA proteins such as MucA22 protein would still have a transmembrane domain which MucP could act upon. Another interesting point is that in the absence of AlgW, a C-terminal truncated MucA peptide is detected (Fig. 4A lane 4 and 4B lane 4). From this data we wonder if a protease other than AlgW can act on the C-terminus of MucA. Prc protease has been suggested to interact with mutant MucA (32). However we did not observe loss of either peptide when *prc* is inactivated in PAO1 (data not shown).

What are the signals that activate AlgW-independent (MucP) mucoidy? Recently mutations have been characterized in RseP that revealed a ligand-binding cleft in the PDZ domain of RseP (16). However, protein substrates have not been identified that interact with RseP. Interestingly, acid stress activates the σ^E stress response in *Salmonella enterica* serovar Typhimurium in an RseP-dependent manner, which does not require DegS (29). Our study and the aforementioned study are the first reports detailing activation of σ^E / AlgU stress response independent of DegS/AlgW. Our data also show that MucD can mediate both AlgW-dependent and -independent pathways.

MucD is a dynamic protein that regulates protein quality control in the periplasm of *P. aeruginosa*. Our data suggest that if proteins that are recognized by MucD accumulate, some

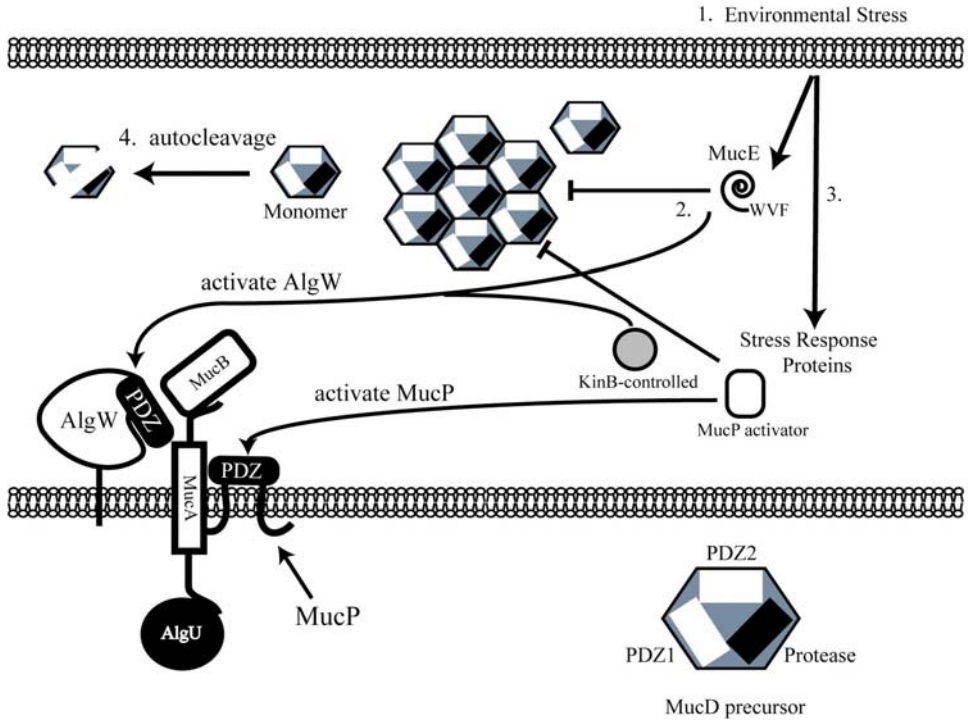

Figure 6. Regulation of alginate production by MucD. In wild type *mucA* strains, MucA must be proteolytically degraded to release AlgU for alginate production. 1. Environmental stress can activate expression of *mucE* (8) which is a periplasmic protein signal that has been shown to activate the AlgW pathway of MucA degradation. 2. If *mucE* expression is high enough MucD can not negatively regulate and AlgW activation will occur. 3. Other stress response proteins could also be unregulated. However some proteins such as those upregulated when *kinB* is inactivated are not suppressed by MucD and can activate AlgW. Other proteins regulated by MucD can activate MucP independent of AlgW activation. 4. Our data suggest MucD undergoes autocleavage in a similar fashion to DegP/HtrA of *E. coli*. Additionally it seems the PDZ domains of MucD influence the stability of the protein. The PDZ domains are not required for suppression of alginate production. We propose when environmental conditions exceed the capacity of MucD regulation, the AlgU pathway will be induced through MucP or AlgW regulated proteolysis.

will activate AlgW-dependent alginate production (such as MucE) and others may activate MucP directly. However, there are signals such as those controlled by KinB that are not mediated by MucD (Fig. 6). Furthermore, it appears MucD is capable of autocleavage in the same fashion as DegP/HtrA. Our data presented here suggest the PDZ domains of MucD negatively regulate autocleavage and that neither PDZ domain is required for suppression of alginate production, Our data also indicate MucP mediates regulated proteolysis and activation of AlgU in the absence of MucD quality control of the periplasm in *P.aeruginosa*. Potentially, conditions that inhibit MucD activity, such as the extreme environment of the CF lung, could activate the AlgW-independent pathway of alginate production directly through MucP.

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CHAPTER 5: Lipotoxin F of *Pseudomonas aeruginosa* is an AlgU-dependent and alginate independent outer-membrane protein involved in resistance to oxidative stress and adhesion to A549 human lung epithelia

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Running title: Lipotoxin LptF in *P. aeruginosa*.

Keywords: Alginate, AlgU/T, Lipotoxin, LptF, MudPIT

SUMMARY

Chronic lung infection with *P. aeruginosa* and excessive neutrophil-associated inflammation are major causes of morbidity and mortality in patients with cystic fibrosis (CF). Overproduction of an exopolysaccharide known as alginate leads to the formation of mucoid biofilms that are resistant to antibiotics and host defenses. Alginate overproduction or mucoidy is controlled by a stress-related ECF sigma factor AlgU/T. Mutation in the anti-sigma factor MucA is a known mechanism for conversion to mucoidy. Recently, we identified inactivation of a kinase (KinB) in nonmucoid strain PAO1 results in overproduction of alginate. Here we report the initial characterization of lipotoxin F (LptF, PA3692), an OmpA-like outer-membrane protein, that exhibited increased expression in the mucoid PAO1*kinB* mutant. The lipotoxin family of proteins was previously characterized to induce inflammation in lung epithelia which may play a role in CF disease progression. Expression of LptF was observed to be AlgU-dependent and upregulated in CF isolates. Deletion of *lptF* from the *kinB* mutant had no effect on alginate production. Deletion of *lptF* from PAO1 caused a differential susceptibility to oxidants that can be generated by phagocytes. The *lptF* and *algU* mutants were more sensitive to hypochlorite than PAO1. However, the *lptF* mutant displayed increased resistance to hydrogen peroxide. LptF also contributed to adhesion with A549 human lung epithelial cells. Our data suggests LptF is an outer-membrane protein that may be important for *P. aeruginosa* survival in harsh environments including lung colonization in CF.

INTRODUCTION

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis (CF) which affects the normal respiratory, gastrointestinal and nutritional functions of the body (25). In CF patients, the respiratory tract system is not only impaired by defective CFTR but also by microbial infections with a variety of pathogens such as *P. aeruginosa* due to decreased mucociliary clearance (14). Chronic lung infection with *P. aeruginosa* leads to increased morbidity and mortality in CF (25). Biofilm formation in CF lungs by *P. aeruginosa* facilitates survival through resistance to host immune responses and increased antibiotic resistance (14). Biofilm formation in the CF lungs is also dependent upon bacterial communication or quorum sensing (QS) (38).

Conversion of *P. aeruginosa* to mucoid phenotype or overproduction of exopolysaccharide alginate has clearly been shown to be protective for survival (14). MucA is a negative regulator of alginate production that sequesters the alginate master regulator, ECF sigma factor AlgU (35), to the inner membrane (33). Mutations in *mucA*, cause constitutive production of alginate (28) due to loss of MucA repression of AlgU. AlgU activates transcription of the *algD* biosynthetic operon (7) which then leads to alginate production (46). Alginate production can also occur independent of *mucA* mutations through proteolytic derepression of MucA by the protease AlgW (32).

The two component response regulator AlgB (PA5483) controls alginate production at the *algD* promoter (46). AlgB and KinB (PA5484) are encoded on the chromosome in an operon and KinB has been shown to phosphorylate AlgB (27). However phosphorylation of AlgB is not required for alginate production (26). AlgB is required for mucoidy (13) and transcriptional activation of the *algD* biosynthetic operon (23). Recently we have observed inactivation of *kinB*

caused strain PAO1 to produce copious amounts of alginate (Fig. 1) (5). Inactivation of *kinB* causes loss of repression by MucA and alginate production that is dependent upon AlgW, AlgB, and the alternative sigma RpoN (σ^{54}) (5). Alginate production provides protection for *P. aeruginosa*, however alginate-independent, AlgU-dependent gene products, are responsible for the detrimental inflammation (12). Of the 5,567 proteins encoded in the PAO1 genome there are 113-186 predicted lipoproteins (1). In mucoid strains, 70% of genes with a >30-fold increase in expression encode lipoproteins (12). AlgU dependent lipoproteins or lipotoxins cause activation of NF-κB in human lung epithelial cells through Toll-like receptor 2 (12). Lipotoxins have been shown to stimulate inflammatory response (10, 12). However, physiological roles of theses lipotoxins have not been characterized.

Here we report inactivation of *kinB* causes upregulation of an outer-membrane protein known as lipotoxin F or LptF (PA3692). The *lptEF* promoter is highly upregulated in mucoid lab strains and CF isolates, and is controlled by AlgU. However *lptF* has no effect on alginate production in the *kinB* mutant. We deleted *lptF* from PAO1 and observed increased resistance to hydrogen peroxide, however increased susceptibility to killing by hypochlorite. Both nonmucoid and mucoid isolates from CF have increased *lptF* promoter expression. Also, PAO1Δ*lptF* exhibits decreased adherence to A549 human lung epithelial cells. The studies presented here suggest LptF in *P. aeruginosa* may be an important survival factor.

METHODS

Bacterial strains, growth conditions, sequencing and oligonucleotides. Bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were grown at 37°C in Lennox broth (LB) or LB agar supplemented, when necessary, with carbenicillin or tetracycline at a concentration of 100 μg/ml and 20 μg/ml respectively. *P. aeruginosa* strains were grown at 37°C in LB or on *Pseudomonas* isolation agar plates (PIA; Difco, Sparks, MD). When necessary, the PIA plates were supplemented with carbenicillin or tetracycline at a concentration of 300 μg/ml and 200 μg/ml respectively. Amplicon sequencing of plasmids and gene deletions were performed by the Marshall University Genomics Core Facility.

Multidimensional Protein Identification Technology (MudPIT) analysis. To identify the proteins present in *P. aeruginosa* total cell lysates, MudPIT with tandem MALDI-TOF mass spectrometry was employed. Strains were streaked on PIA and cultured for 24 hr at 37° C. Cells were harvested and total protein samples were prepared by processing cell lysates with Epicentre Ready preps (Epicentre, Madison, WI). Total protein samples (50 μg) were desalted using C_4 ProteaTip SpinTips (Protea Biosciences, Morgantown, WV) by manufacturer's protocol. The collected samples were lyophilized and dissolved in 100 μl of 50 mM ammonium bicarbonate in 20% acetonitrile for tryptic digestion. The samples were then reduced and alkylated with 10μL of 250mM DTT (60 min/55°C), and 10 μl of 625 mM iodoacetamide (60 min/room temperature/in the dark). Proteolytic digestion was performed in 50 mM ammonium bicarbonate

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Phenotype, genotype, and description [a]	Source
P. aeruginosa		
PAO1	Alg$^-$ Prototroph	P. Phibbs
PAO1Δ*algU*	Alg$^-$, PAO1 in-frame deletion of *algU* (PA0762)	(5)
PAO1Δ*lptF*	Alg$^-$, PAO1 in-frame deletion of *lptF* (PA3692)	This study
PAO1Δ*rpoN*	Alg$^-$, PAO1 in-frame deletion of *rpoN* (PA4462); nonmotile	(5)
PAO1*kinB::aacC1*	Alg$^+$, PAO1 *kinB*:: Gm^R	(5)
PAO1*kinB::aacC1*Δ*algW*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *algW* (PA4446)	(5)
PAO1*kinB::aacC1*Δ*algU*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *algU* (PA0762)	(5)
PAO1*kinB::aacC1*Δ*lptF*	Alg$^+$, PAO1*kinB::aacC1* in-frame deletion of *lptF* (PA3692)	This study
PAO1*kinB::aacC1*Δ*rpoN*	Alg$^-$, PAO1*kinB::aacC1* in-frame deletion of *rpoN* (PA4462); nonmotile	(5)
383	Nonmucoid CF isolate from the sputum of a patient at Children's Hospital, Boston, MA, USA, on 16 June 1980	J. Goldberg (15)
2192	Mucoid CF isolate from the sputum of a patient at Children's Hospital, Boston, MA, USA, on 18 June 1980, isogenic to 383	J. Goldberg (15)
CF149	Alg$^+$ *mucA* mutant, *algU* suppressor mutant	G. Pier (17)
CFO42	Alg$^+$ mucoid isolate (1978)	D. Speert (17)
CFO23o	Alg$^+$ mucoid isolate from same sputum sample (1998) as CFO23s and CFO23s	D. Speert (17)
CFO23s	Alg$^+$ mucoid isolate from same sputum sample as CFO23o and CFO23w	D. Speert (17)
CFO23w	Alg$^+$ mucoid isolate from same sputum sample as CFO23o and CFO23s	D. Speert (17)
E. coli		
DH5α	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Lab strain
TOP10	DH5α derivative	Invitrogen
Plasmids		
pRK2013	*Tra Mob ColE1* Km^R	(9)

pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^R Km^R	Invitrogen
pHERD20T-*lptF*	*lptF* (PA3692) from PAO1 in pHERD20T *Eco*RI/*Hind*III	This study
pEX100T	*Pseudomonas* suicide vector, *sacB*, *oriT*, Cb^R	(37)
pEX100T-*Not*I	*Pseudomonas* suicide vector with *Not*I restriction site fused into *Sma*I of pEX100T, *sacB*, *oriT*, Cbr	(32)
pEX100T-Δ*lptF*	A 1.8-kb fragment flanking *lptF* gene fused with pEX100T-*Not*I with in-frame deletion of *lptF*	This study
miniCTX-*lacZ*	Gene delivery vector for inserting genes at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^R	(18)
miniCTX-P_{lptEF}-*lacZ*	Complete P_{lptEF} promoter (949 bp upstream of ATG of *lptF)* fused *Hind*III/*Eco*RI with *lacZ* for integration at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^R	This study
pMRPQ-1	GFP expression plasmid Cb^R	(6)

[a] Alg^-, non-mucoid phenotype and Alg^+, mucoid phenotype.

[b] The *P. aeruginosa* isolates used in this study were obtained from the following individuals: P.Phibbs, East Carolina University Genetic Stock Center, Greenville, NC.; J. Goldberg, University of Virginia, Charlottesville, VA; G.Pier, Harvard Medical School, Boston, MA.; D. Speert, University of British Columbia, British Columbia, Canada.

buffer using a trypsin to protein ratio of 1:100. The digestion was carried out overnight at 37°C. The digests were cleaned by repeated lyophilizing and reconstituting in a 0.1M acetic acid solution. After final lyophilization, the digests were reconstituted in a strong cation exchange loading buffer (5 mM ammonium formate in 20% acetonitrile, pH 3.0) to be fractionated with ProteaTip spin tips per manufacturer's protocol. The SpinTip was transferred to a new centrifuge tube to collect the sample during elution with 200 µL of elution solution. Eight different elution solutions were used fractionate the peptides (20, 60, 100, 150, 200, 250, 400, 500mM ammonium formate in 20% acetonitrile) in a step-wise manner. The collected fractions were cleaned by repeated lyophilization and reconstitution in a 0.1 M acetic acid solution. After the final lyophilization, the digests were reconstituted in LC run buffer. The fractions were then submitted to LC MALDI spotting and MALDI TOF/TOF spectral analysis as detailed previously to survey the proteome of the *P. aeruginosa* strains. The relative quantity of the protein within the sample was calculated by comparison of number of amino acids identified by MS compared to corresponding to the full length peptide. The ratio of the identified peptides within the sample was also normalized against the various lengths of the expected lengths of the peptides and displayed as relative percent of peptides identified within the sample.

SDS-PAGE, total protein preparation and peptide mass spectrometric sequencing. Total protein preparations were obtained by processing cell lysates with Epicentre Ready preps (Epicentre, Madison, WI). Protein concentrations were determined using Bio-Rad D_C Protein Assay. SDS-PAGE (14% polyacrylamide) was performed to separate total cell lysates for staining with R250 Coomassie stain. Selected up-regulated protein bands were excised from the gel for direct mass spectrometric sequencing. Gel pieces were destained with Protea Silver destaining solution (Protea Biosciences, Morgantown, WV). The pieces were dehydrated and

then rehydrated with acetonitrile and 50 mM ammonium bicarbonate, respectively. Proteins in the gel pieces were reduced and alkylated with 250 mM DTT (60 min/55°C) and 650mM iodoacetamide (60min / room temperature / in the dark), respectively. Digestion was performed with 625 ng trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was performed using 5% formic acid in 50% acetonitrile and with 50 mM ammonium bicarbonate. Three cycles of dehydration, rehydration, and supernatant collection were performed and the recovered peptides were dried down in lyophilizer to be purified with an acetic acid rinse in addition to a final lyophilization.

The LC-MALDI mass spectrometry system utilized was an ABI Tempo LC MALDI spotter with Tempo LC MALDI v.2.00.09 data acquisition and processing software. Lyophilized-digested samples were reconstituted and 5 µl was injected onto a Chromolith CapRod monolith column 150 x 0.1 mm (Merck). The peptides were eluted from the column using an acetonitrile/trifluoroacetic acid gradient (2-72% acetonitrile in 25 min) and spotted directly onto a MALDI plate. The MALDI spots were analyzed using an ABI 4800 MALDI TOF/TOF analyzer operated with 4000 Series Explorer software. The MS acquisition was in reflector mode positive ion mode with 400 laser shots per spectrum performed. The 15 strongest precursors were chosen for MS/MS and the MALDI spot was interrogated until at least 4 peaks in the MS/MS spectra achieved a signal/noise ≥ 70. The resulting MS/MS spectra were analyzed using ABI Protein ProteinPilot software 2.0. The spectral data was compared with the *Pseudomonas* Genome Project Version 2 database for identification of the peptides and corresponding proteins.

Analysis of outer-membrane proteins. *P. aeruginosa* strains were streaked on PIA and cultured for 24 hr at 37 ° C. The cells were scraped from the plates and suspended in PBS. The

cells were harvested by centrifugation at 7000 x *g*. The cell pellet was suspended in 2% sarkosyl with 2 mM PMSF protease inhibitor (phenylmethylsulfonyl fluoride) in PBS. The cells were lysed by sonication for 1 min on ice. The lysate was clearified by low speed centrifugation. The supernatant was taken then centrifuged at 40000 x *g* for 1 hr. The resulting pellet containing outer-membrane proteins was resuspended in TBS. The protein concentration was determined with the Bio-Rad D_C protein assay. The preparations were separated by SDS-PAGE (14% polyacrylamide) and visualized by silver staining with Bio-Rad Silver Stain Plus.

β-galactosidase activity assay of P_{lpteEF}-*lacZ* promoter fusion. The miniCTX-*lacZ* (18) integration gene delivery vector was used for inserting promoter fusions to the CTX phage *attB* site on the *P. aeruginosa* chromosome. 949 bp upstream of the *lptF* start site was cloned into the *Hind*III-*Eco*RI sites of MiniCTX-*lacZ*. The construct was sequenced to show no mutations had occurred during the cloning. MiniCTX-P_{lptEF}-lacZ was transferred to recipient strains by pRK2013-conjugation. Strains with integration into the *attB* site were selected on PIA supplemented with tetracycline (200μg/ml) and were passed through three isolations. The β-galactosidase activity was assayed based on the method as originally described by Miller (29) with the following modification. The cells were grown on PIA with antibiotics for selection in triplicate for 24 h at 37°C and harvested in PBS. Cell density was measured by OD_{600}. The β-galactosidase activity was assayed after toluene permeabilization of the cells. The reported values represent the averages of samples in triplicate of three independent experiments with standard error indicated.

Mutant strain construction. For in-frame deletion of *lptF*, the upstream and downstream sequence fragments (1 kb) flanking *lptF* were PCR amplified and fused via the cross-over PCR method. The PCR products with the in-frame deletion of target gene were the cloned into pCR4-

TOPO. The subcloned in-frame deletion fragment was then digested and ligated into pEX100T-*Not*I vector. The resulting vectors were sequenced to show no mutations had occurred outside of the intended specific gene deletion. A two-step allelic exchange procedure was employed with the pEX100T constructs for gene disruption or in-frame deletion. The single cross-over merodiploid exconjugants were selected based on carbenicillin resistance and for sensitivity on 10% sucrose (*sacB*). After incubation of the merodiploids in the LB broth, the double cross-over recombinants were isolated from the PIA plates supplemented with 10% (wt/vol) sucrose. The disruption or in-frame deletion of target gene was confirmed by antibiotics-resistance assays, PCR amplification of the flanking region of target gene with multiple sets of primers, and amplicon sequencing.

Analysis of alginate production. *P. aeruginosa* strains were grown at 37˚C on PIA plates in triplicate for 24 hr. The resulting bacterial growth was removed from plates and suspended in PBS. The optical density at OD_{600} of the suspension in PBS was measured. The suspensions were assayed for amount of uronic acid comparison with a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich, St. Louis, MO) as previously described (5).

Susceptibility to killing by hydrogen peroxide and hypochloride. Sensitivity to H_2O_2 and NaOCl was determined by measuring the radius of the zone of killing surrounding disks (6mm diameter; BBL). 25 ml of LB agar was poured into 100x15 mm plates. Overnight cultures were diluted with LB, and 100 µl of OD_{600} 0.1 culture was added to 3 ml of molten 0.6% soft agar and gently mixed. The culture-soft agar suspension was then overlaid on the 25ml of LB agar. Discs were soaked with 10 ul of fresh stock solutions of 10% H_2O_2 or 6% NaOCl. The discs were then applied to the soft agar containing plate. The zone of killing was scored after 24 hr incubation at 37° C by measuring the radius.

Cell culture methods. A549 lung epithelial cells (ATCC cat. # CCL-185) were purchased from ATCC. The cells were cultivated in F-12K medium supplemented with 10% fetal bovine serum (ATCC) and antibiotics (pen-strep, MP Biomedicals) in 100x20 mm tissue culture treated dishes (Greiner bio-one) and subcultured every 2-3 days. One day prior to experimental use, they were grown to 80-90% confluency and split at a ratio of 1:1.

A549 epithelial cell adherence assay. Adherence was measured by incubation of A549 cells with GFP-tagged *P.aeruginosa* harboring pMRPQ-1 (6). A549 cells were harvested by treatment with 1 mL trypsin (0.25%, Hyclone) for 10 minutes followed by gentle pipetting to remove any adherent cells. Live harvested cells were quantified by using erythrosin B (10% in PBS, Fisher) exclusion dye and counted on a hemocytometer. 1.5 x 10^5 A549 cells were resuspended in 300 µl of F-12K medium plus 10% fetal bovine serum. GFP-tagged *P. aeruginosa* was added to the cells at a multiplicity of (100:1) and rotated end over end at room temperature in a 1.5 ml microcentrifuge tube for 15 min. The cells were washed twice with 500 ul of FACS buffer (3% BSA, 0.02% sodium azide, 1 mM EDTA in PBS) and analyzed for GFP fluorescence using a Becton Dickinson FACSAria. Ten thousand cells were counted in each sample. Data was analyzed using Flowjo software 8.8.2. Threshold gates were drawn based on a no bacteria control. Results reported as a percentage of PAO1 treated cells. All experiments were conducted in triplicate with three independent trials. Within each trial, data was normalized to the average adherence percentage of PAO1. The average normalized percentage of each trial was then calculated and used in statistical analysis. Student's t-tests were performed to determine reported P-values.

RESULTS

MudPIT detection of peptides in PAO1 *kinB* mutant and PAO1 *kinB/rpoN* double mutant. KinB is a histidine kinase of a two-component signal transduction system with the alginate response regulator AlgB (27). In our previous studies, we observed inactivation of alginate regulator *kinB* in PAO1 caused alginate overproduction (Fig. 1) (5). This suggested KinB is a negative regulator of alginate production in wild type *mucA* strain PAO1. Since inactivation of *kinB* causes mucoidy (PAO1*kinB::aacC1)*, we hypothesized AlgU dependent gene products, as well as genes controlled by KinB-AlgB, would be upregulated. We sought to identify proteins upregulated in the *kinB* mutant to discover members of the KinB regulon. To do so, we subjected total protein extracts to Multidimensional Protein Identification Technology (MudPIT) analysis. MudPIT analysis utilizes two liquid column chromatographic separations and tandem MALDI TOF-MS peptide fingerprinting to identify peptides in a complex sample. Conditions of these experiments allowed survey of the peptides present in the highest concentrations in the proteomes analyzed.

Alginate production by the *kinB* mutant requires *rpoN* (5). Therefore we compared the proteomes of *kinB* mutant and the nonmucoid *kinB/rpoN* double mutant. MudPIT analysis shows peptides in a complex sample that are in the highest concentration. In mucoid PAO1*kinB::aacC1,* AlgD was present but absent in the *kinB/rpoN* double mutant (Table 2). AlgD, or GDP-mannose 6-dehydrognease, is responsible for the initial enzymatic steps

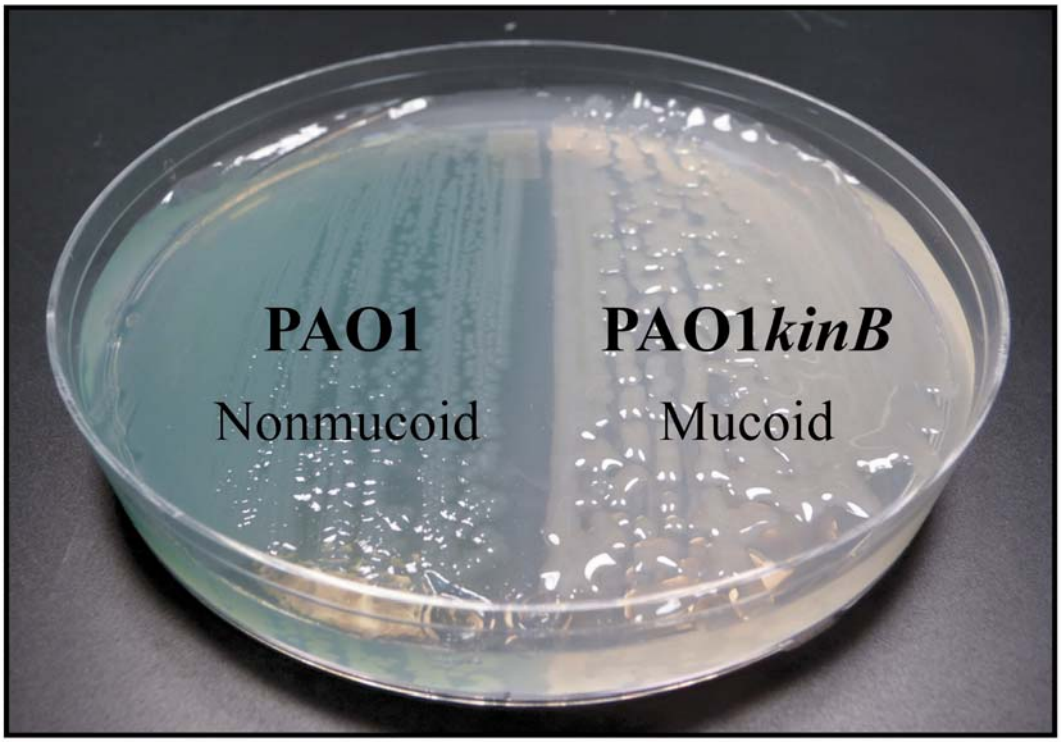

Figure 1. Colony morphologies of *P. aeruginosa* PAO1 and isogenic mucoid variant PAO1*kinB::aacC1*. Inactivation of *kinB* in PAO1 causes alginate overproduction (5).

Table 2. Proteins identified in total protein lysates of mucoid strain PAO1*kinB::aacC1* and nonmucoid strain PAO1*kinB::aacC1*Δ*rpoN* as determined by LC-MALDI TOF-TOF mass spectrometry.

Strain	locus (protein name)	relative % of identified peptides within sample	Description (functional class)
PAO1*kinB::aacC1*			
	PA4739*	16.6	hypothetical protein (unknown)
	PA0594 (SurA)	14.1	peptidyl-prolyl cis-trans isomerase (chaperones & heat shock proteins)
	PA2518 (XylX)	10.7	toluate 1,2-dioxygenase alpha subunit (carbon compound catabolism)
	PA0041*	8.8	probable hemagglutinin (toxins, enzymes, alginate)
	PA3540 (AlgD)	8.6	GDP-mannose 6-dehydrogenase (toxins, enzymes, alginate)
	PA2412	8.3	hypothetical protein (unknown)
	PA2687 (PfeS)	7.8	two-component sensor histidine kinase (regulatory systems)
	PA4385 (GroEL)	7.2	GroEL protein (chaperones & heat shock proteins)
	PA2169*	5.5	hypothetical protein (unknown)
	PA3692 (LptF) *	**5.5**	**outer membrane protein (membrane proteins)**
	PA4277 (TufB)	5.5	elongation factor Tu (post-translational modification)
	PA4922 (Azu)*	1.5	azurin precursor (energy metabolism)
PAO1*kinB::aacC1*Δ*rpoN*			
	PA4922 (Azu) *	15.4	azurin precursor (energy metabolism)
	PA1754 (CysB)	14.0	transcriptional regulator (amino acid biosynthesis and metabolism)
	PA1337 (AnsB)	13.2	glutaminase-asparaginase (amino acid biosynthesis and metabolism)
	PA5339	9.5	hypothetical protein (unknown)
	PA5242 (Ppk)	7.4	polyphosphate kinase (nucleotide biosynthesis and metabolism)
	PA4336	7.3	hypothetical protein (unknown)
	PA4385 (GroEL)	6.3	GroEL protein (chaperones & heat shock proteins)
	PA2952 (EtfB)	5.5	electron transfer flavoprotein beta-subunit (energy metabolism)
	PA4244 (RplO)	3.8	ribosomal protein L15 (post-translational modification)

PA3686 (Adk)	3.3	adenylate kinase (nucleotide biosynthesis and metabolism)
PA0962	2.8	probable DNA-binding stress protein (adaptation)
PA3611	2.3	hypothetical protein (unknown)
PA0888 (AotJ)	2.0	arginine/ornithine binding protein (transport of small molecules)
PA3021	1.4	hypothetical protein (unknown)
PA0329	0.8	hypothetical protein (unknown)
PA2743 (InfC)	0.8	translation initiation factor IF-3 (post-translational modification)
PA0456	0.6	probable cold-shock protein (adaptation)
PA0981	0.6	hypothetical protein (unknown)
PA1804 (HupB)	0.5	DNA-binding protein HU (replication, recombination, modification, and repair)
PA1852	0.2	hypothetical protein (unknown)
PA2622 (CspD)	0.6	cold-shock protein (adaptation)
PA2966 (AcpP)	0.6	acyl carrier protein (Fatty acid and phospholipid metabolism)
PA3031	0.5	hypothetical protein (unknown)
PA3745 (RpsP)	0.6	ribosomal protein S16 (replication, recombination, modification, and repair)

[*] Indicates protein has been shown to be regulated by quorum sensing.
[a] PA loci number designations are according to the Pseudomonas Genome Project http://www.pseudomonas.com.
[b] This value refers to the relative quantity of the protein within the sample. The value is calculated by comparison of number of amino acids identified corresponding to the full length peptide. The ratio of the identified peptides within the sample was also normalized against the various lengths of the expected lengths of the peptides and displayed as percent of peptides identified within the sample.

leading to alginate production in *P. aeruginosa*. Another differentially expressed peptide observed between the two proteomes of the *kinB* and the *kinB/rpoN* double mutant was azurin (PA4922) (Table 2). Azurin is a quorum sensing (QS) regulated redox protein that is located in the periplasm (30, 41). Azurin is secreted by *P. aeruginosa* in response to eukaryotic proteins and induces apoptosis of macrophages (47). In the *kinB* mutant, azurin comprised 1.5% of peptides identified; however, in the *kinB/rpoN* mutant, azurin represented 15.4% of peptides identified.

Five of the twelve peptides identified in the *kinB* mutant have been implicated to be controlled by QS (Table 2). QS regulated proteins were observed in the *kinB* mutant but only one (azurin) in the *kinB/rpoN* double mutant. PA4739 is a small periplasmic hypothetical protein that has been shown to be upregulated in response to QS signals (36) and hydrogen peroxide (34). PA0041 is similar to *Bordetella pertussis* haemagglutinin exoprotein (20). PA0041 was detected in the *kinB* mutant but not the *kinB/rpoN* double mutant (Table 2). Since PA0041 is a secreted protein, it may be a component of the exopolysaccharide matrix of the *kinB* mutant (Fig. 1).

Identification of mucoidy-coupled lipotoxin F. The periplasmic chaperone, SurA, was identified in the *kinB* mutant (Table 2). SurA has been shown to assist in folding of outer membrane proteins OmpA, OmpF and LamB in *E. coli* (21). In our analysis, only one potential outer-membrane protein was observed, PA3692 or LptF (12). Many lipoproteins or lipotoxins have been shown upregulated in mucoid *mucA* mutants (12) and in the presence of the cell wall inhibitor D-cycloserine (44). According to the Pseudomonas Genome Database V2 (http://www.pseudomonas.com) LptF (PA3692) is a conserved OmpA-like lipoprotein. The C-terminal 110 residues are 49% identical to *P. aeruginosa* major porin OprF. We observed LptF

was upregulated in the *kinB* mutant however absent from the *kinB/rpoN* double mutant (Table 2). To validate the observations from the MudPIT analysis, total protein extracts of PAO1 and PAO1*kinB::aacC1* were separated on SDS-PAGE and visualized by Coomassie staining (data not shown). A significantly up-regulated protein was observed in PAO1*kinB::aacC1* total protein extracts with an apparent mass of 27 kDa (Fig. 2 Lane 2). The protein was identified as LptF (PA3692) by direct peptide fingerprint analysis.

LptF is an outer-membrane protein. Computation analysis of the *P. aeruginosa* genome shows LptF has a predicted type II signal peptide for export (24). To confirm LptF is in fact an outer-membrane protein, outer-membrane proteins from PAO1 and PAO1*kinB::aacC1* were prepared by the sarkosyl method. Total protein extracts and sarkosyl insoluble proteins were separated and visualized by silver staining (Fig. 2). LptF is upregulated in the outer-membrane protein fraction of PAO1*kinB::aacC1* however it is also present in PAO1 (Fig. 2 Lanes 3 and 4). Lipotoxins have been shown to activate the host inflammatory response (12) however their physiological functions have not been investigated, and therefore we further characaterized lipotoxin F.

Expression of P_{lptEF} is AlgU-dependent and up-regulated in CF isolates. We reasoned that since LptF was up-regulated in *mucA* mutants (12) and in the mucoid *kinB* mutant, it was likely AlgU-dependent. LptE and LptF are encoded in the genome as an operon (12). Interestingly, the *lptEF* promoter does not contain an AlgU consensus sequence (12). A *lacZ* fusion with the *lptEF* promoter was constructed and integrated into the *P.aeruginosa* chromosome to compare expression of P_{lptEF} in various strains. P_{lptEF} was active in nonmucoid strains PAO1 and PA14 (Fig. 3). P_{lptEF} expression was observed

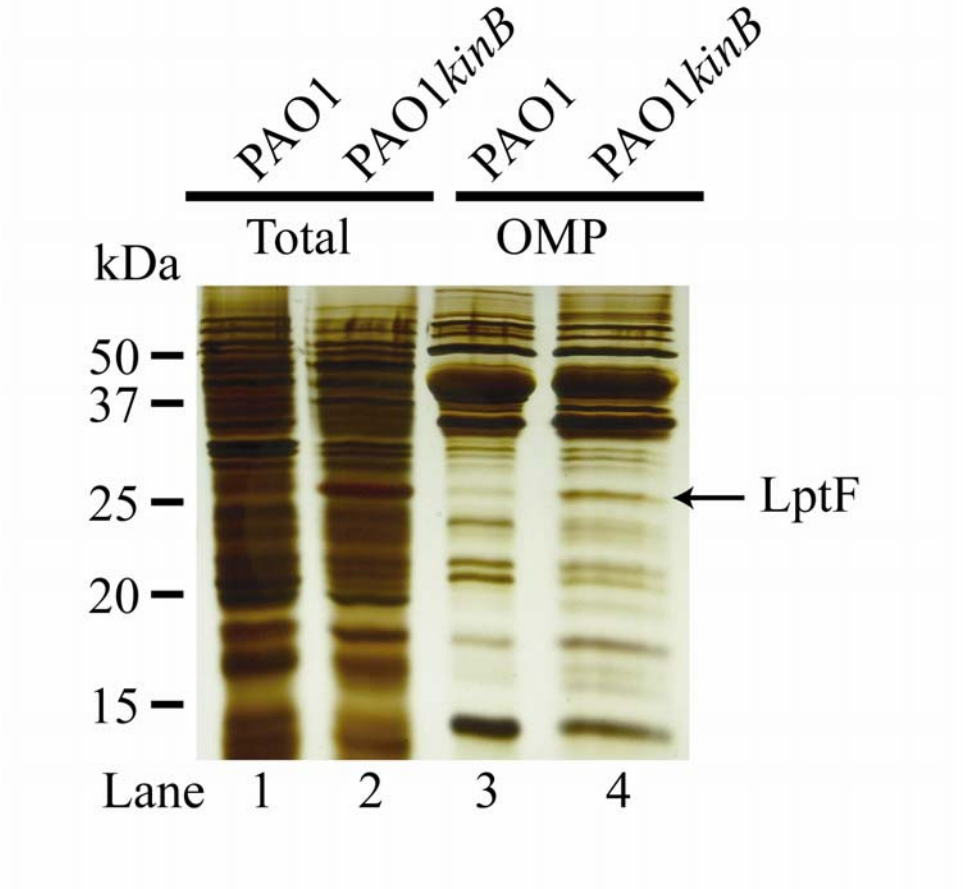

Figure 2. SDS-PAGE of total and outer-membrane proteins from *P. aeruginosa* strains PAO1 and PAO1*kinB::aacC1* reveals LptF is an outer-membrane protein**.** Outer-membrane proteins (OMP) were isolated from total protein lysates by precipitation in 2% sarkosyl. 60 μg of protein preparation was separated and submitted to silver staining. The apparent molecular masses are indicated based on comparison of protein ladder standards (10-250 kDa). The arrow indicates the position of LptF in the separations.

to be AlgU-dependent and can be restored upon expression of AlgU in *trans* (Fig. 3). Also deletion of *algU* from PAO1*kinB::aacC1* caused complete loss of detectable P_{lptEF} (Fig. 3).

Since it was clear *lptEF* expression was AlgU-dependent from previous research and data observed in this study, we hypothesized mucoid CF isolates would have increased expression of the *lptEF* promoter. Strain 383 is a nonmucoid CF isolate (15) and strain 2192 is an isogenic strain to 383 with a *mucA* mutation (15). Interestingly, P_{lptEF} was upregulated in both nonmucoid CF isolate 383 and mucoid CF isolate 2192 (Fig. 3) compared to lab strain PAO1 (Fig. 3). However, no P_{lptEF} expression was detected in nonmucoid CF149. This infers that CF149 may harbor an *algU* mutation. When we sequenced *algU* and *mucA* in CF149, we found this strain carries both *algU* and *mucA* mutations. The *algU* gene of CF149 has a missense mutation (C_{182} to T_{182}) resulting in amino acid change from Ala_{61} to Val_{61}. The *mucA* mutation is deletion of a C at 374 which causes a frameshift with the formation of a premature stop at TGA_{386} (GenBank accession FJ649224). This further suggests P_{lptEF} expression is AlgU-dependent. Several other CF isolates showed high *lptEF* expression (Fig. 3). Even within one CF sputum sample several morphologies were observed and each exhibited a different level of *lptEF* expression (Fig. 3 strains CFO23o,s,w). These results show the *lptEF* promoter is AlgU-dependent and upregulated in CF isolates.

LptF is not required for alginate production. Envelope proteins such as MucE can activate alginate overproduction in *P. aeruginosa* through regulated proteolysis of MucA by the serine protease AlgW (32). Since LptF was highly upregulated in the mucoid *kinB* mutant, we examined if LptF expression plays a role in the signal transduction which leads to AlgW-dependent alginate production of this strain (5). To test this, *lptF* was deleted from PAO1*kinB::aacC1*. However, both the *kinB* mutant and the *kinB/lptF* double mutant

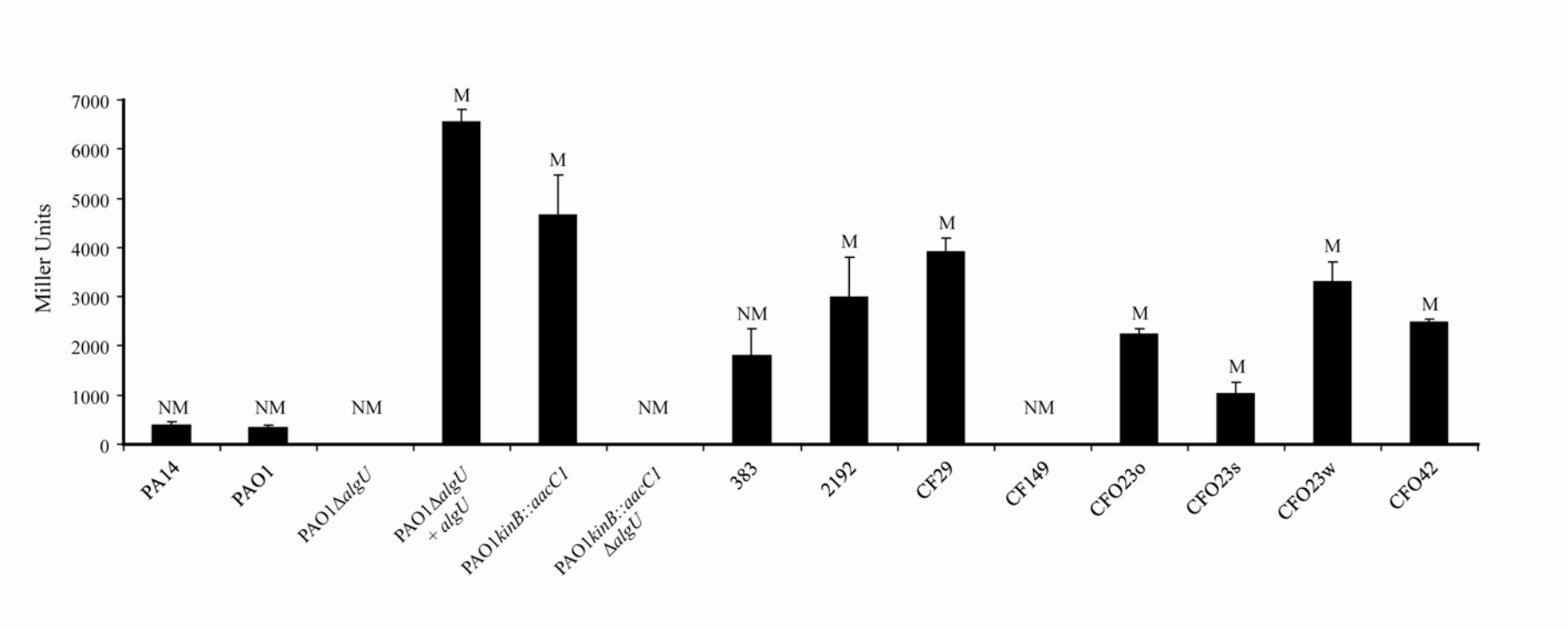

Figure 3. The β-galactosidase activity from P_{lptEF}-*lacZ* reporters constructs integrated to the chromosome at the *attB* site (18) in laboratory and cystic fibrosis isolates. Note expression of the *lptEF* promoter requires AlgU and *lptEF* expression is upregulated in CF isolates. NM indicates the strain is nonmucoid and M indicates the strain is mucoid.

produced approximately 100 μg/ml/OD_{600} of alginate. Furthermore, overexpression of *lptF* in PAO1 from the P_{BAD} promoter of pHERD20T did not stimulate alginate production above the normal nonmucoid level (30 μg/ml/OD_{600}). These results suggested LptF does not activate alginate production. Therefore we concluded that LptF is likely co-expressed with alginate and is not involved in the signaling pathway leading to alginate production.

Deletion of *lptF* caused increased resistance to hydrogen peroxide in PAO1, but increased susceptibility to hypochlorite. In the cystic fibrosis lung, *P. aeruginosa* produces alginate for protection (14). Since LptF is up-regulated along with alginate production, we hypothesized LptF may serve as a protective factor. We first generated a PAO1 *lptF* deletion mutant and observed no changes in growth rate compared to PAO1 showing *lptF* is not an essential gene (data not shown). We next examined if LptF has a protective role against hydrogen peroxide and hypochlorite. To test the role of *lptF* regarding cell membrane integrity, susceptibility assays were performed with hydrogen peroxide and hypochlorite (Table 3). Interestingly, PAO1Δ*lptF* was more resistant to hydrogen peroxide than PAO1 (Table 3). However, deletion of *algU* did not result in the same level of resistance to hydrogen peroxide. Deletion of *lptF* caused significantly increased susceptibility to NaOCl (Table 3). Neutrophils utilize generation of oxidants to kill microbes and mucoid mutants are more resistant hypochlorite killing (22). Deletion of *algU* and *lptF* caused increased susceptibility to NaOCl (Table 3). This data suggests the AlgU-dependent proteins such as LptF may protect *P. aeruginosa* from NaOCl killing.

Deletion of *rpoN* or *lptF* decreases adhesion to A549 lung epithelial cells. Most lipotoxins are small lipoproteins that are likely housed in the inner-leaflet or periplasm. However LptF is an outer-membrane protein (Fig. 2). *E. coli* OmpA can participate in adhesion to surfaces and

Table 3. Altered sensitivity of *P. aeruginosa* strains to hydrogen peroxide and hypochlorite.

	Growth inhibition zone (mean radius [mm] ± SE)[b]	
Strain[a]	10% H_2O_2	6% NaOCl
Lab strain		
PAO1	9.7 ± 0.3	8.2 ± 0.4
PAO1Δ*lptF*	7.8 ± 0.2[c]	10.7 ± 0.3[d]
PAO1Δ*algU*	12.0 ± 0.8	12.2 ± 0.2[d]

[a] PAO1 is wild-type nonmucoid *P. aeruginosa* strain. For statistical analysis isogenic strains were compared to PAO1.
[b] Sensitivity to killing hydrogen peroxide and hypochlorite is expressed as zones of inhibited growth around filter disks impregnated with 10µl of solution indicated. Zones were measured after 24 h at 37°C and are the means of three experiments.
[c] Indicates the strain was more resistant to killing than PAO1. Students unpaired t-test; two tailed.($p<0.05$).
[d] Indicates the strain was more susceptible to killing that PAO1. Students unpaired t-test; two tailed ($p<0.05$)

interactions with cells (40), therefore we were interested to see if LptF also has a role in adhesion. To test this hypothesis, we performed adherence assays with A549 lung epithelial cells. A constitutively GFP-expressing plasmid pMRPQ-1 (6) was conjugated into PAO1, PAO1Δ*rpoN*, PAO1Δ*lptF* and mucoid strain PAO1*kinB::aacC1*. Pili and flagella expression are controlled by *rpoN* (19, 43). TRL5, which is expressed on A549 cells, recognizes flagellin and promotes adherence of bacteria to cell surfaces (16). Thus, PAO1Δ*rpoN* serves as a negative control for adhesion for our experiments. Epithelial cells were incubated with indicated bacteria strains for 15 minutes at room temperature. The cells were then washed twice and analyzed immediately by flow cytometry. Threshold gating was used to determine the percentage of GFP positive cells (Fig. 4A), which is indicative of the adherence of the bacteria to the A549 cells. PAO1 readily adhered to A549 cells, and as expected, deletion of *rpoN* substantially decreased adhesion (Fig. 4B and 4C). In the absence of *lptF*, adhesion to A549 cells decreased to 71.5% ± 7.9 compared to PAO1 (Fig. 4B). This data suggests that *lptF* is required in PAO1 for maximal adhesion to A549 cells. LptF is highly upregulated in PAO1*kinB::aacC1,* which produces copious amounts of alginate (Fig. 1). However, PAO1*kinB::aacC1* adherence is reduced compared to PAO1 (Fig. 4B).

DISCUSSION

P. aeruginosa pulmonary infections cause detrimental and irreversible damage to the CF patient. Alginate overproduction by *P. aeruginosa* occurs in response to the conditions of the CF lung. Lipotoxins are co-expressed with alginate genes. We observed LptF was the only

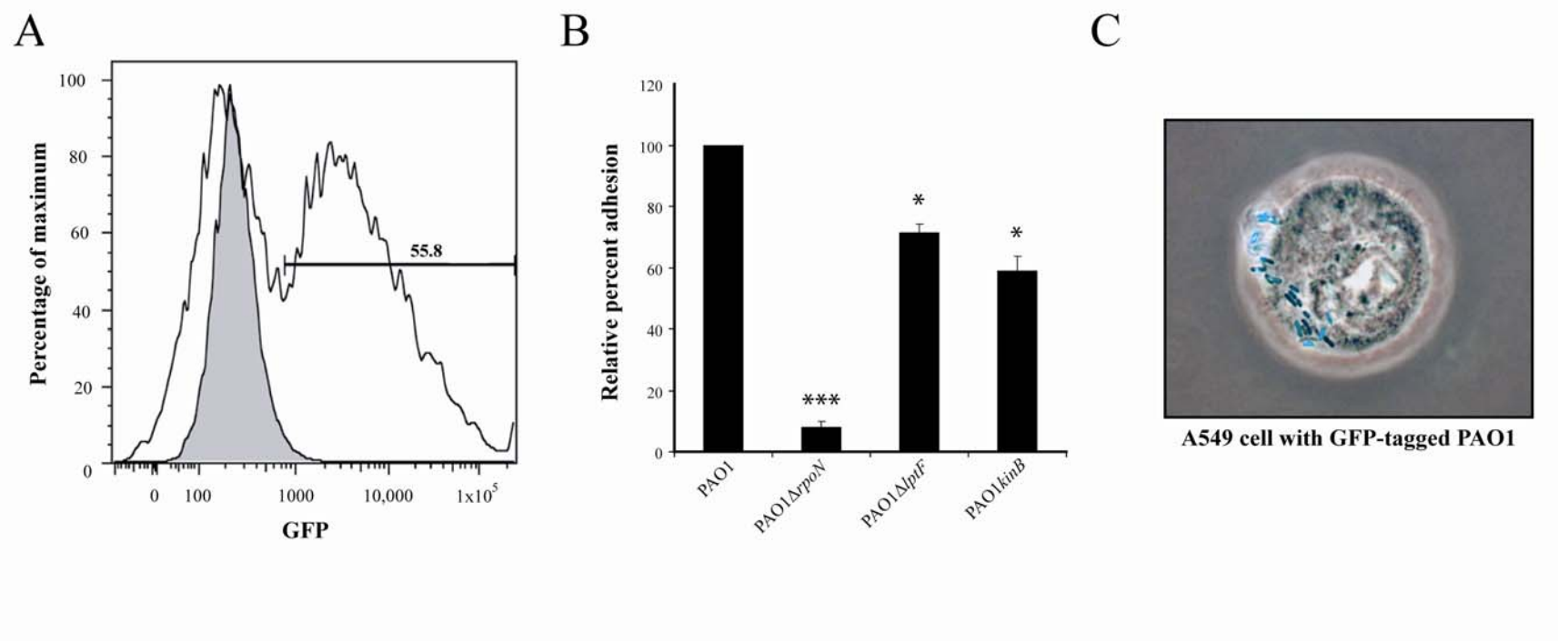

Figure 4. Adherence of *P. aeruginosa* to A549 lung epithelial cells. 4A. Flow cytometry threshold gating of GFP positive A549 cells. GFP positive A549 cells were determined by threshold gating based on A549 cells without GFP-tagged *P. aeruginosa* cells. 55.8 % of A549 cells were positive for GFP-tagged PAO1. 4B. Relative percent adhesion of GFP-tagged *P. aeruginosa* strains to A549 cells. Values were normalized to the observed amount of GFP-tagged PAO1 adhesion to A549 cells. Experiments were performed in triplicate with three independent studies. A student's *t* test was performed for comparison of each of the isogenic mutants to PAO1. Asterisks indicate significant differences (*** $p<0.00001$; *$p<0.001$). 4C. A549 lung epithelial cell with adherent GFP-tagged (pMRPQ-1) PAO1 cells.

lipotoxin identified in MudPIT proteome analysis of the mucoid *kinB* strain. We also confirmed LptF is an outer-membrane protein (Fig. 2). LptF upregulation in mucoid cells (10-12, 44) suggests that LptF may have roles in establishment of mucoid biofilms. Collectively this data warranted that further investigation was necessary.

We first examined *lptF* expression and confirmed *lptF* expression is controlled by AlgU. (Fig. 3). P_{lptEF} expression is upregulated in both nonmucoid and mucoid CF isolates (Fig. 3). Since *lptF* expression is dependent upon AlgU and the *lptEF* promoter does not have an AlgU consensus sequence, there are two possible mechanisms for AlgU expression of *lptF*. Either AlgU drives transcription of LptF directly or indirectly through expression of another transcription factor. Ultimately, LptF expression depends on the master regulator of alginate, AlgU.

To further characterize LptF, we generated an unmarked deletion mutant for downstream analysis. PAO1Δl*ptF* was assayed for survival against killing by hydrogen peroxide and hypochlorite. Our data suggests that LptF has a role in resistance against hypochlorite; however, the deletion of *lptF* causes increased resistance to hydrogen peroxide. This differential suggests LptF protection may be specific for certain niches or environments. In the CF lung *P. aeruginosa* forms biofilms (38) and colonization of the CF lung occurs first by nonmucoid strains (3). These early colonizing strains then establish an immunostimulatory phase of infection (8) resulting in increased inflammation. Mucoid biofilm conversion occurs due to mutations in the anti-sigma factor *mucA* (28). Furthermore, with conversion to mucoidy comes upregulation of the stimulatory lipotoxins. Lipotoxins, like LptF stimulate inflammatory responses through TRL2 (12). Motile strains with flagella activate TRL5 recognition (48). Therefore, immune responses due to the presence of *P. aeruginosa* occurs starting with the initial

infection and continues through the rest of the CF patient life due to inability to eradicate *P. aeruginosa* from the CF lung (4).

Our data shows that deletion of *rpoN,* which controls expression flagella and pili (19, 43), severely attenuated adhesion to A549 epithelial cells. Flagella and pili are both required for early biofilm formation (31). PAO1Δ*lptF* which is motile like PAO1 (data not shown) adheres to A549 cells to a lesser extent than PAO1. This suggests LptF is likely recognized independently by epithelial cells which may allow *P. aeruginosa* to attach to the tissue surface. Alternatively the loss of LptF could result in blockage of transport of extracellular factors necessary to adhere to epithelial cells. PAO1*kinB::aacC1* adhered to A549 cells less than PAO1. Although PAO1*kinB::aacC1* produces alginate there are other factors such as repression of motility factors by AlgU (2, 42) which could affect adherence.

Lipotoxins such as LptF likely not only cause the inflammatory response and detrimental tissue damage in the CF lung, but may also protect *P. aeruginosa* and preserve the biofilm. MudPIT proteomic analysis of the mucoid *kinB* mutant suggests AlgU-dependent LptF is the major lipotoxin expressed in the mucoid strain proteome (Table 2). Unlike most of the other lipotoxins, LptF is an outer-membrane protein (Fig. 2). We also observed *lptF* expression was upregulated in CF isolates (Fig. 3), and LptF may have roles in protection (Table 3) and adhesion to lung epithelia (Fig. 4). Since LptF is highly expressed in mucoid strains that cause chronic infection, it will be interesting to use synthetic peptides to further analyze the activation of the specific inflammatory response to LptF. Recently, azithromycin has been shown to downregulate expression of lipotoxins LptF, LptE, LptD, SlyB, OsmE and PA1323 (39). Also other macrolides have been shown to alter biofilms (45). Therefore, therapeutic treatments with

azithromycin may be able to lessen the potential respiratory tract damage caused by *P. aeruginosa* lipotoxins, such as LptF.

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CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

It is critical to elucidate the mechanisms by which *P.a.* can overproduce alginate because this phenotype directly affects the health and mortality of CF patients. The classical mechanism of conversion to mucoidy has been established; however, data presented here in this dissertation indicates there are other negative regulators of alginate production. These negative regulators are likely part of signal transduction pathways which could be utilized by *P.a.* when infecting the CF lung. In this chapter the data from this dissertation will be summarized and new lines of research will be proposed.

Controllable expression vectors in *Pseudomonas*

In Chapter 2 of this dissertation, the construction and validation of a new set of vectors for controlled gene expression were described. Throughout this dissertation pHERD vectors were extensively utilized in a number of experiments. In Chapter three, pHERD-based vectors were used for complementation studies such as gene expression to examine the effect on mucoid phenotype (Chapter 3, Fig. 2), and for analysis of promoter strength through a reporter assay (Chapter 3, Fig. 3). Next, pHERD vectors were utilized for conditional expression of an HA-eptiope-tagged MucA (Chapter 3, Fig. 4B). By tagging the gene, a commercial antibody could be utilized for detection of the peptide though Western Blot analysis. However, one pitfall to these experiments is that the data is merely an average of all degradation events of the HA-tagged peptide and thus do not provide kinetic data describing the actual degradation. To eliminate this downfall, a novel method for kinetic analysis was developed and performed. Addition of arabinose causes the AraC transcriptional regulator of the PBAD promoter to be deactivated and

allows transcriptional initiation of the PBAD promoter. Furthermore, glucose has been shown to repress PBAD promoter expression due to the catabolite repression. This was utilized to reduce leaky expression of the PBAD promoter, allowing more accurate measurement the amount of HA-tagged MucA over time to determine the rate of degradation (Chapter 3, Fig. 5). Conditional expression of the target gene via the PBAD promoter allowed these experiments to provide useful data. In Chapter 4 the pHERD vectors were used for truncation analysis of MucD to determine the roles of the PDZ domains in suppression of alginate production (Chapter 4, Figs. 1, 2 and 3). Since the pHERD vectors replicate in *E. coli*, Western blot analysis of the pHERD-*mucD* constructs could be performed in *E. coli* as well as *P.a.* (Chapter 4, Fig. 2A). Each of the pHERD-*mucD* constructs were then used for complementation analysis in tandem with Western blotting (Chapter 4, Fig. 2B). This analysis provided an interesting observation regarding the stability of the truncated MucD proteins, which will be discussed in detail later in this chapter. In Chapter 5 of this dissertation LptF was overexpressed to test the hypothesis that it was responsible for the high alginate production in the *kinB* mutant. Overexpression of LptF did not affect alginate production, and therefore showed it was a co-expressed factor that was later shown to be controlled by AlgU. Collectively, the data described in these chapters shows the high utility of the pHERD vectors in *P.a.* However, modification of these vectors would increase their usefulness; therefore, potential modifications to pHERD will be discussed.

pHERD vectors carry *bla* (pHERD20T), *aacC1* (pHERD30T), *tet* (pHERD26T), and *dfRII* (pHERD28T), which encode antibiotic resistance genes for ampicillin, gentamicin, tetracyline, or trimethoprim, respectively. Many genetically altered strains

are produced via transposon insertions. Therefore it would be useful to have pHERD vectors with other antibiotic markers for compatability. Recently a new antibiotic resistance marker has been characterized for resistance to Zeocin, which is a member of the bleomycin family of antibiotics isolated from *Streptomyces*. The pHERD series of vectors could be expanded by insertion of the *Sh ble* gene from *Streptoalloteichus hindustanus*. With this marker Zeocin could be used to select for pHERD. The addition of a new marker to the pHERD series would be beneficial; however, addition of other selection markers could also improve their usefulness.

The *Bacillus subtilis* gene *sacB* encodes levansucrase. Levansucrase hydrolyzes sucrose and synthesizes levans, which are high molecular weight fructose polymers. In most gram negative bacteria, expression of *sacB* in the presence of sucrose kills the organism. This counter-selection marker is especially useful for curing or removing plasmids. To cure a plasmid, the strain is maintained in media lacking antibiotic selection corresponding to the plasmid. Then isolates from the culture are screened for sensitivity to the antibiotic due to the loss of the plasmid. However, most plasmids are maintained as multicopy within bacteria; therefore, it is routine to screen hundreds to thousands of isolates to cure a muticopy plasmid. Addition of a *sacB* gene to the pHERD vectors would allow easy curing by selecting isolates that are resistant to *sacB* and therefore have lost the plasmid.

In some instances the fact that pHERD vectors are multicopy is not optimal due to the fact that some experiments require low expression of the modulated genes. Single or low copy pHERD vectors would also improve their utility. The data presented in this dissertation shows that the PBAD promoter is useful in *P.a.;* however, a decrease in copy

number would better simulate gene expression conditions *in vivo* (Chapter 2). Direct integration of the PBAD promoter and the *araC* gene onto the chromosome of *P.a,* would also improve the series. The PBAD promoter and *araC* gene could be fused onto a chromosomal shuttle vector such as miniCTX and integrated onto the chromosome. Then the *tet* resistance gene from miniCTX could be removed via FLP recombinase (encoded on pFLP2) (3, 4). PBAD promoter expression from a single copy would be very useful for expression of highly regulated toxic genes that even pHERD vectors cannot express. Also, the fact that the expression system is integrated into the chromosome give the geneticist another antibiotic marker for selection.

Throughout this dissertation the hemagglutin (HA) tag has been used for eptitope tagging of proteins expressed from pHERD vectors. By incorporating the coding sequence of HA into the flanking PCR primers the HA tag can be put on either the N- or C-terminus or internally through crossover PCR techniques. In data not shown here, we have also utilized other tags such as the 6X histidine tag which have been useful for purification of proteins by affinity chromatography. Pseudomonas species have a high amount of histidine-rich proteins. Therefore proteins are not effectively isolated with the use of nickel affinity for 6XHis-tagged proteins. Therefore, a translation fusion of a maltose-binding protein would be an improvement for isolation and purification. To isolate small proteins, a translational fusion of maltose binding protein (MBP) could be added in front of the multiple cloning site of pHERD vectors. MBP will bind amylose, which would allow for affinity chromatography purification. Since MBP contains a protease site on its C-terminus, the fusion could be cleaved and thus release the protein of interest.

pHERD vectors have been utilized throughout this dissertation and in other publications (8, 9). However, here several improvements have been suggested to these vectors to better arm the *Pseudomonas* geneticist for testing hypotheses.

Negative regulation of Alginate biosynthesis by KinB and MucD

From the data presented in Chapters 3 and 4 of this dissertation, it was clear that the histidine kinase KinB and the serine protease MucD are both negative regulators of alginate biosynthesis. Both KinB and MucD affect AlgU activity; however, it is evident that they utilize divergent pathways. In this section, the similarities and differences will be discussed and future lines of research will be proposed.

The *kinB* gene was first identified and characterized before the *P.a.* strain PAO1 genome was completely sequenced (7). *kinB* is encoded in an operon with *algB* (7). Based on its homology with *Bacillus subtilis* PhoR, KinB was characterized as a histidine kinase (7). Since most two-component systems such as NtrBC use phosphorylation of the response regulator by the histidine kinase to activate specific gene expression, it would have been expected that KinB phosphorylation of AlgB activates alginate production. However, inactivation of *kinB* in mucoid *mucA* mutant FRD1 does not block alginate production (6). It is clear KinB can phosphorylate AlgB (7); however, phosphorylation of AlgB is not required for alginate production (6). Collectively, these studies suggested that KinB does not play a positive role in alginate production. In Chapter 3 of this dissertation, nonmucoid strain PAO1 was mutagenized with the mariner family transposon pFac (11). Inactivation of *kinB*, as well as an in-frame deletion of *kinB* caused alginate production (Chapter 3, Fig. 1). These data reclassify KinB as a negative

regulator of alginate production. Therefore, in the absence of KinB, alginate production goes uncontrolled.

Since KinB has been shown to phosphorylate AlgB, it was hypothesized that *algB* would be required for alginate production. Alginate production was dependent upon *algB* (Chapter 3, Fig. 1). However, when an AlgB that carried a mutation of the predicted phosphorylation site from an aspartic acid to an asparagine (D59N) was expressed, alginate production could continue to occur (Chapter 3, Table 2). Since AlgB is a NtrC-family response regulator it harbors a σ^{54} interacting domain (http://www.pseudomonas.com/getAnnotation.do?locusID=PA5483). Deletion of *rpoN* (σ^{54}) from the *kinB* mutant also blocks alginate production. Since *P.a.* strain PAO1 has a wild-type *mucA* gene, it could be postulated that one of three main mechanisms could occur and cause alginate production independent of the classical *mucA* mutation. The first possibility is that P_{algD} transcription could be directly activated by RpoN; however, no data supports this possibility since all mucoid strains characterized require *algU*. The second possibility is that P_{algU} transcription could occur and result in a high amount of AlgU in the cells. In data not presented here, it was observed that alginate production in the presence of high expression of AlgU can occur in PAO1Δ*algW*. Therefore, it is likely AlgW may not be required when an ultra-high AlgU expression is occurring. PAO1*kinB* mucoid mutants require *algW* (Chapter 3 Fig. 2B) for alginate production. This data strongly suggests a third possible mechanism whereby derepression of MucA is occurring via proteolyic degradation by the serine protease AlgW.

Since it seemed AlgW was responsible for the activation of AlgU, this hypothesis was tested by promoter-reporter fusions and complementation. PAO1-based strains with

primary mutations or deletions of *kinB* received chromosomal integrations of P_{algU} and P_{algD} fused with *lacZ* as a reporter for promoter expression. To show the mutations were valid, pHERD-based constructs were introduced *in trans* and the mutations were complemented (Chapter 3, Fig. 3). From this data, it was clear that both P_{algU} and P_{algD} activity in *kinB* mutants requires *algU, algB, rpoN,* and *algW*. It was expected that *algU* and *algW* would be required for P_{algU} activity; however, it was unexpected that *algB* or *rpoN* would be required. AlgB and RpoN, as reported in Chapter 1, have been implicated in control of alginate production at the level of P_{algD}. Since *algW, algB* and *rpoN* were required for alginate production it was hypothesized that they may all contribute to proteolysis of MucA. It would be expected that AlgW would play a role in MucA degradation. The hypothesis that *algW, algB* and *rpoN* all affect MucA degradation was tested by examining degradation of a HA-tagged MucA protein expressed from pHERD-based vectors (Chapter 3, Fig. 4). *kinB* double mutant strains of *algW, algB,* and *rpoN* all displayed the same HA-MucA degradation profile with an accumulation of a 19 kDa peptide (Chapter 3, Fig. 4B). Using pHERD conditional expression a higher rate of degradation of HA-MucA was observed in a *kinB* mutant than PAO1 (Chapter 3, Fig. 5). These data established that KinB controls alginate production through AlgW proteolysis; however, this pathway has not been completely elucidated.

The data from Chapter 3 proposed two main models. In Model 1 (Chapter 3, Fig. 6) the *kinB* mutation causes loss of expression of a periplasmic protease that is responsible for chaperoning or degrading AlgW-activating proteins. In Model 2 AlgB/RpoN are responsible for expression of AlgW-activating proteins when *kinB* is inactivated. Since phosphorylation of AlgB by KinB does not seem to be required for

alginate production, KinB could perform phosphatase activity towards AlgB. Thus, when AlgB is un-phosphorylated, AlgB is active, and together with RpoN, increase expression of AlgW activating proteins. Both models indicate that AlgW activating proteins are up-regulated to a level that allows AlgW to cleave MucA and activate alginate biosynthesis. In Chapter 5 proteomic analysis of the *kinB* mutant was performed (Chapter 5, Table 2). Within the list of peptides identified by mass spectrometry, there are no proteins that harbor C-terminal AlgW-activating sequences that have been previously identified (9). It is possible the AlgW-activating protein does not have a "typical" C-terminal sequence. In order to probe and locate the AlgW activating protein total transcriptome analysis must be performed. Transcriptome analysis has not been performed with the *kinB* mutant; however, recent data has shown that cell wall syntesis antibiotics can up-regulate an AlgW-dependent pathway (12). Inhibitors of the cell wall synthesis cause activation of AlgU. However, no protein capable of activating AlgW was recognized or identified (12). Therefore, it is possible that the expression of AlgW activating proteins is low. In this model, it would be expected that AlgW-activing proteins are expressed at a low level but when stress conditions arise, then these proteins could be misfolded or released from the outer membrane to activate AlgW and the AlgU stress response.

The models proposed in Chapter 3 were constructed based on the absence of KinB. However, since KinB is a histidine kinase it is likely that it is capable of sensing environmental conditions and modulating the pathway that is constitutively active. If the KinB pathway is high in the chain of sensory events, then it would be expected that the chaperone/protease MucD could modulate the signals of the KinB pathway. Since MucD is both a protease and a chaperone either of these functions may be play role. However,

data presented in Chapter 4 show that the mucoidy of the *kinB* mutant cannot be complemented by over-expression of MucD (Chapter 4, Table 3). In Chapter 4, the pathway modulated by the negative regulator MucD is characterized. When *mucD* is inactivated, *algW* is not required for alginate production; however, *mucP* is required (Chapter 4, Table 2). Therefore, it is clear that MucD and KinB control divergent pathways. It is also known that the small periplasmic protein MucE can activate AlgW (9), and in Chapter 4, it was shown that overexpression of MucD can block MucE mucoidy (Chapter 4, Table 3). From this data, it can be implied that the KinB pathway does not flow through MucE expression. In fact, in data not shown, this was confirmed by inactivation of *mucE* in the *kinB* strain. Interestingly, a periplasmic protease has been observed in the proteome of the *kinB* mutant. In Chapter 5, Table 2, SurA, a chapterone/protease, was identified in the *kinB* mutant but not in the *kinB/rpoN* double mutant. From this data it can be suggested that SurA may be the negative regulator of the KinB signals but in the absence of *kinB* the signals overcome the regulation of the protease. The role of SurA in the *kinB* mutant was not further explored, however it seems possible that SurA could be a regulator of alginate signals. This could further be investigated by expressing SurA in various mucoid strains that use regulated proteolysis.

The main difference between the *kinB* mutant and the *mucD* mutant is that they utilize different proteases to cleave MucA. When the prototype AlgW-activator MucE is overexpressed, then AlgW activates proteolysis, but intramembrane MucP is also required (9). The requirement of *mucP* was not assessed in the *kinB* mutant. In the absence of *algW,* alginate production still occurs in the *mucD* mutant (Chapter 4 ,Table 2). Interestingly, when *algW* is overexpressed in the *mucD/algW* strain, then alginate

production is lowered. AlgW clearly affects MucA degradation in the *mucD* mutant; however, MucA degradation relies on MucP (Chapter 4 Fig. 4). Data presented in Chapters 3 and 4 shows that KinB and MucD control alginate production through regulated proteolysis of MucA, however each pathway uses different proteases and, as of now, the protease-activating proteins in each of these strains have not been identified.

The homologue of MucD in *E. coli* is DegP. DegP has been extensively characterized by structural models. DegP is capable of forming large oligomeric structures and can either chaperone and refold or degrade periplasmic peptides when necessary. MucD is coded directly downstream of *algU-mucA-mucB*. *mucD* was sequenced and shown to be similar to *degP* of *E. col* (1). In this same study it was shown that inactivation of *mucD* causes alginate production (1). Later it was observed that the proteolytic domain of MucD was required for suppression of alginate production (13, 15). However, these studies did not demonstrate that MucD affected MucA proteolysis. Recently, through the use of C-terminal tagged MucA, it has been shown that inactivation of MucD destabilizes MucA (14). Furthermore, the aforementioned study suggested that AlgW was responsible for degradation of MucA (14). Here in this dissertation we showed that a *mucD/algW* double mutant performs MucA degradation, resulting in high alginate production (Chapter 4).

Data in Chapter 4 shows that MucD mediates MucA degradation. Also the roles of the PDZ domains of MucD in alginate production were investigated. The hypothesis of this section of the study was that since MucD was likely capable of oligomer formation in a fashion similar to DegP, then the PDZ domains would be critical for alginate suppression. However, this hypothesis was found to not be true since MucD

lacking each of the PDZ domains could still suppress alginate production. PDZ2 in DegP is required for oligomerization; however, since MucD without PDZ2 could suppress alginate production (Chapter 4, Figure 2B), then it is likely oligomerization of MucD is not required for alginate suppression. Interestingly MucD with only a protease domain could suppress alginate production (Chapter 4, Figure 2B). However, Western blot analysis revealed that loss of the PDZ domains of MucD causes instability of the peptide (Chapter 4, Figure 2B). The data from this study suggests the PDZ domains may inhibit a phenomenon known as autocleavage. DegP has been shown to autocleave and degrade after its function is completed (5). When MucD lacks either or both of the PDZ domains then the peptide is less stable than full length MucD. In *E. coli* the role of the PDZ domains have not been examined in regards to autocleaveage. Data presented here in this dissertation suggests autocleavage may be mediated by the PDZ domains of MucD.

Chapters 3 and 4 of this dissertation characterized a novel negative regulator of alginate (KinB) and define another novel regulator (MucD). In either model when a negative regulator is inactivated either genetically or potentially by environmental conditions, high amounts of AlgU-mediated gene expression will occur.

Lipotoxin LptF in *P. aeruginosa*.

Using a mass spectrometry approach for identification of proteins highly expressed in a total protein sample, Lipotoxin F or LptF was identified in the proteome of the *kinB* mutant (Chapter 5, Table 2). LptF was then characterized as an outer membrane protein. Originally it was hypothesized that LptF may be causing AlgW activation in the *kinB* mutant. To test this hypothesis, *lptF* was in-frame deleted from the *kinB* mutant and

no observable difference in alginate production resulted. It was interesting that LptF was so highly upregulated in the mucoid mutant. LptF shares high homology with OprF, which is considered the major outer membrane porin of *P.a.* This opened up the question as to why would a protein of high similarity to the major porin be up-regulated in the mucoid strain. First, LptF was confirmed to be an outer membrane protein (Chapter 5, Fig. 2). It was then hypothesized that LptF would be up-regulated in other mucoid strains. To test this, a P_{lptEF}-l*acZ* reporter fusion was constructed. Interestingly, mucoid and nonmucoid CF isolates had higher P_{lptEF}-l*acZ* expression than lab strains (Chapter 5, Fig. 3). From these reporter assays it was also clear that AlgU affected *lptF* expression (Chapter 5, Fig. 3). The focus of the rest of the study turned to characterizing LptF in nonmuciod cells. A PAO1Δ*lptF* strain was constructed and tested for its resistance to oxidants which would be present in the CF lung. Without *lptF,* PAO1 is more sensitive to hypochlorite (bleach), but more resistant to hydrogen peroxide (Chapter 5, Table 3). These data suggest that LptF has a role in resistance to oxidative stress. OmpA is the *E. coli* homologue of OprF of *P.a.* and OmpA has been shown to play a role in biofilm formation and adhesion (10). Using a new method of flow cytometry the role of LptF in adhesion against human lung epithelial A549 cells was examined. The data showed that deletion of *lptF* affected adhesion to A549 cells. Collectively, these data suggest LptF may have multiple roles. However, the question remains to why the bacteria up-regulate LpF as part of the AlgU-stress response associated with alginate production.

SUMMARY AND CONCLUSIONS

The hypothesis of this dissertation was: *P.a. utilizes signal transduction pathways which activate regulated proteolysis of MucA to regulate and control the activity of AlgU, the master regulator of alginate production.* Chapters 3 and 4 show that MucA degradation is controlled by divergent pathways with common features. Furthermore, our data from Chapter 5 shows that genes controlled by AlgU can alter the physiology of the organism. Fig. 1 of this chapter (Page 150) summarizes the pathways elucidated in this dissertation. However, several unknowns still exist and warrant future research. The AlgW-activating proteins controlled by KinB and the MucP activating proteins mediated by MucD need to be identified.

Regulated proteolysis of MucA is the doorway to inducible alginate production. The models studied in this dissertation provide novel data concerning this dynamic mechanism. Mutations in *mucA* will occur and mucoid cells will be selected in the CF lung because they are advantageous for survival in those conditions. However, the data presented here suggests that regulatory networks control inducible alginate production independent of mutations in *mucA*. It is possible that control of alginate production by early *P.a.* strains that infect the CF lung, could permit control of the organism to a point of prolonging the patient-detrimental selection of constitutive mucoid *mucA* mutants. Alginate production has been observed early in the infection process before *mucA* mutations (2); therefore, it is conceivable that inhibiting the alginate pathways could allow eradication of *P.a.* and prolong the life of CF patients.

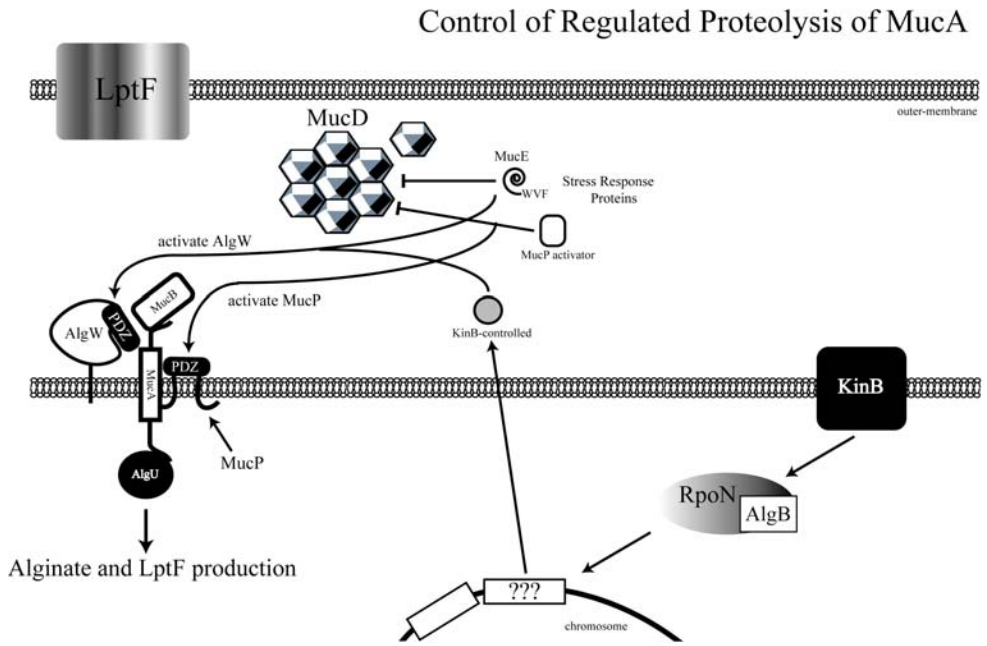

Figure 1. Control of regulated proteolysis of MucA in *P.aeruginosa.* KinB is a novel regulator of alginate production in *P.a.* Alginate production in the absence of *kinB* requires *algB, rpoN,* and *algW.* Data presented here suggests AlgB and RpoN control transcription of factors which can activate AlgW proteolysis of MucA. However these factors are not mediated by the chaperone protease MucD. MucD mediates a divergent set of signals which seem to activate MucP proteolysis of MucA, independent of AlgW. When AlgU is highly upregulated due to being released from MucA, Lipotoxin F is upregulated and expressed by the mucoid cells. LptF was implicated in both resistance to oxidative stress as well as adhesion to lung epithelia. *P.a.* controls AlgU expression by mediating regulated proteolysis of MucA through signal transduction networks modulated by the sensor kinase KinB and the serine protease MucD.

GENERAL DISCUSSION AND CONCLUSIONS REFERENCES

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APPENDIX

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