CHARACTERIZATION OF A TYPE IV PILUS BIOGENESIS OPERON IN
Pseudomonas aeruginosa

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ABSTRACT

KIMBERLY ANN COGGAN: Characterization of a Type IV pilus biogenesis operon in
Pseudomonas aeruginosa
(Under the direction of Matthew C. Wolfgang)

Pseudomonas aeruginosa is an opportunistic bacterial pathogen and frequent cause
of nosocomial infections. Its intrinsic antibiotic resistance makes it challenging to treat.
Additionally, *P. aeruginosa* is the dominant pathogen responsible for chronic pulmonary
infection in individuals with cystic fibrosis (CF). Type IV pili (Tfp) are retractile surface
appendages that promote *P. aeruginosa* virulence by mediating i) bacterial adherence to
host tissue, ii) twitching motility (TM), a form of surface-associated bacterial translocation
that aids in bacterial dissemination, and iii) formation of biofilm communities. In *P.
aeruginosa*, Tfp fibers are primarily composed of a single repeating subunit termed pilin,
which is encoded by the *pilA* gene. In addition, there are several less abundant proteins
associated with the fiber that play key structural and functional roles. The five 'pilin-like'
proteins (FimU, PilV, PilW, PilX, PilE), which share the highly conserved N-terminal α-
helical region of pilin, are encoded by an operon (*fimU-pilVWXY1Y2E* (*fimU*)) that also
encodes the structurally distinct protein, PilY1. The pilin-like proteins and PilY1 are
incorporated into the mature Tfp fiber and are required for Tfp biogenesis; however, PilY1
has additional roles in antagonizing pilus retraction and mediating attachment to host
epithelial cells.

Here we describe the regulatory mechanisms that control expression of the *fimU* Tfp biogenesis operon. Specifically, we identified two linked, but independent networks involving the transcriptional regulators AlgR and Vfr that converge to control *fimU* promoter activity. Additionally, we revealed a positive feedback mechanism that results in *fimU* activation when either the pilin-like proteins or PilY1 is not expressed. We determined that enhanced *fimU* expression involves upregulation of the TCS AlgZ/R. Based on the recently solved PilY1 crystal structure, which revealed an EF-hand-like calcium-binding site within the C-terminus of the protein, we investigated the role of calcium binding in PilY1 function. Both *in vitro* and *in vivo* studies demonstrated the importance of calcium binding and release in the control of pilus extension/retraction. Together, these studies detail the regulatory mechanisms involved in controlling both *fimU* transcription and PilY1 function and provide an enhanced understanding of Tfp biogenesis and regulation.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ABS	AlgR binding site
AC	adenylate cyclase
AHL	acyl-homoserine lactone
bp	base pair
c-di-GMP	cyclic dimeric guanosine mono phosphate
C12	N-dodecanoyl-L-homoserine lactone
C4	N-butyryl_L-homoserine lactone
cAMP	3', 5' cyclic adenosine monophosphate
Cb	carbenicillin
CD	circular dichroism
CF	Cystic Fibrosis
CRP	cAMP receptor protein
CTD	Carboxy Terminal Domain
DGC	diguanylate cyclase
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
EPS	exopolysaccharide
GFP	green fluorescent protein
H-NS	histone –nucleiod silencing
H-T-H	helix-turn-helix
IF	immunofluorescence
IPTG	isopropyl-beta-D-thiogalactosidase

kD	kilodalton
LB	lysogeny broth
M	molar
N-term	amino terminal
nM	nanomolar
PAK	*Pseudomonas aeruginosa* strain K
PAPI	*Pseudomonas aeruginosa* pathogenicity island
PCR	polymerase chain reaction
PDE	phosohodiesterase
PQS	*Pseudomonas* Quinolone signal
QS	Quorom sensing
RSCV	rugose small colony variants
RT	room temperature
SCV	small colony variants
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
T2SS	Type II Secretion System
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
TCS	Two-Component System
TEM	transmission electron microscopy
Tfp or T4P	Type IV pili
TM	Twitching motility

TPS	Two-Partner Secretion
VBS	Vfr binding site
VCS	Vfr consequence sequence
WC	whole-cell
WT	wild-type

CHAPTER 1

Global Regulatory Pathways and Cross-talk Control *Pseudomonas aeruginosa* Environmental Lifestyle and Virulence Phenotype[1,*]

OVERVIEW

Pseudomonas aeruginosa is a metabolically versatile environmental bacterium and an opportunistic human pathogen that relies on numerous signaling pathways to sense, respond, and adapt to fluctuating environmental cues. Although the environmental signals sensed by these pathways are poorly understood, they are largely responsible for determining whether *P*. *aeruginosa* adopts a planktonic or sessile lifestyle. These environmental lifestyle extremes parallel the acute and chronic infection phenotypes observed in human disease. In this review, we focus on four major pathways (cAMP/Vfr and c-di-GMP signaling, quorum sensing, and the Gac/Rsm pathway) responsible for sensing and integrating external stimuli into coherent regulatory control at the transcriptional, translational, and post-translational level. A common theme among these

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pathways is the inverse control of factors involved in promoting motility and acute infection and those associated with biofilm formation and chronic infection. In many instances these regulatory pathways influence one another, forming a complex network allowing *P. aeruginosa* to assimilate numerous external signals into an integrated regulatory circuit that controls a lifestyle continuum.

Introduction

Pseudomonas aeruginosa is a Gram-negative, metabolically versatile bacterium that exists in a wide range of environmental habitats and is a primary agent of opportunistic infection in humans, causing both acute and chronic infections (Driscoll, 2007). *P. aeruginosa* thrives in many habitats including: aquatic sediments, water exposed surfaces, soil, plant roots and leaves, and human and animal sewage (Ringen, 1952; Green, 1974; Pellet, 1983). Numerous epidemiological studies have compared the virulence properties of environmental and clinical isolates of *P. aeruginosa* (Nicas, 1986; Römling, 1994; Ferguson, 2001; Wolfgang, 2003a; Pirnay, 2009). With few exceptions it has been reported that clinical isolates from acute infections, or isolates obtained at the onset of chronic infection, are indistinguishable from environmental isolates. These studies support the following conclusions: 1) environmental strains are the primary source of *P. aeruginosa* infection, and 2) the virulence characteristics of *P. aeruginosa* are pre-existing in the environment. Furthermore, these studies indicate that the environmental lifestyle of *P. aeruginosa* involves a repertoire of phenotypes that otherwise support and contribute to opportunistic human infections.

Many *P. aeruginosa* traits that contribute to human virulence can be linked to a particular function relevant to its environmental lifestyle. It has been hypothesized that the virulence factors used by *P. aeruginosa* (and other opportunistic pathogens) to infect humans evolved as defense mechanisms against eukaryotic predators (Hilbi, 2007). Indeed, it is now well established that *P. aeruginosa* can infect and kill numerous protozoan and non-mammalian organisms that likely inhabit the same environmental

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space (Rahme, 1995; Hilbi, 2007). An important defense mechanism utilized by *P. aeruginosa* is the formation of surface-attached biofilms, multicellular communities encased in a self-produced polysaccharide matrix. Biofilms are intrinsically resistant to harsh conditions and provide protection from both toxic and antibacterial compounds in the environment, as well as predation (Matz, 2005).

In humans, *P. aeruginosa* is associated with both acute and chronic infections in immunocompromised individuals, and is capable of infecting a multitude of sites and tissues, including: lungs, burns, wounds, eyes, ears, and indwelling medical devices (i.e. catheters and ventilators) (Driscoll, 2007). Acute *P. aeruginosa* infections, such as nosocomial pneumonia, are invasive and cytotoxic and frequently result in substantial tissue damage, systemic spread, sepsis, and mortality. The pathogenesis of acute infections relies upon the expression of many surface-exposed and secreted virulence factors, including: toxins, proteases (delivered by a type II secretion system; T2SS), type IV pili (Tfp), flagella and a type III secretion system (T3SS) that can inject a set of eukaryote specific effectors across the plasma membrane of target cells (Sadikot, 2005). The virulence factors important for establishing acute infections are distinct from those critical for chronic infections. Chronic infections are minimally invasive and noncytotoxic. These infections involve the formation of biofilms, which in the context of human infection, protect against assault by the host immune system and provide resistance to antibiotics (Donlan, 2002; Ryder, 2007). Thus, chronic infections rarely result in systemic spread, but instead lead to unrelenting non-productive host inflammation that contributes to the resulting morbidity and mortality (Deretic, 1995).

Chronic pulmonary *P. aeruginosa* infection in individuals with the genetic disease cystic fibrosis (CF) serves as a model for environmental acquisition followed by genetic adaptation to the CF lung and has revealed an inverse relationship between expression of acute and chronic virulence determinants. Early in life, CF patients are plagued with recurrent transient *P. aeruginosa* infections that mimic bronchitis. By adolescence, transient infections are typically replaced by permanent colonization with a single strain that persists for the remainder of the individual's life. During the chronic phase of infection, *P. aeruginosa* adopts a biofilm-like lifestyle, persisting within the thickened mucus of the airways (Lam. J.R., 1980; Singh, 2000). Within the CF lung, *P. aeruginosa* undergoes significant genetic adaptation, including the conversion to a mucoid colony phenotype, resulting from the overproduction of the capsular polysaccharide alginate (Govan, 1996). Additional adaptations include mutation and transcriptional repression of acute virulence factors within subpopulations (Kagami, 1998; Jones, 2010). The accumulation of these adaptations results in the loss of acute virulence in individual isolates, and likely results in the gradual loss of acute virulence within the population as a whole. Persistent inflammation and subsequent lung deterioration in CF patients is the primary cause of mortality (Taccetti, 2005).

The ability of *P. aeruginosa* to adapt to a diverse set of environmental conditions and to cause distinct infections relies on its ability to control gene expression in response to environmental stimuli. *P. aeruginosa* devotes a substantial proportion of its genomic real estate to regulatory systems that sense environmental conditions and integrate information to alter gene expression or behavior accordingly (Stover, 2000). The first *P. aeruginosa* genome was sequenced in 2001, revealing a remarkable number of

transcriptional regulators, and our knowledge of *P. aeruginosa* regulatory pathways has grown exponentially in the subsequent years. Recent work in the field has led to the development of the hypothesis that the regulatory pathways that control the transition from free living (planktonic) to surface-adherent community (sessile) lifestyles in the environment are likely to play a similar role in dictating infection type (acute vs. chronic) in human disease. This review will focus on the regulatory pathways that have emerged as key determinants of *P. aeruginosa* lifestyle: 1) cAMP/Vfr signaling, 2) c-di-GMP signaling, 3) quorum sensing (QS), and 4) the Gac/Rsm pathway. Two of these pathways rely on second messenger signaling molecules cAMP and c-di-GMP (adenosine 3',5'-cyclic monophosphate and bis-(3'-5')-cyclic dimeric guanosine monophosphate). Cyclic AMP is now recognized as a major mediator of acute virulence gene expression and planktonic lifestyle phenotypes in *P. aeruginosa* (Wolfgang, 2003b). In contrast, c-di-GMP appears to play a critical role in controlling chronic infection-related phenotypes and the biofilm lifestyle. The coordination of population behaviors is also essential in determining the commitment to a particular lifestyle. In *P. aeruginosa* it is evident that QS is an important regulator of acute virulence phenotypes (Whiteley, 1999; Wagner, 2004; Schuster, 2007); however, it also plays a significant role in the transition from the planktonic to sessile lifestyle and in the establishment of chronic infection (Singh, 2000; Christensen, 2007). Finally, the Gac/Rsm pathway appears to act as a global regulator of lifestyle by inversely regulating acute virulence factors and planktonic behaviors with chronic infection phenotypes and biofilm formation. Here we will concentrate on recent advances in cyclic nucleotide signaling (cAMP, c-di-GMP), QS, and post-transcriptional control by the Gac/Rsm pathway. We will first summarize each individual pathway and

then highlight particular regulatory interactions between the pathways. Finally, we will discuss the implications of this complex regulatory network in controlling *P. aeruginosa* lifestyle and infection outcome.

cAMP/Vfr signaling

The second messenger signaling molecule, cAMP, regulates multiple virulence factors involved in *P. aeruginosa* infection (Figure 1A) (Wolfgang, 2003b). Cyclic AMP is involved in regulating gene expression in many bacterial species in response to environmental cues (De Lorenzo, 1988; Botsford, 1992; Macfadyen, 1998) and is produced by the enzymatic activity of adenylate cyclase (AC) enzymes. The *P. aeruginosa* PAO1 genome encodes three ACs, the intracellular ACs, CyaA and CyaB, and a secreted effector ExoY (Stover, 2000; Wolfgang, 2003b). ACs are divided into six classes based upon their evolutionary relationships (Alm, 1996b). ExoY belongs to the class II ACs, a group of secreted ACs that function as bacterial toxins (Alm, 1996a). ExoY is secreted via the T3SS directly into the cytoplasm of target host cells and its activity is dependent on a host-encoded cofactor (Alm, 1996a). The role of ExoY in virulence is not well understood, although the AC activity of ExoY does result in actin disruption and rounding of cultured epithelial cells (Cowell, 2005). Mutants lacking either CyaA or CyaB have reduced intracellular cAMP; however, CyaB appears to synthesize the majority of cAMP in *P. aeruginosa* under laboratory growth conditions and during model infection (Wolfgang, 2003b; Smith, 2004). In a murine model of acute pneumonia a *cyaB* mutant is attenuated, indicating a role for cAMP in *P. aeruginosa* virulence (Smith, 2004).   CyaA is a cytosolic class I AC, a class found primarily in

enterobacteria, while CyaB is a member of the class III AC family ubiquitous among both eukaryotes and prokaryotes (Baker, 2004). CyaB consists of a N-terminal MASE2 (membrane associated sensor 2) domain and a C-terminal AC catalytic domain (Nikolskaya, 2003). The MASE2 domain is thought to function as a membrane-anchored sensor that regulates the activity of the C-term AC catalytic domain (Nikolskaya, 2003).

cAMP/Vfr regulated virulence associated genes

In enteric bacteria, the effect of cAMP on gene expression is mediated through the cAMP-receptor protein (CRP), a cAMP-dependent transcriptional regulator. The well-characterized CRP protein from *Escherichia coli,* is primarily involved in regulating genes associated with carbon metabolism in response to glucose availability (Gosset, 2004). CRP functions as a homodimer and is allosterically controlled by binding of cAMP. Following formation of a cAMP-CRP complex, CRP undergoes a conformational change enabling binding of activated CRP to specific DNA sequences within target promoters, facilitating either transcriptional activation or inhibition (Botsford, 1992). In *P. aeruginosa*, cAMP influences gene regulation through its activity as an allosteric activator of the transcription factor, Vfr (<u>v</u>irulence <u>f</u>actor <u>r</u>egulator). Vfr is a member of the CRP family of transcriptional regulators, and shares a high degree of homology to *E. coli* CRP (67% sequence identity) (West, 1994; Gosset, 2004). Vfr coordinately controls the expression of over 200 genes (Suh, 2002; Wolfgang, 2003b) and positively regulates the production of virulence factors important for acute *P. aeruginosa* infections, including: Tfp (West, 1994; Beatson, 2002; Wolfgang, 2003b), the T3SS (Wolfgang, 2003b), the T2SS, secreted toxins, degradative enzymes (West, 1994; Beatson, 2002;

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Wolfgang, 2003b; Ferrel, 2008), and the *las* and *rhl* QS systems, which control hundreds of additional genes, including many virulence factors (Albus, 1997; Croda-García, 2011). In contrast, Vfr negatively regulates the expression of flagellar genes by repressing expression of *fleQ*, which encodes the master regulator of flagellar biogenesis (Dasgupta, 2002).

Allosteric regulation of Vfr: cAMP-dependent/independent mechanisms

A consensus Vfr binding sequence has been proposed (Kanack, 2006), and direct binding of Vfr to DNA sequences within target promoters of several genes including those encoding ToxA (*toxA*), LasR (*las* quorum-sensing regulator), FleQ, RegA and PtxR (*toxA* expression regulators) and CpdA (a cAMP phosphodiesterase) has been demonstrated (Albus, 1997; Dasgupta, 2002; Kanack, 2006; Ferrel, 2008; Fuchs, 2010b). Fuchs *et al*. further identified both cAMP-dependent and -independent Vfr DNA binding mechanisms (Fuchs, 2010a). Vfr binding to the majority of target promoters specifically requires cAMP. However, cAMP is not required for Vfr to bind the *lasR* promoter *in vitro* or for *in vivo lasR* promoter activity (Fuchs, 2010a). Fuchs and colleagues (2010a) identified a single novel Vfr binding site within the *vfr* promoter, and *in vitro* transcription studies demonstrated that both cAMP and Vfr are required for *vfr* positive autoregulation (Fuchs, 2010a). A similar mechanism of autoregulation has been demonstrated for *E. coli crp* (Cossart, 1982; Hanamura, 1992).

Subtle structural differences exist between the cAMP-binding pocket of Vfr and *E. coli* CRP, including the presence of three additional amino acids in the Vfr cAMP-binding pocket (West, 1994; Beatson, 2002). Vfr contains a threonine residue (T133) at a

position relative to serine 128 (S128) of *E. coli* CRP, and threonine substitution of CRP

S128 results in activation of CRP by both cAMP and cGMP (Lee, 1994a). Cyclic AMP-

dependent gene expression in an *E. coli crp* mutant can be restored by *vfr* expression.

However, CRP is not capable of complementing a *P. aeruginosa vfr* mutant for exotoxin

A (ETA) or protease production, which suggests that Vfr and CRP cannot be functionally

interchanged (West, 1994; Suh, 2002). This lack of functional complementation may be

due to differences in cAMP-binding affinity, as Vfr has a higher affinity than CRP for

cAMP (Serate, 2011). Alternatively, the helix-turn-helix (H-T-H) DNA binding motifs of

Vfr and CRP are not identical and may reflect subtle differences in DNA binding site

recognition (Kanack, 2006).

In *P. aeruginosa*, several studies have reported conflicting results regarding the

ability of Vfr to bind cGMP (Beatson, 2002; Fuchs, 2010a; Serate, 2011). A spontaneous

Vfr mutant (Vfr$_{\Delta EQERS}$) that lacks 5 amino acids (EQERS) in the cyclic nucleotide-

binding domain, including two residues critical for cAMP binding, was incapable of

activating Tfp-dependent twitching motility, a form of surface-movement (Mattick, 2002),

but retained the ability to regulate a subset of virulence phenotypes (elastase and

pyocyanin production) (Beatson, 2002). Homology modeling of Vfr$_{\Delta EQERS}$ on a CRP-

cAMP backbone suggests that Vfr$_{\Delta EQERS}$ may have a weaker affinity for cNMP molecules

than *E. coli* CRP or wild type Vfr. Beatson *et al.*, suggest that the Vfr$_{\Delta EQERS}$ mutant is

incapable of regulating twitching motility but retains the ability to regulate other

virulence factors due to altered cyclic nucleotide recognition (Beatson, 2002).

Specifically, the authors suggest that another cyclic nucleotide (cGMP) may be

responsible for regulating twitching motility, and the Vfr$_{\Delta EQERS}$ mutant may no longer

effectively bind this cyclic nucleotide, while regulation of elastase and pyocyanin production is maintained though cAMP autoregulation of Vfr. However, elastase production is also regulated by the *las* QS system (Gambello, 1991). Given that Vfr controls *lasR* expression in a cAMP-independent manner (Fuchs, 2010a), it is likely that Vfr$_{\Delta EQERS}$ no longer responds to cyclic nucleotides, but can still activate cAMP-independent promoters. Fuchs *et al*. (2010a) demonstrated that apo-Vfr was unable to bind promoter DNA in the presence of cGMP and high cGMP concentrations actually inhibited Vfr-DNA complex formation (Fuchs, 2010a). In contrast, Serate and colleagues (2011) reported that cGMP was capable of facilitating Vfr-DNA complexes *in vitro*; although, a significantly higher concentration of cGMP was required compared to cAMP for DNA binding (Serate, 2011). The conflicting results from the Fuchs and Serate studies were obtained utilizing very different experimental assays, which may account for their discrepancies (Fuchs, 2010a; Serate, 2011). Although the effect of cGMP on Vfr activity has not been definitively determined, it is important to note that both of these studies utilized cGMP concentrations that are not likely to be achieved *in vivo*, and thus cGMP is unlikely to be a biologically relevant Vfr ligand.

CbpA: A cAMP-binding protein

Genome analysis studies have identified additional cyclic nucleotide-binding proteins in many bacterial species, suggesting that cAMP-binding proteins are involved in regulating a variety of processes in prokaryotes (Botsford, 1992). Bioinformatic analysis identified a novel cAMP-binding protein in *P. aeruginosa* PAO1, termed CbpA (<u>c</u>AMP-<u>b</u>inding <u>p</u>rotein <u>A</u>). Using structural modeling and functional studies, Endoh and

Engel determined that CbpA is comprised of a C-terminal CAP domain, which binds cAMP, and a N-terminal degenerate CAP domain (Endoh, 2009). A putative Vfr-binding site (VBS) was identified upstream of the *cbpA* translational start site. Mutation of the putative *cbpA*-associated VBS resulted in decreased *cbpA* transcription compared to wild type, indicating that Vfr likely regulates *cbpA* expression. Subsequent EMSA studies confirmed binding to a DNA fragment containing the VBS identified in the *cbpA* promoter (Endoh, 2009). As Vfr regulates *cbpA* transcription, the effect of *cbpA* mutation on Vfr-regulated phenotypes (protease secretion, T3S, twitching motility, and biofilm formation) was investigated. In all cases, *cbpA* mutation had no effect on Vfr-dependent phenotypes, and a *cbpA* mutant was not attenuated in a mouse model of pneumonia. 2D-PAGE determined that CbpA does not affect expression or degradation of proteins, as no significant difference in protein expression profiles between wild type and a *cbpA* mutant was observed. Taken together these results suggest that CbpA does not influence the function of any known Vfr-regulated gene products and has an unknown function in *P. aeruginosa* (Endoh, 2009). Interestingly, CbpA-GFP fusion proteins were used to determine that CbpA levels are growth-phase regulated and CbpA localizes to the flagellated old cell pole in a cAMP-dependent manner (Endoh, 2009), which may provide insight into the function of this protein.

cAMP homeostasis

As cAMP is a membrane-impermeable molecule, in order to maintain homeostasis and reset the cAMP-signaling cascade subsequent to activation, the intracellular cAMP concentration must be tightly controlled. Homeostasis can be

achieved through a number of regulatory mechanisms, including control at the level of cAMP synthesis, degradation, and excretion. Signal attenuation can be achieved via degradation of cAMP to 5'-AMP by the enzymatic activity of cyclic 3'-5'-AMP phosphodiesterases. In *P. aeruginosa* the cAMP phosphodiesterase, CpdA, has been characterized. CpdA was shown to have cAMP phosphodiesterase activity *in vitro* and plays a pivotal role in cAMP homeostasis *in vivo* (Fuchs, 2010b). Furthermore, the cAMP-Vfr complex positively regulates *cpdA* expression. CpdA protein reduces intracellular cAMP levels creating a feedback loop (Fuchs, 2010b). Cyclic AMP homeostasis allows specific cAMP concentrations to be achieved, which may result in a hierarchy of gene expression within the cAMP/Vfr regulon. Upregulation of *cpdA* by cAMP-Vfr at high cAMP levels would promote cAMP degradation and likely limits the expression of cAMP/Vfr-dependent genes to a narrow temporal window.

In *P. aeruginosa* cAMP homeostasis is also regulated at the level of AC activity. In a screen to identify regulators of CyaB function, numerous components of the Chp chemotaxis-like chemosensory system were identified (Fulcher, 2010). The Chp system components are homologous to the *E. coli* chemotaxis system responsible for controlling flagellar motility (Silversmith, 1999). The Chp system was previously shown to control Tfp production and twitching motility (Darzins, 1997; Whitchurch, 2004). Additionally, the Chp system appears critical for *P. aeruginosa* virulence, as mutants are attenuated in a *Drosophila* model of *P. aeruginosa* infection. The reduced virulence displayed by these mutants was more significant than could be attributed to their role in Tfp regulation alone (D'Argenio, 2001). A subset of mutants (*pilG (cheY), pilI (cheW), pilJ (MCP), chpA (cheA), fimL, fimV*) displayed reduced cAMP levels. Increased cAMP levels were

measured in the *pilK (cheR)* and *pilH (cheY)* mutants compared to the parental strain (Fulcher, 2010). These results indicate that Chp gene cluster (*pilGHIJKchpABC*) along with *fimL* and *fimV* affect cAMP intracellular concentrations and exert either a positive (*pilG*, *pili*, *pilJ*, *chpA*, *chpC*, *fimL*, *fimV*) or negative (*pilH*, *pilK*, and *chpB*) regulatory effect. *fimL* mutants resemble *cyaB* mutants for a variety of phenotypes including twitching motility defects, decreased T3SS transcription, as well as decreased cAMP levels (Inclan, 2011). Additionally, it was shown that FimL, CyaB and PilJ (Delange, 2007; Inclan, 2011) are polarly localized; which suggests that FimL functions to link the Chp system and CyaB to regulate the spatial and temporal production of cAMP to specifically influence polarly localized structures (Tfp and T3SS) (Delange, 2007; Inclan, 2011). Western blot analysis determined that CyaB expression was equivalent among all the Chp mutants compared with the parental strain (Fulcher, 2010) and thus Chp does not appear to modulate CyaB expression. To address the possibility that Chp mutants control cAMP levels by controlling cAMP degradation, the effect of PilG and PilH (both CheY homologs) on intracellular cAMP levels was assessed. Using a genetic approach it was demonstrated that PilG and PilH do not regulate cAMP levels via degradation, but specifically affect CyaB activity (Fulcher, 2010). These results support a model whereby the Chp system controls intracellular cAMP pools by affecting CyaB activity, although it remains to be determined whether this effect is direct or occurs through additional proteins.

Environmental signals affecting intracellular cAMP

The signal(s) resulting in activation of the Chp system are not known. However, *in vitro* culture conditions of low calcium (Wolfgang, 2003b) or high osmolarity (Rietsch, 2006) influence intracellular cAMP levels, but whether these represent relevant signals *in vivo* remains to be determined. As the Chp system also regulates Tfp production and twitching motility, the chemoattractants (phophatidylethanolamine and/or phosphatidylcholine) known to affect twitching motility in *P. aeruginosa* may serve as signals that influence cAMP levels (Kearns, 2001; Barker, 2004).

Quorum Sensing

Quorum sensing (QS) is a form of bacterial cell-cell communication utilized by many species to sense population density and coordinate gene expression. QS is achieved by self-production of small diffusible signaling molecules termed autoinducers, such that increases in the bacterial population density results in accumulation of signaling molecules. Once a threshold concentration is achieved, the autoinducers bind their cognate receptors, which directly or indirectly activate gene expression. *P. aeruginosa* encodes three QS systems, two *N*-acyl-homoserine lactone (AHL) based signaling systems (*las* and *rhl)* and a 2-alkyl-4-quinolone (AQ) based signaling system (Figure 1B). These three QS systems are involved in the regulation of virulence factor production, biofilm maturation, and motility phenotypes. The *P. aeruginosa* QS systems are arranged hierarchically with the *las* system positively regulating both the *rhl* (Latifi, 1996; Pesci, 1997) and AQ (Wade, 2005) systems. Additionally, the *rhl* system negatively regulates the AQ system (McGarth, 2004), and each of these systems are further modulated by a

plethora of regulators that function at both the transcriptional and post-transcriptional level.

AHL-dependent QS systems

Each of the *P. aeruginosa* QS systems utilizes a structurally distinct diffusible autoinducer and cognate regulator protein pair to control downstream gene expression. The *las* and *rhl* systems use dedicated autoinducer synthases (LasI and RhlI) to produce the acyl-homoserine lactone (acyl-HSL) molecules *N*-(3-oxododecanoyl)-HSL (C12-HSL) and *N*-butyryl-HSL (C4-HSL), respectively. When sufficient autoinducer concentrations are achieved, the molecules bind and activate their cognate receptors, LasR and RhlR (Juhas, 2005; Schuster, 2006). Together the *las* and *rhl* systems directly or indirectly regulate roughly 10% of the *P. aeruginosa* genome (Schuster, 2006). Transcriptome analysis has revealed significant overlap between the regulons of these two systems (Schuster, 2003). The most abundant class of QS-regulated genes identified by microarray studies were those that encode secreted factors (toxins and extracellular enzymes).

Activation of target genes has been shown to require C12-HSL-dependent multimerization of LasR (Kiratisin, 2002). In contrast, RhlR requires C4-HSL for activation, but is not dependent on C4-HSL binding for dimerization (Ventre, 2003). Both the *las* and *rhl* systems contain a positive feedback loop where their regulators, LasR and RhlR, induce transcription of their cognate synthase genes allowing for rapid signal amplification (Seed, 1995; Latifi, 1996). Further, binding of the LasR-C12-HSL

complex activates transcription of *rhlR* and *rhlI*, accounting for the hierarchical relationship between the two systems (Latifi, 1996; Pesci, 1997).

Biochemical analyses have revealed distinct differences in the DNA-binding properties of LasR and RhlR. Schuster *et al*. (2004) demonstrated that LasR recognition sequences do not require dyad symmetry, and that the presence or absence of dyad symmetry corresponds with cooperative or non-cooperative binding of LasR to promoter sequences (Schuster, 2004b). In contrast, *rhl*-responsive promoters appear to rely on dyad symmetry, highlighting probable determinants that facilitate promoter specificity. Indeed the recognition sequences for LasR and RhlR do not appear to be constrained to a single consensus sequence, rather the quorum-controlled promoters have a high degree of heterogeneity, thus generating a signaling continuum; where some promoters respond to C12-HSL signal alone, both C12- and C4-HSL signals, or C4-HSL signal alone (Schuster, 2007).

AQ-dependent QS system

The third *P. aeruginosa* QS system utilizes an AQ signaling molecule, designated the <u>P</u>seudomonas <u>Q</u>uinolone <u>S</u>ignal (PQS; 2-heptyl-3-hydroxy-4-quinolone). PQS is synthesized by gene products encoded by the *pqsABCD* operon and *pqsH,* which are also involved in the production of other AQs including the immediate PQS precursor, 2-heptyl-4-quinolone (HHQ) (Deziel, 2005; Wade, 2005). PQS binds with high affinity to its cognate LysR-type receptor MvfR (PqsR); together PQS and MvfR control approximately 140 genes, most of which are co-regulated by the *rhl* system (Deziel, 2005). Interestingly, PQS activates *rhlI* expression (McKnight, 2000) while the *rhl*

system negatively regulates the AQ-dependent QS system (McGarth, 2004). Moreover, MvfR is positively controlled by LasR-C12-HSL, indicating that a delicate balance in the ratio of C12-HSL to C4-HSL is necessary for production of PQS (McGarth, 2004).

AQ-dependent signaling is a critical component of the *P. aeruginosa* QS circuit. The exogenous addition of PQS to bacterial cultures promotes biofilm formation and positively influences the production of QS-regulated virulence factors, particularly pyocyanin and proteases (McKnight, 2000; Diggle, 2003). PQS appears to act as an allosteric regulator of MvfR as binding of MvfR to the *pqsABCD* promoter is greatly enhanced by the PQS (Wade, 2005; Xiao, 2006). The *pqsABCD* gene products direct synthesis of the precursor molecule HHQ, which is also capable of potentiating MvfR binding to the *pqsABCD* promoter (Xiao, 2006). Furthermore, a *pqsH* mutant incapable of converting HHQ to PQS, does not display a defect in MvfR-mediated gene expression or virulence, indicating that HHQ is also a functional QS molecule (Xiao, 2006).

Regulators of QS homeostasis

In *P. aeruginosa*, expression of QS controlled genes is largely delayed until the stationary phase of growth. While this observation suggests that the threshold concentration of autoinducers is not achieved until stationary phase, exogenous addition of AHLs or PQS to *P. aeruginosa* strains during logarithmic growth does not result in the immediate activation of QS-dependent gene expression (Whiteley, 1999; Diggle, 2002; Schuster, 2003). In contrast, overexpression of the QS receptor proteins LasR and RhlR during logarithmic growth, in the presence of their cognate autoinducers, results in the advanced expression of many, but not all, QS-controlled genes (Schuster, 2007). Taken

together, these observations suggest that activation of QS genes requires additional factors that likely control receptor expression or function, and/or act as coregulators of some QS-controlled target genes.

The transcriptional regulator RsaL, encoded by the *rsaL* gene, represses *lasI* expression through direct binding to the *lasI* promoter (de Kievet, 1999; Rampioni, 2006). Expression of *rsaL* is positively regulated by LasR-C12-HSL (de Kievet, 1999), creating a negative feedback loop within the *las* system. Negative regulation of *lasI* by RsaL serves to counterbalance the positive feedback loop in which LasR-C12-HSL binds the *lasI* promoter, enhancing *lasI* expression and C12-HSL production. The *rsaL* and *lasI* genes are divergently transcribed and coregulated. LasR binding to the *lasI-rsaL* intergenic region triggers transcription of both genes (Whiteley, 2001; Schuster, 2004b), while RsaL binding results in repression of *lasI-rsaL* transcription (Rampioni, 2007), ensuring tight control of this negative regulator. Rampioni *et al*. (2007) demonstrated that while RsaL and LasR are capable of binding simultaneously to the intergenic region; repression by RsaL supersedes LasR-mediated activation (Rampioni, 2007). RsaL appears to be a global regulator, controlling ~130 genes independent of its effect on *lasI* expression (Rampioni, 2007), including genes whose products are involved in antibiotic resistance and biofilm formation in *P. aeruginosa* (Rampioni, 2009). Evidence for *rsaL* upregulation was found in *P. aeruginosa* RNA harvested from sputum samples obtained from chronically infected CF patients, suggesting that RsaL plays a role in C12-HSL homeostatic control during chronic infection (Son, 2007).

Given that most QS-controlled genes are expressed during stationary phase, it is not surprising that the stationary phase sigma factor, RpoS (σ^S), has been shown to

modulate the expression of approximately 40% of QS-controlled genes (Whiteley, 2000; Schuster, 2004a). Expression of *rpoS* is modestly activated by the *rhl* QS system (Latifi, 1996; Wagner, 2003); however, the presence of RpoS promoter sequences in several QS-controlled genes suggests that RpoS regulates QS gene expression directly (Schuster, 2004a). Furthermore, RpoS appears to have an indirect effect on QS gene expression as it plays a subtle role in activating *lasR* and *rhlR* expression during stationary phase (Schuster, 2004a). While the details of the interplay between QS and RpoS are poorly understood, it is clear that RpoS is involved in restricting QS control of a subset of genes until the onset of stationary phase.

In addition to RpoS, other global regulators elicit significant control over QS. Mutation of the *mvaT* gene results in premature and elevated expression of quorum-regulated genes, demonstrating that MvaT is involved in controlling the magnitude and timing of QS-dependent gene expression (Diggle, 2002). MvaT is a member of the <u>h</u>istone-like <u>n</u>ucleoid <u>s</u>tructuring (H-NS) protein family, involved in transcriptional silencing (Vallet, 2004). MvaT and its homolog MvaU bind specific sequences in AT-rich DNA and oligomerize to form extended protein-DNA complexes that prevent transcription (Castang, 2008). MvaT/U control the expression of approximately 150 genes. Transcriptional profiling and genome wide location analysis (chromatin immunoprecipitation combined with tiled whole genome microarray mapping) revealed that MvaT/U are likely to be involved in silencing as many as 350 genes. Among these, MvaT/U appear to play a direct role in regulating *lasR*, *lasI*, *rsaL*, *mvfR*, and *rpoS*, providing a link to all three *P. aeruginosa* QS systems (Castang, 2008).

While QS homeostasis is subject to control by the above global regulators, other regulators have been identified that appear to specifically modify QS. VqsR (virulence and quorum-sensing regulator), a member of the LuxR-type family of transcriptional regulators, appears to provide another feedback mechanism to finely tune the QS system. Specifically, VqsR has been shown to control production of AHL signaling molecules and virulence factors. Transcriptome analysis of a *vqsR* mutant demonstrated that VqsR regulates the expression of a subset of QS controlled genes, although VqsR has not been shown to bind AHLs (Juhas, 2004). Further, LasR binds the *vqsR* promoter *in vitro* in the presence of C12-HSL (Li, 2007), indicating that *vqsR* expression is under the control of the *las* QS system.

The AraC-type transcriptional regulator VqsM positively regulates the QS systems. Global transcriptome analysis revealed approximately 300 genes that were directly or indirectly influenced by a *vqsM* mutation. Of these, 200 genes were upregulated by VqsM. More than half of the genes positively controlled by VqsM are also activated by QS. This is likely due to VqsM control of several important QS regulators including (*mvfR, rhlR, rsaL, rpoS,* and *vqsR*). Overexpression of *vqsR* in the *vqsM* mutant restored the expression of AHL signaling molecules and most virulence factors, indicating that VqsM regulates QS signaling primarily through modulation of *vqsR* expression (Dong, 2005).

Control of the QS threshold

C12-HSL reaches steady state levels long before *P. aeruginosa* reaches stationary phase and activation of the QS system occurs (Chugani, 2001), indicating that additional

homeostatic mechanisms must control the timing of QS activation. Recently several factors involved in regulating the activation threshold of quorum-regulated genes have been identified. QscR, another LuxR homolog modulates the timing of QS activation by repressing expression of *lasI* (Chugani, 2001). QscR exists as an oligomer or aggregate in cells, but crosslinking experiments demonstrated that QscR is also capable of forming heterodimers with LasR or RhlR. Interaction of QscR with AHL signal molecules resulted in disassociation of the QscR oligomer (Ledgham, 2003). Transcriptome studies demonstrated that QscR controls a discrete regulon independent of RhlR and LasR and can function as either an activator or repressor (Lequette, 2006). Expression of *qscR* is not affected by *lasR* mutation, despite the presence of a well conserved "lux box" upstream of *qscR* (Ledgham, 2003).

Siehnel and colleagues identified a unique regulator (QteE) involved in inhibiting pre-quorum threshold transcription. QteE blocks the expression of QS-regulated genes without affecting transcription or translation of *lasR,* indicating a post-translational control mechanism. Expression of *qteE* prevented LasR accumulation presumably by affecting protein stability. QteE also reduced RhlR accumulation and blocked Rhl-mediated signaling. Overexpression of *lasR* overcame the effects of *qteE* expression, suggesting that the stoichiometry between LasR and QteE is important for QteE control (Siehnel, 2010). Given these results, a model emerges whereby QteE activity exceeds LasR activity at low cell densities, but LasR activity is favored as the cell density increases. However, it has yet to be determined whether QteE affects LasR stability in the presence or absence of bound signal.

QslA was identified in a screen for proteins that interact with LasR (Seet, 2011). QslA homologs are found within several other *Pseudomonas* species, and share approximately 30% sequence identity at the amino acid level; however, none of these homologs have been characterized. The predicted secondary structure of QslA shares a conserved alpha helical structure with TraM and TraM2, anti-activators that inhibit QS activation via protein-protein interactions in *Agrobacterium tumefaciens* (Chen, 2004a). Co-immunoprecipitation assays demonstrated that QslA modulates LasR activity via protein-protein interaction. Further, QslA disrupted binding of LasR to the *lasI-rsaL* intergenic region in EMSA studies, even when a LasR-DNA complex was formed prior to the addition of purified QslA (Chen, 2004a; Seet, 2011). Chromatography analysis determined that QslA (11.8 kDa) exists in solution as a 66 kDa hexamer. Based on previous reports that LasR exists as a dimer (Schuster, 2004b), Seet *et al*. postulate that three pairs of LasR dimers interacts with a QslA hexamer. As both QscR and QslA affect LasR dimerization and DNA-binding respectively, each of these proteins is critical for establishing the QS threshold by modulating free LasR levels. Importantly, at high concentration C12-HSL disrupts QscR inhibition of LasR (and RhlR), while QslA inhibition is not affected by increasing C12-HSL concentrations, suggesting that QslA may control the overall QS threshold. Transcriptome and proteome profiles of *P. aeruginosa* propagated under a variety of experimental conditions demonstrate that QS often controls specific sets of genes in response to particular environmental cues. These results suggest that the QS circuitry may be critical to regulating genes in response to both population density and environmental stimuli.

Gac/Rsm Pathway

The Gac/Rsm pathway appears to play a fundamental role in controlling the course of *P. aeruginosa* infection by inversely regulating the expression of virulence factors associated with acute (T3S, Tfp) and chronic (exopolysaccharides, type VI secretion (T6S)) disease (Figure 1C) (Goodman, 2004; Heurlier, 2004; Burrowes, 2006; Ventre, 2006; Brencic, 2009a; Moscoso, 2011). In contrast to cAMP/Vfr signaling and the QS systems, the Gac/Rsm pathway controls virulence factor expression post-transcriptionally, via alteration of mRNA stability or translation (Smith, 2004). Central to this phenotypic switching mechanism is the GacS/A two component system (TCS). When activated via phosphorylation, GacA promotes the expression of two small untranslated RNAs (sRNAs), RsmY and RsmZ, which sequester the mRNA-binding protein RsmA (Heurlier, 2004; Kay, 2006; Brencic, 2009a). RsmY and RsmZ are comprised of multiple stem-loop structures containing GGA motifs in unpaired regions that facilitate high affinity binding of RsmA (Valverde, 2003). As such, RsmZ and RsmY expression levels are inversely proportionate to cellular levels of free RsmA. Deletion of *rsmY* and *rsmZ* results in phenotypes similar to those of a *gacA* mutant, suggesting that the GacS/A system primarily functions to modulate expression of these sRNA genes (Kay, 2006; Brencic, 2009a). Activated GacA binds a conserved sequence element termed the GacA box (TGTAAGN$_6$CTTACA, N= any nucleotide) present in the *rsmY* and *rsmZ* promoters (Brencic, 2009b). RsmY and RsmZ are also part of a feedback mechanism and inhibit their own transcription by interfering with the GacS/A system, though it is unclear whether this is accomplished by inhibiting GacA phosphorylation, by preventing GacA binding to the GacA box, or by inhibiting GacS/A translation. While the mechanism of

this feedback regulation remains to be determined, it is apparent that RsmY and RsmZ levels are subject to tight homeostatic control.

RsmA: a post-transcriptional regulator

RsmA is a member of the CsrA family of small translational regulatory proteins that have been identified in numerous bacteria and are known to regulate multiple aspects of virulence gene expression (Lapouge, 2008). Transcriptome analyses have defined the RsmA regulon, demonstrating negative control of T6S and exopolysaccharide (EPS) production involved in biofilm formation as well as positive control of T3S, Tfp synthesis, and flagellar motility (Burrowes, 2006; Brencic, 2009a). The mechanism of RsmA mediated translational repression has been extensively characterized. RsmA and RsmA-like proteins of the CsrA family bind GGA motif(s) present within the untranslated 5' sequence of target mRNAs, thus occluding the ribosome binding site and preventing ribosome recruitment (Lapouge, 2008). In contrast, the mechanism by which RsmA positively regulates expression of T3S, Tfp, and flagellar genes remains largely unknown. The *E. coli* RsmA homolog, CsrA, positively affects translation by promoting mRNA stability (Wei, 2001), which may represent a standard mode of positive regulation for this protein family. Alternatively, it has been proposed that positive regulation by RsmA may be indirectly attributed to negative regulation of one or more transcriptional repressors by RsmA (Brencic, 2009a).

Additional regulators of the GacS/A system

Several additional accessory regulators modulate the GacS/A system. Two hybrid sensors lacking cognate sensor kinases, RetS and LadS, have antagonistic roles relative to

one another, and exert negative and positive effects respectively on *rsmZ* expression (Goodman, 2004; Laskowski, 2006; Ventre, 2006). *retS* (<u>r</u>egulator of <u>e</u>xopolysaccharides and <u>T3S</u>) and *rsmA* mutants have similar phenotypes including hyperbiofilm formation, reduced T3S expression and cytotoxicity, and diminished twitching motility (decreased Tfp expression) (Pessi, 2001; Goodman, 2004; Zolfaghar, 2005; Goodman, 2009). LadS (<u>l</u>ost <u>ad</u>herence <u>s</u>ensor) was identified in a transposon library screen for mutants reduced in the ability to form biofilms (Vallet, 2001).

Both RetS and LadS are modular proteins containing N-terminal 7TMR-DISMED2 (7-transmembrane-receptor with diverse intracellular signaling modules extracellular domain 2) and a 7TMR-DISM_7TM (seven transmembrane segments found adjacent to 7TMR-DISM domains) and C-terminal response regulator-like receiver domains, although RetS contains two receiver domains (Goodman, 2004; Ventre, 2006). The N-terminal domains of these proteins have been predicted to bind carbohydrates and their modest (35%) sequence identity suggests they may bind similar, but distinct ligands. RetS and LadS functionally interact with the GacS/A system. Interestingly, RetS activity is independent of conserved phosphorelay residues in RetS and the observation that RetS lacks autophosphorylation activity suggests that RetS exerts its regulatory function directly (Hsu, 2008; Goodman, 2009). RetS has recently been shown to heterodimerize with GacS resulting in suppression of GacS autophosphorylation and thus inhibits phosphotransfer to GacA (Goodman, 2009). This mechanism of sensor kinase inhibition via heterodimerization is novel among signaling proteins. The mechanism for GacA activation, via LadS, has not been fully characterized but recent work in *P. fluorescens* suggests that it also likely makes physical contact with GacS (Workentine, 2009). While

transcriptome analysis of the RetS regulon revealed 397 target genes, LadS only affects the expression of 79, with slightly over half of these overlapping with those inversely affected by RetS (Ventre, 2006). These results indicate that RetS and LadS function to reciprocally regulate numerous gene targets via functionally altering GacS/A output.

The HptB signaling module

The *P. aeruginosa* genome is predicted to encode 12 hybrid-type kinases and 3 putative single domain Hpt proteins in addition to numerous TCS proteins (Stover, 2000; Chen, 2004b). One of these hybrid sensors, PA2284, was identified in a transposon mutant screen for its association with the hyberbiofilm phenotype (Goodman, 2004). This hybrid sensor, along with two additional hybrid sensors, was demonstrated to transfer a phosphoryl group to the histidine phosphotransfer protein (HptB) (Hsu, 2008). HptB then relays the signal (phosphoryl group) to the orphan response regulator PA3346 as demonstrated via *in vitro* and *in vivo* experiments (Figure 1F) (Lin, 2006; Hsu, 2008).

Phosphorelay profiling assays determined that HptB does not interact directly with GacA; instead HptB interacts directly with the response regulator PA3346. PA3346 is a phosphatase consisting of a N-terminal phosphoryl signal receiver domain and a C-terminal eukaryotic type Ser/Thr phosphatase domain (Hsu, 2008). Bioinformatic analysis of the *P. aeruginosa* PAO1 genome revealed that *hptB* (PA3345), PA3346, and PA3347 are clustered closely together and reverse transcription PCR confirmed the operonic structure (Hsu, 2008). Hsu *et al*. further demonstrated via two-hybrid experiments that the output domain of PA3346 interacts directly with PA3347, a protein with similarity to an anti-sigma (σ) factor. These observations suggest that PA3346

regulates PA3347 via dephosphorylation, and is supported by the finding that the conserved serine-56 residue of PA3347 is phosphorylated *in vivo* and the degree of PA3347 phosphorylation inversely correlated with the amount of PA3346 (Hsu, 2008). The activity of PA3346/PA3347 was also shown to specifically impact *rsmY* expression without affecting *rsmZ*. These results led to the hypothesis that an unidentified σ factor is released upon activation of the HptB pathway, and the freed σ factor binds the *rsmY* promoter but not in the *rsmZ* promoter (Figure 1F).

The HptB signaling pathway is involved in the regulation of swarming motility, a form of surface-associated motility, and biofilm formation. A *hptB* mutant is defective for swarming motility while mutants lacking PA3346 and PA3347 display enhanced swarming motility (Hsu, 2008). Biofilm synthesis and disintegration was faster in a *hptB* mutant compared to wild type, while a mutant lacking PA3347 was indistinguishable from the parent PAO1 strain (Hsu, 2008; Bordi, 2010). Introduction of the PA3346 or PA3347 mutations into a *hptB* mutant background ablated the hyperbiofilm phenotype (Bordi, 2010). The inverse phenotypes displayed by the *hptB* mutant and PA3346/PA3347-lacking strains strongly indicate that HptB antagonizes the activity of PA3346/PA3347.

Microarray studies revealed significant overlap between the *hptB* and *retS* mutant transcriptomes (Bordi, 2010). Both *retS* and *hptB* mutants displayed downregulation of T3S genes and upregulation of the *pel* genes; however only the *retS* mutant displayed increased T6S gene expression (Bordi, 2010). HptB overproduction failed to restore expression of T3S and *pel* genes in the *retS* mutant, indicating that HptB does not partner with RetS. As previously mentioned, the HptB pathway specifically alters *rsmY*

expression (Bordi, 2010), although the reason for differential sRNA control is unknown. The upstream regions of *rsmY* and *rsmZ* are disparate and could be recognized by distinct regulatory factors (Brencic, 2009b, a). Secondary mutations in *gacS* or *gacA* suppress the *hptB* phenotype and suggest that HptB functions upstream or at the same level as the GacS/A system (Bordi, 2010). Taken together recent work elucidating the molecular mechanisms by which the Gac/Rsm pathway reciprocally controls acute and chronic virulence factors have uncovered novel means utilized by *P. aeruginosa* to modulate gene expression and phenotypes in response to, as yet, unknown environmental cues.

c-di-GMP signaling

c-di-GMP is an important and ubiquitous secondary signaling molecule in many bacterial species. The intracellular concentration of c-di-GMP within a cell is mediated by the opposing actions of two types of enzymes: diguanylate cyclases (DGC) containing GGDEF-domains that synthesize c-di-GMP; while phosphodiesterases (PDE) containing EAL or HD-GYP domains that degrade c-di-GMP. Several proteins have been identified that contain both GGDEF and EAL-domains, and thus may be capable of both synthesizing and degrading c-di-GMP. Additionally, proteins in various bacterial species have been identified that contain degenerate GGDEF domains, suggesting that these domains are not catalytically active but rather function as allosteric sites (Christen, 2005) and/or c-di-GMP receptors (Newell, 2009). In the *P. aeruginosa* genome, 39 genes contain either a GGDEF, an EAL, or both domains (Figure 1D) (Kulasakara, 2006). Levels of c-di-GMP influence a wide range of phenotypes in diverse bacterial species including cellular responses that effect pathogenesis, including synthesis of adhesins and

EPS mediating biofilm formation, motility, secretion, cytotoxicity, synthesis of secondary metabolites, and environmental stress adaptation. Modulation of c-di-GMP is associated with control of biofilm formation and other group behaviors (Liberati, 2006; Merritt, 2007).

In many bacterial species sessile and motile behaviors are coordinately, but inversely, regulated by c-di-GMP (Tamayo, 2007; Jonas, 2009). Biofilm formation is a surface-associated sessile behavior that requires production of an EPS matrix. Biofilm development requires a number of steps from initial surface attachment, EPS matrix formation, and elaboration of a mature biofilm structure (Ryder, 2007). Cyclic-di-GMP promotes biofilm formation, in part, by positively regulating production of the extracellular matrix at both the transcriptional and allosteric level (Simm, 2004; Hickman, 2005). Bacterial surface appendages are also important for a number of the developmental stages in biofilm formation, including motility toward a surface, initial attachment, and microcolony formation. One class of appendages involved in *P. aeruginosa* biofilm formation are the Cup (<u>c</u>haperone <u>u</u>sher <u>p</u>athway) fimbriae. Cup fimbriae are composed of a multimer of the major fimbrial subunit forming the rod structure, which may or may not contain an attached tip adhesin to facilitate specific binding (Sauer, 2004). Multiple *cup* gene clusters are present in various strains of *Pseudomonas*, and in the PAO1 genome three (*cupA, cupB,* and *cupC*) have been identified (Vallet, 2001). A *cupA* gene cluster mutant is defective in attachment to solid surfaces (Vallet, 2004).

Wsp Chemosensory System

WspR was among the first *P. aeruginosa* proteins identified that influence c-di-GMP formation. WspR consists of a CheY-like phospho-receiver domain and a GGDEF domain that has DGC activity *in vivo* and *in vitro* (D'Argenio, 2002; Hickman, 2005; Güvener, 2007). WspR is a response regulator encoded by the *wsp* operon (*wspABCDEFR*), which comprises a multi-component chemosensory system with homology to the well-characterized chemotaxis (Che) pathway of *E. coli* (Ridgway, 1977; D'Argenio, 2002; Güvener, 2007). The Wsp system consists of a putative methyl-accepting chemotaxis protein (WspA), a hybrid CheA-like histidine kinase (WspE), two CheW-like adaptor proteins (WspB and WspD) predicted to link WspA and WspE, a CheR-like methyltransferase (WspC) and a CheB-like methylesterase (WspF), predicted to modulate the methylation status and activity of WspA. Upon detection of the appropriate signal, WspA is predicted to activate the associated WspE histidine kinase, resulting in phosphorylation of the WspR receiver domain and DGC activation. Consistent with this model, purified WspR shows increased DGC activity following *in vitro* phosphorylation by the phosphate donor acetyl phosphate (Hickman, 2005). While the specific signals detected by the Wsp system are currently unknown (see below), inactivation of *wspF* (a putative methylesterase and negative regulator of Wsp signaling) results in increased cell aggregation, a wrinkly colony phenotype, enhanced biofilm formation, and increased intracellular levels of c-di-GMP. The phenotype of a *wspF* mutant is suppressed in a *wspF, wspR* double mutant, indicating that activation of WspR DGC activity accounts for the profound phenotypic changes. Transcriptome comparison between the wild type PAO1 strain an isogenic *wspF* mutant identified 560 genes

affected by *wspF,* including the *psl* and *pel* operons, which displayed increased transcript levels in the mutant. These findings indicate that the *wsp* chemosensory system modulates intracellular c-di-GMP levels and regulates biofilm formation (Hickman, 2005). Complexes of WspR protein appear transiently in the cytoplasm of bacteria with an active Wsp system, suggesting that Wsp clusters form and dissolve, creating localized concentrations of c-di-GMP (Güvener, 2007). Binding of c-di-GMP to large protein complexes is known to affect their activity, and the protein complexes involved in the synthesis of Pel and Psl EPS are likely targets of WspR-generated c-di-GMP. Güvener and colleagues (2007) determined that the Wsp signal transduction pathway is stimulated in response to solid surface contact, which likely involves signal detection by a surface appendage (Güvener, 2007). Taken together these results demonstrate that WspR localizes in a phosphorylation-dependent manner, indicating that spatial and temporal activation of WspR impacts both its activity and localization.

Cup fimbriae

Independent screens in strains PAO1 and PA14 identified a three-component system involved in the regulation of *cup* fimbriae expression, termed *rocA1 (*regulator of cup)*, rocR, and rocS1* (*sadARS* in PA14) (Kuchma, 2005; Kulasakara, 2005). RocA1, RocS1, and RocR have significant homology to the *Bordetella pertussis* BvgA/S/R system involved in regulation of virulence gene expression (Kulasakara, 2005). RocS1 is a sensor kinase containing a PAS domain that is predicted to localize to the cytoplasm adjacent to the two transmembrane segments. PAS domains were originially identified for their function in sensing intracellular oxygen and/or redox potential (Taylor, 1999).

RocA1 is a classical response regulator containing the HTH motif typical of DNA binding proteins. RocR contains an N-terminal CheY-like phosphoryl receiver domain, suggesting that the aspartate residue present in this domain is phosphorylated by a sensor kinase (Kuchma, 2005). RocR also possesses a degenerate EAL domain suggesting that it may be involved in the hydrolysis of c-di-GMP. Further characterization of the *rocARS* transposon mutants confirmed that RocS1 and RocA1 activate transcription of the *cupB* and *cupC* gene clusters, while deletion of *rocR* resulted in a minimal increase in *cupC-lacZ* transcriptional reporter activity (Kulasakara, 2005). RocA1 is required for RocS1-mediated activation of the *cup* gene clusters. In addition, the observation that RocS1 overexpression in the absence of *rocA1* had no effect on *cup* gene expression supports a mechanism whereby RocA1 activates *cup* gene expression via RocS1 phosphorylation. RocR antagonizes the activity of RocA1, and two-hybrid analysis suggests this regulation is achieved through direct interaction of RocR with RocS1 preventing RocA1 phosphorylation (Kulasakara, 2005). Because RocR contains a degenerate EAL domain, it was initially hypothesized that RocR does not possess catalytic activity; but instead the degenerate domain would serve as a binding site for c-di-GMP and binding would then influence RocR activation. However, recent work has demonstrated the degenerate EAL domain within RocR does possess catalytic activity and facilitates the hydrolysis of c-di-GMP to the dinucleotide 5' pGpG (Rao, 2008). Transcriptome analysis identified additional genes influenced by mutations in the RocA/R/S system, during either planktonic or biofilm growth. Wild type bacteria and *rocR/S* mutants differentially regulated several genes during planktonic growth, but an even more significant difference in the number of differentially regulated genes was observed after 5 days of biofilm

growth. Among the genes displaying the most significantly altered expression in these studies were genes encoding the T3SS, where several T3SS genes (apparatus components and effectors) were elevated in the *rocR/S* mutant biofilm in comparison to the wild type biofilm (Kuchma, 2005). The mechanism by which the RocA/R/S system influences the expression of additional genes is not clear.

The RocA/R/S system regulates later steps in biofilm formation; *rocARS* mutants display initial defects in attachment but by 24 hours display surface attachment comparable to that of wild type (Kuchma, 2005). However, during biofilm growth in a flow-cell, *rocARS* mutants fail to form macrocolonies with defined borders and channel networks, an architecture displayed by the wild type strain by day 5. All three components of the RocA/R/S system appear to be required for mature biofilm formation, as each individual mutant confers the same defect in mature biofilm formation by day 5 during flow cell growth (Kuchma, 2005). As many genes required for formation of the T3SS were downregulated in *rocARS* mutants, the RocA/R/S system may control the switch from cytotoxicity, an acute virulence phenotype, to biofilm formation, a chronic virulence phenotype or an alterative lifestyle during growth in a hostile environment.

P. aeruginosa strain PA14 is a more virulent clinical isolate than PAO1, although both share a similar host range (Rahme, 1995). The PA14 genome contains a pathogenicity island (PAPI-1) that is absent from the PAO1 genome, which carries many genes of unknown function and a fourth *cup* gene cluster (*cupD*) (He, 2004). Directly adjacent to the *cupD* gene cluster, are two divergently oriented sets of genes encoding TCS regulatory systems. The *cupD* gene cluster and these regulatory components are flanked by inverted repeats, suggesting they have been simultaneously acquired. One of

these regulatory components, *pvrR* was previously shown to encode a "phenotype variant regulator" involved in regulating of the frequency of antibiotic resistant variants (Drenkard, 2002). PvrR contains an EAL domain, and therefore likely possesses PDE activity. The second TCS adjacent to the *cupD* gene cluster has been annotated *rcsB* (response regulator) and *rcsC* (sensor kinase) due to sequence homology with TCS systems present in *Salmonella enterica* and *E. coli* (Mikkelson, 2009). Previous studies have demonstrated the contribution of these genes to PA14 virulence in both animals and plants by analyzing transposon (Rahme, 1997) or in-frame deletion mutants (He, 2004). *cupD* gene expression, although not induced under laboratory conditions, is activated by RcsB and is repressed by the putative cognate sensor, RcsC (Mikkelson, 2009; Nicastro, 2009). PvrR shows high similarity (45%) to the response regulator RocR, which down-regulates *cupB* and *cupC* expression (Drenkard, 2002; Kulasakara, 2005). Indeed, Mikkelson (2009) and colleagues demonstrated that *pvrR* expression antagonizes *cupD* gene cluster activation via RcsB. Both RocR and PvrR contain EAL domains within their output domains but lack the classical H-T-H motif, suggesting that they regulate Cup fimbriae expression by modulating c-di-GMP levels rather than by direct DNA binding (Kulasakara, 2005; Mikkelson, 2009). These findings support the theory that high levels of c-di-GMP promote a biofilm-like lifestyle, while low levels of c-di-GMP restrict bacteria to a planktonic mode of growth.

Biofilm Formation and Adherence

FimX is another protein involved in regulating motility in *P. aeruginosa* and is required for twitching motility (Huang, 2003). In addition, Tfp are required for adherence

and biofilm formation in *P. aeruginosa*. FimX contains both GGDEF and EAL domains involved in modulating c-di-GMP levels, although FimX does not appear to possess DGC activity (Kazmierczak, 2006). Through analysis of a series of deletion mutants Kazmierczak *et al*. (2006) demonstrated that both intact GGDEF and EAL domains are required for FimX PDE activity and restrict localization to a single bacterial pole. These results support the hypothesis that the GGDEF domain of FimX functions as an allosteric site for binding of GTP, which activates FimX PDE activity. Consistent with this hypothesis, mutation of the GGDEF domain in FimX, abolishes GTP binding and stimulation of PDE activity (Kazmierczak, 2006). However, recent structural studies indicate that the degenerate GGDEF domain of FimX is incapable of nucleotide binding, while the EAL domain binds c-di-GMP with high affinity inducing a conformational change that may impede FimX binding to its putative partner located at the bacterial pole to regulate Tfp production and twitching motility (Navarro, 2010; Qi, 2011).

Starkey *et al*., identified genes transcriptionally upregulated in response to elevated c-di-GMP levels, revealing a small (35) subset of induced genes (Starkey, 2009). Although most of the identified genes belonged to either the *pel* or *psl* gene clusters, one target belongs to a two-partner secretion (TPS) system encoding a secreted adhesin and its transporter. Borlee *et al*. (2010) renamed PA4625 and PA4624, *cdrA* (cyclic diguanylate-regulated TPS partner A) and *cdrB* respectively, based on the observation that transcription of this TPS system is c-di-GMP-dependent. CdrA is processed at both the C- and N-termini, although following secretion full-length CdrA appears to be the major cell-associated species. Rugose small colony variants (RSCV) are characterized by formation of large cell aggregates and increased biomass, this phenotype can be caused

by *wspF* mutation, which leads to WspR activation (D'Argenio, 2002; Hickman, 2005). A dramatic phenotype was observed when a *cdrA* mutation was introduced into the *wspF* background, the resulting biofilm cells were less tightly packed than the cell clusters observed in a *wspF* mutant, suggesting that CdrA is involved in promoting auto-aggregation. As the *cdrAB* and *psl/pel* gene clusters are co-regulated, Borlee and colleagues hypothesized that CdrA may bind to either Psl or Pel EPS, this hypothesis is supported by the presence of an N-terminal carbohydrate-binding domain. Indeed, as predicted *psl* mutant strains did not auto-aggregate when *cdrAB* was overexpressed. Importantly, the production of CdrA was not affected by *pslBCD* mutation, nor were Psl levels significantly affected by *cdrA* mutation. Furthermore, CdrA was demonstrated to bind directly to Psl (Borlee, 2010). The findings from this study highlight that biofilms are not simply random distributions of secreted polymers with imbedded bacterial cells, but are complex and more ordered than originally appreciated.

Small-colony Variants

In chronic isolates from CF patients, small colony variants (SCV) are often observed and this phenotype has been correlated with poor lung function and enhanced antibiotic resistance (Häussler, 2004). SCVs are characterized by auto-aggregative properties, size, and enhanced biofilm formation. SCV phenotypes may also arise following exposure to antibiotics *in vitro* and from biofilm cultures (Drenkard, 2002).

To investigate the factors involved in the SCV phenotype, Meissner and colleagues (2007) screened 10,000 transposon mutants of the clinical SCV 20265 isolate for reversion to the wild type phenotype. Two transposon insertions mapped to the *cupA*

gene cluster involved in biofilm formation (Vallet, 2001), and two others mapped to PA1119 and PA1120, encoding hypothetical proteins (Meissner, 2007). To determine the involvement of CupA expression in the SCV phenotype, Meissner *et al*. compared CupA expression in SCV 20265 to the wild type strain and the *cupA* gene cluster transposon mutants via immunoblot. CupA expression was elevated in SCV20265 relative to its clonal wild type while the *cupA* gene cluster transposon mutants displayed no CupA expression. CupA expression was also diminished in the other transposon mutants that displayed reversion to the wild type phenotype (Meissner, 2007).

Reversion from the SCV 20265 auto-aggregative phenotype to a wild type phenotype also resulted from insertions in PA1120 and *morA*, both of which possess a transmembrane domain and a C-terminal GGDEF domain. Previously, PA1120 was demonstrated to possess DGC activity and to modulate biofilm formation (Kulasakara, 2005). In addition to a GGDEF domain, the motility regulator MorA also contains an EAL domain. Expression of plasmid-encoded PA1120 restored CupA expression in both the SCV 20265 PA1120 and SCV 20265 *morA* mutants, suggesting that c-di-GMP levels are important for CupA fimbriae expression. Further analyses demonstrated that c-di-GMP levels were elevated (2.7-fold) in the SCV 20265 compared with wild type. Taken together these results demonstrate that alteration of global c-di-GMP levels is sufficient to induce CupA expression. Additionally, the GGDEF domain of MorA, but not the EAL domain, is important for its enzymatic activity as PA1120 overexpression could complement the SCV 20265 *morA* mutant for CupA expression (Meissner, 2007).

Ueda and Woods identified TpbA in a transposon mutant library screen for mutants with enhanced biofilm formation (Ueda, 2009b). A *tpbA* mutant displayed

increased attachment to polystyrene and a defect in swarming motility, but no alteration in rhamnolipid production (Ueda, 2009a). Similar to a *wspF* mutant, the *tpbA* mutant displayed a wrinkly colony phenotype on Congo-red plates, implying increased production of EPS. Transcriptome analysis of a *tpbA* mutant revealed enhanced expression of the *pelACDF* genes and the biofilm matrix adhesin CdrA. The expression of *tpbA* was also upregulated, suggesting auto-regulation (Ueda, 2009a).

In independent transposon mutagenesis screens, Ueda and Wood (2009) as well as Malone and colleagues (2010), identified components of the *yfiBNR* operon as important for biofilm formation and SCV morphology. Ueda and Woods initially identified TpbB (YfiN) due to its ability to suppress the hyperbiofilm phenotype of the *tpbA* mutant. Mutations in *yfiR* also suppressed the autoaggregative phenotype of the *tpbA* mutant (Ueda, 2009a). TpbA is a periplasmic protein with dual phosphatase activity, capable of dephosphorylating both tyrosine and serine/threonine residues (Pu, 2010). TpbB is an integral membrane protein containing a GGDEF domain, previously shown to possess DGC activity (Kulasakara, 2006). In follow-up studies, Pu and Wood demonstrated that TpbB is phosphorylated in a *tpbA* mutant, at both tyrosine and serine/threonine residues (Pu, 2010). Upon incubation of purified TpbA with phosphorylated TpbB, phosphorylation of TpbB is diminished, demonstrating that TpbB is a substrate of TpbA. Further, treatment of biofilm growth cultures with the phosphatase inhibitor trisodium orthovanadate increased biofilm formation, indicating that cellular phosphorylation enhances biofilm formation in *P. aeruginosa* (Ueda, 2009a).

Three independent insertions were mapped to *yfiR*, the first gene of a predicted three-gene operon *yfiBNR* (Malone, 2010). YfiR is a periplasmic protein, previously

identified as a suppressor of the *tpbA* mutant phenotype (Ueda, 2009a). YfiB is a predicted outer-membrane lipoprotein containing a conserved OmpA peptidoglycan-binding domain. As a strong link between c-di-GMP and the SCV phenotype exists, Malone and colleagues hypothesized that the SCV phenotype of the *yfiR* mutant was the result of upregulation of TpbB activity due to release of YfiR-mediated repression. The cellular concentration of c-di-GMP measured in the *yfiR* mutant displayed a significant increase in c-di-GMP when compare to wild type; this observation coupled with the results demonstrating DGC activity of purified TpbB (lacking the transmembrane domain) support the hypothesis that YfiR represses the DGC activity of TpbB (Malone, 2010). Epistasis experiments were performed to determine the regulatory interplay of YfiB, YfiR, and TpbB. Deletion of *yfiB* did not affect attachment or colony morphology while the *yfiRB* double mutant displayed the phenotypes of a *yfiR* single mutant (SCV phenotypes), suggesting that YfiB functions upstream of YfiR. Additional experiments demonstrated that the stoichiometry of YfiR and TpbB are important for tight control of the system, as increased levels of TpbB relative to YfiR resulted in the SCV morphology (Malone, 2010). *In vitro* crosslinking experiments and bacterial two-hybrid analysis attempted to determine the link between YfiB and YfiR function. The crosslinking experiments suggested that oligomerization of YfiR depends upon the presence of YfiB, but not TpbB. The observed YfiR multimer was present at a size consistent with formation of a YfiR homodimer, yet the role of this complex was not defined. Additionally, bacterial two-hybrid analysis failed to demonstrate direct interaction between YfiR and TpbB, or between YfiB and either YfiR or TpbB.

Taken together the results of the studies performed by Wood and colleagues and Malone *et al.,* demonstrate that the activity of the DGC TpbB is tightly controlled. Strong evidence demonstrates that TpbB is phosphorylated on both tyrosine and serine/threonine residues, and this phosphorylation is important for TpbB activity. However, it remains unclear which kinase is responsible for TpbB phosphorylation. Furthermore while it is evident that YfiR and TpbA both control TpbB function through distinct mechanisms, it remains to be determined if these involve discrete signaling pathways. Further biochemical studies are necessary in order to deduce the mechanism by which YfiB integrates with YfiR and TpbB.

SadB/C/BifA pathway

Studies performed by George O'Toole's group have identified several factors that are involved in inversely regulating surface-associated behaviors (Caiazza, 2004, 2007; Kuchma and O'Toole, 2007; Merritt, 2007). Previously, a genetic screen identified surface attachment-defective (*sad*) mutants of *P. aeruginosa* PA14, defined by their inability to form biofilms in a microtiter dish biofilm assay (O'Toole, 1998, 2000). Subsequent characterization of mutants identified by this study revealed a genetic pathway involved in coordinately regulating surface-associated behaviors in *P. aeruginosa* PA14. The *sadB* mutant was originally identified as biofilm-defective in a genetic screen (O'Toole, 1998, 2000). Further investigation demonstrated that the *sadB* mutant did not display a defect in flagellum-mediated swimming motility or Tfp-dependent twitching motility (Caiazza, 2004). However, the *sadB* mutant displayed a significant increase in swarming motility when compared to wild type. Caiazza *et al.*

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further demonstrated that the *sadB* mutant could initiate surface attachment but was unable to transition from reversible to irreversible attachment, an early step in biofilm formation (Caiazza, 2004). Levels of SadB were positively correlated with biofilm formation and irreversible attachment and fractionation studies localized SadB to the cytoplasm.

In subsequent studies, an additional mutant *sadC*, identified in the original genetic screen (O'Toole, 1998), displayed surface-associated phenotypes mimicking the *sadB* mutant, including hyperswarming and a defect in biofilm formation. SadC is a DGC localized to the inner membrane that positively affects global c-di-GMP levels (Merritt, 2007). The *sadC* mutant was deficient in production of EPS, however *pelA/pelG* transcripts were unaffected by *sadC* mutation. Additional work identified yet another component of the pathway involved in regulating *P. aeruginosa* surface-associated behaviors, the phosphodiesterase BifA. A *bifA* mutant displays opposing phenotypes to the *sadB/C* mutants, a *bifA* mutant is deficient in swarming motility and displays increased biofilm formation (Kuchma and O'Toole, 2007). Although, BifA contains both a GGDEF and an EAL domain, BifA does not appear to possess DGC activity. Instead, the GGDEF residues likely bind the allosteric activator GTP, as PDE activity was diminished in GGDEF residue mutants (Kuchma and O'Toole, 2007). Epistasis studies have aided in elucidating the hierarchy of the aforementioned proteins and indicate that BifA and SadC function in the same genetic pathway (Merritt, 2007). Mutating *sadC* in the *bifA* background resulted in significant reduction of the hyperbiofilm phenotype and partial restoration of swarming motility. However, neither of these phenotypes were completely restored to wild type levels, suggesting that other DGCs likely contribute to

overall c-di-GMP levels (Merritt, 2007). In a *sadB* mutant background, expression of *sadC* on a multicopy plasmid results in diminished biofilm formation in comparison to wild type, indicating that *sadB* is likely downstream of *sadC* in the pathway. These results support a model where environmental cues are sensed/received by SadC, resulting in altered c-di-GMP production. BifA can further regulate the signal through the activity of its EAL domain. Lastly, SadB is predicted to be involved in the transmission of the signal to Pel machinery and components of the chemotaxis machinery in order to regulate biofilm formation and swarming motility (Caiazza, 2004, 2007; Merritt, 2007). These findings suggest that the nearly 40 *P. aeruginosa* DGC/PDE enzymes generate specific phenotypic outputs by altering subcellular c-di-GMP pools. Indeed, recent work by Merritt *et al*. (2010) demonstrated that changes in total c-di-GMP levels did not account for the surface-associated behaviors displayed by DGC mutants (Merritt, 2010).

Cross-talk between signaling pathways

Our knowledge of the signaling pathways controlling *P. aeruginosa* is often based on dramatic phenotypic changes resulting from loss-of-function mutations that either fully activate or eliminate signaling under well-controlled laboratory conditions. However, in the environment or host, we hypothesize that a more gradual transition occurs between lifestyles. This concept is supported by the fact that the regulatory pathways discussed above are not insulated from one another; rather there exists substantial cross-talk between the systems indicating a more sophisticated signaling network and the potential for a continuum of phenotypes (Figure 1). To emphasize this point, we will highlight several mechanisms of cross-talk that are relevant to this review.

One of the best-described links between the lifestyle-determining regulatory pathways is the role of the cAMP/Vfr signaling system in controlling the QS response. Specifically, Vfr has been shown to control the QS regulatory cascade by directly activating transcription of *lasR* and *rhlR* (Albus, 1997; Croda-García, 2011). Furthermore, it has been shown that under planktonic growth conditions, Vfr protein levels increase substantially during the transition to stationary phase (Heurlier, 2003), a mechanism that may serve to fine-tune the timing of QS gene expression. While these findings demonstrate a regulatory link between the cAMP/Vfr and QS signaling pathways, recent studies indicate that this regulatory connection is more complicated than originally appreciated. In contrast to other Vfr-regulated targets, the control of *lasR* expression is cAMP-independent (Fuchs, 2010a). As stated previously, *vfr* expression is auto-regulated in a cAMP-dependent manner (Fuchs, 2010a). The existence of cAMP-independent regulation by Vfr implies that either basal Vfr levels are sufficient to drive *lasR* expression in the absence of cAMP, or that there are cAMP-independent mechanisms that can control *vfr* expression. Consistent with the later, it has been shown that activation of the alginate regulatory pathway results in reduced *vfr* expression without altering intracellular cAMP levels (Jones, 2010).

As mentioned above, the cAMP/Vfr pathway is linked to the regulatory pathway for conversion to mucoidy in chronic *P. aeruginosa* CF infection. During the chronic phase of CF lung infection, *P. aeruginosa* frequently converts to the mucoid colony phenotype due to overproduction of the capsular polysaccharide alginate (Govan, 1996). The overproduction of alginate appears to be representative of the chronic infection phenotype, as mucoid conversion promotes persistence in the lungs and enhances

resistance to immune defenses (Deretic, 1995; Pier, 2001). Mucoid conversion results, most frequently, from loss-of-function mutations in *mucA*, which encodes an anti-sigma factor (Boucher, 1997). Inactivation of MucA releases the alternative sigma factor AlgU (AlgT) allowing AlgU to activate the transcription of numerous genes, including the gene encoding the AlgR response regulator (Figure 1E) (Wozniak, 1994; Govan, 1996). Both AlgU and AlgR are necessary for activation of the genes encoding the biosynthetic enzymes required for alginate production, resulting in the mucoid phenotype (Mohr, 1992; Martin, 1994). Loss-of-function mutations in *mucA* are also associated with reduced expression of many acute virulence factors, including: the T3SS, ETA, LasA protease, and Tfp (Figure 1E) (Mohr, 1990; Wu, 2004; Jones, 2010). Recent work by our group demonstrated that *mucA* mutation blocks the production of invasive virulence factors by inhibiting the cAMP/Vfr signaling pathway at the level of *vfr* expression (Jones, 2010). This inhibition is mediated by AlgU and AlgR, but is independent of alginate production and cAMP synthesis. Inhibition of *vfr* expression via *mucA* mutation represents a mechanism for inverse regulation of acute and chronic virulence factors during infection, supporting the hypothesis that acute virulence factors are actively repressed during chronic *P. aeruginosa* infection in the CF lung. The mechanism of *vfr* repression by AlgR has yet to be elucidated (Jones, 2010). In addition to indirectly influencing QS through reduced *vfr* expression, AlgR has been shown to negatively regulate expression of *rhlI* and *rhlR* directly (Morici, 2007). EMSA studies have demonstrated that AlgR binds specifically to the *rhlR-rhlI* promoter, further strengthening the link between chronic human infection and the repression of QS.

The c-di-GMP signaling pathway, via regulation of Alg44, also influences alginate production. It is now documented that increasing c-di-GMP levels by overexpression of DGCs enhances alginate production, and conversely PDE overexpression reduces alginate formation (Merighi, 2007). Alg44, a protein required for alginate biosynthesis, contains a PilZ domain, previously shown to interact with c-di-GMP (Amikam, 2006). Merighi and colleagues (2007) characterized the PilZ domain of Alg44 and demonstrated that mutation of residues within the PilZ domain diminished binding of c-di-GMP and alginate production. Localization studies determined that Alg44 associates with the membrane and protein localization is not affected by mutations in the PilZ domain. These results are consistent with Alg44 functioning as a regulator of the alginate polymerase (Alg8), a transmembrane protein (Merighi, 2007). The precise role of c-di-GMP binding on Alg44 function has yet to be elucidated.

Although early reports correlated elevated c-di-GMP levels with increased expression of polysaccharide synthesis genes, the mechanism facilitating this effect was unknown. FleQ was previously shown to repress expression of the *pel* operon (Dasgupta, 2002). Despite the absence of characterized c-di-GMP binding domains, FleQ bound radiolabeled c-di-GMP *in vitro,* and c-di-GMP binding inhibited FleQ-binding to the *pel* promoter transcript (Hickman, 2008). Because FleQ also activates genes involved in flagellar (acute virulence factor) biosynthesis, this regulation represents another mechanism for inverse control of acute (flagella) and chronic (Pel) virulence factors. Initial observations of Vfr-mediated repression of *fleQ* expression were perplexing, as Vfr is known to positively regulate virulence factors involved in acute infection. However, FleQ repression of chronic phenotype-associated factors suggests that Vfr is

also involved in downregulating acute virulence factors (flagella) and unexpectedly indirectly mediates expression of chronic virulence factors (Pel polysaccharide) through repression of *fleQ*.

Both mutations in *retS*, or high intracellular c-di-GMP levels, can induce biofilm formation suggesting that the Gac/Rsm pathway and c-di-GMP may intersect to control the expression of chronic virulence factors. Recently, work by Moscoso and colleagues has further established the link between the Gac/Rsm pathway and c-di-GMP by demonstrating that *retS* mutants themselves display high levels of c-di-GMP (Moscoso, 2011). RetS inversely controls the expression of the genes encoding the T3SS and the T6SS, which are associated with chronic infections (Goodman, 2004; Mougous, 2006; Brencic, 2009a). WspR DGC activity is finely controlled, however, mutation of a single residue within the I-site (WspR^{R242A}) results in a constitutively active enzyme. Overexpression of WspR^{R242A} enhanced biofilm formation and elevated EPS production. Additionally, the influence of c-di-GMP levels on the T3SS/T6SS switch was investigated by expressing WspR^{R242A} in a *pelA* background. The roughly twofold increase in c-di-GMP levels observed in this strain coincided with a reduction in PcrV (T3SS) production and an increase in Hcp-1 (T6SS) production. Overexpression of another DGC, TpbB (YfiN) similarly resulted in a switch between the expression of the T3SS and the T6SS. Therefore, artificial increases in c-di-GMP, via DGC overexpression, inversely controls expression of the T3SS and the T6SS. Consistent with this result, overexpression of the PDE PA2133 in a *retS* mutant reverted the T3SS and T6SS expression profiles. In the absence of RsmY and RsmZ, DGC overexpression is no longer able to induce a switch from T3SS to T6SS expression indicating that RsmY and RsmZ

are required for the c-di-GMP-dependent switch between T3SS and T6SS (Moscoso, 2011). These findings establish a direct link between the Gac/Rsm and c-di-GMP dependent pathways.

One final cross-talk mechanism is Gac/Rsm pathway control of cAMP/Vfr signaling through modulation of *vfr* expression. Microarray analysis revealed a twofold decrease in *vfr* expression in a *rsmA* mutant compared to PAO1 wild type. Additionally, analysis of *vfr* transcriptional fusions demonstrated decreased *vfr* expression in the *rsmA* mutant compared to PAO1 (Burrowes, 2006). Consistent with these findings, many of the acute virulence factors activated by RetS depend upon Vfr for expression (Goodman, 2004). In general, a consistent theme has emerged, whereby the Gac/Rsm pathway appears to promote expression of acute virulence factors though a Vfr-dependent mechanism, but represses chronic phenotypes through a Vfr-independent mechanism.

Cross-talk amongst the regulatory signaling pathways described here most often occurs at the transcriptional level (i.e. Vfr control of QS and AlgR control of QS), where different pathways regulate overlapping sets of genes. However, cross-talk can also occur via post-transcriptional regulation (i.e. c-di-GMP binding: Alg44 and FleQ). Collectively, the studies reviewed here suggest that *P. aeruginosa* signaling pathways are arranged in complex circuits with extensive cross-talk at multiple levels, thereby providing cells the ability to generate a finely tuned and progressive response to a range of stimuli.

The aforementioned pathways play a critical role in determining *P. aeruginosa* lifestyle by detecting various environmental conditions and controlling global phenotypic changes. In many cases, the environmental cues that influence these signaling pathways remain unknown. However, in some instances potential signals have been elucidated. For

example, the cAMP/Vfr signaling pathway is responsive to low calcium levels and osmotic stress (Wolfgang, 2003 and Rietsch, 2006). It has been hypothesized that the Gac/Rsm pathway may respond to carbohydrate signals, as the hybrid sensors RetS and LadS contain carbohydrate-binding domains (Goodman, 2004 and Ventre, 2006). Recent work by O'Callaghan and colleagues (2011) demonstrated that oxygen availability, detected through the anaerobic response regulator Anr, modulates *rsmY/Z* expression and thus Gac/Rsm-dependent phenotypes. QS is controlled by both the cAMP/Vfr and Gac/Rsm pathways and thus signals that trigger these pathways, ultimately influence QS gene expression. In addition, membrane perturbations and the stringent response have previously been shown to influence QS (Mohr, 1991; Yu, 1997), indicating that nutrient starvation/availability plays a substantial role in determining *P. aeruginosa* social behavior. The signals influencing c-di-GMP levels remain largely unknown, although recent work demonstrates that the availability of specific amino acids influence c-di-GMP levels and surface-associated behaviors (Bernier, 2011). Furthermore, surface contact or attachment itself appears to represent an important cue for c-di-GMP production (Güvener, 2007). Given the plethora of enzymes involved in c-di-GMP synthesis and degradation, it is plausible that many additional environmental cues contribute to regulation of c-di-GMP levels. The physiological relevance of these signals, in the context of a complex environmental or host niche, has yet to be determined and represents an important and daunting future direction for the field. Current progress in this area is severely hampered by the lack of tractable model systems and the fact that many of the signals mentioned are likely to have pleiotropic effects.

Concluding Remarks

While it is convenient to think of *P. aeruginosa* infection as a switch between acute and chronic phenotypes it has become clear that infection, like environmental lifestyle, is a finely-balanced continuum and involves substantial overlap and cross-talk between environmental detection systems. We envision a model (Figure 2) whereby the transition from acute to chronic virulence phenotype during infection closely parallels the gradual changes observed during the shift from a planktonic to sessile lifestyle within an environmental reservoir. Further, a similar transition from a sessile to planktonic lifestyle can occur within the environment enabling dissemination to new environmental niches. We hypothesize that this sessile to planktonic switch also occurs durig chronic human disease, although the mechanism and implications are less clear.

In the planktonic environmental lifestyle of *P. aeruginosa,* individual or small groups of free-living bacteria have the capacity to evade or defend against bacterivorous protozoan and metazoan predators (Hilbi, 2007). To do so, *P. aeruginosa* relies on cAMP/Vfr signaling to facilitate expression of many traditional "virulence factors" like the T3SS, which in an environmental reservoir is an important defense mechanism against phagocytosis by predators. Additionally, in this planktonic phase *P. aeruginosa* expresses appendages which mediate motility, providing yet another mechanism for avoiding predation and facilitating dispersal. The cAMP/Vfr signaling cascade controls the QS pathway. Once a quorum is reached, the bacteria begin to exhibit population behaviors, such as the secretion of degradative enzymes and toxins, which in significant concentrations can liberate nutrients from environmental sources and provide defense against predators and competing bacterial species. Similarly, in acute human infection the

virulence phenotype of *P. aeruginosa* is dominated by the expression of virulence factors that are cytotoxic and invasive (T3SS, T2SS, flagella, Tfp) which cause tissue damage, sepsis and contribute to bacterial dissemination. The cAMP/Vfr and QS pathways largely control expression of these virulence factors.

In times of limited nutrients or substantial predation, *P. aeruginosa* can adapt a sessile biofilm lifestyle as a defense mechanism for evading predators and restricting metabolic demands. Upon attachment to a surface, the pathways leading to a sessile lifestyle (c-di-GMP, Gac/Rsm) become activated. Attachment is initially dependent upon the expression of cAMP/Vfr-regulated genes, such as those necessary for Tfp expression and QS. As the bacterial population transitions to the sessile lifestyle these pathways are ultimately repressed and those involved in EPS production and biofilm formation are activated. During persistent or chronic human infection, *P. aeruginosa* frequently transitions to a noncytotoxic and minimally invasive virulence phenotype, adopting a biofilm lifestyle, which protects against host immune system insults and provides resistance to antibacterial therapy.

The last aspect of this model is that at any given time individuals residing in the environmental sessile community can transition from this lifestyle to once again become motile to seek new nutrient sources and more favorable environmental conditions. Similarly, we envision that individual members of a biofilm community, within the context of infection, can leave the biofilm and reemerge as individual motile bacteria. This transition may facilitate spread to a new colonization site within the CF lung and the renewed expression of acute virulence factors may contribute to the frequent bouts of exacerbation experienced by CF patients with chronic *P. aeruginosa* infection.

Additionally, in the case of chronic biofilm infection of catheters, this reemergence of motile and cytotoxic bacteria may explain the periodic development of septicemia. Analogous to the environmental lifestyle transition, we hypothesize that the conversion to a chronic virulence phenotype occurs via a continuum, where the bacterial population largely transitions from cAMP/Vfr and QS-dominated phenotypes to c-di-GMP and Gac/Rsm-dominated phenotypes.

While this review primarily focuses in the regulatory pathways used by *P. aeruginosa* to generate a phenotypic continuum in response to extracellular signals, it is important to note that genetic variability also contributes to dynamic phenotypes within *P. aeruginosa* populations. This is best exemplified in chronic *P. aeruginosa* infections. Current evidence supports the idea that *P. aeruginosa* infection in CF largely involves a clonal population; however, there is abundant evidence that this population undergoes diversification through the accumulation of mutations (Belete, 2008). Early evidence suggested that these mutations are in fact selected for (Schurr, 1996; Kagami, 1998). The majority of documented loss-of-function mutations accumulate in the alginate regulatory pathway (*mucA*), QS system (*lasR*) and cAMP signaling system (*vfr* and *cyaB*) as well as the downstream targets controlled by these systems (Whitchurch, 1996; Mathee, 1997; Kagami, 1998; Whitchurch, 2002). In general, these mutations reduce the capacity of individual isolates to cause acute and invasive infection (Belete, 2008), supporting the notion that acute and persistent infections involve distinct and potentially mutually exclusive phenotypic strategies. However, the maintenance of wild type alleles within the overall *P. aeruginosa* population suggests the need to maintain a full phenotypic repertoire. In particular, the spread of chronic infection (e.g. during exacerbation) to

otherwise naive sites within the CF lung may require the capacity to initiate an acute

virulence phenotype.

In opportunistic pathogens, regulatory systems must promote changes in gene

expression to facilitate transition from an environmental reservoir to the host. Temporal

control and spatial localization may allow ubiquitous signals to elicit specific responses.

The ability of *P. aeruginosa* to occupy diverse environmental habitats and cause a wide

variety of infections depends on a complex series of global regulatory networks that

integrate diverse extracellular signals to coordinate phenotypic change. Intense study

over the past decade has generated a wealth of information about the complex lifestyle of

P. aeruginosa; however, many questions remain to be answered.

Type IV pili

Pili are colonization factors expressed by a vast array of bacterial species. The different types of pili were originally classified based upon their morphology and are also now categorized based upon their mechanisms of assembly (Pelicic, 2008). Type IV pili (Tfp) are filamentous surface appendages that facilitate attachment to a variety of surfaces in a diverse group of bacteria, including both pathogenic and non-pathogenic species. Tfp are the only class of pili present in both Gram-positive and Gram-negative bacteria and have been identified in numerous bacterial phyla including Proteobacteria, Cyanobacteria, and Firmicutes (Mattick, 2002). Among the different types of pili, only Tfp are able to retract, a property that promotes intimate interactions between bacteria and eukaryotic host cells and possibly other surfaces as well. In addition, the repeated cycles of pilus fiber extension and retraction facilitate a type of surface movement termed twitching motility that is involved in bacterial colonization and spread.

In *P. aeruginosa,* the process of Tfp assembly and function involves several gene products (Mattick, 2002). The structural fiber is primarily composed of repeating pilin subunits, encoded by the *pilA* gene (Strom, 1986). However, several less abundant fiber-associated proteins are also required for Tfp biogenesis and function (described in more detail below). Pilin (PilA) subunits are produced as pre-pilins containing an amino-terminal leader sequence that is cleaved prior to assembly of the mature protein into the pilus fiber. The length of the leader sequence and mature pilin is used to further classify the different subtypes of Tfp, as multiple subtypes can be expressed in some species. Type IVa pilins have short leader sequences and shorter pilin subunits (150-160aa) while Type IVb pilins possess longer leader peptides and can have either long (≈200aa) or very

short ($\approx$30aa) pilin subunits (Craig, 2004). The prepilin peptidase, PilD, mediates cleavage of pre-pilins and also processes the structurally related Xcp pilin-like proteins involved in *P. aeruginosa* Type II secretion (Bally, 1992). The mature pilin protein contains a highly conserved, extended and hydrophobic N-terminal α-helical region followed by a globular C-terminal domain that terminates with a disulfide bonded loop (DSL) that varies in size among different pilin alleles (Parge, 1995; Craig, 2003; Kus, 2004). Despite differences in length, the type IVa and type IVb pilins share a conserved architecture (Craig, 2003). Mature pilin subunits are predicted to assemble such that the N-terminal α-helical regions form the core of the pilus fiber, while the globular C-terminal domains are exposed on the outside surface (Burrows, 2005).

Pilin subunit assembly occurs on the periplasmic surface of the inner membrane in an energy-dependent manner. PilC is an inner-membrane protein that serves as the scaffold upon which the growing pilus fiber is assembled (Hobbs, 1993a). The ATPase, PilB, localizes to the inner membrane and provides the energy for pilin monomer assembly and extrusion of the growing pilus fiber through a pore in the outer membrane. A second ATPase, PilT, drives the disassembly and subsequent retraction of the pilus fiber (Whitchurch, 1991). Loss-of-function mutations in *pilB* result in a non-piliated, non-twitching phenotype (Nunn, 1990), whereas inactivation of *pilT* impedes Tfp retraction, resulting in both hyperpiliation and abrogation of twitching motility (Whitchurch, 1991). Mutants lacking *pilT* are defective for T3S-depdendent cytotoxicity *in vitro* (Zolfaghar, 2003), presumably due to the inability to form productive interactions with eukaryotic cells via Tfp retraction. *pilT* mutants also displayed reduced virulence in a mouse model of infection, which is likely due to a defect in dissemination as the mutant strain

efficiently infected lungs but not peripheral organs (Comolli, 1999). The outer membrane pore is a dodecameric ring-like structure comprised of PilQ subunits, a protein belonging to the super family of secretins (Collins, 2001; Wolfgang, 2003a). The *pilQ* gene is located in an operon along with four other genes (*pilMNOPQ)*. This operon encodes an important transmembrane complex important for pilus function, although few mechanistic details exist. PilM is a cytoplasmic protein with structural similarity to the actin-like domain of GspL, a component of the Type II secretion system (T2SS). PilN and PilO are bitopic inner membrane proteins whose periplasmic domains interact to form a heterodimer (Sampaleanu, 2009). PilP is an inner membrane lipoprotein with no homology to the evolutionarily related T2SS. Based upon co-fractionation studies, these proteins (PilM/N/O/P) are predicted to form an inner membrane complex (Ayers, 2009), which likely directs alignment of the PilQ secretin with the Tfp biogenesis machinery (PilB/T and PilC), although mechanistic details to confirm this prediction are lacking.

In *P*. *aeruginosa*, five 'pilin-like' proteins (FimU, PilV, PilW, PilX, PilE) share the highly conserved N-terminal α-helical region of pilin as well as the pre-pilin leader sequence (Winther-Larsen, 2005). The pilin-like proteins are required for Tfp biogenesis (Russell, 1994; Alm, 1995, 1996b) and are present in mature Tfp fibers at much lower abundance than pilin itself (Giltner, 2010). The *fimU-pilVWXY1Y2E* operon (*fimU* operon) encoding the pilin-like proteins also encodes the structurally distinct protein PilY1. Like the pilin-like proteins, PilY1 is associated with Tfp fibers and is necessary for Tfp biogenesis (Heiniger, 2010; Orans, 2010). However, PilY1 has several unique properties (described in more detail below) not shared by the other Tfp components. Recent localization studies demonstrate that the pilin-like proteins are incorporated into

the pilus fiber at significantly reduced concentrations when compared to the structural subunit, PilA (Giltner, 2010). Furthermore, the proteins of the *fimU* operon are predicted to interact to form a structural complex that is required for pilus biogenesis (Giltner, 2010; Heiniger, 2010), as specific stoichiometic ratios were necessary for pilus expression and function (Giltner, 2010). Due to homology with *Neisseria* PilC, PilY1 is predicted to localize to the tip of the pilus fiber where it can facilitate attachment. However, PilY1 localization has yet to be experimentally determined, but based upon the similar Tfp expression defect of *fimU-pilE* and *pilY1* mutants (Heiniger, 2010), we hypothesize that PilY1 localization involves interaction with the pilin-like proteins.

Several functions have been attributed to PilY1, such as antagonizing pilus retraction and mediating attachment to human airway epithelial cells (Heiniger, 2010; Orans, 2010). PilY1 is a bipartite protein, where the C-terminal portion of PilY1 functions in Tfp biogenesis and the N-terminal portion is predicted to mediate attachment to host tissues. The PilY1 C-terminus shares homology with the PilC proteins of *Neisseria* species (Jonsson, 1991; Wolfgang, 1998; Heiniger, 2010; Orans, 2010) and contains an EF-hand-like calcium-binding site that is conserved in the C-terminal domains of pilus biogenesis proteins related to PilY1. We recently demonstrated that calcium binding and release by PilY1 is necessary for control of pilus extension and retraction (Orans, 2010). The 2 μM calcium binding affinity of PilY1 lies in a range where the binding and release of calcium would be physiologically relevant (Orans, 2010), and suggests that the opposing forces of the calcium-bound and unbound state are likely responsible for Tfp-mediated twitching motility.

The molecular mechanisms underlying attachment of *P. aeruginosa* to host cells and the identity of the bacterial adhesin have remained controversial. Previous studies suggested that *P. aeruginosa* pilin was directly involved in binding the GalNAcβ1-Gal moiety of non-sialylated glycosphingolipids asialo-GM1 that is abundant on the apical surface of mammalian epithelial cells (Lee, 1994b; Sheth, 1994). However other studies established that *P. aeruginosa* binds preferentially to the basolateral surfaces of epithelial cells (Fleiszig, 1997; Lee, 1999), an area where asialo-GM1 is not well localized. These results suggested that asialo-GM1 is likely not the primary *P. aeruginosa* host cell receptor.

More recent studies have demonstrated that PilY1, presumably through interactions of the N-terminus, is required for *P. aeruginosa* Tfp-associated adherence to the basolateral surface of differentiated human airway epithelial cells (Heiniger, 2010) and *in vitro* studies suggest attachment may be achieved through an interaction between integrins and the arginine-glycine-aspartic acid (RGD) binding motif present in PilY1 (Johnson, 2011). The limited sequence similarity between the N-termini of *P. aeruginosa* PilY1 and the Neisserial PilCs (Rudel, 1995; Morand, 2001; Orans, 2010) suggests this region likely dictates the precise tissues targeted by these bacteria *in vivo*.

The regulatory process governing Tfp biogenesis features multiple levels of control including transcriptional regulation of several structural components. The PilS/R two-component signal transduction system (TCS) controls *pilA* transcription (Hobbs, 1993b; Koga, 1993) and the RpoN alternative sigma factor controls *pilS/R* transcription (Strom, 1993). However, the signal to which PilS/R responds is not known (Boyd., 1994). The atypical sensor-regulator pair AlgZ/AlgR has also been implicated in controlling Tfp

biogenesis and function, as mutants lacking either component are deficient for twitching motility (Whitchurch, 1996). Because AlgZ/R did not affect *pilA* expression, it was concluded that the system must regulate some other component involved in Tfp biogenesis. Later, AlgZ/R was shown to positively regulate the *fimU* operon, with AlgR recognizing two distinct sites in the *fimU* promoter region (Belete, 2008). AlgR can bind DNA in either a phosphorylation-dependent (*hcnA*) (Cody, 2009) or -independent manner (*algD)* (Wozniak, 1994). The fact that *algZ* mutants are defective for twitching motility suggests that AlgR phosphorylation is necessary for activation of the *fimU* operon, but this prediction has not been experimentally confirmed.

Another aspect of twitching motility regulation involves Vfr, the *P. aeruginosa* homolog of the *E. coli* catabolite repressor protein (Crp) (Beatson, 2002), and its allosteric regulator, the second messenger-signaling molecule adenosine 3', 5'-cyclic monophosphate (cAMP). In most cases, Vfr activity is cAMP-dependent (Fuchs, 2010a). cAMP is synthesized by the enzyme adenylate cyclase *and* the *P. aeruginosa* genome encodes two adenylate cyclases (Wolfgang, 2003b). Recent work by our laboratory showed that the *chp* gene cluster, which encodes a putative chemotactic sensory system, regulates the major *P. aeruginosa* adenylate cyclase, CyaB (Fulcher, 2010). The Chp system was first implicated in controlling twitching motility and Tfp production (Darzins, 1997; Whitchurch, 2004), although the mechanism for mediating a Tfp-dependent chemotactic response in *P. aeruginosa* is unknown. Fulcher *et al*. demonstrated that the Chp system controls intracellular cAMP levels by modulating CyaB activity (Fulcher, 2010). Furthermore, Tfp production is cAMP-dependent, thus linking the Chp system to control of Tfp expression (Fulcher, 2010).

The work presented here further investigates the function of PilY1 in pilus retraction and Tfp production. Additionally, we investigate the mechanisms of transcriptional regulation that influence expression of the *fimU* Tfp biogenesis operon. Lastly, we describe *fimU* dysregulation and attempt to elucidate the mechanism responsible for this altered regulatory state.

Figure 1. Model of the regulatory pathways and cross-talk that modulate *P. aeruginosa* lifestyles.
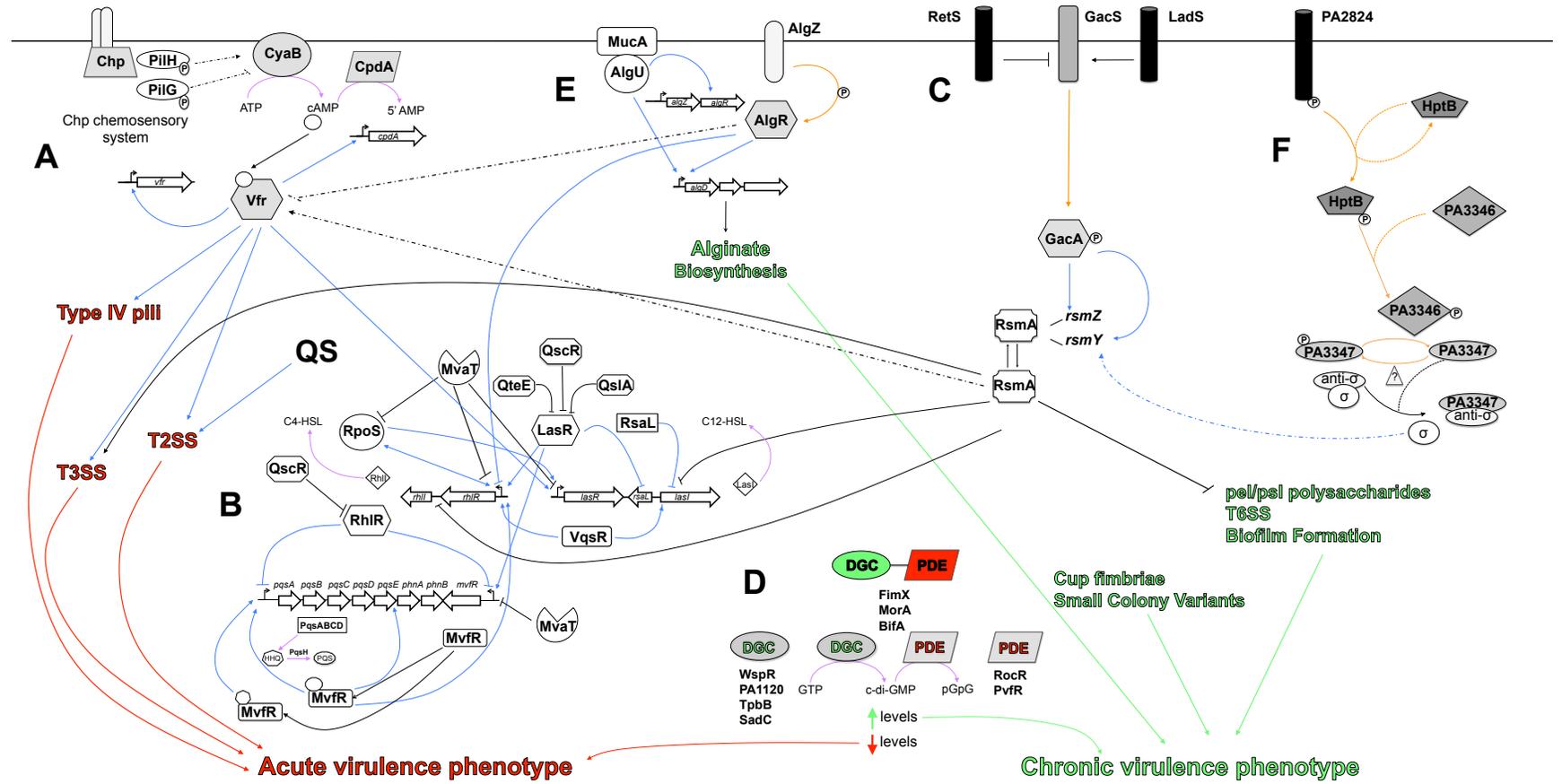
Figure 2. *Pseudomonas aeruginosa* **exhibits parallel lifestyle extremes in the environment and human host.**
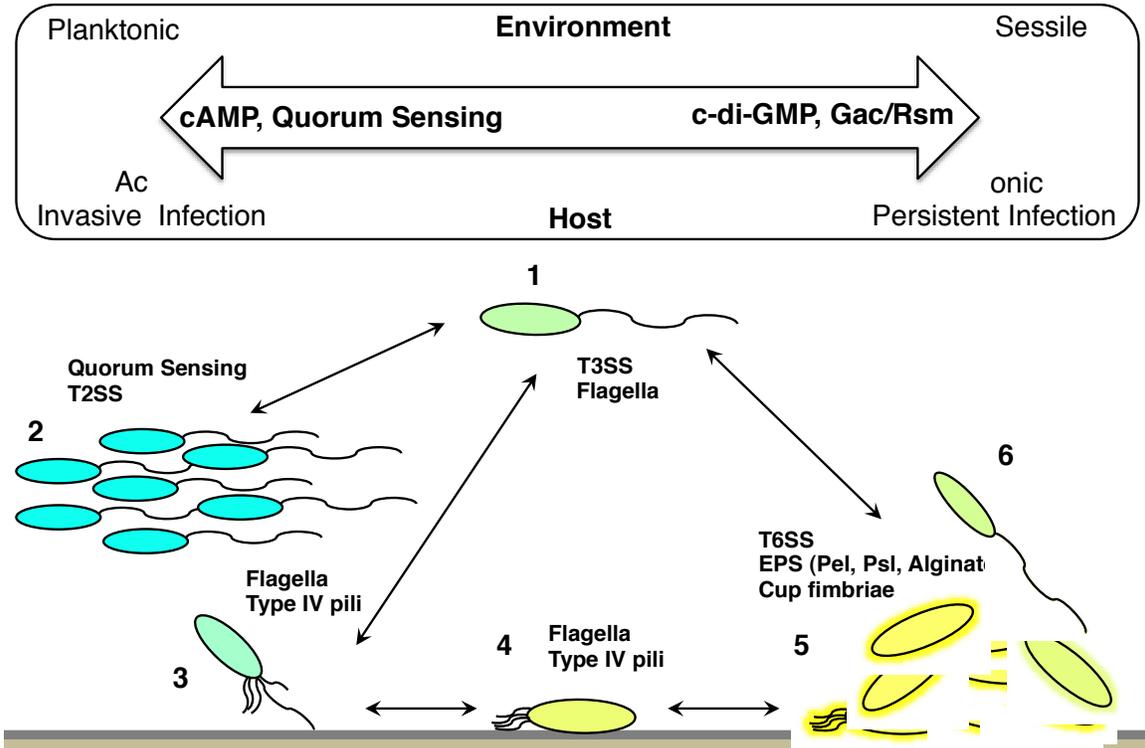
Figure 1.1 Model of the regulatory pathways and cross-talk that modulate *P. aeruginosa* lifestyles.

Lines depict direct regulatory mechanisms within given signaling pathways as well as cross-talk between regulatory systems. Arrows represent positive regulation and T-bars indicate negative regulation. Blue lines represent transcriptional regulation while purple lines represent enzymatic reactions. Orange lines depict post-translational modification events. Black lines illustrate post-translational regulation events. Dashed lines indicate unknown mechanisms of regulation. For more details see text references to this figure.

A. cAMP/Vfr signaling. The Chp chemosensory system modulates the enzymatic activity of CyaB, an adenylate cyclase that synthesizes the majority of intracellular cAMP. The phosphodiesterase CpdA degrades intracellular cAMP. When present in sufficient quantities, cAMP binds to and activates the transcription factor Vfr. cAMP/Vfr regulates numerous virulence factors, primarily those associated with an acute virulence phenotype, in addition to *lasR*, the master regulator of the quorum sensing hierarchy. Both *vfr* and *cpdA* are transcriptionally regulated by cAMP/Vfr creating a feedback loop to maintain cAMP homeostasis.

B. Quorum Sensing. Schematic representation of the AHL- and AQ-dependent QS systems in *P. aeruginosa*. LasR activates *lasI* expression to produce C12-HSL. The LasR/C12-HSL complex positively influences expression of the second AHL-dependent QS system (RhlRI/C4-HSL) and many other target genes. The AHL-dependent systems are transcriptionally and post-transcriptionally regulated by numerous regulators including two LuxR homologues (VqsR and QscR), RsmA, and the alternative sigma factor, RpoS. The RhlR/C4-HSL complex negatively regulates the AQ-dependent QS system.

C. Gac/Rsm pathway. The GacS/A TCS positively regulates expression of two sRNA molecules, RsmY and RsmZ, which bind and sequester the RNA-binding protein RsmA. Activation of the HptB signaling cascade promotes chronic virulence factor expression by specifically activating *rsmY* expression. GacS activity is antagonized by the hybrid sensor kinase RetS and promoted by the orphan sensor kinase LadS. Free RsmA facilitates the expression of acute virulence factors, such as the T3SS, and represses expression of chronic virulence factors including the T6SS and EPS (Pel and Psl) involved in biofilm formation.

D. c-di-GMP signaling. c-di-GMP levels inversely control functions involved in motility (acute) and biofilm formation (chronic). Synthesis and degradation of c-di-GMP is facilitated by diguanylate cyclases and phosphodiesterases. Multiple DGCs and PDEs encoded within the *P. aeruginosa* genome appear to be spatially localized to alter local c-di-GMP concentrations and influence protein function and gene expression by largely unknown mechanisms.

E. MucA signaling. The anti-sigma factor MucA sequesters the sigma factor AlgU. During chronic CF infection, *mucA* mutation results in constitutive AlgU activation. AlgU increases expression of *algR* and together AlgU and AlgR activate the alginate biosynthetic operon. AlgR negatively regulates QS by inhibiting *rhlR* expression. AlgR inhibits *vfr* expression via an unknown mechanism resulting in decreased expression of many acute virulence factors.

F. HptB signaling. Upon activation by an unknown signal the sensor kinsase PA2284 transfers a phosphoryl group specifically to HptB. HptB then relays the signal to the response regulator PA3346. Phosphorylated PA3346 functions as a Ser/Thr phosphatase and dephosphorylates PA3347. Phosphorylation is hypothesized to modulate the binding

activity of PA3347. In the unphosphorylated state PA3347 is thought to bind an anti-sigma factor, resulting in the release of an unidentified sigma factor, which specifically regulates *rsmY* expression leading to expression of genes associated with swarming motility and biofilm formation.

Figure 1.2 *Pseudomonas aeruginosa* exhibits parallel lifestyle extremes in the environment and human host.

The interplay of four global regulatory pathways (cAMP, c-di-GMP, Quorum sensing and Gac/Rsm) appears to create a phenotypic continuum that controls transition between a planktonic and sessile lifestyle within the environment; and plays an analogous role in human infection by inversely controlling acute and chronic infection phenotypes.

1. In the environment *P. aeruginosa* can exist as planktonic cells or small groups of free living motile organisms, providing the means to colonize new environmental niches. **2.** As the population increases, cells associate as a quorum, producing QS signal molecules and exhibit social behaviors (degradative enzyme and toxin secretion), which promote nutrient acquisition and group survival among environmental predators. **3.** Upon interaction with a solid surface, *P. aeruginosa* can attach via Tfp or flagella. **4.** Following loose-attachment, *P. aeruginosa* may exhibit surface motility utilizing Tfp or flagella to move toward nutrients. Upon generation of an intimate surface attachment, *P. aeruginosa* may initiate microcolony formation. **5.** Eventually *P. aeruginosa* becomes sessile and produces exopolysaccharides that encase the bacteria in a complex matrix, which protects the bacteria from environmental fluctuations and provides a physical barrier against predators. **6.** Unknown environmental signal(s), or stochastic processes, cause members of the sessile community to become motile, leave the biofilm, and return to a planktonic lifestyle.

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CHAPTER TWO:

A *P. aeruginosa* Tfp Biogenesis Operon is Controlled Synergistically by Two Global Regulatory Systems, cAMP/Vfr and the Two-Component System AlgZR[2•]

SUMMARY

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a striking proportion of nosocomial infections annually. One virulence factor required for *P. aeruginosa* infection is Type IV pili (Tfp), which are retractile surface appendages that promote bacterial adherence to host tissue. Tfp also mediate twitching motility, a form of surface-associated translocation believed to aid in bacterial dissemination. Tfp are composed of a major structural protein, pilin. Several less abundant fiber-associated proteins also play structural and functional roles in Tfp biogenesis, including the pilin-like proteins (FimU, PilV, PilW, PilX, PilE) and PilY1, which are encoded by the *fimU* operon. PilY1 also mediates Tfp-dependent adherence to host tissues. Expression of Tfp, as well as other virulence factors required for acute *P. aeruginosa* infection, is controlled by the

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*Attributions: TEM and IF images were taken by Hanne Winther-Larsen. All EMSAs were performed by Peter Intile. The DNase I footprinting was performed by Evan Brutinel[2]

transcription factor Vfr and its allosteric effector, cAMP. Our investigation into the basis for reduced Tfp production in cAMP/*vfr* mutants revealed a defect in *fimU* operon transcription. We showed that cAMP/Vfr activation of the *fimU* operon occurs via direct binding of Vfr to a specific promoter sequence. We also refined the role of the response regulator AlgR in *fimU* promoter regulation by demonstrating that AlgR phosphorylation is required for binding to the *fimU* promoter region *in vitro*. Subsequently, we showed that Vfr regulates the *algZ* promoter (operon encoding *algR*) revealing an indirect regulatory loop that also affects *fimU* operon transcription. Overall, these results reveal two linked, but independent regulatory systems involved in controlling Tfp biogenesis and highlights the layers of complex regulation involved in controlling virulence factors associated with acute and chronic *P. aeruginosa* infection.

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen responsible for causing a wide range of infections, ranging from localized acute respiratory infections to life-threatening chronic lung infections in individuals with the genetic disease cystic fibrosis (CF) (Lam. J.R., 1980; Deretic, 1995; Govan, 1996). Acute *P. aeruginosa* infections occur at various sites within the host and are characterized by an intense inflammatory response, extensive tissue damage and sepsis. In contrast, chronic *P. aeruginosa* infections, such as colonization of the CF lung, are non-invasive and cause cumulative damage over time. The ability of *P. aeruginosa* to cause infection relies on the expression of a multitude of surface-exposed and secreted virulence factors (Rahme, 1995; Rahme, 2000) and it is thought that particular virulence factors are associated with acute and chronic modes of infection (Coggan, 2011).

Acute infection is largely mediated by the Type III secretion system (T3SS) toxins, which are injected directly into host cells, as well as the toxins, degradative proteases and lipases secreted by the Type II secretion system (T2SS). Acute virulence is also mediated by Type IV pili (Tfp), which are filamentous appendages associated with the bacterial surface that mediate interactions with host cells. Chronic infection is typified by a biofilm-like mode of growth, where bacteria exist as microcolonies encased in one of several exopolysaccharides (Costerton, 1999; Donlan, 2002; Drenkard, 2002; Ryder, 2007). In the case of CF, chronic isolates commonly exhibit a "mucoid" colony phenotype, resulting from overproduction of the exopolysaccharide alginate (Pedersen, 1992). Medical device-associated biofilm formation is also a major source of nosocomial infection and is difficult

to eradicate due to the substantial resistance of exopolysaccharide-encased biofilms to antibiotic treatment and host defenses (Høiby, 2010).

Despite the association of specific *P. aeruginosa* factors with either acute or chronic infection "lifestyles", Tfp have the potential to contribute to both modes of infection. In addition to their role in host cell attachment (Heiniger, 2010), Tfp mediate a form of surface-associated motility (twitching motility) that promotes bacterial spread within infected tissue (Comolli, 1999). Tfp are also important for the initiation of biofilm formation (initial surface attachment) (Giltner, 2006) and they play a role in maintaining biofilm structure (Mikkelsen, 2011). The various functions of Tfp fibers are mediated by repeated cycles of extension and retraction. The Tfp fiber is primarily composed of repeated pilin (or PilA) subunits, which are produced as pre-pilins containing a N-terminal six amino acid leader sequence that is cleaved prior to assembly (Wade, 2005). Rapid assembly and disassembly at the inner membrane, an energy-dependent process driven by the ATPases PilB and PilT, facilitates extension and retraction of the pilus fiber through a pore in the outer membrane (Mattick, 2002). In addition to the major Tfp structural subunit pilin, there are several less abundant proteins FimU, PilV, PilX, and PilE (termed "pilin-like" proteins due to their homology to pilin) that are required for assembly of a functional pilus fiber (Russell, 1994; Alm, 1995, 1996b, a). These proteins were recently shown to be present in the mature pilus fiber (Giltner, 2010), where they are believed to form a complex at regular intervals along the length of the fiber. Another minor component of Tfp is PilY1, which is important for regulating pilus extension and also hypothesized to be the putative adhesin facilitating attachment to the basolateral surface of human epithelial cells (Heiniger, 2010; Orans, 2010). There are several other biogenesis factors necessary for Tfp assembly (i.e.

PilM, PilN, PilO, PilP, and PilQ), but these proteins are not associated with the extracellular pilus fiber but instead are located within the periplasm and/or inner membrane.

Multiple signal transduction pathways in *P. aeruginosa* have been implicated in reciprocally controlling expression of virulence factors associated with acute and chronic infection. AlgR, a two-component system response regulator, is involved in regulating multiple virulence systems. Like many TCS, the genes encoding AlgR and its putative cognate sensor kinase AlgZ are immediately adjacent to one another in an operon (*algZR*), such that the *algZ* promoter drives transcription. AlgZ and AlgR are both required for twitching motility and Tfp production, such that phosphorylation of AlgR by AlgZ is required for activity (Whitchurch, 1996, 2002; Belete, 2008). AlgR controls transcription of the *fimU-pilVWXY1Y2E* operon (*fimU*), which encodes the Tfp pilin-like proteins (Belete, 2008). AlgR also mediates the conversion to mucoidy (alginate overproduction) that occurs during chronic infection in the CF lung. Loss-of-function mutations in *mucA*, which encodes an anti-sigma factor (Schurr, 1996; Boucher, 1997; Mathee, 1997), initiates a cascade of regulatory events resulting in activation of the *algR* promoter Mohr, 1991 #236;Mohr, 1992 #194;Yu, 1997 #237}. Subsequently, AlgR, along with the alternative sigma factor AlgU (AlgT), directly activate transcription of the alginate biosynthetic genes (*algD* operon) (Mohr, 1991; Martin, 1994). Unlike the requirement in Tfp regulation, AlgR activation of *algD* is phosphorylation independent, suggesting that AlgZ is not required for alginate production (Ma, 1998).

Vfr, the global transcriptional regulator coordinately controls expression of several acute virulence factors (Wolfgang, 2003; Jones, 2010). Vfr activity is dependent upon the second messenger 3', 5'- cyclic AMP (cAMP), which is synthesized by two adenylate

cyclase enzymes (CyaA and CyaB) in *P. aeruginosa* (West, 1994; Beatson, 2002a; Wolfgang, 2003; Fuchs, 2010a). Vfr coordinately controls the expression of over 200 genes, many of which are involved in *P. aeruginosa* virulence (Suh, 2002; Wolfgang, 2003). cAMP/Vfr have been implicated in twitching motility and in Tfp production. Initially, *vfr* was identified in a screen for twitching-motility defective mutants (Beatson, 2002b) and it was later demonstrated that Tfp production requires Vfr and cAMP (Fulcher, 2010; Jones, 2010). Recently our group showed that *mucA* mutation results in down regulation of invasive virulence factors via inhibition of *vfr* expression, an effect that involves both AlgU and AlgR (Jones, 2010). Thus, when over-expressed due to *mucA* mutation, AlgR not only mediates the switch to a chronic infection phenotype via activation of alginate production but also represses acute virulence factors through inhibition of cAMP/Vfr signaling.

Although the cAMP/Vfr signal transduction system has been linked to Tfp biogenesis and function, the exact mechanism by which these regulators control Tfp is not known. To better understand this aspect of Tfp regulation, we sought to identify the specific defect in cAMP signaling mutants that accounts for loss of Tfp biogenesis and twitching motility. Further we investigated how cAMP/Vfr Tfp regulation integrates with the complex regulatory networks controlling Tfp production.

RESULTS

Vfr and intracellular cAMP control Tfp abundance

It was previously reported that Tfp production and function in *P. aeruginosa* is Vfr-dependent (Beatson, 2002a) and requires production of the second-messenger signaling molecule cAMP (Fulcher, 2010). However, the nature of the defect and the mechanism(s) by which Vfr and cAMP regulate Tfp have not been determined. To more thoroughly characterize the Tfp defect in strains lacking Vfr or cAMP production, we evaluated the abundance and morphology of Tfp fibers by transmission electron microscopy (TEM) (Fig. 1A). TEM images of the wild-type strain show both flagella (thick fibers) and abundant Tfp (thin fibers) extending from the bacterial surface. In contrast, the *vfr* mutant and a *cyaA*, *cyaB* (*cyaAB*) double mutant, which lacks the two ACs that synthesize cAMP, showed a substantial reduction in Tfp. The length, diameter (relative to flagella), and overall morphology of Tfp produced by the *vfr* and *cyaAB* mutants were indistinguishable from Tfp produced by the wild-type strain. Both mutants displayed approximately one Tfp fiber per cell as assessed by evaluating bacteria in multiple TEM images (data not shown). Despite the dramatic reduction in Tfp, both the *vfr* and *cyaAB* mutants could be distinguished from a *pilA* mutant, which lacks the major pilus structural subunit and is non-piliated.

To confirm the TEM results, Tfp were labeled with pilin-specific antibody and visualized using immunofluorescence (IF) microscopy. This technique, which involves growing the bacteria on poly-lysine coated glass coverslips, was previously used for direct immunovisualization of bacterial surface Tfp (Winther-Larsen, 2005; Heiniger, 2010) and provides a means for assessing Tfp abundance on live bacteria. When examined by IF, the

wild-type strain displayed an extensive network of Tfp fibers and the *pilA* mutant lacked

any appreciable Tfp staining (Fig. 1B). The *cyaAB* and *vfr* mutants both displayed

dramatically less Tfp staining relative to the wild-type strain. The fact that bacteria-

associated Tfp could be detected in the *cyaAB* and *vfr* mutants by IF indicates that the

reduction in surface fibers observed by TEM using the corresponding strains (Fig. 1A) was

not due to Tfp shedding.

As pilin is the major structural component of Tfp, we also evaluated the relative

amounts of pilin recovered in sheared pilus preparations, which correlates with surface Tfp

abundance. As previously demonstrated (Beatson, 2002a; Fulcher, 2010), the *vfr* and *cyaAB*

mutants displayed dramatically reduced levels of surface pilin when compared to the wild-

type strain (Fig. 2A, upper panel), and no pilin was detected in preparations from a *pilA*

mutant. Similar levels of pilin were detected in all strains as determined by immunoblotting

whole cell lysates and probing with a pilin-specific antibody (Fig. 2A, lower panel),

indicating that the Tfp defect in the *vfr* and *cyaAB* mutants lies at the level of fiber

assembly rather than reduced intracellular pilin pools.

Vfr and cAMP control expression of the *fimU* Tfp biogenesis operon

Our comprehensive assessment of Tfp abundance on the surface of strains lacking

cAMP or Vfr consistently showed reduced fiber production. Based on these results, we

hypothesized that cAMP and Vfr are required for the expression of one or more genes

involved in Tfp assembly. We chose to examine the role of cAMP/Vfr in regulating the

fimU Tfp biogenesis operon due to the following evidence: i) the *fimU* operon showed the

greatest cAMP/Vfr-dependent response among the Tfp biogenesis genes that were down-

regulated in *cyaAB* and *vfr* mutants (Wolfgang, 2003), ii) altered expression of the *fimU* operon affects Tfp-associated phenotypes in multiple strains (Lizewski, 2004; Belete, 2008), and iii) deletion of the *fimU* operon abolished Tfp production without affecting intracellular pilin pools (Heiniger, 2010), a phenotype similar to that of *cyaAB* and *vfr* mutants (Fig. 2A).

As a readout for *fimU* operon expression, we initially evaluated the level of PilW, a protein encoded by the *fimU* operon. The *cyaAB* and *vfr* mutants produced substantially less PilW than the wild-type strain, as determined by western blot of whole cell lysates (Fig. 2B). To determine if altered expression of the *fimU* operon accounts for reduced PilW in the cAMP/Vfr mutants, we engineered a *fimU* transcriptional reporter by fusing the putative *fimU* promoter region to the β-galactosidase encoding *lacZ* gene and introduced the construct onto the chromosome of the relevant strains. The *cyaAB* and *vfr* mutants showed a significant (P < 0.0001) reduction in *fimU* reporter activity compared to that of the wild-type strain (Fig. 2C). *fimU* reporter activity was restored in the *cyaAB* and *vfr* mutants using plasmid-based expression of either the major AC, *cyaB* (pPa-*cyaB*) or *vfr* (pPa-*vfr*), respectively (Fig. S1). The relative contribution of cAMP and Vfr to PilW protein levels (Fig. 2B) and *fimU* promoter activation (Fig. 2C) closely paralleled the reduction in Tfp displayed by these mutants (Fig. 1AB and 2A). These results demonstrate that both cAMP and Vfr are required for optimal expression of the *fimU* operon. In addition, reduced expression of the Tfp biogenesis factors encoded by the *fimU* operon contributes to the Tfp production defect in mutants lacking cAMP and/or Vfr.

Multiple regulatory systems control expression of the *fimU* operon

The two-component system AlgZ and AlgR (AlgZR) was previously shown to control Tfp production by directly regulating the *fimU* operon (Lizewski, 2004; Belete, 2008). Both the putative sensor kinase (AlgZ) and the response regulator (AlgR) have been shown to control Tfp function, albeit in different strains and by different assays, the dependence on AlgZ suggests that AlgR phosphorylation is required for control of Tfp. (Whitchurch, 2002, 2004; Belete, 2008). However, in the case of alginate production, AlgR activation is AlgZ-independent (and therefore phosphorylation-independent) (Yu, 1997). To determine whether AlgZR and cAMP/Vfr have a similar regulatory role in controlling Tfp production, we assessed the relative abundance of surface and total pilin in single mutants lacking either *algZ* or *algR* to assess their individual contribution as well as a double *algZR* mutant. All three mutants lacked detectable surface Tfp (Fig. 2A, top panel) but retained the capacity to synthesize pilin (Fig. 2A, bottom panel).

Next, we assessed the impact of the *algZR* mutations on *fimU* operon expression. The *algZ*, *algR* and *algZR* mutants all showed reduced PilW by Western blot of whole cell lysates (Fig. 2B) and a significant (P < 0.0001) reduction in *fimU* reporter activity compared to the wild-type strain (Fig. 2C). Altered *fimU* reported activity was restored in each of the mutants when complemented with plasmid-encoded versions of the corresponding mutated gene or genes (Fig. S1), indicating that the defects are not due to secondary mutations or polar effects of the mutations. The reduction in *fimU* promoter activity associated with the AlgZR mutants is consistent with previous reports and likely accounts for the role of this system in controlling Tfp function (Whitchurch, 1996; Lizewski, 2002; Whitchurch, 2002; Lizewski, 2004). These results confirm that AlgZ and

AlgR are each individually required for Tfp production and that their Tfp phenotype is equivalent to that of cAMP/*vfr* mutants. Further, these results indirectly support the data that AlgR regulation of Tfp production is AlgZ dependent (Whitchurch, 2002).

Expression of *algZ* is both cAMP- and Vfr-dependent

Based on the evidence that AlgZR positively regulates the *fimU* operon and that *algZ* expression was reduced in a *vfr* mutant (Wolfgang, 2003), we hypothesized that decreased expression of *algZ* could account for the defect in Tfp production observed in the cAMP and *vfr* mutants. We engineered an *algZ* transcriptional reporter system (P_{algZ}-*lacZ*) by fusing the putative *algZ* promoter region to the *lacZ* gene and integrated the construct into the chromosome of the wild-type and mutant strains. P_{algZ}-*lacZ* promoter activity was significantly reduced in the *vfr* and *cyaAB* mutants ($P < 0.0001$), and the defect was complemented by plasmid-based expression of *vfr* (pPa-*vfr*) or *cyaB* (pPa-*cyaB*), respectively (Fig. 3). These results demonstrate that expression of *algZ* is dependent on both cAMP and Vfr and this dependence could explain the reduction in expression of the *fimU* operon and Tfp production observed in the AC and *vfr* mutants.

Two putative Vfr binding sites are required for *algZ* promoter activity

Kanack *et al*. demonstrated that a probe corresponding to the *algZ* promoter region was shifted by Vfr in EMSA and identified a putative Vfr-binding site based on the Vfr sequence recognition data available at the time; however this Vfr-binding site was never confirmed experimentally (Kanack, 2006). Here, we identified another putative Vfr-binding site (5'- AAG**TGCGA**CGA**:**TGA**TAGCA**GGC) further upstream of the *algZ* coding

sequence. This second site more closely matches the Vfr-binding consensus sequence (VCS) (5'- ANWW**TGNGA**WNY:AGW**TCACA**T) that was published following characterization of additional Vfr-dependent transcripts (Fuchs, 2010a). For clarity, we have designated the upstream sequence as Vfr Binding Site 1 (VBS1) and the previously predicted sequence proximal to the *algZ* open reading frame (Kanack, 2006) as VBS2 (Fig. 4A). The probe used by Kanack *et al*. contains both of the putative Vfr-binding sites, so theoretically either VBS1 or VBS2 could account for shifting of the *algZ* promoter fragment. However, only a single shift product was reported (Kanack, 2006). To examine the Vfr binding site(s) within the *algZ* promoter region, we first generated a P$_{algZ}$ DNA EMSA probe encompassing both putative Vfr binding sites and observed that increasing concentrations of cAMP-saturated Vfr resulted in two distinct shift products, consistent with Vfr binding to both predicted sites (Fig. 4B). We next determined which of the Vfr-binding sites directly influences *algZ* transcription by generating point mutations in each of the putative Vfr-binding sites (Fig. 4C) in the P$_{algZ}$-*lacZ* transcriptional reporter strain (Fig. 4D). Mutations in either site resulted in significantly reduced reporter activity (P < 0.0006) that was indistinguishable from that of a *vfr* mutant. Simultaneous disruption of both sites did not result in a further loss of promoter activity. These results suggest that both VBS1 and VBS2 are required for Vfr-dependent transcriptional activation of the *algZ* promoter. Based on the known Vfr binding site sequences, both VBS1 and VBS2 are predicted to confer low affinity binding. As such, it is possible that simultaneous binding to both sites is necessary to form a stable Vfr-DNA complex. Additional *in vitro* studies are necessary to confirm whether Vfr directly recognizes both predicted sites and if disruption of one site reduces Vfr affinity for the second.

Both Vfr and AlgZR are required for expression of the *fimU* operon

Based on the findings above, we hypothesized that cAMP and Vfr indirectly control expression of the *fimU* operon by controlling *algZ* expression and ultimately the activity of the AlgZR TCS. To determine the order in which the cAMP/Vfr and AlgZR systems work to regulate *fimU* operon expression, we conducted a series of epistasis experiments. Plasmid-based expression of *algZR* (pPa-*algZR*) from a synthetic IPTG-inducible promoter was sufficient to restore wild type *fimU* promoter reporter activity in an *algZR* mutant background (Fig. S1, Fig. 5A). However, when expressed under identical conditions, pPa-*algZR* did not restore wild-type activity of the *fimU-lacZ* promoter reported in an isogenic *vfr*, *algZR* triple mutant (Fig. 5A). Similarly, plasmid-expressed *vfr* restored *fimU* promoter activity in a *vfr* mutant, but not in the *vfr*, *algZR* mutant (Fig. 5A). Consistent with these results, plasmid-based expression of either *vfr* or *algZR* did not restore PilW production, as assessed by Western blot of whole cell lysates (Fig. 5B), or Tfp production, as measured by recovery of pilin in sheared surface fractions (Fig. 5C). These results indicate that the cAMP/Vfr and AlgZR systems do not act in a strictly linear pathway to control *fimU* operon expression.

Interestingly, the *vfr*, *algZR* mutant had significantly lower (P < 0.0001) *fimU-lacZ* promoter activity than either the *vfr* or *algZR* mutants alone and complementation of the triple mutant with either *vfr* or *algZR* resulted in a slight, but significant increase in promoter activity (Fig. 5A). Overall, these results suggest that Vfr and AlgZR act synergistically to control *fimU* promoter activity.

cAMP/Vfr directly regulates transcription of the *fimU* operon

As expression of the *fimU* operon was dependent on expression of both AlgZR and Vfr, we hypothesized that Vfr has a direct role in regulating transcription of the *fimU* operon. To test this hypothesis, we assessed the ability of purified Vfr to directly bind the *fimU* promoter by EMSA. For these assays we used the same upstream promoter region of *fimU* that was used to generate the P_{fimU}-*lacZ* transcriptional reporter (Fig. 6A). Shifting of the P_{fimU} probe was observed with increasing concentrations of cAMP-saturated Vfr (Figure 6B, lanes 2-9). To determine the role of cAMP in Vfr-binding, we generated cAMP-free Vfr (apo-Vfr) by denaturing Vfr in 6 M urea, dialyzing the protein to remove cAMP and then refolding in the absence of cAMP (Fuchs, 2010a). Apo-Vfr did not shift the P_{fimU} probe (Fig. 6C, lane 2), but shifting was restored by the addition of cAMP to the binding reaction (Fig. 6C, lanes 3-9), thus demonstrating that Vfr requires cAMP for binding to the P_{fimU} probe.

Vfr binds to a single site within the *fimU* promoter *in vitro*

Previous bioinformatic analyses did not identify a Vfr-binding site upstream of the *fimU* operon (Kanack, 2006). To define the Vfr binding site located within the *fimU* promoter region, we performed DNase I footprinting of P_{fimU}. In the presence of cAMP-saturated Vfr, a 46-bp sequence was protected from DNase I cleavage (Fig. 7). Within the protected region, enhanced cleavage sites were detected at -130 and -119 bp (Fig 7, Fig. 6A). The spacing of DNase I hypersensitivity sites within P_{fimU} are consistent with the previously reported Vfr footprints of the P_{fleQ}, P_{lasR}, P_{txR}, P_{regA}, $P_{cpdA,}$ and P_{vfr} promoters (Albus, 1997; Dasgupta, 2002; Kanack, 2006; Ferrel, 2008; Fuchs, 2010b, a). The enhanced

DNase I cleavage is attributed to distortion of the helical DNA structure, and occurs at positions 5 and 6 within the conserved half sites of both CRP and Vfr binding regions (Kanack, 2006). By aligning the position of the hypersensitivity sites with those of previously published Vfr footprints, we were able to determine the Vfr binding site (5'-ACG**TGAGC**TAT:GCA**GCACT**TCC-3') within the protected region of the *fimU* promoter (Fig. 7, Fig. 6A).

The proposed Vfr binding site (5'-ACG**TGAGC**TAT:GCA**GCACT**TCC-3'; underlined sequences represent conserved Vfr half-sites) identified in the *fimU* promoter shares modest conservation with the proposed consensus binding sequence (5'-Ann**TGnGA**WCY:AnT**TCACA**TTT, dimeric Vfr predicted to bind two half-sites) (Fuchs, 2010a). The upstream half-site (TGAGC) within the *fimU* promoter matches well with the consensus upstream half-site; however, the downstream half-site (GCACT) contains more variability compared to the downstream consensus half-site (TCACA), further illustrating the significant variability found in Vfr target binding sites.

To further confirm the Vfr binding site within the *fimU* promoter, we performed site-directed mutagenesis to alter the predicted half-sites (Fig. 8A). Initially, we mutated the half-sites such that they more closely mirrored the Vfr consensus half sites (TGAGC>TGAG**A**) and (GCACT>**T**CACT). We then constructed a new version of the *fimU* reporter containing the mutated half-sites (P$_{fimU\,M1}$ –*lacZ*) to test the effect of these alterations on reporter activity. Altering the Vfr half-sites to more closely resemble the consensus Vfr half-sites resulted in significantly elevated reporter activity, but to a level that was only slightly greater than that observed with the P$_{fimU}$ reporter (Fig. 8B). Mutating the P$_{fimU}$ Vfr half-sites such that they were more divergent from the consensus half-sites

(TGAGA>T**C**AG**C**) and (GCACT>G**G**ACT) in the P*fimU* M2 *lacZ* reporter (Fig. 8A) resulted

in a significant (P < 0.0001) decrease in reporter activity compared to P*fimU* (Fig. 8B). None

of the P*fimU* half-site mutations affected reporter activity in the *vfr* mutant background (data

not shown).

In order to determine whether mutations in the predicted Vfr binding site altered

DNA binding, we generated DNA probes containing the mutations corresponding to P*fimU*

M1 and P*fimU* M2 (Fig. 8A) and assessed Vfr binding by EMSA. Vfr appeared to recognize

P*fimU* M1 with no detectable change in binding affinity (Fig. 8C), whereas Vfr failed to bind

P*fimU*M2 (Fig. 8D). Therefore, altering the binding site to more closely resemble the

consensus Vfr binding sequence (P*fimU* M1) had little effect on Vfr binding affinity, which is

consistent with the modest increase in reporter activity we observed (Fig. 8B). In contrast,

mutations in the putative Vfr binding site (P*fimU* M2) that eliminated Vfr-dependent *fimU*

transcription (Fig. 8B) also eliminated Vfr binding *in vitro*. Taken together, these results

confirm the identity of the Vfr binding site within the *fimU* promoter, and support the

finding that cAMP-saturated Vfr binds directly to the *fimU* promoter region to activate

transcription.

AlgR affinity for the *fimU* promoter is dramatically enhanced by the phosphodonor

phosphoamidate

Belete et *al*. identified two putative AlgR binding sites (designated ABS1 and

ABS2) upstream of the *fimU* ORF (Fig. 6A) and demonstrated AlgR binding to the *fimU*

promoter *in vitro* (Belete, 2008). Our results showing that both AlgZ and AlgR are required

for activation of the *fimU* promoter (Fig. 2C) suggest that AlgZ likely functions as the

cognate sensor kinase responsible for phosphorylation and subsequent activation of AlgR. This hypothesis is supported by previous work indicating that an *algZ* mutant was defective for Tfp-dependent function (Whitchurch, 2002). Further, an AlgR substitution mutant (AlgR D54N), lacking the phospho-accepting aspartate residue at amino acid position 54 was defective for Tfp production and function *in vivo* and unable to bind the *fimU* promoter *in vitro* (Whitchurch, 2002; Belete, 2008). Despite these findings, direct evidence that phosphorylation specifically increases AlgR DNA binding activity is lacking. To confirm AlgR binding to the *fimU* promoter and to assess the impact of phosphorylation, we purified full-length AlgR as an amino-terminal histidine fusion and assessed its ability to bind to and alter the electrophoretic mobility of the P$_{fimU}$ probe in the presence and absence of small molecule phosphodonor compounds. In the absence of a phosphodonor, AlgR was only able to shift P$_{fimU}$ at high concentrations (351 nM), such that less that 50% of the probe was shifted under these conditions (Fig. 9A). The non-specific probe, which is devoid of identifiable AlgR binding sites, was shifted by AlgR with similar kinetics, suggesting that binding to P$_{fimU}$ in the absence of AlgR phosphorylation is not biologically relevant (Fig. 9A). In contrast, the addition of the phosphodonor phosphoamidate (PAM; 50 mM) resulted in a specific shift of P$_{fimU}$ by AlgR (Fig. 9B). Although the exact increase in affinity could not be calculated due to the poor binding activity of unphosphorylated AlgR, we estimate that the K$_{eq}$ decreased by at least an order of magnitude in the presence of PAM. In addition to PAM, acetyl phosphate (AcP) is commonly used as a phosphodonor *in vitro* and has been reported to specifically phosphorylate purified AlgR (Deretic, 1992). However, the addition of AcP did not result in increased AlgR affinity for the P$_{fimU}$ probe (data not shown).

These results confirm that AlgR binds the *fimU* promoter and that phosphorylation is required for specific high affinity binding. Further, these findings support the hypothesis that AlgZ functions as the cognate sensor kinase responsible for AlgR phosphorylation. Although Belete *et al.* report two shifted products, our results demonstrate a single shift product (Fig. 9B). While two putative AlgR binding sites have been identified (Belete, 2008), our results suggest that only a single site is occupied or that AlgR binding is cooperative such that both sites become occupied simultaneously. Further studies are required to determine the individual contributions of the two putative AlgR binding sites.

Vfr and AlgR simultaneously bind the *fimU* promoter

As demonstrated, AlgR and Vfr are necessary for synergistic activation of the *fimU* promoter (Fig. 5) and both proteins are capable of independently binding upstream of the *fimU* coding sequence (Fig. 6 and 9). To determine whether both proteins simultaneously bind the *fimU* promoter and whether binding is cooperative, we assessed the kinetics of co-binding by EMSA. Initially, the P*fimU* probe was incubated with a constant, but non-saturating, concentration of cAMP-saturated Vfr and increasing concentrations of AlgR in the presence of PAM (Fig. 10A). As previously shown (Fig. 6), the addition of cAMP-saturated Vfr resulted in the expected P*fimU* shift product (Fig. 10A, lane 3). The further addition of AlgR in the presence of phosphodonor resulted in the formation of a second discrete concentration-dependent supershift product (Fig. 10A, lanes 4-9), indicating that both proteins simultaneously bound the P*fimU* probe. The apparent equilibrium constant for the AlgR-dependent supershift product was nearly identical to that of AlgR in the absence of Vfr (compare Fig. 9B and 10A), indicating that Vfr did not influence AlgR binding.

To determine the impact of AlgR binding on Vfr recruitment to the *fimU* promoter, we conducted a second EMSA study in which the P*fimU* probe was incubated with a non-saturating constant amount of AlgR (in the presence of PAM) and increasing concentrations of cAMP-saturated Vfr (Fig. 10B). AlgR formed the expected shift product (Fig. 10B, lane 3), while the addition of Vfr resulted in formation of a second discrete shift product. The apparent Keq for the second shift product was slightly lower than that of Vfr in the absence of AlgR, suggesting that AlgR binding may slightly increase the binding affinity of Vfr. Overall, these results demonstrate that both AlgR and Vfr can simultaneously bind the *fimU* promoter and binding of either protein appears to be largely independent of the other. However, recognition of the *fimU* promoter by AlgR and Vfr together has a positive synergistic effect on *fimU* transcription.

fimU operon complementation does not restore the Tfp defect of *vfr* mutants

To determine whether the Tfp defect of *algZR* and *vfr* mutants is attributed solely to the lack of *fimU* transcription, we complemented the *algZR* and *vfr* mutants with plasmid-based expression of the entire *fimU* operon (pPa-*fimU-pilE*). When expressed under conditions that restored Tfp production in a *fimU-pilE* mutant (Fig. 11A, lane 3), pPa-*fimU-pilE* was sufficient to restore Tfp production in the *algZR* mutant (Fig. 11A, lane 9) but not in the *vfr* mutant (Fig. 11A, lane 6). As a further control, we confirmed that plasmid-based expression of *vfr* (pPa-*vfr*) and *algZR* (pPa-*algZR*) were sufficient to restore Tfp production in the *vfr* and *algZR* mutants, respectively (Fig. 11A, lanes 5 and 8). Further, we showed that pPa-*fimU-pilE* complementation restored PilW expression to both the *vfr* and *algZR* mutants by Western blot of whole-cell lysates (Fig. 11B). Taken together, these results

suggest that the Tfp defect of *vfr* mutants involves additional defects other than reduced

transcription of the *fimU* operon, whereas the Tfp defect of *algZR* mutants can be solely

attributed to the role of the AlgZR TCS in regulating the *fimU* operon.

DISCUSSION

The fact that regulation of Tfp production and function is extremely complex and multi-factorial is perhaps not surprising given the number of components involved, their various functions and their genetic arrangement. There are distinct mechanisms governing the structural components of Tfp as well as the factors involved in processing and assembly; these components may be regulated at the level of transcription and/or function (e.g. fiber extension and retraction/chemosensing). While this study builds upon the work of multiple investigators (Whitchurch, 1991, 1996; Kanack, 2006; Belete, 2008), we present several novel results that contribute to the understanding of the regulatory scheme controlling Tfp. We identified two independent, but linked, regulatory systems involving the transcription factors AlgR and Vfr that control expression of the *fimU* operon. These findings suggest a mechanistic explanation for the Tfp biogenesis and twitching motility defect of *algZR* and *vfr* mutants in that the pilin-like proteins and PilY1 are not synthesized in the absence of *fimU* operon transcription. However, as shown in Figure 10, the Tfp defect in the *vfr* mutant is not solely attributed to *fimU* operon control as complementation of *fimU* does not restore Tfp expression. The possibility that Vfr controls additional factors necessary for Tfp biogenesis and expression is also supported by microarray analysis that identified several Tfp biogenesis-related genes with altered expression in a *vfr* mutant (Wolfgang, 2003). Several categories of Tfp biogenesis-related genes displayed altered expression levels in this study: i) Chp chemosensory system genes (*pilG, pilJ, pilK*) that regulates twitching motility and cAMP production, ii) essential pilus biogenesis machinery components (*pilNOPQ* and *pilZ, pilU*), iii) additional regulators of Tfp biogenesis and/or function (*fimV, pilS*), and iv) *pilD*, the prepilin peptidase. The altered expression of most of

these genes (except *pilS*) does not alter intracellular pilin levels, but instead controls Tfp production at the level of assembly and/or function and could contribute to the Tfp biogenesis and twitching motility defect of *vfr* mutants.

Vfr activates the *fimU* and *algZ* promoters

In this study we specifically focused on the mechanism of Vfr regulation of *fimU*. We demonstrate that cAMP/Vfr acts as both a positive regulator of the *fimU* and *algZ* promoters. Previous bioinformatics analysis predicted a VCS for the *algZ* promoter, but failed to predict a VCS upstream of the *fimU* promoter (Kanack, 2006). Further, Vfr was shown to bind a DNA probe containing the approximately 300 bp region upstream of the *algZ* promoter via EMSA (Kanack, 2006). Here, we identified an additional Vfr-binding site located further upstream of the *algZ* promoter and showed binding in EMSA. Furthermore, mutation of each site alone ($algZ_{M1}$ and $algZ_{M2}$) or in combination ($algZ_{M3}$) had a dramatic effect on P_{algZ}–*lacZ* reporter activity. This result is consistent with the microarray analysis of a *vfr* mutant, which provides evidence for Vfr-dependent regulation of *algZ* expression (Wolfgang, 2003). Follow-up studies will extend these *in vivo* results using *in vitro* EMSA to determine the contribution of the Vfr binding site mutations to Vfr affinity for the P_{algZ} EMSA probe. Additionally, DNase I footprinting studies will be necessary to confirm the precise location of the Vfr-binding sites within the *algZ* promoter region but are beyond the scope of this investigation.

Despite the fact that the *fimU* promoter lacked a predicted VCS, our study provides the following evidence for Vfr-dependent regulation of *fimU* transcription: i) P_{fimU}–*lacZ* reporter activity was significantly decreased in both *cyaAB* and *vfr* mutants, ii) Vfr shifted

the P*fimU* probe in a concentration- and cAMP-dependent manner *in vitro*, and iii) mutation to the Vfr half-sites identified by DNase I footprinting diminished P*fimU*–*lacZ* reporter activity and abolished binding of Vfr *in vitro*. In fact, the VBS (5'-AACG**TGAGC**TAT:GCA**GCACT**T) we identified within the *fimU* promoter region shares reasonable consensus with the previously identified VCS (5'-ANWW**TGNGA**WNY:AGW **TCACA**T). The upstream Vfr half-site (TGAGC) shares considerable conservation with the upstream VCS half-site (TGNGA). While the downstream *fimU* Vfr half-site (GCACT) deviates significantly from the downstream VCS half-site (TCACA). Given the results of previous studies that have characterized the Vfr binding-sites for several Vfr-dependent targets (Kanack, 2006; Fuchs, 2010a), it is clear that Vfr-binding sites display significant variability and thus, it is not surprising that several Vfr-dependent genes are directly regulated by Vfr activity that were not previously identified via bioinformatics.

Cooperative binding of Vfr and AlgR to the *fimU* promoter

Given the requirement of both transcriptional regulators Vfr and AlgR for activation of the *fimU* promoter, we attempted to determine whether the binding of either of these regulators required the presence of the other. Using co-binding EMSAs, we determined that AlgR and cAMP-saturated Vfr are capable of binding the P*fimU* probe simultaneously. While binding of either Vfr or AlgR to the *fimU* promoter was largely independent of the presence of the other, a noticeable synergism between AlgR and Vfr is responsible for activation of the *fimU* promoter.

AlgR phosphorylation is required for activation of the *fimU* promoter

Previous work by Belete *et al*. demonstrated that AlgR positively regulates the *fimU* operon and identified two AlgR binding sites within the *fimU* promoter region (Belete, 2008). We extended these findings by addressing the requirement for AlgR phosphorylation by analyzing the Tfp phenotype and P*fimU*–*lacZ* reporter activity of mutants in which either *algZ* or *algR* was inactivated. Both *algZ* and *algR* single mutants were defective for Tfp production and displayed P*fimU*–*lacZ* reporter activity equivalent to an *algZR* double mutant, suggesting that AlgZ activity is required for AlgR regulation of *fimU*. Although previous studies have demonstrated that AlgR can be phosphorylated *in vitro* using the *E. coli* CheA kinase as a phosphodonor (Deretic, 1992; Whitchurch, 2002), these studies did not directly address the role of AlgR phosphorylation in activation of the *fimU* operon. Our *fimU* promoter EMSA studies using phosphoamidate as a phosphodonor resulted in a significant reduction of the Keq value. Taken together, these results provide strong evidence that AlgR phosphorylation is necessary for regulation of the *fimU* operon. Future studies will address the biochemical characterization of AlgZ, as the functionality of this protein has long been speculated without experimental support. Due to the absence of conserved kinase motifs (D/F and G boxes required for ATP binding), it has been suggested that AlgZ does not function as a sensor kinase but it may instead function as a phosphatase (Yu, 1997). However, experimental results supporting either function have been limited to the characterization of *algZ* mutant phenotypes.

Based on the response regulators homologous to *P. aeruginosa* AlgR, it would be predicted that phosphorylation affects DNA binding (Nikolskaya, 2003). However, it is not clear if this is the case for AlgR since it appears to bind nearly identical recognition sequences. In our study, unphosphorylated AlgR bound the P$_{fimU}$ probe at high concentrations, and phosphorylation of AlgR dramatically reduced the amount of AlgR required to shift the P$_{fimU}$ probe. This result suggests that, regardless of phosphorylation status, AlgR recognizes the *fimU* promoter, but phosphorylation serves to alter DNA affinity. Interestingly, promoters known to be regulated by unphosphorylated AlgR are associated with chronic infection phenotypes *(algC, algD)*(Ma, 1998) whereas phosphorylated AlgR regulates genes more often associated with acute phenotypes (*fimU* operon and *hcnA*) (Belete, 2008; Cody, 2009). Perhaps phosphorylated AlgR contributes to gene regulation in coordination with an additional regulator, and phosphorylation affects this interaction. Thus, AlgR phosphorylation may serve as a signal responsible for the commitment to a particular virulence phenotype. The signal(s) that result in AlgR phosphorylation may be involved in this commitment, and if AlgZ is the cognate sensor kinase, expression of *algZ* may also serve as a method of regulating virulence phenotype.

Concluding Remarks

Collectively the results of our study demonstrate that the transcriptional regulator, AlgR activates the *fimU* operon in a phosphorylation-dependent manner, presumably via phosphorylation by the cognate putative sensor kinase AlgZ. Furthermore, we extend previous phenotypic observations implicating Vfr in Tfp expression by demonstrating that Vfr directly regulates the *fimU* operon in a cAMP-dependent manner. In addition, Vfr

indirectly influences *fimU* expression via control of *algZ* expression and we predict that this influences AlgR activity, although the contribution of Vfr-dependent *algZ* expression to *fimU* regulation was not directly tested.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria (*E. coli* and *P. aeruginosa*) were routinely grown at 37°C in LB medium. pMMB-based expression plasmids were maintained in *P. aeruginosa* with 150 μg ml^{-1} carbenicillin (Cb), except where indicated. Bacterial growth in liquid culture was assessed by optical density at 600 nm (OD$_{600}$).

The *algZ* and *algR* deletion allele plasmids (pEXGmΔ*algZ* and pEXGMΔ*algR)* were constructed by amplifying the upstream and downstream regions (~500 bp) flanking the target genes; the resulting PCR products were digested with restriction endonucleases and then ligated into pEX18GM. In all constructs, the mutant alleles were introduced onto the chromosomes of the appropriate *P. aeruginosa* strains as previously described (Wolfgang, 2003) and were confirmed by DNA sequencing of PCR products.

Construction of N-terminal His$_6$-tagged AlgR. A plasmid for overexpression of recombinant 6-Histidine tagged AlgR in *E. coli* was constructed as follows. AlgR was amplified from PAK genomic DNA using primers NdeAlgR_5'(TACAAAAAAGCAGGCTCATATGAATGTCCTGATTGTCGATGAC) and BamHI-AlgR_3' (TACAAGAAAGCTGGGTGGATCCTCAGAGCTGATGCATCAGACGCCTGAC). The resulting PCR fragment was gel extracted and purified using a kit (Qiagen). Both purified PCR fragment and pET28a vector were digested using NdeI and BamHI and subsequently ligated together. After confirmation via sequencing the resulting plasmid was transformed into *E. coli* BL21 (DE3) strains for protein overexpression.

β-galactosidase assays. Assays for β-galactosidase activity were performed as previously described (Fuchs, 2010b).

Isolation of surface Tfp. *P. aeruginosa* was grown on LB agar plates until confluent. Bacteria were collected and suspended in 1 ml 0.15 M NaCl/0.2% formaldehyde and vortexed vigorously for 1 min to release surface Tfp. Bacterial cells were removed by centrifugation at $12,000 \times g$ for 5 min. Supernatants were transferred to new tubes and adjusted to 0.1M $MgCl_2$ and incubated at 4°C for 12 h. Following centrifugation at 12,000 x *g* for 5 min, the resulting Tfp pellets were suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and were separated by SDS-PAGE (18% polyacrylamide) and pilin visualized by Coomassie Blue staining.

Immunoblotting. Whole-cell lysates were prepared from bacteria grown in LB broth to mid-exponential growth phase ($OD_{600} = 1$). Lysates were normalized based on total protein. Strains harboring plasmids were grown in the presence of 30 µg ml^{-1} Cb and the indicated amount of IPTG. Pilin samples were collected by centrifugation and suspended in 100 ml of SDS-PAGE sample buffer and incubated at 95°C for 10 min. The lysate was diluted 1:10 and whole cell lysates and Tfp fractions were separated on 18% (pilin) or 7.5% (PilY1) SDS-polyacrylamide gels and transferred to nitrocellulose or PVDF membranes, respectively. Membranes were probed with PKL1 anti-pilin mouse monoclonal antibody (Yu, 1994)(1:30 000 dilution, gift of Randall Irvin, University of Alberta), or anti-PilW rabbit serum (Giltner, 2010)(1:8,000 dilution). Horseradish peroxidase-conjugated secondary antibodies were used for the detection of specific antibody-antigen complexes. Blots were developed with chemiluminescence reagents (Millipore) and visualized via autoradiography.

Electrophoretic Mobility Shift Assays (EMSAs). DNA promoter probes were generated by PCR using the indicated oligonucleotides and end labeled using 10 μCi of [-^{32}]ATP (GE Healthcare) and 10 U of T4 polynucleotide kinase (New England Biolabs). EMSAs were performed as previously described (Brutinel, 2008). Briefly, probes (0.25 nM each) were incubated in binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 100 μg/ml bovine serum albumin) containing 5 μg/ml poly(2'-deoxyinosinic-2'-deoxycytidylic acid) [poly(di-dC); Sigma] for 5 min at 25°C. As noted in figure legends, cAMP-saturated Vfr, apo-Vfr, or His$_6$-AlgR protein was then added at concentrations (indicated in figure legends) for a final reaction volume of 20 μl and incubated for an additional 15 min at 25°C. Apo-Vfr and cAMP-saturated Vfr were generated as previously described (Fuchs, 2010a, b). Where indicated (in figure legends) 50mM PAM and 1mM MgCl$_2$ was incubated with His$_6$-AlgR. Samples were subjected to electrophoresis on a 5% polyacrylamide glycine gel (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging and data analyses were performed using an FLA-700 Phosphorimager (Fuji-film) and MultiGauge v3.0 software (Fujifilm). EMSAs were repeated a minimum of two times, and representative gels are shown.

AlgR Overexpression and Purification. An overnight culture of *E. coli* strain BL21 carrying the pET28a-AlgR overexpression construct was sub-cultured (1:100) into 1 L LB containing 25 μg ml^{-1} kanamycin and grown at 37°C to an OD$_{600}$ of ~ 0.8. The flask was then chilled to room temperature (RT) and induced to 0.5 mM IPTG and grown at RT overnight on a rotary shaker (~30 rpm). Cells were collected and washed twice via resuspension in 40 mL of binding buffer (50 mM NaH$_2$PO$_4$ Buffer, 300 mM NaCl, 20 mM Imidazole, pH 8.0). Cells were washed with binding buffer twice and resuspended in a final

volume of 20 mL binding buffer. 0.5 mg mL^{-1} lysozyme was added and cells were incubated on ice for 30 minutes. Cells were lysed via sonication and centrifuged at 10,000 x g for 30 minutes to remove cellular debris. The soluble fraction was then incubated with 3 ml of Ni^{2+} charged resin at 4°C for two hours with gentle rotation. The resin was loaded onto a 20 mL BioRad disposable column, washed twice with wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 40 mM Imidazole, pH 8.0), then eluted with elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 100 mM Imidazole, pH 8.0). Fractions containing significant amounts of 6x-His-AlgR, as determined by SDS-PAGE analysis, were concentrated using a Centriprep concentrator and subsequently loaded on a Sephadex 75 size elusion column. Fractions from a peak corresponding to AlgR (27 kD) were concentrated to ~ 3 mL, loaded into a Slid-a-Lyzer (Pierce), dialyzed twice against 2L of buffer (20 mM Tris, 500 mM NaCl , 1 mM DTT, .5% Tween 20, pH 8.0) then dialyzed into storage buffer (20 mM Tris,, 500 mM NaCl , 1 mM DTT, .5% Tween 20, 50% glycerol pH 8.0). Protein concentration was determined by Bradford assay.

FIGURE LEGENDS

Figure 1. *P. aeruginosa* **ACs and Vfr are required for Tfp production. A)** Visualization of Tfp (thin fibers) produced by wild-type (WT) and mutant strains of *P. aeruginosa* by TEM. Few Tfp fibers (indicated by arrows) were detected in association with the double AC mutant and *vfr* mutant. Tfp fibers were absent from the negative control mutant (*pilA*). **B)** Assessment of Tfp abundance on wild-type and mutant *P. aeruginosa* strains by IF microscopy. *P. aeruginosa* cells (green) were identified with Hoechst stain and Tfp (red) were detected with pilin-specific antiserum and Alexa Red-conjugated goat anti-rabbit IgG (Molecular Probes). Representative IF micrographs are shown at 100 x magnification.

Figure 2. Multiple regulators are required for Tfp production and expression of the *fimU* **Tfp biogenesis operon. A)** Coomassie Blue-stained SDS-polyacrylamide gel showing pilin from purified pilus fractions isolated from the wild-type (WT) and indicated mutant strains (surface). Immunoblot of whole-cell lysates from the above strains probed with pilin-specific monoclonal antibody (WC) or **B)** PilW-specific antiserum. **C)** Activity of the *fimU* promoter as measured by β-galactosidase assay in the indicated strains harboring a chromosomal P$_{fimU}$-*lacZ* transcriptional reporter. Values are the means ± standard error of the mean (SEM) ($n \geq 4$). When compared pairwise, values for the mutants were significantly different ($P < 0.0001$) than that of the wild-type strain.

Figure 3. cAMP/Vfr activates transcription of *algZ***.** Activity of the *algZ* promoter as measured by β-galactosidase assay in the indicated strains harboring a chromosomal P$_{algZ}$-*lacZ* transcriptional reporter. The wild-type (WT) or indicated mutant strains containing

vector, pPa-*cyaB,* or pPa-*vfr* expression plasmids. Strains containing expression vectors were grown in LB broth containing 30 µg ml^{-1} carbenicillin (Cb) and 50 µM IPTG. Values are the means ± standard error of the mean (SEM) ($n \geq 3$). Values for the *cyaAB* and *vfr* mutants containing empty vector were significantly different than that of WT ($P \leq 0.0001$).

Figure 4. Binding of cAMP-saturated Vfr activates transcription of *algZ in vitro*. A) Diagram of the *algZ* promoter region. Numbering (in bp) is relative to the *algZ* translational start site. Also depicted is the divergently transcribed gene, *argH*. Translational start sites are indicated in bold type and marked with an arrow depicting the direction of translation. Partial coding sequences of *algZ* and *argH* are highlighted in grey. The proposed Vfr-binding sites VBS1 (centered between -177 and -166 bp) and VBS2 (centered between - 65 and -76 bp) are indicated in bold and underlined. The locations of the oligonucleotides (*algZ* probe 5' and *algZ* probe 3') used to generate the P$_{algZ}$ reporter fragment and P$_{algZ}$ EMSA probes are indicated. **B)** Specific and non-specific probe (0.25 nM were incubated in the absence (lane 1) or presence (lanes 2-8) of various concentrations of cAMP-Vfr (9-271 nM) for 15 minutes followed by electrophoresis and phosphorimaging. Mobility of the P$_{algZ}$ probe, which encompasses both putative Vfr binding sites VBS1 and VBS2, was retarded in a Vfr concentration-dependent manner, indicating the formation of specific protein-DNA complexes. Bands representing shifting of the non-specific probe (~ 200 bp) are indicated by an asterisk (*). Arrows indicate the two cAMP-Vfr dependent shift complexes, Shift 1 and Shift 2.

C) Site-directed mutagenesis of two putative Vfr binding sites (VBS1 and VBS2) within the *algZ* promoter region. The sequence of each VBS in the wild-type *algZ* promoter is highlighted in grey. The base changes used to create three mutated versions of the *algZ* promoter (*algZ*$_{M1}$, *algZ*$_{M2}$, and *algZ*$_{M3}$) are indicated in bold type. *algZ*$_{M1}$ contains mutations in VBS1, *algZ*$_{M2}$ contains mutations in VBS2, and *algZ*$_{M3}$ contains mutations in both VBS1 and VBS2. **D)** Promoter activity in strains containing either the wild-type *algZ* promoter reporter (P$_{algZ}$) or one of the three mutated *algZ* promoter reporters (P$_{algZ\,M1}$, P$_{algZ\,M2}$, or P$_{algZ\,M3}$), as measured by β-galactosidase assay. Reporter constructs were either in a wild-type (WT) or *vfr* mutant strain background. Values are the means ± standard error of the mean (SEM) ($n \geq 3$). Values for all of the mutants were significantly different ($P < 0.0006$) when compared pairwise to that of the wild-type strain.

Figure 5. Both AlgR and cAMP/Vfr are required for *fimU* transcription and Tfp production. A) The wild-type (WT) or indicated mutant strains containing either vector, pPa-*vfr*, or pPa-*algZR* expression plasmids were grown as previously described. Values are the means ± standard error of the mean (SEM) ($n \geq 3$). The value for the *algZR*/*vfr* mutant containing any expression plasmid was statistically different than that of the WT strain ($P \leq 0.0001$). **B)** Immunoblot of whole-cell lysates probed with PilW-specific antiserum. **C)** Coomassie Blue-stained SDS-PAGE gel showing pilin from purified pilus fractions isolated from the WT and indicated mutant strains.

Figure 6. cAMP/Vfr directly regulates the *fimU* operon. A) Diagram of the *fimU* promoter region. Numbering (in bp) is relative to the translational start site of *fimU*, which

is indicated in bold type and marked with an arrow depicting the direction of translation. The partial coding sequence of *fimU* is highlighted in grey. The locations of the oligonucleotides (*fimU* probe 5' and *fimU* probe 3') used to generate the P$_{fimU}$ reporter fragment and P$_{fimU}$ EMSA probes are indicated. The boxed region represents the sequence protected by cAMP-Vfr in DNase I footprinting assays. The identified VBS (centered at -130 and -119 bp) is indicated in bold and underlined. The previously identified AlgR binding sites (ABS1 and ABS2) are indicated. **B)** Specific (P$_{fimU\ WT}$) and non-specific (Non-Sp) probes (0.25 nM) were incubated in the absence (lane 1) or presence (lanes 2-8) of various concentrations of cAMP-Vfr (9-271 nM) for 15 minutes followed by electrophoresis and phosphorimaging. Mobility of the P$_{fimU\ WT}$ probe was retarded in a Vfr concentration-dependent manner, indicating the formation of specific protein-DNA complexes. The non-specific probe shift is indicated by an asterisk (*). **C)** Apo-Vfr (68 nM) incubated with specific (P$_{fimU\ WT}$) and non-specific (Non-Sp) probes (0.25 nM) in the absence (lane 2) or presence (lanes 3-9) of increasing concentrations of cAMP for 15 minutes followed by electrophoresis and phosphorimaging. Mobility of the P$_{fimU\ WT}$ probe was retarded in a cAMP-concentration dependent manner, indicating that cAMP is required for formation of a Vfr-DNA complex.

Figure 7. Vfr protected region of the *fimU* operon. DNase I footprinting of the *fimU* promoter region. Samples contained a DNA fragment (0.4 nM) corresponding to (~ -300 bp) relative to the translational start site. The top strand of the DNA probe was radiolabeled on one end and was incubated in the absence or presence of cAMP-Vfr (114, 13, or 1 nM) prior to DNase I treatment. DNase I-generated fragments were separated by electrophoresis

and Maxim-Gilbert sequencing ladders were made using the same DNA. The region protected by Vfr binding is boxed, and the predicted half-sites are indicated by lines to the right of the sequence.

Figure 8. Mutation of the putative Vfr binding site influences *fimU* transcription.

A) Site-directed mutagenesis of the putative Vfr binding half-sites within the *fimU* promoter region. The sequence the VBS in the wild-type *fimU* promoter is highlighted in grey. The base changes used to create two mutated versions of the *fimU* promoter (*fimU*$_{M1}$ and *fimU*$_{M2}$) are indicated in bold type. P$_{fimU\,M1}$ contains mutations to make the sequence more closely resemble the VCS reported by Fuchs et *al*. (Fuchs, 2010a). P$_{fimU\,M2}$ contains mutations predicted to disrupt Vfr binding. **B)** Promoter activity in strains containing either the wild-type *fimU* promoter reporter (P$_{fimU}$) or one of the two mutated *fimU* promoter reporters (P$_{fimUM1}$ or P$_{fimUM2}$), as measured by β-galactosidase assay. Reporter constructs were either in a wild-type (WT) or *vfr* mutant strain background. Values are the means ± standard error of the mean (SEM) ($n \geq 3$). Values for all of the mutants were significantly different ($P \leq 0.0002$) when compared pairwise to that of the wild-type strain.

Figure 9. AlgR requires phosphorylation for *fimU* promoter recognition. Specific (P$_{fimU}$ $_{WT}$) and non-specific (Non-Sp) probes (0.25 nM) were incubated in the absence (lane 1) or presence (lanes 2-8) of various concentrations of AlgR (11-271 nM) for 15 minutes followed by electrophoresis and phosphorimaging. Reactions were performed in the absence **A)** or presence **B)** of the small molecule phosphodonor PAM (50 mM). Mobility of the P$_{fimU\,WT}$ probe was retarded in an AlgR concentration-dependent manner, indicating the

formation of specific protein-DNA complexes. The non-specific probe shift is indicated by an asterisk (*).

Figure 10. AlgR and Vfr bind to the P*fimU* WT probe. A) P*fimU* WT probe incubated in the absence (lane 1) or presence (lanes 2-9) of AlgR and Vfr. Lanes 2 and 3 show the concentrations of AlgR and Vfr, respectively resulting in ~90% shifting of the P*fimU* WT probe. Lanes 4-9 depict incubation of the P*fimU* WT probe and Vfr (68 nM) with increasing concentrations of AlgR (11-351 nM). **B)** P*fimU* WT probe incubated as in panel A, except AlgR concentration was held constant (176 nM) while cAMP-saturated Vfr was added at increasing concentrations (2.25-68 nM). Shifted complexes consisting of *fimU* with each of the individual proteins (Vfr shift and AlgR shift) or both proteins (AlgR/Vfr shift) are indicated.

Figure 11. The *fimU* operon is not sufficient to restore the Tfp defect of a *vfr* mutant. A) Tfp production as determined by Coomassie Blue-stained SDS-PAGE gels of purified pilus fractions from WT and indicated mutant strains. **B)** Immunoblots from whole-cell lysates of WT and indicated mutant strains probed with PilW-specific antiserum.

Supplemental Figure 1. Restoration of P*fimU* –*lacZ* reporter activity in mutants.
The wild-type (WT) or indicated mutant strains containing either vector, pPa-*cyaB*, pPa-*vfr*, pPa-*algZ*, pPa-*algR* or pPa-*algZR* expression plasmids were grown in LB broth containing 30 μg ml^{-1} carbenicillin (Cb) and 50 μM IPTG. Values are the means ± standard error of the mean (SEM) ($n \geq 3$).

Table I. Strains and plasmids used in this work

Strain or plasmid	Description or relevant characteristic(s)[a]	Reference or source
P. aeruginosa strains		
PAK	_P. aeruginosa_ strain K, wild type	(Takeya, 1966)
PAK::P_fimU_-_lacZ_	PAK with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilA_::P_fimU_-_lacZ_	_pilA_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_fimU-pilE_::P_fimU_-_lacZ_	_fimU-pilVWXY1Y2E_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_cyaAB_::P_fimU_-_lacZ_	_cyaAB_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_vfr_::P_fimU_-_lacZ_	_vfr_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_algZ_::P_fimU_-_lacZ_	_algZ_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_algR_::P_fimU_-_lacZ_	_algR_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_algZR_::P_fimU_-_lacZ_	_algZR_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_algZR_Δ_vfr_::P_fimU_-_lacZ_	_algZRvfr_ with chromosomal _fimU_ promoter reporter	This study
PAK::P_fimU$_{M1}$_-_lacZ_	PAK with chromosomal _fimU$_{M1}$_ promoter reporter	This study
PAK::P_fimU$_{M2}$_-_lacZ_	PAK with chromosomal _fimU$_{M2}$_ promoter reporter	This study
PAKΔ_vfr_::P_fimU$_{M1}$_-_lacZ_	_vfr_ with chromosomal _fimU$_{M1}$_ promoter reporter	This study
PAKΔ_vfr_::P_fimU$_{M2}$_-_lacZ_	_vfr_ with chromosomal _fimU$_{M2}$_ promoter reporter	This study
PAK::P_algZ_-_lacZ_	PAK with chromosomal _algZ_ promoter reporter	This study
PAKΔ_cyaAB_::P_algZ_-_lacZ_	_cyaAB_ with chromosomal _algZ_ promoter reporter	This study
PAKΔ_vfr_::P_algZ_-_lacZ_	_vfr_ with chromosomal _algZ_ promoter reporter	This study
PAK::P_algZ$_{M1}$_-_lacZ_	PAK with chromosomal _algZ$_{M1}$_ promoter reporter	This study
PAK::P_algZ$_{M2}$_-_lacZ_	PAK with chromosomal _algZ$_{M2}$_ promoter reporter	This study
PAK::P_algZ$_{M3}$_-_lacZ_	PAK with chromosomal _algZ$_{M3}$_ promoter reporter	This study
PAKΔ_vfr_::P_algZ$_{M1}$_-_lacZ_	_vfr_ with chromosomal _algZ$_{M1}$_ promoter reporter	This study
PAKΔ_vfr_::P_algZ$_{M2}$_-_lacZ_	_vfr_ with chromosomal _algZ$_{M2}$_ promoter reporter	This study
PAKΔ_vfr_::P_algZ$_{M3}$_-_lacZ_	_vfr_ with chromosomal _algZ$_{M3}$_ promoter reporter	This study
Plasmids		
pDONR201	Gateway cloning vector, Km^r	Life Technologies
mini-CTX-_lacZ_	Plasmid for chromosomal integration at the ϕCTX phage site of transcriptional fusions; Tc^r	(Hoang, 1998)
mini-CTX-P_fimU_-_lacZ_	_fimU_ promoter region (bp to bp) in EcoRI and BamHI sites of mini-CTX-_lacZ_; Tc^r	This Study
mini-CTX-P_algZ_-_lacZ_	_algZ_ promoter region (bp to bp) in EcoRI and BamHI sites of mini-CTX-_lacZ_; Tc^r	This Study
pEX18Gm	Suicide vector, Gm^r	(Hoang, 1998)
pEXGmΔ_fimU-pilE_	pEX18Gm containing _fimU-pilE_ deletion allele; Gm^r	(Heiniger, 2010)
pEXGmΔ_pilA_	pEX18Gm containing _pilA_ deletion allele; Gm^r	(Fulcher, 2010)
pEXGmΔ_cyaA_	pEX18Gm containing _cyaA_ deletion allele; Gm^r	(Wolfgang, 2003)
pEXGmΔ_cyaB_	pEX18Gm containing _cyaB_ deletion allele; Gm^r	(Wolfgang, 2003)
pEXGmΔ_vfr_	pEX18Gm containing _vfr_ deletion allele; Gm^r	(Wolfgang, 2003)
pEXGmΔ_algZ_	pEX18Gm containing _algZ_ deletion allele; Gm^r	This Study
pEXGmΔ_algR_	pEX18Gm containing _algR_ deletion allele; Gm^r	This Study
pEXGmΔ_algZR_	pEX18Gm containing _algZR_ deletion allele; Gm^r	(Jones, 2010)
pMMB67EH	Empty _P. aeruginosa_ expression vector; Ap^r	(Furste, 1986)
pMMBV1GW	Gateway-adapted version of pMMB67EH; Ap^r	(Fulcher, 2010)
pPa-_cyaB_	_P. aeruginosa cyaB_ carried on pMMBV2; Ap^r	(Fulcher, 2010)
pPa-_vfr_	_P. aeruginosa vfr_ carried on pMMBV1; Ap^r	(Fuchs, 2010)
pPa-_algZ_	_P. aeruginosa algZ_ carried on pMMBV1; Ap^r	This study
pPa-_algR_	_P. aeruginosa algR_ carried on pMMBV3; Ap^r	This study
pPa-_algZR_	_P. aeruginosa algZR_ carried on pMMBV1; Ap^r	(Jones, 2010)
pPa-_fimU-pilE_	_P. aeruginosa fimU-pilE_ carried on pMMBV1; Ap^r	(Heiniger, 2010)

[a] Ap^r, ampicillin resistance marker; Km^r, kanamycin resistance marker; Gm^r, gentamicin resistance marker; Tc^r, tetracycline resistance marker
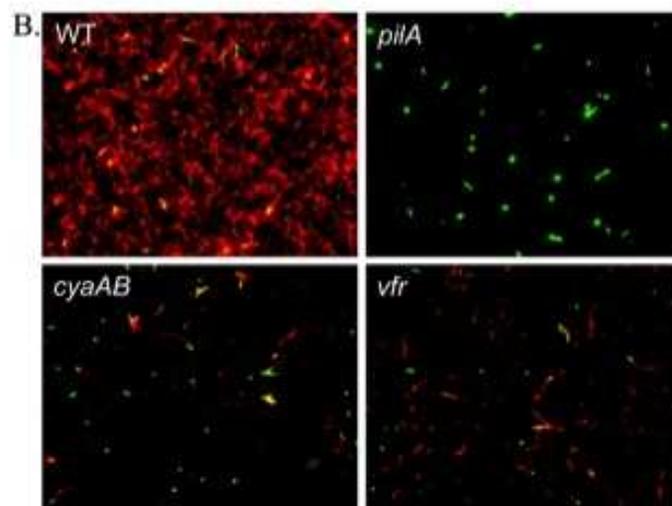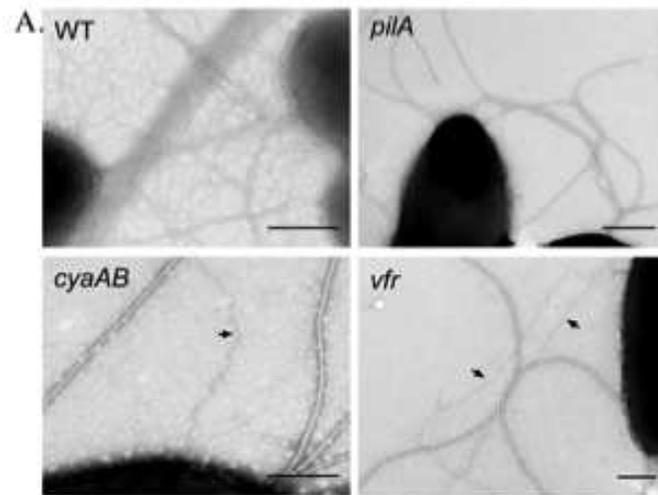
Figure 1.

Figure 2.

A. Surface .... Pilin

WC .... Pilin

B. WC .... PilW

wt pilA cyaAB vfr algZ algR algZR fimU-pilE

C.

β-galactosidase activity
(Miller Units)

WT pilA cyaAB vfr algZ algR algZ/R

Figure 3.

P$_{algZ}$-*lacZ*

	WT	cyaAB		vfr	
vector	+	+	-	+	-
pPa-cyaB	-	-	+	-	-
pPa-vfr	-	-	-	-	+

Figure 4.

A.

CAGGACTGGTTGGTCTTCTCTACGCT**CAT**GGTATCTCTCGCTGCAAC **-218**

algZ probe 5'

AAGAAACGGACAGGCCGCGCGGCCTGCGTGACGGGCAAAG**TGCG** **-174**

ACGATGA**TAGCA**GGCCGGGAGGAAAAACCGGCGGCTCGTCGACGA **-129**

VBS1

AGCCGGAACGAGCGCTGTTTCGCGGTTT**TTTGC**GGCAGA**TGGCA**TT **-82**

VBS2

TCGGTGCTTTATTTCGCCCGTACGCCCGCGGCTGTCTTGCGGGCGC **-26**

algZ

CACGGGCCTTGCGGAAACTCTGTCG**ATG**CCTATCCGATTCAAGCCC **21**

GGCCGCTCGACCCGGCCGGCCGTCCAAGACGACTTCTTCATTCCC **66**

GAGCTCTGCGAAGCCGAAGCCCTGTTCACCCTGGTGCTGCT **107**

algZ probe 3'

Figure 4.

B.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

Shift 2
Shift 1
*
*
P$_{algZ\ WT}$
Non-Sp

| Vfr (nM) | - | 271 | 9 → 271 |

126

Figure 4.

C.

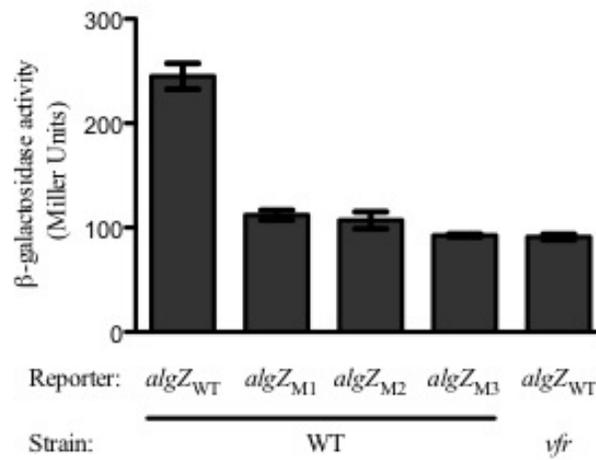| Promoter	VBS1	VBS2
WT	TGCGA:TAGCA	TTTGC:TGGCA
*algZ*_{M1}	**GG**CAA:TTACT	**WT**
*algZ*_{M2}	**WT**	**GA**TTT:TGACA
*algZ*_{M3}	**GG**CAA:TTACT	**GA**TTT:TGACA

D.

Figure 5.
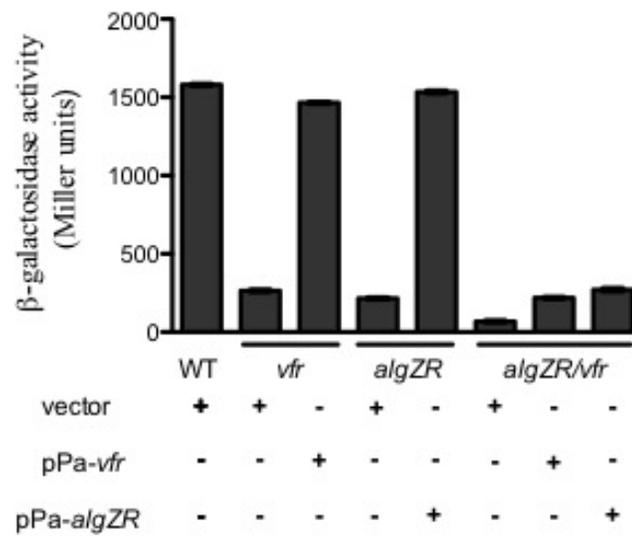
A.

B. PilW   WC

C. Pilin   Surface

Pilin   WC

Figure 6A.

GAATTCAGAGGGCTGGCTGCTTGCGGAAGACGGTGGGGAACGGCTTCTCGTG **-306**

fimU probe 3'

GGACGCGCGCTTGCAAAGAAGGAAAGGCTCCTCTGGAACCGTTCGAACCGCCT **-253**

CAGGTTCCTCCCCAGCGGCCAGCTCAACATTCACAACGGGCGGTTTCTGCTCT **-200**

GCGGAAGGCATACCGTTGCCTGGCAACTGATCCTCAACCGGCAGGGCCGGCTC **-166**

CGTACCGCGAGCGCCAGGGAGAACCGGGAGAAACG**TGAGC**TATGCA**GCACT**T **-114**

CCGGATGATCATGGACCAGTTGTCCTGC**CCCTCGGGCC**GACCGTCAACGACCG **-61**
ABS1

CAAGACGCTCCCAGGC**CCGTTTGGC**ATGCTTTCCAGGCGTAGACCCCCTGGAG **-8**
ABS2

CAACCGC**ATG**TCATATCGTTCCAACTCGACCGGCTTCACCCTGATCGAGTTGCT **47**

GATCATCGTCGTCCTGCTGGCCATCATGGCGAGCTTCGCCATTCCGAACTTCAA **101**

GCAGCTGACAGAACGCAACGAACTGCAGAGCGCCGCCGAGGAACTCAATGCG **153**
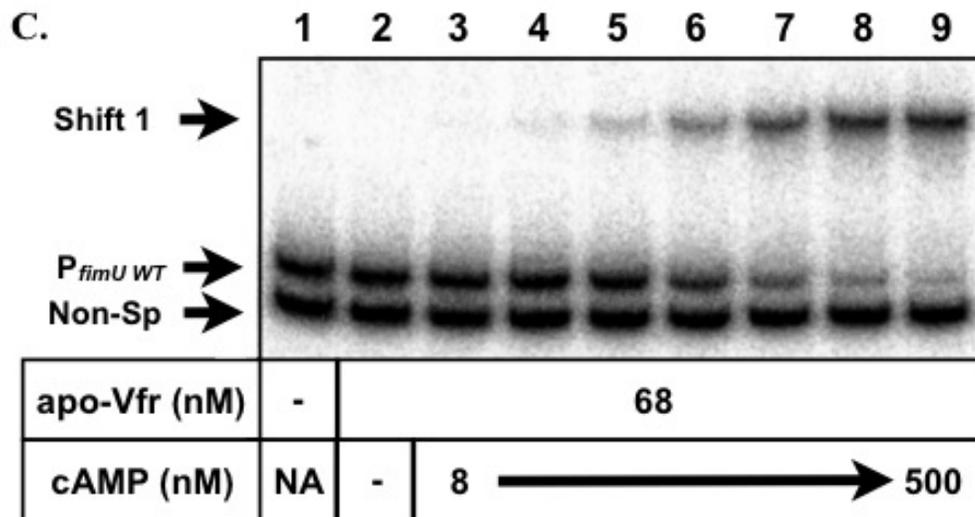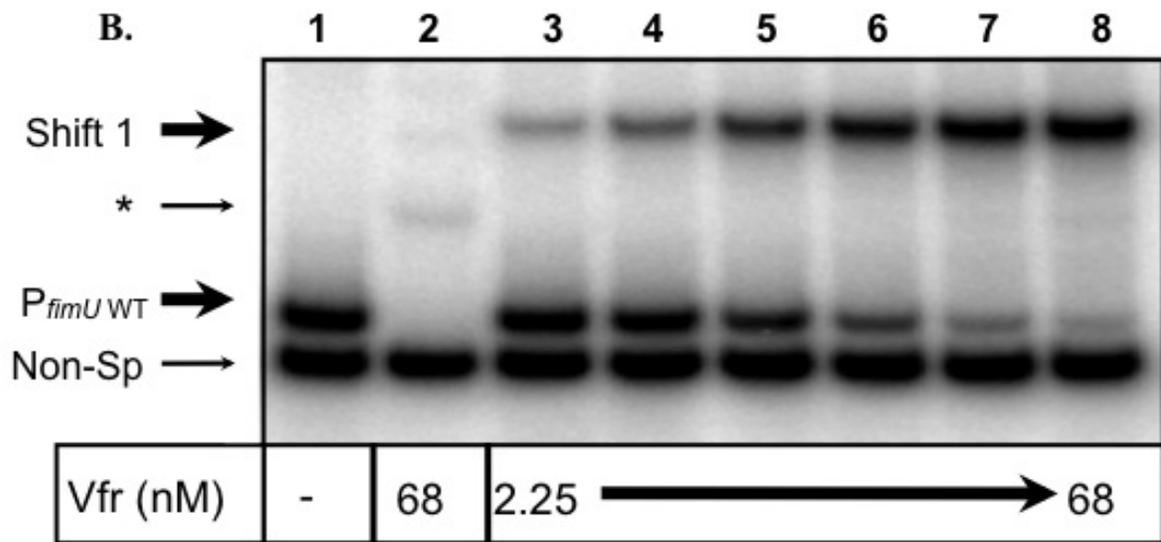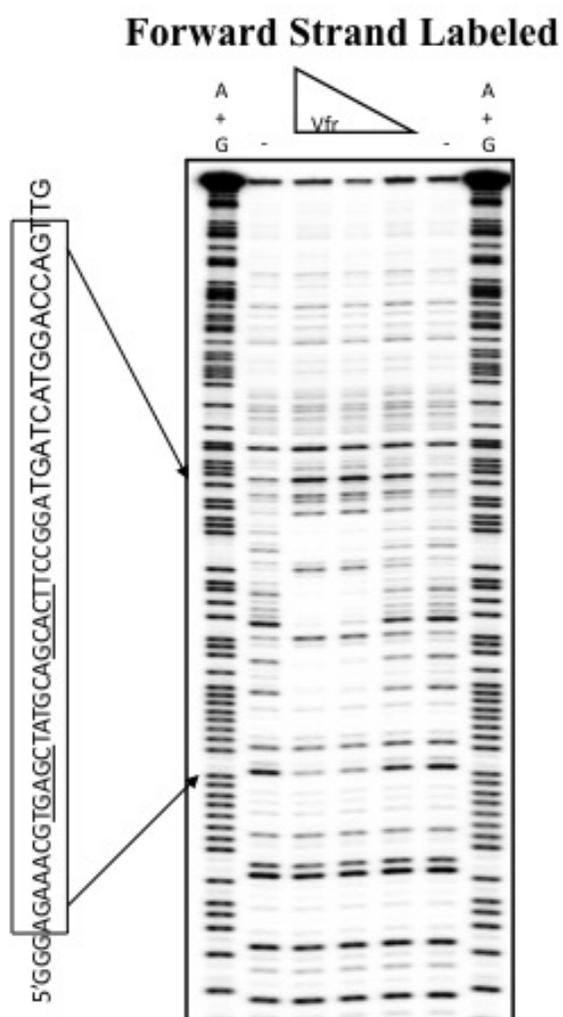
fimU probe 3'

Figure 6.

B.

| Vfr (nM) | - | 68 | 2.25 ——————————→ 68 |

C.

| apo-Vfr (nM) | - | 68 | | | | | | | |
| cAMP (nM) | NA | - | 8 ——————————→ 500 | | | | | | |

Figure 7.

Forward Strand Labeled

5'GGGAGAAACGTGAGCTATGCAGCACTTCCGGATGATCATGGACCAGTTG

Figure 8.

A.

Promoter	*fimU* VBS
WT	TGAGC:GCACT
fimU$_{M1}$	TGAG**A**:**T**CACT
fimU$_{M2}$	**T**CAGC:G**G**ACT

B.

Figure 8.

Figure 9.

A.

− PAM

B.

+ PAM

Figure 10.

A.

AlgR (nM)	-	351	-	11 → 351					
Vfr (nM)	-	-	68	68					

B.

AlgR (nM)	-	-	176	176					
Vfr (nM)	-	68	-	2.25 → 68					

Figure 11.

A.

Surface
Pilin

	WT	fimU-pilE		vfr			algZR		
vector	+	+	-	+	-	-	-	+	-
pPa-*vfr*	-	-	-	-	+	-	-	-	-
pPa-*algZR*	-	-	-	-	-	-	-	+	-
pPa-*fimU-pilE*	-	-	+	-	-	+	-	-	+

B.

PilW

WC

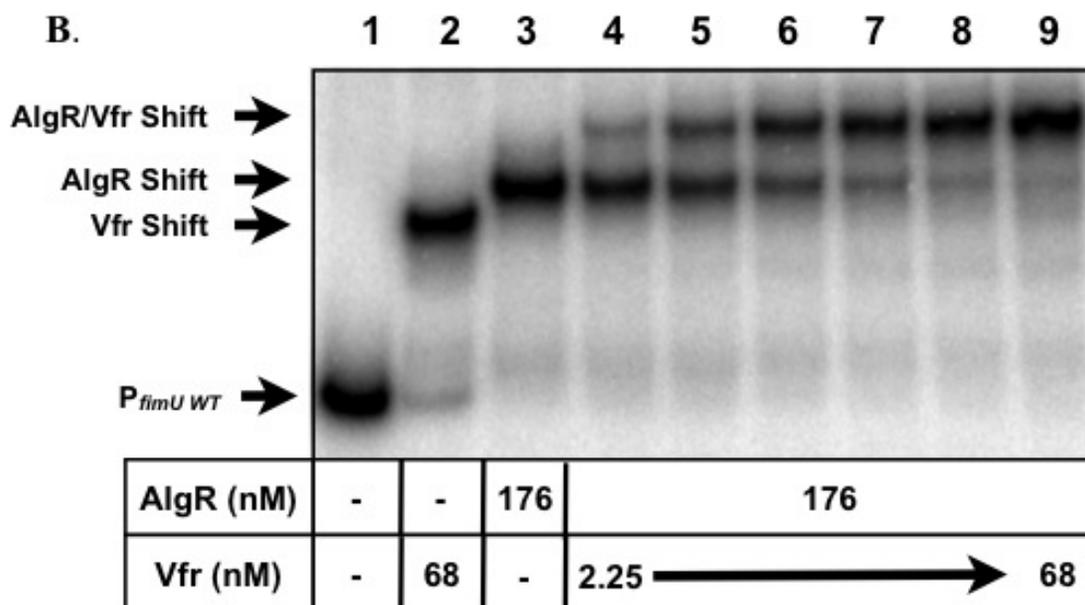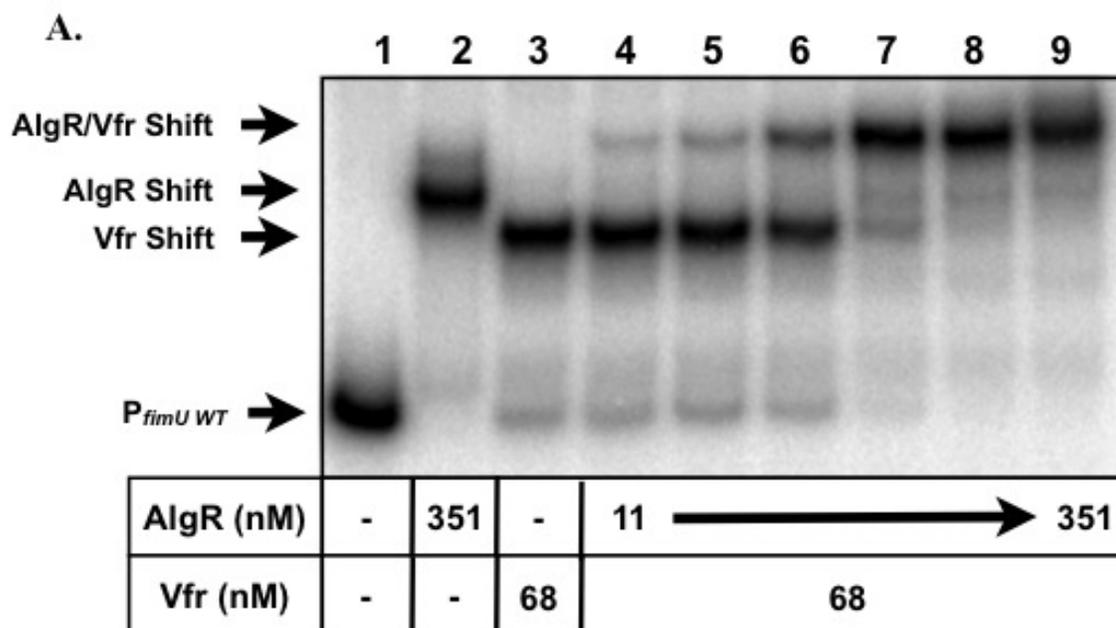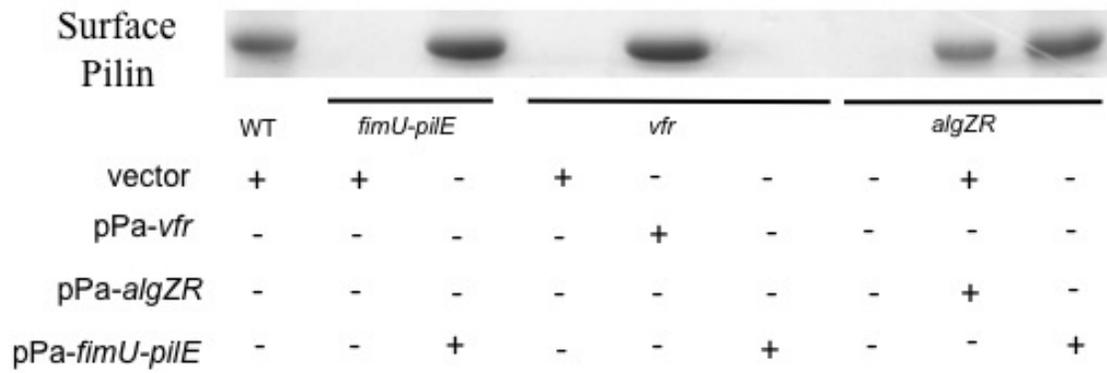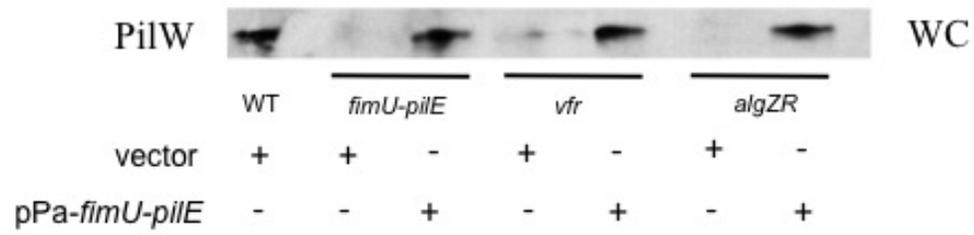|	WT	fimU-pilE		vfr		algZR	
vector	+	+	-	+	-	+	-
pPa-*fimU-pilE*	-	-	+	-	+	-	+

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CHAPTER 3:

Crystal Structure Analysis Reveals *Pseudomonas* PilY1 as an Essential Calcium-Dependent Regulator of Bacterial Surface Motility[3][•]

SUMMARY

Several bacterial pathogens require the "twitching" motility produced by filamentous type IV pili (T4P) to establish and maintain human infections. Two cytoplasmic ATPases function as an oscillatory motor that powers twitching motility via cycles of pilus extension and retraction. The regulation of this motor, however, has remained a mystery. We present the 2.1 Å resolution crystal structure of the *Pseudomonas aeruginosa* pilus-biogenesis factor PilY1, and identify a single site on this protein required for bacterial translocation. The structure reveals a modified β-propeller fold and a distinct EF-hand calcium-binding site conserved in pathogens with retractile T4P. We show that preventing calcium binding by PilY1 using either an exogenous calcium chelator or

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mutation of a single residue disrupts *Pseudomonas* twitching motility by eliminating surface pili. In contrast, placing a lysine in this site to mimic the charge of a bound calcium interferes with motility in the opposite manner – by producing an abundance of non-functional surface pili. Our data indicate that calcium binding and release by the unique PilY1 EF-hand loop identified in the crystal structure controls the opposing forces of pilus extension and retraction. Thus, PilY1 is an essential, calcium-dependent regulator of bacterial twitching motility.

INTRODUCTION

The pathogenesis of bacterial infections typically depends on microbial adherence to host tissue, aggregation of the pathogen at the site of infection, and subsequent dissemination to other anatomical sites within the host. Type IV pili (T4P) are thin surface filaments involved in biofilm formation, bacterial aggregation and microbial adherence to biotic and abiotic surfaces. T4P biogenesis requires more than a dozen proteins conserved across a range of bacterial species (Craig and Li, 2008). A subset of bacteria, including the opportunistic human pathogen *Pseudomonas aeruginosa,* cyclically extend and retract their T4P, facilitating a form of surface movement termed twitching motility (Merz et al., 2000; Skerker and Berg, 2001; Wolfgang et al., 1998a). Retractile T4P are required for *P. aeruginosa* adherence to host tissue, virulence and dissemination of infection (Comolli et al., 1999; Mattick, 2002a; Tang et al., 1995).

The pilin subunits that form T4P are incapable of self-assembly. Instead, pilus extension and retraction requires repeated rounds of pilin polymerization and depolymerization, both of which demand ATP hydrolysis (Chiang et al., 2008; Jakovljevic et al., 2008). In *P. aeruginosa*, the cytoplasmic ATPase PilB drives the assembly of pilin monomers into mature T4P (Chiang et al., 2008; Turner et al., 1993), while the structurally related cytoplasmic ATPase PilT disassembles T4P back into pilin monomers (Chiang et al., 2008; Morand et al., 2004; Whitchurch et al., 1991). Loss-of-function mutations in PilT result in the loss of twitching motility due to the inability of formed pilus fibers to retract (Whitchurch et al., 1991). The C-terminal half of the 117-kDa *P. aeruginosa* PilY1 protein shares sequence homology with the C-terminal domain (CTD) of the PilC proteins of pathogenic *Neisseria* species; in contrast, the N-terminal regions of the PilCs and PilY1 are

divergent in sequence (Alm et al., 1996). While the PilC proteins from *Neisseria* have been implicated in antagonizing pilus retraction by the pilus biogenesis ATPase PilT, the N-terminal domains of these proteins appear critical for host cell attachment (Rudel et al., 1995; Wolfgang et al., 1998b). Thus, we hypothesized that the conserved CTDs of PilY1 and PilC may play a common role in T4P biogenesis rather than adhesion, and pursued a structural and functional analysis of the CTD from *Pseudomonas aeruginosa* PilY1.

RESULTS

PilY1 Exhibits a Modified β-Propeller Fold

Examination of the primary sequence of PilY1 from *P. aeruginosa* strain PAK revealed conservation between its C-terminal 550 residues and the same regions of the PilC proteins from *Neisseria*, and a predicted structural homology to the eight-bladed β-propeller fold exhibited by a range of proteins (Kelley and Sternberg, 2009; Subramaniam, 1998). Thus, we overexpressed, purified and crystallized the PilY1 CTD (amino acids 614-1163) for examination by x-ray diffraction. Native diffraction data were collected to 2.1 Å resolution (Table I); however, attempts to determine the structure using existing β-propeller search models or by isomorphous replacement methods were not successful. PilY1 CTD contains only six methionine residues, which upon selenomethionine substitution failed to produce an adequate signal to support structure determination by anomalous dispersion (Hendrickson et al., 1990). To overcome this problem, a series of single leucine-to-methionine (L-M) substitutions were placed in the PilY1 CTD to provide additional sites for single wavelength anomalous dispersion (SAD) phasing (Supplemental Figures 2.1- 2.2). After several attempts, three L-M substitutions (at positions 712, 812, 823) were successfully combined, crystallized, and generated clear selenomethionine fluorescence signal upon examination in the x-ray beam. The structure was determined by SAD phasing using crystals containing nine total selenomethionine residues per protein monomer.

The structured 505 residues of the PilY1 CTD exhibit a seven-bladed modified β-propeller fold composed of 31 β-strands and 9 α-helices (Figure 2.1A; Supplemental

Figures 2.1- 2.2; residues at the termini, as well as 713-721 and 1061-1068 were disordered and are not in the refined model). Blades I through IV are 4-stranded antiparallel β-sheets and align well with blades of a canonical β-propeller enzyme, quinohemoprotein alcohol dehydrogenase from *Comamonas testosteroni* (RCSB 1KB0; overall RMSD of 3.6 Å over 290 equivalent Cα positions with 15% sequence identity; Figure 2.1B) (Kelley and Sternberg, 2009). This β-propeller enzyme was identified by Phyre to align with the PilY1 predicted structure (E-value 0.014, 95% certainty) (Kelley and Sternberg, 2009). Blades V through VII of the PilY1 CTD, however, deviate from the standard β-propeller fold and align poorly with established β-propellers. Blade V contains a fifth strand (β20) that is shared with blade VI. Blade VI is composed of six strands, including β20, and maintains an insert containing a short helix and β-turn-β motif (α9, β26-27). Finally, blade VII is truncated and composed of three relatively short strands.

WD40 β-propeller proteins mediate protein-protein interactions via surface contacts at the propeller center (Smith et al., 1999). Superposition of the PilY1 CTD structure onto that of the seven-bladed β-propeller WDR5, complexed with a lysine 4-containing histone H3 peptide, revealed an RMSD of 3.9 Å over 261 equivalent Cα positions sharing only 12% sequence identity (Holm et al., 2008). Blades I-IV of the PilY1 CTD align well with the first four blades of WDR5, while the last three blades of each protein deviate more significantly in structure (Supplemental Figure 2.3). However, helices 4 and 7 of PilY1 appear to block the surface site used by WDR5 to interact with peptide (Smith et al., 1999). Both helices are predicted to be conserved in the C-terminal domains of the related *Neisseria* PilC proteins (Supplemental Figure 2.2) (Subramaniam, 1998). These

observations suggest that PilY1 and related pilus biogenesis factors do not mediate protein-protein interactions using the canonical WD40 surface-binding site.

PilY1 Contains a Unique Calcium Binding Loop

A strong $|F_o|-|F_c|$ electron density peak in the PilY1 CTD structure was interpreted as a calcium atom based on the observed coordination by three aspartic acid side chains (851, 855, 859), one asparagine side chain (853), one main-chain carbonyl oxygen of valine 857, and one water (Figure 2.2A; see also Supplemental Figure 2.1). The calcium binding observed in PilY1 is nearly identical to that seen in the canonical EF-hand Ca-binding motifs (Figure 2B; Supplemental Figure 2.4) (Chattopadhyaya et al., 1992). PilY1 and EF hands both employ seven contacts to the bound calcium ion, including one from a water molecule and one bidentate contact via an acidic side chain. PilY1 is unique, however, in that it achieves this calcium chelation using a stretch of only nine amino acids (851-859) in a loop between two β-strands (β 13 and β 14) rather than the twelve residues between two α-helices typically observed in the EF-hands (Chattopadhyaya et al., 1992). In addition, the carboxylic acid group of the bidentate Asp-859 residue is rotated nearly 90° with respect to the equivalent residue in EF-hand proteins (Chattopadhyaya et al., 1992). Asp-859 forms a hydrogen bond with His-797, which is conserved in PilY1 proteins of known sequence. The calcium-chelating residues observed in the structure are conserved in the PilY1s of known sequence and in the related PilCs, with the exception that Asn-853 is replaced with an aspartic acid in the *Neisseria* proteins (Figure 2.2C; Supplemental Figure 2.2). This modified EF-hand motif is also present in PilY1 orthologues found in other bacteria that utilize T4P, including the human pathogen *Kingella kingae*. Thus, a novel calcium-binding

site appears to be conserved in the C-terminal domains of pilus biogenesis proteins related to PilY1.

Measurement of calcium binding affinity using a fluorescence competition assay established that purified PilY1 CTD exhibits a K_d of 2.6 μM for calcium (Figure 2.2D), which is similar to the affinities reported for traditional EF-hand proteins (Weber et al., 1994). Titration with increasing concentrations of magnesium chloride reveals that this cation does not bind to PilY1. Mutation of the calcium chelating residue Asp-859 to alanine eliminates specific calcium binding by the protein (Figure 2.2D). Removal of calcium either by Chelex-100 or by mutating a chelating residue did not change the overall structure (as measured by circular dichroism spectropolarimetry; Supplemental Figure 2.6) or melting temperature (Supplemental Figure 2.7) of the PilY1 CTD (Suzuki et al., 1985). Additional mutations D851A, N853, and D855A were made to PilY1, all of which eliminated calcium binding and did not affect the overall secondary structure (Figure Ad 2.1A-C, 2.2A-C). Thus, calcium binding appears not to play a role in the overall structure of the PilY1 C-terminal domain.

Calcium Binding and Release by PilY1 are Essential for Functional Pili

Expression of mutant versions of PilY1 in *P. aeruginosa* revealed that calcium binding and release are essential for pilus production and twitching motility. To examine the phenotypic consequences of altering the PilY1 calcium-binding site on T4P production and function, we engineered plasmid-encoded versions of full-length wild-type PilY1 and PilY1 carrying the D859A substitution. Both proteins were expressed to native levels in a *P. aeruginosa* strain in which the chromosomal *pilY1* gene was deleted (Supplemental Figure

2.8). The PilY1^{minus} strain is devoid of surface T4P, lacks measurable twitching motility, and is phenotypically indistinguishable from a non-piliated PilA^{minus} strain, which does not produce the major T4P subunit, pilin (Figures 2.3A-B). Complementation of the PilY1^{minus} strain with plasmid-expressed PilY1 restored twitching motility and pilus production to wild type levels (Figures 2.3A-B). In contrast, complementation of the PilY1^{minus} strain with full-length PilY1 D859A resulted in significantly less twitching motility ($p<0.001$) (Figure 2.3A). The level of surface T4P produced by the PilY1 D859A expressing strain was also dramatically reduced compared to wild type and likely accounts for the observed defect in twitching motility (Figure 2.3B).

PilY1 was previously shown to traffic to the surface T4P fraction (Alm et al., 1996) (Supplemental Figure 2.8), where it presumably functions through a direct association with the pilus fiber. To determine whether the defect in twitching observed with the D859A form of PilY1 was caused by disrupted pilus assembly/extension or increased pilus retraction, the PilY1 D859A mutant was expressed in a mutant strain lacking the PilT-retraction ATPase (PilT^{minus}, PilY1^{minus} strain). These bacteria regained the ability to produce normal levels of T4P, and the mutant PilY1 protein was capable of trafficking to the sheared pilus fraction (Figures 2.3C-F). This result indicates that the loss of surface pili observed with the PilY1 D859A mutant is caused by PilT-mediated pilus retraction. In the presence of PilT, the D859A form of PilY1 is incapable of antagonizing PilT-mediated pilus retraction despite normal trafficking of the protein. We conclude that the calcium-binding site in the PilY1 CTD is necessary for normal pilus function by antagonizing the retractile activity of PilT.

We then created a mutation in full-length PilY1 designed to mimic calcium binding and to potentially render the protein calcium-insensitive. Specifically, a lysine residue was

150

substituted for the bidentate calcium chelating Asp-859 residue (D859K). While a lysine

residue is not equivalent to a divalent metal ion, modeling of the D859K substitution

mutation indicates that the α-amino group of lysine would be expected to form a salt bridge

with the two proximal aspartic acids at 851 and 855 (Supplemental Figure 9). Purified

D859K PilY1 CTD does not bind calcium, and is not distinct in structure or melting

temperature from the wild type CTD (Supplemental Figures 2.5- 2.7). When expressed to

native levels in the *P. aeruginosa* PilY1^{minus} strain (Supplemental Figure 2.8), the full-

length PilY1 D859K mutant was significantly reduced for bacterial twitching motility

($p<0.001$) compared to the same strain expressing wild type PilY1 (Figure 2.3A).

Interestingly, the level of surface pilus production in the PilY1 D859K expressing strain

was equivalent to that of the wild type strain (Figure 2.3B), indicating that reduced fiber

production does not account for the twitching motility defect. PilY1 D859K was capable of

trafficking to the surface T4P fraction when expressed in both a *pilY1* mutant background

(Supplemental Figure 2.8) and a PilT^{minus}, PilY1^{minus} strain (Figures 2.3C-F). Thus, using

the crystal structure as a guide, we produced a single-site mutation that dramatically alters

pilus function without affecting pilus production. Based on these data, we conclude that the

release of calcium by PilY1 is required for PilT-dependent fiber retraction and twitching

motility.

Finally, we tested whether the calcium chelator EGTA impacts *P. aeruginosa* pilus

production. We found that EGTA reduces the levels of surface pili present on bacteria

expressing wild-type PilY1 while the non-retractile PilT^{minus} strain is blind to calcium

chelation (Figure 2.4A). Similarly, the D859K form of PilY1 is insensitive to the effects of

EGTA and supports the production of wild-type levels of surface pili (Figure 2.4A). These

data show that the PilY1 D859K mutant, mimicking the calcium bound state of PilY1, has increased capacity to antagonize PilT-mediated pilus retraction. Taken together, these cell-based studies support the conclusion that both calcium binding and calcium release by the unique site in PilY1 are essential for the proper regulation of pilus retraction dynamics and twitching motility.

DISCUSSION

Based on the structural and functional data presented here, we propose that the PilY1 C-terminal domain exists in two critical states: calcium-bound, which inhibits PilT-mediated pilus retraction, and calcium-free, which does not inhibit PilT and allows pilus retraction to proceed. We further propose that the interconversion between these two states in wild-type PilY1 is required for the cycles of pilus extension and retraction necessary for twitching motility (Figure 2.4B, *top*). The D859A mutant form of PilY1 appears to mimic the calcium-free state of the protein, and is not capable of antagonizing PilT-mediated retraction; thus, the equilibrium is shifted toward retraction, similar to that seen with the PilY1^{minus} strain (Figure 2.4B). Calcium chelation by EGTA also shifts the equilibrium toward retraction. In contrast, the D859K variant mimics the calcium-bound state, shifting the equilibrium toward pilus extension and producing an effect similar to that seen with the PilT^{minus} strain (Figure 2.4B). The relatively moderate 2 μM calcium binding affinity exhibited by PilY1 is in the range where cycles of calcium binding and release would be expected to be physiologically relevant. Thus, we conclude that PilY1 acts as a calcium-dependent regulator of the opposing forces responsible for T4P-mediated twitching motility. However, we stress that this is a functional hypothesis; the physical contacts between PilY1 and other factors necessary to achieve this regulation have yet to be elucidated. It is interesting to note that calcium binding by bacterial pseudopilins has recently been shown to be critical for functional type II secretion, a process believed to be analogous to T4P biogenesis and function (Korotkov et al., 2009). Thus, calcium may play an important role in several aspects of pilus biogenesis and regulation.

Twitching motility mediated by PilY1's influence on the PilB/PilT oscillatory motor would be expected to be coordinated with the significant >100 pN retractile motion exerted by the system once target cell adhesion occurs (Maier et al., 2004; Maier et al., 2002). Surface translocation by twitching motility likely involves relatively moderate affinity contacts between a surface and T4P (Maier et al., 2004). For example, a disulfide loop in pilin, the major pilin subunit of the pilus fiber, has been proposed to be a point of contact between surfaces and T4P (Farinha et al., 1994). We propose that the cycles of pilus extension and retraction actuated by PilY1's C-terminal domain may control twitching motility involving these moderate affinity contacts. Once target host cell contact has been achieved, however, the T4P system would be expected to convert to a rapidly retracting, PilT-dominated state in which nearly nN forces are exerted (Sokurenko et al., 2008; Thomas et al., 2006). The function of this mode would be to quickly draw the bacterial cell into close contact with host tissue cells. It is tempting to speculate that PilY1 may serve as the switch between twitching and rapid retraction. The sequence divergent N-terminal domains of PilY1 orthologues are proposed to perform target cell-specific adhesion; once that is achieved, perhaps the conserved C-terminal domains switch to the calcium-free state to facilitate rapid PilT-mediated retraction (Kallstrom et al., 1997; Kirchner et al., 2005; Rudel et al., 1995).

The C-terminal domain of *P. aeruginosa* PilY1 shares significant sequence identity with the equivalent regions of the *Neisseria* PilC's (Figure 2.2C), and similar levels of identity in PilY1 homologues from other human, plant, and marine infectious bacteria (Subramaniam, 1998). We find that each PilY1 homologue contains a predicted calcium-binding site highly similar in sequence to that observed in the PilY1 C-terminal domain (see

Supplemental Figure 2.2). Thus, calcium binding and release may play an equally important

role in pilus biogenesis and motility in organisms that maintain a PilY1 homologue. Taken

together, the structural and functional data we outline provide new insights into the

complex functional regulation of the T4P essential to a range of human pathogenic bacteria.

METHODS

Structure Determination. PilY1 residues 615-1163 were cloned from genomic DNA from the PAK strain of *P. aeruginosa* (GenBank #EU234515), and overexpressed recombinantly in *E. coli*. The protein was purified using Ni-affinity and size-exclusion chromatography steps to >95% purity, and crystallized in 0.3 M sodium malonate (pH 6.5), 20% (w/v) PEG 3350, 50 mM DTT, 30 mM ammonium phosphate, and 4-8% trifluoroethanol. Residues 614-1163 of PilY1 contained only five methionine residues; as such, crystals containing selenomethionine-substituted protein failed to produce sufficient signal to allow structure determination. L712M, L813M and L823M mutations were successfully generated, combined, expressed, purified and crystallized in PilY1 specimen sufficient for x-ray data collected. Diffraction data to 2.8 Å resolution were collected at the peak wavelength (0.979 Å) for selenium single-wavelength anomalous dispersion (SAD) phasing (Table 1), and indexed and scaled in space group C2 using HKL3000 (Minor et al., 2006; Otwinowski and Minor, 1997). Automated model building correctly built 30% of the final structure; complete building and refinement was accomplished using COOT and CNS, respectively (Brunger et al., 1998; Cowtan, 2004). Once the selenomethionine-substituted structure was refined at 2.8 Å resolution, it was used in molecular replacement to solve the 2.1 Å native structure, which was refined using COOT and CNS to the final statistics shown in Table 1 (Brunger et al., 1998; Cowtan, 2004).

Calcium Binding and Biophysical Studies: Calcium was removed from purified PilY1 using Chelex-100 (BioRad) prior to biophysical studies (Suzuki et al., 1985). A binding curve for Oregon Green® 488 BAPTA-5N was measured on a PHERAstar (BMGLabtech) at 488 nm

and the K_d established at 10.1 μM. WT, D851A, N853A, D855A, D859A, and D859K

PilY1 CTD was serial diluted 2 fold to calculate PilY1 CTD K_d for calcium binding, if

applicable. For biophysical characterizations via circular dichroism spectropolarimetry, a

wavelength scan from 195-260 was performed at 22 ^{o}C. Thermal denaturation protein

samples were measured 210 nm at a range of 10 ^{o}C to 85 ^{o}C.

Isolation of Surface T4P: *P. aeruginosa* strains were spread on LB agar plates and grown

until confluent. Bacteria were collected and suspended in 1 ml 0.15M NaCl/0.2%

formaldehyde and vortexed vigorously for 1 minute to release surface T4P. Bacterial cells

were removed by centrifugation at 12,000 x *g* for 5 minutes. T4P fractions were separated

by SDS-PAGE (18% polyacrylamide) and pilin visualized by GelCode Blue stain (Pierce).

Twitching Motility: Bacteria were stab inoculated to the bottom of 100 mm tissue culture-

treated plates containing 5 ml LB agar (1% w/v). Plates were incubated for 40 hours at

37°C in a humidified incubator. Twitching motility was quantified by measuring the

diameter of the subsurface zone of bacterial spread.

Western Blots: Whole cell lysates and T4P fractions were separated on 18% (pilin) or 7.5%

(PilY1) SDS-polyacrylamide gels and transferred to nitrocellulose or PVDF membranes,

respectively. Membranes were probed with PKL1 anti-pilin monoclonal antibody or anti-

PilY1 CTD rabbit serum. Horseradish peroxidase-conjugated secondary antibodies were

used for the detection of specific antibody-antigen complexes. Blots were developed with

chemiluminescence reagents and visualized via autoradiography.

CREDITS

FIGURE LEGENDS

Figure 2.1 Crystal structure of the *P. aeruginosa* PilY1 CTD. A. 2.1 Å resolution crystal structure of the *P. aeruginosa* PilY1 C-terminal domain, which begins at residue 644 and ends at 1148 and encompasses seven β-blades (I-VII; colored red, orange, yellow, green, blue, magenta and violet). **B.** Superposition of the seven-bladed PilY1 CTD β-propeller (red through violet) on the eight-bladed β-propeller of the quinohemoprotein alcohol dehydrogenase from *Comamonas testosteroni* (1KB0; grey).

Figure 2.2 PilY1 calcium-binding site. A. The PilY1 calcium binding site is composed of aspartic acids 851, 855 and 859, as well as asparagine 853, the main chain carbonyl oxygen of valine 857, and a water molecule (red sphere). Distances are in Å. **B.** Superposition of the nine-residue calcium-binding site of PilY1 (green) on the canonical twelve-residue site in human calmodulin (CaM; cyan). The calcium atoms (Ca) and water molecules (W) are depicted as spheres. Viewed in roughly the same orientation as 2A. **C.** Sequence conservation in the calcium binding sites of the PilY1 homologues PilC1 and PilC2 from *Neisseria meningitidis* (*N.men.*) and *N. gonorrhoeae* (*N.gon.*). **D.** The PilY1 CTD binds to calcium with a Kd of 2.6 μM (black), while the Asp-859-Ala mutant form of the CTD exhibits only non-specific calcium binding (grey). Error represents standard error of the mean.

Figure 2.3 The PilY1 calcium-binding site is essential for T4P production and function.
A. Twitching motility of *P. aeruginosa* (wt), or a *pilY1* mutant expressing either empty vector (-), PilY1, or mutant versions of PilY1 (D859A or D859K). The twitching motility zones produced by bacteria expressing mutant PilY1 were significantly (*, $p < 0.001$) reduced compared to those of bacteria expressing wild type PilY1. **B.** Coomassie Blue-

stained gel of pilin isolated from the bacterial surface. **C-F**. Abolishing T4P retraction

allows conditional localization of PilY1 to the bacterial surface. **C.** Coomassie-stained gel

of pilin isolated from the bacterial surface. **D**. Western blot of T4P-containing surface

fractions probed with PilY1- specific antiserum. **E.** Western blot of whole cell lysates

probed with a pilin-specific antibody. **F.** Western blot of whole cell lysates probed with a

PilY1-specific antiserum.

Figure 2.4 Calcium chelation impacts T4P production. A. Pilus preparations from a *pilT*

mutant or a *pilY1* mutant expressing either PilY1, or mutant versions of PilY1 grown in the

presence (+) or absence (-) of the calcium chelator EGTA**. B.** Summary of the impact

specific mutants have on pilus state. Wild-type PilY1 appears to balance extension and

retraction to produce twitching motility (boxed), while gene deletions, PilY1 site mutants,

and the calcium chelator EGTA all significantly disrupt the equilibrium between pilus

extension and retraction.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 2.1 Topology diagram of the *P. aeruginosa* PilY1 CTD fold showing the positions of secondary structural elements (strands as arrows, helices as cylinders), disordered regions (dotted lines), the calcium binding site (green sphere), and the locations of the seven leucine-to-methionine mutations examined during structure determination. Colored as shown in Figure 1A.

Supplemental Figure 2.2 Sequence alignment of the PilY1 CTDs from three *Pseudomonas* strains (PAK, the subject of this study; PA01, PA14) along with the PilC1's from two *Neisseria* strains, *N. meningitidis* (NEIME) and *N. gonorrhoeae* (NEIGO). Secondary structure elements, disordered regions, the calcium binding site, and the leucine-to-methionine mutations are indicated as in Supplemental Figure 1.

Supplemental Figure 2.3 A. Seven-bladed β-propeller structure of WDR5 (purple) complexed with an H3 peptide containing lysine-4 (yellow). **B.** Seven-bladed β -propeller structure of the PilY1 CTD (cyan). **C.** Superposition of the β -propeller structures showing that the site employed by WDR5 for peptide binding is blocked by β helices 4 and 7 in PilY1.

Supplemental Figure 2.4 Comparison of the ligand-to-calcium atom distances in PilY1 and human calmodulin (CaM), as well as the sequences of the calcium binding sites in the two proteins.

Supplemental Figure 2.5 Calcium binding curve for purified *P. aeruginosa* PilY1 D859K C-terminal domain.

Supplemental Figure 2.6 Circular dichroism spectropolarimetry wavelength scans for

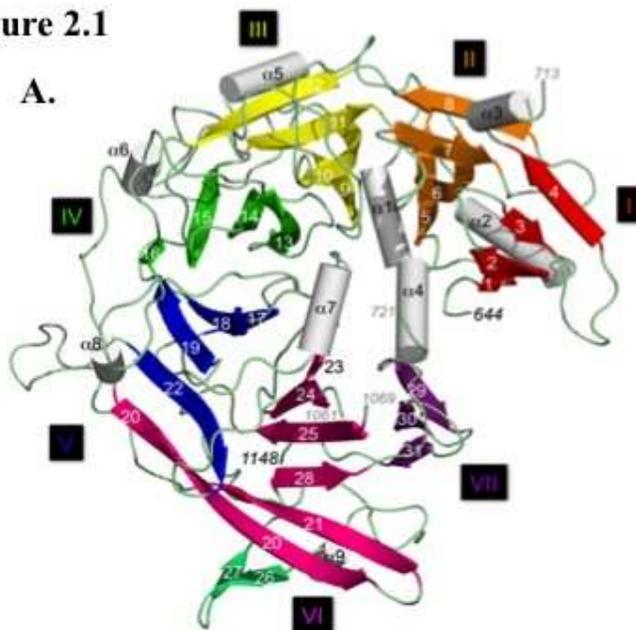purified *P. aeruginosa* PilY1 C-terminal domain proteins in the presence and absence of calcium.

Supplemental Figure 2.7 Melting temperature for purified *P. aeruginosa* PilY1 C-terminal domain proteins in different conditions as monitored by circular dichroism spectropolarimetry.

Supplemental figure 2.8 A-D. Modulation of calcium binding influences T4P production without altering pilin availability. **A.** Coomassie-stained gel of pilin isolated from the bacterial surface. **B**. Western blot of T4P-containing surface fractions probed with PilY1-specific antiserum. **C.** Western blot of whole cell lysates probed with a pilin-specific antibody. **D.** Western blot of whole cell lysates probed with PilY1-specific antiserum.

Supplemental Figure 2.9 Model of the Asp-859-Lys (D859K) mutation in the PilY1 C-terminal domain calcium binding site.
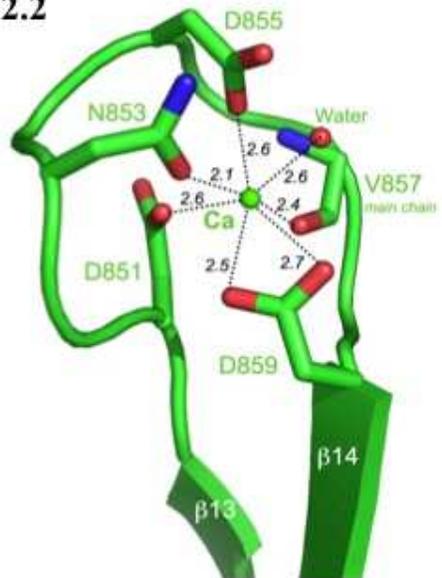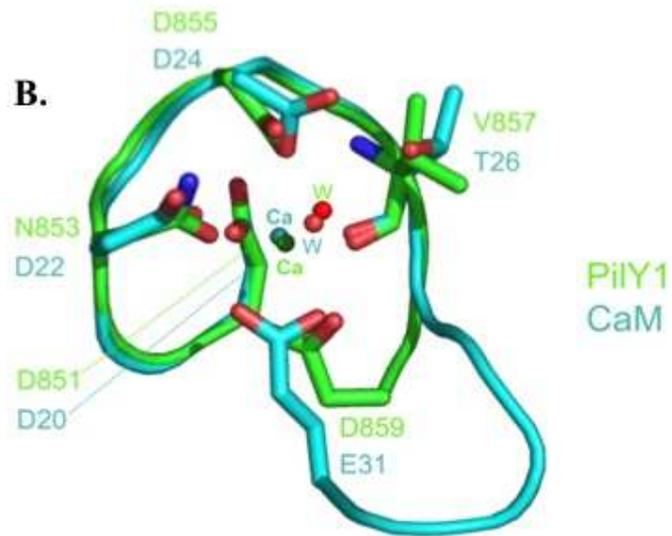
Figure 2.1

Figure 2.2

A.

D855

N853

Water

2.6

2.1

2.6

2.6

Ca

2.4

V857
main chain

D851

2.5

2.7

D859

β14

β13

B.

D855
D24

V857
T26

N853
D22

W

Ca

W

Ca

D851
D20

PilY1
CaM

D859
E31

Figure 2.2

C.

```
P.aer. PilY1    851-DNNSDGVAD-859
N.men. PilC1    728-DKDLDGTVD-736
N.men. PilC2    738-DKDLDGTVD-746
N.gon. PilC1    736-DKDLDGTAD-744
N.gon. PilC2    741-DKDLDGTVD-749
```
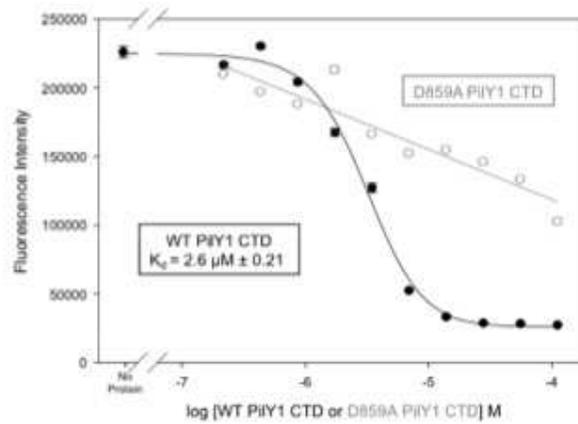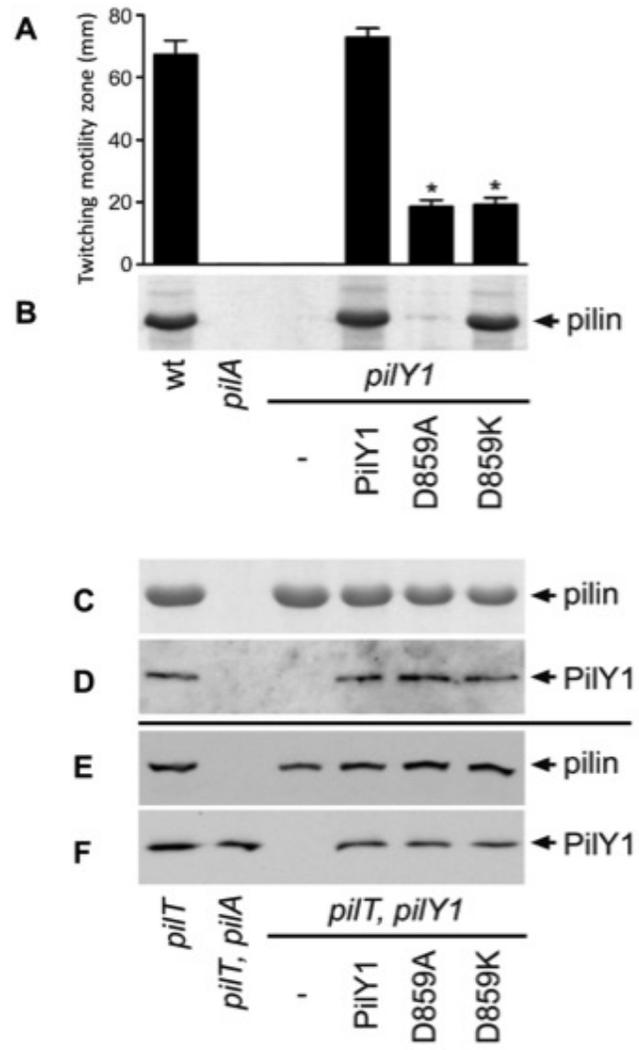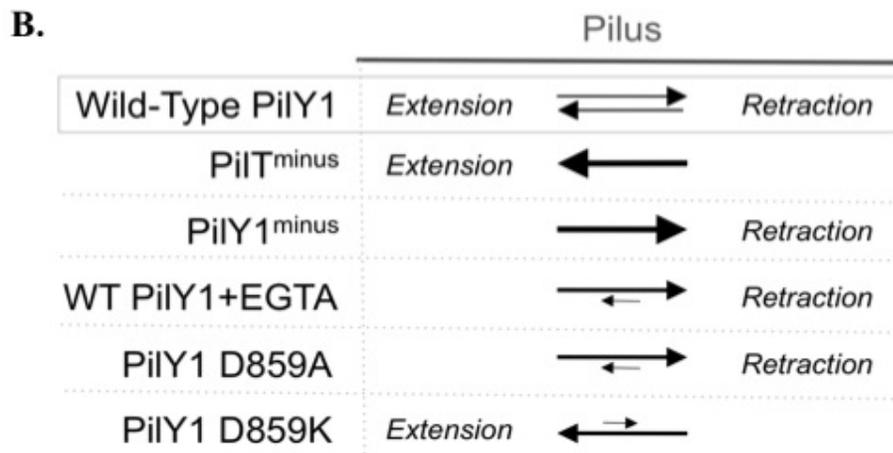
D.

Figure 2.3

Figure 2.4

A.

pilin ←

pilT   *pilY1*

PilY1   D859A   D859K

- +   -   +   -   +   -   +   EGTA

B.

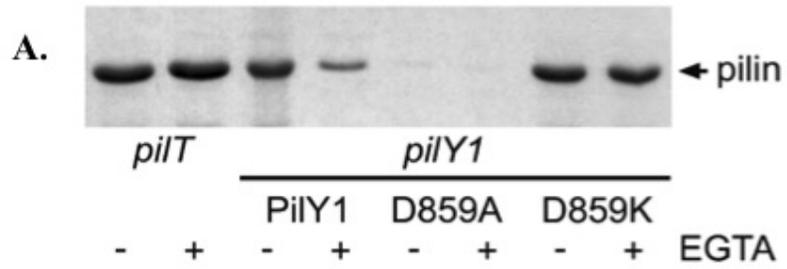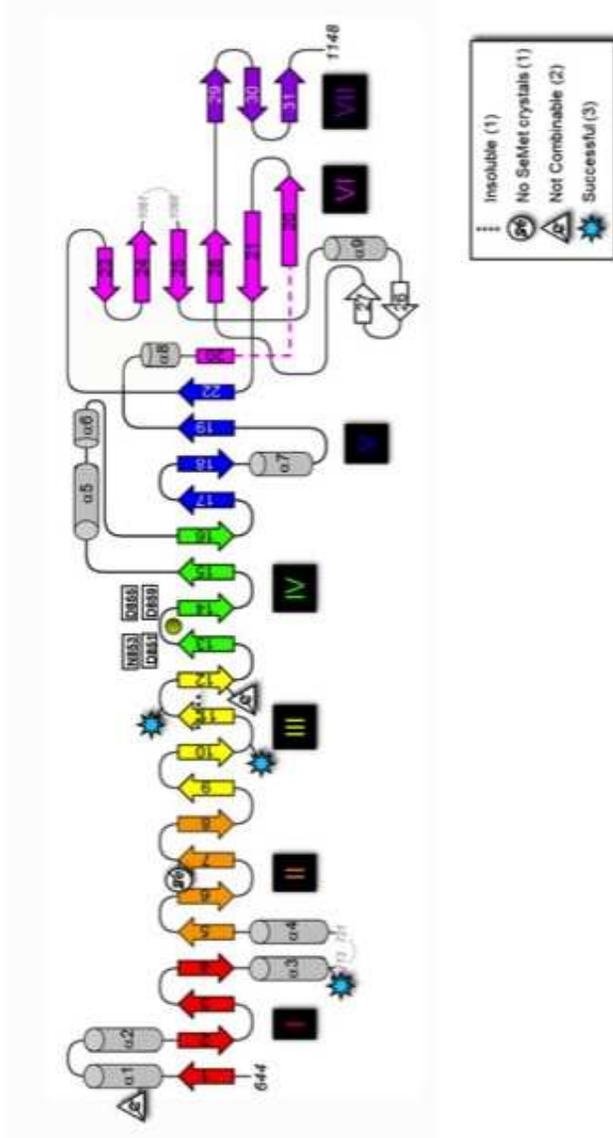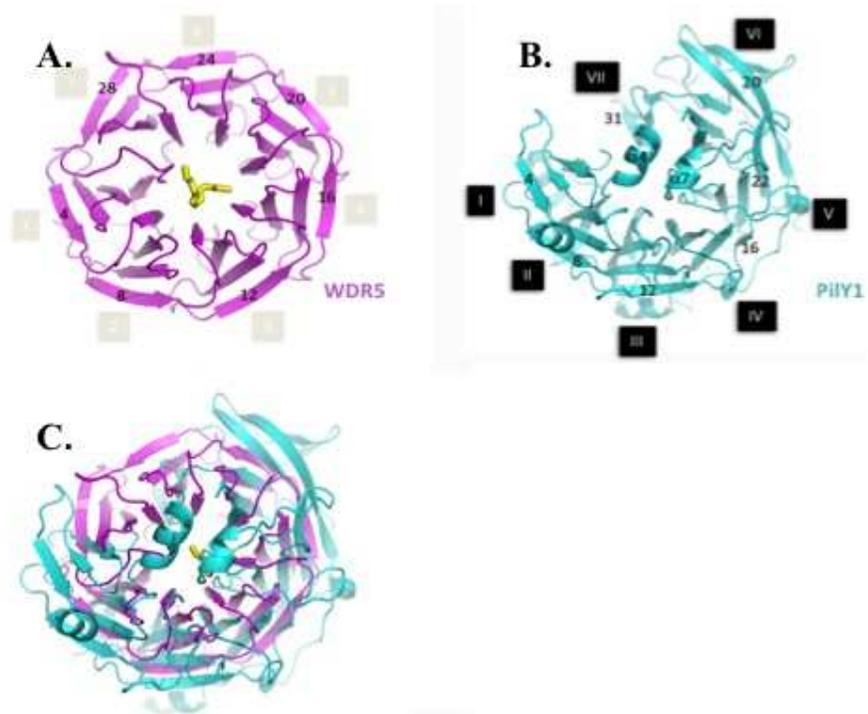|	Pilus		
Wild-Type PilY1	*Extension*	⇄	*Retraction*
PilT^minus	*Extension*	←	
PilY1^minus		→	*Retraction*
WT PilY1+EGTA		⇄	*Retraction*
PilY1 D859A		⇄	*Retraction*
PilY1 D859K	*Extension*	⇄	

Supplemental Figure 2.1

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Supplemental Figure 2.2

Supplemental Figure 2.3

Supplemental Figure 2.4

PilY1 Structure	Distance to Ca (Å)	Distance to Ca (Å)	Calmodulin Structure
D851	2.6	2.4	D20
N853	2.1	2.6	D22
D855	2.6	2.4	D24
V857 O	2.4	2.4	T26 O
D859	2.5	2.4	E31
	2.7	2.4	
water	2.6	2.4	water

```
PilY1    851-DNNSDGVA---D-859
CaM       20-DKDGDGTITTKE-31
```

Supplemental Figure 2.5

D859K C-Terminal PilY1 Calcium Binding Curve
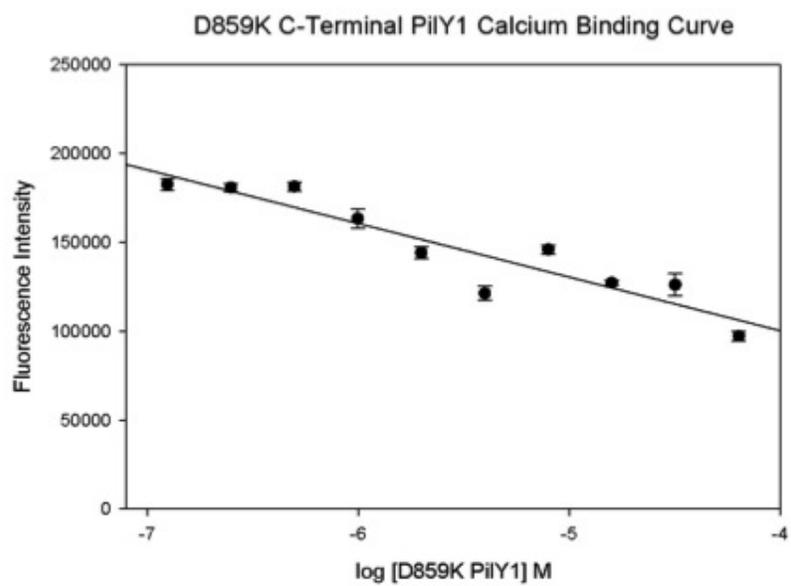
Supplemental Figure 2.6

CD WT PilY1 CTD and D859A PilY1 CTD +/- Ca
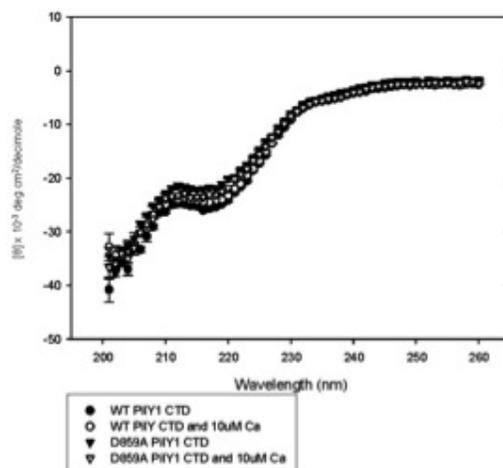
CD WT PilY1 CTD and D859K PilY1 CTD +/- Ca
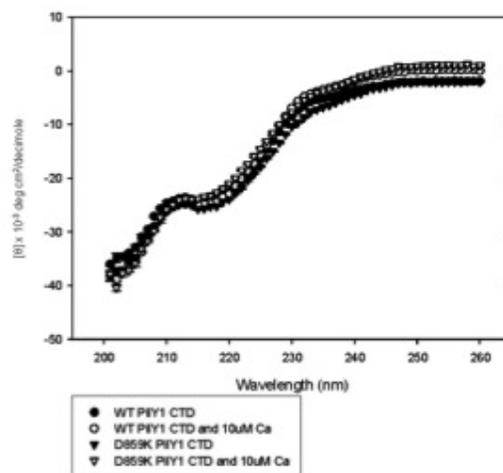
Supplemental Figure 2.7

Melting Temperature (T_m) Measured by CD for PilY1 CTD Proteins	
Protein	T_m (°C)
WT PilY1 CTD	41.0 ± 0.3
WT PilY1 CTD plus 10μM Ca	41.9 ± 0.4
D859A PilY1 CTD plus 10μM Ca	43.5 ± 0.3
D859K PilY1 CTD plus 10μM Ca	43.4 ± 0.3

Supplemental Figure 2.8

Supplemental Figure 2.9
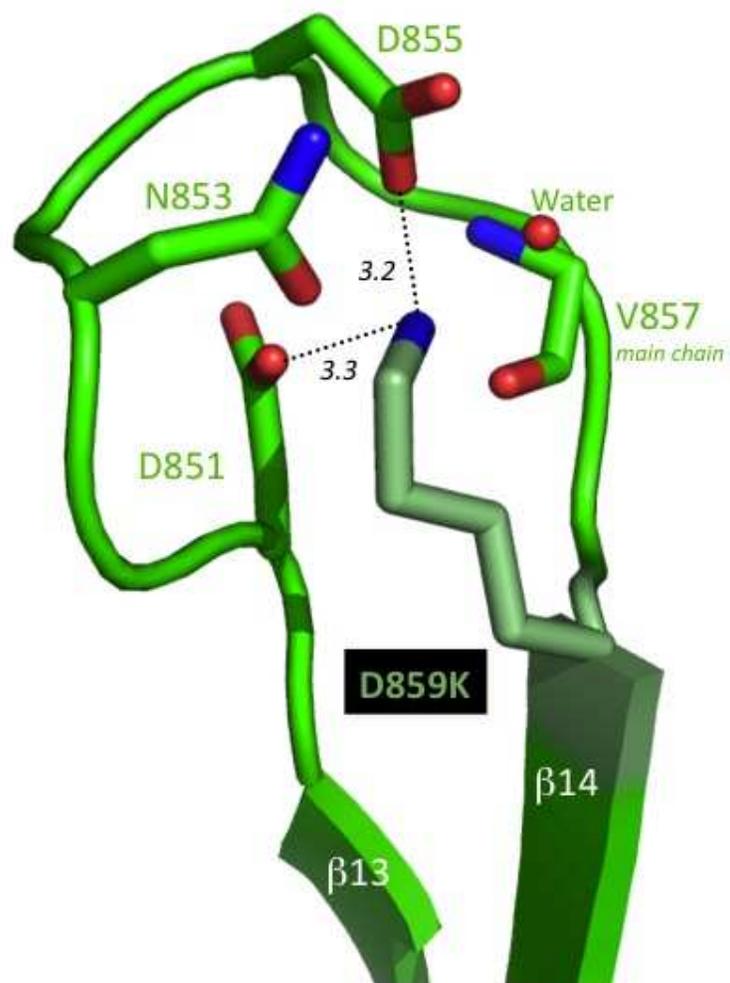
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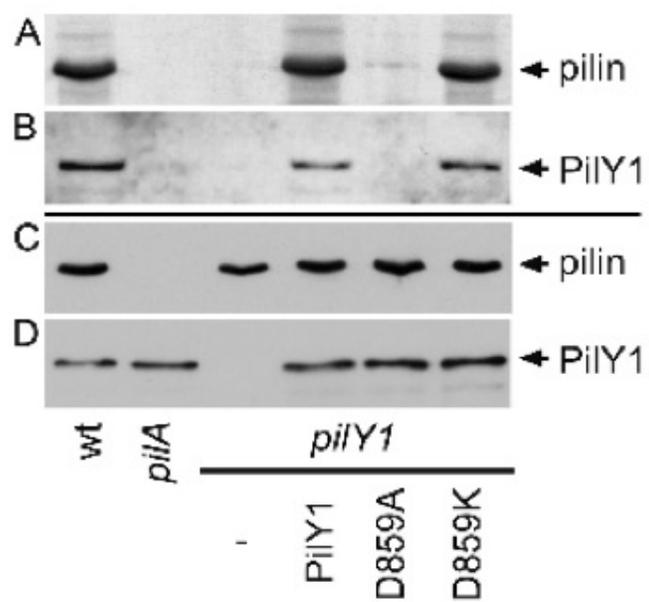
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CHAPTER FOUR:

A Positive Feedback Mechanism Enhances *fimU* Operon Activation via Enhanced Expression of the Two-Component System, AlgZR[•]

SUMMARY

Pseudomonas aeruginosa is an environmental bacterium responsible for a significant proportion of nosocomial infections and is the primary cause of morbidity and mortality in patients with the genetic disorder cystic fibrosis (CF). Localized or disseminating *P*. *aeruginosa* infection can occur following tissue damage due to surgery, severe wounds, or burns. Type IV pili (Tfp) are retractile surface appendages that promote *P*. *aeruginosa* virulence by mediating i) bacterial adherence to host tissue and ii) twitching motility, a form of surface-associated bacterial translocation that may aid in bacterial dissemination. Tfp undergo repeated cycles of extension and retraction via a complex dynamic process requiring multiple proteins. While the major subunit of the pilus fiber is pilin (*pilA)*, several additional fiber-associated proteins play key structural and functional roles. The *fimU* operon encodes multiple factors required for Tfp biogenesis, specifically the pilin-like proteins (FimU, PilV, PilW, PilX, PilE) and PilY1, which is involved in Tfp biogenesis and additionally functions as an adhesin mediating Tfp-dependent adherence to host tissues. In this report we demonstrate that in the absence of *pilY1*, transcription of the

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fimU operon is strongly induced. Using several additional Tfp assembly mutants we determined that *fimU* transcriptional activation is not a general response to the inability to assemble Tfp, as none of our Tfp assembly mutants mimicked the *pilY1* mutant phenotype. Additional studies established that the effect of *pilY1* mutation on *fimU* transcription involves elevated expression of the *fimU* regulators AlgZR. Overall, although the mechanism by which Tfp dysregulation alters *fimU* transcriptional regulation remains unknown, our data begin to define a regulatory feedback mechanism that links PilY1 localization to *fimU* transcription.

INTRODUCTION

Pseudomonas aeruginosa is both a ubiquitous environmental bacterium and an important opportunistic pathogen responsible for a significant proportion of nosocomial infections. In addition, *P. aeruginosa* is also the leading cause of morbidity and mortality in individuals with the genetic disorder cystic fibrosis (CF) (Govan, 1996; Lyczak, 2002). The pathogenesis of *P. aeruginosa* infections is complex and requires the expression of several virulence factors. Adherence to host tissues represents a critical first step in the infection process for many bacteria. In *P. aeruginosa*, attachment to host cells is mediated by multiple attachment factors including the filamentous surface appendages type IV pili (Tfp). Tfp also allow the bacteria to traverse solid surfaces in a process termed twitching motility (Mattick, 2002). Both the attachment and motility functions of Tfp are believed to contribute to biofilm formation during chronic *P. aeruginosa* infections by mediating attachment to both biotic and abiotic surfaces and the subsequent formation of complex biofilm structures (O'Toole, 1998; Mikkelsen, 2011).

The assembly and function of Tfp in *P. aeruginosa* is a complex and dynamic process requiring a vast number of gene products (Figure 1A)(Mattick, 2002). The structural Tfp fiber is primarily composed of a single repeating subunit termed pilin, which is encoded by the *pilA* gene (Strom, 1986). Pilin subunits are produced as pre-proteins containing an N-terminal leader sequence that is cleaved prior to pilus assembly (Strom, 1991). Although the major structural protein of the Tfp fiber is pilin, several additional fiber-associated proteins play key structural and/or functional roles. In *P. aeruginosa* five 'pilin-like' proteins (FimU, PilV, PilW, PilX, and PilE) share the highly conserved N-

terminal α-helical region of pilin as well as the pre-pilin leader sequence (Winther-Larsen, 2005). The pilin-like proteins are incorporated into the mature Tfp fiber at much lower abundance than the major subunit (Giltner, 2010) and are required for Tfp biogenesis (Russell, 1994; Alm, 1995, 1996). The Tfp fiber is assembled at the periplasmic surface of the inner membrane and extruded through a pore complex, composed of the secretin protein PilQ, which is embedded in the outer membrane (Wolfgang, 2000; Collins, 2001). Two cytoplasmic membrane-associated ATPases, PilB and PilT, facilitate the extension and retraction of Tfp fibers during twitching motility (Turner, 1993; Chiang, 2008). PilB drives the assembly of pilin monomers into mature fibers, whereas PilT mediates Tfp disassembly and retraction (Whitchurch, 1991; Chiang, 2008). Inactivation of *pilT* blocks retraction of Tfp fibers, resulting in both hyperpiliation and abrogation of twitching motility (Whitchurch, 1991).

The operon encoding the pilin-like proteins (*fimU-pilVWXY1Y2E* (*fimU* operon)) also encodes the structurally unrelated protein PilY1 (Figure 1B). Like the pilin-like proteins, PilY1 is associated with Tfp fibers and is conditionally required for Tfp assembly (Heiniger, 2010; Orans, 2010). Together, the proteins encoded by the *fimU* operon are predicted to form a functional complex within or associated with the Tfp fiber. Although mutants lacking either *pilY1* or the entire *fimU* operon cannot assemble Tfp, this defect can be suppressed by *pilT* mutation (Heiniger, 2010), suggesting that PilY1 and the pilin-like proteins act at similar steps in the Tfp biogenesis pathway. Additionally, pilin levels in *pilY1* and *fimU* operon mutants are unaltered, demonstrating that the biogenesis defect in these mutants lies in the process of assembly (Heiniger, 2010).

Although the pilin-like proteins and PilY1 appear to have overlapping functions in Tfp biogenesis, PilY1 has been shown to have additional roles in antagonizing pilus retraction and mediating attachment to host epithelial cells (Heiniger, 2010; Orans, 2010). The C-terminal portion of PilY1 is predicted to contribute to the Tfp biogenesis function of this protein. The PilY1 C-terminus shares homology with the PilC proteins of *Neisseria* species (Jonsson, 1991; Wolfgang, 1998; Heiniger, 2010; Orans, 2010) and contains an EF-hand-like calcium-binding site. Mutational analysis demonstrated that calcium binding and release by PilY1 is required for controlling pilus extension and retraction (Orans, 2010). The N-terminal domain of PilY1 is predicted to mediate attachment of *P. aeruginosa* to the basolateral surface of differentiated human airway epithelial cells, possibly through an interaction between integrins and the arginine-glycine-aspartic acid (RGD) binding motif present in PilY1 (Johnson, 2011). The limited sequence similarity between the N-terminal domain of *P. aeruginosa* PilY1 and the Neisserial PilCs (Rudel, 1995; Morand, 2001; Orans, 2010) suggests this region may contribute to the distinct tissue tropisms displayed by these bacteria.

Transcription of the *fimU* operon is controlled by the AlgZ/AlgR two component regulatory system (TCS) and the cAMP receptor protein Vfr, in a cAMP-dependent manner (Lizewski, 2002; Wolfgang, 2003; Belete, 2008) (Chapter 2). In contrast, the *pilA* gene (encoding pilin) is controlled by another TCS, PilS/R. Based on these different regulatory schemes, we hypothesize that transcription of the *fimU* operon and the Tfp biogenesis machinery genes (i.e. *pilA*, *pilB*, *pilT*, and *pilQ*) can be modulated independently. This additional layer of Tfp regulation may allow alteration of PilY1-dependent Tfp phenotypes (adherence and twitching motility) in response to changes in environmental conditions. In

Neisseria meningitidis, transcription of *pilC,* the *pilY1* homolog, is downregulated following host cell contact (Taha, 1998; Morand, 2004). Given the orthology between PilY1 and PilC, it is enticing to speculate that *P. aeruginosa* is also responsive to interaction with host cells. Previous reports have demonstrated that deletion of *pilY1* results in increased autolysis and increased secretion of secondary metabolites including pyocyanin and 4-hydroxy-2-alkylquinolines (Bohn, 2009). Additionally, *pilY1* mutants have altered expression of several genes, including activation of *lipC,* a secreted lipase (Martinez, 1999). Although these studies did not address the role of host cell adherence in gene regulation, these results suggest that altered PilY1 expression and/or localization may serve as a feedback signal responsible for altering bacterial gene expression in response to cell contact.

Deletion of *pilY1* or the *fimU-pilE* operon results in activation of the *fimU* promoter

In the absence of *pilY1,* Tfp are not assembled on the bacterial surface (Alm, 1996; Heiniger, 2010). Despite the lack of surface Tfp fibers on a *pilY1* mutant, the strain expresses a wild-type level of pilin (Heiniger, 2010). Unexpectedly, in-frame deletion of *pilY1* had a dramatic effect on *fimU-pilE* transcription. Using a previously described *fimU-lacZ* transcriptional reporter (P$_{fimU}$-*lacZ*, Chapter 2), we found that deletion of *pilY1* resulted in a greater than 15-fold increase in β-galactosidase activity relative to the wild-type parent strain ($P \leq 0.0001$) (Figure 2A, lanes 1 and 2). Transcriptional upregulation was suppressed by expression of *pilY1 in trans* (Figure 2A), indicating that the transcriptional effect is due to loss of the PilY1 protein and not alteration of the *fimU-pilE* transcript.

Because the proteins of the *fimU-pilVWXY1Y2E* operon, which also encodes *pilY1*, are predicted to form a complex necessary for Tfp biogenesis (Heiniger, 2010), we hypothesized that disruption of this structural complex initiates *fimU* dysregulation. Therefore we asked whether the altered *fimU* transcription observed in a *pilY1* mutant was specifically due to inactivation of *pilY1*. We tested a mutant in which the entire *fimU-pilE* operon was deleted to determine whether the pilin-like proteins encoded by this operon play a role in *fimU* promoter regulation. Like the *pilY1* mutant, the *fimU-pilE* mutant displayed significantly ($P < 0.0001$) elevated *fimU* transcription when compared to the parent strain (Figure 2A); this phenotype could be complemented *in trans* with a plasmid encoding the entire *fimU-pilE* operon (Figure 2A), but not a plasmid encoding *pilY1* alone (Figure 2A). These results suggest that the transcriptional upregulation of the *fimU-pilE* operon is not

due to the absence of *pilY1* specifically, but instead may be a general response to the inability to form a structural complex necessary for Tfp biogenesis as absence of the pilin-like proteins encoded by this operon (i.e. FimU, PilV, PilW, PilX, and/or PilE) results in *fimU* dysregulation. However, in the absence of Tfp biogenesis, pilin subunits accumulate in the periplasm, and the accumulation of improperly folded and/or localized proteins may trigger *fimU* transcriptional upregulation.

pilY1 and *fimU-pilE* mutation is not a general signal for promoter activation

In order to rule out the possibility that mutation of genes within the *fimU* operon does not result in global dysregulation of transcription we utilized a previously constructed cAMP/Vfr-dependent transcriptional reporter system (P$_{lacP1}$-*lacZ)* (Fulcher, 2010) to test the effect of both *pilY1* and *fimU-pilE* mutation. The P$_{lacP1}$-*lacZ* reporter consists of an artificial promoter derived from the *Escherichia coli lac*P1 promoter fused to the *lacZ* gene; this construct was integrated into the vacant ΦCTX phage attachment site on the *P. aeruginosa* chromosome. Neither *pilY1* or *fimU-pilE* mutation had any effect on P$_{lacP1}$-*lacZ* reporter activity (Figure 2B), indicating that the transcriptional activation observed in these mutants is likely specific to the *fimU* promoter.

Inactivation of other Tfp assembly factors do not cause elevated *fimU* promoter activity

In an effort to understand the mechanism responsible for elevated *fimU* transcription regulation in the *pilY1* and *fimU-pilE* mutants, we determined whether particular Tfp assembly factors had a similar impact on P$_{fimU}$–*lacZ* reporter activity. Initially we

hypothesized that the increase in *fimU* transcription resulted from an accumulation of pilin in the periplasm, as the buildup of misfolded pilus proteins triggers a transcriptional response in *E. coli* (Jones, 1997; Hung, 1999; Nevesinjac, 2005). To test this hypothesis we assessed P*fimU*–*lacZ* reporter activity in a *pilA* mutant. We observed that the *pilA* mutant had *fimU* reporter activity equivalent to that of the wild-type strain, suggesting that the transcriptional upregulation observed in the *pilY1* mutant (Figure 2) was not due to accumulation of pilin subunits within the periplasm.

Given this result, we theorized that *fimU* transcriptional upregulation might be due to a specific defect in Tfp biogenesis. Therefore, we inactivated several components of the Tfp biogenesis system (*pilB, pilT, pilD*, and *pilQ)*. We evaluated *pilT* based on our finding that PilY1 plays a role in antagonizing pilus retraction (Heiniger, 2010; Orans, 2010). We also evaluated the role of the opposing ATPase required for pilus extension by inactivating *pilB*. We investigated the effect of *pilD*, which encodes the prepilin peptidase that processes pilin and pilin-like proteins prior to assembly and export. In the absence of *pilD*, uncleaved pilin and pilin-like protein subunits accumulate within the periplasm (Giltner, 2010), resulting in a defect in pilus assembly (Nunn, 1990; Koga, 1993). Therefore we hypothesize that accumulation of both pilin and the pilin-like proteins within the cytoplasm might phenocopy the *pilY1* and *fimU-pilE* mutants. Lastly, we inactivated the *pilQ* gene, encoding the secretin protein that forms the pore complex through which the pilus fiber is extended. In the absence of *pilQ,* pilin subunits accumulate intracellularly, which might also mimic the *pilY1* phenotype. In addition, the Tfp biogenesis defect of a *pilQ* mutant can be suppressed by *pilT* deletion, which is also the case for the *pilY1* and *fimU-pilE* mutants.

In terms of P$_{fimU}$–*lacZ* reporter activity, the *pilT* mutant displayed a modest increase (approximately twofold) relative to that of the wild-type strain (Figure 3); however, this level was substantially less than that of the *pilY1* mutant. The *pilB* mutant had reduced *fimU* transcription compared to the wild-type strain (Figure 3). In contrast, inactivation of *pilD* or *pilQ* had minimal effect on *fimU* transcription (Figure 3, lanes 5 and 6). Although these results suggest that pilus retraction and extension have opposing effects on *fimU* transcription, the effects observed were opposite to what we would have predicted. Originally we hypothesized that a *pilB* mutant would mimic a *pilY1* mutant, as both mutants favor retraction. Similarly, we envisioned that a *pilT* mutant might display diminished P$_{fimU}$ –*lacZ* reporter activity, as the Tfp in a *pilT* mutant are fully extended. However, the exact opposite results were observed, suggesting that the signal detected in *pilY1* or *fimU-pilE* mutants is more complicated than Tfp extension or retraction. Overall, none of the candidate genes related to Tfp function we tested affected *fimU* transcription to the same degree as observed in either a *pilY1* or *fimU-pilE* mutant.

Tfp assembly mutants do not suppress the *fimU* transcriptional activity of *pilY1* mutants

The altered *fimU* transcription observed in the *pilB* and *pilT* Tfp assembly mutants suggested that these factors act on the same pathway. However, the observed effects on *fimU* transcriptional activity were not as profound as the dramatic phenotype seen in either *pilY1* or *fimU-pilE* mutants. We hypothesized that, in the absence of these proteins, a specific signal is generated that results in *fimU* transcriptional upregulation and, therefore, these mutations may suppress the transcriptional upregulation of a *pilY1* mutant. We

inactivated *pilA, pilB,* or *pilT* in the *pilY1* P*fimU-lacZ* reporter strain and assayed *fimU*

transcriptional activity (Figure 4). Inactivation of *pilA* or *pilB* in the *pilY1* background did

not affect *fimU* transcription relative to the *pilY1* mutant (Figure 4). In contrast, the

pilT/pilY1 double mutant displayed a nearly 50% reduction in P*fimU-lacZ* reporter activity

compared to a *pilY1* mutant (Figure 4, lanes 2 and 8). However, the P*fimU-lacZ* reporter

activity of the *pilT/pilY1* double mutant was still higher than that of the wild-type strain

(Figure 4, lanes 1 and 8), demonstrating that the hyperpiliated, non-retractile phenotype of a

pilT mutant only partially suppressed the *fimU* phenotype of a *pilY1* mutant. Initially, we

hypothesized that a *pilB* mutation would be most likely to suppress the *fimU* transcriptional

upregulation of a *pilY1* mutant, and that a *pilT* mutation would not suppress the *pilY1*

phenotype. Taken together, these results suggest that defects in Tfp biogenesis influence

fimU transcription. However, it remains unclear what the signal responsible for altered *fimU*

transcription is and how this signal is relayed.

Regulators of *fimU* transcription suppress the *pilY1* mutant *fimU* phenotype

As our evaluation of factors affecting Tfp biogenesis failed to implicate

dysregulation of surface Tfp fibers in the *pilY1* mutant phenotype, we next investigated the

impact of two transcription factors known to regulate the *fimU-pilE* operon, AlgR and Vfr.

We have shown that AlgR regulates the *fimU* operon in a phosphorylation-dependent

manner (Chapter 2). Vfr directly activates the *fimU* operon and indirectly affects *fimU*

transcription via activation of the *algZ* promoter in a feedback loop that ultimately

influences AlgR levels (Chapter 2). Inactivation of *algZR* in the *pilY1* mutant resulted in a

reduction in P*fimU-lacZ* reporter activity to levels equivalent to that of the *algZR* double

mutant strain (Figure 5). This result suggests that AlgZR is involved in the *fimU* transcriptional upregulation observed in a *pilY1* mutant, or, alternatively, that AlgZR is absolutely required for P*fimU*-*lacZ* activity; either of these mechanisms could account for the suppression of the *pilY1* phenotype. Inactivation of *vfr* in a *pilY1* mutant background significantly reduced P*fimU* –*lacZ* reporter activity compared to that of the *pilY1* mutant (Figure 5, lanes 2 and 6). However, the P*fimU* activity of the *vfr/pilY1* double mutant was still greater than that of the wild-type strain ($P < 0.0001$). Given that Vfr positively regulates *algZ* transcription, the suppression of P*fimU* –*lacZ* reporter activity in a *vfr/pilY1* mutant is most likely due to decreased *algZ* expression.

Deletion of *pilY1* causes *algZ* promoter activation

Because AlgZR is involved in *fimU* transcriptional upregulation in a *pilY1* mutant, we wanted to determine if altered *algZ* transcription contributes to this phenotype. To test this possibility, we assayed a P*algZ*-*lacZ* transcriptional reporter strain in which *pilY1* was inactivated. Similar to the results from the P*fimU*-*lacZ* transcriptional reporter, inactivation of *pilY1* resulted in a greater than tenfold increase in *algZ* transcription (Figure 6) compared to the wild-type strain. Again, the *pilY1* phenotype was suppressed by *vfr* mutation (Figure 6). However the P*algZ*-*lacZ* transcriptional reporter activity of the *vfr/pilY1* double mutant was significantly higher ($P <0.001$) than that of the wild-type strain, indicating that a transcriptional regulator other than Vfr is likely influencing *algZ* transcriptional activation.

DISCUSSION

Intimate interaction between *P. aeruginosa* Tfp and host cells represents a critical event in initiating pathogenesis. Further, because Tfp play integral roles in adherence and biofilm formation, they are crucial virulence factors during both acute and chronic infections. Recent studies have indicated that PilY1 participates in regulating multiple surface behaviors associated with both acute and chronic phenotypes (Kuchma, 2010). Based on the observation in *N. meningitidis* that host cell contact results in a rapid transient increase in *pilC1* transcription (Taha, 1998; Morand, 2004), we hypothesized that *P. aeruginosa* attachment to host surfaces could serve as a sensing mechanism for altering gene expression. As a putative Tfp-associated adhesin, PilY1 is perfectly localized to function as a sensor of changing environmental conditions. Therefore it is plausible that PilY1 localization can modulate a variety of signaling pathways. In an effort to investigate whether regulation of *pilY1* is analogous to the regulation of *pilC1* in *N. meningitidis*, we used a strain lacking *pilY1* as a model for the Tfp "dysregulation" that occurs during host cell contact.

We determined that elevated transcription of the *fimU* Tfp biosynthetic operon occurred when *pilY1* or the entire operon was deleted. This result is in agreement with previous microarray analysis of a *pilY1* mutant that showed the genes of the *fimU* operon were the most strongly induced (Bohn, 2009). We further showed the effect of Tfp dysregulation was not PilY1-specific, as *in trans* complementation with *pilY1* did not restore *fimU* transcription to wild-type levels in the *fimU-pilE* mutant. Importantly, none of the proteins encoded by the *fimU* operon are predicted to have DNA binding domains or

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putative transcriptional regulatory function, thus the mechanism resulting in altered *fimU* transcription remains elusive. Taken together, these results suggest that the signal responsible for altering *fimU* transcription may be altered localization of PilY1 and/or other minor pilins. Pilus retraction does not elicit a response that mimics *pilY1* deletion, as a *pilB* mutant displayed decreased rather than increased *fimU* transcription compared to the wild-type strain. However, pilus retraction and extension do appear to have opposing effects as a *pilT* mutant had elevated *fimU* promoter activity compared to the wild-type strain. Our study indicates that upregulation of *fimU* transcription is not a general response to the lack of assembled Tfp, as none of the Tfp assembly mutants tested in this study displayed transcriptional activation of *fimU* that mimicked the *pilY1* mutant phenotype.

The transient induction of *pilC1* transcription in response to host cell contact in *N. meningitidis* is mediated by the CgrA transcriptional regulator (Jamet, 2010). *P. aeruginosa* possesses a CgrA homolog, previously shown to regulate expression of the phase-variable *cupA* fimbrial genes, through a poorly understood interaction with CgrC (McManus, 2011). Our studies showed that the *fimU* transcriptional upregulation displayed by a *pilY1* mutant likely results from enhanced expression of AlgZR, the known *fimU* regulator. In contrast, Vfr does not seem to play a role in the feedback mechanism controlling *fimU* transcription with the minor effect on *fimU* promoter activity being attributed to altered *algZR* expression. An unknown regulator is likely responsible for elevated *algZR* expression, as a *vfr/pilY1* double mutant had increased *algZ* promoter activity compared to the wild-type strain, indicating that enhanced *algZR* transcription occurs independent of Vfr control. We hypothesize that inactivation of *algZR* would abolish *pilY1* induction in response to host cells but this remains to be tested.

Despite upregulation of *pilC1* in *N. meningitidis,* piliation is not altered, leading researchers to question the mechanism/purpose of PilC1 upregulation. It has been hypothesized that basal levels of PilC1 are sufficient to promote piliation, whereas increased PilC1 levels may be necessary to properly localize PilC1 to the tip of the Tfp fiber. Our results in *P. aeruginosa* support the hypothesis that proper localization of PilY1, or any of the other proteins encoded by the *fimU* operon, is necessary for *fimU* feedback. Together the proteins of the *fimU* operon are predicted to form a complex required for Tfp biogenesis, although the makeup of this putative complex is not understood at the molecular level. Our results suggest that disruption of this complex serves as the signal for altered *fimU* transcription.

We envision a model where, through a sensing mechanism mediated by PilY1 and other pilin-like proteins, intimate contact between *P. aeruginosa* and host cells triggers a specific gene expression program responsible for promoting mucosal infection. We predict that upon interaction with a host cell, the *fimU* operon is transiently upregulated in order for proper localization of PilY1 to the tip of the Tfp fiber, which allows optimal interaction with host receptors. Following attachment, Tfp retraction brings the bacteria closer to the host cell, which induces the Type III secretion system. Previous studies provide evidence that PilY1 dysregulation is not limited to the *fimU* operon alone but rather induces a more global response, as expression of 76 genes was differentially regulated in a *pilY1* mutant compared to the wild-type strain (Bohn, 2009). Strikingly, of the additional 71 differentially regulated genes, only eight had been previously characterized, and the vast majority of genes were classified as conserved hypotheticals or genes of unknown function (Bohn, 2009).

However, the mechanism by which absence of a Tfp structural protein/complex alters transcription of either *fimU* or *algZ* is currently unclear. Future studies will be aimed at determining whether a similar upregulation of *fimU* transcription occurs following contact with well differentiated human airway epithelial cells as well as abiotic surfaces.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. During routine passage, strains were grown at 37°C in LB medium. For complementation experiments, vector, pPa-*pilY1*, and pPa-*fimU-pilE* were maintained in *P*. *aeruginosa* with 150 μg ml^{-1} carbenicillin (Cb), except where indicated. Bacterial growth in liquid culture was assessed by optical density at 600 nm (OD$_{600}$). *P. aeruginosa* double mutant strains *pilA/pilY1*, *pilB/pilY1*, *pilT/pilY1*, *algZR/pilY1*, and *vfr/pilY1* were constructed by introducing the appropriate deletion allele (encoded by a pEXGm plasmid) onto the chromosome of the *pilY1* mutant using a previously described method (Wolfgang, 2003). Chromosomal deletion alleles were confirmed by DNA sequencing of PCR products. An *algZ* transcriptional reporter plasmid was constructed by PCR amplifying bp -300 to +100 relative to the *algZ* translational start codon from PAK chromosomal DNA with oligonucleotides *algZ* rep 5' (5'-TACAAAAAAGCAGGCTGAATTCTCAGCGGTAGAGACGCTTGTCGAAGTC- 3') and *algZ* rep 3' (5'-TACAAGAAAGCTGGGTGGATCCTCAGCAGCACCAGGGTGAACAGGGC-3') tailed with attB1 or attB2 sequences for Gateway cloning into pDONR201 (Life Technologies). Promoter fragments were removed from pDONR201 by digestion with EcoRI and BamHI and ligated into the corresponding restriction sites of mini-CTX-*lacZ* (Hoang, 1998). The resulting plasmids were used to integrate the promoter-*lacZ* fusions onto the chromosome at a vacant CTX phage attachment site of wild-type and mutant strains as described previously (Hoang, 1998).

β-galactosidase assays. Overnight cultures of strains containing P$_{fimU}$ or P$_{algZ}$ promoter-*lacZ* fusions were diluted 1:100 into LB and grown to an OD$_{600}$ of 1.0. Cb (30 μg/ml) and

isopropyl-β-D-thiogalactopyranoside (IPTG; 50 µM) were added as indicated in the figure

legends. For all samples, β-galactosidase activity measurements were carried out as

described previously (Fuchs, 2010). Each assay was repeated at least three times.

Statistical analysis. The two-tailed unpaired t test was used for data comparison where

appropriate using Prism v5.0b (GraphPad Software).

FIGURE LEGENDS

Figure 1. Major components of the *P. aeruginosa* Type IV pilus (Tfp) biogenesis system.

A) Tfp are dynamic surface fibers that mediate twitching motility through extension and retraction. The Tfp fiber is primarily composed of pilin subunits. Assembly (extension) occurs at the inner membrane and is driven by the PilB ATPase. Extending fibers are extruded through an outer membrane complex (PilQ). Retraction (disassembly) of the pilus is mediated by the PilT ATPase. The putative adhesin PilY1 is associated with the pilus fiber. **B)** The *pilY1* gene is part of an operon with six additional Tfp-associated genes. Five of these genes (depicted in blue) encode proteins (FimU, PilV, PilW, PilX, and PilE) with homology to the highly conserved amino-terminus of pilin and are thus termed "pilin-like" proteins. The *pilY2* gene is believed to be a pseudogene (depicted in white), In *N. gonohorreae*, pilin-like proteins are required for inclusion of the PilY1 homolog PilC into the sheared pilus fraction (Winther-Larsen, 2005).

Figure 2. The *pilY1* mutant has dramatically increased expression of the Tfp minor pilin operon. A.) The wild-type (WT) or indicated mutant strains containing the chromosomal P_{fimU}-*lacZ* reporter and either empty vector, the *pilY1* or *fimU-pilE* expression plasmid were grown in LB broth containing 30 μg ml^{-1} carbenicillin (Cb) and 50 μM IPTG to mid-log growth phase ($OD_{600} = 1.0$) and assayed for β-galactosidase activity. Values are the means ± standard error of the mean (SEM) ($n \geq 4$). All values were significantly different than WT when compared pair-wise, ($P < 0.0018$). **B.)** The wild-type (WT) or indicated mutant strains containing the chromosomal P_{lacP1}-*lacZ* reporter were grown as

described in A. Values are the means ± SEM ($n \geq 3$). None of the values were significantly different than WT when compared pair-wise.

Figure 3. Tfp mutants display varying levels of *fimU* transcriptional activity. The wild-type (WT) or indicated mutant strains containing the chromosomal P*fimU*-*lacZ* reporter were grown and assayed as described in Figure 1. Values are the means ± SEM ($n \geq 4$). All values were significantly different than WT when compared pair-wise ($P \leq 0.0053$).

Figure 4. Secondary mutations do not fully suppress the *fimU* upregulation of a *pilY1* mutant. The wild-type (WT) or indicated mutant strains containing the P*fimU*-*lacZ* reporter were grown in LB broth to mid-log growth phase ($OD_{600} = 1.0$) and assayed for β-galactosidase activity. Values are the means ± SEM ($n = 3$). All values except were significantly different than WT when compared pair-wise ($P \leq 0.001$).

Figure 5. *fimU* transcriptional upregulation in the *pilY1* mutant is mediated by AlgZ/AlgR. The wild-type (WT) or indicated mutant strains containing the P*fimU*-*lacZ* reporter were grown in LB broth to mid-growth phase ($OD_{600} = 1.0$) and assayed for β-galactosidase activity. Values are the means ± SEM ($n = 3$). All values were significantly different than WT when compared pair-wise ($P \leq 0.01$).

Figure 6. The *pilY1* mutant displays markedly elevated *algZ* transcription. The wild-type (WT) or indicated mutant strains containing the P*algZ*–*lacZ* reporter were grown in LB broth to mid-growth phase ($OD_{600} = 1.0$) and assayed for β-galactosidase

activity. Values are the means ± standard error of the mean (SEM) ($n = 3$). All values were

significantly different than WT when compared pair-wise ($P \leq 0.01$).

Table I. Strains and plasmids used in this work

Strain or plasmid	Description or relevant characteristic(s)[a]	Reference or source
P. aeruginosa strains		
PAK	_P. aeruginosa_ strain K, wild type	(Takeya, 1966)
PAK::P_fimU-lacZ_	PAK with chromosomal _fimU_ promoter reporter	Chapter 2
PAKΔ_pilA_::P_fimU-lacZ_	_pilA_ with chromosomal _fimU_ promoter reporter	Chapter 2
PAKΔ_fimU-pilE_::P_fimU-lacZ_	_fimU-pilVWXY1Y2E_ with chromosomal _fimU_ promoter reporter	Chapter 2
PAKΔ_pilY1_::P_fimU-lacZ_	_pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_vfr_::P_fimU-lacZ_	_vfr_ with chromosomal _fimU_ promoter reporter	Chapter 2
PAKΔ_algZR_::P_fimU-lacZ_	_algZR_ with chromosomal _fimU_ promoter reporter	Chapter 2
PAKΔ_pilT_::P_fimU-lacZ_	_pilT_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilB_::P_fimU-lacZ_	_pilB_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilD_::P_fimU-lacZ_	_pilD_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilQ_::P_fimU-lacZ_	_pilQ_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_algZR_Δ_pilY1_::P_fimU-lacZ_	_algZR/pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilA_Δ_pilY1_::P_fimU-lacZ_	_pilA/pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilB_Δ_pilY1_::P_fimU-lacZ_	_pilB/pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_pilT_Δ_pilY1_::P_fimU-lacZ_	_pilT/pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAKΔ_vfr_Δ_pilY1_::P_fimU-lacZ_	_vfr/pilY1_ with chromosomal _fimU_ promoter reporter	This study
PAK::P_algZ-lacZ_	PAK with chromosomal _algZ_ promoter reporter	This study
PAKΔ_vfr_::P_algZ-lacZ_	_vfr_ with chromosomal _algZ_ promoter reporter	This study
PAKΔ_pilY1_::P_algZ-lacZ_	_pilY1_ with chromosomal _algZ_ promoter reporter	This study
PAKΔ_vfr/pilY1_::P_algZ-lacZ_	_vfr/pilY1_ with chromosomal _algZ_ promoter reporter	This study
PAK::P_lacP1-lacZ_	PAK with chromosomal _lacP_1 promoter reporter	(Fulcher, 2010)
PAKΔ_cyaAB_::P_lacP1-lacZ_	_cyaAB_ with chromosomal _lacP_1 promoter reporter	(Fulcher, 2010)
PAKΔ_fimU-pilE_::P_lacP1-lacZ_	_fimU-pilVWXY1Y2E_ with chromosomal _lacP_1 promoter reporter	This study
PAKΔ_pilY1_::P_lacP1-lacZ_	_pilY1_ with chromosomal _lacP_1 promoter reporter	This study
Plasmids		
pDONR201	Gateway cloning vector, Km[r]	Life Technologies
mini-CTX-_lacZ_	Plasmid for chromosomal integration at the ⟩CTX phage site of transcriptional fusions; Tc[r]	(Hoang, 1998)
mini-CTX-P_fimU-lacZ_	_fimU_ promoter region (bp to bp) in EcoRI and BamHI sites of mini-CTX-_lacZ_; Tc[r]	Chapter 2
mini-CTX-P_algZ-lacZ_	_algZ_ promoter region (bp to bp) in EcoRI and BamHI sites of mini-CTX-_lacZ_; Tc[r]	Chapter 2
pEX18Gm	Suicide vector, Gm[r]	(Hoang, 1998)
pEXGmΔ_algZR_	pEX18Gm containing _algZR_ deletion allele; Gm[r]	(Jones, 2010)
pEXGmΔ_fimU-pilE_	pEX18Gm containing _fimU-pilE_ deletion allele; Gm[r]	(Heiniger, 2010)
pEXGmΔ_pilA_	pEX18Gm containing _pilA_ deletion allele; Gm[r]	(Fulcher, 2010)
pEXGmΔ_pilB_	pEX18Gm containing _pilB_ deletion allele; Gm[r]	(Wolfgang, 2003)
pEXGmΔ_pilD_	pEX18Gm containing _pilD_ deletion allele; Gm[r]	(Wolfgang, 2003)
pEXGmΔ_pilQ_	pEX18Gm containing _pilQ_ deletion allele; Gm[r]	(Wolfgang, 2003)
pEXGmΔ_pilT_	pEX18Gm containing _pilT_ deletion allele; Gm[r]	(Heiniger, 2010)
pEXGmΔ_pilY1_	pEX18Gm containing _pilY1_ deletion allele; Gm[r]	(Heiniger, 2010)
pEXGmΔ_vfr_	pEX18Gm containing _vfr_ deletion allele; Gm[r]	(Wolfgang, 2003)
pMMB67EH	Empty _P. aeruginosa_ expression vector; Ap[r]	(Furste, 1986)
pMMBV1GW	Gateway-adapted version of pMMB67EH; Ap[r]	(Fulcher, 2010)
pPa-_fimU-pilE_	_P. aeruginosa fimU-pilE_ carried on pMMBV1; Ap[r]	(Heiniger, 2010)
pPa-_pilY1_	_P. aeruginosa fimU-pilE_ carried on pMMBV1; Ap[r]	(Heiniger, 2010)

[a] Ap[r], ampicillin resistance marker; Km[r], kanamycin resistance marker; Gm[r], gentamicin resistance marker, Tc[r], tetracycline resistance marker
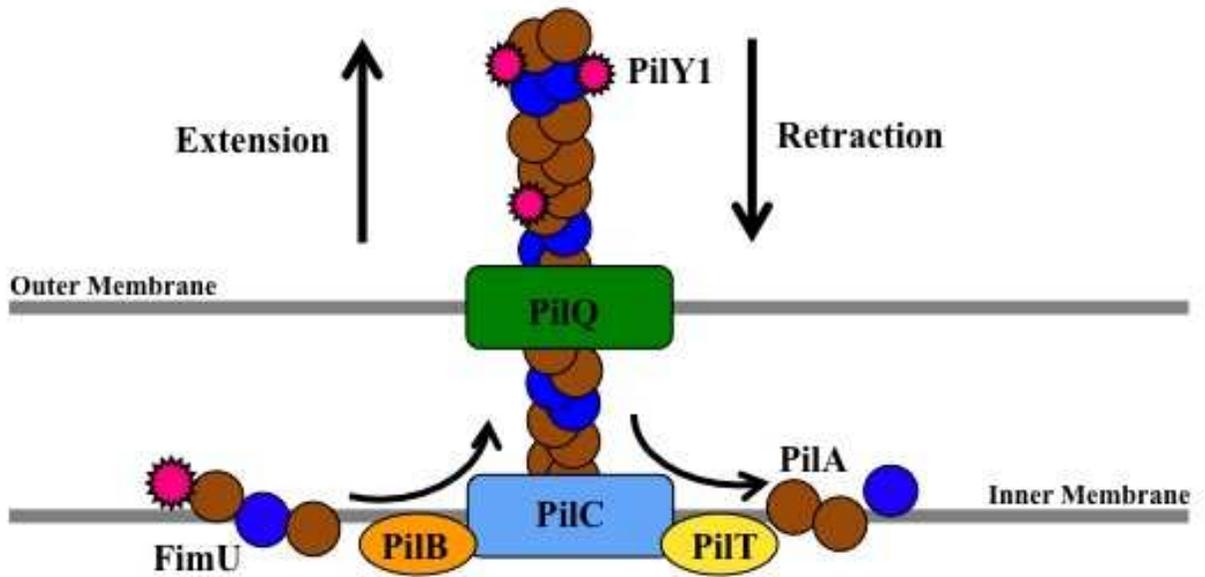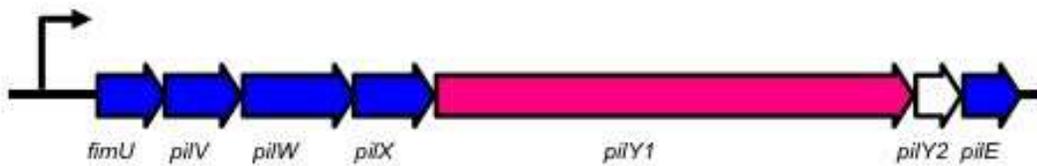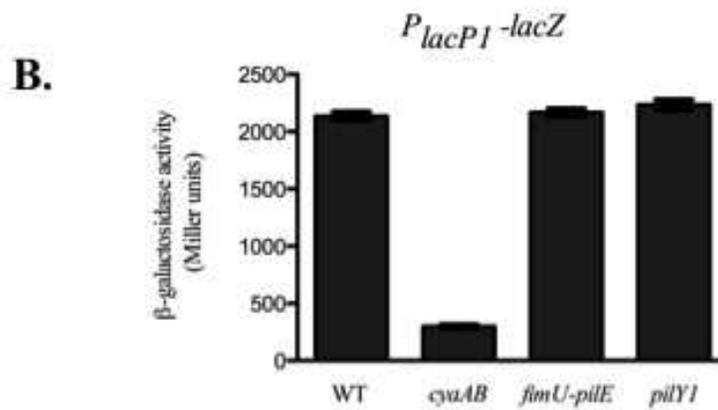
Figure 1A.

Figure 1B.

Figure 2.

A.

P$_{fimU}$-lacZ

β-galactosidase activity (Miller units)

	WT	pilY1		fimU-pilE		
vector	+	+	-	+	-	-
pPa-pilY1	-	-	+	-	-	+
pPa-fimU-pilE	-	-	-	-	+	-

B.

P$_{lacP1}$-lacZ

β-galactosidase activity (Miller units)

WT   cyaAB   fimU-pilE   pilY1

Figure 3.

Figure 4.

P_{fimU}-lacZ

Figure 5.

P*fimU*-lacZ
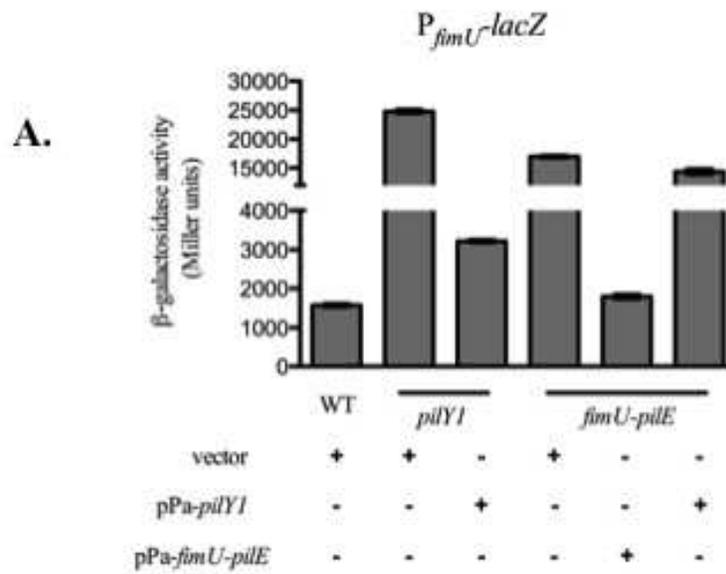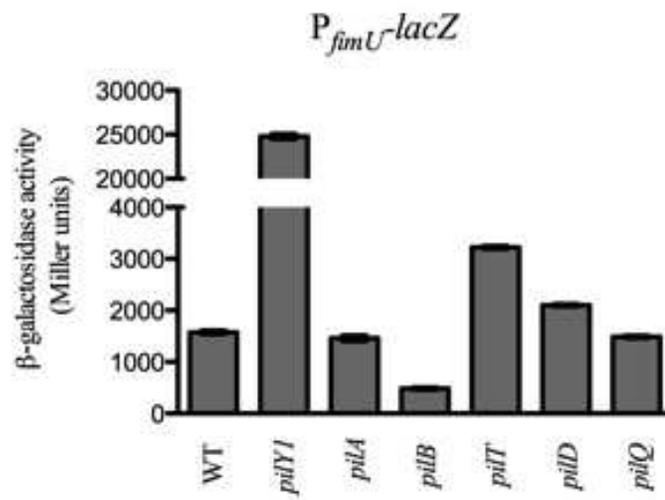
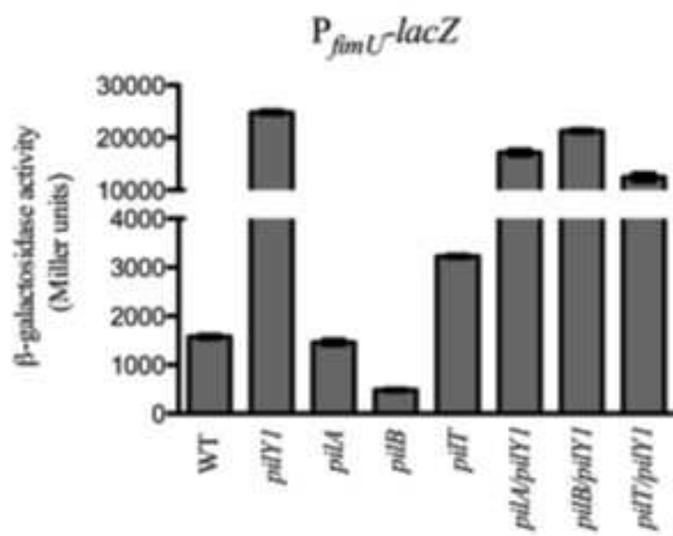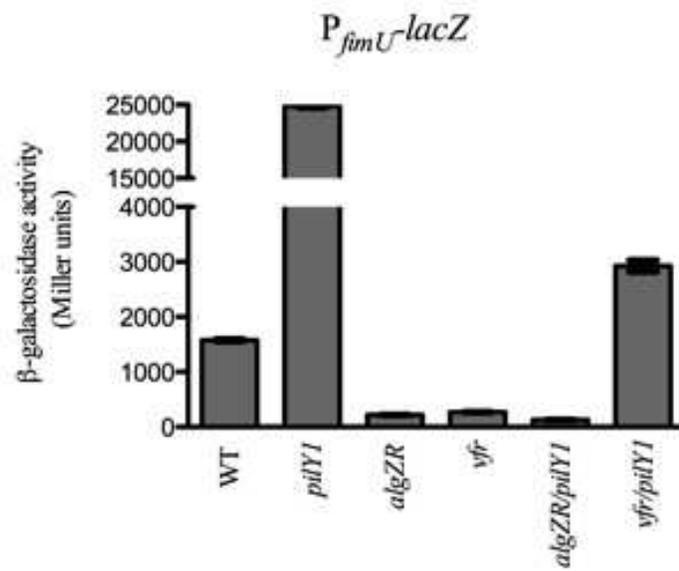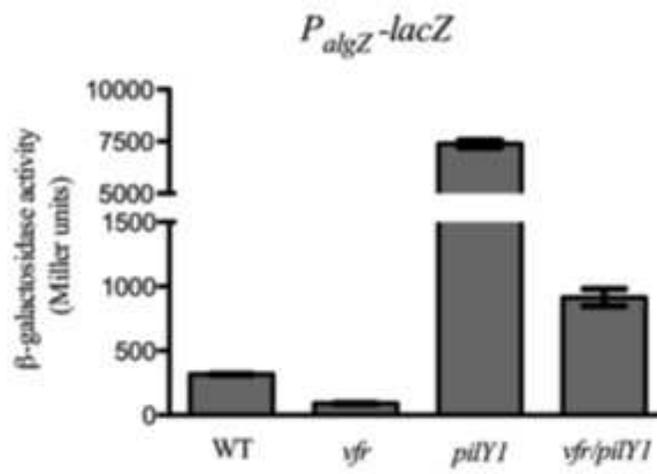**Figure 6.**

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CHAPTER FIVE:

Summary and Future Directions

Pseudomonas aeruginosa is a ubiquitous and metabolically versatile bacterium characterized by its ability to inhabit a number of diverse habitats. In addition, *P. aeruginosa* is a formidable opportunistic pathogen, responsible for a significant proportion of nosocomial infections annually and is the predominant cause of morbidity and mortality in individuals with the genetic disease cystic fibrosis (CF). The intrinsic antibiotic resistance of this bacterium coupled with the continued emergence and spread of multi-drug resistant strains make *P. aeruginosa* infections increasingly difficult to treat in the immunocompromised population.

The pathogenesis of *P. aeruginosa* infections is multi-factorial, requiring the expression of a number of virulence factors. An early and critical step in bacterial pathogenesis is the interaction of bacteria with host cells, which facilitates attachment and host colonization. In *P. aeruginosa,* initial attachment to airway epithelium is mediated by both flagella and type IV pili (Tfp) (Bucior, 2012). However, the bacterial adhesin responsible for mediating attachment to the basolateral surface of differentiated airway epithelial cells is associated with Tfp fibers (Heiniger, 2010). Thus, the expression of Tfp is critical for pulmonary bacterial infection (Tang, 1995). The research described here was directed toward understanding both the regulated expression of the Tfp biogenesis operon

fimU-pilVWXY1Y2E (*fimU)* and the function(s) mediated by the proteins encoded therein. We chose to focus on the proteins encoded by the *fimU* operon due to their essential role in Tfp biogenesis (FimU, PilV, PilW, PilX, PilE, and PilY1), and the ability to mediate bacterial attachment to eukaryotic cells (PilY1). In addition, the fact that the *fimU* operon and *pilA* are differentially regulated suggests that expression of PilY1 and the pilin-like proteins can be modulated independently of the Tfp structural subunit and thus may serve as a general mechanism for responding to environmental conditions. Understanding the regulatory mechanisms that control expression of the *fimU* operon will aid in characterizing the function of proteins encoded by this operon as well as the process of Tfp biogenesis in *P. aeruginosa* and other bacteria with Tfp.

Transcriptional regulation of the *fimU* operon

        In Chapters 2 and 4, we revealed the convergence of two linked, but independent regulatory mechanisms involving AlgZR and Vfr that control expression of the *fimU* operon. These findings demonstrate previously unrecognized layers of a complex regulatory networks controlling this critical *P. aeruginosa* virulence system. Although *fimU* promoter regulation by AlgR was previously reported (Belete, 2008), we further demonstrated that phosphorylation of AlgR is required for *fimU* activation, based on the following evidence: i) mutants lacking the kinase (AlgZ) are deficient in *fimU* transcriptional activity and Tfp production, and ii) AlgR bound the *fimU* promoter in EMSA with greater affinity in the presence of the small molecular phosphodonor, phosphoramidate. In terms of Vfr regulation, it was known that Vfr mutants lacked Tfp (Beatson, 2002) and the finding that a *vfr* mutant had dramatically reduced *fimU* operon

expression (Wolfgang, 2003) suggested that altered levels of the pilin-like proteins contributed, at least in part, to the Tfp defect. During our investigation of this possibility, we revealed two mechanisms by which Vfr regulates the *fimU* operon: i) binding to the *fimU* promoter (direct), and ii) binding to the *algZR* promoter, which ultimately controls levels of AlgR, the other transcriptional activator of the *fimU* operon (indirect). Despite the fact that AlgR and Vfr independently recognize the *fimU* promoter in EMSA and DNase I footprinting assays, our epistasis studies demonstrated that neither AlgZ/R nor Vfr alone were sufficient to activate expression of the *fimU* operon. These results demonstrate that *fimU* transcription requires multiple regulatory signals and represents a key focal point of Tfp regulation. Additionally, the complex regulation of the *fimU* operon, apart from *pilA* regulation, suggests that a complex formed by the minor pilins (and PilY1) may serve as a biologically relevant sensor responding to environmental cues.

Characterization of the Tfp-associated bacterial adhesin PilY1

Our interest in the function of PilY1 in Tfp biogenesis stems from the fact that, although the Tfp ATPases (PilB and PilT) are known to drive cycles of pilus extension and retraction, the regulation of these opposing forces has remained a mystery. In pathogenic *Neisseria* species, PilC has been shown to antagonize pilus retraction (Wolfgang, 1998) and we hypothesized that if the homologous protein in *P*. *aeruginosa* (PilY1) was performing a similar function, it would shed light on regulation of Tfp function. In collaboration with the Redinbo group, we solved the crystal structure of the C-terminal portion of PilY1 and revealed a distinct EF-hand-like calcium-binding site. This site was conserved in several pathogenic bacteria with retractile Tfp, suggesting a function in pilus extension and/or

retraction (Orans, 2010). To investigate the functional role of the PilY1 EF-hand-like calcium-binding site, we attempted to alter function via site-directed mutagenesis or calcium chelation. We showed that calcium binding and release by PilY1 was required for PilY1-dependent control of pilus extension and retraction *in vivo*. The experimentally determined calcium-binding affinity of PilY1 (2 μM) is likely in a biologically relevant range, and also supports the hypothesis that calcium binding and release by the conserved C-terminal domain is important for pilus biogenesis and motility in organisms with a PilY1 homologue.

Because the PilY1 EF-hand-like calcium-binding site is a structural motif common to retractile Tfp of many pathogenic bacterial species, it is possible that this region may play an important role in multiple aspects of pilus biogenesis, function, and regulation. Indeed, subsequent studies have identified an additional calcium-binding site within PilY1 that appears to regulate RGD-dependent biding of PilY1 to human integrins *in vitro* (Johnson, 2011). Follow-up studies will be directed at extending these observations to determine if the RGD motif of PilY1 is important for *P. aeruginosa* interaction with integrins *in vivo*. We plan to generate transformed cell lines that express a single class of human integrins and evaluate adherence of RGD mutant strains and whether calcium affects binding. We will also determine if bacterial adherence is blocked by antibodies directed against integrins, thus providing further evidence for the specificity of the PilY1/integrin interaction. Additionally, we would test the effect of PilY1 calcium-binding site mutations on *P. aeruginosa* binding to primary airway cells to determine whether calcium binding also regulates PilY1-mediated adhesion.

Another avenue of investigation regarding PilY1 function is to characterize the relationship between PilY1 and the pilin-like proteins encoded by the *fimU* operon. Because each of these proteins is necessary for Tfp biogenesis (Giltner, 2010; Heiniger, 2010), we hypothesize that the proteins may form a complex that mediates Tfp assembly. To date, the only evidence for localization of the *fimU* operon proteins is that they have been reported to be incorporated into the growing pilus fiber (Giltner, 2010). To investigate the possibility that the proteins of the *fimU* operon form a structural complex, we would like to investigate the interaction of the pilin-like proteins (FimU, FimV, PilW, PilX, and PilE) with PilY1 initially using *in vitro* pulldown experiments. However, to date we have been unsuccessful at expressing recombinant full-length His-tagged PilY1, possibly due to the relatively large size of this polypeptide. In the future, we will undertake experiments to investigate the interactions between the pilin-like proteins, as they are much smaller and are thus likely more amenable to overexpression and purification methods.

Biological significance of *fimU* operon dysregulation

In Chapter 4 we addressed dysregulation of the *fimU* operon. While investigating the function of PilY1 we made an observation that deletion of *pilY1* resulted in significant induction of *fimU* transcription. We felt this result was reminiscent of the induction of *pilC1* in *Neisseria meningitidis* following interaction with host cells. As *pilY1* is a homolog of *pilC1*, we hypothesize that a similar response to cell contact might occur in *P. aeruginosa*. To test this hypothesis, we will construct P*fimU*-*gfp* fusions to investigate the *fimU* transcriptional response following interaction with eukaryotic cells, including airway epithelial cells as we have previously used these cells to model *P. aeruginosa* pulmonary

infection and demonstrated Tfp-dependent attachment (Heiniger, 2010). However, as an important pathogen during burn wound infection and other types of nosocomial infection, it would be interesting to evaluate *fimU* promoter activity during interaction of *P. aeruginosa* with other relevant cell types.

In an effort to identify additional Tfp defects that replicated the phenotype of a *pilY1* mutant, we used a genetic approach assessing P*fimU*-*lacZ* reporter activity in a limited number of mutants known to affect Tfp biogenesis. However, none of the Tfp assembly mutants we tested resulted in *fimU* upregulation to the same degree observed in a *pilY1* mutant. In the future we will take a more comprehensive approach and conduct a screen to identify genes involved in *fimU* dysregulation. We will create a transposon (Tn) insertion mutant library in a *vfr* mutant containing the chromosomal P*fimU*-*lacZ* reporter and screen for clones with enhanced activity (i.e. darker blue vs. parent strain), thus mimicking the phenotype of the *pilY1* mutant. We chose the *vfr* mutant background for the following reasons: i) to increase the sensitivity of the screen as *vfr* mutant P*fimU*-*lacZ* reporter activity is less than that of wild-type, ii) to help identify Vfr-independent regulation that contributes to the *pilY1* mutant phenotype, and iii) to eliminate Tn hits in genes that increase Vfr expression/activity. In this screening scheme, we would expect that Tn insertion into other genes within the *fimU* operon would yield the correct phenotype, and therefore serve as a positive control. Given that there are five potential targets within this operon, a significant proportion of Tn hits may map to this region. To rule out these candidates, we will determine if the Tn mutant phenotype can be complemented by plasmid-encoded *fimU*-*pilE*. As an alternative screening approach, we could use a *pilY1* single mutant and screen for Tn mutants that restore P*fimU*-*lacZ* reporter activity to wild-type levels. Because *algZR* mutation

in the *pilY1* mutant background abolishes P*fimU*-*lacZ* reporter activity, we expect Tn mutants

in this screen to map to the *algZR* region. For each these screens we would utilize a

"promoter out" transposase (Goodman, 2004), which allows for both insertional

inactivations and enhanced activation, as we expect that enhanced expression of a

transcriptional regulator may be necessary for *fimU* upregulation. With either screening

approach we envision two categories of Tn hits: i) transcriptional regulators that enhance

P*fimU*-*lacZ* activity, which may include transcriptional regulators that control *algZR*

expression, and ii) those that indirectly recapitulate the *pilY1* mutant phenotype, which will

likely include genes encoding structural proteins that simulate the signal detected in a *pilY1*

mutant. Although the set of Tfp assembly mutants tested in our study did not identify any

mutants that fully phenocopied the *pilY1* mutant phenotype, we hypothesize that Tn hits in

additional genes are likely involved in Tfp biogenesis.

One possible clue for the mechanism responsible for *fimU* dysregulation in the

absence of PilY1 is that, in many ways, this pathway parallels the stress response triggered

by off-pathway P pili proteins in *E. coli*. In this system, the TCS consisting of the sensor

kinase CpxA and the response regulator CpxB senses misfolded P pilus assembly

intermediates and subsequently activates expression of both assembly and regulatory

factors necessary for P pilus expression (Cosma, 1995). Additional reports have

demonstrated a role for the Cpx stress response in expression of the type IV bundle-forming

pili of enteropathogenic *E. coli* (EPEC) (Nevesinjac, 2005). Taken together, these findings

suggest that Cpx stress response activation may play a universal role in the expression of

surface-localized organelles. We hypothesize that the phenotypes of *pilY1* and *fimU* operon

mutants reflect an "off-pathway" response, where mislocalization or inability to form a complex triggers a stress response resulting in upregulation of Tfp biogenesis components.

Our finding that *fimU* dysregulation is mediated by AlgZR may provide additional support for the hypothesis that this regulation is linked to stress response in *P. aeruginosa*. AlgR has been implicated in the response to heat-shock and osmotic stress in microarray studies showing AlgR-dependent expression of multiple targets (Lizewski, 2004). In addition, expression of *algR* was altered independently of *algZ* expression by the extreme heat shock sigma factor, AlgU (Martin, 1994; Schurr, 1995). Although we cannot know at present if the stress signals regulating *algZR* in these cases are related in any way to the signals inducing *fimU* dysregulation, it will be of interest to evaluate the involvement of factors from the various stress response pathways.

Conclusion

Taken together this work demonstrates that the proteins encoded by the *fimU* operon are necessary for Tfp biogenesis. Additionally, the *fimU* operon is subject to complex regulation and dysregulation. The regulation of these accessory proteins differs from the regulation of the structural protein, PilA.

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