CHARACTERIZATION OF THE PILS-PILR TWO COMPONENT SYSTEM

CHARACTERIZATION OF THE PILS-PILR TWO COMPONENT REGULATORY SYSTEM OF *PSEUDOMONAS AERUGINOSA*

By

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TITLE: Characterization of the PilS-PilR Two Component Regulatory System of *Pseudomonas aeruginosa*

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To the friends and family that got me here—thank you.
This is for you.

LAY ABSTRACT

Pseudomonas aeruginosa is a Gram negative bacterium and a common cause of hospital acquired infections. The World Health Organization recently ranked *P. aeruginosa* as one of the top "priority pathogens" for which new treatments are desperately needed, in part due to its intrinsic resistance to many antibiotics. Among the key features that contribute to the infectivity of *P. aeruginosa* are its Type IV pili (T4P), which are flexible, retractile surface appendages involved in cell surface attachment, movement across solid surfaces and other important functions. Production of the major pilin protein, PilA, which forms most of the pilus, is tightly controlled by the two-component regulatory system, PilS-PilR, where PilS is a sensor and PilR is a regulator that directly controls pilin expression. The aim of this work was to identify the signal(s) detected by the sensor, as well as additional genes or systems under PilSR control. We showed that the pilin protein interacts directly with the sensor to control its own expression, and that dysregulation of the PilS-PilR two-component system impairs both pathogenicity and other forms of motility. Together, the data presented here provide insight into how PilS-PilR control expression of systems required for virulence of *P. aeruginosa* and highlight the potential of these proteins as possible therapeutic targets.

ABSTRACT

Two-component regulatory systems are an important means for most prokaryotes to adapt quickly to changes in their environment. Canonical systems are composed of a sensor kinase, which detects signals that trigger autophosphorylation, and a response regulator, which imparts changes within the cell, usually through transcriptional regulation. The opportunistic pathogen, *Pseudomonas aeruginosa,* expresses a plethora of two-component systems including the PilS-PilR sensor-regulator pair, which directs transcription of the major component of the type IV pilus (T4P) system, *pilA,* in response to an unknown signal. T4P are surface appendages that are required for full virulence, as they perform several important functions including twitching motility, cell surface attachment, surface sensing, and biofilm formation. While loss of pili is known to decrease virulence, the effect of surplus surface pili on pathogenicity was unknown. In other T4P-expressing bacteria, PilR regulates the expression of non-T4P related genes, but its regulon in *P. aeruginosa* was undefined. Here, we identify PilA as an intramembrane signal for PilS, regulating its own expression. When PilS-PilR function is altered through the use of activating point mutations, which induce hyperpiliation, pathogenicity in *C. elegans* was significantly impaired compared to both wild type and non-piliated strains of *P. aeruginosa.* This phenotype could be recapitulated using other hyperpiliation-inducing mutations, providing evidence that over production of surface pili likely prevents productive engagement of contact-dependent virulence factors. Last,

transcriptomic analyses revealed that expression of over 50 genes – including several involved in flagellar biosynthesis and function – is modulated by PilSR, suggesting coordinate regulation of motility in *P. aeruginosa*. Together, this work provides new information on the control of *pilA* transcription and suggests novel roles for surface pili and the PilSR two component system in virulence and swimming motility, respectively. The knowledge gained from this work could be applied to the development of a PilS or PilR based anti-virulence therapeutic.

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Ap	ampicillin
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
BME	β-mercaptoethanol
bp	base pair
BACTH	bacterial adenylate cyclase two-hybrid
c-di-GMP	3', 5'-cyclic-di-gluanylate
C-terminal	carboxyl-terminal
cAMP	cyclic adenosine monophosphate
CFU	colony forming units
cyt	cytoplasmic
Da	Dalton
DNA	deoxyribonucleic acid
EBP	enhancer binding protein
FRT	flippase recognition target
g	gravity
Gm	gentamicin
GS	*Geobacter sulfurreducens*
h	hour(s)
IM	inner membrane
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Kn	kanamycin
LB	Lennox broth
min	minute(s)
N-terminal	amino-terminal
NBT	nitro-blue tetrazolium
NGM	nematode growth media
OD	optical density
OM	outer membrane
ONPG	ortho-Nitrophenyl-β-galactoside
PA	*Pseudomonas aeruginosa*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDB	Protein Data Bank
PEG	polyethylene glycol
PG	peptidoglycan
pH	power of hydrogen
PIA	*Pseudomonas* isolation agar
PP	periplasm
RR	response regulator
RNA	ribonucleic acid
RNAseq	ribonucleic acid sequencing
s	second
SDS	sodium dodecyl sulfate
SK	sensor kinase
TCS	two-component system
T3S	type III secretion
T4P	type IV pili/pilus
TM	transmembrane segments
WT	wild type

CHAPTER ONE

Introduction

Overview

Pseudomonas aeruginosa was first described in 1872 (1) as a ubiquitous environmental bacterium, capable of colonizing a wide range of niches including soil, water and plants and later, abiotic surfaces such as catheters and contact lenses (2). Since its first description, *P. aeruginosa* has become better known for its role as an opportunistic pathogen and common cause of nosocomial infections in immunocompromised patients, and particularly those with cystic fibrosis (3, 4). Its intrinsic antibiotic resistance to many frontline antibiotics used in the clinic and its propensity to form biofilms both within a host and on medical equipment make *P. aeruginosa* difficult to treat (5-7). The World Health Organization (WHO) recently listed it as a "priority pathogen" for which new treatments are needed (8).

One of the key attributes *P. aeruginosa* uses to adhere to surfaces and cause infections in a host is the Type IVa pilus (T4P) system (6, 9-12). T4aP are long, flexible, retractile surface fibres that extend from the poles of *P. aeruginosa* **(Figure 1.1A).** They can also be found on other Gram negative (13-15) and positive species (16), as well as on some archaea (17). Some bacterial species carry the less well characterized T4bP system, which predominates in enteropathogenic bacteria such as *Salmonella enterica* (18). T4a and T4b pili are distinguished from one another by differences in the major pilin subunits, particularly in the leader sequences of the pre-pilin (19) and in the proteins that make up the assembly systems. This work focuses on the T4aP system, which will be referred to as T4P throughout, for simplicity.

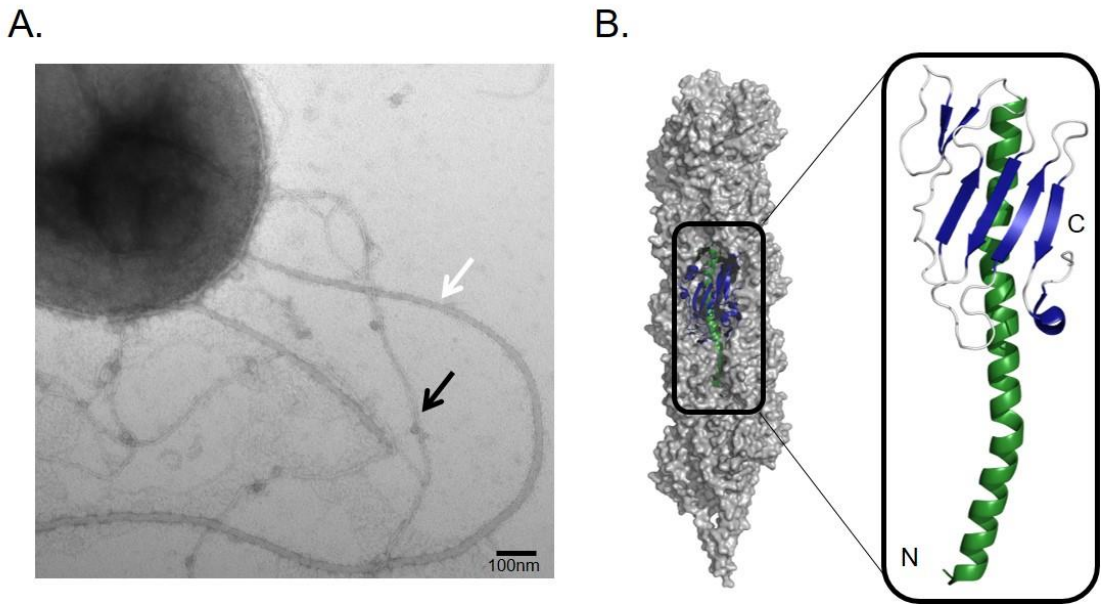

Figure 1.1 Type IVa pili of *P. aeruginosa* A) Negative stained transmission electron micrograph of polar surface pili (black arrow), in contrast to the single, thicker polar flagellum. Magnification: 150000x **B)** The pilus is a helical fibre (PDB ID: 1OQW) composed of hundreds of subunits of the major pilin protein (PDB: 2HIL) arranged with the hydrophobic N-terminus (green) forming the pilus core and the globular C-terminal head groups (blue) decorating the exterior.

T4P have many important functions including being a major contributor to infectivity and pathogenicity, thus the T4P system is an attractive target for future antivirulence strategies, but a clearer understanding of how pilin expression is controlled will be required.

Type IV pilins

The pilus fibre is composed predominantly of repeating subunits of the major pilin protein, PilA, assembled into helical fibre (20, 21) **(Figure 1.1B)** and to

a much lesser extent, a collection of minor pilin-like proteins, so named because of their low abundance relative to PilA (22). *P. aeruginosa* strains in particular will express one of 5 major pilin types (groups I-V), which are categorized by the size and sequence of the C-terminal head groups of the pilin and the presence or absence of accessory proteins located immediately downstream of the *pilA* gene (23, 24). Most common lab strains, including PAK, belong to group II and require no accessory proteins. Groups 1 and 3-5 require either one or two accessory proteins to facilitate post translational modification of the pilin (24, 25).

Pilin proteins share a conserved overall lollipop-like architecture, in which the N-terminus forms a highly conserved, hydrophobic α-helix—the lollipop stick—and the C-terminus forms a more variable globular domain composed of 4-7 antiparallel β-strands (24, 26-28). One exception is the short pilins from *Geobacter* species, which lack a C-terminal head group (29). Surface pili in *G. sulfurreducens* are conductive and therefore, the presence of a truncated pilin may allow for more efficient electron transfer along the length of the fibre (30).

Characteristic of most pilin-like proteins is the C-terminal disulfide bonded loop that can vary in length across different strains, and thus can be used as an identifier for different pilin types, and is essential for normal pilus assembly and function (31, 32). The disulfide bonded loop was reported to confer adhesive properties to the pilin subunits, but when part of an assembled fibre, only those loops at the tip of the pilus are exposed for adherence (33). Newer data however, using atomic force microscopy pulling experiments, suggest that the pilus fibre

itself has adhesive properties along its length, particularly to hydrophobic surfaces (34, 35).

The minor pilins (MP) mentioned above, FimU, PilV, PilW, PilX and PilE contribute indirectly to adherence to surfaces as well, though they are not yet known to be adhesive themselves. Instead, they form a tip complex at the distal end of the pilus fibre that not only primes pilus assembly (22, 36) but also promotes extracellular display of the putative adhesin and surface sensor PilY1, which is co-transcribed with the MPs in a polycistronic operon **(Figure 1.2A)** (11, 36-39). While the MPs are structurally similar to the major pilin protein, they are functionally distinct and are present at lower amounts than the major pilin. PilVWX are thought to form a core complex that provides a scaffold for PilY1, while FimU and PilE act as connectors to bridge the MP complex to the polymerized major pilins in the fibre (28, 36). PilVWX are also capable of negatively regulating swarming motility (40), and contributing to virulence independent of their role in T4P biogenesis (Marko, V *et al.* In preparation).

The N-terminal α-helix of PilA and the MPs can be further divided into two subdomains, α1-N, containing highly conserved hydrophobic residues 1-28 of the mature pilin, and α1-C, residues 29-52 (21). The α1-C segment is amphipathic and tends to be less conserved than the nearly invariant α1-N domain, possibly due to being embedded in the highly variable C-terminal globular domain of the protein, or as a result of needing to perform alternative functions. For example, the α1-C segment of *G. sulfurreducens* is comparatively rich in aromatic amino

acids that are essential to the bacterium's ability to transfer electrons from cellular respiration to a final external electron acceptor (41).

For a short sequence, the α1-N region of the pilin performs an impressive number of functions. The first ~25 amino acids of PilA are near invariant across most T4P-expressing bacteria, suggesting a conserved role for this region. The α1-N region of multiple pilin subunits forms the hydrophobic core of the pilus fibre, and the C-terminal head groups to decorate the pilus exterior (21, 42) **(Figure 1.1B).** It also functions as a transmembrane (TM) segment, storing pilin subunits in the inner membrane to be used in repeated rounds of pilus extension (43-45) and as we highlight here, α1-N plays an important regulatory role in pilin autoregulation (46, 47).

The major pilin proteins of both *P. aeruginosa* and *N. gonorrheoae* display an S-shaped kink in their N-terminal helices **(Figure 1.1B)** (26, 48) due to proline and glycine/proline residues at positions 22 and 42, respectively (48, 49). This domain controls the extent of packing of PilA subunits in the pilus, ensuring the formation of a flexible fibre (26), efficient disassembly (50) and may contribute to the tilted angle of the N-terminal helix of the major pilin when it is anchored in the inner membrane to allow for easier extraction from the membrane during pilus assembly (43). More recent computational analyses have also indicated that the flexibility in the α1-N domain provided by residue P22 allows for stretching of the α1-N domain during the application of force to the pilus fibre as a possible means of mechanosensing (51).

The major pilin protein is expressed first as a prepilin, with a short, positively charged leader sequence including a conserved -1 Gly residue that is removed by the prepilin peptidase PilD during processing (52, 53). PilD then methylates the +1 residue, normally a phenylalanine (54). These steps are essential for pilin polymerization. This type of leader, cleaved at the cytoplasmic face of the membrane, has been designated 'type III' to distinguish it from those of other secreted or inner membrane proteins (type I) or lipoproteins (type II) both of which are cleaved at the exterior face of the membrane. The invariant Glu residue at position +5 relative to the PilD cleavage site (32) plays an essential role in pilus assembly (54), a supplementary role in N-terminal methylation after cleavage (55) and a role in regulation of *pilA* transcription (46, 47). It does not, however, affect cleavage by PilD (55).

The Type IV pilus assembly system

Production of a T4P requires the coordinated effort of over 40 gene products that are spread throughout the genome in several clusters **(Figure 1.2A)**. Together, these products form a megadalton sized cell envelope-spanning complex that enables pilus extrusion. High resolution crystallographic studies on a complex this large remain a challenge, but recent reports using electron cryo-tomography of *M. xanthus* and *Thermus thermophilus* revealed for the first time the structures of T4P assembly complexes in both open and closed states (56, 57).

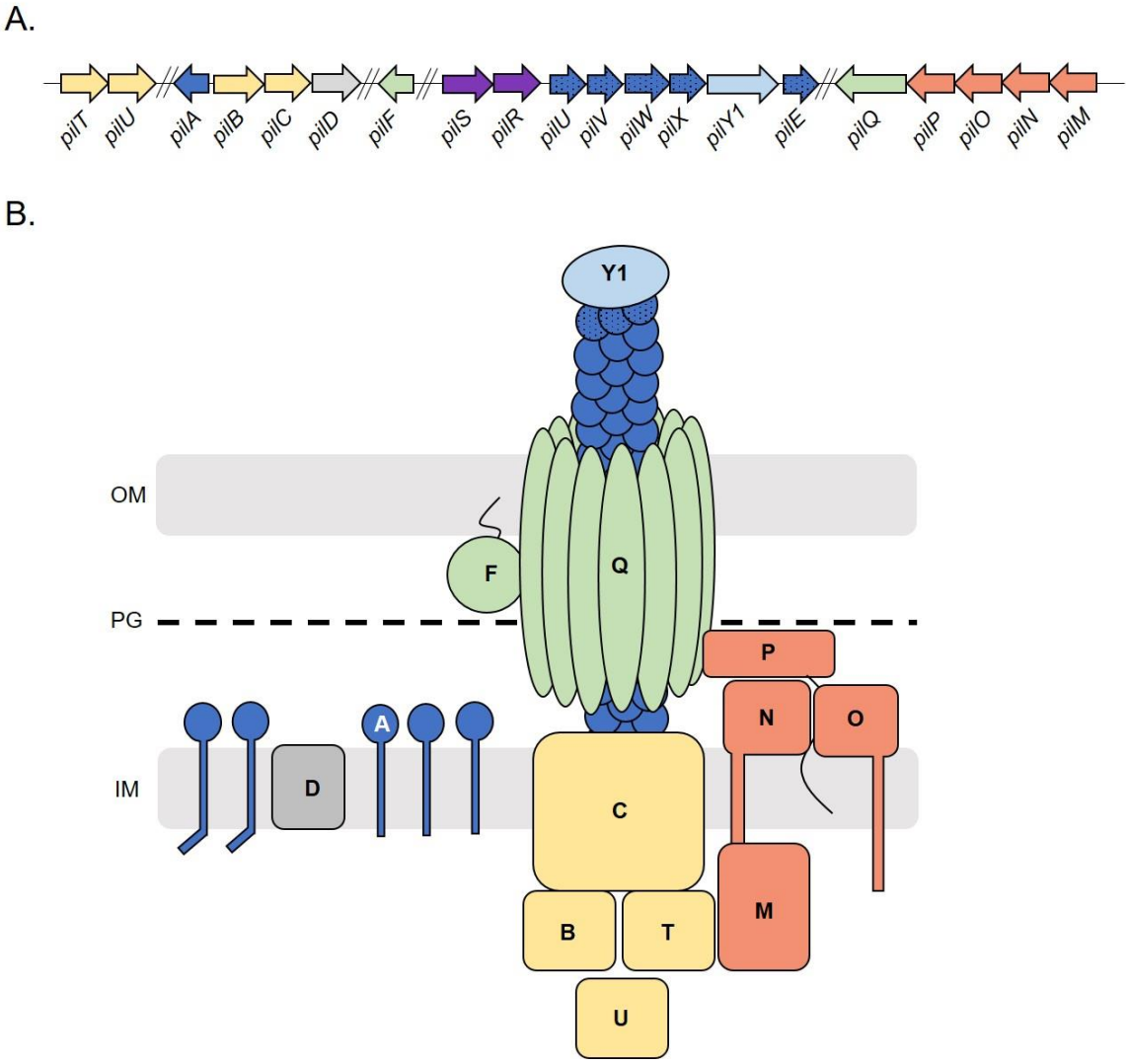

Figure 1.2 The T4P system is composed of >40 gene products. **A)** A simplified schematic of the genetic organization of select T4P genes that comprise the assembly and regulatory systems demonstrates genetic distribution throughout the genome. Line breaks denote large spaces between gene clusters. **B)** The simplified cartoon schematic of the T4P assembly system highlights the 4 main subcomplexes: i) the pilus (blue), ii) the outer membrane secretin (green), iii) The inner membrane alignment subcomplex and iv) the motor subcomplex. Genes and proteins are not drawn to scale.

Using a combination of deletion mutants and modelling with previously determined crystal structures of the assembly complex components, both studies were able to assign densities in the tomograms to specific T4P assembly proteins and to identify differences in overall topology of the T4P system when the pilus fibre was (open), or was not (closed) present. In *T. thermophilus*, some of the conformational changes made by the system to make room for an extending pilus fibre could be discerned. In particular, the monomers of the outer membrane secretin displayed a 30Å shift in the N-terminal domains in *T. thermophilus* (57) and the cytoplasmic ATPases that power pilus assembly and retraction alternate binding with the remainder of the T4P system, depending on whether the pilus is extending or retracting (56, 57). These studies provided tremendous insight into the organization and stoichiometry of the T4P systems of their respective species, but have also revealed previously undefined conformational changes that occur between the open and closed states of the system.

A typical T4aP system **(Figure 1.2B)** is composed of 4 subcomplexes; i) the pilus fibre itself (blue), ii) the outer membrane secretin subcomplex (green), iii) the inner membrane alignment subcomplex (red) and iv) the motor subcomplex (yellow). Universal nomenclature for the T4P assembly system components has not been developed, so for simplicity, the *P. aeruginosa* nomenclature will be used throughout this report.

The pilus fibre is a flexible surface fibre composed predominantly of polymerized PilA subunits (20) plus a small number of minor pilins that form a

putative tip complex to promote surface display of the adhesin-like protein, PilY1 (22, 36, 58). T4P are typically 5-7nm in diameter and can extend up to 1-2µm in length. The PilA subunits are tightly packed, forming a helix with approximately 4 subunits per turn, though the kink in the N-terminus produced by residue P22 of PilA likely provides additional flexibility to the fibre (21, 59). The conserved N-termini of PilA subunits pack together in the fibre with the positively charged, methylated F1 neutralizing the charge of the invariant E5 residue of the adjacent subunit, creating a hydrophobic core, ensuring proper alignment of sequential subunits, and resulting in helical assembly of the pilus (48, 60).

During pilus assembly pilin subunits, which are housed in the inner membrane, are inserted at the base of the growing fibre, causing extrusion of the pilus out of the cell as more subunits are added. Conversely, during pilus retraction, pilin subunits are removed from the base of the fibre at a rate of 1000 subunits s^{-1} and returned to the inner membrane for use in future extension cycles (44). The pilus itself is thought to have some adhesive properties (33-35, 61) but also promotes surface display of the putative tip adhesion, PilY1 (12).

To enable pilus extrusion through the peptidoglycan (PG) and the outer membrane, an outer membrane secretin complex is required. In most T4P expressing gram negative bacteria, PilQ fills this role. In *M. xanthus* (56), *T. thermophilus* (57), and *N. meningitidis* (62), the secretin is a 12-14 subunit ring of PilQ monomers that assemble in the outer membrane to form a gated 60-80Å pore through which the pilus can extend. Interestingly, recent structural analysis

of the *P. aeruginosa* PilQ secretin using cryoelectron microscopy revealed that it is composed of 14 PilQ subunits (63) and gated on the periplasmic side of the pore by N-terminal loop regions that can be displaced towards the walls of the pore upon pilus assembly (63). It is currently unknown however, whether displacement of the loops is a single occurrence upon initiation of pilus assembly or if it has a dynamic role in pilus "ratcheting" during extension. Outer membrane targeting and multimerization of PilQ is dependent on the pilotin lipoprotein, PilF (64, 65). Retargeting PilF to the inner membrane can result in PilQ multimer formation in the inner membrane.

PilQ is mostly periplasmic with its C-terminal region, known as the secretin domain, embedded in the OM. The periplasmic region contains 4 subdomains; 2 N-terminal AMIN domains which have been identified as putative septal PG binding domains; N0; and N1, which is required for interactions with the inner membrane alignment subcomplex via PilP (66).

Recent work revealed that the AMIN domains of PilQ are required for polar recruitment and localization of PilQ and its partners to future sites of cell division, where they are preinstalled in nascent poles during synthesis (67). Recruitment of the alignment subcomplex protein, PilO to the septum was dependent on the presence and proper localization of PilQ, suggesting that similar to in *M. xanthus*, assembly and recruitment of the T4P system in *P. aeruginosa* is dependent on proper localization and integration of the outermost components, rather than the inner (68). In *M. xanthus*, the authors proposed a model in which the alignment

subcomplex members docked onto a fully formed outer membrane secretin, while in *P. aeruginosa*, data supports association of the inner and outer membrane components at the poles, prior to PilQ being flipped to the OM.

The alignment subcomplex of *P. aeruginosa* consists of PilMNOP (69), where PilP is an inner membrane lipoprotein that interacts with both PilQ and the bitopic inner membrane proteins PilN and PilO (70). PilQ interacts directly with the C-terminal domain of PilP through its N0 domain (70). While the alignment subcomplex may align the motor complex with the OM secretin to maximize T4P assembly efficiency as the name suggests, it also modulates T4P retraction, as specific point mutations in the coiled-coil regions of PilNO caused hyperpiliation while abrogating twitching motility, similar to other retraction deficient mutants (71). Cysteine crosslinking analysis revealed that PilN and PilO form a tetramer of homo- and heterodimers in vivo and that dynamic rearrangement of their interaction interfaces is important for normal T4P function (71).

The N-terminus of PilN extends into the cytoplasm and interacts with the only cytoplasmic member of the alignment subcomplex, PilM (72). PilM is an actin-like protein with structural similarities to the cell division protein FtsA (73). In addition to binding to the N-terminus of PilN, PilM interacts with members of the motor complex, PilC, PilB and PilT (72).

The motor complex is made up of PilC, PilB, PilT and PilU. PilC is the inner membrane platform protein on which the pilus fibre is assembled and

disassembled, and in *P. aeruginosa,* is essential for T4P expression. Loss of *pilC,* even in a retraction-deficient *pilT* background, results in complete abolishment of detectable surface pili (74). PilU is also thought to be an ATPase that helps modulate pilus retraction and may localize to the leading poles of twitching cells. However, the exact role of PilU remains elusive (75) Similarly, in *M. xanthus*, the assembly ATPase PilB interacts with both the platform protein, PilC and the alignment subcomplex protein, PilM (76). Upon binding to the platform, which may be mediated by conformational changes in PilM, PilB is able drive clockwise rotation of PilC in a manner that is thought to support the sequential addition of pilins to a right handed helix, while PilT, when bound to PilC, pulls down on the membrane domains of PilC, causing it to rotate counter clockwise, facilitating depolymerisation of a right handed helical pilus (77, 78). However, the signal that allows the ATPases to bind and release PilC remains elusive, but could perhaps involve connection with the putative pilus length control protein FleP (79). Together, the ATPases and other proteins in the T4P system work in a tightly regulated, coordinated fashion to produce retractile T4P that can perform many biological functions.

Functions of the Type IV pilus system

T4P have a wide range of biological functions in both environmental and pathogenic bacteria (reviewed in (27)), including enabling bacteriophage infection (80), promoting DNA uptake (81), transfer of electrons in *Geobacter* species through a chain of aromatic residues in the α1-C domain, unique to *Geobacter*

pilins (30) and often serving as a notable virulence factor (12, 14, 82-85) for pathogenic bacteria like *P. aeruginosa, K. kingae* and *Neisseria* species. In *P. aeruginosa* especially, pili must be both present and fully functional to promote full pathogenicity as loss of the retraction ATPase *pilT* results in a cell that still expresses pili but is also reduced in its ability to adhere and infect a host (10, 86, 87).

T4P promote attachment to a wide breadth of surface types, both biotic and abiotic surfaces through the pilus itself and the adhesin, PilY1, as described above (2, 14, 84, 88, 89) and also support the initial steps of biofilm formation and maintenance (6, 13, 83, 89). However, considering the diversity in T4P major pilin sequences, the exact mechanism and properties of PilA or PilY1 that allow for adherence to varied surfaces remain elusive. These adhesive properties enable T4P to perform two of the functions most pertinent to the work presented herein; twitching motility and surface sensing.

Twitching motility is driven by retraction of the pilus fibre (90) and is preferentially activated when the bacteria are present on solid or semi-solid surfaces (91). Twitching motility results from the coordination of three key steps; extension, adherence and retraction. Using energy derived from the assembly ATPase, PilB, pilins from the inner membrane can be added to the base of the growing fibre (76, 77, 92), extending it outwards at the pole of the cell. Pili can be retracted without adhering to a surface but these unattached pili will not facilitate twitching motility. Upon attachment of the pili to a surface, the retraction ATPase,

PilT provides energy to begin disassembling the pilus fibre from the base, returning pilin subunits to the inner membrane (44, 90). Retraction of the fibre pulls the cell body towards the point of pilus attachment, making the T4P system function much like a molecular grappling hook. While an individual pilus fibre is reasonably strong, multiple fibres can bundle and retract in a coordinated fashion to increase retraction forces to upwards of 100pN (93), making the T4P system one of the strongest molecular motors identified to date.

More recent studies have identified a potential new role for T4P as surface sensors (9, 11, 38, 94). Upon initial attachment to a surface, intracellular levels of the messenger molecule cAMP are increased, dependent on both the Chp chemosensory protein PilJ and functional T4P. Increased cAMP results in increased expression and surface exposure of PilY1, dependent on T4P assembly. It is thought that PilY1, which has a motif analogous to the mechanosensing Von Willebrand Factor domain, undergoes shear forces that result in the deformation of this domain which signals in an unknown manner through the pilus and alignment subcomplex to the SadC diguanylate cyclase, which upregulates a second messenger molecule, c-di-GMP. Increased c-di-GMP levels promote a transition from a motile to sessile state by increasing expression of genes associated with biofilm formation and repressing those involved in T4P and flagellar dependent swarming motility (38, 95).

Under shear forces, the pilus and pilins themselves can undergo significant deformation, exposing new epitopes not normally exposed on the

exterior of the fibre (96). Persat et al. (9) proposed that chemosensory proteins in *P. aeruginosa* may detect these deformed pilins as a means of sensing surface attachment.

To drive an increase in c-di-GMP production in response to surface attachment or external shear forces, T4P must be functional, as a retraction-deficient *pilT* mutant of *P. aeruginosa* was no longer able to upregulate c-di-GMP production either through attachment to a surface or the application of mechanical shear forces (94). Thus, cell body-surface contact is required for signal transduction, or in the absence of pilus retraction, deformed pilins and/or PilY1 fail to signal surface attachment through intracellular proteins. While the details of surface-associated signal cascades remain under investigation, the end result of surface attachment is the upregulation of several virulence factors, aiding in the persistence of *P. aeruginosa* in a host (9, 11).

Two Component System Regulation

Two component system (TCS) regulation is a ubiquitous way for bacteria, archaea, fungi and some higher order plant species to rapidly respond to changes in their intra- or extracellular environments and are used to regulate a wide variety of cellular functions [reviewed in (97)]. *P. aeruginosa* in particular, expresses a higher-than-average number of TCSs, a significant number of which have been implicated in various virulence pathways for the bacterium (98, 99).

As the name suggests, two-component systems have two main elements, a sensor histidine kinase (SK) and a response regulator (RR). Both partners typically function as homodimers, although in special cases, such as the GacS-RetS-LadS-PA1611 system, different sensor kinases can form functional heterodimers to integrate signals from various pathways to fine-tune virulence factor expression and the transition from motile to sessile lifestyles (100, 101). The SK detects an environmental signal and passes the information to the RR through a phosphorelay. The RR then interacts with its substrate, usually DNA at

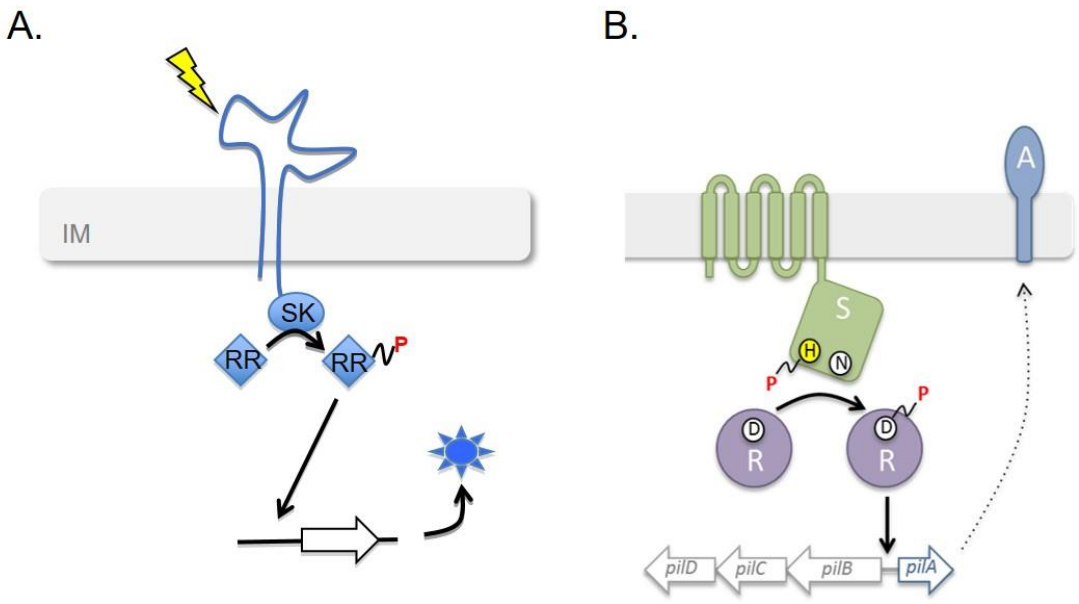

Figure 1.3. Comparison of a canonical TCS with the PilS-PilR system. A) The SK of a canonical TCS has 2 TM segments connected by a large periplasmic loop for signal input. Upon stimulation, the SK is autophosphorylated and activates its RR by phosphorylation to regulate gene expression. **B)** PilS differs from the usual SK in that it has 6 TM segments connected by very short loops. The "extra" TM segments may be used for signal input, to then allow for PilR phosphorylation and activation of *pilA*

promoter element. Generally, the phosphorylated RR is the active form, though there are cases where the unphosphorylated RR is active (102-104).

In a typical TCS **(Figure 1.3A)**, the SK is an inner membrane protein with 2 TM segments connected by a large, periplasmic loop, which often serves as the site of signal input. These elements are followed by a relatively conserved cytoplasmic N-terminus that contains a kinase domain and ATP binding domain (97, 105). Upon signal detection, the SK autophosphorylates on a conserved His residue adjacent to its ATP-binding domain. Whether phosphorylation occurs from the same (in cis) or opposing (in trans) monomer of the SK dimer is dependent on the organization of the cytoplasmic helix bundle loops flanking the conserved histidine residue (106). The high energy phosphate is usually transferred directly to a conserved Asp residue on the RR, although some systems contain intermediate steps in the phosphorelay which increase its dynamics in response to various signals (97). Upon phosphorylation, the RR, which can bind to specific DNA sequences regardless of its phosphorylation state through a DNA-binding helix-turn-helix domain, becomes active and through interactions with the alternate sigma factor σ^{54} (RpoN) and the RNA polymerase holoenzyme, activates transcription of specific genes in response to the initial stimulus (97).

While most SKs detect periplasmic signals, some SKs are cytoplasmic and therefore detect intracellular signals. For example, CheA and NtrB are soluble cytoplasmic chemotaxis sensor and nitrogen-uptake regulatory sensors,

respectively, and likely respond to intracellular cues and signals (107, 108). Similarly, FleS is predicted to be a cytoplasmic SK that controls flagellar gene expression and likely detects intracellular signals issued by FleQ (79, 109). Conversely, there are some sensor kinases, usually with more than 2 TM segments, that detect intramembrane signals. These SKs are more common in Gram positive bacteria, which lack an outer membrane, and often detect perturbations to the membrane itself (110, 111). As will be described in the following section, PilS of *P. aeruginosa* is also an intramembrane-sensing SK (46).

In contrast to the signal input domain, which is highly variable due to the diversity of signals that can be detected, the histidine kinase domain has a higher degree of conservation. All kinase domains of SKs contain a conserved His that functions as the site of ATP-dependent autophosphorylation (97). The His residue is typically located in the cytoplasm within a dimerization interface. Immediately adjacent to the site of phosphorylation in many families of SKs is a conserved phosphatase motif. In HisKA family SKs, the motif is D/ExxN/T, where amino acid substitution at the first residue disrupts both kinase and phosphatase activity, while mutation of the N/T residue disrupts only phosphatase activity (112). Dual function SKs allow for fine-tuning of the cognate RR activity.

In the classical two-component system, RRs are cytoplasmic proteins that function as transcriptional activators. In general, they can be divided into a receptor (or regulatory) domain and an effector domain. The receptor domain

contains a conserved Asp residue, which acquires a phosphate group from the His residue of its cognate SK. Phosphorylation at this site results in a conformational change in the protein, within the effector domain that enables activation of the RR (113). Interestingly, despite a high degree of conservation in the receptor domain of RRs and the kinase domains of many SKs, these are also the regions that are critical for insulation of TCS pairs from unwanted cross talk from other systems. Specific amino acids in these regions add specificity for the cognate SK or RR (114).

Regulation of *pilA* transcription

Overview

The use of T4P can be energetically costly, as several hundred to thousands of pilin subunits are required for each pilus fibre and 2 molecules of ATP appear to be required for the addition or removal of each (77). As described above, *P. aeruginosa* and presumably many other T4P-expressing bacteria reduce this cost by recycling pilin subunits (44) and by using predominantly "inexpensive" amino acids in the synthesis of the pilins (27, 115). A third method for reducing the metabolic cost associated with twitching motility is to tightly control expression of the pilin protein itself.

Transcription of the major pilin gene, *pilA*, is strictly regulated by the PilSR TCS in *P. aeruginosa* (116, 117). PilSR functions similarly to a canonical TCS in that PilS, the inner membrane SK detects a signal and undergoes

autophosphorylation at a conserved cytoplasmic histidine residue, His319 (118). The high energy phosphate group can then be transferred to an aspartic acid, D54 on the cytoplasmic response regulator, PilR (119). Phosphorylation of PilR activates the protein, allowing it to work in concert with the alternative sigma factor RpoN (σ^{54}) and RNA polymerase to increase *pilA* transcription **(Figure 1.3B)**. Like most enhancer binding proteins (EBPs) or transcriptional activators, PilR binds to DNA upstream of the *pilA* promoter, even when inactive (unphosphorylated), but is generally incapable of separating the DNA strands to enable transcription until it is phosphorylated (120).

Most other T4P expressing bacteria including *M. xanthus* (47), *K. kingae* (121), *G, sulfurreducens* (103), *D. nodosus* (122) and many commensal *Neisseria* species (123) use PilSR to regulate transcription of *pilA*, though the mechanism of action can vary. For example in *M. xanthus*, PilS is a negative regulator of *pilA* transcription as deletion of the *pilS* gene results in a PilA-overproducing strain (47). In *P. aeruginosa*, PilS is a positive regulator whose loss abolishes *pilA* transcription (116). This difference indicates that while PilS from these two species may have opposing effects, strict regulation of *pilA* transcription is necessary, as over- or under-producing PilA could have negative consequences. PilS from *P. aeruginosa, M. xanthus, K. kingae* and *D. nodosus* each share between 24-37% identity, mainly in the conserved kinase and ATP binding domains (103, 124). However, as we describe in Chapter 2, PilS from these species is likely able to interact directly with a conserved segment of PilA to

allow for pilin autoregulation (46, 47, 125), thus the interaction likely relies on key structural features of PilS rather than specific amino acid contacts.

PilSR in pathogenic *Neisseria* species present an interesting exception to the paradigm of *pilA* transcriptional regulation. While commensal *Neisseria* species use the standard PilSR system for controlling *pilA* transcription (123), most pathogenic *Neisseria* species still contain remnants of the *pilS-pilR* TCS in their genome that can regain functionality when transferred to *P. aeruginosa* (126). However, the system is inactive, with transcription of the pilin gene *pilE* under σ^{70} control instead (123, 127, 128). This organization may be beneficial for obligate pathogens that require constitutive expression of pili for colonization (129).

The sensor kinase PilS

PilS is a 59 kDa inner membrane sensor histidine kinase (116) and is a dual function enzyme, acting as both a kinase and a phosphatase on its cognate response regulator, PilR (118). In contrast to the canonical SK described above, which typically contains 2 TM segments connected by a large periplasmic loop region, topological analyses of PilS have revealed that it has 6 TM segments (5 in the case of *G. sulfurreducens*) with both N- and C- termini in the cytoplasm (130) **(Figure 1.3B)**. The TM segments are connected by very short loops on both the periplasmic and cytoplasmic faces. This atypical topology coupled with lack of a large periplasmic domain suggested that PilS may detect either an intramembrane or cytoplasmic signal. In addition to the putative role of the TM

segments in signal detection, they are also required for integration of PilS into the inner membrane and are important but not absolutely required for polar targeting of PilS, as the TM segments can be substituted with those of MalG and still localize the protein to the poles (131). Following the TM segments is an extended cytoplasmic linker region that is critical for localization of PilS to the poles of *P. aeruginosa* (131). The linker region has also been hypothesized to transduce signals from the signal receiving domain in the inner membrane, to the cytoplasmic kinase domain, and perhaps to be a site for signal input (118).

While the complete mechanism for polar localization has not yet been determined, it was thought to be dependent on a *P. aeruginosa*-specific factor because expression of PilS in *E. coli* resulted in circumferential labeling (131). The putative hub protein FimV was recently identified as an important localization factor for PilS as in a *fimV* mutant, PilS exhibits circumferential localization. Interestingly, loss of the PG binding LysM motif in FimV partially disrupts this localization, suggesting that PilS localization relies on FimV's putative ability to bind septal PG (132). FimV is thus the first protein identified as being required for PilS polar localization, as loss of numerous other T4P components (*pilA, pilB, pilT, pilU, pilC, pilD, pilG, pilY1*), or the flagellin (*fliC*) had no effect (131).

Also in the cytoplasm following the linker are the conserved histidine kinase motif and ATP binding domains, characteristic of histidine kinases. The conserved His residue used for autophosphorylation is His319 and a putative D/ExxN/T phosphatase motif (in the ExxN form) is immediately adjacent at

residues 320-323 in *P. aeruginosa* (112, 124). Interestingly, while any amino acid substitutions at the conserved His319 site impair autophosphorylation and by extension, PilS kinase function, an H319P substitution also may disrupt PilS phosphatase activity (118). PilS is a positive regulator of *pilA* transcription in most T4P expressing bacteria, with the exception of *M. xanthus* (47), but its overexpression has a negative impact on pilin levels in *P. aeruginosa*. This effect is lost in PilS H319P but not other H319 substitutions (118). It was hypothesized based on these data that overexpression of PilS increases PilR dephosphorylation, which serves to repress *pilA* transcription. Overexpression of PilS and PilR together can also counter this phenotype (118).

The primary function of PilS is to phosphorylate PilR in response to a previously unknown signal and by extension, regulate the expression of the major pilin, PilA (116). However, *in vitro* kinase assays provided evidence that PilS phosphorylates 2 additional response regulator-like proteins (133), GcbA, a diguanylate cyclase that can facilitate biofilm dispersal and partially regulate swimming motility (134, 135), and the sensor-regulator hybrid, SagS, which also aids in biofilm formation and also the acquisition of biofilm-associated tolerance to antibiotics (136, 137). In addition to providing potential evidence for crosstalk *in vivo,* this also raises the possibility that PilS, and perhaps by extension PilR, control genes in addition to *pilA* and phenotypes other than twitching motility.

The response regulator PilR

PilR is the cognate cytoplasmic response regulator for PilS and controls the transcription of the major pilin gene (119). It is an NtrC-like transcriptional activator that when activated, works in combination with RpoN and RNA polymerase to transcribe the genes with in its regulon (119). Like most RRs, PilR is composed of two main domains; the N-terminal receiver, or REC domain, and the C-terminal regulatory domain (97, 119).

The REC domain contains the conserved Asp residue for phosphorylation by PilS, Asp54 (119) and bears strong similarity to NtrC, with ~71% similarity in the first half of the protein, and 62% overall (119). Despite the high similarity of the N-terminal region of PilR to NtrC, and numerous other NtrC family RRs, the REC domain has a critical role in insulating PilR from crosstalk from other non-cognate SKs, as described above (114). Key structural and amino acid differences allow PilR to receive only signals from its cognate SKs while overall sequence similarity between PilR and other NtrC family RRs alludes to a common mechanism for SK driven phosphorylation.

Following the N-terminal receiver domain of most NtrC-family proteins, including PilR, is an ATPase domain within the central region of the protein. This domain has been hypothesized to hydrolyze ATP when the RR is in its active, phosphorylated form. The energy derived from ATP hydrolysis allows for the opening of the DNA double helix at the site(s) where the RR is bound with the RNA polymerase holoenzyme to the promoter regions of the genes in the RR's

regulon (138). In the case of *P. aeruginosa pilA* this binding occurs from ~80 to 120bp upstream of the *pilA* start codon (120). DNA footprinting analyses in *P. aeruginosa* showed that PilR protected this region of DNA while leaving the -24 and -12 sites recognition sites for RpoN unobstructed (120). Within the 40 bp region, a consensus sequence was identified in *P. aeruginosa*. Mutational analyses revealed three repeats of a 5'-$(N)_{4-6}$(C/G)TGTC-3' consensus sequence on the non-coding strand each separated by ~10 bp, and a fourth NifA-like recognition site on the coding strand; 5'-TGT-$(N)_{11}$-ACA-3, each of which is required for *pilA* transcription (120). This consensus sequence was based solely on the *pilA* promoter region of *P. aeruginosa* and so while all 4 sites are required for binding at the *pilA* promoter, it is possible that other sequences, and numbers of motifs, can be tolerated for PilR binding at genes other than *pilA*.

Both the active and inactive forms of PilR – phosphorylated and unphosphorylated, respectively – can bind to the consensus sequence described above. For *P. aeruginosa* PilR and those from most other species, it is phosphorylation of PilR that allows for activation of transcription of the genes under its control of which upwards of 50 have been tentatively identified in *G. sulfurreducens* using DNA microarray and bioinformatics approaches (139, 140). Interestingly, in *G. sulfurreducens,* a PilR D54N point mutant was not phosphorylated, but when expressed *in trans*, supported higher than WT levels of *pilA* transcription, suggesting that in *G. sulfurreducens,* activation of gene expression is not entirely reliant on PilR phosphorylation (103). It is still possible

that PilR D54N could be phosphorylated at an alternate Asp residue, but this was not tested.

In the TCS field, it is rare that only a single gene is regulated by a TCS; instead, there are usually several genes being controlled like in the case of PilS-PilR in *G. sulfurreducens*. Many PilR-regulated genes were potentially involved in flagellar motility, but no phenotypic studies were performed to validate this finding. To further support the notion that PilR regulates additional, non T4P-associated genes, recent work in *Lysobacter enzymogenes* revealed a role for PilR in modulating intracellular c-di-GMP levels and by extension, controlling production of an antifungal compound (141). Prior to the work described in this thesis, no comparable studies had been performed in *P. aeruginosa* representing a gap in knowledge for how PilSR works and affects overall cellular function.

Hypothesis and Research Aims

T4P are key virulence factors for *P. aeruginosa* and a number of other bacterial species. In *P. aeruginosa,* studies in hosts from *C. elegans* to mice and humans have described the importance of PilA and surface pili in establishing infection. The PilSR TCS was identified almost 25 years ago in *P. aeruginosa, G. sulfurreducens, M. xanthus, D. nodosus,* and *K. kingae* as the system responsible for controlling transcription of the major pilin gene, *pilA.* In the intervening years, additional studies in *P. aeruginosa* defined PilS' 6 TM topology, its dual activities, and its polar localization, but the signal to which its responds, and the effects of dysregulating the activity of PilSR remained unclear.

In *G. sulfurreducens* and *D. nodosus,* microarray and bioinformatics studies identified non-T4P associated genes dysregulated by the loss of PilR but few phenotypic tests were performed and comparable studies have not been performed in *P. aeruginosa*. The aim of the work presented here was to identify the factors that activate *pilA* transcription through PilSR and to better characterize the scope of genes and phenotypes regulated by PilSR. The overarching hypothesis guiding this work was that PilSR detects fluctuating pilin levels in the inner membrane to regulate not only *pilA* transcription, but also the expression of other genes, possibly associated with virulence and motility.

This hypothesis was addressed through the following research aims:

1. **To identify the signal to which PilS responds**

   PilS topology revealed "extra" transmembrane segments compared to a canonical TCS, raising the possibility that PilS detected an intramembrane signal. Previous evidence that PilA expression in *M. xanthus* is autoregulated (47) and that *P. aeruginosa* can alter *pilA* transcription in response to fluctuating intracellular pilin levels (91) suggested that PilA may be able to regulate its own expression through direct intramembrane interactions with PilS. These findings are discussed in **Chapter 2.**

2. **To determine the effects of dysregulating PilS and PilR function and resulting hyperpiliation on pathogenicity using a *C. elegans* slow killing assay**

Numerous studies have clarified the importance of T4P in establishing infection in a variety of hosts, including *C. elegans*, and showed that loss of T4P is detrimental to virulence (142). However, minimal work has been done to assess the effects of pilin overproduction on pathogenicity. Some studies addressed this question indirectly by showing that retraction deficient strains, which overproduce PilA, are less virulent than WT but suggested that it was loss of twitching motility that impaired pathogenicity (10, 86, 87). In **Chapter 3,** we generated mutants that mimic a constitutively activated PilSR system and show that they overproduce surface pili and are less pathogenic in nematodes, but have wild type twitching motility. Based on these and additional data using other hyperpiliated mutants, we conclude that overproduction of surface pili rather than loss of twitching impairs virulence, possibly by disrupting engagement of contact-dependent virulence factors.

3. **To define the spectrum of genes and associated phenotypes that are controlled by PilSR in *P. aeruginosa***

In *G. sulfurreducens,* approximately 50 genes with putative PilR binding sites upstream of their start codons were identified, and several were differentially expressed in a *pilR* mutant compared to WT (139, 140). Prior to this work, *pilA* was the only known member of the PilR regulon of *P. aeruginosa* but based on the data from *G. sulfurreducens*, we hypothesized that PilR would contribute to the regulation of multiple genes, potentially including novel motility-related genes. The PilR regulon of *P. aeruginosa* is described in **Chapter 4**.

CHAPTER TWO

Type IV pilins regulate their own expression via direct intramembrane interactions with the sensor kinase PilS

Preface

Chapter two consists of the following publication:

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Attributions: Experiments were designed by SLNK and LLB and were performed by SLNK. The manuscript was written by SLNK and LLB.

Title page and author list

Type IV pilins regulate their own expression via direct intramembrane interactions with the sensor kinase PilS

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Abstract

Type IV pili (T4P) are important virulence factors for many pathogens, including *Pseudomonas aeruginosa*. Transcription of the major pilin gene – *pilA* – is controlled by the PilS-PilR two-component system in response to unknown signals. The absence of a periplasmic sensing domain suggested that PilS may sense an intramembrane signal, possibly PilA. We suggest that direct interactions between PilA and PilS in the inner membrane reduce *pilA* transcription when PilA levels are high. Overexpression *in trans* of PilA proteins with diverse and/or truncated C-termini decreased native *pilA* transcription, suggesting that the highly conserved N-terminus of PilA was the regulatory signal. Point mutations in PilA or PilS that disrupted their interaction prevented autoregulation of *pilA* transcription. A subset of PilA point mutants retained the ability to interact with PilS but could no longer decrease *pilA* transcription, suggesting that interaction between the pilin and sensor kinase is necessary but not sufficient for *pilA* autoregulation. Furthermore, PilS's phosphatase motif was required for the autoregulation of *pilA* transcription, supporting the hypothesis that under conditions where PilA is abundant, the PilA-PilS interaction promotes PilR dephosphorylation and thus downregulation of further *pilA* transcription. These data reveal a clever bacterial inventory control strategy in which the major subunit of an important *P. aeruginosa* virulence factor controls its own expression.

Importance

Although two-component systems are a ubiquitous means of rapid bacterial adaptation to changing environments, identification of the specific signals detected by sensor kinases can be challenging. Also, little is known about the diverse, poorly characterized family of sensor kinases that detect intramembrane signals. We show that the type IV pilin, PilA, is an inhibitory intramembrane ligand for the PilS sensor kinase that controls *pilA* expression, and characterize the mechanism of signal transduction. Since the conserved N-terminal domain of PilA alone can repress *pilA* expression, peptides corresponding to this short region could have potential as therapeutic agents to suppress T4P biogenesis.

Introduction

A wide variety of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa,* use Type IV Pili (T4P) for attachment to surfaces and host tissues, biofilm formation, DNA uptake, and twitching motility (6, 82, 85, 143) T4P are retractile surface appendages made predominantly of thousands of subunits of the major pilin protein, PilA (21), which are rapidly polymerized and depolymerized by a complex assembly machine. Also incorporated into T4P are small amounts of the minor pilins FimU, PilV, PilW, PilX and PilE (*P. aeruginosa* nomenclature), which prime pilus assembly (22, 36)

PilA and the minor pilins share a similar lollipop-like topology, with a highly conserved, hydrophobic N-terminal α-helix packed against a variable C-terminal antiparallel β-sheet (26, 31). The first ~24 residues of mature pilins anchor the

subunits in the inner membrane until they are polymerized by the assembly machinery (43). When pili are retracted, subunits are disassembled from the base of the pilus and returned to the inner membrane for reuse in subsequent cycles of extension. In *P. aeruginosa*, high levels of PilA in the inner membrane lead to decreased *pilA* transcription, while depletion of PilA inner membrane pools significantly elevates *pilA* transcription (91, 144). These data suggest that *pilA* expression is responsive to levels of intracellular PilA, although the sensory mechanism is unknown.

pilA transcription in *P. aeruginosa* and many other species is dependent on the PilS-PilR two-component regulatory system (47, 116, 119, 121). Two-component systems (TCSs) allow bacteria to rapidly detect and adapt to changes in their environment (97). The sensor kinase (SK) detects physical or chemical signals, typically via an extracytoplasmic domain flanked by two transmembrane (TM) segments (97). Upon detection of an activating signal, the protein undergoes autophosphorylation on a conserved His residue in the cytoplasmic kinase domain. The phosphate is transferred to a cytoplasmic response regulator, which regulates gene expression in response to the stimulus (145).

In the PilS-PilR TCS, PilR is the cytoplasmic response regulator that activates *pilA* transcription (120). Its cognate SK PilS is atypical, with 6 TM segments connected by very short loops and no obvious external signal input domain (130). When PilS is absent, *pilA* transcription is significantly reduced, while loss of PilR abrogates *pilA* transcription (116). Interestingly, overexpression

of full-length PilS also decreases pilin expression, while overexpression of its cytoplasmic kinase domain alone leads to constitutive *pilA* transcription, a common result of decoupling sensor kinases from their signal input domains (118, 120, 146).

Recently, a conserved E/DxxN/T phosphatase motif adjacent to the phosphorylated His was identified in the HisKA family of SKs to which PilS belongs, indicating that many SKs can have dual kinase and phosphatase activities to fine tune regulation (112). PilS has a canonical ExxN motif at position 320-323, beside the H319 phosphorylation site. Coupled with the observation that PilS overexpression reduces *pilA* transcription, the presence of this motif suggests that PilS could have intrinsic phosphatase activity on phospho-PilR.

Among the most significant challenges in the TCS field is identification of the specific signal(s) to which SKs respond. The observation that *pilA* transcription is inversely correlated with levels of PilA in the inner membrane – coupled with the unusual 6 TM topology of PilS – suggested that it recognizes an intramembrane signal, possibly PilA itself. Here, we show that direct intramembrane interactions between the conserved, hydrophobic N-terminus of PilA and one or more TM of PilS downregulate *pilA* transcription, and define the sequence elements on PilA involved in interaction and regulation. Based on analysis of PilS mutants, we propose that PilA-PilS interactions likely maintain PilS in a phosphatase state when membrane pools of PilA are high, providing a sensitive feedback mechanism for pilin inventory control.

Results

Overexpression of heterologous pilins reduces native PilA levels by decreasing pilA transcription

P. aeruginosa strains carry one of five *pilA* alleles (groups I-V) (24), encoding pilins that vary in size and sequence of the C-terminal domain (31), plus the presence or absence of post-translational modifications (147, 148) and accessory proteins (147, 149). However, the first ~24 amino acids of mature pilins are highly conserved **(Figure S2.1),** while the C-terminal domains are diverse (150). In contrast, PilS in *P. aeruginosa* strains is invariant (151), regardless of the strain's pilin type. These data – plus PilS's lack of an extramembranous signal recognition domain – suggested that the highly conserved hydrophobic N-terminus of PilA could be the PilS ligand. To test whether diverse pilins could suppress PilA expression in a heterologous strain, V5-epitope tagged pilins of groups I, III, IV and V were overexpressed in strain PAK (group II). Intracellular levels of native PAK PilA were monitored using PAK PilA-specific antisera. Overexpression of each of the heterologous pilins from an inducible vector reduced native PilA levels by >50% **(Figure 2.1A)**.

G. sulfurreducens (GS) – which regulates *pilA* transcription using a similar PilSR TCS (140) – has unusually short type IV pilins that retain the characteristic conserved hydrophobic N-terminal α-helix, but lack a globular C-terminal domain. This architecture is thought to promote efficient electron transfer through pilus "nanowires" (29). The first 24 residues of the mature GS pilin are 92% identical to

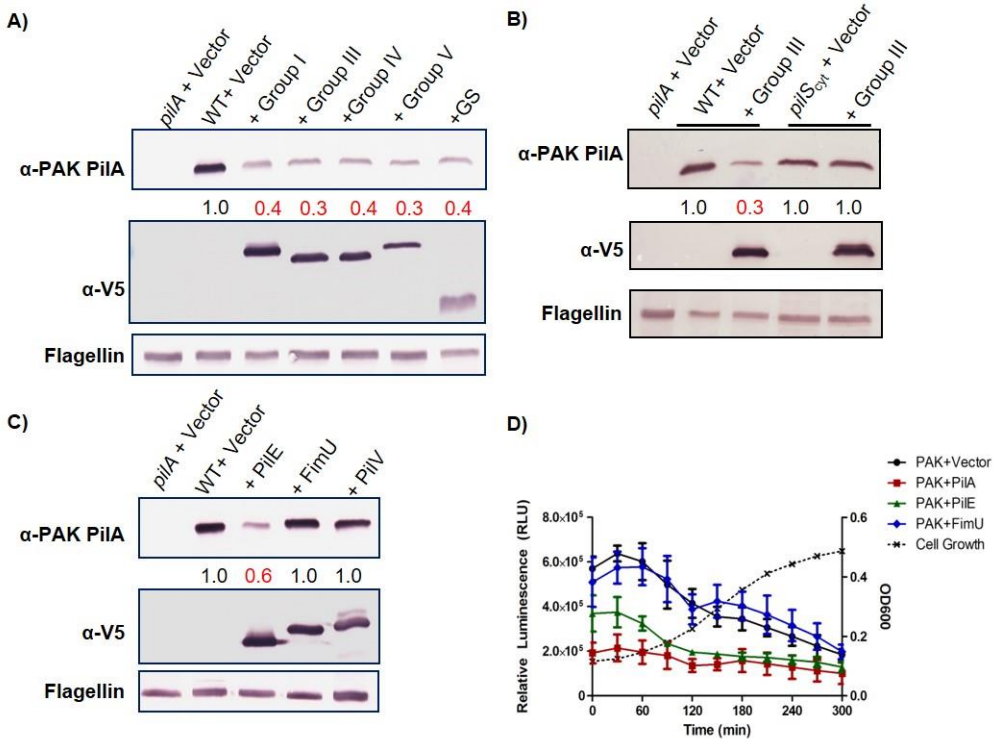

Figure 2.1 Overexpression of heterologous pilins reduces native PilA levels. A) V5 epitope tagged pilins from PA1244 (Group Ⅰ), PA14 (Group Ⅲ), PA5196 (Group Ⅳ), PA1457 (Group Ⅴ) and the *G. sulfurreducens* PilA were overexpressed in the PAK (Group Ⅱ) strain. Heterologous PilAs overexpressed to similar extents as demonstrated by α-V5 blot, reduced native PilA levels. **B)** In the absence of the TM segments of PilS (PilS$_{cyt}$), overexpression of Group Ⅲ PilA no longer affects native pilin levels. **C)** From the overexpression of the minor pilins – FimU, PilV and PilE—only PilE caused a reduction in native pilin levels. For all blots, the flagellin band acted as a loading control. Numbers represent relative expression of native PilA in recombinant strains compared to one carrying the empty vector control, as measured by densitometry and a one-way ANOVA statistical test (n = 3). **D)** PilA and minor pilins FimU and PilE were overexpressed in PAK+pMS402 p*pilA*, and relative luminescence as a function of *pilA* promoter activity was recorded. Mean and SEM of three independent experiments are shown

those of *P. aeruginosa* PilA (**Figure S2.1**). When the GS pilin was overexpressed in *P. aeruginosa*, native pilin expression was reduced to the same extent as with heterologous *P. aeruginosa* pilins **(Figure 2.1A),** showing that the N-terminal segment of a pilin is sufficient for repression of PilA expression. This autoregulatory phenotype is dependent on the TM segments of PilS. The chromosomal copy of PilS was replaced with an N-terminal truncation lacking its TM segments—residues 3-176 ($PilS_{cyt}$)—which alone can support near-WT levels of PilA expression. However, overexpression of Group III PilA in this background could no longer reduce native PilA levels, suggesting a dependence on the PilS TM segments for pilin autoregulation **(Figure 2.1B).**

Although overexpression of heterologous pilins reduced native PilA levels, T4P function was unaffected if the heterologous subunits were competent for assembly. GS can assemble *P. aeruginosa* pilins (152), but the reverse was not true, even when pilus retraction was blocked to maximize the capture of assembled pili **(Figure S2.2A)**. When the assembly-incompetent GS pilins were expressed from an inducible vector in *P. aeruginosa*, twitching motility decreased in an inducer concentration-dependent manner **(Figure S2.2B)**.

To address whether overexpression of pilin-like proteins with divergent N-termini could impact PilA levels, each of the minor pilins — FimU, PilV, PilW, PilX or PilE — was overexpressed in strain PAK. However, the levels of PilW and PilX were lower than those of other minor pilins and they were therefore excluded. Of the three remaining minor pilins, only PilE reduced native PilA levels, to ~60% of

the vector control. Neither FimU nor PilV had an effect on PilA levels when overexpressed (**Figure 2.1C**). Thus, specific sequences in the pilin N-terminus are required for *pilA* regulation.

To test if PilA autoregulation occurred at the level of transcription, the *pilA* promoter was cloned upstream of a luciferase (*lux)* reporter (pMS402-p*pilA*). *pilA* promoter activity was monitored in PAK carrying the pBADGr vector or pBADGr-*pilA*, pBADGr-*pilE* – both of which reduced native pilin levels when overexpressed – or pBADGr-*fimU*, which had no effect on PilA levels **(Figure 2.1D)**. In agreement with the Western blot data in **Figure 2.1C**, overexpression of FimU had no impact on *pilA* transcription. However, *pilA* transcription was significantly decreased in both PilA and PilE overexpression strains **(Figure 2.1D)**. Due to the inherent leakiness of the pBADGr promoter, differences in *pilA* transcription as a result of protein overexpression can be seen even at t = 0, when expression from pBADGr is first induced with arabinose. Together, these data suggest that the conserved N-termini of PilA and PilE, but not FimU, contain the appropriate sequence information needed for downregulation of *pilA* transcription.

PilA and PilS interact directly in the inner membrane

We next tested our hypothesis that direct PilA and PilS interaction led to decreased *pilA* transcription, using a bacterial two-hybrid assay (153). T18-pilin fusions of PAK PilA, *G. sulfurreducens* PilA, and minor pilins PilE, FimU and PilV were co-expressed with T25-PilS in *E. coli* BTH 101, and potential interactions

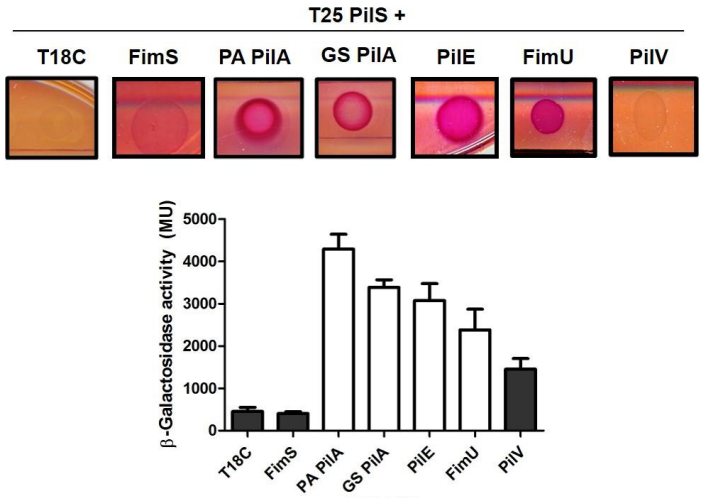

Figure 2.2. PilA and select minor pilins interact with PilS. T25-PilS interacts with PilA from both *P. aeruginosa* and *G. sulfurreducens*, PilE, and to a lesser extent, FimU, each tagged with the T18 domain of adenylate cyclase. PilV did not interact with PilS in either the McConkey agar plate assay or the β-galactosidase activity assay (grey bars). β-galactosidase activity resulting from protein-protein interactions was measured by ONPG-hydrolysis as described in the Methods.

detected by monitoring β-galactosidase activity. PilA from *P. aeruginosa* and *G. sulfurreducens,* as well as the minor pilin, PilE, interacted with PilS **(Figure 2.2)** ($p<0.01$), correlating with their ability to reduce *pilA* transcription when overexpressed in *P. aeruginosa*. Unexpectedly, FimU – which had no effect on *pilA* transcription – interacted with PilS, suggesting that interaction and regulation are separable phenotypes. Based on the results of a one-way ANOVA analysis ($p>0.05$) and a negative result in the McConkey plate assay, PilV did not interact with PilS (**Figure 2.2, grey bars**).

E5 and P22 of PilA are important for interaction and regulation

The ability of PilA, PilE and FimU to interact with PilS, even though FimU did not repress *pilA* transcription, implied that specific pilin N-terminal residues

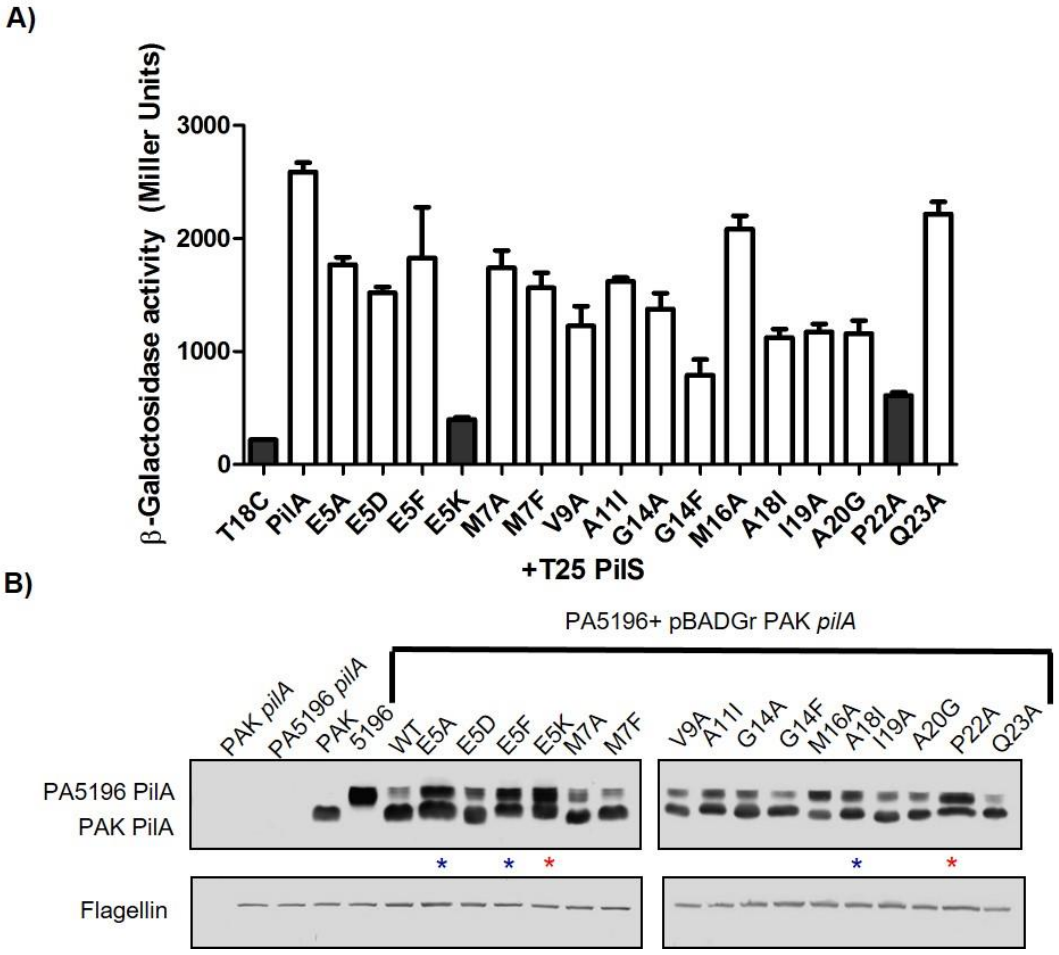

Figure 2.3. PilA residues E5 and P22 are important for PilA-PilS interaction and PilA autoregulation. Point mutations in the conserved N-terminus of PAK PilA were generated using site directed mutagenesis. **A)** T18-PilA fusions co-expressed with T25-PilS in the β-galactosidase liquid assay were tested for interaction. PilA E5K and P22A fail to interact with PilS (grey bars, $p<0.01$). **B)** The same set of PAK PilA point mutants were overexpressed in strain PA5196 (Group IV) and both PAK mutant and native PA5196 PilA levels were detected by Western blot with α-PAK and α-PA5196 PilA-specific antibodies. Overexpression of PAK WT and most mutant PilA derivatives decreased PA5196 PilA levels, while E5A, E5F and M16A interacted with PilS but their overexpression resulted in near-WT levels of native PilA (blue stars) and E5K and P22A transformants did not interact with PilS and also had near-WT levels of PA5196 PilA (red stars).

were important for discrimination of the regulatory signal. To dissect the residues required for both PilA-PilS interaction and subsequent regulation of *pilA* transcription, a series of point mutations were generated in the PAK T18-PilA fusion and the inducible pBADGr-*pilA* construct, respectively.

We targeted residues E5, A20 and P22, important for a number of T4P-related functions (26, 32, 47, 54, 55, 154), plus additional residues conserved between PilA and PilE, but not FimU **(Figure S2.1)**, suggesting that they could be regulatory positions. In general, the native residue was substituted to Ala, but E5A, E5D, E5F and E5K substitutions were also made to establish which R-group characteristics were important at this position. The stability of each mutant T18-PilA fusion was verified by α-PilA Western blot (**Figure S2.3).** Though its expression is slightly decreased compared to the other T18-PilA fusions, T18-PilA E5K is present in high enough abundance to support interaction with WT T25-PilA **(Figure S2.3B).** Only substitutions at E5 or P22 disrupted PilA-PilS interaction in the BACTH assay **(Figure 2.3A)**. Interestingly, only PilE and FimU have a P22 residue, supporting a role for a kinked N-terminal helix in pilin-PilS interactions. A positive charge at position E5 was non-permissive, as E5K – but not E5A, E5F or E5D – abolished PilA-PilS interaction **(Figure 2.3A)**.

The effects of PilA point mutant overexpression were also tested directly in *P. aeruginosa*. To more easily differentiate between plasmid and chromosomally encoded pilins, PAK PilA point mutants were overexpressed in strain PA5196 (Group IV). Because PA5196 pilins are glycosylated with D-arabinofuranose (147,

148) they are of larger mass than those of PAK, allowing the two to be readily separated on 18% SDS PAGE. Co-incubation of Western blots with non-cross reactive α-PAK PilA and α-PA5196 PilA antibodies allowed for the simultaneous detection of both pilins. PAK PilA point mutants were all stably expressed in PA5196 (**Figure 2.3B**, lower band). Overexpression of most PAK PilA derivatives reduced native pilin levels to the same extent as the WT pilin, while PAK PilA E5K and P22A failed to reduce PA5196 PilA levels (**Figure 2.3B,** upper band), consistent with their inability to interact with PilS. Notably, PAK PilA E5A and E5F could interact with PilS (**Figure 2.3A**) but had no effect on PA5196 PilA expression. The M16A and A18I point mutants had intermediate phenotypes, in that their overexpression reduced levels of PA5196 PilA, but to a lesser extent than the WT PAK pilin. Together, the data suggest that PilA-PilS interaction is necessary but not sufficient for modulating PilA expression levels, and that specific intermolecular contacts that depend on PilA N-terminal conformation (P22) and charge (E5) are required for proper signal transduction.

PilS and its homologs in other species contain a conserved, positively charged arginine residue in the first predicted TM (PilS R24 in *P. aeruginosa*), that we hypothesized might interact with PilA E5. R24 is not required for PilS activity, but an R24E substitution leads to loss of *pilA* autoregulation **(Figure 2.4A)**. As predicted, PilS R24E failed to interact with PilA **(Figure 2.4B)**. Interestingly, charge-swapped PilA E5K and PilS R24E variants also failed to interact **(Figure 2.4B)**, suggesting that the R24 has another role, potentially

formation of a salt bridge with a second conserved charged residue in TM3 of PilS, D86. Charge alteration at either R24 or D86 disrupted the PilA-PilS interaction, but PilA interaction was restored with a PilS R24E D86K double charge-swapped mutant, suggesting that the charged TM residues control PilS conformation and thus its ability to interact with pilins **(Figure S2.4).**

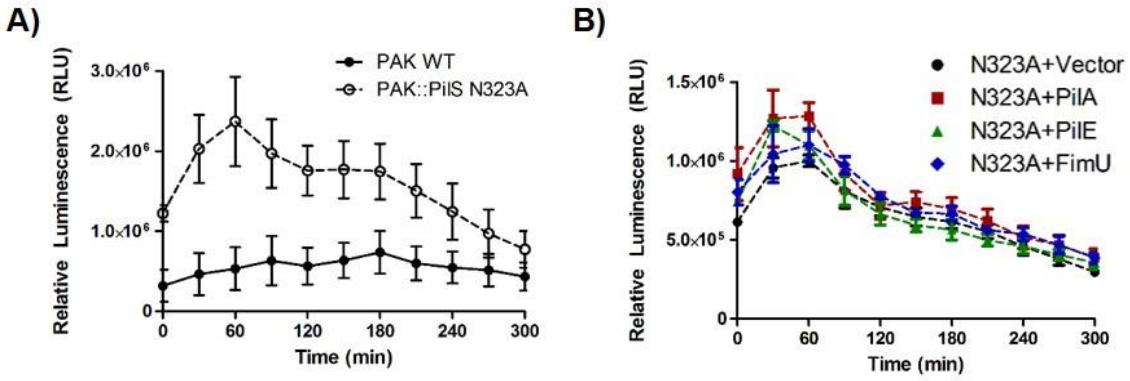

Figure 2.5 A conserved PilS phosphatase motif is required for PilA autoregulation. A chromosomal PilS N323A substitution was made to disrupt the putative PilS phosphatase motif. **A)** The PilS N323A mutant has significantly higher than WT levels of *pilA* transcription. **B)** When PilA, PilE or FimU are overexpressed in the PilS N3232A background, *pilA* promoter activity is comparable to the empty vector control. Error bars represent the standard error calculated from 3 independent experiments.

A PilS phosphatase motif is required for PilA autoregulation

In earlier studies (118), PilS was predicted to have kinase and potentially phosphatase activities. We identified a canonical ExxN phosphatase motif (112) adjacent to the H319 residue that is the site of PilS phosphorylation, and engineered a PAK chromosomal point mutant expressing PilS N323A, a substitution shown previously to disrupt SK phosphatase – but not kinase –

activity (112). WT PAK and the PilS N323A mutant were each transformed with the pMS402-p*pilA lux* reporter to test the effect of this mutation on *pilA* transcription. The PilS N323A mutant had a ~3-fold increase in basal *pilA* transcription compared to WT (**Figure 2.5A)**. These data suggest that PilS N323A could lack phosphatase activity, or have increased kinase activity.

To distinguish between these possibilities, the PilS N323A mutant was co-transformed with pMS402-p*pilA* and empty pBADGr vector, or pBADGr-*pilA, -pilE* or *-fimU,* and the ability of the pilins to modulate *pilA* transcription tested. Unlike in the WT background, as shown in **Figure 2C**, overexpression of PilA or PilE failed to reduce *pilA* transcription in the PilS N323A point mutant **(Figure 5B)**, even though the pilins interact with PilS N323A (**Figure S5**). Together, these data suggest that PilA-PilS interactions likely downregulate *pilA* transcription by promoting PilS phosphatase activity on PilR.

Discussion

Two-component systems are widely used by bacteria for rapid adaptation to changes in their intra- and extracellular environments. However, the signals to which most SKs respond remain unknown (97). In this work, we provide evidence that PilA is a protein ligand for the SK, PilS, which controls pilin expression. We propose an auto-regulatory model for the control of *pilA* transcription that is dependent on intrinsic PilS phosphatase activity (**Figure 2.6).**

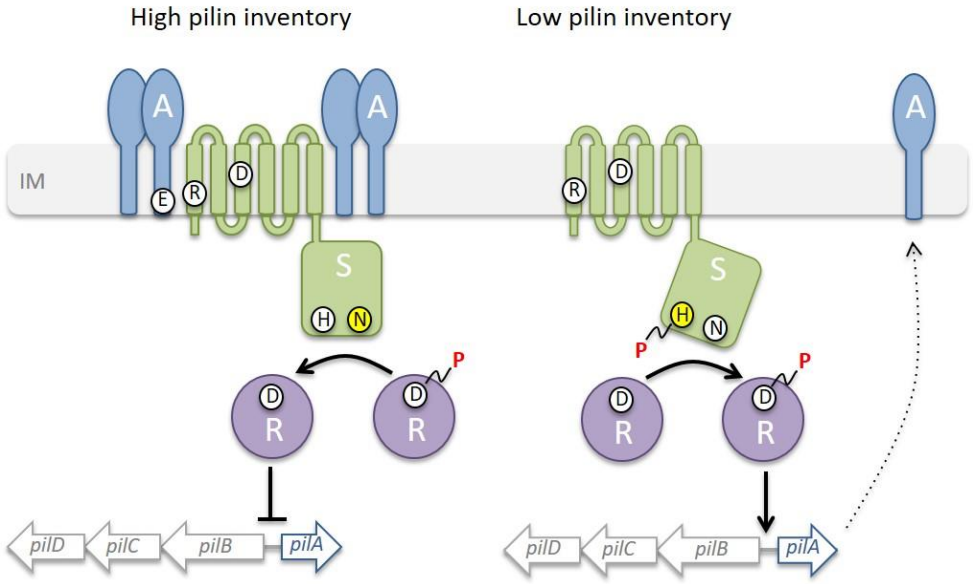

Figure 2.6 Model of *pilA* transcriptional autoregulation. (Left) When PilA levels in the inner membrane are high, PilA-PilS interactions occur more frequently and PilS dimers bound to PilA adopt a phosphatase-active conformation, deactivating PilR and thus reducing *pilA* transcription. (**Right)** When inner membrane pools of PilA are low, PilA-PilS interactions are less frequent. PilS remains in a kinase state and continues to upregulate *pilA* transcription until PilA levels rise**.**

When levels of intracellular PilA are low, *pilA* transcription is significantly increased (91). In our model, depletion of PilA in the inner membrane leads to fewer PilA-PilS interactions. In the absence of such interactions, PilS adopts a kinase conformation and phosphorylates PilR, activating *pilA* transcription until intracellular PilA inventory increases. Conversely, when PilA levels are high**,** *pilA* transcription is dramatically reduced (91). While changes in PilA protein levels under the same conditions are more modest due to inherent pilin stability (91) we propose an autoregulatory role for PilA. PilS-PilR may also impact PilA regulation beyond the transcriptional level, accounting for the difference in magnitude

between transcriptional and protein levels but these putative effects are beyond the scope of this study. We propose that PilA-PilS interaction promotes a PilS phosphatase conformation, preventing further transcription until intracellular pilin inventories become depleted. Although PilA interacts with PilS N323A (**Figure S2.5**), presumably the SK no longer dephosphorylates PilR, and *pilA* transcription is elevated even when intracellular PilA levels are high**.** Similarly, our model explains why overexpression of only the cytoplasmic portion of PilS does not impair *pilA* transcription – lack of inhibitory signal input via interaction of PilA with the TM segments of PilS likely prevents propagation of the conformational changes that favor phosphatase activity. Furthermore, overexpression of a heterologous pilin in the presence of only the cytoplasmic portion of PilS no longer causes a reduction in native pilin levels, indicating not only a dependence on PilS for PilA autoregulation, but a dependence on the TM segments specifically **(Figure 2.1B)**.

While an activating signal for *pilA* transcription has not been identified, this work suggests that PilA is an inhibitory signal for its own transcription. The opposite paradigm was identified in *Streptococcus bovis*, where the kinase activity of the 8-TM SK BovK is partially controlled by its product, the lantibiotic HJ50, via interactions at the periplasm-inner membrane interface (155). Unlike PilA, HJ50 acts as an inducer of its own expression when it interacts with BovK, which – like PilS – lacks a notable signal detection domain (155).

Autoregulation in TCSs has also been demonstrated in some Gram-negative systems. The most relevant example is the PhoPQ TCS of *E. coli*, which controls the transcription of a diverse set of genes that combat cellular stress caused by low magnesium (156-158), low pH, or antimicrobial peptides (156-158). MgrB is a small membrane peptide whose expression is positively regulated by PhoPQ. When MgrB is highly expressed, transcription of the entire PhoPQ regulon is decreased, while the regulon is upregulated in *mgrB* mutants (159). MgrB interacts directly with PhoQ (159). Interestingly, autoregulation appears to be the only function of MgrB, whereas PilA has both autoregulatory and structural roles.

Intramembrane-signal sensing SKs have been best studied in Gram-positive bacteria, where they often sense perturbations in the membrane itself (105, 160). Only a few Gram-negative SKs that rely on intramembrane interactions for signal transduction have been identified, and most require accessory proteins for signal transduction. For example, *E. coli* UhpB is a predicted 8 TM SK that modulates expression of a sugar phosphate transport protein, in response to extracellular glucose-6-phosphate (161). Unlike PilS, UhpB cannot detect its signal directly. Instead, UhpC, a single TM inner membrane protein, binds glucose-6-phosphate and interacts with UhpB in the inner membrane to drive downstream transcriptional activation (161). Thus, PilS represents one of the first Gram-negative SKs that directly detect its ligand, PilA, in the inner membrane without the use of accessory proteins.

The N-terminal amino acid sequence of PilA is highly conserved – even among distantly related species (16, 32, 162) – while the C-terminal domains of pilins, both major and minor, can be extremely divergent (150). Heterologous *P. aeruginosa* pilins, plus the naturally truncated pilin from *G. sulfurreducens,* interacted with PilS **(Figure 2.2)** and reduced chromosomal PilA expression in *P. aeruginosa* **(Figure 2.1A),** suggesting that the N-terminus of PilA mediates both the interactions and their regulatory consequences. However, pilin-PilS interaction alone is not enough for regulation, as specific point mutants of PilA (E5A or E5F) or minor pilins (FimU) interacted with PilS but failed to decrease PilA expression **(Figures 2.1C, 2.2,** and **2.3).** It is possible, although less likely, that these pilins interact with PilS at a different site than the WT pilin, leading to their inability to reduce *pilA* transcription. Although minor pilin-PilS interactions are probably physiologically irrelevant due to the low abundance of minor pilins *in vivo* (22, 163), the effects of their overexpression on *pilA* transcription gave insight into sequence specificity of PilS interaction and regulation. PilE and FimU each share 14 of 24 N-terminal residues with PilA, but the pattern of conservation is different **(Figure S2.1)**, implicating both overall similarity and the presence of specific residues in regulation. For example, P22 creates a kink in the N-terminus and controls pilin orientation in the membrane (43). The P22A mutation abolished PilA-PilS interactions and PilA autoregulation, and PilV, which lacks a proline at position 22, failed to interact stably with PilS. Very low levels of β-galactosidase

activity in this sample could be indicative of a weak or transient interaction, although the plate-based assay supports lack of interaction (**Figure 2.2)**.

Also important for PilA-PilS interaction and autoregulation of *pilA* transcription was the highly conserved E5 residue, present in PilA, PilE and FimU – all of which interact with PilS – but also in PilV, which did not (**Figure 2.2**). Thus, multiple contacts are important for pilin-PilS interaction. Rare charged residues in TM segments are typically buried within multi-TM proteins or involved in protein-protein interactions (164, 165). PilA E5K or PilS R24E substitutions disrupted PilA-PilS interactions and dysregulated PilA expression, but the PilA E5K and PilS R24E charged-swapped pair failed to interact **(Figure 2.4B)**. Instead, interaction with WT PilA was restored when PilS R24E and D86K substitutions were combined **(Figure 2.4B)**, suggesting that these residues form a salt bridge that stabilizes a PilS conformation amenable to PilA binding.

Identification of the signals detected by SKs remains a significant challenge. While we show here that PilA is an inhibitory signal for PilS, there may also be an activating signal, as hypothesized previously (47, 118). Recent studies showed that T4P and a number of other *P. aeruginosa* virulence factors are significantly upregulated upon surface contact, through increases in intracellular cAMP production (38). Increased cAMP production leads to activation of *pilSR* expression by the cAMP-binding regulatory protein Vfr (166), which could account for increased *pilA* transcription upon surface interaction. Alternatively,

cAMP itself, or other molecules associated with the surface-responsive Chp chemotaxis system (11) could activate PilS and thus increase PilA levels.

Each pilus contains thousands of subunits whose synthesis consumes cellular resources, but the subunits can be recycled back to the inner membrane upon disassembly to mitigate demand (44). We suggest that the inventory control mechanism identified here is used by T4P-expressing bacteria to regulate expression of major pilins in response to their levels in the inner membrane. The ability of the naturally truncated GS pilin to impair expression of the heterologous *P. aeruginosa* pilin gene suggests that it may be possible to design PilA N-terminal-mimetic peptides with the potential to block T4P expression and function.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are summarized in Tables S2.1-S2.2. All vectors were constructed using standard cloning techniques and introduced into *E. coli* or *P. aeruginosa* using heat shock and electroporation respectively. All *E. coli* and *P. aeruginosa* strains were grown in Lennox broth (LB) media (Bioshop) or LB 1.5% agar plates in the presence of appropriate antibiotics. Antibiotic concentrations for *E. coli* strains were Gentamycin; 15µg/mL, Ampicillin; 100µg/mL, or Kanamycin; 50µg/mL. For the comparable *P.*

aeruginosa strains, Gentamycin; 30µg/mL, or Kanamycin; 150µg/mL were used. Deletion and FRT mutants were made as described in (167).

Site directed mutagenesis

Site-directed mutagenesis of PAK *pilA* was performed using the QuikChange protocol (Agilent Technologies) Following PCR amplification, reactions were then treated with 1 µL of 10U/µL FastDigest DpnI (Fermentas) for 2h in a 37°C water bath and transformed into chemically competent DH5α cells. Nucleotide substitutions were verified by DNA sequencing (McMaster Institute for Molecular Biology and Biotechnology (MOBIX), Hamilton, ON, Canada).

Lux-pilA luminescent reporter assay

pilA transcription in various *P. aeruginosa* strains was measured in 96-well plate liquid cultures similar to the method described in (168). Briefly, bacterial strains carrying the pMS402+p*pilA* plasmid were grown for 16 h at 37°C in 5 mL LB-Kan 150µg/mL. Cultures were then diluted 1:20 in fresh media and grown to a standardized OD_{600} = 0.15 and the OD_{600} and relative luminescence of each culture was measured at 15 min intervals using a Synergy 4 microtitre plate reader (BioTek) for 5h. See supplemental methods for additional details.

Twitching motility assay

Twitching motility assays were performed as described previously (150). Briefly, 1% LB agar plates were stab inoculated to the plate-agar interface with a single *P. aeruginosa* colony and incubated at 37°C for 24 h. The agar was then

removed and plates were stained with 1% (w/v) crystal violet for 20 min and washed with water to remove excess dye. Twitching zone diameter was measured in ImageJ and statistical significance was determined using a one-way ANOVA test.

Sheared surface protein analysis

Sheared surface protein preparations were conducted as described in (74). Additional details are available in the supplemental methods.

Western blot analysis

See the supplemental methods section for details on protein sample preparation. Protein samples were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then blocked in 5% (w/v) milk solution in phosphate buffered saline (PBS). α-5196 PilA and α-PAK PilA rabbit polyclonal antibodies (Cedarlane Laboratories, Burlington, ON, Canada) were used at 1:7500 dilutions. α-V5 monoclonal primary antibody and alkaline phosphatase-conjugated goat α-rabbit secondary antibody (both Sigma Aldrich, Oakville, ON) were used at 1:3000 dilutions. Blots were developed using manufacturers' instructions. Blots were scanned and densitometry was performed using ImageJ (http://imagej.nih.gov/ij/, NIH, Bethesda, MD) and data from at least 3 independent experiments. One-way ANOVA analysis was used to determine significance of native pilin decreases in Graphpad Prism 5.01 (La Jolla, CA).

Bacterial two-hybrid β-galactosidase activity assay

Chemically competent *E. coli* BTH 101 cells were co-transformed with derivatives of the pUT18C and pKT25 plasmids – expressing the T18 and T25 domains of adenylate cyclase respectively – and interactions determined using a 96-well β-galactosidase assay as described in (169) or on MacConkey agar supplemented with 1% maltose. A one-way ANOVA statistical analysis was performed on β-galactosidase assay results of 4 independent experiments with a Dunnett post-test to determine significance. Additional details can be found in the supplemental methods.

Supplemental

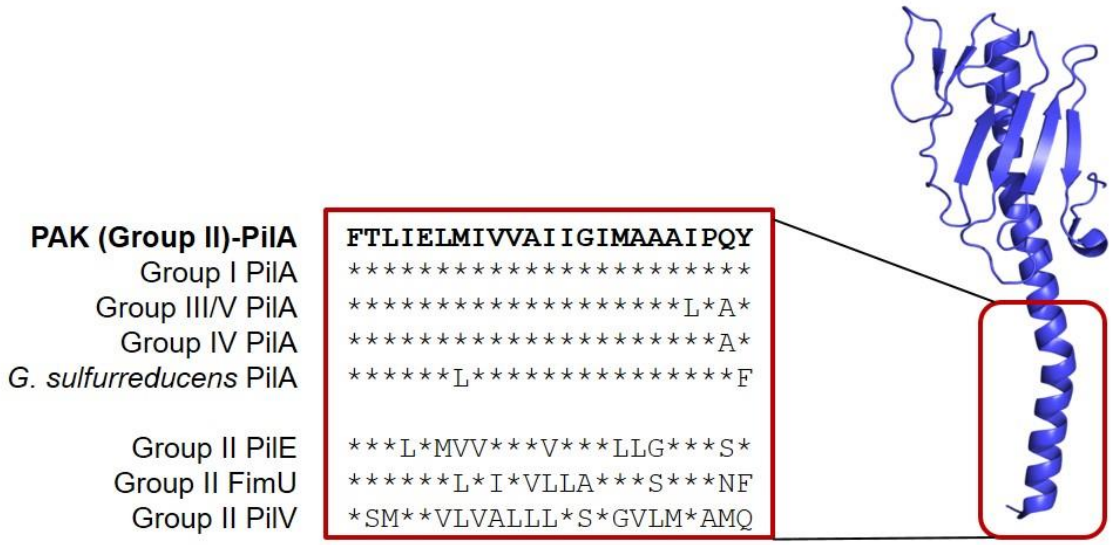

Figure S2.1: Major and minor pilins have conserved N-termini. An amino acid sequence alignment of residues 1-24 of the mature major and minor pilins overexpressed in Figure 1 shows high sequence identity with the native PAK PilA sequence. Heterologous PilAs from other sources are near identical in this domain, while minor pilins are more divergent. Stars (*) denote an identical residue between the sequence and that of PAK PilA in a given position.

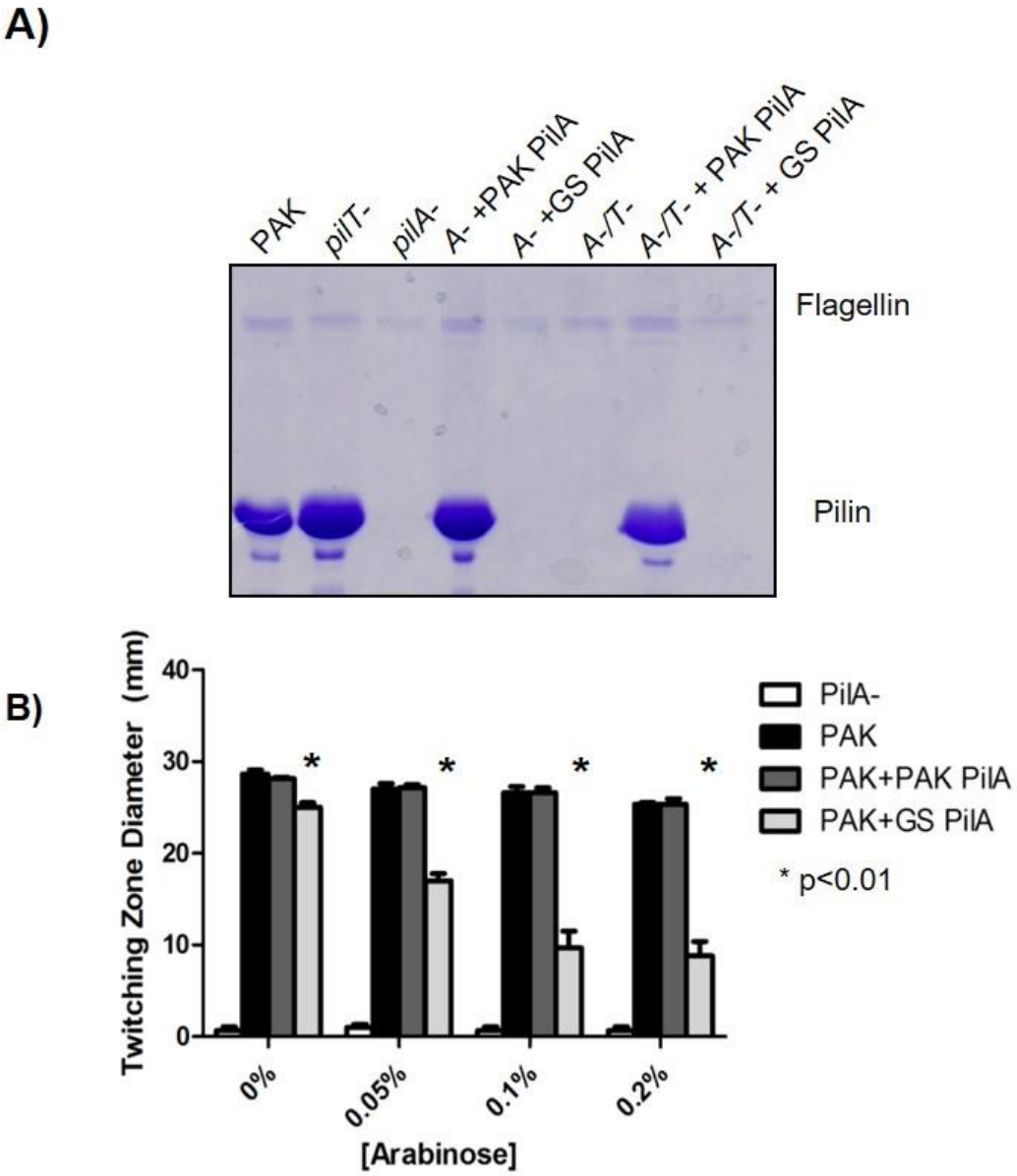

Figure S2.2: Overexpression of non-functional pilins can impair normal T4P function.

A) SDS-PAGE of a sheared surface protein preparation examining surface piliation after complementation of a PAK *pilA* deletion with either the native or *G. sulfurreducens* (GS) *pilA* in both a retraction-proficient and deficient (*pilT-)* background. *G. sulfurreducens* PilA cannot be assembled, even under retraction-deficient conditions, while complementation with PAK PilA can fully restore

surface piliation in both the presence and absence of normal pilus retraction. **B)** Results of a twitching stab assay of PAK strains overexpressing either PAK PilA or GS PilA at increasing levels of arabinose induction. Overexpression of the functional PAK pilin does not affect twitching motility, regardless of the level of induction. However, overexpression of a pilin that cannot be assembled, like GS PilA, can impair twitching motility at higher concentrations.

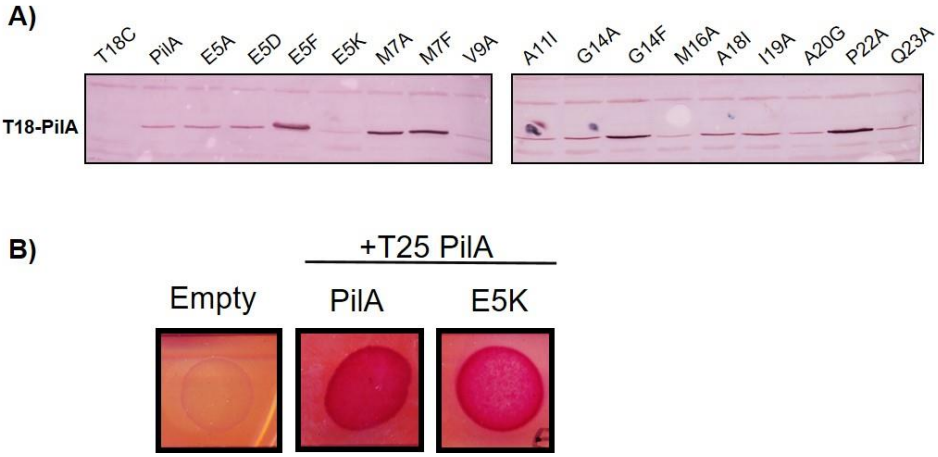

Figure S2.3: T18-PilA mutant fusions are stable. A) α-PAK PilA (1:7500 dilution) Western blot was performed to determine the stability of all T18-PilA point mutants generated in *E. coli* DH5α. While there is some difference in protein levels, each of the point mutants are present and lower abundance mutants do not necessarily affect β-galactosidase output, as mutations such as PilA M16A or Q23A are lowly expressed but interact robustly with PilS in the BACTH assay. **B)** Despite being present at a lower abundance than most other T18-PilA variants, PilA E5K is still present in high enough abundance to reveal interactions in the BACTH assay where they are present.

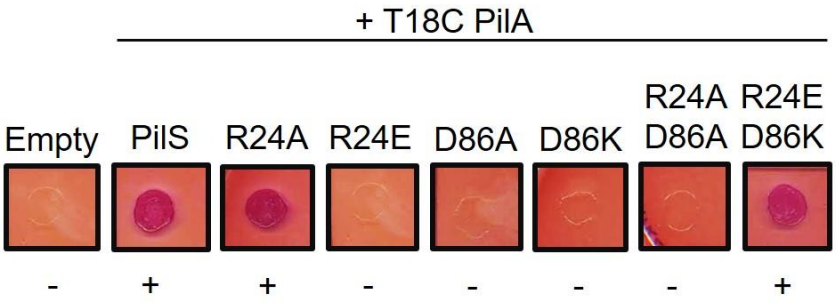

Figure S2.4: PilS residues R24 and D86 are important for PilA-PilS interaction. Individual charge swaps at positions R24 and D86 of PilS both disrupted the PilA-PilS interaction, which was restored in the BACTH plate assay when the mutations were combined.

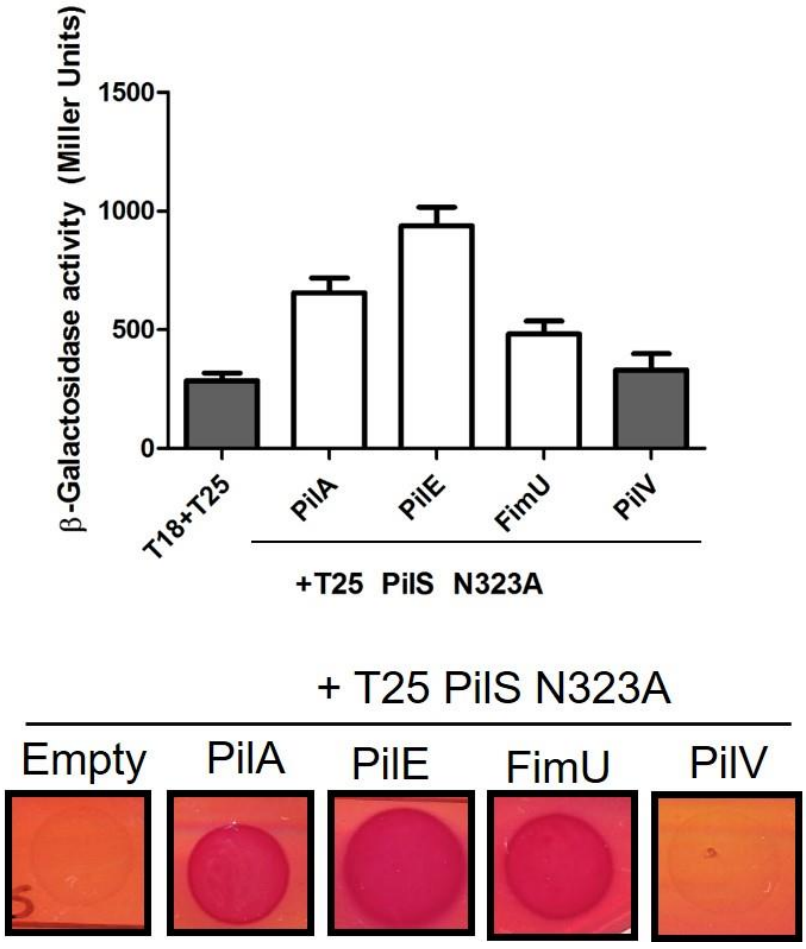

Figure S2.5: PilS N323A interacts with PilA, PilE and FimU in the BACTH assay. Phosphatase-deficient PilS N323A was N-terminally tagged with T25 and assessed in the BACTH assay for its ability to interact with T18-major and minor pilin fusions. PilS N323A interacts with PilA, PilE and FimU (white bars, $p<0.05$), but not PilV, comparable to their interactions with WT PilS in Figure 2.

Table S2.1: Primers used in this study

Primer Name	Oligonucleotide Sequence (5'→3')
F1A_For	ATG AAA GCT CAA AAA GGC GCT ACC TTG ATC GAA CTG ATG
F1A_Rev	CAT CAG TTC GAT CAA GGT AGC GCC TTT TTG AGC TTT CAT

E5A_For	GGC TTT ACC TTG ATC GCC CTG ATG ATC GTG GTT G
E5A_Rev	CAA CCA CGA TCA TCA GGC CGA TCA AGG TAA AGC C
E5D_For	GGC TTT ACC TTG ATC GAC CTG ATG ATC GTG GTT G
E5D_Rev	CAA CCA CGA TCA TCA GGT CGA TCA AGG TAA AGC C
E5F_For	GGC TTT ACC TTG ATC TTT CTG ATG ATC GTG GTT
E5F_Rev	ACC CAC GAT CAT CAG AAA GAT CAA GGT AAA GCC
E5K_For	GGC TTT ACC TTG ATC AAA CTG ATG ATC GTG GTT G
E5K_Rev	CAA CCA CGA TCA TCA GTT TGA TCA AGG TAA AGC C
M7A_For	ACC TTG ATC GAA CTG GCG ATC GTG GTT GCG ATC
M7A_Rev	GAT CGC AAC CAC GAT CGC CAG TTC GAT CAA GGT
M7F_For	ACC TTG ATC GAA CTG TTT ATC GTG GTT GCG ATC
M7F_Rev	GAT CGC AAC CAC GAT AAA CAG TTC GAT CAA GGT
V9A_For	ATC GAA CTG ATG ATC GCG GTT GCG ATC ATC GGT
V9A_Rev	ACC GAT GAT CGC AAC CGC GAT CAT CAG TTC GAT
V9F_For	ATC GAA CTG ATG ATC TTT GTT GCG ATC ATC GGT
V9F_Rev	ACC GAT GAT CGC AAC AAA GAT CAT CAG TTC GAT
A11I_For	CTG ATG ATC GTG GTG ATC ATC ATC GGT ATC CTG
A11I_Rev	CAG GAT ACC GAT GAT GAT CAC CAC GAT CAT CAG
G14A_For	GTG GTG GCG ATC ATC GCT ATC CTG GCG GCA ATT
G14A_Rev	AAT TGC CGC CAG GAT AGC GAT GAT CGCCAC CAC
G14F_For	GTG GTT GCG ATC ATC TTT ATC TTG GCT GCA ATT
G14F_Rev	AAT TGC AGC CAA GAT AAA GAT GAT CGC AAC CAC
M16A_For	GCG ATC ATC GGT ATC GCG GCG GCA ATT GCC ATT
M16A_Rev	AAT GGC AAT TGC CGC CGC GAT ACC GAT GAT CGC
A18I_For	ATC GGT ATC CTG GCG ATA ATT GCC ATT CCC CAG
A18I_Rev	CTG GGG AAT GGC AAT TAT CGC CAG GAT ACC GAT

I19A_For	GGT ATC CTG GCG GCA GCT GCC ATT CCC CAG TAT
I19A_Rev	ATA CTG GGG AAT GGC AGC TGC CGC CAG GAT ACC
A20G_For	ATC CTG GCG GCA ATT GGC ATT CCC CAG TATA CAG
A20G_Rev	CTG ATA CTG GGG AAT GCC AAT TGC CGC CAG GAT
P22A_For	GCG GCA ATT GCC ATT GCC CAG TAT CAG AAC TAT G
P22A_Rev	CAT AGT TCT GAT ACT GGG CAA TGG CAA TTG CCG C
Q23A_For	GCA ATT GCC ATT CCC GCG TAT CAG AAC TAT GTT G
Q23A_Rev	CAA CAT AGT TCT GAT ACG CGG GAA TGG CAA TTG C
PilS N323A For	GCC CAT GAG ATC CGC GCC CGC CTG GGC GCG ATC
PilS N323A Rev	GAT CGC GCC CAG CGG GGC GCG GAT CTC ATG GGC
Lux-pPilA For	GTC GTG GGA TCC ACA GTC GAA TAT CTCCAT TGA TAT GTA TAG G
Lux-pPilA Rev	AGT CGT GGA TCC CTG AGA GGA GAA GGA AAT CGC AGA G
BACTH PilA For	GCG TCT AGA ACT GAT GAA AGC TCA AAA AGG CTT TAC
BACTH PilA Rev	CAT GAG CTC TCT GTT ATC ACA ACC TTT CGG AGT G
BACTH PilS For	GCA ACT GGA TCC GTG CGC AGT GCG CGC TGA ACG GC
BACTH PilS Rev	ACT TCT GAA TTC TCA GCC GGG TGG GTG CGT TTG AGT CG
BACTH FimU For	GCA TCT AGA CTT CAC CCT GAT CGA GTT GCT GAT
BACTH FimU Rev	CAT GAA TTC TCA ATA GCA TGA CTG GGG CGC CT
BACTH PilV For	GCA TCT AGA CTT CAG CAT GAT CGA AGT GCT GGT CG
BACTH PilV Rev	CAT GGT ACC TCA TGG CTC GAC CCT GAG G
BACTH PilE For	GCA TCT AGA CTT CAC GTT GCT GGA AAT GGT GGT GGT
BACTH PilE Rev	CAT GGT ACC TCA GCG CCA GCA GTC GTT GAC
$PilS_{cyt}$ F1	GTT GAA TTC GCC GGA AAA CCA GGA TC
$PilS_{cyt}$ R1	TCA GGA TCC GCG CAC GGT CGC CCT GGT CCG
$PilS_{cyt}$ F2	TCA GGA TCC CAG GAG CAG ACC GAA ACG CTG
$PilS_{cyt}$ R2	TCA AAG CTT GGC ATC CAG TTC CTC TGA CTC

Table S2.2: Strains and plasmids used in this study

Strain	**Characteristics**	**Source**
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
E. coli BTH 101	F-, *cya-99*, *araD139, galE15, galK16, rpsL1 (Str r)*, *hsdR2, mcrA1, mcrB1.*	Euromedex
PAK WT	WT, Group II T4P	(J. Boyd)
PA5196	WT, Group IV T4P, Rectal clinical isolate	(24)
PA5196 *pilA*::FRT	PA5196 with an FRT scar in *pilA*	(24)
PAK *pilA::*FRT (NP)	FRT scar in *pilA*	(This study)
PAK *pilT*::FRT	FRT scar at position 540 *pilT,* T4P retraction-deficient strain	(56)
PAK *pilA::*FRT/*pilT::*FRT	FRT scar in *pilA and* FRT scar at position 540 in *pilT*	(This study)
PAK $pilS_{cyt}$	PAK with a chromosomal deletion of nucleotides corresponding to amino acid residues 3-176 of PilS	(This study)
Vector	**Characteristics**	**Source**
pEX18Ap	Suicide vector used for gene replacement	(54)
pPS856	Source of FRT-flanked gentamicin cassette	(54)
pFLP2	Suicide vector encoding flp recombinase	(54)
pEX18Gm	Suicide vector used for gene replacement	(54)
pBADGr	Broad host range arabinose inducible vector used for complementation; ori *araC-*P_{BAD} Gm^r mob^+	(9)
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE* gene; *ori* of pRO1614	(55)
pUT18C	Derived from pUC19, $p_{lac,}$ Amp^r, Contains *B. pertussis* CyaA (225-399) (T18 domain) for N-terminal tagging	(32)
pKT25	Derived from pSU40, $p_{lac,}$ Kan^r, Contains *B. pertussis* CyaA (1-224) (T25 domain) for N-terminal tagging	(32)
pMS402::P*pilA*	pMS402 vector with *lux* genes under control of the *pilA* promoter, cloned into the *BamHI* site	(This Study)
pBADGr+ PA1244 *pilA*-V5	pBADGr expressing PA1244 (Group I) PilA with a C-terminal V5 epitope tag	(This Study)

pBADGr+ PAK *pilA*	pBADGr expressing PAK (Group II) PilA	(25)
pBADGr+PA14 *pilA*-V5	pBADGr expressing PA14 (Group III) PilA with a C-terminal V5 epitope tag	(This Study)
pBADGr+PA5196 *pilA*-V5	pBADGr expressing PA5196 (Group IV) PilA with a C-terminal V5 epitope tag	(This Study)
pBADGr+ PA1457 *pilA*-V5	pBADGr expressing PA1457 (Group V) PilA with a C-terminal V5 epitope tag	(This Study)
pBADGr+*fimU*-V5	pBADGr expressing the mature PAK minor pilin FimU with a C-terminal V5 epitope tag	(This Study)
pBADGr+*pilV*-V5	pBADGr expressing the mature PAK minor pilin PilV with a C-terminal V5 epitope tag	(This Study)
pBADGr+*pilE*-V5	pBADGr expressing the mature PAK minor pilin PilE with a C-terminal V5 epitope tag	(This Study)
pKT25+*pilS*	BACTH plasmid expressing PAK PilS with an N-terminal T25 tag	(This Study)
pKT25+*pilS* R24E	BACTH plasmid expressing PAK PilS R24E with an N-terminal T25 tag	(This Study)
pKT25+*pilS* D86A	BACTH plasmid expressing PAK PilS D86A with an N-terminal T25 tag	(This Study)
pKT25+*pilS* D86K	BACTH plasmid expressing PAK PilS D86K with an N-terminal T25 tag	(This Study)
pKT25+*pilS* N323A	BACTH plasmid expressing phosphatase-deficient PAK PilS N323A with an N-terminal T25 tag	(This Study)
pUT18C+*pilA*	BACTH plasmid expressing the full length PAK PilA with an N-terminal T18 tag	(This Study)
pUT18C+*pilA* F1A	pUT18C+*pilA* with a PilA F1A mutation (numbered according to mature pilin)	(This Study)
pUT18C+*pilA* E5A	pUT18C+*pilA* with a PilA E5A mutation	(This Study)
pUT18C+*pilA* E5D	pUT18C+*pilA* with a PilA E5D mutation	(This Study)
pUT18C+*pilA* E5F	pUT18C+*pilA* with a PilA E5F mutation	(This Study)
pUT18C+*pilA* E5K	pUT18C+*pilA* with a PilA E5K mutation	(This Study)
pUT18C+*pilA* M7A	pUT18C+*pilA* with a PilA M7A mutation	(This Study)
pUT18C+*pilA* M7F	pUT18C+*pilA* with a PilA M7F mutation	(This Study)
pUT18C+*pilA* V9A	pUT18C+*pilA* with a PilA V9A mutation	(This Study)
pUT18C+*pilA* A11I	pUT18C+*pilA* with a PilA A11I	(This Study)

	mutation	
pUT18C+*pilA* G14A	pUT18C+*pilA* with a PilA G14A mutation	(This Study)
pUT18C+*pilA* G14F	pUT18C+*pilA* with a PilA G14F mutation	(This Study)
pUT18C+*pilA* M16A	pUT18C+*pilA* with a PilA M16A mutation	(This Study)
pUT18C+*pilA* A18I	pUT18C+*pilA* with a PilA A18I mutation	(This Study)
pUT18C+*pilA* I19A	pUT18C+*pilA* with a PilA I19A mutation	(This Study)
pUT18C+*pilA* A20G	pUT18C+*pilA* with a PilA A20G mutation	(This Study)
pUT18C+*pilA* P22A	pUT18C+*pilA* with a PilA P22A mutation	(This Study)
pUT18C+*pilA* Q23A	pUT18C+*pilA* with a PilA Q23A mutation	(This Study)
pUT18C+ GS *pilA*	pUT18C carrying the *G. sulfurreducens pilA* gene	(This Study)
pUT18C+ *fimS*	pUT18C carrying the PAK *fimS* gene	(This Study)
pBADGr+*pilA* F1A	pBADGr+ PAK *pilA* with a PilA F1A mutation (numbered according to mature pilin)	(This Study)
pBADGr+*pilA* E5A	pBADGr+ PAK *pilA* with a PilA E5A mutation	(This Study)
pBADGr+*pilA* E5D	pBADGr+ PAK *pilA* with a PilA E5D mutation	(This Study)
pBADGr+*pilA* E5F	pBADGr+ PAK *pilA* with a PilA E5F mutation	(This Study)
pBADGr+*pilA* E5K	pBADGr+ PAK *pilA* with a PilA E5K mutation	(This Study)
pBADGr+*pilA* M7A	pBADGr+ PAK *pilA* with a PilA M7A mutation	(This Study)
pBADGr+*pilA* V9A	pBADGr+ PAK *pilA* with a PilA V9A mutation	(This Study)
pBADGr+*pilA* A11I	pBADGr+ PAK *pilA* with a PilA A11I mutation	(This Study)
pBADGr+*pilA* G14A	pBADGr+ PAK *pilA* with a PilA G14A mutation	(This Study)
pBADGr+*pilA* M16A	pBADGr+ PAK *pilA* with a PilA M16A mutation	(This Study)

pBADGr+*pilA* A18I	pBADGr+ PAK *pilA* with a PilA A18I mutation	(This Study)
pBADGr+*pilA* I19A	pBADGr+ PAK *pilA* with a PilA I19A mutation	(This Study)
pBADGr+*pilA* A20G	pBADGr+ PAK *pilA* with a PilA A20G mutation	(This Study)
pBADGr+*pilA* P22A	pBADGr+ PAK *pilA* with a PilA P22A mutation	(This Study)
pBADGr+*pilA* Q23A	pBADGr+ PAK *pilA* with a PilA Q23A mutation	(This Study)
pBADGr+ GS *pilA*	pBADGr carrying the *G. sulfurreducens pilA* gene	(This Study)
pEX18Ap+*pilA::*Gm FRT	Gent-FRT insertion in *pilA*	(25)
pEX18Gm::*pilS* N323A	pEX18Gm plasmid carrying PAK *pilS* with an N323A point mutation	(This Study)
pEX18Gm::*pilS* R24E	pEX18Gm mating plasmid containing 500bp up and downstream of PAK *pilS* at position R24 (72bp), where an R→E substitution was made	(This Study)

Supplemental Methods

Pilin overexpression experiments

Heterologous pilin genes of interest were cloned into the pBADGr vector at the EcoRI and HindIII sites, in most cases with a C-terminal V5 epitope tag (See Supplemental Table S2). Sequence-verified plasmids were transformed by electroporation into either PAK or PA5196 WT *Pseudomonas strains* and selected for on LB 1.5% agar plates supplemented with 30µg/mL gentamicin. Single colonies were selected and grown overnight with shaking at 37°C in liquid LB with gentamicin. The following day, samples were diluted 1:20 in fresh LB containing 30µg/mL gentamicin and 0.1% arabinose to induce protein expression

from pBADGr. Cultures were grown at 37°C with shaking to a standardized OD_{600}=0.6 (~3 h). At this time, cells from 1mL of the standardized cultures were collected by centrifugation and the supernatants discarded. Cell pellets were resuspended in 100µL of 1X SDS sample buffer and boiled for 10 min in preparation for Western blot analysis.

Lux-pilA luminescent reporter assay

pilA transcription in various *P. aeruginosa* strains was measured in 96-well plate liquid cultures as described in (168) with some modifications. Bacterial strains carrying the pMS402+p*pilA* plasmid, which has the luciferase (*lux)* genes under control of the *pilA* promoter, were grown with shaking for 16 h at 37°C in 5mL LB-Kan 150µg/mL media. Gent 30µg/mL was also added to those cultures containing pBADGr constructs. Following incubation, cultures were diluted 1:20 in 5mL fresh media containing Kan 150µg/mL and as needed, Gent 30µg/mL. Cultures were grown to a standardized OD_{600}= 0.15. Standardized cultures (100µL) were added in triplicate to a white wall, clear-bottom 96-well plate (3632 Costar, Corning Inc). At (t = 0 min), 0.1% arabinose was added to each well to induce protein expression from the pBADGr vector where applicable, and the plate was sealed. Plates were incubated in a Synergy 4 microtiter plate reader (BioTek) with moderate agitation at 37°C and OD_{600} and luminescence were measured every 15 min for 5 h, with the first read occurring immediately after arabinose was added and the plate sealed. Relative luminescence was

normalized to OD_{600} measured at the same time points. Data presented represent the mean ± standard error of at least 4 independent experiments.

Sheared surface protein analysis

Sheared surface protein preparations were conducted as described in (74). Briefly, strains of interest were streaked in a grid-like pattern on LB 1.5% agar plates (150 mm x 15 mm) containing antibiotic where appropriate and grown overnight at 37°C. Cells were gently scraped using sterile glass coverslips and resuspended in 4.5mL PBS. Cell suspensions were vortexed vigorously for 30 s to shear off surface appendages and cells were pelleted by centrifugation at 16000 x g for 5 min. Supernatants were transferred to new 1.5mL Eppendorf tubes and re-centrifuged at 16000 x g for 20 min to remove any remaining cellular debris. Supernatants containing sheared surface proteins were transferred to new Eppendorf tubes and 5M NaCl and 30% polyethylene glycol were added, to final concentrations of 0.5M and 3% respectively. Solutions were incubated on ice for 90 min, inverting tubes occasionally to precipitate proteins. Following incubation, precipitated proteins were collected by centrifugation. Pellets were resuspended in 150µL 1x SDS sample buffer (80mM Tris (pH 6.8), 5.3% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue and 2% (w/v) SDS) and boiled for 10 min in preparation for 15% SDS-PAGE analysis (3).

Bacterial Two-hybrid β-galactosidase activity assay

To test for interactions between pilins and the sensor kinase PilS, chemically competent *E. coli* BTH 101 cells were co-transformed with derivatives

of the pUT18C and pKT25 plasmids—expressing protein fusions with the T18 and T25 domains of adenylate cyclase respectively. Interactions between pilins and PilS were determined using a 96-well β-galactosidase assay as described in (169) or on MacConkey agar indicator media supplemented with 1% maltose. For the β-galactosidase assay, 5 mL liquid LB cultures supplemented with kanamycin and ampicillin were inoculated with the co-transformed BTH 101 cells and grown overnight with shaking at 37°C. Samples were diluted 1:20 in 5 mL of fresh media and induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) and grown to a standardized OD_{600}=0.6 at 30°C for optimal protein expression. Cells from 1 mL of standardized culture were pelleted by centrifugation, resuspended in 400 µL PBS and were lysed with silica bead matrix by FastPrep (MP Biomedicals). Following lysis, tubes were centrifuged for 1min at 16000 x g to pellet the beads and cellular debris. Sample supernatants (50 µL, or an appropriate dilution in PBS totalling 50µL) were pipetted into a 96-well plate and 50 µL of 2x reaction buffer (200mM sodium phosphate buffer, pH 7.0, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 14.4nM o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma Aldrich)) was added to each well. Plates were incubated at 37°C for 30min. The reaction was stopped with 1M sodium carbonate and A_{420} and A_{550} were measured using a Multiscan GO microplate reader (Thermo Scientific). β-galactosidase activity was calculated in Miller Units (MU), using the following equation:

$MU=1000\cdot((A_{420}-(1.75\cdot A_{550}))/(OD_{600}\cdot t\cdot v))$, where A is absorbance, t is time in

minutes and v is the lysate volume in mL. A one-way ANOVA statistical analysis was performed with a Dunnett post-test to determine significance, where the T18C-T25 empty vector control was used as the control value. Reported data represents the mean of at least 4 independent experiments +/- standard error.

CHAPTER THREE

Hyperpiliation of *Pseudomonas aeruginosa* reduces pathogenicity by preventing engagement of contact-dependent secretion systems

Preface

Chapter three consists of the following manuscript for submission

Kilmury SLN, Moskal D, MacNeil LT and Burrows LL. 2017. Hyperpiliation of *Pseudomonas aeruginosa* reduces pathogenicity by preventing engagement of contact-dependent virulence secretion systems

Attributions: DM was an undergraduate student mentored by SLNK. DM generated select deletion mutants. LTM provided worms and assisted with methodology. SLNK performed experiments. LLB and SLNK designed experiments. SLNK and LLB wrote the manuscript.

Title page and author list

Hyperpiliation of *Pseudomonas aeruginosa* reduces pathogenicity by preventing engagement of contact-dependent secretion systems

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Running Title: Hyperpiliation impairs *Pseudomonas* pathogenicity

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Key Words: Type IV Pili, virulence, hyperpiliation, Type III Secretion

Abstract

Type IV pili (T4P) are well-established virulence factors for many Gram negative and Gram positive pathogens, including the opportunist, *Pseudomonas aeruginosa*. While T4P expression is clearly required for establishment of productive infection in various hosts, the roles of T4P regulation and function in virulence are more ambiguous. The retraction ATPase, *pilT* is an important player in pathogenicity, attributed to its role in pilus retraction and twitching motility. However, here we show using a *Caenorhabditis elegans* slow killing model that hyperpiliation - rather than loss of retraction - decreases virulence. Hyperactivating point mutations in the PilS-PilR two-component system that controls transcription of the major pilin PilA increase levels of surface pili. Although twitching motility of these hyperpiliated strains is similar to wild type, they display significant defects in pathogenicity in *C. elegans* that can be reversed by deleting *pilA* or otherwise impairing pilus assembly. Hyperpiliated *pilO* and *pilT* mutants showed similar PilA dependent impairments in virulence, showing that the phenotype is robust. Pilus retraction is important for engagement of the Type III secretion system, and loss of *pscN*, encoding the T3SS ATPase, reduces virulence of *P. aeruginosa* PAK towards *C. elegans*. Our data support a model in which a surfeit of pili may act as a physical barrier between the bacteria and host cells, preventing engagement of contact dependent virulence factors.

Importance

Pseudomonas aeruginosa is recognized as a serious contributor to hospital acquired infections and is particularly problematic due to its intrinsic resistance to many front-line antibiotics. In addition to traditional antimicrobials, strategies to combat this and other multidrug resistant bacteria include development of anti-virulence therapeutics. We show here that pathogenicity of *P. aeruginosa* is impaired when the amount of type IV pili expressed on the cell surface is increased, and that this is independent of the bacteria's ability to twitch. A better understanding of how T4P facilitate interaction with a host cell—or in this case, how they prevent it—will improve the design of therapeutics targeting components involved in regulation of Type IV pilus function to reduce the clinical burden of *P. aeruginosa* and other Type IV pili expressing bacteria.

Introduction

Type IV pili (T4P) are versatile surface appendages used by bacteria and archaea for cell surface attachment, biofilm formation, DNA uptake, electron transfer and movement across solid and semi solid surfaces using twitching motility (6, 30, 82, 85, 143). These functions make T4P important virulence factors for many pathogens, including the model organism *Pseudomonas aeruginosa*. Recent studies of *P. aeruginosa* T4P also implicated them and/or their associated mechanosensory adhesin, PilY1, in detecting surface attachment and initiating a signal cascade for virulence factor upregulation (9, 11).

T4P are composed predominantly of hundreds to thousands of repeating subunits of the major pilin protein, PilA, whose expression is autoregulated by the PilS-PilR two-component system (TCS) (21, 46, 118, 119). The lollipop-shaped monomers are anchored in the inner membrane when they are not part of an assembled pilus fibre (43) and are proposed to be polymerized into a growing pilus by the platform protein, PilC and the assembly ATPase, PilB, using mechanical energy generated through ATP hydrolysis (77, 78, 92). Deletion of *pilB* causes loss of pilus assembly. Conversely, when pili are retracted, moving the cell body towards the point of pilus attachment, pilin subunits are thought to be depolymerized by PilC using mechanical energy generated by the retraction ATPase, PilT (162). When *pilT* is absent, pili can be assembled but not retracted, resulting in a hyperpiliated strain that is no longer capable of twitching motility (90, 162).

Many studies have confirmed the role of retractile T4P fibres in initiating and establishing *P. aeruginosa* infection in a variety of hosts and models (12, 21, 170-172)**.** Similar findings have been reported for other T4P-expressing bacteria such as *Dichelobacter nodosus* (87), *Neisseria meningitidis* (173), *N. gonorrhoeae* (174), and *Kingella kingae* (14), *Xanthomonas citri* (175) and *Xylella fastidiosa (176)*. Most of these studies focused on comparing the pathogenicity-associated phenotypes of wild type strains to those of their non-piliated counterparts. Few studies have addressed the effects of pilus over-production on pathogenicity. Those studies that have characterized the virulence

of hyperpiliated plant or mammal pathogens—typically through the use of retraction-deficient *pilT* mutants—have predominantly done so in the context of the strains' ability—or inability—to twitch. Virulence of *pilT* (or homologs thereof) mutants were tested for *D. nodosus* in sheep (87), *Pantoea ananatis* in onion seedlings (88) , *P. aeruginosa* in murine and human infection models (10, 86), *Acidovorax avenae* in seed transmission assays (83) and *N. meningitidis* in mice (177). In each case, *pilT* mutants were reported to be less virulent and/or defective in attachment compared to their parent strains. These studies generally attributed the loss of pathogenicity to a loss of twitching motility, and in most cases, the contribution of excess surface pili to loss of virulence was not considered.

In addition to its role in driving pilus retraction, *pilT* has also been implicated in promoting function of the Type III secretion (T3S) system (178-180). The T3S machinery in *P. aeruginosa* forms a needle-like structure that allows for direct injection of the effector proteins ExoS, ExoT, ExoY and ExoU from the cytoplasm of the bacterium into the cytoplasm of eukaryotic host cells, where they can cause apoptosis or lysis (181). T4P are required for full function of T3S (178-180), and more recently it was suggested that not only pili but functional pilus retraction was required, as loss of PilT or a second putative retraction ATPase in *P. aeruginosa*, PilU, reduced the efficiency of T3S, though the latter had a less significant impact (179).

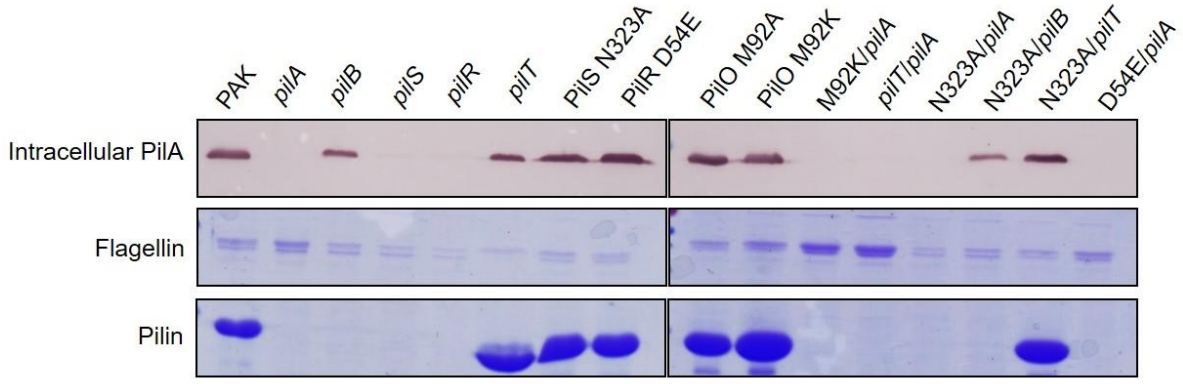
Here, we tested strains of *P. aeruginosa* with mutations in various structural, assembly and regulatory components of T4P that induce hyperpiliation for virulence in a *C. elegans* slow killing model. We show that all the hyperpiliated mutants tested are less pathogenic than wild type (WT) PAK, and that these virulence defects can be reversed by deletion of the major pilin gene, *pilA*. These mutants included those with specific point mutations in PilS and PilR that induce hyperpiliation without abolishing twitching motility. Thus, we propose a model in which hyperpiliation—and not loss of twitching motility, as previous studies have suggested—reduces the infectivity of *P. aeruginosa*, potentially by impairing the ability of the bacteria to engage contact-dependent virulence factors such as the T3S system.

Results

Increased activity of PilS-PilR causes hyperpiliation without loss of pilus function

The PilS-PilR two-component system controls the transcription of the major pilin gene, *pilA* (116, 118, 119) and we showed previously that this system can be constitutively activated using a specific point mutation in the conserved phosphatase motif of PilS, PilS N323A (46, 112). To determine whether hyperactivation of PilS-PilR could also lead to increased surface piliation, we also generated a hyperactive PilR point mutant, PilR D54E, which substitutes the conserved phosphorylation site to mimic an active, phosphorylated state (182, 183). Both the PilS N323A and PilR D54E proteins were expressed from their native loci on the chromosome to ensure normal expression levels. Similar to the

A.



B.

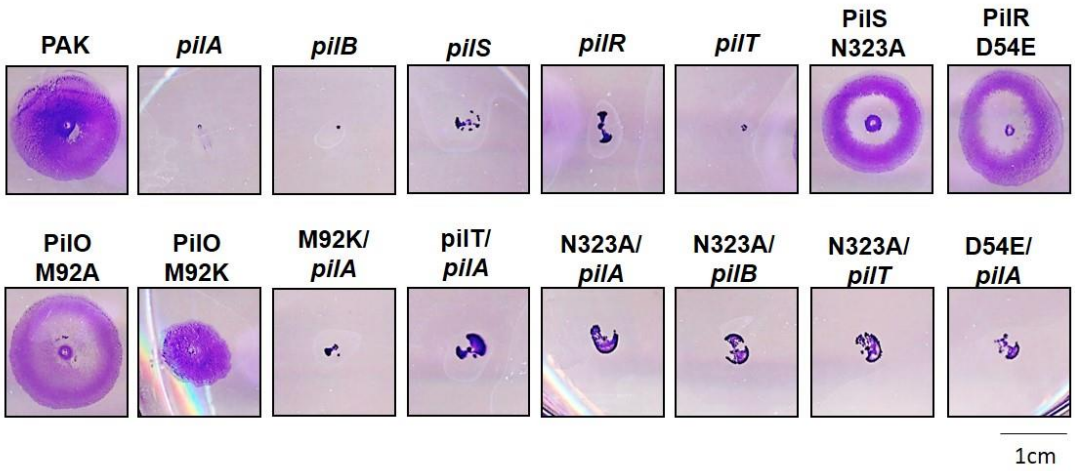


Figure 3.1. Specific point mutations in PilS and PilR induce hyperpiliation. **A)** Mutation of PilS N323A, a conserved phosphatase motif or PilR D54E the site of phosphorylation results in hyperactivation of the regulatory proteins and by extension an increase in PilA expression as shown by α-PAK PilA Western blot. Sheared surface protein preparations indicate that these strains, like a retraction deficient *pilT* mutant, are hyperpiliated compared to WT PAK. The flagellin band is provided as a sample loading control. **B)** In contrast to *pilT*, hyperpiliated PilS and PilR point mutants can twitch, as shown by plate based twitching motility assay. These data are representative of ≥3 independent experiments. Numbers shown indicate zone diameter in millimeters ± standard error

PilS N323A mutant, the PilR D54E mutant had increased expression of PilA **(Figure 3.1A).** Sheared surface protein analyses revealed that – like a mutant lacking the retraction ATPase, *pilT*, which has increased surface pili (162), or a mutant with an M92K mutation in the alignment subcomplex protein, PilO, which was previously found to produce excess surface pili (184) **–** both the hyperactive PilS and PilR mutants were hyperpiliated compared to the WT PAK strain **(Figure 3.1B).** Surface piliation was lost in all strains when the pilin gene, *pilA,* or the gene encoding the assembly ATPase, *pilB*, was deleted. In contrast to the retraction-deficient *pilT* mutant that fails to twitch, or the PilO M92K mutant, which has impaired twitching, the hyperactive PilS and PilR mutants retain the ability to twitch to WT levels, suggesting that these pili are fully functional **(Figure 3.1C).** This set of hyperpiliated mutants, representing a range of pilus functionality, was used in further studies.

Hyperactive PilS-PilR mutants display pilin-dependent reductions in pathogenicity in C. elegans

To clarify the roles of piliation versus twitching motility in pathogenicity of *P. aeruginosa*, we used a *C. elegans* slow killing model. As we hypothesized based on the results of previous studies (84, 85), non-piliated strains including *pilA, pilS* and *pilR* all had slightly decreased pathogenicity compared to WT PAK. Interestingly, hyperactivation of PilS or PilR via the N323A or D54E point mutations, respectively, significantly decreased pathogenicity, with the point mutants causing 50% killing on average 4 days later than the WT strain **(Figure**

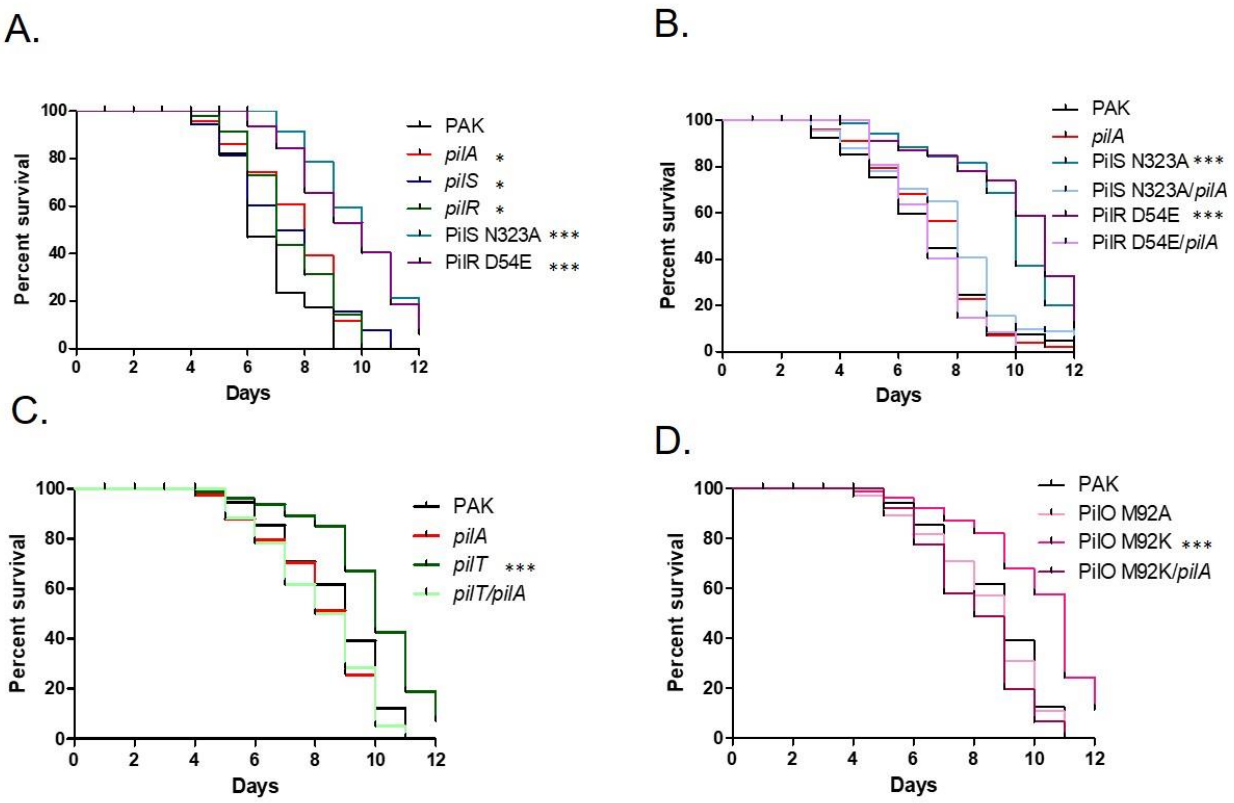


Figure 3.2. Hyperpiliated strains of PAK exhibit pilin-dependent reduced pathogenicity towards *C. elegans* **A)** Mutants lacking surface pili (*pilA, pilS* and *pilR*) have slightly reduced pathogenicity compared to WT PAK. However, hyperactive PilS and PilR point mutants—N323A and D54E respectively—have significantly reduced virulence. **B)** Deletion of *pilA* from the PilS N323A or PilR D54E hyperactive strains returns pathogenicity to near WT levels. **C)** Retraction deficient *pilT* mutants have reduced pathogenicity towards *C. elegans* that is reversed by deleting *pilA* in combination. **D)** An M92K but not M92A substitution in the alignment subcomplex protein PilO was shown previously to result in hyperpiliation of the strain**.** Only the M92K mutant exhibits reduced virulence compared to WT. As above, this pathogenicity defect is reversed through loss of *pilA* in the PilO M92K/*pilA* combination mutant. Data shown are representative of ≥3 independent trials. Stars denote statistical significance compared to WT using the Gehan-Breslow-Wilcoxon test (*p<0.05, ***p<0.005).

3.2A). These trends were consistent in the PA14 strain of *P. aeruginosa,* which is more virulent towards *C. elegans* than PAK **(Supplemental Figure S3.1)**. To clarify if this phenotype was dependent on increased pilin expression versus altered expression of the as-yet undefined PilS-PilR regulon, *pilA* was deleted from both the PilS N323A and PilR D54E backgrounds. In both cases, the pathogenicity of the combination mutants was comparable to that of PAK, suggesting that loss of pathogenicity in the single mutants is PilA-dependent **(Figure 3.2B)**.

Other mutations that cause hyperpiliation similarly reduce virulence

To demonstrate the role that surface pili specifically—and their overproduction—have in virulence in *C. elegans*, we compared the pathogenicity of a deletion mutant of the retraction ATPase, *pilT*, with a double mutant lacking both *pilT* and *pilA*. As expected, the *pilT* mutant was hyperpiliated and unable to twitch **(Figure 3.1B, C).** In the slow killing assay, loss of *pilT* significantly reduced the pathogenicity of the bacteria, and this reduction in virulence was reversed to wild type levels by loss of *pilA* in combination **(Figure 3.2C)**. As neither of these mutants twitch, but have drastically different levels of surface pili, loss of twitching motility is not necessarily correlated with a loss of pathogenicity.

Each of the single mutants tested thus far either directly or indirectly modulates transcription of the pilin gene due to their effects on pilin levels in the inner membrane (91, 118, 119). To separate the contributions of surface piliation from regulation of *pilA* transcription in pathogenicity, we sought to assess

pathogenicity of strains that were hyperpiliated due to mutations in genes outside of the known regulatory network of PilS-PilR. Our group previously identified two point mutations in the T4P alignment subcomplex protein, PilO, that differently affect surface piliation (184). PilO M92A has no detectable impact on surface piliation or motility compared to WT PAK, but another substitution at the same position, M92K, results in a hyperpiliated phenotype coupled with reduced motility (184), **(Figure 3.1B)**. Consistent with the results above, the hyperpiliated M92K mutant, but not the M92A point mutant which has WT piliation, was significantly impaired in its ability to kill *C. elegans,* with the time to 50% worm death increased by an average of 2 days **(Figure 3.2D)**. This phenotype was reversed by deletion of *pilA* in the PilO M92K background, further supporting the hypothesis that hyperpiliation is detrimental to pathogenicity in a *C. elegans* model.

Overproduction of surface pili reduces swarming motility

After measuring the effects of hyperpiliation of *P. aeruginosa* on twitching motility—which varied depending on the mutation —and pathogenicity in a *C. elegans* slow killing model, we next tested whether swarming motility, which is a partially pilus dependent phenotype in *P. aeruginosa* (95, 185) was affected by either the loss or overexpression of surface pili. Using PilS N323A and PilR D54E as representative strains for the hyperpiliated phenotype, we measured swarming motility over 48h and found that *pilA, pilS* and *pilR* mutants all exhibited some swarming motility but had a different morphology than WT PAK swarming,

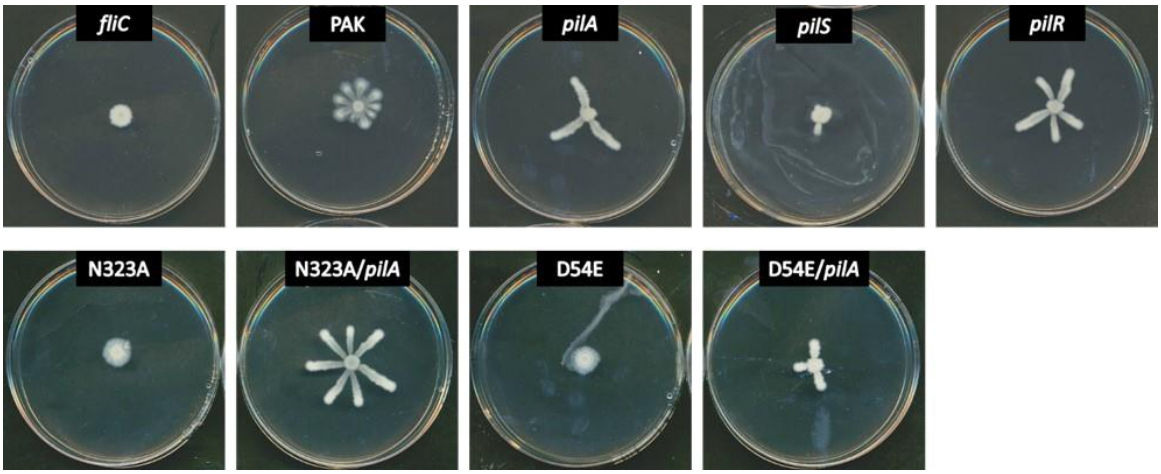


Figure 3.3 Swarming motility is abolished in hyperpiliated strains. *pilA*, *pilS* and *pilR* mutants are still capable of swarming, albeit in a different pattern than that of WT, due to loss of pili in each strain. Hyperactive PilS and PilR point mutants N323A and D54E respectively, do not swarm, but motility can be restored to that of a *pilA* mutant upon deletion of *pilA* from the hyperpiliated strains.

forming fewer but longer tendrils extending from the point of inoculation **(Figure 3.3)**. Conversely, the hyperpiliated PilS N323A and PilR D54E strains are both deficient in swarming motility, more closely resembling the unflagellated *fliC* negative control. Abrogation of swarming motility in these strains is at least partially pilin dependent, as deletion of *pilA* from either PilS N323A or PilR D54E backgrounds restored swarming motility back to *pilA* levels **(Figure 3.3)**. Together, these data indicate not only that surface pili are required for WT swarming motility in PAK but also, like pathogenicity, hyperpiliation is more detrimental to the ability of *P. aeruginosa* to swarm than not having any pili at all.

Pathogenicity can be restored in pilin-expressing avirulent mutants by disrupting pilus assembly

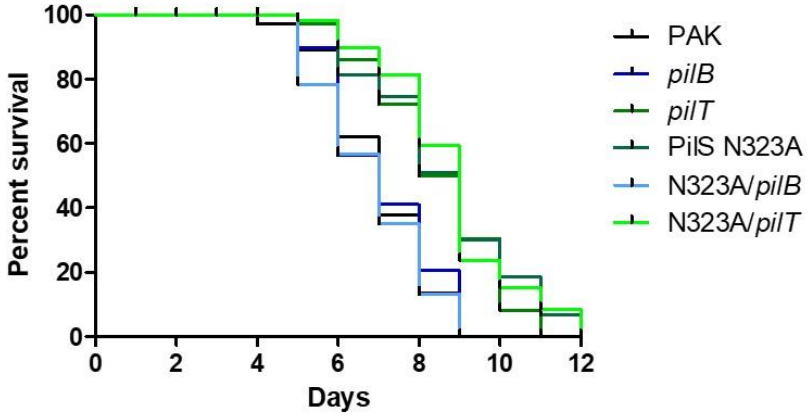


Figure 3.4. Attenuation is dependent on overexpression of surface pili, not intracellular pilin levels. Deletion of the assembly ATPase PilB alone does not have a significant effect on pathogenicity in *C. elegans* although it has WT levels of intracellular pilins. Introduction of this mutation into a virulence-deficient, hyperactive PilS N323A point mutant restores pathogenicity to near WT levels. Conversely, combination of the already hyperpiliated *pilT* mutant with PilS N323A does not further reduce pathogenicity.

The data above showed that loss of *pilA* from various hyperpiliated strains returns pathogenicity to near WT levels. However, this phenotype did not directly address whether the reversion was due to the loss of piliation, or due to the loss of the PilA protein specifically. To separate these possibilities, we made a mutant that combined the hyperpiliated, less pathogenic PilS N323A mutation with a deletion in the assembly ATPase PilB. In this strain, PilS N323A should still be constitutively active (46), but pilus assembly is blocked (92). Similar to a *pilA* mutant, a single *pilB* mutant has WT pathogenicity **(Figure 3.4)**. However, when combined with PilS N323A, deletion of *pilB* had the dominant phenotype,

reversing the loss of pathogenicity in the PilS N323A mutant back to near WT, suggesting that it is loss of pili and not just loss of pilins that result in pathogenicity reversal **(Figure 3.4)**. Conversely, when the PilS N323A mutation was combined with a deletion of *pilT*, there was no change in pathogenicity compared to either of the single mutants **(Figure 3.4)**. Together, the data suggest that the loss of pathogenicity is due to a surfeit of surface pili.

Overexpression of pilA in trans, nor decreased growth or colonization account for decreased pathogenicity of hyperpiliated strains of P. aeruginosa

After establishing that hyperpiliation was correlated with decreased pathogenicity in *C. elegans,* we asked if overexpressing *pilA in trans* in the WT was sufficient to decrease pathogenicity, as PilS N323A, PilR D54E, and to a lesser extent, *pilT,* all have increased PilA expression compared to WT (46, 91) (**Figure 3.1A**). *pilA* was expressed *in trans* in PAK from the arabinose-inducible plasmid, pBADGr, at 0, 0.1 and 0.2% arabinose and surface piliation evaluated. Regardless of the arabinose concentration tested, overexpressing *pilA* in WT PAK did not affect surface pili levels **(Figure 3.5A).** When the same concentrations of arabinose were supplied in the media for the slow killing assay, there were no differences in virulence for a recombinant strain expressing PilA from pBADGr **(Figure 3.5B).** This result suggests that the loss of pathogenicity in PilS N323A or PilR D54E is not simply the result of having an increased number of pilins.

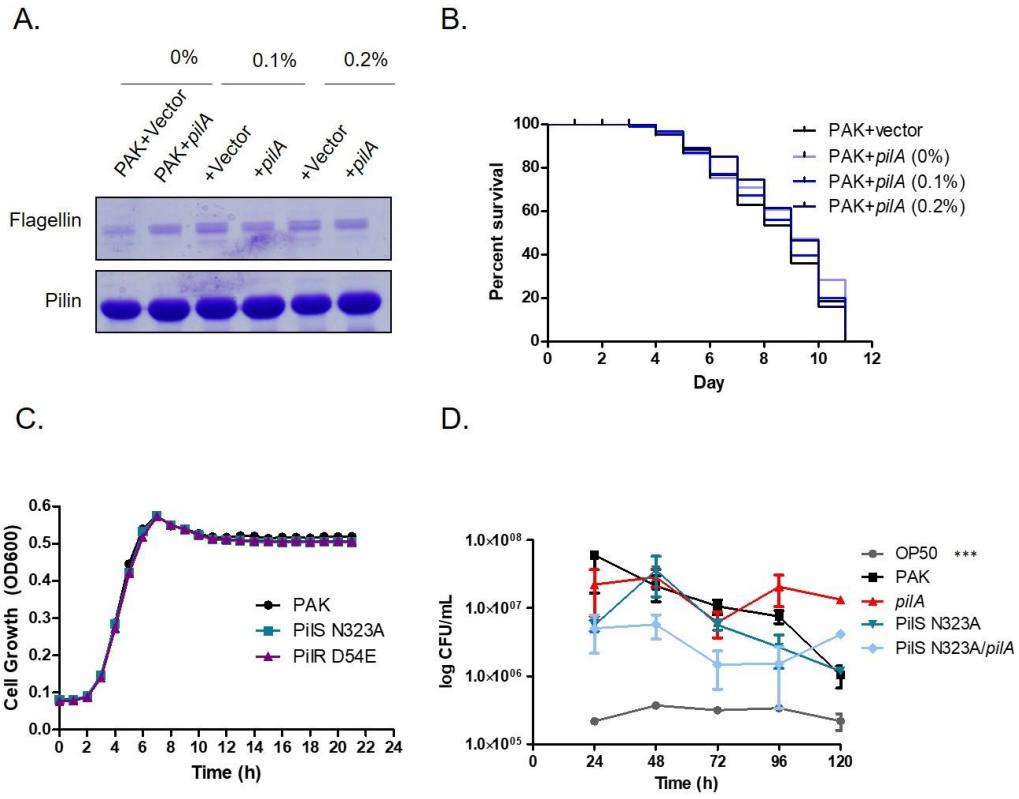


Figure 3.5. Reversion of pathogenicity is not the result of pilin overexpression, growth defects, or altered gut colonization. A) PilA was overexpressed *in trans* from an inducible vector in WT PAK at the indicated concentrations of the inducer, arabinose. Sheared surface protein preparations showed that surface piliation was unaltered at any of the arabinose concentrations tested. **B)** Overexpression of *pilA in trans* with 0, 0.1 or 0.2% arabinose had no effect on pathogenicity in the slow killing assay. **C)** Growth curves in a liquid form of the slow killing media showed no growth defects that could contribute to reduced pathogenicity. **D)** Bacterial persistence in the gut of *C. elegans* was measured over the course of 5 days for WT PAK, *pilA,* PilS N323A and PilS N323A *pilA.* No significant differences in persistence of the 4 strains were apparent.

To verify that the constitutively active regulatory mutations, PilS N323A and PilR D54E had no growth defects that might account for decreased pathogenicity, we grew the mutants and WT overnight in liquid slow killing media to replicate the nutrient conditions of the pathogenicity assay. The liquid media lacked agar and FUDR, required in the solid media to prevent hatching of *C. elegans* eggs. Growth curves were performed over 24h and no detectable differences in growth were observed **(Figure 3.5C).** We conclude that growth defects do not account for the differences in pathogenicity between PAK and the hyperactive PilS and PilR mutants.

We next established whether hyperpiliation impaired *P. aeruginosa's* colonization of the gut of *C. elegans.* Using PilS N323A as a representative strain, we performed colonization assays comparing PAK, *pilA*, PilS N323A and the PilS N323A *pilA* double mutant. We hypothesized that if hyperpiliation affected the ability of PAK to colonize *C. elegans*, PilS N323A would show defects compared to WT, which would be reversed in the PilS N323A *pilA* double mutant. However, over the course of 5 days, there were no significant differences in bacterial colonization between the *P. aeruginosa* strains tested **(Figure 3.5D)**. If anything, PilS N323A *pilA* trended towards decreased colonization but was not defective in *C. elegans* killing. In contrast, *E. coli* OP50 levels remained low at each time point, as it does not accumulate in or colonize the gut (142).

Impaired Type III secretion contributes to the decreased pathogenicity of hyperpiliated mutants

After ruling out several hypotheses regarding the possible cause(s) of decreased pathogenicity in hyperpiliated *P. aeruginosa* strains, we considered changes in the nature of the interaction between the bacteria and *C. elegans*. The T3SS injectasome relies on intimate cell-cell contact between bacteria and host, and is important in a number of infection models (181). While T3S confers virulence in several animal and tissue models (181), its role in killing of nematodes remains open to debate and may be strain dependent. For example, studies using PA14 suggested that T3S has a negligible role in *C. elegans* slow killing (186), whereas others using the less pathogenic PAO1 strain indicated that loss of T3S diminishes pathogenicity (187). We tested the contribution of T3S to

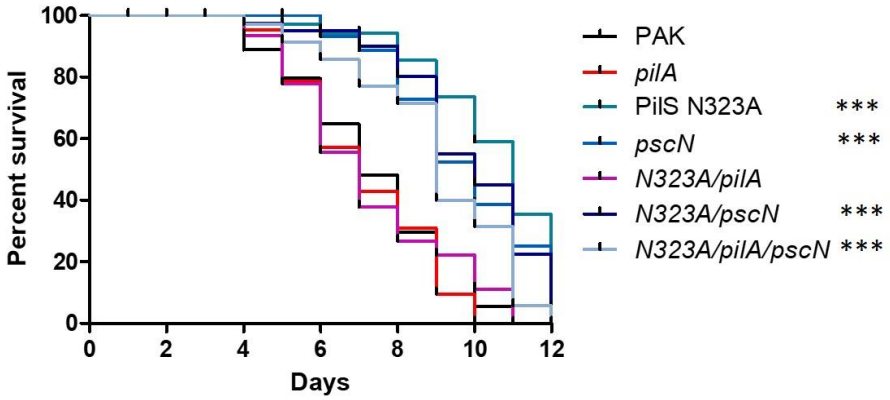


Figure 3.6. Hyperpiliation may impair engagement of the Type III Secretion system. Deletion of the T3S ATPase, PscN, which inactivates T3S, is able to decrease the pathogenicity of PAK, indicating that T3S is involved in the slow killing of *C. elegans.* When this mutant is combined with PilS N323A, no additive effect on pathogenicity is observed, but in combination with PilS N323A *pilA,* pathogenicity is reduced to that of a single *pscN* mutant.

slow killing in *C. elegans* by PAK by generating a single deletion mutant of the T3S system ATPase, *pscN,* which prevents secretion of ExoSTUY effectors as shown in *Yersinia enterocolitica* (188) and a number of other T3S expressing pathogens. Loss of *pscN* significantly reduced the pathogenicity of PAK in the slow killing assay **(Figure 3.6).**, When this mutation was introduced into the PilS N323A hyperpiliated mutant that is already less pathogenic, loss of *pscN* did not further alter pathogenicity. However, deleting *pscN* in the PilS N323A *pilA* double mutant – which has WT virulence –decreased its pathogenicity **(Figure 3.6).** Together, these preliminary data suggest that T3S is involved in *C. elegans* slow killing by the PAK strain, and that hyperpiliation of *P. aeruginosa* may block activation or engagement of T3S and thus, reduce infectivity.

Discussion

Type IV pili are important virulence factors for *P. aeruginosa* and a number of other T4P-expressing bacteria (82, 85, 189). Several studies have investigated the contribution of T4P to virulence but limited work to date has been performed to determine the effects of over production of surface pili in these models. Studies of hyperpiliated strains, mainly retraction deficient *pilT* mutants, usually attributed their results to loss of twitching motility, without considering the role that hyperpiliation may play (10, 86). In this work, we demonstrate that hyperpiliation, rather than loss of twitching motility or loss of *pilT* specifically, in *P. aeruginosa* substantially reduces pathogenicity towards *C. elegans*. We propose that excess

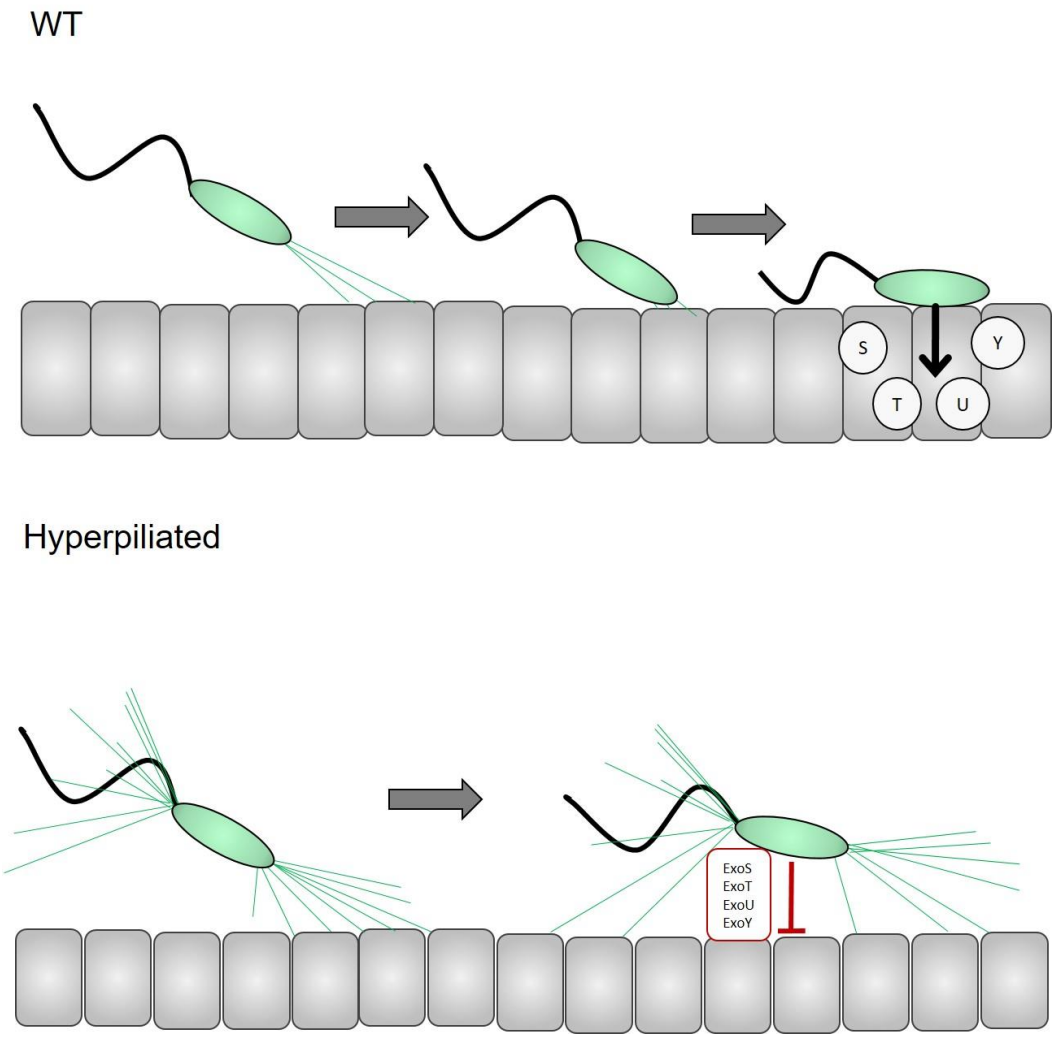


Figure 3.7. Model of pathogenicity impairment caused by hyperpiliation of *P. aeruginosa*. Under normal conditions, pili can be used to attach to the surface of the host cell and retract normally to bring the cell body in contact with the epithelial layer to allow for activation of T3S. In the absence of surface pili, cell-cell contact may be reduced slightly but can still occur by chance, allowing for irreversible attachment and subsequent activation of the T3S system. However, in the event of hyperpiliation of *P. aeruginosa*, the pili may impose a physical barrier that prevents irreversible attachment from taking place. Cells can still adhere and persist in the gut due to this attachment but the decrease in cell-cell contact may reduce activation of the T3S system and by extension, reduce pathogenicity of *P. aeruginosa.*

surface pili act as a physical barrier between the bacterial and host cells, allowing for reversible attachment but preventing intimate cell-cell interactions and therefore engagement of contact-dependent virulence factors such as the T3SS **(Figure 3.7).**

We used a set of hyperpiliated mutant strains that display a range of twitching motility phenotypes so we could separate the contributions of excess surface pili from twitching motility to pathogenicity. Although *pilT*, PilS N323A, PilR D54E and PilO M92K mutants are all hyperpiliated compared to WT **(Figure 3.1B),** only *pilT* is completely deficient in twitching motility. PilS N323A and PilR D54E have excess but fully functional pili, allowing for WT twitching motility, while PilO M92K retains only partial twitching motility, suggesting a role for this mutation in modulating extension and retraction dynamics (184) **(Figure 3.1C)**. Regardless of their twitching motility phenotypes, each showed a comparable defect in pathogenicity in *C. elegans* (**Figure 3.2A-D)**. These data emphasize the importance of the autoregulation of *pilA* expression by PilS-PilR (46). When too many pili are produced, even if they are fully functional, there can still be other effects on the bacteria such as loss of pathogenicity, swarming motility **(Figure 3.3)** or other unintended consequences due to the dysregulation of other members of the PilS-PilR regulon (*in preparation)*.

Overexpression of PilA *in trans* in WT was not sufficient to cause hyperpiliation **(Figure 3.5A)** nor reduce pathogenicity (**Figure 3.5B)**, probably because the normal PilS-PilR regulatory circuit remains intact. Therefore, as

pilins are expressed from the plasmid, chromosomal expression of *pilA* decreases due to their ability to suppress PilR activation via PilS (46). Using PilS N323A and PilS N323A *pilA* as representative hyperpiliated and non piliated mutants respectively we showed there were no significant differences in either growth rates or colonization capacity that would account for the decreased pathogenicity of PilS N323A and by extension, other hyperpiliated strains **(Figure 3.5C, D).**

These data led to the conclusion that it is physical manifestation of surface hyperpiliation itself that impairs *P. aeruginosa*'s ability to kill *C. elegans*. All hyperpiliated mutants with reduced pathogenicity could be reverted back to WT virulence by deleting *pilA* **(Figure 3.2A-D)**. Notably, the *pilT* and *pilT pilA* mutants, neither of which is capable of twitching **(Figure 3.1C)** have divergent pathogenicity phenotypes. To ensure that loss of PilA per se in the hyperpiliated backgrounds was not specifically increasing virulence, we showed that loss of the assembly ATPase, PilB, in the hyperpiliated PilS N323A background reverted pathogenicity to WT **(Figure 3.4).** This strain still produces PilA, therefore these data support the idea that it is loss of surface pili, not pilins, that increase pathogenicity in hyperpiliated strains. These data indicated that – in contrast to previously published studies (10, 87, 88, 174) – loss of twitching motility is likely not the cause of lost infectivity in *C. elegans* slow killing. Rather, it is the increased level of surface pili that is detrimental. Additional work is needed to determine if this holds true in other infection models

Interestingly, hyperpiliation rather than the absence of pili appears to have a more significant impact on pathogenicity in the slow killing model., In the *C. elegans* slow killing model, *P. aeruginosa* is being actively ingested rather than needing establish contact with the host—a stage that normally relies on T4P (85). Furthermore, this difference in pathogenicity between non- and hyperpiliated strains is obvious despite the proposed role of T4P in surface detection, upregulation of virulence and surface display of the putative mechanosensory protein PilY1 (11, 38). Recent work however, has indicated that *pilT* is also required for c-di-GMP response to shear forces and surface sensing (94), suggesting that hyperpiliation may also disrupt signal cascades required for activation of virulence.

After establishing that a surfeit of surface pili causes a decrease in pathogenicity in *C. elegans*, we aimed to address the mechanism through which this occurs, ruling out reduced colonization **(Figure 3.5D).** Injection of the T3S effector, ExoS, requires T4P (178) and is dependent on the presence and function of the retraction ATPase, PilT (179). However, there has been some debate in the field as to whether T3S is involved in the slow killing of *C. elegans.* Studies in PA14 indicate that while T3S is expressed during infection of *C. elegans,* it is not required for full pathogenicity (186), but also suggest that loss of full T3S by deleting the effector *exoU* can sensitize PA14 for the identification of other virulence factors (142). These data could indicate that T3S plays a supplemental role in *C. elegans* slow killing. Conversely, in PAO1, T3S plays a

major role in pathogenicity as loss of T3S function severely reduces PAO1 pathogenicity (187). We made a T3S deficient mutant in PAK by deleting the ATPase *pscN*. Supporting the PAO1 data, PAK lacking T3S also has significantly reduced virulence against *C. elegans* **(Figure 3.6)**. Given the increased virulence of PA14 towards *C. elegans* relative to PAK and PAO1 **(Figure S3.1)**, it is possible that T3S is also involved in its pathogenicity as well, but that other, more potent virulence factors are able to kill *C. elegans* before T3S effectors have an obvious effect.

Using the PAK strain, we sought to determine if loss of T3S engagement in hyperpiliated strains caused decreased pathogenicity of such mutants. To do so we deleted *pscN* in PilS N323A and PilS N323A *pilA* mutants. If hyperpiliation already blocks T3S, we hypothesized that loss of *pscN* in PilS N323A would not further decrease pathogenicity. Similarly, loss of surface pili would once again allow for engagement of T3S in PilS N323A *pilA,* which has WT virulence, but removing *pscN* in that background would decrease virulence. Pathogenicity of PilS N323A *pscN* was similar to the individual PilS N323A and *pscN* mutants, showing no additive effects **(Figure 3.6)**, while the triple mutant had decreased virulence compared to the PilS N323A *pilA* strain **(Figure 3.6).** Together, these data support a model in which overproduction of surface pili, regardless of their functionality, prevent engagement of the T3S and therefore reduce pathogenicity in *C. elegans*. These data also contradict earlier findings that a functional type IV pilus system is required for T3S engagement (178, 179), as the PilS N323A *pilA*

mutant has no functional pili, yet is more pathogenic (presumably due to use of its T3SS) than the triple mutant that lacks PscN.

This model is further supported by the swarming motility data in **Figure 3.3.** While no clear correlation between swarming motility and pathogenicity has been made, swarming and T3S have been positively correlated in both lab and clinical strains (175, 185). The PilS N323A and PilR D54E strains of PAK do not swarm **(Figure 3)** and based on slow killing data **(Figure 3.2A),** are also the least pathogenic of the strains tested. Interestingly, deletion of *pilA* from these two backgrounds restores swarming motility near to that of a *pilA* mutant, which retains some motility but in a different pattern than WT, as pili play a supplemental rather than essential part in PAK swarming motility (95).

Taken together, these data challenge existing models that loss of twitching motility causes loss of pathogenicity in *C. elegans* and other animal models. Instead we suggest that the presence of too many surface pili is detrimental, as they interfere with direct contact between the bacterial and host cells, which in turn prevents *P. aeruginosa*'s ability to engage the contact dependent secretion system, T3S. With the recent call for the development of antivirulence strategies rather than new antibiotics (190), these data demonstrate that inducing surface expression of T4P may be an equally, if not better target for therapeutics than trying to fully inhibit pilus biogenesis.

Methods

Strain construction and growth conditions

Bacterial strains and plasmids used in this study are summarized in **Table S3.1.** Vectors were constructed using standard cloning techniques and the restriction enzymes indicated in the primer table, **Table S3.2,** and were introduced into either chemically competent *Escherichia coli* or *Pseudomonas aeruginosa* Strain K (PAK) using heat shock or electroporation, respectively. Deletion constructs were prepared by ligating 500 bp up and downstream of the gene being deleted and cloning the resulting fusion into the pEX18Gm suicide vector. Unless otherwise indicated, strains were grown in Lennox broth (LB) (Bioshop) or on LB 1.5% agar plates. Where necessary, gentamicin was added to the media for selection at a concentration of 15µg/mL or 30µg/mL for *E. coli* and *P. aeruginosa* respectively. Deletion mutants in *P. aeruginosa* were generated using the method described in (167).

Sheared surface protein preparations

Sheared surface protein preparations were performed as described previously (74). Briefly, strains of interested were streaked in a grid-like pattern on a 150mm x 15mm LB 1.5% agar plate and grown overnight at 37°C. Cells were scraped from the plate and resuspended in 4.5mL PBS. Intracellular samples were prepared by diluting the remaining cell suspensions to OD_{600}=0.6 in PBS, pelleting 1mL of cells and resuspending pellets in 100µL 1x SDS sample

buffer (100µL of 1X SDS sample buffer (80mM Tris (pH 6.8), 5.3% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue and 2% (w/v) SDS). Surface appendages were sheared from the 4.5mL of aliquoted suspension by vortexing them for 30 s and centrifuging for 5 min at 16 000 x g. Supernatants were transferred to a new tube and centrifuged again for 20 min to remove any remaining cellular debris. Supernatants were transferred to a fresh tube and 5M NaCl and 30% polyethylene glycol (MW=8000Da) were added to final concentrations of 0.5M and 3% respectively. Samples were incubated on ice for 1.5 h, inverting occasionally to facilitate protein precipitation and following incubation, were centrifuged at 16 000 x g for 30 min to pellet sheared proteins. Supernatants were discarded and after allowing the protein pellets to air dry, they were resuspended and boiled in 150 µL 1x SDS sample buffer in preparation for SDS-PAGE analysis.

Western blotting

Prepared samples were separated on 15% SDS PAGE and transferred to nitrocellulose membrane. Following transfer, membranes were blocked in a 5% (w/v) skim milk powder in phosphate buffered saline (PBS). α-PAK PilA primary monoclonal antibodies (Cedarlane Laboratories, Burlington ON, Canada) and goat-α-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma Aldrich, Oakville, ON, Canada) were used at 1:7500 and 1:5000 dilutions respectively and blots were developed according to manufacturer's instructions. Data are representative of at least 3 independent experiments.

Twitching motility assays

Twitching motility assays were performed as described previously (150). Briefly, a single colony of each strain of interest was stab inoculated to the agar-plastic interface of an LB 1% (w/v) agar plate. Plates were incubated at 37°C for 24-48h. Following incubation, the agar was carefully removed from the plate and discarded and twitching zones were visualized by staining the plastic plate with 1% (w/v) crystal violet. Plates were imaged using a standard computer scanner and twitching zones were measured using ImageJ (http://imagej.nih.gov/ij/, NIH, Bethesda, MD). At least 3 independent replicates were performed.

Slow killing pathogenicity assays

Slow killing (SK) assays were performed as described previously (191). *Caenorhabditis elegans* strain N2 populations were propagated and maintained on Nematode Growth Media (NGM) plates inoculated with *E. coli* OP50. Eggs were harvested to obtain a synchronized population by washing worms and eggs from NGM plates with M9 buffer. Worms were degraded by adding buffered bleach, leaving only eggs intact. Eggs were washed with M9 buffer and resuspended in M9 buffer with rocking overnight to allow eggs to hatch into L1 larvae. Synchronized L1 worms were plated on NGM plates for 45h to develop into L4 worms. During this process, slow killing plates supplemented with 100µM 5-Fluoro-2′-deoxyuridine (FUDR) were prepared and inoculated with 100µL of a 5mL LB overnight culture of bacterial strains of interest and incubated at 37°C for 16-18h. Harvested and washed L4 worms (~30-40) were dropped by Pasteur

pipette onto each SK plate. Using a dissecting microscope, plates were scored daily for dead worms, which were picked and removed. Survival curves were prepared using Graphpad Prism 5.01 (La Jolla, CA) and statistically significant differences in pathogenicity between strains were identified using Gehan-Breslow-Wilcoxon analysis. Representative curves of at least 3 independent experiments are shown.

Swarming motility assays

Swarming motility assays were performed similarly to (95) with slight modifications. Briefly, 5mL liquid LB cultures were inoculated with *P. aeruginosa* strains of interest and grown overnight at 37°C with shaking. Swarming plates were prepared on the day the assays were to be performed, with 10x M8 salts (12.8% w/v $Na_2HPO_4 \cdot 7H_20$, 3% KH_2PO_4, 0.5% NaCl, pH 7.4), diluted to a final 1x concentration with water, 0.5% agar, and autoclaved together. After autoclaving and cooling to ~60°C, media was supplemented with 2mM $MgSO_4$, 0.2% glucose, 0.05% L-glutamic acid and trace elements (composition available upon request). Media was pipetted into 100mmx15mm petri dishes in 20mL aliquots per plate and allowed to solidify upright at room temperature for 1.5 h. From each of the prepared overnight cultures, 3.5µL of inoculum was then spotted in the centre of each plate, which were then incubated upright at 30°C in a humidity controlled incubator for 48 h. Plates were imaged using a standard computer scanner and where required, surface area was calculated using ImageJ

(http://imagej.nih.gov/ij/, NIH, Bethesda, MD). Representative images of 4 independent experiments are shown.

Growth curves

Growth curves were performed by growing *P. aeruginosa* strains of interest in a 5mL overnight culture of liquid slow-killing assay media, omitting agar and FUDR. The following day, cultures were diluted 1:20 in fresh slow killing media (50µL culture in 950µL fresh media) and samples were plated in a 100 well honeycomb plate (Oy Growth Curves Ab Ltd) in 300 µL technical triplicates. Growth (OD_{600}) was measured at 1 h intervals for 24 h using a Bioscreen C plate reader (Oy Growth Curves Ab Ltd) set with continuous shaking at 37°C, and curves were generated using Graphpad Prism 5.01 (La Jolla, CA)**.** Mean and standard error of 3 independent biological replicates (9 total samples) are shown.

Pseudomonas colonization assays in C. elegans

Colonization assays were performed using the methods described in **(192).** Briefly, worms and bacterial strains were prepared as for the Slow Killing assay. Synchronized L4 worms were seeded onto slow killing plates containing 30µg/mL gentamicin that had been preincubated overnight at 37°C with the *P. aeruginosa* strains of interest. For counter selection against residual *E. coli*, all *P. aeruginosa* strains were transformed with the pBADGr plasmid, which confers gentamicin resistance. *C. elegans* was allowed to feed on the *P. aerginosa* strains for 5 days. On each day, 10 worms were removed from the plate and washed 3 times in M9 buffer containing 1mM sodium azide to prevent expulsion of bacteria from the

gut. A sample of the final wash was serially diluted and plated on LB 1.5% agar plates containing 30µg/mL gentamicin to estimate how many *P. aeruginosa* colony forming units (CFUs) remained on the exterior of the worms. Worms were then lysed by vortexing for 30 s in the presence of 4-6 stainless steel, 3.175 mm diameter beads (Lysing matrix S**,** MP Biomedicals, Canada)**.** Lysates were serially diluted and plated on LB 1.5% agar with gentamicin to determine *P. aeruginosa* CFU/mL combined on the interior and exterior of the worms. These plates were grown overnight at 37°C and colonies were counted. To estimate *P. aeruginosa* CFU/mL inside *C. elegans*, the supernatant CFU/mL was subtracted from the lysate CFU/mL—which should contain internalized bacteria as well as remaining bacteria in the final wash. This difference, representing internalized bacteria was plotted as CFU/mL over time. Measurements were made at 24 h intervals over 5 days. Experiment was repeated in triplicate and a one-way ANOVA statistical test with Dunnett's post-test was used to assess significance at each time point, using PAK as the reference sample.

Supplemental

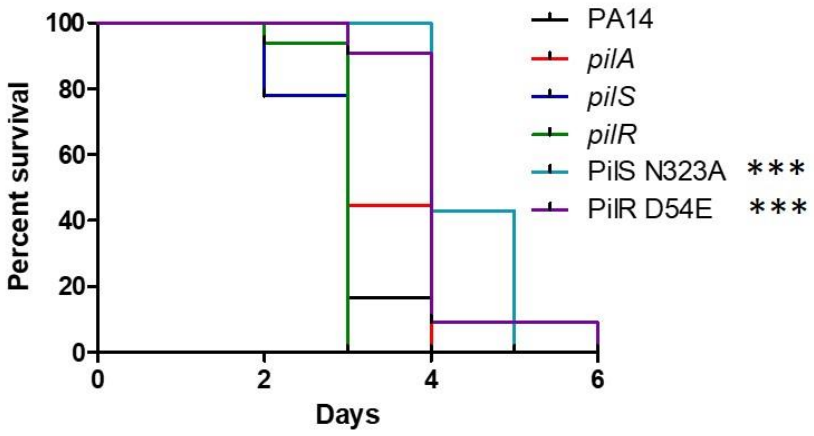


Figure S3.1: Pathogenicity of PA14 is reduced under conditions of hyperpiliation. Slow killing assays performed with the pathogenic PA14 strain and hyperpiliated PilS N323A and PilR D54E strains show the same trends as assays performed with PAK. Hyperpiliated strains are less pathogenic compared to WT (n=3).

Table S3.1 Strains and plasmids used in this study

Strain	Characteristics	Source
E. coli strains		
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
E. coli SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^R)	(193)
E.coli OP50		(L.T. MacNeil)
P. aeruginosa strains		
PAK WT	WT, Group II T4P	(J. Boyd)
PA14 WT	WT, Group III T4P	(G.A. O'Toole)
PAK *pilA*	PAK with a chromosomal *pilA* deletion	(This study)
PAK *pilB*	PAK with chromosomal deletion of *pilB*	(This study)
PAK *pilS*	PAK with a chromosomal deletion of *pilS*	(This study)
PAK *pilR*	PAK with a chromosomal deletion of *pilR*	(This study)
PAK *pilT*	PAK with a chromosomal deletion of *pilT*	(This study)
PAK PilS N323A	PAK with a chromosomal substitution of PilS phosphatase motif residue N323 to alanine	(46)
PAK PilR D54E	PAK with a chromosomal substitution of PilR phosphorylation site D54 to glutamic acid	(This study)
PAK PilO M92A	PAK with a chromosomal substitution of residue M92 to alanine in PilO	(184)
PAK PilO M92K	PAK with a chromosomal substitution of residue M92 to lysine in PilO	(184)
PAK PilO M92K/*pilA*	PilO M92K strain with *pilA* deleted from the chromosome	(This study)
PAK *pilA*::FRT/*pilT*::FRT	FRT scar in *pilA and* FRT scar at position 540 in *pilT*	(46)

PAK PilS N323A/*pilA*	PAK with disrupted PilS phosphatase motif and clean *pilA* deletion	(This study)
PAK PilR D54E/*pilA*	PAK with altered PilR phosphorylation site and *pilA* deletion	(This study)
PAK PilS N323A/*pilB*	PAK with disrupted PilS phosphatase motif and deletion of *pilB*	(This study)
PAK PilS N323A/*pilT*	PAK with disrupted PilS phosphatase motif and deletion of *pilT*	(This study)
PAK *pscN*	Clean chromosomal deletion of the Type III secretion ATPase *pscN*	(This study)
PAK *pscN*/PilS N323A	Deletion of Type III secretion ATPase and chromosomal substitution of PilS residue N323 to alanine	(This study)
PAK *pscN*/PilS N323A/*pilA*	Deletion of *pscN* and *pilA* combined with PilS N323A stubstitution	(This study)
PA14 *pilA*	PA14 with clean deletion of *pilA*	(This study)
PA14 *pilS*	PA14 with deletion of *pilS*	(This study)
PA14 *pilR*	PA14 with deletion of *pilR*	(This study)
PA14 PilS N323A	PA14 with chromosomal substitution of PilS residue N323 to alanine	(This study)
PA14 PilR D54E	PA14 with chromosomal substitution of PilR residue D54 to glutamic acid	(This study)
C. elegans species		
C. elegans N2	WT Bristol strain	(L.T. MacNeil)
Vector	**Characteristics**	**Source**
pEX18Gm	Suicide vector used for gene replacement	(54)
pBADGr	Broad host range arabinose inducible vector used for complementation; ori *araC-*P_{BAD} Gm^r mob^+	(9)
pBADGr+ PAK *pilA*	pBADGr expressing PAK PilA	(25)

Table S3.2: Primers used in this study

Primer Name	Sequence (5'→3')
pilA F1	GCG GAA TTC GTG TTG GCG GAC CAG CTT
pilA R1	GCA CCC GGG GCC TTT TTG AGC TTT CAT
pilA F2	GCA CCC GGG CCG AAA GGT TGC TCT AAG TAA
pilA R2	ATT GCA TGC ATT GCC GAG GCC CGG
pilB F1	ATA GAG CTC CCA CGA GAA AGC GC
pilB R1	ATA GGA TCC CAG GCC GCT CAG T
pilB F2	ATA GGA TCC GGA GGA AGT CAA CCG
pilB R2	ACT AAG CTT GCC AGA CTG TTC CCC
pilS F1	GTT GAA TTC GCC GGA AAA CCA GGA TC
pilS R1	GTT GGA TCC CAG ACG GAG GAT GCG TTG
pilS F2	GTT GGA TCC GGA AGG CGG CGG CTG C
pilS R2	GTT AAG CTT ACT GAT GTA GAC CGG CGC
pilR F1	GTC AGA ATT CCT CCC GTC GCC GCC AGG C
pilR R1	TGA CGG ATC CGA CGA TCA GGG CTT TTT G
pilR F2	GTC AGG ATC CCG CCT GAA AAA GCT GGG C
pilR R2	TGA CAA GCT TGG CCT GGA ACT GCC CGT G
pilT F1	CAA CGA GCT CAC CGG GTG GTC GAC GTG T
pilT R1	CAA CCT GCA GGC CGC CCT TTT CCA C
pilT F2	CAC CCT GCA GAG CCT GGA AAT CAC CGA C
pilT R2	CAC CAA GCT TCC TGC TGG CGC CGG CCC A
PilR D54E F	AC CTG TGC CTC ACC GAG ATG CGC CTG CCG GAC
PilR D54E R	TC CGG CAG GCG CAT CTC GGT GAG GCA CAG GTC
pscN F1	ATT GAA TTC GGT GGG CGA TCA GCG CCT
pscN R1	ATT GGA TCC CGA TGG CGT GGC GCA TCC
pscN F2	ATT GGA TCC AGC GAT TAC GCA CAG GCC
pscN R2	GGC AAG CTT TTC CAG TTC GCC TTC CTC

CHAPTER FOUR

Pseudomonas aeruginosa PilSR regulates *fleSR* connecting twitching and swimming motilities

Preface

Chapter 4 consists of the following manuscript for submission:

Kilmury, S.L.N. and Burrows LL. 2017. *Pseudomonas aeruginosa* PilSR regulates *fleSR* connecting twitching and swimming motilities

Attributions: SLNK made deletion and point mutants, performed phenotypic motility assays, isolated RNA and performed luminescent reporter assays. RNAseq was performed by the Farncombe Metagenomics Facility (McMaster University, Hamilton, ON). SLNK and LLB designed the experiments and performed data analysis. The manuscript was prepared by SLNK and LLB.

Title page and author list

Pseudomonas aeruginosa PilSR regulates *fleSR* connecting twitching and swimming motilities

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Running Title: Loss of PilS-PilR reduces FleSR expression

Key words: Type IV pili, twitching motility, swimming motility, flagella, two-component systems

Abstract

Expression of Type IV pili (T4P) is tightly coordinated at multiple levels, including at the level of pilin transcription. The PilS-PilR two-component regulatory system (TCS) regulates expression of the major pilin gene, *pilA,* in *Pseudomonas aeruginosa* and a number of other T4P-expressing bacteria, likely to manage the metabolic cost associated with T4P-related functions such as twitching motility. Similarly, biosynthesis and function of the single polar flagellum of *P. aeruginosa* is regulated by a hierarchical system involving multiple, interconnected regulatory proteins including FleQ, the FleS-FleR TCS, FliA, and the alternate sigma factor, σ^{54} (RpoN). Previous studies in *Geobacter sulfurreducens* and *Dichelobacter nodosus* implicated PilR in regulation of additional, non-pilin related genes. Because T4P are implicated in bacterial surface sensing, it is important to distinguish between phenotypes arising from loss of PilSR – leading to loss of PilA expression - from lack of PilA itself, which activates the PilSR system. Here we used RNAseq analysis to identify 48 genes in addition to *pilA* that have altered expression in the absence of *pilR*, including 11 genes that were inversely dysregulated by loss of *pilA* versus *pilR*. These analyses revealed that the PilSR TCS regulates transcription of *fleS-fleR,* and thus many of the genes in the FleSR regulon. As a result, deletion mutants of *pilSR* have defects in swimming motility that are independent of the loss of PilA. Together, these data suggest that PilSR has a broader role in regulation of *P. aeruginosa* virulence factor expression than previously appreciated, revealing a

novel, direct connection between the regulation of the Type IV pilus system and flagellar biosynthesis and function.

Introduction

Many cellular programs in prokaryotic species rely on the use of two-component regulatory systems (TCS) to tightly control their activity. Typically composed of an inner membrane histidine sensor kinase and a cytoplasmic response regulator, TCSs allow bacteria to respond rapidly to chemical or physical changes in their intra- or extracellular environments, altering specific gene expression in response to a stimulus (97). The opportunistic pathogen *Pseudomonas aeruginosa* expresses a higher than average number of two-component systems (98) that control diverse functions, including several motility phenotypes.

Flagellum-dependent swimming motility, for example, is controlled through a regulatory cascade that includes the transcriptional regulator, FleQ (194) and the FleS-FleR TCS, which like many TCSs also requires the alternate sigma factor RpoN (σ^{54})(109). FleQ controls transcription of *fleS-fleR* in addition to multiple other flagellar, adhesion and biofilm-associated genes, often in a c-di-GMP dependent manner (194, 195). FleSR has been implicated in the expression of 20 or more flagellar biosynthetic genes in *P. aeruginosa*, as well as additional genes not previously known to be involved in flagellar assembly or function (79).

The other major motility system in *P. aeruginosa* is the type IV pilus (T4P) system, which is used for twitching motility across solid and semi solid surfaces (143, 196) and a number of other important functions. In contrast to the single polar flagellum of *P. aeruginosa*, there are generally ~7-10 pili extended from the pole of a cell that can retract, either independently or in a coordinated bundle, to drive twitching motility (90, 197, 198). Pili can be extended from either pole but typically a single pole is used at one time to allow for directional twitching motility (199). The pilus fibre is made of up predominantly of hundreds to thousands of repeating subunits of the major pilin protein, PilA (20), the expression of which could be energetically costly to the cell if not tightly controlled.

pilA transcription is regulated by another TCSs –PilS-PilR–in *P. aeruginosa* and some other T4P-expressing bacteria (47, 117, 121, 122, 139). PilS is an atypical inner membrane sensor kinase (SK) in that it has 6 transmembrane segments rather than the 2-4 that are commonly present in SKs (116, 118, 130) Recent work by our group showed that PilS interacts directly with PilA through one or more of its transmembrane segments to allow for autoregulation of *pilA* transcription in response to inner membrane pilin levels (46). PilR is the cytoplasmic response regulator that can bind in conjunction with σ^{54} upstream of the *pilA* promoter to activate transcription (119, 120).

In contrast to the response regulator FleR, which has a well-defined regulon in *P. aeruginosa* (79), the potential suite of genes controlled by PilR is poorly characterized. Genetic and *in silico* analyses for PilR of *Geobacter*

sulfurreducens (139, 140) and *Dichelobacter nodosus* (122) have been performed, but comparable studies are lacking in *P. aeruginosa*. Screening of the *G. sulfurreducens* genome for putative PilR binding sites revealed 54 putative binding sites that co-occurred with hypothesized σ^{54}-dependent promoters; a number of these sites were upstream of genes required for T4P and flagellar biosynthesis or cell wall biogenesis (140). Those data, in association with the work performed in *D. nodosus*, which identified several surface-exposed proteins as being controlled by PilR, suggest that PilR of *P. aeruginosa* likely has additional functions beyond controlling *pilA* transcription. However, each of the cited studies focused mostly on characterization of pilus-related functions and did not look at other phenotypic consequences of loss of *pilR*.

In this work, we hypothesized that PilR of *P. aeruginosa* likely controls the expression of several other genes in addition to *pilA*. We used RNAseq analysis to identify genes that were ≥3-fold dysregulated by loss of *pilR*. Because T4P have important roles in surface sensing and control of downstream events such as biofilm formation, we included a *pilA* mutant in our analysis to distinguish those genes that are controlled by PilR from those that are affected by the loss of PilA, as *pilR* mutants also lack PilA. In addition to several genes that are co-regulated with *pilA*, we identified several flagellar genes, including the *fleSR* TCS, which is downregulated in the absence of *pilR.* We show that the consequence of this downregulation is a previously unidentified defect in swimming motility in both *pilS* and *pilR* mutants. This work defines the PilR regulon and provides evidence

for a novel, direct connection between regulation of the *P. aeruginosa* T4P and flagellar motility systems.

Results

Some genes are inversely dysregulated by loss of PilA versus PilR

RNAseq analysis was performed with the goal of identifying genes in addition to *pilA* that might be controlled by the PilSR TCS. However, in designing this experiment we first had to consider that i) *pilR* mutants also lack the PilA protein, ii) loss of PilA contributes to a decrease in intracellular levels of the messenger molecule cAMP (166) and iii) there are upwards of 200 genes in *P. aeruginosa* that are at least partially cAMP-dependent, including the gene encoding the cAMP-binding virulence factor regulator, Vfr (166). Therefore, to be able to separate those genes that are affected by loss of PilA and by extension, decreased cAMP, from those that are truly regulated by *pilR*, we sought to identify differentially expressed genes with altered expression in *pilA*, *pilR,* or both, compared to WT PAK. Those genes that are similarly dysregulated by loss of *pilA* or *pilR* are most likely to be genes affected by the absence of PilA **(Figure 4.1)**. Here, we used a ≥3-fold change cutoff to obtain a manageable list of genes for follow up. Genes that were similarly dysregulated by loss of *pilA* or *pilR* are summarized in **Table S4.1** and were not further examined here. However, this class of genes includes several T4P-associated genes such as *tsaP,* and minor pilins *fimU, pilW* and *pilY1*, previously identified as being Vfr dependent. 12/34

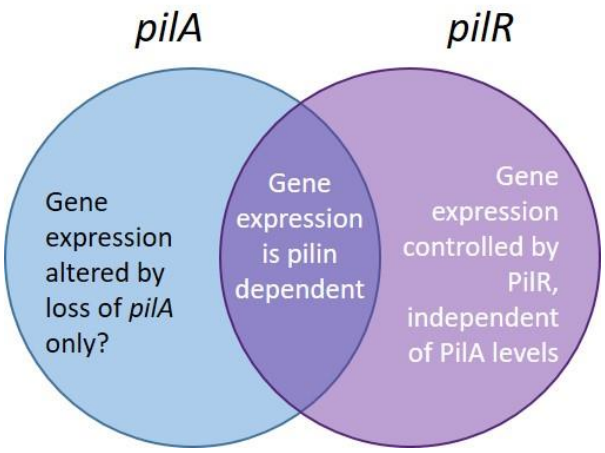
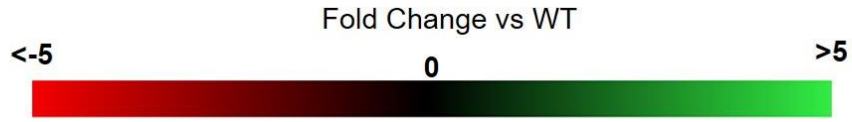


Figure 4.1. RNAseq experimental design. An RNAseq experiment designed to identify members of the PilR regulon required also identifying genes that are dysregulated by loss of *pilA,* as *pilR* mutants also lack the pilin protein. Genes dysregulated only by loss of *pilA* are likely pilin dependent but require regulators other than *pilR*. Genes coordinately dysregulated in both *pilA* and *pilR* compared to WT may be due to loss of PilA directly. Some genes may be inversely dysregulated in *pilA* and *pilR* mutants, suggesting that they, like *pilA*, are upregulated in the absence of PilA, in a *pilR*-dependent manner. Genes dysregulated only by loss of *pilR* and not *pilA* are dependent on PilR expression and are the focus of this study.

genes in this category were also determined previously to be Vfr dependent (166).

Among the genes whose expression was decreased in the *pilR* mutant were 12 genes that – somewhat counterintuitively – displayed increased expression in the *pilA* mutant, even though *pilR* mutants also lack PilA (119) (**Figure 4.2, Table S4.2)**. All but 2 of these genes are classed as hypothetical proteins or are unannotated in the PAO1 genome. Similar to PilA itself, expression of these genes appears to be increased in the absence of PilA, which activates PilSR in response to depletion of pilins (46). This expression pattern



Inversely Regulated Genes

PA Number	Gene Name	Product	Fold Change (*pilA* vs WT)	Fold Change (*pilR* vs WT)
PA0507		acyl-CoA dehydrogenase		
PA0951a		unannotated		
PA0952		hypothetical protein		
PA0952a		unannotated		
PA1441		hypothetical protein		
PA1512	*hcpA*	secreted protein (T6S)		
PA4027		hypothetical protein		
PA4683		hypothetical protein		
PA5228		hypothetical protein		
PA5228a		unannotated		
PA5267	*hcpB*	secreted protein (T6S)		

Pilus Associated Genes

PA Number	Gene Name	Product	Fold Change (*pilA* vs WT)	Fold Change (*pilR* vs WT)
PA0020	*tsaP*	T4P secretin associated protein		
PA4525	*pilA*	major pilin of T4P system		
PA4526	*pilB*	T4P assembly ATPase		
PA4528	*pilD*	Type IV prepillin peptidase		
PA4547	*pilR*	response regulator (T4P regulator)		
PA4550	*fimU*	type IV minor pilin *fimU*		
PA4552	*pilW*	type IV minor pilin *pilW*		
PA4554	*pilY1*	T4P putative adhesin *pilY1*		

Flagellum Associated Genes

PA Number	Gene Name	Product	Fold Change (*pilA* vs WT)	Fold Change (*pilR* vs WT)
PA1077	*flgB*	flagellar basal body rod protein		
PA1078	*flgC*	flagellar basal body rod protein		
PA1079	*flgD*	flagellar basal body rod modification protein		
PA1080	*flgE*	flagellar hook protein		
PA1081	*flgF*	flagellar basal body rod protein		
PA1082	*flgG*	flagellar basal body rod protein		
PA1083	*flgH*	flagellar L-ring protein precursor		
PA1092	*fliC*	flagellin		
PA1098	*fleS*	Sensor histidine kinase (flagellar regulator)		
PA1099	*fleR*	Response regulator (flagellar regulator)		
PA1100	*fliE*	flagellar hook basal body complex protein		
PA1101	*fliF*	flagellar M-ring outer membrane protein precursor		
PA1441	*fliK*	flagellar hook length control		
PA1967		hypothetical protein		
PA4310	*pctB*	chemotactic transducer		
PA4326		hypothetical protein		

Figure 4.2: Multiple T4P and non-T4P genes are affected by loss of *pilR*. The heat maps highlight select genes of interest that are ≥3-fold dysregulated by the loss of *pilR*, or both *pilA* and *pilR*. In addition to *pilA*, several other T4P

associated genes have altered expression in *pilA* and/or *pilR* mutants. Expression of eleven genes, mostly hypothetical or unannotated, is increased in a *pilA* mutant but decreased in *pilR*, which also lacks PilA. Of the genes dysregulated in a *pilR* mutant, a substantial number were associated with flagellar biosynthesis and function, including those encoding the FleS-FleR TCS.

suggests that the products of these genes could be previously unidentified contributors to T4P biogenesis and/or function.

RT-PCR was used to validate the expression of select genes, including the currently unannotated ORF PA0951a **(Figure 4.3)**, which was among the genes whose expression was most strongly affected by loss of *pilA* and *pilR*. There was a 5-fold increase in transcription in a *pilA* mutant but negligible expression in *pilR,* by both RNAseq and RT-PCR **(Table S4.2, Figure 4.3)**. Adjacent ORFs PA0952 and PA0952a exhibited comparable expression patterns in RNAseq **(Table S4.2, Figure 4.3);** together these genes may comprise an operon that is co-transcribed.

Loss of PilR alone dysregulates expression of 37 genes ≥3-fold

Of interest to this work were 37 genes with significantly altered expression (≥3-fold) in *pilR* mutants, while being unaffected by loss of *pilA*. Prior to this work, *pilA* was the only known gene controlled by the PilS-PilR TCS in *P. aeruginosa* (116), though studies in *G. sulfurreducens* and *D. nodosus* implied the possibility of additional members in the PilR regulon (122, 139, 140). The list of *pilR*

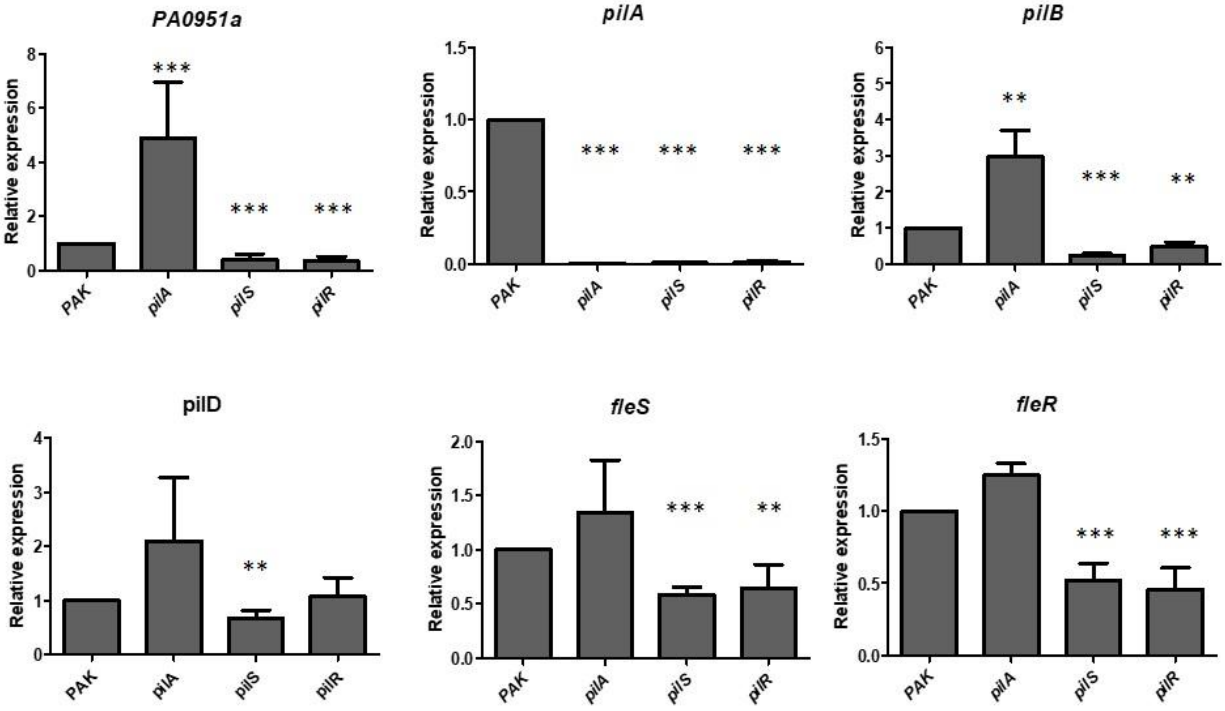


Figure 4.3. RT-PCR validation of select genes from RNAseq. *pilA* transcription was validated as a control for RNA quality, as *pilA, pilS* and *pilR* mutants should have negligible *pilA* transcription. *pilB* transcription was increased in a *pilA* mutant and decreased in *pilS* and *pilR* mutants. Effects on *pilD* transcription were modest but *pilS* mutants still had significantly decreased *pilD* transcription compared to WT. PA0951a had inversely dysregulated expression in *pilA* and *pilR* mutants in RNAseq, which were confirmed by RT-PCR. *fleS-fleR* were significantly downregulated in *pilS* and *pilR*, but not *pilA*. Mean and standard error of >3 experiments are shown.

dependent genes (at ≥3-fold change in expression) is summarized in **Table S4.3.** Genes in this group belong to several functional categories, including 5 putative chemotactic transducers, 2 biofilm associated chemosensory proteins, 6 hypothetical proteins, and several metabolic enzymes.

However, motility-associated genes were common. , The genes encoding the T4P assembly ATPase, *pilB* and prepilin peptidase, *pilD*, which share a

divergent promoter with *pilA*, were downregulated in *pilR,* but unaffected by loss of *pilA* **(Figure 4.2)**, even though previous studies list them as being under control of σ^{70} and not PilSR (200). When RT-PCR was used to validate this result, *pilB* was still significantly decreased in both *pilS* and *pilR* mutants **(Figure 4.3).** *pilD* expression trended towards being decreased in both as well, but was only statistically significant in the *pilS* mutant.

Multiple flagellum biosynthetic genes are downregulated in a pilR mutant

In addition to the few T4P associated proteins that appeared to be downregulated in a *pilR* mutant, independent of the loss of PilA, RNAseq also revealed a substantial number of flagellum biosynthetic genes with decreased expression in *pilR* but not *pilA* **(Figure 4.2, Table S4.3).** Among these genes were *fleS-fleR*, encoding the FleSR TCS, part of a regulatory hierarchy that controls the expression of a number of genes associated with flagellum biosynthesis and function (79, 109). Each had 3.5-fold decreased expression in *pilR* compared to WT. This trend was verified using RT-PCR, though the decrease was closer to 2-fold. Of the flagellar genes identified by RNAseq in **Figure 4.2**, 12 of 14 (excluding *fleS* and *fleR*) are known to be *fleR* dependent (79). The remaining two, *fliE* and *fliF,* are FleQ dependent, but showed decreased (≥2-fold) transcription in a *fleR* mutant in a previous study (79). These data suggest that PilSR positively regulate *fleSR* expression, leading to a decrease in expression of other FleSR-dependent genes when PilR is absent.

Swimming motility is impaired by loss of pilS-pilR

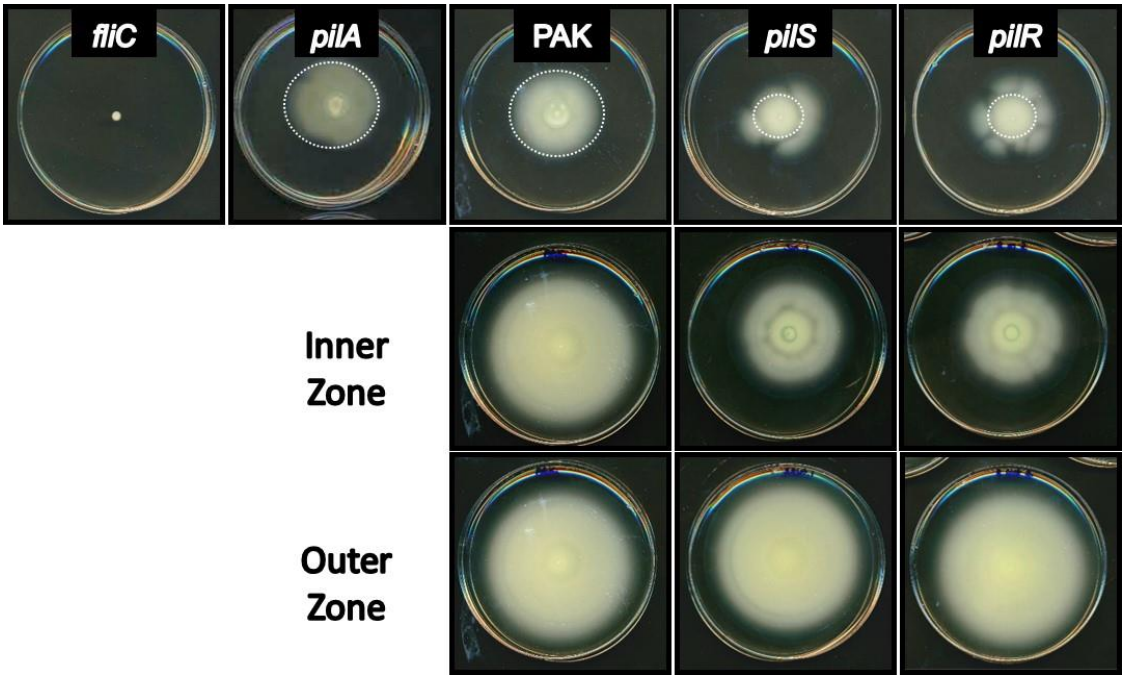


Figure 4.4. Swimming motility is impaired in *pilS* and *pilR* mutants. Loss of *pilS* or *pilR* results in decreased swimming motility (~40% of WT) in a plate based assay. *pilA* mutants swim comparably to WT, indicating that the swimming defect is not PilA dependent. *pilS* and *pilR* mutants appear to acquire suppressors that overcome these defects resulting in asymmetrical flares. Reinoculation of swimming plates with cells from the interior of swimming zones—inside the dotted white circles of *pilSR* (inner) recapitulate the original phenotype, while cells taken from the flares (outer, from flares outside the white circle, except for WT) swim to WT levels.

We next tested if the downregulation of *fleS-fleR* and associated flagellar genes in their regulon in the *pilS* and *pilR* backgrounds had an impact on swimming

motility, using a plate-based assay. A *fliC* mutant, which lacks the flagellin subunit, does not swim and was used as a negative control. *pilA* mutants no longer express the major pilin protein or surface pili, and are able to swim comparably to WT PAK, as T4P are not known to contribute to swimming motility. In contrast, both *pilS* and *pilR* mutants exhibit significant swimming defects ($p<0.005$), swimming uniformly only to about 40% of WT **(Figure 4.4, dashed line)**. Interestingly, for the *pilS* and *pilR* mutants, we routinely observed flares extending beyond these uniform zones with increased swimming motility. These regions were hypothesized to be the result of selection for suppressor mutations that could overcome the effect of *pilS* or *pilR* deletion on swimming.

To test this idea, we isolated cells from the inner swimming zones of *pilS* and *pilR* plates (inside the dashed line, **Figure 4.4**) and cells from the putative suppressor mutants (flares outside the dashed line) and reassessed their ability to swim after culturing them overnight. As controls, we took samples from the WT zone close to the point of inoculation (inner) and from the outer edge of the swimming zone (outer). Repeating the swimming assays with these samples revealed no difference in swimming between inner and outer samples from PAK. However, *pilS* and *pilR* cells taken from the inner swimming zones recapitulated the original swimming motility phenotype of the mutants, while cells taken from the outer flares had WT motility **(Figure 4.4),** indicating that they likely acquired mutation(s) that allow for full motility in the absence of *pilSR*.

We next wanted to determine if other flagellum-dependent phenotypes such as swarming motility were affected by loss of *pilS-pilR,* using both the original mutants, and the suppressors isolated from the swimming experiments performed above. *pilS-pilR* mutants in PAO1 were previously reported to be non-swarmers (185), though in our hands, the mutants in strain PAK are still capable of partial swarming motility, with an altered morphology compared to WT. The PAK *pilS-pilR* mutants swarm comparably to a *pilA* mutant, with fewer irregular tendrils **(Figure S4.1).** Interestingly, *pilS-pilR* mutants isolated from the outer flares of the swimming plates in **Figure 4.4** had swarming motility comparable to those isolated from the inner zones and the parent *pilS* and *pilR* strains, indicating that absence of *pilS* or *pilR* was the dominant phenotype for swarming.

FleS-FleR impact twitching motility and pilA expression

RNAseq analysis revealed that PilS and PilR affect the expression of *fleSR* and by extension, swimming motility. We next wanted to determine if this was part of a reciprocal regulatory pathway in which FleSR might contribute to regulation of *pilS-pilR* and the PilSR regulon. We tested this hypothesis by determining if FleSR affected *pilA* expression and/or T4P function. A double deletion of both *fleS* and *fleR* was made in the PAK background, and twitching motility was assessed. Loss of *fleSR* reduced twitching motility by a modest but significant degree ($p<0.005$), with strains twitching to approximately 80% of WT (**Figure 4.5A).** Interestingly, when *pilA* transcription was monitored using a *lux-pilA* reporter assay, *fleS-fleR* mutants had increased *pilA* transcription compared to

WT over the extent of the 5h time course measured **(Figure 4.5B)**. Therefore, while FleS-FleR contribute to twitching motility and *pilA* transcription, it is not yet clear if this occurs through regulation of *pilS-pilR* directly. Additional experiments, measuring *pilS-pilR* transcription in *fleS-fleR* mutants will be required.

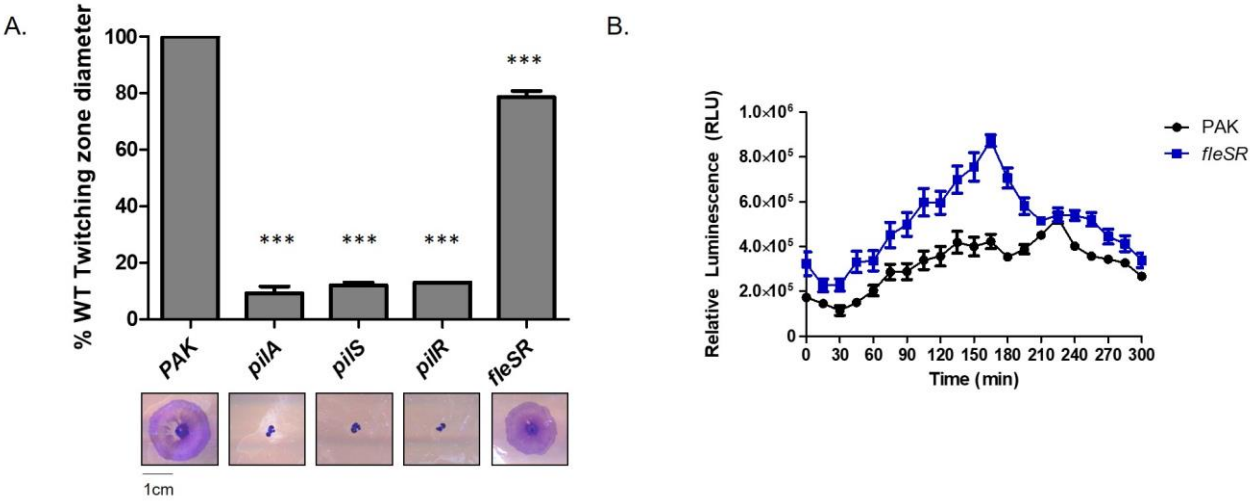


Figure 4.5. Loss of *fleSR* reduces twitching motility but increases *pilA* transcription. A) Loss of *fleSR* reduces twitching motility by approximately 20%. Mean and standard error of 6 independent replicates are shown. Significance determined by one-way ANOVA. **B)** A *lux-pilA* luminescent reporter assay measuring *pilA* promoter activity indicated that *pilA* transcription is increased over 5h. Mean and standard error of 4 biological replicates are shown.

Discussion

Two component systems control a plethora of phenotypes in prokaryotic species, allowing for quick responses by the cell to changes in its intra- and extracellular environments. These systems are not only important for survival of the bacterium, but also in many cases for coordinating virulence programs. Most

TCS explored to date control the transcription of multiple genes but prior to this work, in *P. aeruginosa* PilR had only a single known target, *pilA* (119). Microarray and bioinformatics analyses on the *G. sulfurreducens* PilR regulon provided evidence that PilR, like most other response regulators, regulates multiple genes ,including those required for soluble Fe(III) uptake—which is a pilin independent phenotype—flagellar assembly and function, and cell envelope biogenesis (139, 140). Based on those data, we explored the PilR regulon of *P. aeruginosa* and performed phenotypic assays to determine if the dysregulation of any genes contributed to alterations in swimming, swarming, and twitching motility, all phenotypes associated with virulence in specific hosts (6, 10, 87, 88).

The *G. sulfurreducens* microarray study and comparable *D. nodosus* studies failed to account for the confounding factor that by deleting *pilR*, PilA is not expressed. This was an important consideration in designing our RNAseq experiment, as loss of PilA results in a decrease of cellular cAMP and by extension, a downregulation of cAMP-dependent genes in the Vfr regulon (166). To that end, almost half the genes similarly dysregulated by loss of both *pilA* and *pilR* were previously identified by Wolfgang et al. to be Vfr dependent (166) To separate genes that are dependent on PilR from those that are affected by loss of PilA and/or decreased cAMP, we included both *pilA* and *pilR* mutants for comparison to WT **(Figure 4.1).** Here we focused on those genes that were dysregulated by loss of *pilR* but the genes in the latter category are summarized in **Figure S4.1**.

Interestingly, we identified 12 genes with increased transcription in a *pilA* mutant but significantly decreased transcription in the absence of *pilR*, even though *pilR* mutants lack PilA **(Figure 4.2)**. While this initially seemed counterintuitive, we realized that these genes likely encode proteins that are co-regulated with PilA. At high intracellular concentrations, PilA represses its own transcription by interacting directly with PilS and promoting its phosphatase activity on PilR (46). Conversely, when PilA is absent, *pilA* promoter activity is significantly increased, presumably in an attempt to replenish intracellular PilA pools (91). In the absence of *pilA*, PilS is hypothesized remain in a kinase state, activating PilR and increasing transcription of other genes in its regulon. In the absence of PilR, expression of those genes would be reduced **(Figure 4.2, Table S4.2)**. Interestingly, a disproportionate number of the genes in this category encoded for hypothetical proteins or were unannotated in the PAO1 and PAK genomes, the latter of which possibly encoding regulatory RNAs. Additional studies will be required to determine if, in addition to being co-regulated with *pilA*, these genes play a role in T4P biogenesis and function. The only two genes in this group that have been characterized are *hcpA* and *hcpB*, which encode secreted proteins associated with the Type VI secretion system. They are paralogs of one another and are possibly the result of a gene duplication event (124). Regardless, this may represent a previously unknown link between T4P and Type VI secretion.

Of particular interest were the genes identified by RNAseq as being affected only by loss of *pilR*, independent of the presence or absence of PilA. As reported by Krushkal *et al* for *G. sulfurreducens* (140), a substantial proportion of the 48 PilR-dependent genes were those previously associated with at least one form of motility. Among them were genes for the T4P assembly ATPase PilB and the prepilin peptidase, PilD **(Figure 4.2)**. RT-PCR analyses confirmed that *pilB* but not *pilD* was significantly dysregulated in the absence of *pilS* or *pilR* **(Figure 4.3)**. *pilBCD* are contiguous genes with *pilD* furthest from the divergent *pilA-pilB* promoter, but it does not appear that *pilBCD* are co-transcribed as polar insertions in one gene did not affect transcription of the remaining 2 genes (200) and *pilC* did not have enough reads assigned to it from RNAseq to determine differential expression (124). *pilB* is thought to be constitutively expressed from a σ^{70} dependent promoter region (200) and was not classified previously as being PilR or σ^{54} dependent. However, the changes in *pilB* expression observed here may not reflect direct regulation by PilR. Instead, when PilR facilitates DNA helix opening for *pilA* transcription by the σ^{54} holoenzyme, it may coincidentally enhance transcription from the divergent *pilB* promoter.

Of the 36 genes found to be strictly PilR dependent, 18 were annotated as involved in various aspects of biosynthesis, function and regulation of the flagellum (**Figure 4.2, Table S4.3)**. The genes encoding the FleSR TCS were among them (**Figure 4.3**), and all but two of the other flagellar genes downregulated by loss of *pilR* are known members of the FleSR regulon (79). It is

likely that expression of those genes are not controlled directly by PilSR but rather are downregulated because of decreased *fleS-fleR* expression in the mutants. As predicted from the expression data, swimming motility was decreased to about 40% of WT in both *pilS* and *pilR* mutants, while swimming of a *pilA* control was equivalent to WT **(Figure 4.4)**. These data confirm that the swimming deficit in the *pilS* and *pilR* mutants is not PilA dependent, but rather, likely linked to decreased *fleS-fleR* expression. Interestingly, by carefully analyzing the swimming data, we identified suppressor mutants that overcame the defects caused by *pilS-pilR* deletion, forming flares beyond the periphery of the majority of swimming cells. Bacteria isolated from these flares swam to WT levels. Work is ongoing to identify these suppressor mutations; we hypothesize that they may have occurred in, *fleS-fleR, fleQ,* or their promoter regions, as such mutations could increase activity or expression of *fleS-fleR*. These mutations are specific for flagellar function, as swarming motility (95) of the *pilS* and *pilR* mutants and their highly motile suppressors, all of which lack PilA, was comparable to a *pilA* mutant **(Figure S4.1)**,

Although *pilS-pilR* were not previously considered members of the FleSR regulon (79), twitching motility was modestly but significantly reduced to about 80% of WT in the absence of *fleSR* **(Figure 5A)**, while, *pilA* promoter activity was increased compared to WT **(Figure 5B)**. This phenotype is reminiscent of that of a *pilT* mutant, which is defective in pilus retraction and has no twitching motility, but exhibits increased *pilA* transcription due to unopposed pilus assembly and

depletion of PilA subunits from inner membrane pools (90, 91). Whether FleSR might affect pilus function will be the topic of future work.

During the characterization of the FleSR regulon, two genes (PA3713 and PA1096/*fleP)* not previously known to participate in motility phenotypes were identified. Mutants in those genes were significantly impaired in swimming, and in the case of *fleP,* in twitching motility (79). FleP was proposed to control pilus length, as when it was deleted, surface pili were significantly longer than those of WT cells, resulting in a form of hyperpiliation (79). In this study, *fleP* and PA3713 did not appear as differentially expressed genes as the number of assigned reads were too low for them to be included. They may have decreased expression in *pilS-pilR* mutants due to their FleSR dependence, but additional studies will be required to test this directly. However, deletion of *fleS-fleR* may indirectly affect twitching and *pilA* expression due to decreased *fleP* transcription.

Both the PilSR and FleSR TCSs are required for the full virulence of *P. aeruginosa* (reviewed in (99)), as each is involved in several virulence-associated phenotypes. PilSR and FleSR each contribute to surface attachment and biofilm formation (6, 201), and are important for twitching and swimming motilities. Both PilSR and FleSR are required for swarming motility in most strains of *P. aeruginosa* due to their involvement in pilus and flagellum function (95, 185, 201), (**Figure S1)**. Given the significant overlap in phenotypes controlled by.

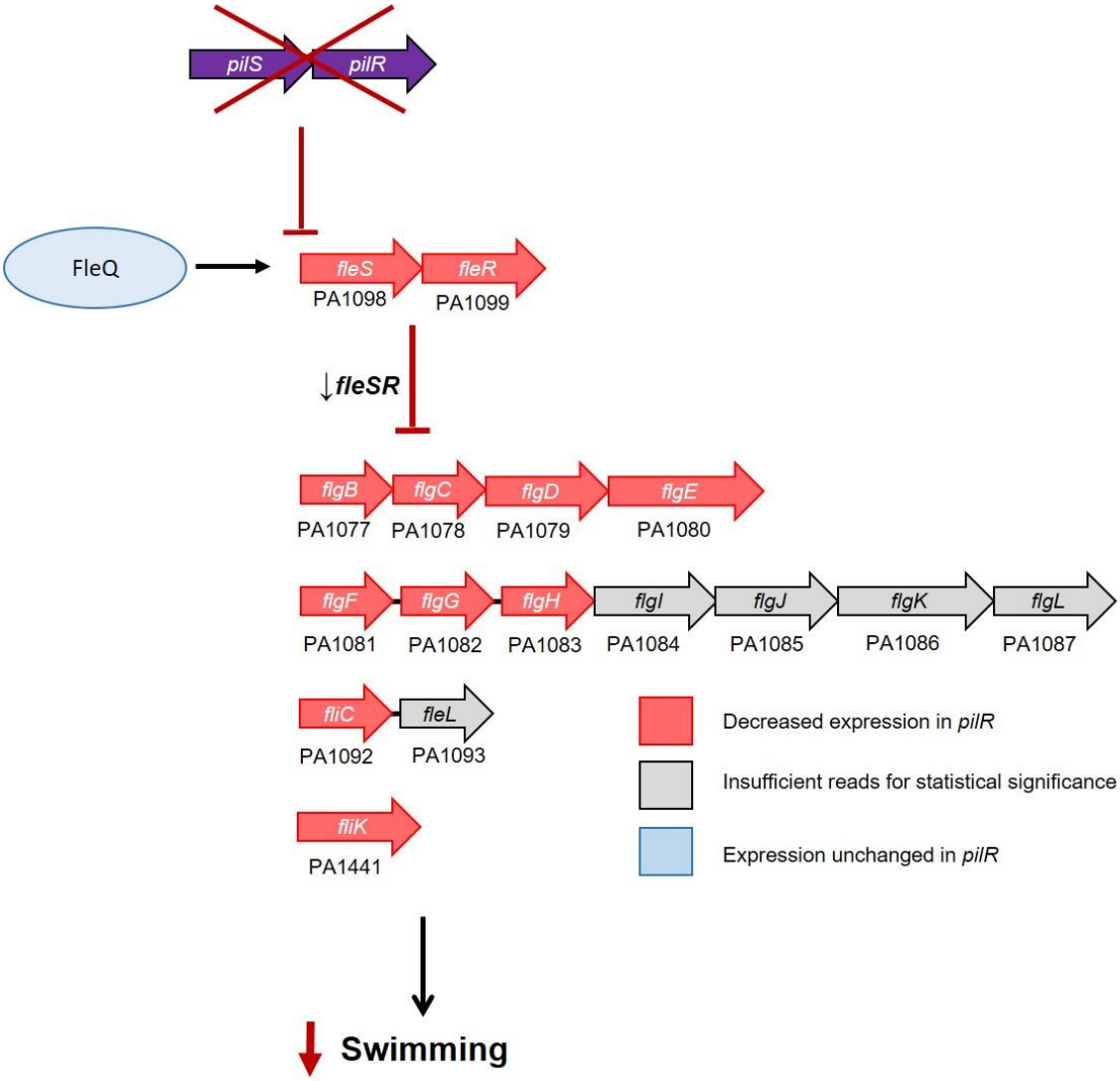


Figure 4.6. Model for *pilSR* dependent regulation of *fleSR* and the *fleSR* regulon. Under conditions in which *pilSR* expression is decreased (low cAMP) or perhaps when their activity is low (high intracellular PilA), *fleSR* transcription is decreased. As a result, in *pilSR* mutants, as is expression of the the *fleSR* regulon. Genes in red are those that had decreased expression in a *pilR* mutant in RNAseq. Grey genes did not have sufficient reads assigned to them from RNAseq to accurately report differential expression. FleQ (blue) was not differentially expressed between WT or *pilR* indicating that *pilSR* fits into the flagellar regulatory hierarchy after FleQ but before *fleSR*, as *fleQ* itself, and most FleQ dependent genes were unaffected by loss of *pilR*.

PilSR and FleSR, it is perhaps not surprising that expression of the two systems may be linked. From our RNAseq analysis and subsequent phenotypic assays, we propose a model in which PilSR positively regulates *fleS-fleR* transcription, perhaps independently of PilA depletion **(Figure 4.6).** The hierarchy for flagellar biosynthesis proposed by Dasgupta *et al* (79), proposes that transcription of *fleS-fleR* is predominantly dependent on FleQ. Since FleQ was not differentially expressed in *pilR*, we can infer that PilS-PilR likely promotes *fleS-fleR* transcription directly, rather than by first modulating expression of FleQ.

The question that remains is why – and under what conditions – would this regulatory circuit be active*?* Twitching motility is normally used on solid or semi solid surfaces (196) while flagella are typically used in liquid and lower viscosity conditions so one might expect that when one system is turned off, the other would be turned on, in response to environmental conditions. Since this appears to not be the case, it is possible that the regulatory integration of these two systems is an adaptation to life as an opportunistic pathogen. T4P and flagella are typically expressed during the acute phase of infection (85, 109) and during the transition to the chronic infection phase, motility systems are downregulated in favour of those promoting Type VI secretion and biofilm formation (7, 99). In fact, clinical isolates of *P. aeruginosa* taken from chronically colonized patients are often found to be both non-flagellated and non piliated (202). Lack of the immunogenic flagellum may help *P. aeruginosa* escape phagocytosis (202) and in other species aflagellate bacteria were better able to evade the inflammatory

response of the host (178). Perhaps placing *fleS-fleR* under control of PilSR may facilitate a more rapid transition to the chronic disease state.

This study supports previous work in *G. sulfurreducens* that identified putative PilR binding sites upstream of several genes, in addition to *pilA*. Using RNAseq analysis, we identified 48 genes in addition to *pilA* that are affected directly by loss of *pilR*. Our data show that the role of *P. aeruginosa* PilSR extends beyond just controlling expression of the major pilin gene and support a model in which PilSR can regulate *fleS-fleR* transcription downstream of FleQ. This work identifies a novel mechanism for the regulation of two diverse motility systems, which may have implications in the transition from acute to chronic disease states in a host.

Methods

Bacterial strains and growth conditions

Unless otherwise specified, *Pseudomonas aeruginosa* PAK strains were grown in Lennox Broth (LB) (Bioshop) or on LB 1.5% agar plates at 37°C. Where the antibiotic kanamycin was used, it was introduced at a final concentration of 150µg/mL. Mutants were generated by homologous recombination, using standard mating techniques described in (167). The strains and plasmids used in this study are outlined in **Table 4.1.** Plasmids were prepared using standard cloning techniques and introduced into *P. aeruginosa* using electroporation.

Table 4.1. Strains and plasmids used in this study

Strain	Description	Source
PAK WT	WT Group II strain of *P. aeruginosa*	(J. Boyd)
pilA	PAK with chromosomal deletion of *pilA*	(This study)
pilS	PAK with chromosomal deletion of *pilS*	(This study)
pilR	PAK with chromosomal deletion of *pilR*	(This study)
fliC	PAK wit FRT insertion in *fliC*	(This study)
fleSR	PAK with chromosomal of the full *fleS-fleR operon*	(This study)
Plasmid		
pMS402-p*pilA*	*pilA* promoter cloned into the BamHI site of pMS402, putting lux genes under control of *pilA* promoter	(46)

RNA isolation, library preparation, cDNA synthesis and analysis

To isolate RNA, cells from strains of interest were streaked in triplicate onto half of an LB 1.5% agar plate (100x15mm petri dishes) and grown overnight at 37°C. Cells were scraped from the plates and resuspended in 1.5mL RNAprotect Bacteria Reagent (Qiagen) to maintain integrity of isolated RNA. Cells were chemically lysed using 1mg/mL lysozyme in 10mM Tris-HCl and 1mM EDTA, pH 8.0 and RNA isolated using the RNeasy mini kit (Qiagen) according to manufacturers' instructions. An on-column DNase treatment was performed to minimize potential DNA contamination. Purified RNA was eluted into 50µL nuclease free water and quantified.

The following steps were performed by the Farncombe Metagenomics Facility (McMaster University, Hamilton, ON, Canada). For RNAseq analysis,

ribosomal RNA was depleted from 9 RNA samples (3x WT PAK, 3x *pilA* and 3x *pilR*) using the Ribo-zero rRNA depletion kit (Illumina) and cDNA libraries prepared by the NEBnext Ultra Directional Library Kit. Libraries were sequenced using paired end 75bp reads on the Illumina MiSeq platform. Reads were aligned to the PAO1 reference genome with 98% of reads mapped and normalization and differential gene expression were calculated using the Rockhopper software (203). q-values for each identified gene are reported in **Tables S1-3.**

RT-PCR analyses

RNA was isolated and DNase treated as described above. To generate cDNA for RT-PCR analysis, qScript cDNA synthesis kit (QuantaBio) was used according the manufacturer's instructions, using 600ng of RNA as the starting template. cDNA was diluted 1:100 and 5µL of diluted cDNA was used for RT-PCR. PCR samples were prepared using PerfeCTa SYBR Green Super mix (QuantaBio) according to manufacturer's instructions and gene specific primers. *rpsL* (30S ribosomal protein S12, PA4268) was used as a control housekeeping gene. RT-PCR was performed using a Lightcycler 480 plate reader (Roche) and relative expression levels of genes of interest were calculated using the PFAFFL equation (204). Data presented are mean and standard error of 4 independent experiments. Statistical significance was determined using a one-way ANOVA.

Motility assays

Swimming. Swimming motility plate assays were performed similarly to (205), with some modifications. Overnight 5mL cultures of strains of interest were grown

at 37°C in LB with shaking. On the day of inoculation, LB 0.25% agar plates were prepared and allowed to solidify at room temperature for 1.5h. Cell cultures were standardized to an OD_{600}=1.0 and 2µL were spotted onto the centre of each plate. Plates were incubated upright for 16h at 37°C and swimming zone diameters were quantified using ImageJ (http://imagej.nih.gov/ij/, NIH, Bethesda, MD). Where applicable, swimming zone diameters were defined at the outer most part of the swimming zone that was still uniform in appearance. Images are representative of 4 independent experiments. To determine statistical significance, a one-way ANOVA analysis with Dunnett's post-test was performed, using WT as the control strain.

Twitching. Twitching motility assays were performed as described in (150). Briefly, strains of interest were stab inoculated to the bottom of an LB 1% agar plate with a P10 pipette tip and plates were incubated upside down at 37°C for 16-24h. Following incubation, agar was carefully removed and the plastic petri dish was stained with 1% crystal violet for 20min. Excess dye was washed away with water and twitching zone diameters were quantified using ImageJ ((http://imagej.nih.gov/ij/, NIH, Bethesda, MD). A one-way ANOVA statistical test was used to determine significant differences in twitching compared to WT.

Swarming. Swarming motility assays were performed as described in (132). Briefly, strains of interest were grown overnight in 5mL LB cultures at 37°C. On the day the assay was to be performed, 0.5% agar plates with M8 buffer, supplemented with 2mM $MgSO_4$, 0.2% glucose, 0.05% L-glutamic acid and trace

metals, were prepared and allowed to solidify at room temperature for 1.5h. After this time, 3.5µL of culture were spotted onto the centre of a single plate and plates were incubated upright in a humidity-controlled 30°C incubator for 48h. Plates were imaged using a standard computer scanner. Figures shown are representative of 3 independent experiments.

lux-pilA reporter assay

Luminescent reporter assays were performed as in (46). Strains of interest were transformed by electroporation with the pMS402-p*pilA* plasmid, which contains the luciferase genes under control of the *pilA* promoter. Strains were grown overnight in 5mL LB cultures supplemented with 150µg/mL kanamycin. The following day, a 1mL aliquot of a 1:20 dilution of cultures were prepared and 100µL samples were plated in triplicate in a white walled, clear bottom 96-well plate (3632 Costar, Corning Inc). Luminescence and OD_{600} were measured at 15min intervals over 5h using a Synergy 4 microtitre plate reader (BioTek) programmed to shake continuously and incubate the plate at 37°C. Luminescence was normalized to OD_{600} and relative luminescence was plotted against time. Mean and standard error of >4 biological replicates are shown.

Supplementary data

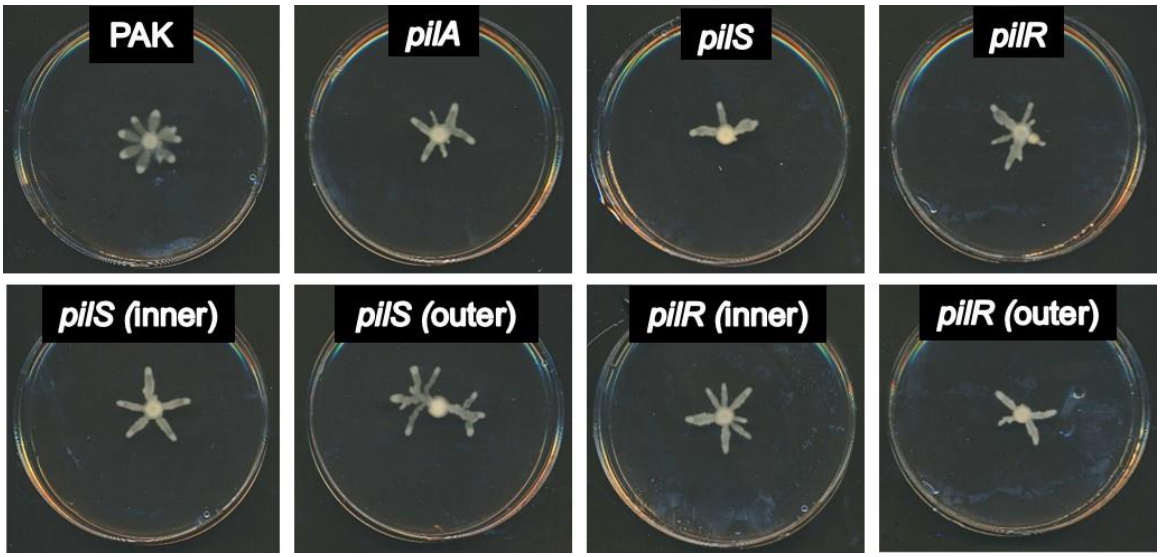


Figure S4.1. Putative suppressors that restore swimming do not affect swarming motility in *pilSR mutants.* Cells from *pilS* and *pilR* mutants with putative suppressor mutations that restore swimming motility (outer) were inoculated in a swarming assay for comparison to *pilS* and *pilR* mutants that do not have suppressors (inner). Strains with possible suppressors still exhibit *pilS* and *pilR*-like swarming motility patterns indicating that the *pilSR* phenotype is dominant.

Table S4.1 Genes cooperatively dysregulated by loss of *pilA* and *pilR*

PA Number	Gene Name	Product	Fold Change (*pilA* vs WT)	Fold Change (*pilR* vs WT)	qValue (pilA/*WT)*	qValue (*pilR*/WT)
PA0020	*tsaP*	T4P secretin associated protein	-2.56	-2.65	0.080003	0.002698
PA0320	*carO*	calcium-regulated OB fold protein	-2.40	-3.84	4.95E-04	1.27E-13
PA0391		hypothetical protein	-2.96	-2.92	2.86E-06	1.43E-08
PA0407	*gshB*	Glutathione synthetase	-2.79	-2.76	0.001543	1.58E-05
PA0413	*chpA*	Component of chemotactic system	-2.51	-2.39	5.34E-04	2.32E-05
PA0572		Hypothetical protein	-3.67	-3.63	2.60E-10	9.33E-14
PA0602		Binding protein component of ABC transporter	-2.73	-3.04	0.003606	7.69E-07
PA0613		Hypothetical protein	-2.30	-3.38	0.002407	4.29E-12
PA0662	*argC*	N-acetyl-gamma-glutamyl phosphate reductase	-2.26	-2.68	0.001543	7.97E-08
PA0663		Hypothetical protein	-2.02	-2.51	0.009526	1.03E-06
PA0664		Hypothetical protein	-2.25	-3.02	0.001859	2.03E-08
PA0812		Hypothetical protein	-2.29	-2.77	0.003342	1.44E-07
PA0824		Hypothetical protein	-2.32	-3.02	0.002088	2.42E-09
PA0852	*cbpD*	Chitin binding protein	-5.07	-3.66	3.43E-11	1.05E-06
PA1028	*amaA*	L-pipecolate oxidase	-2.60	-3.12	0.126972	1.83E-04
PA1296		2-hydroxyacid dehydrogenase	2.11	2.84	0.189054	0.001719
PA1871	*lasA*	Protease	-2.74	-2.11	0.003288	0.016141
PA2204		Binding protein component of ABC transporter	-2.20	-2.64	0.005116	1.88E-06
PA2453		Hypothetical protein	-2.14	-3.15	0.003851	1.63E-10
PA2782	*bamI*	Biofilm associated metzincin inhibitor	-11.07	-5.70	3.23E-152	3.74E-48
PA2783	*mep72*	metalloprotease	-12.17	-12.17	9.44E-49	4.00E-65
PA2916		Hypothetical protein	-2.08	-3.62	0.007118	7.10E-13
PA3278		Hypothetical protein	-3.15	-4.60	4.81E-07	1.66E-27
PA3450	*lsfA*	1-cys peroxiredoxin	-2.26	-2.61	0.013348	5.48E-05
PA3792	*leuA*	2-isopropylmalate synthase	-2.50	-2.68	1.54E-04	5.23E-07
PA4525	***pilA***	**Major pilin of T4P system**	**-2333.25**	**-212.52**	**0**	**0**
PA4550	*fimU*	Type IV minor pilin *fimU*	-2.01	-4.27	0.047982	3.89E-20

PA4552	*pilW*	Type IV minor pilin *pilW*	-2.12	-4.28	0.064459	9.38E-16
PA4554	*pilY1*	T4P putative adhesin *pilY1*	-2.03	-3.05	0.105150	1.95E-07
PA4704	*cbpA*	cAMP binding protein A	-3.36	-2.93	2.63E-09	2.66E-08
PA5027		Hypothetical protein	-2.16	-3.40	0.007990	7.24E-13
PA5137		Hypothetical protein	-2.42	-2.89	0.00115	2.10E-08
PA5332	*crc*	Catabolite repression control protein	-3.53	-3.49	6.45E-11	1.80E-12
PA5355	*glcD*	Glycolate oxidase subunit	-2.71	-2.30	0.069706	0.059996
PA5472		Hypothetical protein	-2.22	-3.30	0.002248	1.08E-10

Table S4.2 Genes differentially dysregulated by loss of *pilA* and *pilR*

PA Number	Gene Name	Product	Fold Change (*pilA* vs WT)	Fold Change (*pilR* vs WT)	qValue pilA/WT	qValue *pilR*/WT
PA0507		Acyl-CoA dehydrogenase	4.89	-3.42	6.27E-12	1.95E-07
PA0951a		Unannotated	5.17	-64.65	0.039446	0
PA0952a		Unannotated	8.55	-10.17	3.06E-25	1.36E-14
PA0952		Hypothetical	4.78	-49.02	0.001324	0
PA1441		Hypothetical	1.76	-3.04	0.797948	6.97E-07
PA1512	*hcpA*	Secreted protein (T6S)	3.04	-3.25	0.028525	1.82E-09
PA4027		Hypothetical	5.47	-2.07	8.11E-17	0.143421
PA4683		Hypothetical	5.12	-4.46	1.83E-04	7.16E-26
PA5228		Hypothetical	2.06	-4.76	0.529977	6.51E-20
PA5228a		Unannotated	1.92	-9.31	0.772088	7.42E-110
PA5267	*hcpB*	Secreted protein (T6S)	2.64	-4.96	0.09538	1.51E-04

Table S4.3 Genes dysregulated in *pilR* compared to WT

PA Number	Gene Name	Product	Fold Change (*pilR* vs WT)	q Value *pilR*/WT
PA0534	*pauB1*	FAD-Dependent oxidoreductase	-3.16	4.88E-11
PA1077	*flgB*	flagellar basal body rod protein	-3.01	3.29E-07
PA1078	*flgC*	flagellar basal body rod protein	-3.01	3.29E-07
PA1079	*flgD*	flagellar basal body rod modification protein	-3.01	3.29E-07
PA1080	*flgE*	flagellar hook protein	-2.73	1.22E-04
PA1081	*flgF*	flagellar basal body rod protein	-3.00	1.04E-06
PA1082	*flgG*	flagellar basal body rod protein	-3.00	1.04E-06
PA1083	*flgH*	flagellar L-ring protein precursor	-3.00	1.04E-06
PA1092	*fliC*	Flagellin	-3.91	2.86E-16

PA1098	*fleS*	Sensor histidine kinase (flagellar regulator)	-3.56	3.01E-15
PA1099	*fleR*	Response regulator (flagellar regulator)	-3.56	3.01E-15
PA1100	*fliE*	flagellar hook basal body complex protein	-2.94	9.20E-07
PA1101	*fliF*	flagellar M-ring outer membrane protein	-2.94	9.20E-07
PA1422	*gbuR*	regulatory protein	-3.56	1.71E-09
PA1423	*bdlA*	biofilm dispersion (chemosensor)	-6.52	6.69E-46
PA1441	fliK	flagellar hook length control	-3.04	6.97E-07
PA1452	*flhA*	flagellar biosynthesis	-2.91	3.56E-08
PA1453	*flhF*	flagellar biosynthesis	-2.56	9.38E-07
PA1561	*aer*	aerotaxis receptor	-2.94	1.51E-09
PA1697		hypothetical protein	-3.60	1.54E-13
PA1966		hypothetical protein	-2.60	2.38E-04
PA1967		hypothetical protein	-3.56	4.72E-13
PA2274		hypothetical protein	5.35	1.25E-14
PA2654		chemotaxis transducer	-3.71	7.59E-17
PA2787		glutamate carboxypeptidase	-2.33	2.32E-05
PA2867		chemotaxis transducer	-3.06	3.11E-10
PA2954		hypothetical protein	-2.40	5.26E-06
PA2955		hypothetical protein	-2.40	5.26E-06
PA3350	*flgA*	flagellar basal body P-ring biosynthesis protein	-2.20	1.91E-04
PA3417		pyruvate dehydrogenase E1 component	2.80	0.004487328
PA4309	*pctA*	chemotactic transducer	-2.60	1.65E-04
PA4310	*pctB*	chemotactic transducer	-3.24	7.36E-12
PA4326		hypothetical protein	-3.18	5.14E-10
PA4524	*nadC*	nicotinate-nucleotide pyrophosphorylase	-7.08	1.00E-34
PA4526	*pilB*	T4P assembly ATPase	-4.68	1.24E-17
PA4528	*pilD*	Type IV prepillin peptidase	-3.83	3.30E-12
PA4547	***pilR***	**response regulator (T4P regulator)**	**-48.82**	**0**

CHAPTER FIVE

Discussion, future directions, and conclusions

Discussion

Type IV pilin expression is autoregulated

Use of the T4P system for twitching motility can be very energetically costly to the cell, with hundreds to thousands of major pilin subunits being required for each extension cycle (196). Although the subunits can be reused (44), *P. aeruginosa*, *M. xanthus, G. sulfurreducens* and other species tightly regulate production of PilA using the PilSR TCS to reduce this potential cost PilSR (47, 117, 139). One of the important but unanswered questions prior to this work and similarly, one of the major challenges in the study of two-component systems in general, was identifying the signal(s) to which sensor kinases such as PilS respond (97).

Previous studies in both *P. aeruginosa* and *M. xanthus* hinted at the possibility that PilA might regulate its own expression (47, 91). Upon deletion of the *pilA* gene in *P. aeruginosa*, or loss of the retraction ATPase PilT, which depletes pilin pools by trapping them outside the cell, *pilA* promoter activity was significantly elevated. Conversely, deletion of the assembly ATPase, *pilB,* causes an increase in membrane-associated pools of PilA while *pilA* transcription decreases (91). In *M. xanthus,* mutations at the conserved E5 residue of PilA which prevent pilin assembly resulted in increased *pilA* promoter activity using a β-galactosidase reporter, while other mutations outside of the conserved N-terminal α-helix did not have this effect (47).

From these studies, it appeared that *P. aeruginosa* could detect levels of intracellular pilin and adjust *pilA* transcription accordingly to maintain a steady state, and furthermore, that specific mutations in the N-terminus of PilA might impair this feedback. However, the mechanism of feedback was obscure. Because PilS was known to control *pilA* transcription and because topological studies revealed that it had 6 TM segments, atypical for a sensor kinase, which normally has only 2-4 (97, 118, 130), we hypothesized that PilS could detect membrane bound PilA directly **(Chapter 2)**. Using a combination of heterologous pilin overexpression and a BACTH assay, PilA and PilS were found to interact directly through the conserved N-terminal α-helix of PilA and one or more of the TM segments of PilS. This interaction is thought to convert PilS to its phosphatase conformation, resulting in a decrease in *pilA* transcription until membrane pools of PilA are depleted. PilS is localized to the poles of *P. aeruginosa* cells in a manner dependent on the cytoplasmic linker region of PilS and the hub protein FimV (131, 132). This may improve PilS's function as a possible energy-saving SK as maintaining localization where pilins are thought to be most concentrated would allow it to respond only to a true depletion of pilins rather than to decreases in the pilin concentration gradient as one moves farther away from the poles.

While this work addressed the nature of the signal detected by PilS in *P. aeruginosa,* it has bigger implications for the areas of T4P regulation and TCSs. With the high degree of sequence conservation in the N-terminus of PilA and

evidence of pilin autoregulation in other T4P-expressing bacteria, it is likely that autoregulation through direct PilA-PilS interactions is the mechanism used for controlling *pilA* transcription in a wider range of bacterial species. Indeed, we showed that pilins from *G. sulfurreducens*, which consist of little more than the N-terminal α-helical region of a typical type IV pilin, could suppress expression of native pilins when overexpressed in *P. aeruginosa*.

Diverse pilin genes and their corresponding accessory proteins are often acquired through horizontal gene transfer, while PilS is not currently known to be comparably mobile (23). Adaptation of a conserved N-terminal region in the pilin has made it possible for pilin genes—with their appropriate accessory proteins—to be transferred between different strains of *P. aeruginosa* and still be recognized by the T4P assembly system, which is highly conserved across *P. aeruginosa* strains. Interestingly, it seems that the PilSR regulatory system has capitalized on this conserved region as well since PilS is nearly identical across *P. aeruginosa* strains and can detect and respond to increased levels of PilA from any of the 5 groups, or the truncated pilin from *G. sulfurreducens* **(Chapter 2)**. This level of conservation in the pilin may allow for more broad distribution of various pilin types while still being able to maintain the same mechanism for regulation across multiple T4P-expressing species.

Furthermore, this work unveiled an unusual mechanism for TCS regulation: the product of a TCS directly inhibiting its own expression through

intramembrane interactions. Most of the previously identified intramembrane signals of TCSs are related to cell envelope integrity, and are more prevalent in Gram positive bacteria (110). Systems such as PhoPQ (159) or BovKR (155) that use autoregulation to control gene expression, do so through the expression of regulatory peptides or as a means for upregulating, rather than downregulating its own expression respectively. However, the work described herein provides evidence for autoregulation via intramembrane sensing, which should prompt a search for other examples of this interesting feedback strategy. Investigating the contributions of this mechanism for regulation of other inner membrane proteins may be a valuable starting point as unchecked expression of these proteins—particularly those that encode transporters or pore-formers—could potentially disrupt integrity of the inner membrane.

Overexpression of surface pili is detrimental to P. aeruginosa virulence

In addition to characterizing PilSR's role in regulating overall pilin levels to mitigate the potential metabolic cost of protein production, we uncovered its unexpected contribution to controlling the level of surface piliation – and thus pathogenicity – of *P. aerugi*nosa. The role of T4P in virulence has been established in many bacteria on many different hosts (9, 10, 83-85, 87, 88, 99) but almost no studies have focused on the effects of pilus overexpression. Those that have addressed the adherence capabilities or virulence of hyperpiliated strains such as *pilT* or *pilU* mutants attributed their lack of pathogenicity to a loss of retraction or twitching motility, rather than to having too many surface pili (10,

86, 87). More recent work however, has started to link *pilT* mutants and their resultant hyperpiliation to defects in contact-dependent virulence factors such as T3S (178, 179) and exolysin-mediated killing (206). Furthermore, loss of pilus retraction also impairs surface sensing by *P. aeruginosa*, which is required for upregulation of multiple virulence programs (9, 11, 94) Prior to the work presented herein, it was difficult to separate twitching and hyperpiliated associated phenotypes, as a surplus of surface pili is usually the result of deficits in pilus retraction (75, 77, 90, 184, 207). As retraction is the driving force for twitching motility, these mutants typically had significantly impaired twitching motility (90).

In **Chapter 3,** we provided evidence contradicting the paradigm that twitching motility is required for virulence. The activity of TCSs can be modulated in a number of different ways, through the use of specific amino acid residues. Kinase or phosphatase motifs of the sensor kinase component can be mutated to selectively diminish one activity or the other (112, 118), while the conserved Asp at the site of phosphorylation on the response regulator can be altered to mimic phosphorylation, usually through a D→E substitution (182, 208). We capitalized on both of these options to create PilS N323A and PilR D54E chromosomal point mutants, disrupting the PilS phosphatase motif and the site of PilR phosphorylation, respectively. In both cases, we demonstrated that these mutants overproduced PilA, but that both strains also produced a surfeit of surface pili. Interestingly, since these mutations did not affect pilus retraction,

both twitched at WT levels, unlike most other hyperpiliated mutants identified to date.

When assessed for their ability to kill *C. elegans* in a slow killing assay, both PilS and PilR point mutants were less pathogenic than WT, similar to a retraction-deficient *pilT* mutant, despite having normal twitching motilities. This result provided evidence that at least in this model, twitching motility had a negligible effect on virulence. Through deletion of *pilA* or the assembly ATPase *pilB,* we determined that it was the presence of an overabundance of surface pili that was responsible for reducing virulence, as deletion of either of these two genes reverted pathogenicity of the hyperpiliated strains to WT.

As loss of T3S reduced pathogenicity of *P. aeruginosa* in *C. elegans* in WT and strains carrying the PilS N323A and *pilA* double mutations, we hypothesized that hyperpiliation creates a physical barrier between *P. aeruginosa* cells and the host epithelium, preventing cell-cell contact required for engagement of T3S and other virulence factors including type VI secretion and exolysin mediated killing (179, 181, 206, 209). Our results mean that the data showing that loss of *pilT* impairs injection of the T3S effector ExoS into hosts (179, 180, 210) may require reinterpretation. Furthermore, while both *pilT* and *pilU* are thought to be involved in cytotoxicity in a mouse model, the *pilU* mutants are less hyperpiliated than *pilT* and had more modest effects on the pathogenicity associated phenotypes tested (86). With fewer surface pili than a *pilT* mutant, it is possible that *pilU* mutants are still partially capable of engaging contact-dependent virulence factors.

Together, these data improve our knowledge of the role of T4P in pathogenicity. While they are known virulence factors, in *C. elegans,* overproduction of surface pili is surprisingly more detrimental to virulence than loss of pili all together, presumably because non-piliated mutants are still capable of contacting the host cell surface, the gut of the worm. While it appeared counterintuitive that *pilA* mutants were nearly as pathogenic as wild type in this model, we surmise that the route of infection (ingestion of bacteria by the worms) means that T4P-mediated host adherence is less critical. We also provide an alternate explanation for the loss of virulence in *pilT* mutants, suggesting that it is decreased engagement of contact dependent virulence factors as a result of hyperpiliation, rather than a direct result of diminished retraction, that causes this effect. This information also provides us with expanded alternatives for developing pilus-based antivirulence therapeutics, as compounds that either abrogate or increase expression of surface pili may be equally successful at impeding pathogenicity.

The PilSR TCS regulates multiple genes including those involved in flagellar biosynthesis

When the PilSR TCS was first described, it was thought to control only expression of *pilA* (117). In the 20 years since this first description, there has been very limited work done to explore the full suite of genes controlled by PilSR, particularly in *P. aeruginosa*. In *G. sulfurreducens,* which produces T4P capable of electron transfer (13), DNA microarray and bioinformatics studies identified

several genes with altered expression as a result of loss of PilR as well as many with putative PilR dependent promoters (139, 140). Among these genes were several involved in T4P biogenesis, flagellar biogenesis and function, and cell envelope maintenance. However, in those studies the authors focused predominantly on the transcriptional aspects rather than the phenotypic consequences of PilR-dependent dysregulation. One exception to this was the characterization of *G. sulfurreducens'* inability to reduce soluble Fe (III) in the absence of PilR. Even though this was a T4P independent phenotype, it is specific to *Geobacter* species.

In **Chapter 4**, we sought to define the *P. aeruginosa* PilR regulon and associated phenotypes. Previous studies indicated that loss of PilA can result in a decrease in intracellular levels of the secondary messenger cAMP, which contributes to the expression of over 200 genes, including many involved in virulence (166). Interestingly, in **Chapter 3**, neither *pilA* nor *pilR* mutants are significantly impaired in pathogenicity in *C. elegans,* despite this. It is possible that loss of *pilA* only modestly decreases expression of cAMP virulence genes, as described in (166), still allowing for sufficient expression for near-WT virulence. As *pilR* mutants also lack PilA, we had to include a *pilA* mutant to separate those genes that are truly regulated by PilR from those that are only affected by loss of the pilin. This was a detail not heavily considered in previous studies, though the effects of loss of PilA on cAMP levels in *G. sulfurreducens* has not been characterized and may be negligible in this species.

We used RNAseq analysis to identify differentially expressed genes in *pilA* mutants, *pilR* mutants or both compared to WT and to obtain a manageable number of genes to follow up with and characterize, we elected to use a 3-fold cut off for differential expression. As expected, many of the genes that were dysregulated by both loss of *pilA* and *pilR* matched those identified as being cAMP dependent (166) and thus, were not the focus of this study.

Instead, we focused our attention on genes that were dysregulated only by loss of PilR and on a somewhat unexpected category, genes with increased expression in a *pilA* mutant but decreased expression in a *pilR* mutant. Interestingly, this is the same pattern exhibited by *pilA*, since its expression is autoregulated (211). Promoter activity is significantly increased in a *pilA* mutant (166) but lost in a *pilR* mutant, as PilR is required for expression (119). Thus, the genes that match this pattern may be ones controlled by PilSR in response to PilA levels. Such genes may be co-regulated with PilA and could be potential candidates for novel T4P associated genes.

We also identified several genes whose expression was dependent on PilR but largely insensitive to loss of *pilA*. While the genes identified were from a range of functional categories, a disproportionate number were related to flagellar motility, including *FleSR,* encoding a TCS that controls expression of a number of flagellar genes and whose expression decreased with the loss of *pilR.* FleSR regulates most of the flagellar genes that exhibited decreased expression in a *pilR* mutant (79) and therefore it is likely that their reduction is related to

decreased *FleSR* expression rather than loss of PilR directly. The decrease in flagellar gene expression was sufficient to impair swimming motility to about 40%

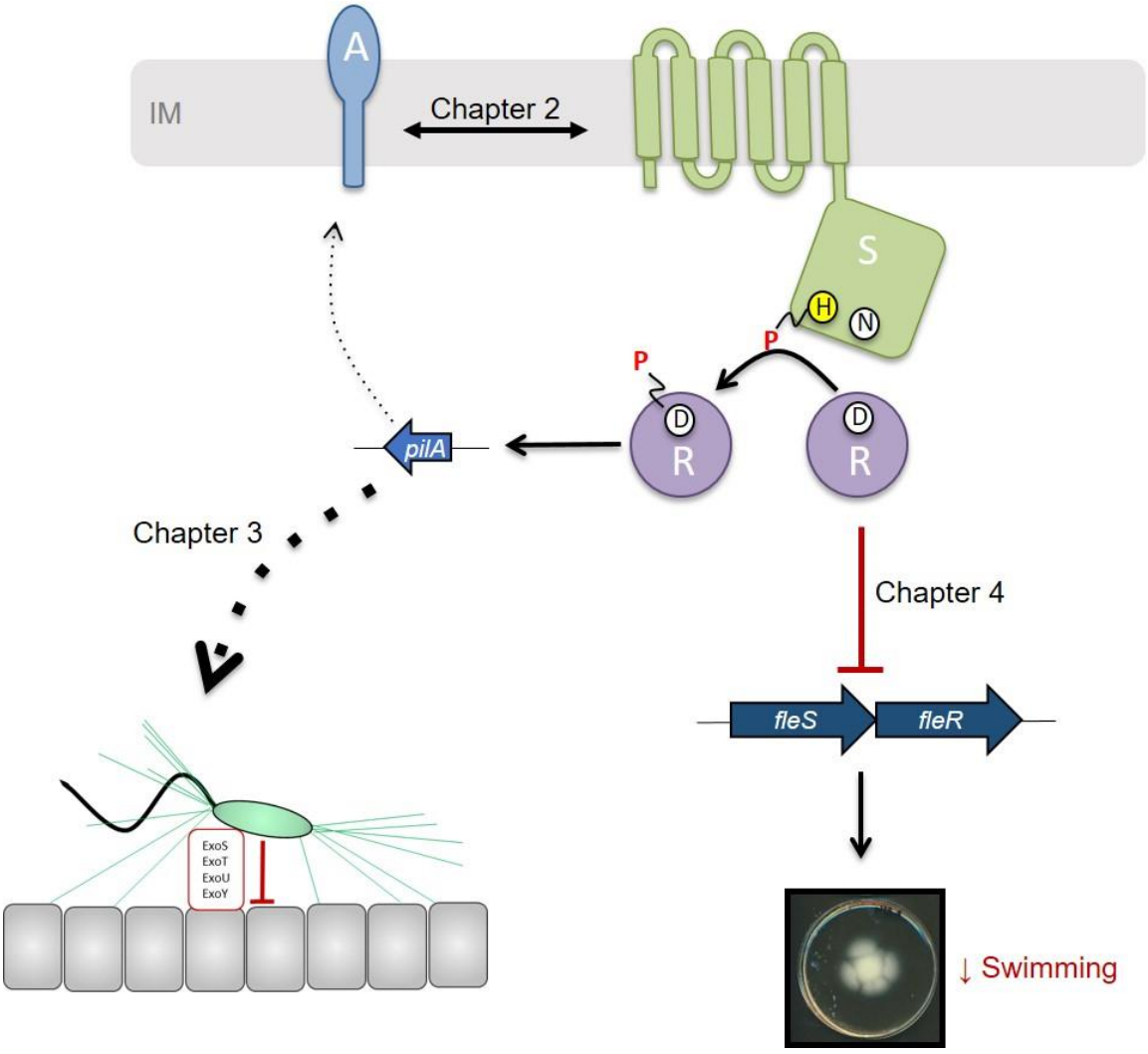


Figure 5.1. Model for integrated regulation of *piA*, swimming and virulence. PilS-PilR controls *pilA* transcription. Upon sufficient expression of PilA to restore intramembrane pools, PilA and PilS interact within the IM to switch PilS to its phosphatase state and block *pilA* transcription **(Chapter 2).** However, if PilA and surface pili are overproduced through specific PilSR point mutations or by blocking pilus retraction, virulence in *C. elegans* is compromised, likely due to blocking engagement of contact-dependent virulence factors like T3S **(Chapter 3).** Conversely, in the absence of *pilS-pilR* and presumably their activity, the expression of the *fleSR* TCS and multiple other genes is reduced, leading to a reduction in swimming motility and possibly impairing other currently untested phenotypes **(Chapter 4).**

of WT in both *pilS* and *pilR* mutants, though this deficit could be overcome by suppressor mutations. Prior to this work, direct connections between regulation of PilA and components of the flagellar system had not been identified, but our findings may hint at how the two systems can be co-regulated and thus turned off rapidly during the transition to chronic infection in the host.

The data further revealed that genes in addition to *pilA* may be co-regulated by PilSR in *P. aeruginosa*, while other genes are affected by loss of *pilR* independently of PilA. The subset of genes dysregulated by alterations in PilSR activity or expression may impact several different phenotypes and could lead to differences in virulence **(Figure 5.1).**

Future directions

Characterization of the PilA-PilS interaction interface

After determining that PilS can detect levels of PilA in the inner membrane through direct interactions through their respective TM segments, we began to identify specific amino acid residues on both PilA and PilS that were required to support this interaction. On PilA, specific substitutions at residues E5 and P22 of the mature pilin disrupted PilA-PilS interaction and interrupted autoregulation of *pilA* transcription **(Chapter 2).** From these data, we hypothesized that the charge at position E5 and the kink in the N-terminal α-helix created by P22 were both important for interaction. Site directed mutagenesis also revealed that the charges at residues R24 and D86 of PilS were required for interaction with PilA,

likely by forming a salt bridge that stabilizes PilS in a conformation amenable to interaction. To date though, the TM segment(s) of PilS that directly contact PilA, and the nature of their interface remains unknown. Future work will use mutational analysis in combination with the BACTH assay to identify first which TM segments are required. Previous topological analyses of PilS suggested combinations of TM segments that can be deleted while retaining protein stability and proper orientation of the cytoplasmic C-terminal domain of the protein (130). We will use this as a framework for making internal deletions of PilS TM segments, testing the resulting constructs for interaction with PilA using the BACTH assay. PilS-PilS interactions can be measured as a control for protein expression and stability, as homodimerization occurs through the cytoplasmic domain of the protein (97). If we identify which TM segments are required for interaction, we can then use SDM to define key residues involved.

Using BACTH and Western blot analyses, we have shown that PilA from *G. sulfurreducens* can interact with PilS from *P. aeruginosa* to repress native PilA levels when overexpressed **(Chapter 2)**. Additional BACTH data has provided evidence that PilA and PilS from *G. sulfurreducens* interact, as do PilS from *G. sulfurreducens* and PilA from *P. aeruginosa* (data not shown), implying that *G. sulfurreducens* may use a similar autoregulatory mechanism for *pilA* transcription. We will perform similar interaction experiments with PilA and PilS from *Myxococcus xanthus,* as PilA expression is also autoregulated in this species, but PilS has been characterized as a negative regulator of *pilA* transcription (47), in

contrast to its positive regulatory role in *P. aeruginosa, G. sulfurreducens* and *K. kingae* (116, 121, 140). PilA from all 3 of these species are 98% identical in their N-terminal 25 amino acids while PilS homologues share only 24% identity, most of which is in the conserved kinase motif and ATP binding domain. It would be ideal to perform a structural comparison of PilS from these and other pathogenic species, as they can all detect the same ligand, despite limited sequence conservation. This would also enable us to see if there are any notable structural differences that could account for the different activities of PilS from *P aeruginosa* and *M. xanthus.*

Developing a clearer picture of the PilA-PilS interaction interface will enable the rational design of peptide mimetics to cause dysregulation of PilS activity. Development of peptides to disrupt protein-protein interactions, including those that occur within the membrane is already ongoing in other systems (212-214). With the information gleaned from **Chapter 3** that increased PilA production from PilSR is sufficient for reducing virulence, we could develop either inhibitory peptides to mimic PilA binding but with higher affinity than PilA or alternatively, create peptides that block the PilA binding site while allowing PilS to remain in its kinase state, possibly enabling constitutive activation and by extension a reduction in virulence. Small molecule inhibitors or activators may also be considered but with the added challenges that small molecules are rarely sufficient to disrupt a full protein-protein interaction interface (212) and at present, no known allosteric binding sites have been identified on PilS.

Determine the effects of hyperpiliation on virulence in other more complex animal models

In the *C. elegans* slow killing model, overexpression of surface pili by point mutations in PilSR, deletion of the retraction ATPase, *pilT,* or disrupting the normal assembly and retraction dynamics in *P. aeruginosa* reduces virulence of the bacterium. Interestingly, this effect is more pronounced than the decrease in pathogenicity associated with complete loss of surface pili in this model **(Chapter 3).** In the slow killing assay, bacterial colonization relies on the bacteria first being ingested by the host, as opposed to attaching to open wounds or adhering to lung epithelial cells in many vertebrate and human models or infections. As a result, we will investigate the contribution of hyperpiliation to loss of pathogenicity in more complex animal models. Because PAK is not ideal for animal studies, we produced hyperpiliated PilS N323A and PilR D54E mutants in the highly pathogenic PA14 strain, for which there are mouse models of acute (215) and chronic (216) infection, and confirmed that hyperpiliation of this strain similarly reduces pathogenicity in *C. elegans* **(Chapter 3)**.

Identification of other contact-dependent phenotypes that may be affected by P. aeruginosa hyperpiliation

Hyperpiliation of *P. aeruginosa* can significantly impair its ability to infect and kill *C. elegans,* independently of both the strain used and its ability to twitch **(Chapter 3)**. We presented preliminary data suggesting that this loss of pathogenicity was due to decreased engagement of contact-dependent virulence

factors such as T3S. However, T3S was reported previously (186) to be expressed unnecessary for slow killing by PA14, suggesting other contact dependent virulence factors may be impeded by hyperpiliation. Thus, we are currently testing if Type VI secretion is also i) required for full *P. aeruginosa* virulence in *C. elegans* slow killing and ii) impaired when *P. aeruginosa* is hyperpiliated. Further, we will assess whether the PilS N323A or PilR D54E mutants are deficient in any other contact dependent phenotypes including phage susceptibility and competence.

Functional characterization of hypothetical genes inversely regulated by pilA and pilR

Of the genes dysregulated by loss of *pilR*, one subset stood out as being particularly interesting. Transcription of 11 genes was increased upon deletion of *pilA*, but significantly decreased when *pilR* was deleted, even though *pilR* mutants also lack PilA **(Chapter 4**). We hypothesized that these genes may be those co-regulated with *pilA*, as this same phenotype is observed in the absence of the *pilA* gene (91). All but 2 are either unannotated or encode for hypothetical proteins and therefore could have any number of novel functions. Future work will be directed at trying to determine what role these previously uncharacterized proteins have through the use of deletion mutants; and more specifically, we will determine if any affect T4P biogenesis and/or function.

For those genes that are affected only by the loss of PilR, and not PilA, I plan to determine if their regulation is dependent on signalling through both PilS

and PilR or if PilR activity is perhaps being modulated by alternate SKs. PilS may phosphorylate non-cognate response regulators (133) and therefore PilR may be capable of receiving signal from other sensor kinases as well. We will use RT-PCR to test transcription of any genes of interest we select for follow up, comparing WT, *pilS,* and *pilR* backgrounds to see if they are differently regulated in *pilS* versus *pilR.* Once complete, I will follow up with some hits from this subset to determine what functions they have. This work will expand our knowledge of the roles of PilSRand may also highlight additional virulence factors that are under control of this TCS.

How do PilSR and FleSR coordinately regulate motility?

In **Chapter 4**, we revealed that amongst the genes regulated by PilS and PilR were those encoding the FleSR TCS. As a result of their downregulation in *pilS* and *pilR* mutants, swimming motility is decreased. As this is the first report of direct regulation of one motility system by the other at a transcriptional level, there remain many unanswered questions about the mechanism. In future work, we will determine if regulation is reciprocal, testing if loss of *FleSR* also affects expression of *pilS-pilR*. We approached this question indirectly in **Chapter 4** by measuring *pilA* transcription and twitching motility. Since components of the FleSR regulon affect T4P function, we could not determine if aberrant T4P expression and function in the *FleSR* mutant was caused by altered PilSRlevels or altered expression of proteins such as FleP, which affect both swimming and

twitching motilities (79) We will use RT-PCR with *pilSR* specific primers to determine if there are differences in expression between WT PAK and a *fleSR* double mutant. We will identify conditions under which this regulation may occur *in vivo*. Most of the work done to establish the decreased swimming motility phenotype was performed on cells grown on solid or semi solid plates. Therefore, it would be interesting to see if the magnitude of dysregulation caused by loss of *pilR* is different between solid and liquid states.

From our data in **Chapter 4**, *pilS* and *pilR* mutants easily acquire suppressor mutations that allow cells to overcome the effects of *pilSR* deletion and regain normal swimming motility. Work is ongoing to identify these mutations and determine if they are conserved mutations, or at least mutations occurring in the same genes in all cases. We will begin by sequencing some of the more likely candidate genes to have acquired suppressors; namely FleSR themselves, which could cause hyperactivation; *fleQ*, resulting in overexpression of *fleSR;* or the promoter region of *fleS*, that too could increase expression. If these experiments show no change from the parental strain, we can do whole-genome sequencing of the mutant and parent strains to identify novel SNPs. With this information, we may uncover new regulatory circuits that are important for swimming motility.

Conclusions

The T4P system is an important virulence factor for a number of Gram negative and Gram positive bacteria, including the opportunistic pathogen, *P.*

aeruginosa. T4P expression and biogenesis is tightly regulated at several stages, including at the transcriptional level, where expression of the major pilin gene, *pilA* is tightly controlled by the PilSR TCS. As this is a well conserved system for regulating *pilA* transcription, a full understanding of its mechanism and scope of activity would provide valuable insight into the regulation of a major contributor to pathogenicity, and allow for the development of novel antivirulence therapeutics using PilS or PilR as targets. At the outset of this work, the signal to which PilS responded was unknown, but with the knowledge that PilA represses its own expression by interacting with PilS, we could use this interaction as a target for the development of small molecule inhibitors or peptide mimetics to turn off pilin expression. We also uncovered a novel mechanism where a membrane protein regulates its own expression through direct intramembrane interactions with its sensor kinase, without the use of intermediary proteins. Hyperpiliation of *P. aeruginosa* decreases pathogenicity in a *C. elegans* slow killing model, due excess pili and not lack of pilus retraction. Based on our preliminary data, we hypothesized that hyperpiliation blocks contact dependent virulence factors such as T3S. Thus, dysregulation of PilSR function – either increasing or decreasing – can result in decreased pathogenicity. We showed that PilSR participates in the regulation of many genes other than *pilA*, including the flagellar TCS, FleSR, affecting swimming motility. To our knowledge, this is the first report of regulation of T4P being directly interconnected with that of flagellar biosynthesis. Together, the work presented in this thesis paint a picture of a TCS with a considerably

broader function than was previously assumed. PilSR now has a well-defined inhibitory ligand—PilA—and its activity can be modulated to reduce virulence, both directly and by downregulating other known virulence associated phenotypes such as swimming motility. Collectively, these data suggest that PilSR may make a valuable target, as we can impair multiple virulence-associated systems simultaneously.

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