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# The Role of PILJ and Its Structural Domains in the Localization and Function of the CHP Chemosensory System in Pseudomonas Aeruginosa

Vibhuti Hemantkumar Jansari
*University of Wisconsin-Milwaukee*

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by

Vibhuti H. Jansari

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ABSTRACT

THE ROLE OF PILJ AND ITS STRUCTURAL DOMAINS IN THE LOCALIZATION AND FUNCTION OF THE CHP CHEMOSENSORY SYSTEM IN *PSEUDOMONAS AERUGINOSA*

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Bacteria detect environmental signals using membrane-bound methyl-accepting chemotaxis proteins (MCPs), which are part of a larger complex of chemosensory proteins. *Pseudomonas aeruginosa* has four functionally distinct chemosensory protein complexes. The Chp chemosensory system regulates type IV pili mediated twitching motility and intracellular levels of cAMP by modulating the activity of an adenylate cyclase, CyaB. The Chp system is also proposed to be involved in type IV pili mediated directional twitching motility towards phosphatidylethanolamine (PE). PilJ is the only MCP predicted to be associated with the Chp system. In this study we investigated different domains of PilJ in order to decipher their roles in signal transduction and localization of the Chp chemosensory system.

Our results show that both the periplasmic and transmembrane domains are involved in signal transduction. A PilJ periplasmic domain mutant ($PilJ_{\Delta 74\text{-}273}$) showed a partial but significant decrease in cAMP levels highlighting the importance of this domain in regulation of this phenotype. However, when sufficient intracellular cAMP is provided, twitching motility occurs in the presence or absence of PE independent of the periplasmic domain. To maintain the inner membrane localization while assaying the role of the transmembrane domains of PilJ, Tsr-PilJ chimeric proteins were used. These fusion proteins showed impaired signal transduction.

Through localization studies, we discovered that the role of the PilJ transmembrane domains is not limited to signal transduction. Using fluorescence microscopy, an examination of the intracellular localization of C-terminus truncations of PilJ revealed that the second transmembrane domain plays a role in polar localization of PilJ. This is the first report where the localization of a MCP is impacted by a domain other than the cytoplasmic domain. Determining the internal localization cues for this MCP was important as MCPs are reported to be instrumental in chemosensory cluster formation and localization. Indeed, PilJ is needed for PilI foci formation as demonstrated by fluorescence microscopy studies. Taken together, these studies suggest that the PilJ transmembrane domains are important for both signal transduction and localization. Establishing the importance of PilJ for the potential nucleation of the Chp system paves the path for future work to identify potential partners that would directly or indirectly control the polar localization of PilJ.

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*I dedicate this work to my parents as a small measure of compensation for the time that we could have spent together but lost forever.*

# Chapter One

# Introduction

### 1.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative rod-shaped bacterium that belongs to class Gamma proteobacteria, family Pseudomonadaceae. It is a free-living bacterium ubiquitously found in soil and water. It can survive under conditions of minimal nutritional availability and this property allows it to thrive in almost any environmental niche. It has a large bacterial genome of 6.3 million bps (1). The large number of genes include those involved in regulation, compound efflux systems, catabolism, transport, four chemotaxis-like systems and others which are consistent with its abilities as a nutritionally versatile and multidrug resistant bacterium (1).

*P. aeruginosa* is an opportunistic human pathogen. *Pseudomonas* infections are common in patients with organ transplants, acute leukemia, burn patients, cystic fibrosis (CF), cancer, AIDS or in patients hospitalized for prolonged periods. It is the most common cause of nosocomial infections and the most fatal infections include endocarditis, meningitis, septicemia, respiratory system infections and many others (2).

*P. aeruginosa* can move across surfaces using polar cell surface structures called type IV pili at a speed of about 500 cell lengths per hour. Type IV pili mediate different kinds of surface motility like twitching, crawling, walking or slingshot motility (3). It undergoes swimming motility in liquid with the help of a single polar flagellum. Along with multiple forms of motility, there are multiple chemosensory systems. Each type of motility is under the control of its cognate chemosensory system. This makes *P. aeruginosa* more complex than *E. coli,* which has one chemotaxis system that controls flagellar-based swimming motility.

### 1.2 Chemosensory systems in Bacteria

Bacteria can sense a variety of environmental signals such as concentrations of toxins, nutrients, pH levels, oxygen availability, osmolarity and others (4). Prokaryotes utilize histidine-aspartate phosphorelay (HAP) systems commonly referred as two component signal transduction

systems to carry out such sensory functions and transduce signals inside the cell to generate an appropriate response. The HAP systems consist of a histidine kinase (HK) and a response regulator (RR) (5). The HK is trans-autophosphorylated with a phosphate from an ATP molecule. This phosphate is then transferred to the RR to bring about a physiological response (6). The HAP systems are very sensitive and react to minute changes at very low concentrations of signaling molecules.

Chemosensory systems are modified two-component signal transduction systems, which involve a HK and RR (4, 7). Here the presence of signaling molecules is detected by dedicated transmembrane receptors known as methyl-accepting chemotaxis proteins (MCPs) (8). MCPs form mixed trimers of homodimers in the inner membrane and bind to signaling molecules through their periplasmic domain (9). This causes a series of conformational changes in the transmembrane and HAMP domains of the MCP that results in signal transduction to the HK, which trans-autophosphorylates (10, 11). This phosphate group is then transferred to the RR of the system thus causing the cell to respond to stimuli (4). The interaction between the MCP and HK is facilitated by a coupling protein (CheW). Addition or removal of methyl groups from the MCPs by a methyltransferase or a methylesterase respectively, allows the system to adapt to the current stimulus state (12). Adaptation prevents system saturation and renders it sensitive to any further changes in the concentration of the stimuli and thus generates a fresh response.

### 1.2.1 Assembly of a chemosensory system

The well-studied chemosensory system in *E. coli* (Che) controls flagellar based motility and is made up of methyl-accepting chemotaxis proteins (MCPs), a histidine kinase (HK), a coupling protein (CheW), a response regulator (RR), a methyltransferase and a methylesterase. The MCPs form homodimers that come together to form trimers of dimers that group together to form hexagonal arrays (11). Larger arrays localize to cell poles while smaller arrays are found at

non-polar locations. Homodimers form trimers by interaction in the trimer interaction domain of the cytoplasmic region of the MCP. The HK and coupling proteins interact with the MCP at its signaling region thus stabilizing the trimers of dimers (13). Electron microscopy density mapping suggested the stoichiometry of the MCPs: HK: coupling proteins in the *E. coli* chemotaxis signaling complex is 6: 1: 1, where there are 2 trimers of MCP homodimers, 1 HK and 1 monomer of the coupling protein, although these ratios are dependent on the sample and preparation (14-16).

**1.2.2 MCP structure**

To understand the MCP structure, two *E. coli* MCPs, Tsr and Tar are widely studied. A MCP has four major domains called the periplasmic domain, transmembrane domains (TM1 and TM2), the HAMP (Histidine kinase, Adenylate cyclase, Methyl-accepting proteins, Phosphatases) domain and the cytoplasmic region (Fig. 1.1). The periplasmic ligand-binding domain in a MCP homodimer is made of four α-helices arranged in parallel that generate 2 ligand-binding sites at the dimer interface (11). The transmembrane domain consists of TM1 and TM2 where TM2 is connected to the periplasmic domain and the highly conserved HAMP domain. Studies in Tar have shown that upon ligand (aspartate) binding there is a 1.4Å piston like displacement of the helix 4 of the periplasmic domain. This results in the bending of TM2 helices, which further translates into slight conformational change in the helices of the HAMP domain (11).

In the *E. coli* MCP Tsr, each monomer has a 50 residue HAMP domain made up of 2 α-helices joined by a non-helical connector (CTR), which become a four α-helix bundle in the homodimer (17-20). The highly conserved cytoplasmic region is the largest and the most dynamic section of a MCP. It extends from the HAMP domain and is composed of the methylation helices, the flexible bundle and the signaling domain (Fig. 1.1). It is formed of four

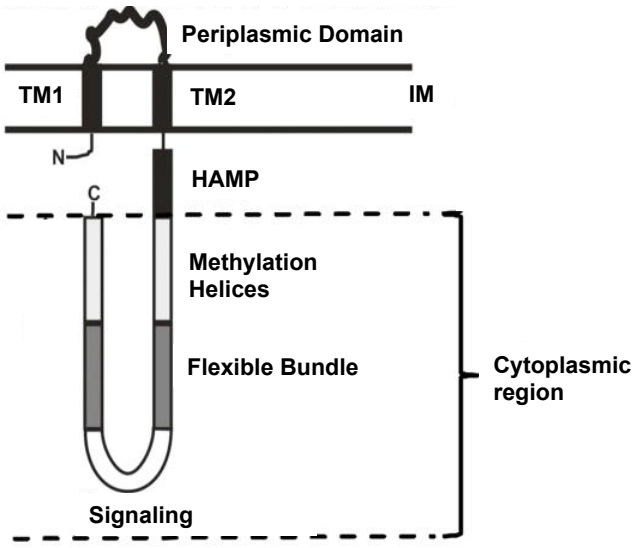


Fig. 1.1. A schematic of four different structural domains in a MCP monomer. A MCP can be divided into the periplasmic domain, the transmembrane domains (TM1 and TM2), the HAMP domain and the cytoplasmic region (modified from (21)).

α-helices in the homodimer with a hairpin turn at the distal end (22). The methylation helices are also referred to as the adaptation region and contain specific residues that are modified by the methyltransferase (CheR) and the methylesterase (CheB) to control adaptation (23, 24). The activity of methyltransferase is constitutive and methylation of an MCP causes increased auto-transphosphorylation of the HK. Conversely, demethylation of the MCP by the methylesterase results in decreased HK phosphorylation (11). The methylesterase is activated upon phosphorylation by the HK and thus it sets up a negative feedback loop allowing the system to adapt to the current levels of a stimulus and be sensitive to further changes. The flexible bundle allows the cytoplasmic domain to bend in a way to either decrease or increase its flexibility thus promoting or demoting downstream signaling (25, 26). The signaling domain is highly conserved and it acts as a platform for interaction with the HK and the coupling proteins (13).

### 1.3 Chemosensory systems in *Pseudomonas aeruginosa*

*P. aeruginosa* has very intricate chemosensory machineries with over 20 chemosensory genes segregated in five distinct chemosensory gene clusters and 26 predicted methyl-accepting chemotaxis protein (MCP)-like genes scattered throughout the genome (1). The chemosensory systems from *P. aeruginosa* are homologous to the Che system of *E. coli*. The Che1 system of *P. aeruginosa* is also functionally homologous to the *E. coli* Che system and is involved in regulating flagellar-based motility also called swimming motility (27-30). The Che2 system function is unknown. The Wsp chemosensory system responds to surface sensing and is implicated in biofilm formation by modulation of intracellular c-di-GMP levels (31, 32). The Chp chemosensory system regulates levels of intracellular cAMP, type IV pilus biogenesis and type IV pili mediated twitching motility (33-35).

#### 1.3.1 Che1 chemotaxis system

The *P. aeruginosa* Che1 chemotaxis system controls flagellar-based motility, and is

functionally homologous to the chemotaxis system found in *E. coli* (see section 1.2). 23 of the MCPs scattered throughout the genome are predicted to contribute to this system, allowing the cells to respond to many compounds including amino acids, inorganic phosphate and α-ketoglutarate (36, 37). This system differs from *E. coli* in that it is predicted to use CheV, a coupling protein that also contains a C-terminal response regulator domain. The role of CheV protein varies depending on the bacterial species. In *Helicobacter pylori* CheV proteins are thought to function as phosphate sinks, while in *Bacillus subtilis* CheV is involved in adaptation (38, 39).

Importantly, the Che1 system of *P. aeruginosa* localizes to the poles of the cells, which is the same localization pattern shown by the flagellum. This co-localization is expected to be important for function. Keeping the CheY response regulator in close proximity to the flagellar motor is thought to ensure efficient signal transduction. This polar localization pattern contrasts with the pattern seen in the peritrichously flagellated *E. coli* (40-42). While large clusters of chemotaxis proteins were initially localized to the poles of the cell in *E. coli,* more recent work revealed that the MCPs are inserted in lateral cell membranes and form small signaling complexes throughout the membrane. It is postulated that when sufficient MCPs aggregate to form large signaling complexes, they are forced to the poles to accommodate this complex (40).

It has recently been determined that partitioning systems may function to localize chemosensory systems in polarly- flagellated gamma- proteobacteria. Traditionally, Par systems are responsible for segregation of chromosome and plasmids after cell division. Par systems are made of two proteins ParA (ATPase) and ParB in addition to a DNA binding site *parS* (43). In *Rhodobacter sphaeroides* and *Vibrio* spp*.,* chemotaxis systems are localized via a partitioning (Par) system (44-46). A homologue of ParA causes the segregation of a chemoreceptor Tlps in *R. sphaeroides* (47). ParC, a ParA-like protein in *V. cholerae* is important for chemotaxis protein

localization and function. After cell division, ParC localizes to the new pole, which is followed by the chemotaxis proteins at that cell pole. Deletion of *parC* results in mislocalized chemotaxis proteins and decreased chemotaxis (44).

### 1.3.2 Wsp chemosensory system

In *P. aeruginosa,* the membrane associated Wsp system is involved in auto-aggregation and biofilm formation. It helps control biofilm formation by increasing the levels of an intracellular signaling molecule, c-di-GMP. The Wsp system has two characteristics that separate it from the conventional chemotaxis systems. First, it responds to surface association instead of small soluble chemotactic compounds (48). Secondly, it localizes along the periphery of the cell. This is unlike the three other chemosensory systems in *P. aeruginosa*, which are found exclusively at the poles (21, 28). Localization studies with a chimeric MCP that had the N-terminus of PctA (a polar MCP of *P. aeruginosa*) and the C-terminus of WspA (the MCP of the Wsp system) revealed that the C-terminus of WspA is important for its punctate localization since this chimera was also punctate in localization (21). Further investigation highlighted that the trimer interaction domain of WspA cytoplasmic region was involved in its punctate localization (21). In *E. coli,* the interaction between the highly conserved trimer interaction domains of the receptors allows formation of strongly bonded trimers of dimers. This results in formation of larger groups of MCPs, which are pushed to the poles due to their large nature (42). In contrast, the interactions between the WspA molecules is not very strong and it forms less dense aggregates, localizing to punctate locations in the cell (21). The response regulator of the Wsp system, WspR forms cytoplasmic clusters and catalyzes c-di-GMP production upon phosphorylation (48). Previous analysis of the response regulator WspR-YFP localization patterns in Δ*wspA*, in conjuction with genetic studies on WspA point mutants (in the trimer interaction domain) suggested that punctate localization of the WspA is important for its

function and transmitting the signal to WspR to catalyze the production of c-di-GMP (21, 48).

### 1.3.3 Chp chemosensory system in *Pseudomonas aeruginosa*

The Chp cluster (PA0408-PA4116) is one of five clusters of chemosensory protein encoding genes in *P. aeruginosa*. The Chp system controls T4P mediated twitching motility and regulates the levels of intracellular cAMP by modulating the activity of an adenylate cyclase, CyaB (29, 34, 35). The Chp gene cluster contains *pilGHIJK* and *chpA-D* genes (34). PilJ is the only predicted MCP of the Chp system and *pilJ* mutants are defective in twitching motility (33)(R. Alexander and I. Zhulin, personal communication). The signal (which is proposed to be surface association (49)) is believed to be received by PilJ and is transmitted to ChpA (HK). The CheW-like coupling proteins, PilI and ChpC aid the interaction between these proteins (Fig. 1.2) ChpA is a large HK with eight phosphotransfer domains (Xpts), six of which contain histidine (Hpts), one contains serine (Spt) and one contains threonine (Tpt). In addition, it has a dimerization domain, a CheW-like scaffolding domain, a catalytic HATPase_c domain and a C-terminal receiver domain (ChpArec) (34, 50). Biochemical and genetic studies have revealed the pathway for the transfer of phosphate within ChpA before it is transferred to the response regulators. During signal transduction, in the presence of the catalytic HATPase_c domain, Hpts 4, 5, 6 get phosphorylated and transfer the phosphate to ChpArec rapidly in a reversible manner (50). Hpts 2, 3 function downstream of ChpArec. They do not react with ATP but can reversibly procure a phosphate from ChpArec. ChpArec can auto-dephosphorylate and hence is predicted to act as a phosphate sink. Genetic studies suggested that the response regulator PilG can receive a phosphate from Hpts 4, 5 or 6 but likely obtains it from Hpt2 and loses it to Hpt3. Rapid transfer of phosphate from Hpt3 to PilH was also reported (50). Thus, during signal transduction, ChpA trans-autophosphorylates and transfers the phosphate to either of the two response regulators, PilG and PilH (Fig. 1.2). The phosphorylated response regulators bring about changes

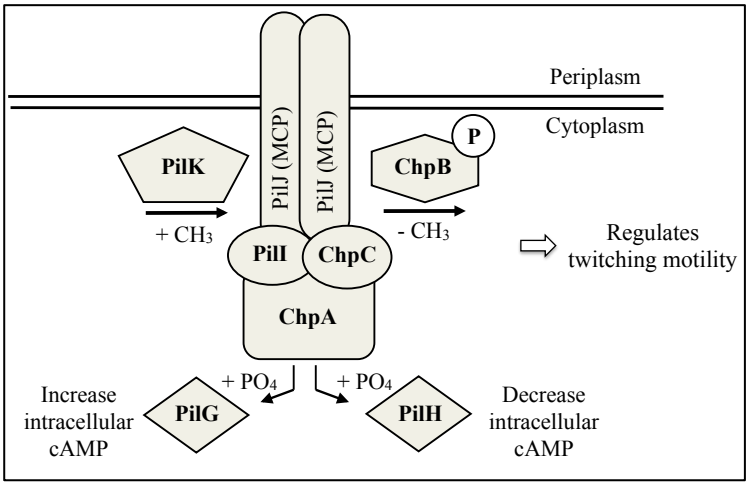


Fig. 1.2. A schematic of the proteins involved in the Chp chemosensory system. The predicted proteins are methyl accepting chemotaxis protein (PilJ), a methyltransferase (PilK), a methylesterase (ChpB) and two CheW homologues (PilI and ChpC) that act as coupling proteins between the MCP (PilJ) and the histidine kinase (ChpA). PilG and PilH are the two response regulators which when phosphorylated bring about changes in CyaB activity (an adenylate cyclase). The Chp system is also involved in regulating twitching motility.

in cell behavior as discussed below. ChpB, the methylesterase and PilK, the methyltransferase of the Chp system control the methylation status of the MCP and thus regulate adaptation. Increased cAMP levels in a Δ*chpB* (methylesterase) suggests that ChpB is phosphorylated by ChpA during adaptation, thus making it too a response regulator of the Chp system (35, 51, 52). Phosphorylated PilG or PilH cause either upregulation or downregulation of CyaB activity respectively. The intracellular cAMP (an important second messenger molecule) levels generated due to CyaB activity regulate type IV pilus biogenesis among other virulence factors by binding to the transcriptional activator Vfr (virulence factor regulator) (53). Independent of cAMP levels and pilus biogenesis, the Chp chemosensory system regulates twitching motility, which is mediated through extension and retraction of type IV pili (3, 35, 54, 55).

### 1.4 Type IV pili

Type IV pili (T4P) are surface structures commonly found in bacteria (3, 56). They participate in host-cell adhesion, surface motility, DNA uptake by transformation, biofilm formation, phage attachment and pathogenesis (57, 58). They are capable of adhering to a variety of biotic and abiotic surfaces. This property of adherence promotes biofilm formation and virulence in bacteria (3). Upon surface contact, the transition from planktonic to sessile state is facilitated by T4P (59). Many Gram-negative mutants lacking T4P are shown to have reduced virulence (60), and correspondingly they are widely studied in human pathogens like *Neisseria* and *Pseudomonas* species as drug and vaccine targets.

#### 1.4.1 Type IVa pilus structure in Gram-negative bacteria

There are two main types of T4P found in nature, type IVa (T4aP) and type IVb (T4bP). T4aP is broadly distributed amongst bacteria whereas T4bP is commonly found in enteric pathogens (60). T4P are polar structures, 5-7nm in diameter and several μm long that can produce forces approximately 100pN (61). T4P are composed of four main protein complexes

that span from the inner membrane to the outer membrane through the periplasmic space (Fig. 1.3).

The outer membrane complex comprises of a secretin protein PilQ which forms a dodecamer >1MDa in size. This multimer forms a pore in the outer membrane that allows the pilus to exit the cell membrane (62). A lipoprotein pilotin PilF is associated with PilQ and is responsible for the proper oligomerization of the secretin in the outer membrane (63). The motor complex is associated with the inner membrane and includes the platform protein PilC and the ATPases PilB, PilT and PilU (64-66). PilC is an inner membrane platform protein with two cytoplasmic domains and that possibly assembles as a tetramer. It is necessary for T4P assembly and disassembly and potentially interacts with the ATPases PilB, PilT and PilU (64-69). PilB is a bipolar hexameric ATPase predicted to be involved in pilus assembly by providing energy for addition of pilin monomers at the pilus base. PilT is similar to PilB except for that it is predicted to be involved in pilus disassembly (65, 66). PilU is a unipolar ATPase, which is thought to have resulted from a gene duplication event of *pilT*. PilU is important for pilus function but its role in pilus depolymerization is yet undefined (66). A lack of ATPases has been shown to cause loss of twitching motility (69, 70).

The alignment complex made up of PilM, PilN, PilO and PilP is predicted to bridge the outer membrane complex with the motor complex to ensure proper positioning of these complexes so the pilus can exit the cell membrane as its shaft grows (71, 72). Also affiliated with the alignment complex is the peptidoglycan binding protein FimV that promotes the multimerization of the outer membrane secretin (73) and potentially helps it pass through the peptidoglycan layer (35).

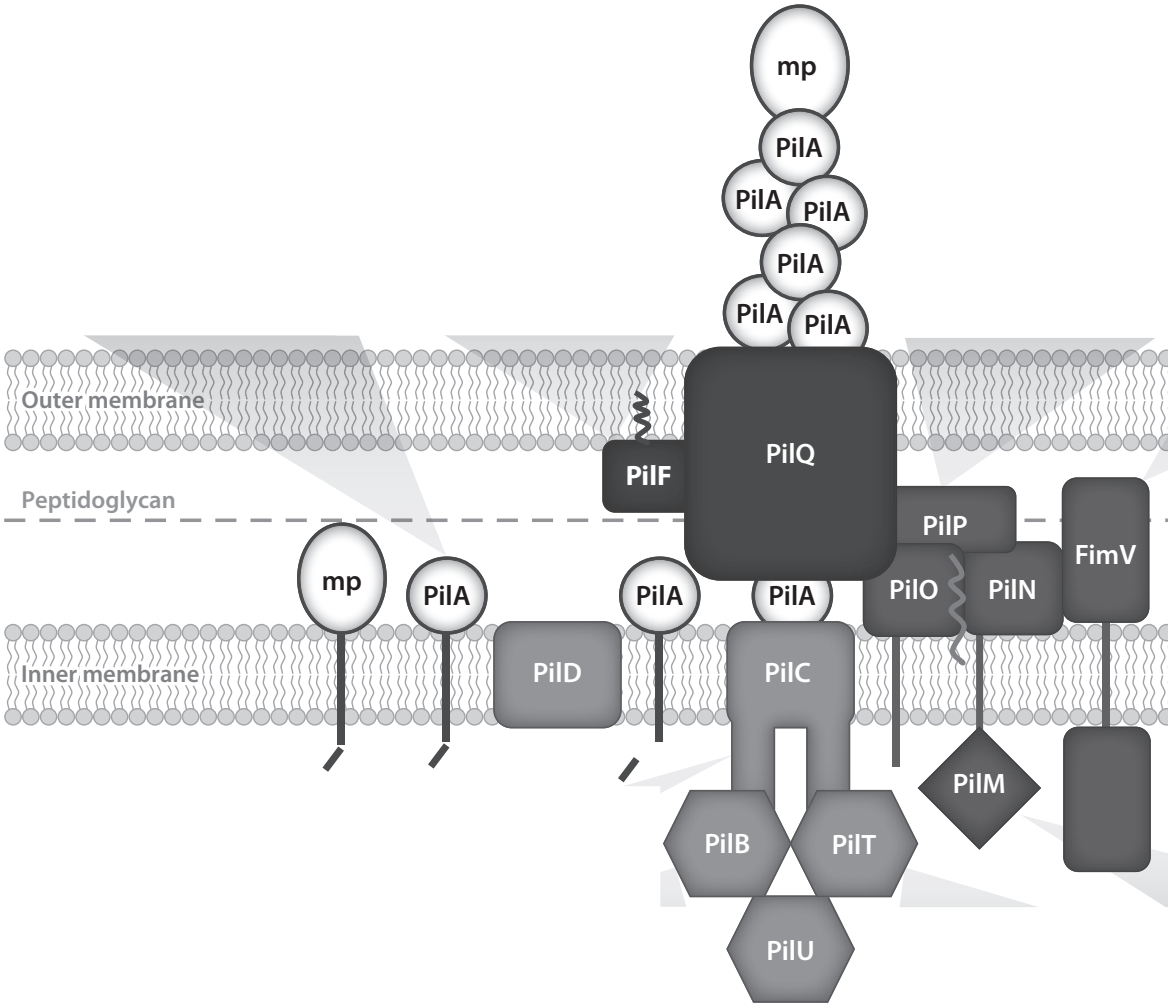


Fig. 1.3. A schematic of the proteins involved in the type IV pilus assembly. T4P structure is made of four major complexes. The outer-membrane complex is made of a dodecameric secretin (PilQ) and a pilotin protein (PilF). The inner-membrane complex consists of a platform protein (PilC), an extension ATPase (PilB), and two retraction ATPases (PilT and PilU). The alignment complex is made of PilMNOP and FimV. The fourth complex is the pilus shaft itself made of major pilin (PilA) and different minor pilins (mp). A prepilin peptidase (PilD) cleaves the N-terminal signal sequence of the prepilins thus preparing them for assembly in the pilus shaft (modified from (3)).

The fourth complex is the T4P shaft. It is made of helically arranged pilin subunits. The pilus consists of major pilin PilA (74) and minor pilins like FimU, PilEVWX (3). It is believed that minor pilins are incorporated during initiation of pilus assembly and hence are found at the distal end of the pilus shaft (75, 76). The pilins are first introduced in the inner membrane by the Sec system (77, 78) as prepilins. Here, PilD a membrane bound prepilin peptidase cleaves the type III signal sequence at the N-terminus, before the pilin gets added into the pilus (79).

### 1.4.2 Type IV pilus mediated motility

Organisms such as *P. aeruginosa*, and *Neisseria* spp. exhibit T4P mediated surface motility known as twitching motility. Synchronous extension and retraction of T4aP (T4P) moves the cell along a surface in response to a stimulus (80, 81) and is required for virulence and biofilm formation (82). The extension and retraction of pilus occurs with the help of the two ATPases that function at the base of the pilus assembly (PilB and PilT respectively) (65, 66). Surface association regulates pilus gene expression as well as pilus production (49). When the cell contacts a surface, the pilus shaft attaches to the substratum, which is followed by the initiation of pilus disassembly. Since the pilus tip is attached and the pilus assembly is fixed, as the pilus shaft shortens the cell moves towards the pilus attachment point. Thus pili act as molecular grappling hooks for motility. During retraction, the pilins removed from the pilus assembly are inserted back in the inner membrane, which make them readily available for another cycle of pilus extension (61, 83).

### 1.4.3 Regulation of T4P biogenesis and twitching motility

A model recently proposed by Persat et. al, (2015) states that T4P act as mechanosensors that get activated upon surface contact. When the cell is in liquid, the pili are not attached to a surface and hence not actuated. But upon surface contact, the pilus attachment followed by

retraction leads to development of a tension along the pilus shaft. This is believed to cause an uncharacterized modification at the base of the pilus assembly that results in a direct interaction between PilA (pilin) and the periplasmic domain of PilJ (the MCP), thus causing signal transduction through the Chp system (84). Signal transduction results in the modulation of intracellular cAMP levels and regulation of twitching motility (33-35). We have previously shown that when the periplasmic domain of PilJ is deleted, there is a slight cAMP dependent decrease in twitching motility, which is restored when sufficient cAMP is provided. This suggests that the classical signal transduction pathway through the PilJ periplasmic domain is involved in the regulation of cAMP levels but not twitching motility (54).

PilJ transduces the signal to ChpA (the HK) that stimulates CyaB activity through its response regulator PilG, resulting in the production of intracellular cAMP (35). cAMP is a small intracellular signaling molecule involved in the regulation of a variety of cellular processes including motility, virulence, and biofilm formation (53). cAMP forms a complex with the CRP homologue Vfr (Virulence factor regulator) and upregulates the expression of >200 virulence related genes including *pilMNOPQ, pilBTU* and others (53, 85, 86). Additionally, the Chp system also has cAMP independent role in regulating twitching motility (35, 54, 55). Phosphotransfer from ChpA to the two response regulators PilG and PilH result in upregulation and downregulation of cAMP production respectively (35, 50). Earlier reports suggested that PilG controls PilB activity causing pilus extension and PilH controls PilT activity resulting in pilus retraction (33, 87, 88). However, this hypothesis is not yet confirmed. It is also speculated that PilG is the response regulator and PilH functions as a phosphate-sink thus controlling the phosphorylation of PilG to modulate the output (35). Such a regulatory control of a signal transduction system with two response regulators on swimming motility has been seen before in

*Sinorhizobium meliloti* (89). *S. meliloti* has two response regulators CheY1 and CheY2. In the absence of an attractant, CheA (HK) transphosphorylates both response regulators and CheY2-P slows down the flagellar motor. In the presence of an attractant, CheA is inactive and CheY2-P transfers its phosphate back to CheA, which then transfers the phosphate to CheY1 (90). CheY1 autodephosphorylates continuously and thus acts as a phosphate sink (89).

In *P. aeruginosa* another two-component system made of a signal sensor PilS and a response regulator PilR along with an alternate sigma factor RpoN ($\sigma^{54}$) is involved in the transcription of *pilA* (91). PilS is an inner membrane polarly localized histidine kinase that potentially senses PilA as a signal and gets phosphorylated. The phosphate is transferred to PilR, the response regulator thus upregulating *pilA* transcription.

Additionally, FimV, a T4P alignment complex protein also functions in CyaB activation and twitching motility potentially through its interaction with a scaffolding protein FimL (92, 93). However, FimV is epistatic to FimL for the twitching motility phenotype. FimL is an accessory protein of the Chp system with an unknown function that is shown to be important for T4P function and likely functions upstream of Vfr (93, 94).

**1.4.4 Type IV pilus localization**

T4P are bipolar cell surface thread-like structures made up of helically arranged pilin subunits. A recent report shed light on the process of insertion of this multi-protein complex in the cell membrane when there is no peptidoglycan hydrolase associated with the complex. The model proposes the involvement of a number of different mechanisms that work to localize T4P proteins at cell poles (95). The first is one is the T4P alignment complex proteins, FimV. FimV is an inner membrane protein with a periplasmic peptidoglycan (PG) binding domain, 1 transmembrane domain and a cytoplasmic domain (96). While the peptidoglycan-binding

domain of FimV is important for the polar localization of the outer membrane secretin PilQ and the alignment complex protein PilO, FimV is also required for localizing PilS, PilG, FimL and PilMNOP to the poles (55, 93, 95). FimL and PilG directly interact with each other and FimV interacts with FimL but not PilG. Thus FimL is proposed to be the scaffold that links FimV (member of T4P alignment complex) to PilG (RR of the Chp system) (93). This is proposed to promote co-localization of T4P and the Chp system thus facilitating signaling and upregulation of cAMP dependent virulence mechanisms in response to surface association (93).

Another set of proteins involved in the polar localization of T4P is the Poc (polar organelle coordinator) complex. *P. aeruginosa* PocA-PocB-TonB3 are homologous to the *E. coli* ExbB-ExbD-TonB system that powers siderophore uptake through the outer membrane (97). Although all three Poc complex proteins were present in the insoluble cellular fraction, they were not polarly localized. Cowles et. al (2013) found that deletion of the protein PocA results in mislocalization of T4P and twitching motility was also impaired. Δ*pocB* and Δ*tonB* mutants had no surface pili. Subsequent fluorescence microscopic studies have shown that PocA is important for the polar localization of the outer membrane secretin PilQ, the ATPase PilT and the alignment complex protein PilO. Although FimV and PocA are involved in the polar localization of T4P, the role of FimV is independent of PocA as FimV is polarly localized in Δ*pocA* (95).

**1.5 Concluding Remarks**

The *P. aeruginosa* Chp chemosensory system localizes to the poles, regulates intracellular cAMP levels and T4P function (twitching motility). The regulation of cAMP levels is critical in virulence gene expression including genes for T4P biogenesis (33, 86). T4P assist in establishment of a sessile state of growth for the development of chronic antibiotic resistant infections of *P. aeruginosa* (59). Mutant lacking T4P have reduced virulence (60) and are

studied as drug and vaccine targets. Given the contributions of the Chp system to *P. aeruginosa* virulence it is important to further understand the factors that affect its function.

Localization of *P. aeruginosa* Wsp chemosensory system has been previously shown to be important for its function (21, 48). Hence we think that the localization of Chp system is likely important for its function. So far nothing is known about the localization cues for the Chp system (beyond PilG) and the significance of its polar localization for its function. MCPs generally localize to the cell poles and tether other chemotaxis proteins of the same system (98). Given the known roles of MCPs in localizing chemotaxis systems, it becomes important to study the localization cues for PilJ to understand localization of Chp system. PilJ is the only known MCP of the Chp system, is polarly localized and is known to be required for production of T4P (99). We determined the role of PilJ in mediating signal transduction for the distinct outputs of this system. Since the Chp chemosensory cluster does not encode any Par-like protein candidates it is likely that the polar localization of the Chp system occurs through a different mechanism. This work highlights the potential roles of PilJ N-terminus (periplasmic and transmembrane domains) in both function and localization of this MCP.

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## Chapter Two

# Twitching motility and cAMP levels: signal transduction through a single methyl-accepting chemotaxis protein

Vibhuti H. Jansari, Vishwakanth Y. Potharla, Geoff T. Riddell and Sonia L. Bardy*

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## 2.1 Introduction

*Pseudomonas aeruginosa* is an opportunistic pathogen that exhibits swimming and twitching motility. The Chp chemosensory system controls type IV pili (T4P) mediated twitching motility and regulates intracellular levels of adenosine 3', 5'-cyclic monophosphate (cAMP) by altering CyaB activity (1-4). CyaB is one of two intracellular adenylate cyclases that is responsible for synthesis of the majority of cAMP (4). cAMP is an allosteric activator of Vfr, thereby regulating T4P as well as a number of other virulence factors (5-7). An additional Chp-based signaling mechanism involved in regulation of PilY1 expression and c-di-GMP levels has recently been suggested based on interactions between PilJ and FimS (8).

Although the Chp chemosensory system has homology to the *Escherichia coli* chemotaxis system, signal transduction is more complex. Encoded within the *chp* gene cluster are two CheY-like proteins (PilG and PilH), two CheW coupling proteins (PilI and ChpC), a methyltransferase protein (PilK), a methylesterase protein (ChpB), a complex CheA/Y hybrid protein (ChpA), and one methyl-accepting chemotaxis protein (MCP) PilJ (3). PilJ is predicted to have two transmembrane domains and a periplasmic domain of 269-273 amino acids (9, 10). The periplasmic domain of PilJ has recently been proposed as the site of interaction with the major pilin (PilA) leading to modulation of CyaB activity (11). This proposal follows the classical signal transduction model reported in *E. coli,* where ligands bind to the periplasmic domain of MCPs, ultimately resulting in altered kinase activity (12-14).

While the *P. aeruginosa* genome contains 26 annotated MCPs, PilJ is the only MCP known to function within the Chp chemosensory system. Deletion of *pilJ* results in loss of surface piliation and twitching motility, and reduction in cAMP levels (3, 4, 8, 9, 15). Piliation and a basal level of motility can be restored through exogenous cAMP (4). The CheY-like

proteins, PilG and PilH are involved in regulation of both twitching motility and CyaB activity. Deletion of *pilG* results in reduced cAMP and surface piliation, and loss of twitching motility, while Δ*pilH* had increased cAMP and surface piliation, and decreased twitching (4, 6). PilG and PilH function upstream of ATPases PilB and PilT/U respectively, and likely regulate extension and retraction of T4P (6). PilG and PilH also modulate CyaB activity, affecting intracellular levels of cAMP and T4P biosynthesis (4, 16). Exactly how PilG and PilH mediate these functions remains unclear.

*P. aeruginosa* shows directed twitching towards dioleoyl phosphatidylethanolamine (18:1 PE) (17). While little is known about PE directed twitching motility, the response is mediated through T4P and requires the extracellular phospholipase PlcB (18, 19). This preferential migration is specific to unsaturated long chain fatty acid ($C_{16-20}$) and dilauroyl PE (12:0) (17, 19). There have been limited investigations into the Chp chemosensory system role in directional twitching; the *pilJ* mutant is null for twitching and correspondingly cannot exhibit directional twitching, and the *pilH* mutant is non-viable under directional twitching assay conditions (15, 17).

We determined if intracellular cAMP levels, twitching motility, and directional twitching to PE were modulated by classical signal transduction through PilJ. Deletion of the PilJ periplasmic domain reduced intracellular cAMP levels and resulted in a slight cAMP-dependent reduction in twitching, but had no effect on directional twitching. We also found that PilJ is not required for directional twitching provided sufficient cAMP is present to partially restore surface piliation and twitching motility.

## 2.2 Materials and Methods

### 2.2.1 Bacterial Strains and Growth Conditions

All *P. aeruginosa* mutants listed (Table 2.1) were derived from PAO1. *P. aeruginosa* and *E. coli* were grown at 37°C in LB, unless otherwise stated. *E. coli* S17-1 was used for conjugation of plasmids (Table 2.1) into *P. aeruginosa*. Tetracycline and gentamicin were used at 10 μg $ml^{-1}$ (*E. coli*) or 50 μg $ml^{-1}$ (*P. aeruginosa*).

### 2.2.2. Construction of *P. aeruginosa* mutants and expression strains

In-frame deletions of *pilA, cyaB, pilJ, pilT* and the putative periplasmic domain of PilJ ($pilJ_{\Delta 74\text{-}273}$) were constructed using splicing by overlap extension (SOE) PCR, using primers listed in Table 2.2. Colony PCR of PAO1 was used for the initial amplification of each PCR product. Fusion constructs were sequenced to ensure the final deletion alleles were in-frame and no other mutations were introduced. The deletion alleles were cloned into pEX18Tc, transferred into *E. coli* S17-1, then introduced into *P. aeruginosa* by conjugation. Merodiploids were selected on 75 μg $ml^{-1}$ tetracycline and 5 μg $ml^{-1}$ chloramphenicol. Resolution of the merodiploids was achieved through 10% sucrose counter selection. Following screening on tetracycline and sucrose, the deletions were confirmed by PCR.

Expression plasmids were generated by amplifying *cyaB* or *pilJ-his* using the primers listed (Table 2.2). The resulting PCR products were cloned into pJN105 and sequenced. To generate $cyaB_{R412H}$ codons were altered based on the QuikChange mutagenesis kit (Stratagene). The codon change was confirmed through sequencing.

### 2.2.3 Motility Assays

To assay twitching motility, nine individual colonies were stab inoculated into LB agar (1% agar), and incubated at 37°C for 40 hours. Directional twitching assays were performed as

**Table 2.1**. Strains and plasmids used in this study.

| Strain | Description | Reference or Source |
|---|---|---|
| *P. aeruginosa* strains | | |
| PAO1 | Wild type | C. Harwood |
| $pilJ_{\Delta 74\text{-}273}$ | In-frame deletion of 200 amino acids (G74 – Q273) of PilJ | This study |
| Δ*pilJ* | In-frame deletion of *pilJ* | This study |
| Δ*cyaB* | In-frame deletion of *cyaB* | This study |
| Δ*cyaBpilT* | In-frame deletion of *pilT* in Δ*cyaB* strain | This study |
| Δ*pilT* | In-frame deletion of *pilT* | This study |
| Δ*pilJpilT* | In-frame deletion of *pilT* in Δ*pilJ* strain | This study |
| Δ*pilA* | In-frame deletion of *pilA* | This study |
| | | |
| *E. coli* strains | | |
| DH5α | *fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | New England Biolabs |
| S17-1 | *Thi pro hsdR recA RP4-2*(Tc::Mu) (KM::Tn7) | D. Saffarini |
| Plasmids | | |
| pEX18Tc | Suicide cloning vector; SacB, $Tet^R$ | (21) |
| pEX18Tc$pilJ_{\Delta 74\text{-}273}$ | pEX18Tc-based plasmid for deletion of PilJ periplasmic domain | This study |
| pEX18TcΔ*pilJ* | pEX18Tc-based plasmid for deletion of *pilJ* | This study |
| pEX18TcΔ*cyaB* | pEX18Tc-based plasmid for deletion of *cyaB* | This study |
| pEX18TcΔ*pilT* | pEX18Tc-based plasmid for deletion of *pilT* | This study |
| pEX18TcΔ*pilA* | pEX18Tc-based plasmid for deletion of *pilA* | This study |
| pJN105 | araC-$P_{BAD}$ cassette in $pBBR_1$MCS-5, $Gm^R$ | (22) |
| pJN105-*pilJ*<br>pJN105-*pilJ-his*<br><br>pJN105- $pilJ_{\Delta 74\text{-}273}$-*his* | *pilJ* cloned into EcoRI-XbaI sites of pJN105, $Gm^R$<br>C-terminal 6X His-tagged *pilJ-his* gene cloned into pJN105<br>C-terminal 6X His-tagged $pilJ_{\Delta 74\text{-}273}$-*his* gene cloned into pJN105 | This study<br>This study<br><br>This study |
| pJN105-$cyaB_{R412H}$ | $cyaB_{R412H}$ cloned into EcoRI-XbaI sites of pJN105, $Gm^R$ | This study |

**Table 2.2**: Primers used in this study.
[a]Restriction enzyme sites are underlined.

| | Primer Name | Oligonucleotide Sequence (5'-3')[a] |
|---|---|---|
| In-frame deletions | Peri_UPFor | GCCCTGCAGCCAAGGACGCCGAGACCAGCGC |
| | Peri_UPRev | GAAGATGTTGTTCGCCGCTTCCCGGACCGCCGCC GCTTCGGTGGCGTTCTTCGC |
| | Peri_DNFor | GCGAAGAACGCCACCGAAGCGGCGGCGGTCCGG GAAGCGGCGAACAACATCTTCAGC |
| | Peri_DNRev | GCCCTGCAGGGATCAGCGCCGCGAGGGTC |
| | *pilJ*_UPFor | CGAATTCCCGACCGAGATGTACAAGCTGACC |
| | *pilJ*_UPRev | CCGCCGCGCCTATGCATTTGGCCCCCGCCCGGAC C |
| | *pilJ*_DNFor | GGGCGGGGGCCAAATGCATAGGCGCGGCGGCCG CC |
| | *pilJ*_DNRev | GGAATTCGGCTCGAGCTCCGGATGGCTC |
| | *cyaB*_UPFor | AGGTACCCTTCCGCGATGATCCGCTGG |
| | *cyaB*_UPRev | GCGCTGGAGAGGATCCCTG |
| | *cyaB*_DNFor | GCGACCTCTCCTAGGGACGTTCGTCGAACGCCGC CGGCA |
| | *cyaB*_DNRev | ACTGCAGGTCGTCACCAGCCTGCTGG |
| | *pilA*_UPFor | CGCGAATTCCGTCCTGCGGTTTGCG |
| | *pilA*_UPRev | CAAGCCACCTTCGATCACCGAATCTCTCCGTTGA TTATG |
| | *pilA*_DNFor | CATAATCAACGGAGAGATTCGGTGATCGAAGGT GGCTTG |
| | *pilA*_DNRev | GTCCTGCAGCGGCGGCGACCTTACC |
| | *pilT*_UPFor | TGGATCCTTGCCGGCGCCGATGAACG |
| | *pilT*_UPRev | GCGGCGGATCGGCGCCGCCAGGAGGGACTCCCC AATTACAAGCAAG |
| | *pilT*_DNFor | TGCTTGTAATTGGGGAGTCCCTCCTGGCGCCGAT CCGCCGC |
| | *pilT*_DNRev | GAAGCTTGACAGGTCCATCCACACCTG |
| Expression | *cyaB*-For | AGGAATTCATGAAGCCTACCCTCCCCGAC |
| | *cyaB*-Rev | ATTCTAGATTAGAGGATGACCTTGTCGCG |
| | $cyaB_{R412H}$-For | CAGGTGGTGGACTCGCACCGCGACCTCGGCGCC |
| | $cyaB_{R412H}$-Rev | GGCGCCGAGGTCGCGGTGCGAGTCCACCACCTG |
| | *pilJ*-Rev<br>*pilJ-his*-For<br>*pilJ-his*-Rev | ATTCTAGATCAGGCCTGCTCCACGCC<br>AGGAATTCCGAGAAGAACGACCGCAACCAG<br>ATCTAGATCAATGGTGGTGATGGTGGTGGGCCTG CTCCACGCCCTCCGGC |

described previously (19, 20). The phospholipid or stearic acid gradient was formed by spotting PE (18:1) or stearic acid (10 mg $ml^{-1}$ in chloroform) onto the agar and incubating at 30°C for 24 hours. All strains were grown in LB until early stationary phase and concentrated to $9x10^9$ cells $ml^{-1}$ in MOPS buffer (pH 7.6). The concentrated cells were spotted 5 mm from the edge of the phospholipids, and incubated at 37°C for 16 hours. For each assay three independent colonies were assayed in triplicate. Images of colonies were obtained using an Olympus SZX16 stereomicroscope and used to determine the length of the leading and lagging zones.

**2.2.4 Surface Pilus Preparation**

T4P were sheared from bacterial cell surface by vortexing as previously described (4). Cells were grown on LB agar, scraped from the plates, resuspended in 0.15M NaCl + 0.2% formaldehyde (final $OD_{600}$ = 23), and vortexed to shear the pili off the bacterial surface. Bacterial cells and debris were removed by centrifugation. The pili were precipitated through overnight incubation at 4°C in 100 mM $MgCl_2$. Pili were resuspended in SDS-PAGE loading dye, resolved on a 15% polyacrylamide gel and stained with a Coomassie brilliant blue G-250 - perchloric acid solution (23).

**2.2.5 β-galactosidase assay**

To determine intracellular levels of cAMP, *P. aeruginosa* strains containing the cAMP reporter construct *lac*P1-*lacZ* were grown on LB agar (4). Surface grown cells were resuspended in LB, and β-galactosidase assays were performed as previously described (16).

**2.2.6 Immunoblotting**

Whole cell lysates were prepared from mid-log phase ($OD_{600}$ ~ 0.5) cultures, and loading was normalized based on $OD_{600}$. Protein samples were separated via 15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Membranes were probed with anti-PilA (gift

from L. Burrows, McMaster University; 1:10,000) followed by peroxidase conjugated goat anti-rabbit (1:100,000). Immunoblots were developed with Pierce SuperSignal West chemiluminescent kit and a Fotodyne Luminary system.

### 2.2.7 Statistical Analysis

Data was analyzed using ANOVA followed by TukeyHSD, or a Student's *t*-test as indicated in the figure legend. Analysis was done using R (3.2.3).

## 2.3 Results and Discussion

The involvement of the Chp chemosensory system in regulating twitching motility and intracellular cAMP, along with the recent proposal that PilJ - PilA interactions allow T4P to function as a mechanosensor, led us to investigate the role of the periplasmic domain of PilJ in signal transduction. PilJ is the sole MCP encoded within the Chp gene cluster, and deletion of *pilJ* results in a non-twitching phenotype and reduced levels of intracellular cAMP (3, 4, 9, 15). In the well-studied MCPs of *E. coli*, the periplasmic domain is the site of ligand binding and is required for classical signal transduction (14, 24). We generated an in-frame deletion of the putative periplasmic domain (G74-Q273) and determined the phenotypes of the resulting strain ($pilJ_{\Delta 74\text{-}273}$) with regards to intracellular cAMP levels, twitching motility, and directional twitching towards PE.

### 2.3.1 The putative periplasmic domain of PilJ regulates cAMP levels

As previously reported (25), deletion of full-length *pilJ* resulted in a loss of twitching motility and reduced levels of intracellular cAMP (~20% of wild type) (Fig. 2.1A and D). This reduction in cAMP was similar to that seen in Δ*cyaB* ($p$=0.926). The intermediate twitching motility levels of Δ*cyaB* contrast with Δ*pilJ* which was null for twitching. This difference highlights the importance of PilJ in controlling twitching motility separate from cAMP levels, as

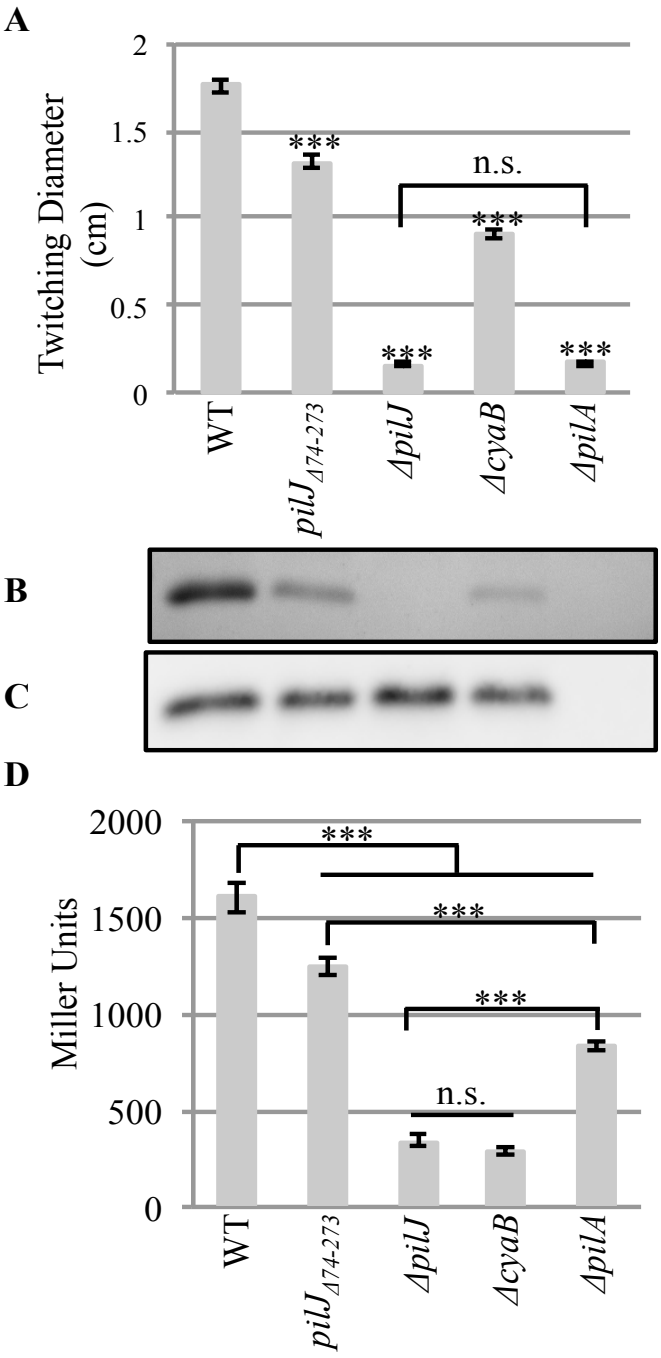

A
Twitching Diameter (cm)
2
1.5
1
0.5
0
***
n.s.
***
***
***
WT
pilJ Δ174-273
ΔpilJ
ΔcyaB
ΔpilA
B
C
D
Miller Units
2000
1500
1000
500
0
***
***
***
n.s.
WT
pilJ Δ174-273
ΔpilJ
ΔcyaB
ΔpilA

Fig. 2.1. Deletion of the putative periplasmic of PilJ (*pilJ*$_{\Delta 74\text{-}273}$) reduces twitching motility and intracellular cAMP levels. A) Diameter of twitching motility zones for the indicated strains. Nine colonies were assayed to determine the average and standard error of the mean. Asterisks (***) indicate values significantly different from wild type ($p<0.001$) as determined by ANOVA followed by TukeyHSD. Non-significant differences are indicated by n.s. B) SDS-PAGE showing the surface levels of PilA obtained from the indicated strains. C) Whole cell levels of PilA as determined by western blotting for the indicated strains. D) Indicated strains were tested for β-Galactosidase activity, which is indicative of intracellular levels of cAMP bound to Vfr represented as Miller Units. Three colonies were assayed in triplicate to determine the average and the standard error of the mean. Asterisks (***) indicate significantly different values ($p<0.001$) as determined by ANOVA followed by TukeyHSD. Non-significant differences are indicated by n.s.

previously reported (4). Analysis of surface piliation showed that a residual level of pilus biogenesis occurs even with the low levels of cAMP present in *cyaB* mutant (Fig. 2.1B). Deletion of the major type IV pilin (*pilA*) resulted in a complete loss of twitching motility, and a ~50% reduction in cAMP levels relative to wild type. Although earlier studies reported that *pilA* deletion resulted in cAMP levels similar to those found in Δ*pilJ* (11), our data show significant differences in cAMP levels between Δ*pilJ* and Δ*pilA* ($p$<0.001, Fig. 2.1D), suggesting PilJ plays a larger role in regulating cAMP than PilA.

To determine the role of the periplasmic domain of PilJ, we tested a mutant that lacks this domain. In contrast to *pilJ* deletion mutants, surface grown cells of $pilJ_{\Delta 74\text{-}273}$ retained twitching motility, surface pilin levels and intracellular cAMP albeit at significantly reduced amounts relative to wild type (Fig. 2.1A, B and D). The reduction of surface piliation was not due to inavailability of the major pilin subunit as whole cells retained wild type levels of PilA (Fig. 2.1C). The reduction in cAMP suggests that the PilJ periplasmic domain is involved in signal transduction affecting CyaB activity. Surface contact is critical for signal transduction through the periplasmic domain as liquid grown $pilJ_{\Delta 74\text{-}273}$ had wild type levels of cAMP (data not shown). The mechanosensor model suggests that PilA interacts with PilJ periplasmic domain to trigger signal transduction (11). However, deletion of the PilJ periplasmic domain did not reduce cAMP to the same levels as the *pilA* or *pilJ* deletions, suggesting that additional regions of PilJ are important in regulation of cAMP levels. It is not clear if the periplasmic domain is needed for ligand binding or if its deletion causes a conformational change in PilJ thereby altering ChpA activation and reducing cAMP levels. It is unlikely that regulation occurs through direct interaction between the PilJ periplasmic domain and CyaB, as Δ*pilG* has reduced cAMP similar to Δ*cyaB* indicating signal transduction through the Chp system is required (4).

The reduced twitching motility seen in *pilJ*$_{\Delta 74\text{-}273}$ was partially complemented through plasmid-based expression of wild type PilJ (Fig. 2.2A), which corresponded with a slight but insignificant increase in cAMP ($p>0.05$, Fig. 2.2D). This is in contrast to wild type *P. aeruginosa,* where overexpression of PilJ resulted in a decrease in twitching motility and cAMP relative to the parent strain ($p<0.05$) (Fig. 2.2A and D). Expression of *pilJ* in Δ*pilJ* restored twitching, cAMP and surface piliation to levels similar to or above wild type (Fig. 2.2).

As chemosensory systems are sensitive to stoichiometry (26), we verified that the impaired signal transduction in *pilJ*$_{\Delta 74\text{-}273}$ was not due to altered levels of expression or polar effects on the downstream genes. Multiple attempts to quantify levels of epitope-tagged chromosomally encoded PilJ and PilJ$_{\Delta 74\text{-}273}$ were unsuccessful. We therefore overexpressed C-terminal 6×His-tagged versions of PilJ or PilJ$_{\Delta 74\text{-}273}$ in Δ*pilJ* background. When protein expression was induced to similar levels using 0.03% arabinose, twitching motility and cAMP levels were reduced in Δ*pilJ* (*pilJ*$_{\Delta 74\text{-}273}$-His) relative to the wild type (Δ*pilJ* (*pilJ*-His)) (Fig. 2.3). In an attempt to separate the twitching motility and cAMP defects, we expressed a CyaB point mutant to restore intracellular levels of cAMP. CyaB$_{R412H}$ synthesizes cAMP independent of Chp signal transduction and is reported to give cAMP levels most similar to wild type (27). Expression of *cyaB*$_{R412H}$ in *cyaB* and *pil* mutants resulted in increased cAMP (Fig. 2.4D), but did not always correspond to large increases in twitching motility (Fig. 2.4A). The small increase in twitching motility in the absence of PilJ (Δ*pilJ* (*cyaB*$_{R412H}$)) may result from unregulated activity of the extension and retraction ATPases (PilB/T) (6, 28).

The inability of increased cAMP to restore wild type levels of twitching motility in Δ*pilJ* again highlights the importance of full length PilJ in controlling twitching motility separate from cAMP levels. This separation of cAMP and twitching motility is not seen in *pilJ*$_{\Delta 74\text{-}273}$, where

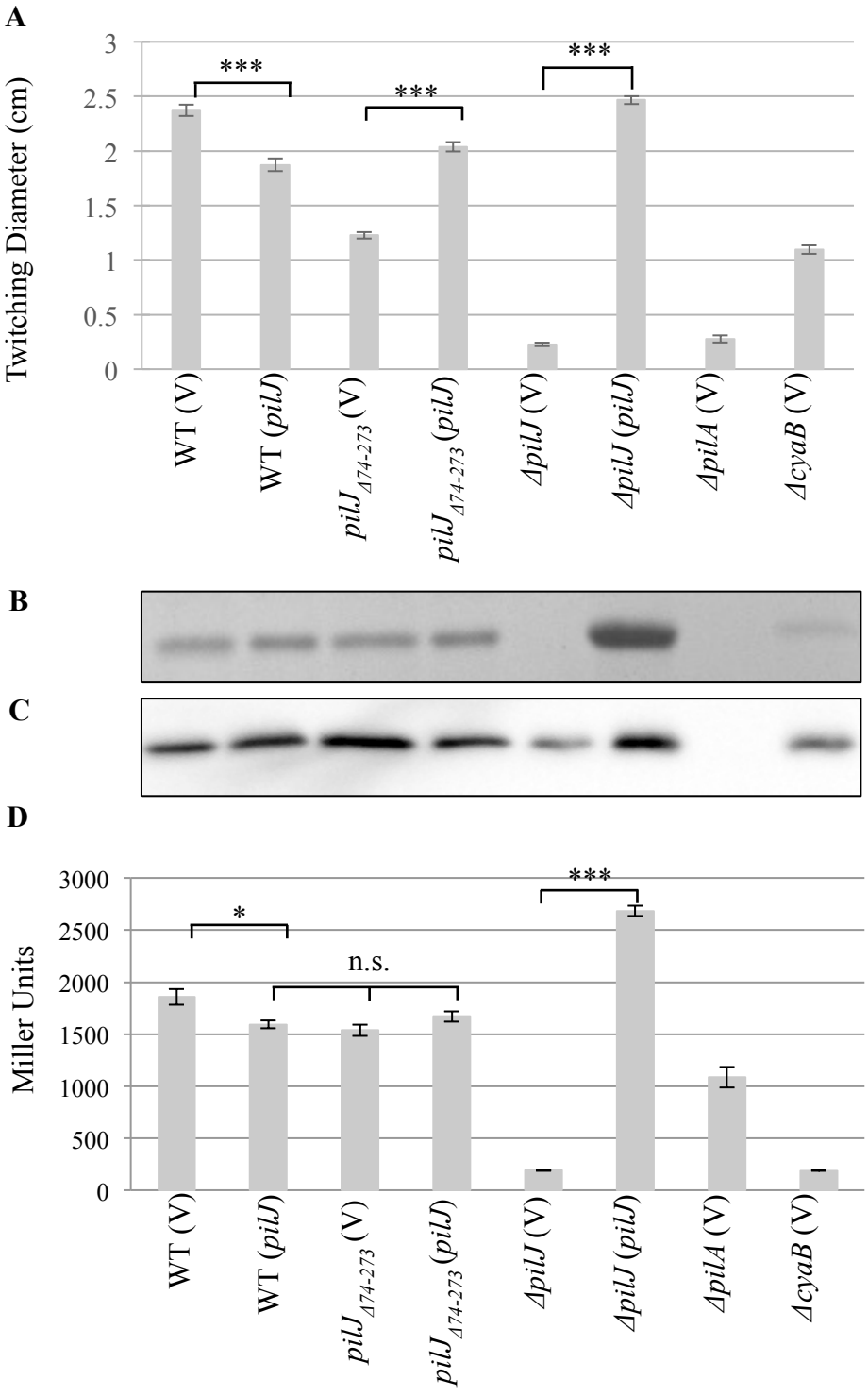

A
Twitching Diameter (cm)
3
2.5
2
1.5
1
0.5
0
***
***
***
WT (V)
WT (pilJ)
pilJ Δ74-273 (V)
pilJ Δ74-273 (pilJ)
ΔpilJ (V)
ΔpilJ (pilJ)
ΔpilA (V)
ΔcyaB (V)
B
C
D
Miller Units
3000
2500
2000
1500
1000
500
0
*
n.s.
***
WT (V)
WT (pilJ)
pilJ Δ74-273 (V)
pilJ Δ74-273 (pilJ)
ΔpilJ (V)
ΔpilJ (pilJ)
ΔpilA (V)
ΔcyaB (V)

Fig. 2.2. Expression of full-length *pilJ* partially restores twitching motility and surface piliation in $pilJ_{\Delta 74\text{-}273}$. A) Zones of twitching motility for the indicated strains. Nine colonies were assayed to determine the average and standard error of the mean. B) SDS-PAGE showing the surface levels of PilA obtained from the indicated strains. C) Whole cell levels of PilA as determined by western blotting for the indicated strains. D) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented as Miller Units. Three colonies were assayed in triplicate to determine the average and the standard error of the mean. The strains contained either pJN105 (V) or pJN105-*pilJ* (*pilJ*) as indicated. In panels A and D asterisks (***, $p<0.001$; *, $p<0.05$) indicate values significantly different from the isogenic controls as determined by ANOVA followed by TukeyHSD. Non-significant differences are indicated by n.s.

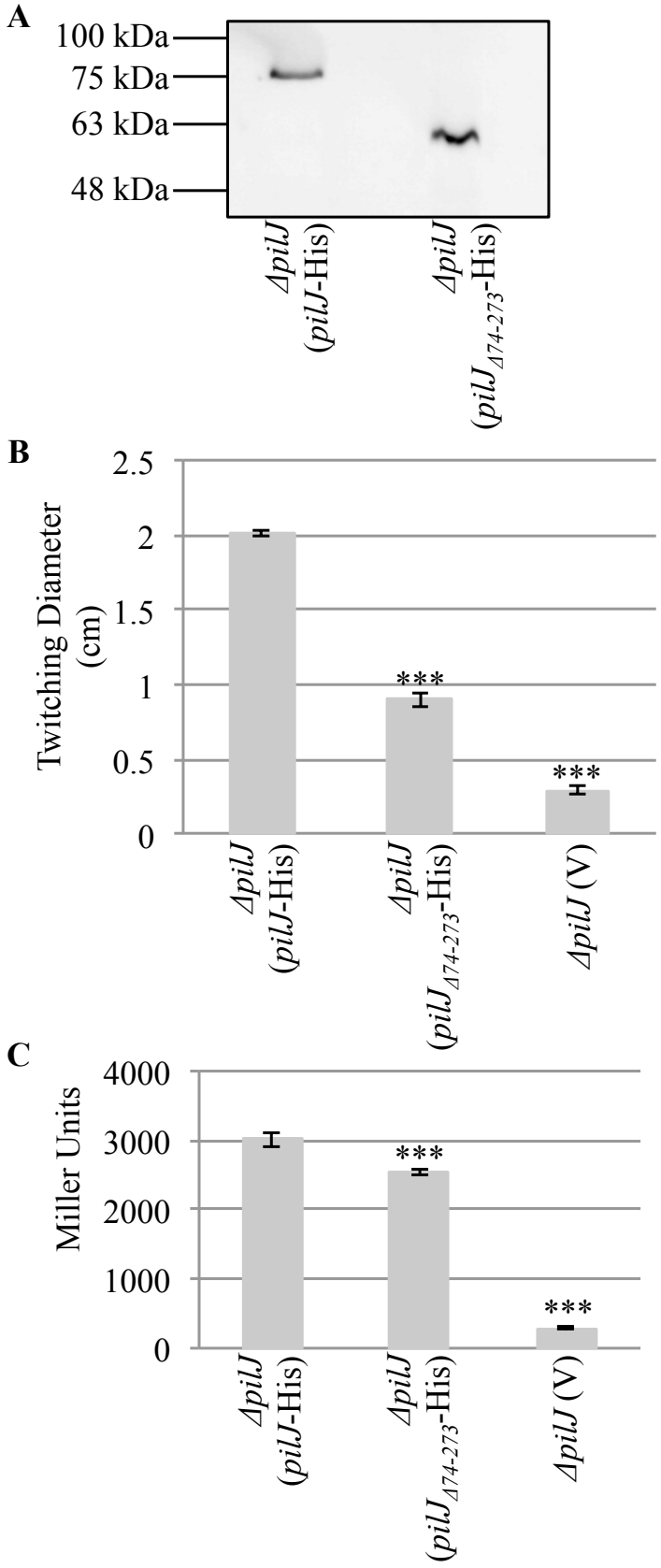

A
100 kDa
75 kDa
63 kDa
48 kDa
ΔpilJ (pilJ-His)
ΔpilJ (pilJΔ474-273-His)
B
Twitching Diameter (cm)
2.5
2
1.5
1
0.5
0
***
***
ΔpilJ (pilJ-His)
ΔpilJ (pilJΔ474-273-His)
ΔpilJ (V)
C
Miller Units
4000
3000
2000
1000
0
***
***
ΔpilJ (pilJ-His)
ΔpilJ (pilJΔ474-273-His)
ΔpilJ (V)

Fig. 2.3. Expression of *pilJ*$_{\Delta 74\text{-}273}$-His results in reduced twitching motility and intracellular cAMP when compared to expression of full length *pilJ*-His. A) Anti-His western blotting of membranes demonstrated similar expression levels of full-length PilJ-His and PilJ$_{\Delta 74\text{-}273}$-His. B) Diameter of twitching motility zones for the indicated strains. Nine colonies were assayed to determine the average and standard error of the mean. C) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented as Miller Units. Three colonies were assayed in triplicated to determine the average and standard error of the mean. The asterisks in panel B and C indicated significantly different values (***, $p<0.001$) from wild type (Δ*pilJ* (*pilJ*-His)) as determined by ANOVA followed by TukeyHSD.

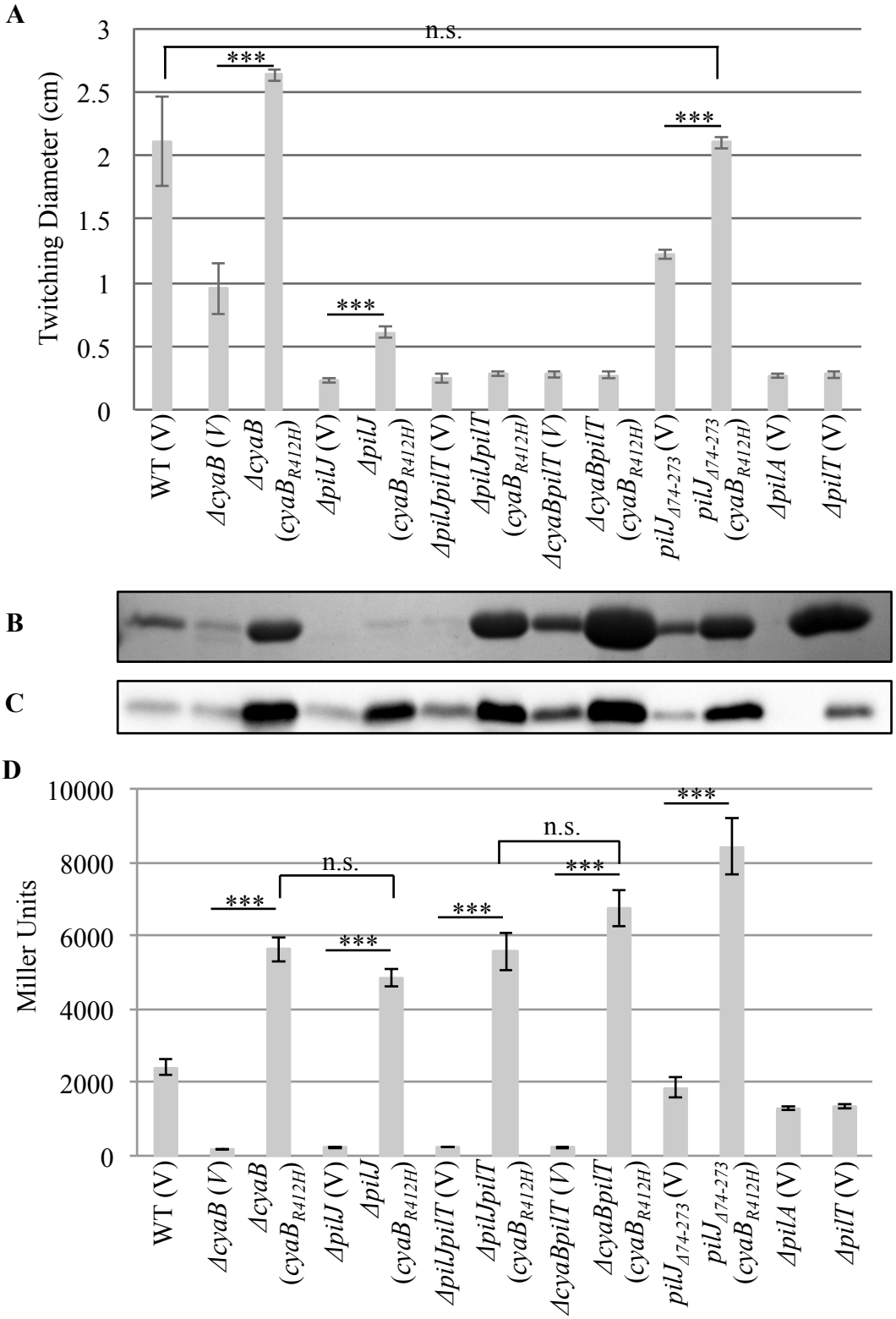
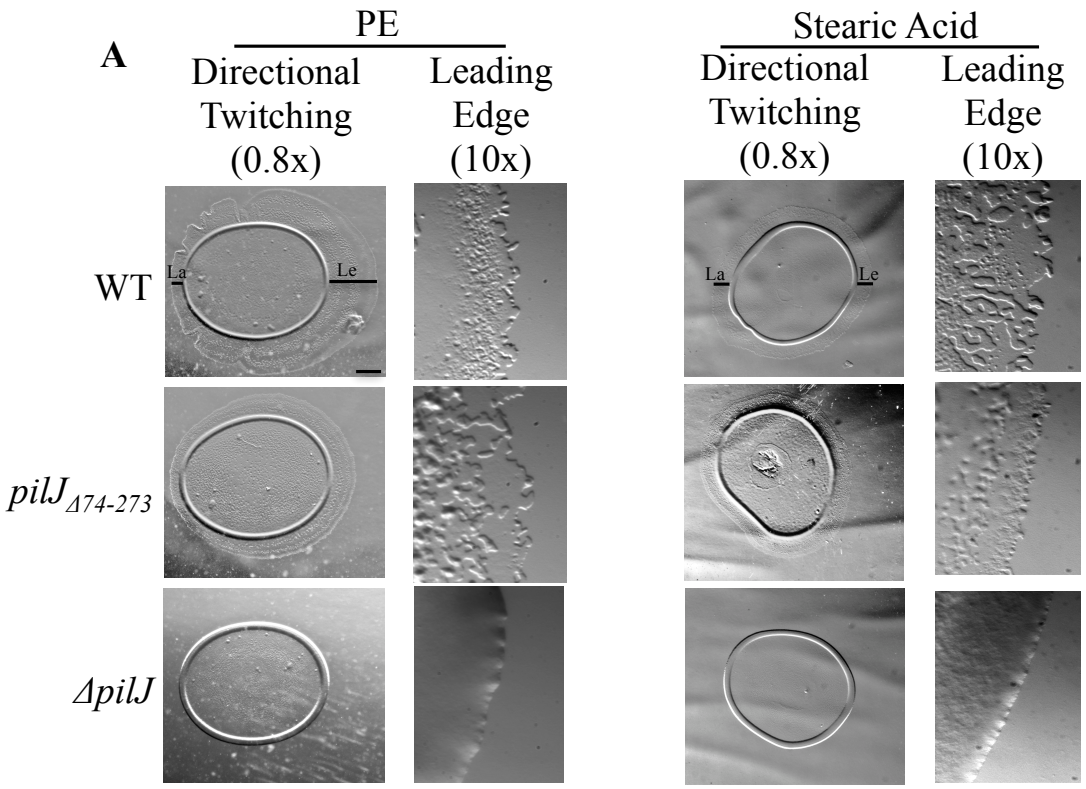

A
Twitching Diameter (cm)
3
2.5
2
1.5
1
0.5
0
n.s.
***
***
***
WT (V)
ΔcyaB (V)
ΔcyaB (cyaB R412H)
ΔpilJ (V)
ΔpilJ (cyaB R412H)
ΔpilJpilT (V)
ΔpilJpilT (cyaB R412H)
ΔcyaBpilT (V)
ΔcyaBpilT (cyaB R412H)
pilJ Δ474-273 (V)
pilJ Δ474-273 (cyaB R412H)
ΔpilA (V)
ΔpilT (V)
B
C
D
Miller Units
10000
8000
6000
4000
2000
0
n.s.
n.s.
***
***
***
***
***
***
WT (V)
ΔcyaB (V)
ΔcyaB (cyaB R412H)
ΔpilJ (V)
ΔpilJ (cyaB R412H)
ΔpilJpilT (V)
ΔpilJpilT (cyaB R412H)
ΔcyaBpilT (V)
ΔcyaBpilT (cyaB R412H)
pilJ Δ474-273 (V)
pilJ Δ474-273 (cyaB R412H)
ΔpilA (V)
ΔpilT (V)

Fig. 2.4. Providing cAMP through a *cyaB* point mutant restores wild type levels of twitching motility in $pilJ_{\Delta 74\text{-}273}$. A) Zones of twitching motility for the indicated strains. Nine colonies were assayed to determine the average and standard error of the mean. B) SDS-PAGE showing the surface levels of PilA obtained from the indicated strains. C) Whole cell levels of PilA as determined by western blotting for the indicated strains. D) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented as Miller Units. Three colonies were assayed in triplicate to determine the average and the standard error of the mean. The strains contained either pJN105 (V) or pJN105-$cyaB_{R412H}$ ($cyaB_{R412H}$) as indicated. In panels A and D asterisks (***, $p<0.001$) indicate significantly different values as determined by ANOVA followed by TukeyHSD. Non-significant differences are indicated by n.s.

expression of $cyaB_{R412H}$ increases cAMP and restores wild type levels of twitching (Fig. 2.4A and D). This showed that the periplasmic domain is not involved in the regulation of twitching motility, provided sufficient cAMP is present. Additionally, $pilJ_{\Delta 74\text{-}273}$ retains directional twitching towards PE, as evidenced by a leading: lagging ratio >3 (Fig. 2.5). A positive result for directional twitching is defined as a ratio >2. As a control, these strains were assayed against stearic acid (17); both wild type and $pilJ_{\Delta 74\text{-}273}$ twitched uniformly resulting in leading: lagging edge ratios of ~1 (Fig. 2.5B). These data indicate that while the periplasmic domain plays an important role in regulating cAMP levels, twitching motility occurs independent of this domain, both in the presence and absence of a proposed chemoattractant. Non-classical signal transduction has previously been reported in *E. coli* MCPs, where the phenol sensing response is mediated by the transmembrane and HAMP domains (29). It is possible that the PilJ transmembrane and/or HAMP domains are involved in the regulation of twitching motility. This is the first time a defined domain within PilJ has been identified as having a distinct role in signal transduction.

When examining the surface pilin and whole cell pilin levels of these strains, several observations were made (Fig. 2.4B and C). Δ*pilJ* ($cyaB_{R412H}$) has minute amounts of surface piliation. These levels of surface pilin are significantly less than Δ*cyaB* ($cyaB_{R412H}$), despite having similar levels of cAMP. This difference is likely due to an extension deficiency as comparable strains lacking the retraction ATPase PilT had unequal amounts of surface piliation (Fig. 2.4B, compare Δ*pilJpilT* ($cyaB_{R412H}$), and Δ*cyaBpilT* ($cyaB_{R412H}$)). Additionally, whole cells with increased cAMP had high levels of PilA protein compared to wild type (Fig. 2.4C), and this increase is not simply due to increased surface piliation (compare Δ*pilJ* ($cyaB_{R412H}$) and Δ*pilJpilT* (V), Fig. 2.4B and C). PilA expression is thought to be regulated transcriptionally and


A
PE
Stearic Acid
Directional Twitching (0.8x)
Leading Edge (10x)
Directional Twitching (0.8x)
Leading Edge (10x)
WT
pilJΔ74-273
ΔpilJ
La
Le
La
Le

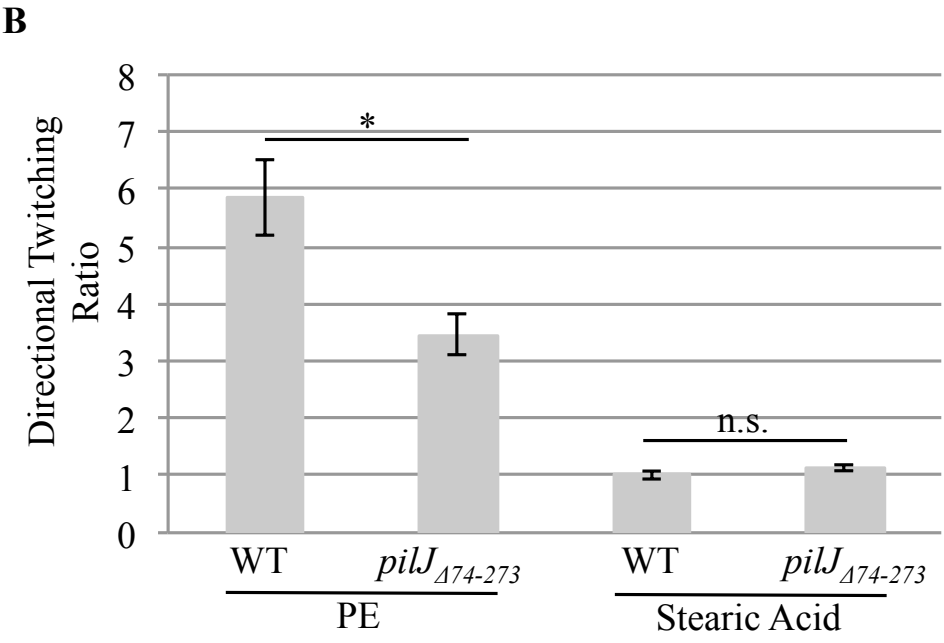

B
Directional Twitching Ratio
0
1
2
3
4
5
6
7
8
*
n.s.
WT
pilJΔ74-273
WT
pilJΔ74-273
PE
Stearic Acid

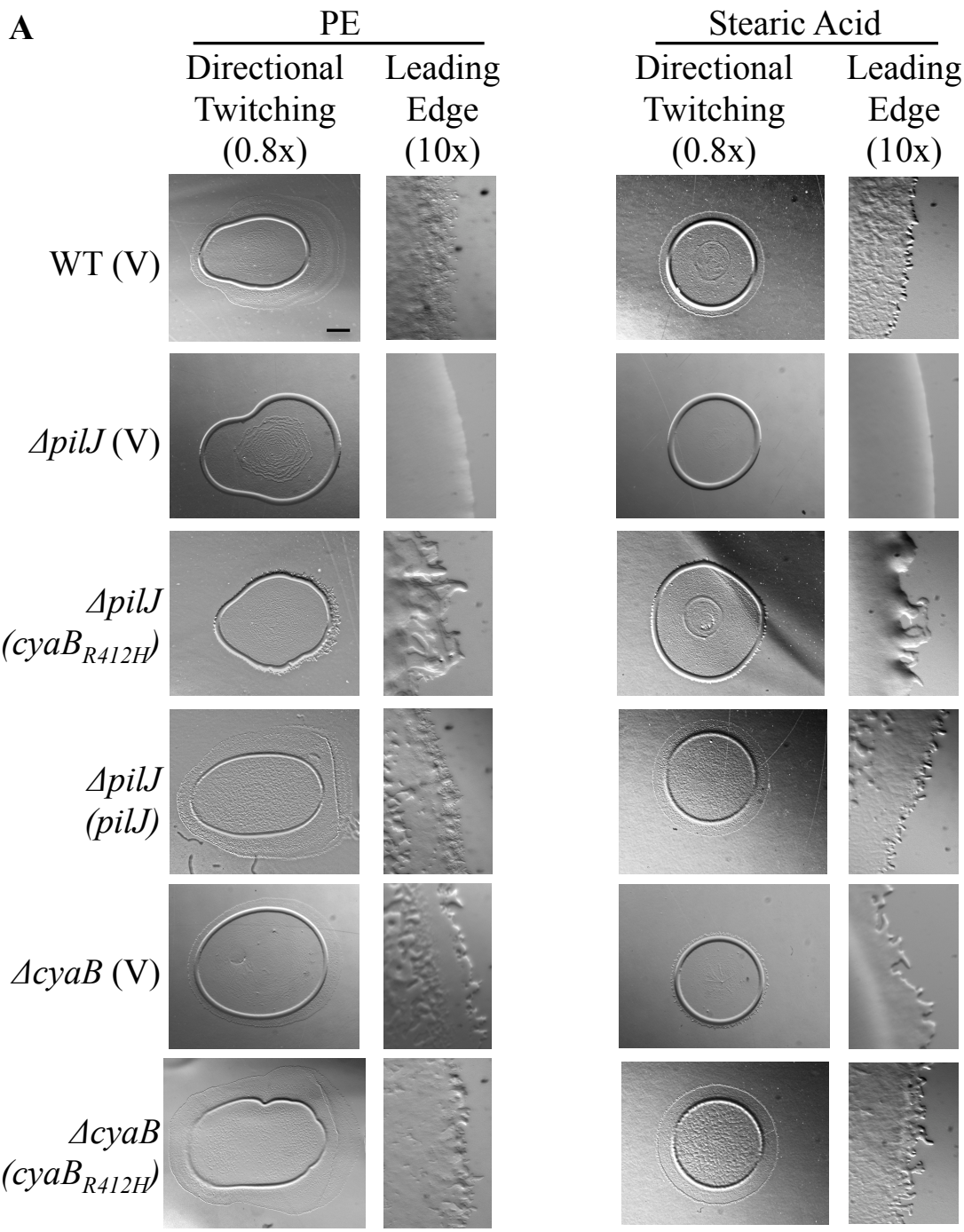
Fig. 2.5. The putative periplasmic domain of PilJ is not required for directional twitching to PE. A) Representative images of directional twitching results for the indicated strains. The PE or stearic acid was deposited on the plate to the right of where the *P. aeruginosa* culture was placed. Black bars on the wild type strain indicates the leading edge (Le) and lagging edge (La) that were measured to determine the directional twitching ratio. Scale bar = 1mm (0.8x magnification). B) Directional twitching ratios for both wild type and the $pilJ_{\Delta 74\text{-}273}$ strains. Directional twitching ratios were calculated by dividing the length of the leading edge by the length of the lagging edge. Three independent colonies were analyzed in triplicate. A ratio greater than 2 indicates directional twitching. Significantly different values were determined using a student's T-test (paired, *, $p<0.05$).

post- translationally. Transcriptional regulation occurs through a two-component system PilR-PilS, but the signal sensed by PilS remains unidentified (30, 31). Additionally, *pilA* promoter activity is affected by deletion of *chpA* and *pilG* (6). Previous studies did not address if the different promoter activity is due to altered levels of cAMP, or altered levels of PilA in the inner membrane (32). Our data show a correlation between increased levels of PilA in whole cells and cAMP levels that are significantly higher than wild type. Further studies will be done to determine the relationship behind this pattern.

### 2.3.2 Excess cAMP enables directional twitching towards phosphatidylethanolamine in the absence of PilJ

Because expression of $cyaB_{R412H}$ partially restores twitching motility in Δ*pilJ*, this strain was tested for directional twitching towards PE. Δ*pilJ* ($cyaB_{R412H}$) demonstrated directional twitching (ratio >2) but with a distinct morphology (Fig. 2.6A). The rafts extending from the edge of the Δ*pilJ* ($cyaB_{R412H}$) colony were thicker than the rafts seen on the other strains assayed. This same morphology was seen on the edges of Δ*pilJ* ($cyaB_{R412H}$) colonies showing non-preferential twitching towards stearic acid (Fig. 2.6A). Therefore, although twitching is restored by providing extra cAMP to Δ*pilJ*, the appearance of this motility is dramatically different from wild type.

There are a number of possible explanations for the retention of directional twitching in Δ*pilJ* ($cyaB_{R412H}$). Earlier studies focused on the role of PE as a chemoattractant (18, 19), and it is possible that PE functions as a chemoattractant independent of PilJ. This would suggest that there is another MCP in the Chp system. Previous studies however failed to identify an alternate MCP functioning in directional twitching (Bardy, Vasil, and Maddock, unpublished). Although unlikely, it is possible that signal(s) enter the Chp system in an MCP independent manner. ChpA


A
PE
Stearic Acid
Directional Twitching (0.8x)
Leading Edge (10x)
Directional Twitching (0.8x)
Leading Edge (10x)
WT (V)
ΔpilJ (V)
ΔpilJ (cyaB R412H)
ΔpilJ (pilJ)
ΔcyaB (V)
ΔcyaB (cyaB R412H)

**B**

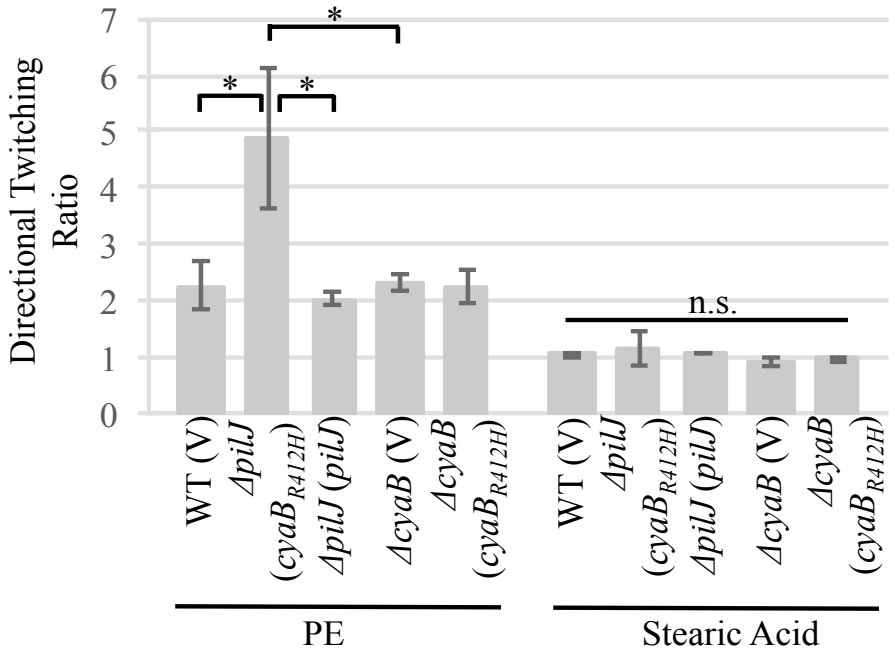



Fig. 2.6. PilJ is not absolutely required for directional twitching to PE provided sufficient cAMP is present. A) Representative images of directional twitching results. The PE or stearic acid was deposited on the plate to the right of where the *P. aeruginosa* culture was placed. Scale bar = 1 mm (0.8x magnification) B) Directional twitching ratios were calculated by dividing the length of the leading edge by the length of the lagging edge. Three independent colonies were analyzed in triplicate. A ratio greater than 2 indicates directional twitching. Significantly different values were determined by ANOVA followed by TukeyHSD (*, $p<0.05$).

is an atypical histidine kinase with 6 putative histidine phosphotransfer sites, and putative serine/threonine phosphotransfer sites (3). This large number of phosphotransfer sites may allow for a high degree of regulation or multiple points of signal recognition. Alternatively, it is possible that the directional twitching seen with Δ*pilJ* (*cyaB*$_{R412H}$) is because PE has other properties that result in increased movement. PE may function as a surfactant, similar to rhamnolipids (33), thereby resulting in increased motility at higher concentrations. This is supported by early studies on directional twitching by *P. aeruginosa*, wherein uniform concentrations of PE enhanced swarm expansion (17). It is also possible that PE triggers signal transduction altering cAMP levels, thereby increasing pilus biogenesis resulting in increased movement. Additional studies are required to further understand this phenotype.

In this study, we have begun to dissect the different domains of PilJ to understand the mechanisms of signal transduction. The periplasmic domain appears to be important for wild type cAMP levels, and subsequently for twitching motility. This is in agreement with the mechanosensor model, where the periplasmic domain of PilJ was postulated to be important in signal transduction through interaction with PilA in response to surface contact (11). We propose however that classical signal transduction is not the only mechanism regulating cAMP as *pilJ*$_{\Delta 74\text{-}273}$ had cAMP levels higher than those detected in Δ*pilA* indicating that other domains of PilJ are involved.

This is the first reported mutation within the Chp signal transduction system that allows the separation of cAMP levels from twitching motility. A number of recent studies have reported on the roles of second messengers in regulating surface behaviors, including twitching motility, swarming, and biofilm formation (4, 8). The ability to separate signal transduction regarding cAMP levels from signal transduction regulating twitching motility will allow us to tease apart the exact roles for each of these outputs in surface sensing and the resultant lifestyle changes.

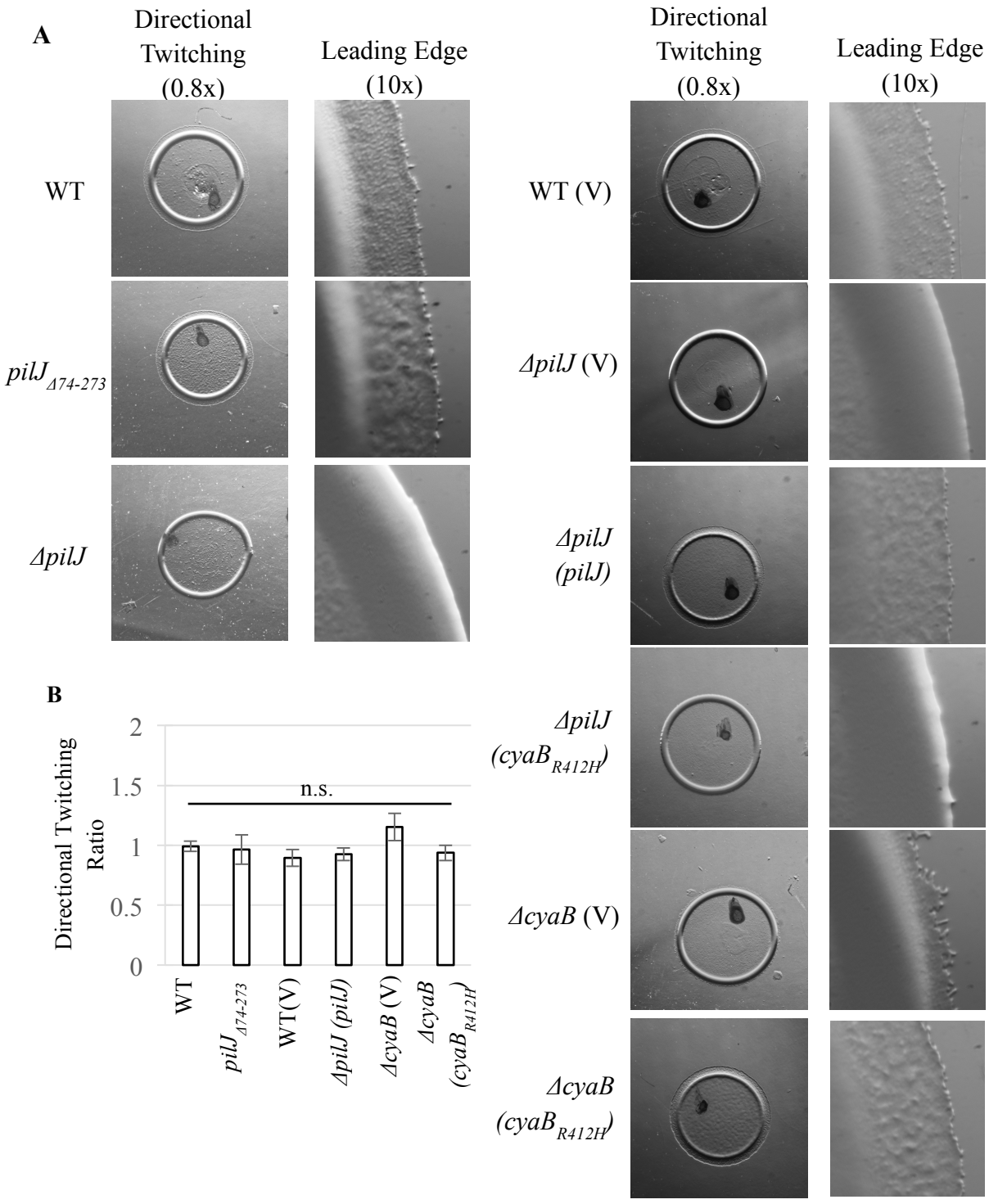

A
Directional Twitching (0.8x)
Leading Edge (10x)
WT
pilJ Δ74-273
ΔpilJ
Directional Twitching (0.8x)
Leading Edge (10x)
WT (V)
ΔpilJ (V)
ΔpilJ (pilJ)
ΔpilJ (cyaB R412H)
ΔcyaB (V)
ΔcyaB (cyaB R412H)
B
Directional Twitching Ratio
2
1.5
1
0.5
0
n.s.
WT
pilJ Δ74-273
WT(V)
ΔpilJ (pilJ)
ΔcyaB (V)
ΔcyaB (cyaB R412H)

Fig. 2.7. Chloroform does not trigger a directional twitching response. A) Representative images of directional twitching results in response to chloroform. The chloroform was deposited on the plate to the right of where the *P. aeruginosa* culture was placed. B) Ratios were calculated by analyzing three independent colonies, each tested in triplicate. A ratio less than 2 indicates that directional twitching did not occur. In comparison to PE/stearic acid based directional twitching assays results all strains showed a reduced twitching on TPM media with chloroform, with Δ*pilJ* (*cyaB*$_{R412H}$) little to no twitching motility. As such, no ratio was calculated for Δ*pilJ* (*cyaB*$_{R412H}$).

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# Chapter Three

## Deciphering the domains involved in polarity and signal transduction in PilJ, the methyl-accepting chemotaxis protein of the Chp chemosensory system

## 3.1 Introduction

Dedicated chemosensory systems respond to specific external stimuli and regulate variety of cellular processes including the function of bacterial motility structures such as flagella and type IV pili (T4P). These motility structures are also used as adhesins and are thus important during pathogenesis (1). In rod shaped bacterial cells, flagella can have a variety of localization patterns such as the peritrichous pattern seen in *Escherichia coli* or the single polar flagellum of *Pseudomonas aeruginosa*. Flagella are necessary for chemotaxis via swimming motility and help to establish an infection (2). T4P are polar structures required for surface-based motility which includes twitching, crawling and sling-shot motility (3). They stabilize more intimate attachment of the bacterium with the host cell in the initial stage of infection and are also required for biofilm formation, transformation etc. (1).

*P. aeruginosa*, a Gram-negative opportunistic pathogen is well- studied for its pathogenicity and environmental versatility. It has many chemosensory proteins that are organized into four functionally distinct chemosensory clusters. The *chp* gene cluster (PA0408-PA4116) contains *pilGHIJK* and *chpA-D* genes (4, 5). The Chp chemosensory system regulates T4P mediated twitching motility, intracellular levels of cAMP and T4P biogenesis by modulating CyaB activity (4, 6, 7). cAMP is a second messenger molecule that is presumed to modulate gene expression by allosteric regulation of a cAMP receptor protein, Vfr (virulence factor regulator) (8). cAMP-Vfr upregulates expression of genes encoding virulence factors that include T4P assembly, the PilS-PilR two component system that regulates pilin expression, type II secretion system, quorum sensing, type III secretion system, flagellar biosynthesis and many secreted toxins (8-12). *P. aeruginosa* T4P are polarly localized and the Chp chemosensory system that controls T4P function is also polar (6, 13, 14). However, the mechanisms that regulate the polar localization of the Chp chemosensory proteins are not completely explored.

Methyl accepting chemotaxis proteins (MCPs) are transmembrane receptors that bind chemoeffectors in the periplasm thus triggering signal transduction through the associated chemosensory system (15). An increase in the production of T4P upon surface association is previously reported (16). This is in agreement with the mechanosensor model for surface sensing by T4P, which proposes that upon surface association, there is an unknown modification at the base of the pilus. This modification is thought to modulate the interaction between pilin monomers and PilJ. This interaction is believed to be sensed as a signal by PilJ through its periplasmic domain (17). The signal is transduced to ChpA (HK) and PilI and ChpC, the coupling proteins, are expected to mediate the interaction between PilJ and ChpA. ChpA gets autophosphorylated and after passing through numerous Hpts in ChpA the phosphoryl group is transduced to the response regulators, PilG and PilH. When phosphorylated, PilG and PilH respectively upregulate or downregulate the activity of CyaB and trigger pilus extension and retraction. ChpB, the methylesterase and PilK, the methyltransferase regulate adaptation of the Chp system by modulating the methylation status of the MCP. During adaptation, ChpB is phosphorylated by ChpA making it too a response regulator of the Chp system (7, 18, 19).

Intracellular localization of MCPs is shown to be important for normal function of a chemosensory system (20). MCPs are also important for the chemosensory protein cluster formation (20, 21). The deletion of the MCPs results in a non-functional chemotaxis system where the CheW and CheA are diffuse in the cytoplasm (22). Previous reports in *E. coli* have shown that MCPs generally localize to the cell poles and tether other chemosensory proteins with them (21-24). The MCPs are inserted in lateral cell membranes and form small signaling complexes. Once they form large aggregates, they are forced to the poles to accommodate this complex (25).

In *P. aeruginosa*, studies on the punctate chemosensory system Wsp revealed that the

sole MCP (WspA) is responsible for this localization. Chimeric proteins generated from WspA fused with a polar MCP revealed that the localization cue of WspA lies in its cytoplasmic domain (26). Additionally, point mutations in the trimer interaction domain in the cytoplasmic domain showed that punctate localization is essential for downstream signaling to the response regulator (WspR-YFP) for the production of c-di-GMP (20, 26). PilJ is the sole MCP predicted to be part of the Chp chemosensory system and has polar localization in a *pilG* deletion (one of the response regulators) (27). Based on the localization studies from *E. coli* and the Wsp system in *P. aeruginosa*, and the homology of the Chp system with the *E. coli* chemotaxis system, we propose that PilJ is important for the localization of PilI (coupling protein) and ChpA (HK) of the Chp system. We also investigated the internal polar localization cue for PilJ. If the cytoplasmic domain is responsible for polar localization of PilJ then we expect to lose the polarity of PilJ upon deletion or mutation of its cytoplasmic region.

In this study we found that PilJ is important for PilI foci formation. This points to the possibility of PilJ acting as a nucleating protein for the aggregation of PilJ-PilI-ChpA, the core chemosensory protein complex. We were also able to identify the PilJ domains that are involved in foci formation and polar localization. The cytoplasmic domain is involved in wild type levels of foci formation of PilJ-mCherry, but not localization. Upon additional deletion of the second transmembrane domain there was a greater decrease in foci formation, and of the foci that were formed, only 46% were polar. Hence we conclude that the cytoplasmic domain, most likely the trimer interaction domain, is required for wild type levels of foci formation, while the second transmembrane domain is important for polar localization of PilJ at wild type levels.

Previous studies have shown that the periplasmic domain of PilJ is involved in signal transduction for regulation of cAMP levels but not twitching motility (Chapter 2)(28). This data, combined with the non-classical signal transduction pathway that has been previously reported

in *E. coli* (29), leads us to propose the possibility of another domain of PilJ mediating signal input. In this study, using chimeric constructs generated by fusing PilJ with an *E. coli* MCP, Tsr, we show that the native amino acids in the periplasmic and the two transmembrane domains of PilJ are important for localization and signal transduction.

## 3.2 Materials and Methods

### 3.2.1 Bacterial strains, plasmids and growth conditions

All strains and plasmids are listed in Table 3.1. *Pseudomonas aeruginosa* PAO1 (WT) and *Escherichia coli* (DH5α) were cultured in Luria Bertani (LB) medium supplemented with 1.5% agar at 37°C overnight, unless otherwise stated. *Escherichia coli* S17-1 was used for conjugation of plasmids with PAO1. *P. aeruginosa* transformants were selected and maintained using tetracycline (75 μg $ml^{-1}$) or gentamicin (100 μg $ml^{-1}$) and *E. coli* transformants were selected and maintained using tetracycline (10 μg $ml^{-1}$) or gentamicin (10 μg $ml^{-1}$) unless mentioned otherwise.

### 3.2.2 Generation of *P. aeruginosa* deletion mutants and chromosomal fluorescent tag insertions

In-frame deletion mutants of *pilJ*, *pilI*, *pilA*, *cyaB* and fluorescent gene fusions of *pilI-cfp*, *pilJ-mCherry, $pilJ_{N398}$-mCherry, $pilJ_{N346}$-mCherry* and *$pilJ_{N306}$-mCherry* were generated by splicing by overlap extension (SOE) PCR using primers listed in Table 3.2 (30). For generating deletion mutants, approximately 1Kb sequences upstream and downstream of the gene were amplified, followed by an overlap PCR to obtain an allele with the gene deletion. For generating mCherry tagged constructs, approximately 1Kb sequences from upstream and downstream of the site of insertion were amplified. *mCherry* was amplified from pmCherry (Clonetech

**Table 3.1. Strains and plasmids used in this study.**

| Strains | Description | Reference or Source |
|---|---|---|
| *P. aeruginosa* | | |
| PAO1 | Wild type | C. Harwood |
| Δ*pilJ* | In-frame deletion of *pilJ* | This study |
| Δ*pilI* | In-frame deletion of *pilI* | This study |
| Δ*pilA* | In-frame deletion of *pilA* | This study |
| Δ*cyaB* | In-frame deletion of *cyaB* | This study |
| PilI-CFP | In-frame chromosomal fusion of *pilI-cfp.* | This study |
| Δ*pilJ*PilI-CFP | In-frame chromosomal fusion of *pilI-cfp* in Δ*pilJ* | This study |
| PilJ-mCherry | In-frame chromosomal fusion of *pilJ-mCherry* | This study |
| Δ*pilI*PilJ-mCherry | In-frame chromosomal fusion of *pilJ-mCherry* in Δ*pilI* | This study |
| $PilJ_{N398}$-mCherry | In-frame chromosomal fusion of 398 N-terminal amino acids from *pilJ* to *mCherry* | This study |
| $PilJ_{N346}$-mCherry | In-frame chromosomal fusion of 346 N-terminal amino acids from *pilJ* to *mCherry* | This study |
| $PilJ_{N306}$-mCherry | In-frame chromosomal fusion of 306 N-terminal amino acids from *pilJ* to *mCherry* | This study |
| *E. coli* | | |
| DH5α | *fhuA2*Δ*(argF-lacZ)U169 phoA glnV44* Φ*80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | New England Biolabs |
| S17-1 | *Thi pro hsdR recA RP4-2*(Tc::Mu) (KM::Tn7) | D. Saffarini |
| Plasmids | | |
| pEX18Tc | Suicide cloning vector; SacB, $Tet^R$ | (31) |
| pEX19Gm-CFP | Suicide cloning vector; SacB, $Gm^R$, *cfp* cloned into KpnI-XbaI sites | (20) |
| pEX18TcΔ*pilJ* | pEX18Tc-based plasmid for deletion of *pilJ* | This study |
| pEX18TcΔ*pilI* | pEX18Tc-based plasmid for deletion of *pilI* | This study |
| pEX18TcΔ*pilA* | pEX18Tc-based plasmid for deletion of *pilA* | This study |
| pEX18TcΔ*cyaB* | pEX18Tc-based plasmid for deletion of *cyaB* | This study |

| | | |
|---|---|---|
| pEX19Gm*pilI-cfp* | pEX19Gm-based plasmid for insertion of *pilI-cfp* | This study |
| pEX19Gm*pilI-cfp*Δ*pilJ* | pEX19Gm-based plasmid for insertion of *pilI-cfp* in Δ*pilJ* | This study |
| pEX18Tc*pilJ-mCherry* | pEX18Tc-based plasmid for insertion of *pilJ-mCherry* | This study |
| pEX18Tc$pilJ_{N398}$-*mCherry* | pEX18Tc-based plasmid for insertion of $pilJ_{N398}$-*mCherry* | This study |
| pEX18Tc$pilJ_{N346}$-*mCherry* | pEX18Tc-based plasmid for insertion of $pilJ_{N346}$-*mCherry* | This study |
| pEX18Tc$pilJ_{N306}$-*mCherry* | pEX18Tc-based plasmid for insertion of $pilJ_{N306}$-*mCherry* | This study |
| pJN105 | araC-$P_{BAD}$ cassette in $pBBR_1MCS$-5, $Gm^R$ | (32) |
| pJN105-*pilJ-his* | C-terminal 6X His-tagged *pilJ-his* gene cloned into pJN105 | (28) |
| pJN105- $pilJ_{\Delta 74-273}$-*his* | C-terminal 6X His-tagged $pilJ_{\Delta 74-273}$-*his* gene cloned into pJN105 | (28) |
| pJN105- *tsrJ-his* | C-terminal 6X His-tagged *tsrJ-his* gene cloned into pJN105 | This study |
| pJN105- $tsrJ_{\Delta 55-164}$-*his* | C-terminal 6X His-tagged $tsrJ_{\Delta 55-164}$-*his* gene cloned into pJN105 | This study |
| pJN105-*pilJ-mCherry* | C-terminal mCherry-tagged *pilJ-mCherry* gene cloned into pJN105 | This study |
| pJN105- $pilJ_{\Delta 74-273}$-*mCherry* | C-terminal mCherry-tagged $pilJ_{\Delta 74-273}$-*mCherry* gene cloned into pJN105 | This study |
| pJN105- *tsrJ-mCherry* | C-terminal mCherry-tagged *tsrJ-mCherry* gene cloned into pJN105 | This study |
| pJN105- $tsrJ_{\Delta 55-164}$-*mCherry* | C-terminal mCherry-tagged $tsrJ_{\Delta 55-164}$-*mCherry* gene cloned into pJN105 | This study |
| pJN105- *pilJ-Q-mCherry* | C-terminal mCherry-tagged *pilJ-Q-mCherry* gene cloned into pJN105 | This study |

**Table 3.2. Primers used in this study.**

| | Primer name | Oligonucleotide sequence (5'-3') |
|---|---|---|
| In-frame deletions | | |
| | *pilJ*_UPFor | CGAATTCCCGACCGAGATGTACAAGCTGACC |
| | *pilJ*_UPRev | CCGCCGCGCCTATGCATTTGGCCCCCGCCCGGACC |
| | *pilJ*_DNFor | GGGCGGGGGCCAAATGCATAGGCGCGGCGGCCG CC |
| | *pilJ*_DNRev | GGAATTCGGCTCGAGCTCCGGATGGCTC |
| | *pilI*_UPFor | CAGGAATTCGGCTCGCGATTGGGTCGC |
| | *pilI*_UPRev | CCCAACCGGTGAAGCTGTGCGGGGCCTGGTCTTT CC |
| | *pilI*_DNFor | GGAAAGACCAGGCCCCGCACAGCTTCACCGGTTG GG |
| | *pilI*_DNRev | CAGAAGCTTGATGTTGTTCGCCGCTTC |
| | *pilA*_UPFor | CGCGAATTCCGTCCTGCGGTTTGCG |
| | *pilA*_UPRev | CAAGCCACCTTCGATCACCGAATCTCTCCGTTGA TTATG |
| | *pilA*_DNFor | CATAATCAACGGAGAGATTCGGTGATCGAAGGTG GCTTG |
| | *pilA*_DNRev | GTCCTGCAGCGGCGGCGACCTTACC |
| | *cyaB*_UPFor | AGGTACCCTTCCGCGATGATCCGCTGG |
| | *cyaB*_UPRev | GCGCTGGAGAGGATCCCTG |
| | *cyaB*_DNFor | GCGACCTCTCCTAGGGACGTTCGTCGAACGCCGC CGGCA |
| | *cyaB*_DNRev | ACTGCAGGTCGTCACCAGCCTGCTGG |
| In-frame insertions | | |
| | *pilJmCh*_UPFor | AGGAATTCCGAGAAGAACGACCGCAACCAG |
| | *pilJFLmCh*_UPRev | TCCTCGCCCTTGCTCACCATGGCCTGCTCCACGCC CTCCG |
| | *pilJmCh*_DNFor | ATGGACGAGCTGTACAAGTAGGCATAGGCGCGG CGGCCGC |
| | *pilJmCh*_DNRev | AATCTAGAGGCTCGAGCTCCGGATGGCTC |
| | $pilJ_{N398}mCh$_UPRev | TCCTCGCCCTTGCTCACCATCTCCACCAGTTCGCG GAGCTG |
| | $pilJ_{N346}mCh$_UPRev | CTCGCCCTTGCTCACCATGTTCTTCTCGGCGGTCT C |
| | $pilJ_{N306}mCh$_UPRev | TCCTCGCCCTTGCTCACCATGCGCCCGCCGGCCA GGTTCT |
| | *mChFL*_For | GAGGGCGTGGAGCAGGCCATGGTGAGCAAGGGC GAGGA |
| | $mCh_{N398}$_For | CAGCTCCGCGAACTGGTGGAGATGGTGAGCAAG GGCGAGGA |
| | $mCh_{N346}$_For | GAGACCGCCGAGAAGAACATGGTGAGCAAGGGC GAG |

| | | |
|---|---|---|
| | *mCh*$_{N306}$_For | AGAACCTGGCCGGCGGGCGCATGGTGAGCAAGG GCGAGGA |
| | *mCh*_Rev | GCGGCCGCCGCGCCTATGCCTACTTGTACAGCTC GTCCAT |
| | *pilIcfp*_UPFor | CAGGAATTCGATGACTCTCCGACCGAG |
| | *pilIcfp*_UPRev | CAGGGTACCAACGGAGGCGGAGGCGGAGGTACG GCGACGTCGAGG |
| | *pilIcfp*_DNFor | CAGTCTAGAACAGCTTCACCGGTTGGG |
| | *pilIcfp*_DNRev | CAGAAGCTTGATGTTGTTCGCCGCTTC |
| | *pilIcfppilJ*_DNRev | CAGAAGCTTCTCGAGCTCCGGATGGCT |
| Expression | | |
| | *pilJ*_For | GGAATTCATGAAGAAAATCAACGCAGGCAAT |
| | *pilJexp*_Rev | TCCTCGCCCTTGCTCACCATGGCCTGCTCCACGCC CTCCG |
| | *mChFor_pilJ*Rev | GAGGGCGTGGAGCAGGCCATGGTGAGCAAGGGC GAGGA |
| | *mChexp*_Rev | CATACTAGTCTTGTACAGCTCGTCCATG |
| | *tsrJhis*_For | GCTCGAATTCATGTTAAAACGTATCAAAATTG |
| | *tsrJhis*_Rev | CACTAGTTCAATGGTGGTGATGGTGGTGGGCCTG CTCCACGCCCTCCGGC |
| | *tsrJ*$_{\Delta 55\text{-}164}$*-his*_UPRev | GTAAGCCACATACTGCTTATTCAGCGTGGATTGC TG |
| | *tsrJ*$_{\Delta 55\text{-}164}$*-his*_DNFor | CAGCAATCCACGCTGAATAAGCAGTATGTGGCTT AC |
| | mutA523S_*pilJ*For | CCAGACCAACATCCTC**AGC**CTGAACGCCGCGATC C |
| | mutA523S_*pilJ*Rev | GGATCGCGGCGTTCAG**GCT**GAGGATGTTGGTCTG G |
| | mutS531E_*pilJ*For | ACATCCTCAGCCTGAACGCCGCGATCCAGGCG**GA G**ATGGCCGG |
| | mutS531E_*pilJ*Rev | CCGGCCAT**CTC**CGCCTGGATCGCGGCGTTCAGGC TGAGGATGT |
| | mutD545T_*pilJ*For | TGGTAGCG**ACC**GAGGTACAGCGACTGGCGGAAC AGTCCTCGGC |
| | mutD545T_*pilJ*Rev | GCCGAGGACTGTTCCGCCAGTCGCTGTACCTC**GG TC**GCTACCA |
| | mutR553Q_*pilJ*For | ACAGCGACTGGCGGAA**CAG**TCCTCGGCGGCGAC CA |
| | mutR553Q_*pilJ*Rev | TGGTCGCCGCCGAGGA**CTG**TTCCGCCAGTCGCTG T |

Note: Underline indicates the restriction enzyme cleavage sites

**Bold** text indicates the codons mutated

Laboratories, Inc) and two consecutive overlap PCRs were carried out to generate an allele with the mCherry insertion. Each allele was cloned into pEX18Tc suicide vector. Similarly, to generate the *pilI-cfp* chromosomal construct, approximately 1Kb gene sequences upstream and downstream of the point of CFP insertion were amplified. These fragments were cloned into pEX19Gm-CFP suicide vector, which contains a copy of the *cfp* gene in its multiple cloning site. The 1Kb upstream and downstream fragments were cloned upstream and downstream of *cfp* in the suicide vector. All constructs were sequenced to ensure no unwanted mutations were introduced, transformed into *E. coli* S17-1 and introduced into *P. aeruginosa* by conjugation. Merodiploids were selected on LB containing tetracycline 75 μg $ml^{-1}$ or gentamicin 50 μg $ml^{-1}$ with chloramphenicol 5 μg $ml^{-1}$, then resolved by counter selection on 10% sucrose and confirmed by PCR.

### 3.2.3 Generation of His-tagged plasmid based expression constructs

Expression constructs *pilJ*-His and $pilJ_{\Delta 74\text{-}273}$-His were previously generated (28). The *tsrJ-his* was amplified from *tsrJ*-His chromosomal version using primers listed in Table 3.2. To generate *tsrJ*-His, the N-terminus (1-1287 bps) of *tsr* from *E. coli* RP437 and the C-terminus (997-2046 bps) of *pilJ* from *P. aeruginosa* PAO1 were amplified followed by an overlap PCR using the two fragments as template (Fig 3.6B). $tsrJ_{\Delta 55\text{-}164}$-His was generated using *tsrJ*-His as a template. Two fragments corresponding to 1-162 bps and 493-2337 bps of *tsrJ-his* were amplified by separate PCR, which was followed by an overlap PCR between the two to obtain an allele with the periplasmic deletion now $tsrJ_{\Delta 55\text{-}164}$-His. Thus the periplasmic fragment of 163-492 bps (corresponding to amino acids 55-164) was deleted by a SOE PCR. All alleles described above were cloned into pJN105 and sequenced to ensure the absence of any unwanted mutations.

### 3.2.4 Generation of mCherry-tagged plasmid based expression constructs

The *pilJ-mCherry* was amplified from the chromosomal PilJ-mCherry strain listed in

Table 3.1. $pilJ_{\Delta 74\text{-}273}$, *tsrJ* and $tsrJ_{\Delta 55\text{-}164}$ were amplified from $pilJ_{\Delta 74\text{-}273}$ (28), *tsrJ*-His (Table 3.1) and $tsrJ_{\Delta 55\text{-}164}$-His (Table 3.1) respectively and fused to *mCherry* amplified from pmCherry (Clonetech Laboratories, Inc) by SOE PCR. All mCherry tagged alleles were cloned into pJN105 and sequenced to ensure the absence of any unwanted mutations. All primers that were used for the generation of the above mentioned constructs are listed in Table 3.2.

### 3.2.5 Generation of mCherry- tagged PilJ quadruple point mutant plasmid based expression construct

Based on the QuikChange II site-directed mutagenesis protocol (Stratagene), four rounds of site-directed mutagenic PCR were used to generate the four point mutations (A523S, S531E, D545T and R553Q) in *pilJ-his*pJN105 (Table 3.1). Specific mutagenic primers listed in table 3.2 were designed with the mutations in their sequence. Following amplification, the PCR reactions were treated with DpnI for 4hrs at 37°C to destroy the methylated template DNA. The resulting amplicon was then transformed into DH5α and sequenced to confirm the presence of the point mutation. The newly mutated *pilJ-his* with the four point mutations was termed as *pilJ-Q-his*. *pilJ-Q* was amplified from *pilJ-Q-his* and fused to *mCherry* (Clonetech Laboratories, Inc) by SOE PCR to obtain *pilJ-Q-mCherry* which was cloned into pJN105 and sequenced to ensure the absence of any unwanted mutations (Table 3.1)

### 3.2.6 Twitching motility assay

To measure twitching motility phenotype, nine individual colonies of each strain were stab inoculated in to LB agar (1% agar) or LB with gentamicin (50 μg $ml^{-1}$) and incubated for 40hr at 37°C. At the end of incubation, the agar was removed and the diameter of the twitching zone was measured. The error bars represent standard error of the mean. Statistical analysis was done using ANOVA followed by TukeyHSD. Analysis was done using R (3.2.3).

### 3.2.7 β-galactosidase assay

To determine the intracellular levels of cAMP, strains containing the reporter *lac*P1-*lacZ* were used (7). Three individual colonies of each strain were each tested in triplicate. Surface grown cells were re-suspended in LB broth and β-galactosidase assays were performed as previously described (10). The error bars represent standard error of the mean. Statistical analysis was done using ANOVA followed by TukeyHSD. Analysis was done using R (3.2.3).

### 3.2.8 Immunoblotting

Whole cell lysates were prepared from surface grown cells and loading was normalized based on $OD_{600}$. Proteins were separated using 10% SDS-PAGE and transferred to PVDF membrane. Tagged proteins were detected using the primary antibodies which include rabbit anti-CFP (BioVision, Inc. 1:1000), rabbit anti-mCherry (BioVision, Inc. 1:1000) or mouse anti-His (Sigma Aldrich Co., 1:3000) followed by horseradish peroxidase conjugated goat anti-rabbit (1:10,000) or sheep anti-mouse (1:10,000) secondary antibodies as appropriate. Immunoblots were developed using Pierce SuperSignal West Femto Maximum Sensitivity Chemiluminescent substrate kit (Thermo Fisher Scientific) and a Fotodyne Luminary system.

### 3.2.9 Intracellular localization by fluorescence microscopy

To detect intracellular localization of tagged proteins, a colony of surface grown cells was re-suspended in 100 ul PBS. Cells were allowed to incubate on ice for 15 mins before imaging. For imaging, poly-lysine pre-coated coverslips were used to promote adherence and cells were imaged using a Nikon Eclipse 90i microscope with a Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera. 200 cells were counted for each strain and observed for the presence of fluorescent foci and the localization pattern.

## 3.3 Results and Discussion

### 3.3.1 PilJ is required for PilI-CFP foci formation

Because PilJ is the only known MCP of the Chp system, we hypothesized that it is important for the assembly of the Chp system and dictates its polar localization. The polar localization of PilJ had previously been determined using fluorescent microscopy (14). To determine the role of PilJ in the localization of the Chp system, we generated a *pilI-cfp* fusion in its native chromosomal site that functioned as a marker for Chp system localization. PilI is, based on its analogy with the *E. coli* chemotaxis system, a coupling protein of the Chp system that is proposed to mediate the interaction between PilJ and ChpA (the histidine kinase) thus allowing for signal transduction. It is also essential for twitching motility as the Δ*pilI* strain is null for twitching (4, 6, 33). The localization of PilI-CFP was examined in both WT and Δ*pilJ* cells for fluorescent foci formation and localization patterns (Fig. 3.1A). Bright spots that are contained within the cell (not wider than the cell width) were considered as foci. Foci present at the ends of the rod-shaped cell located within the cell curvature are considered polar. The foci that localize within the cell to an area that lacks the membrane curvature are classified as non-polar. Deletion of *pilJ* results in a drastic reduction in PilI-CFP foci formation (3% cells with foci compared to 92% wild type cells expressing PilI-CFP) (Fig. 3.1C). Western blotting revealed equivalent levels of PilI-CFP expression in both wild type and Δ*pilJ*, confirming that the reduction in number of foci in the Δ*pilJ*PilI-CFP strain was not due to a lack of expression or protein instability (Fig. 3.1B).

To determine if PilJ and PilI were interdependent in their localization, a *pilJ-mCherry* chromosomal fusion was similarly generated in the native chromosomal site. This allowed us to study the effect of Δ*pilI* deletion on PilJ localization. There was a slight decrease in PilJ-mCherry foci formation upon deletion of *pilI* (69% cells with foci compared to 96% in wild type

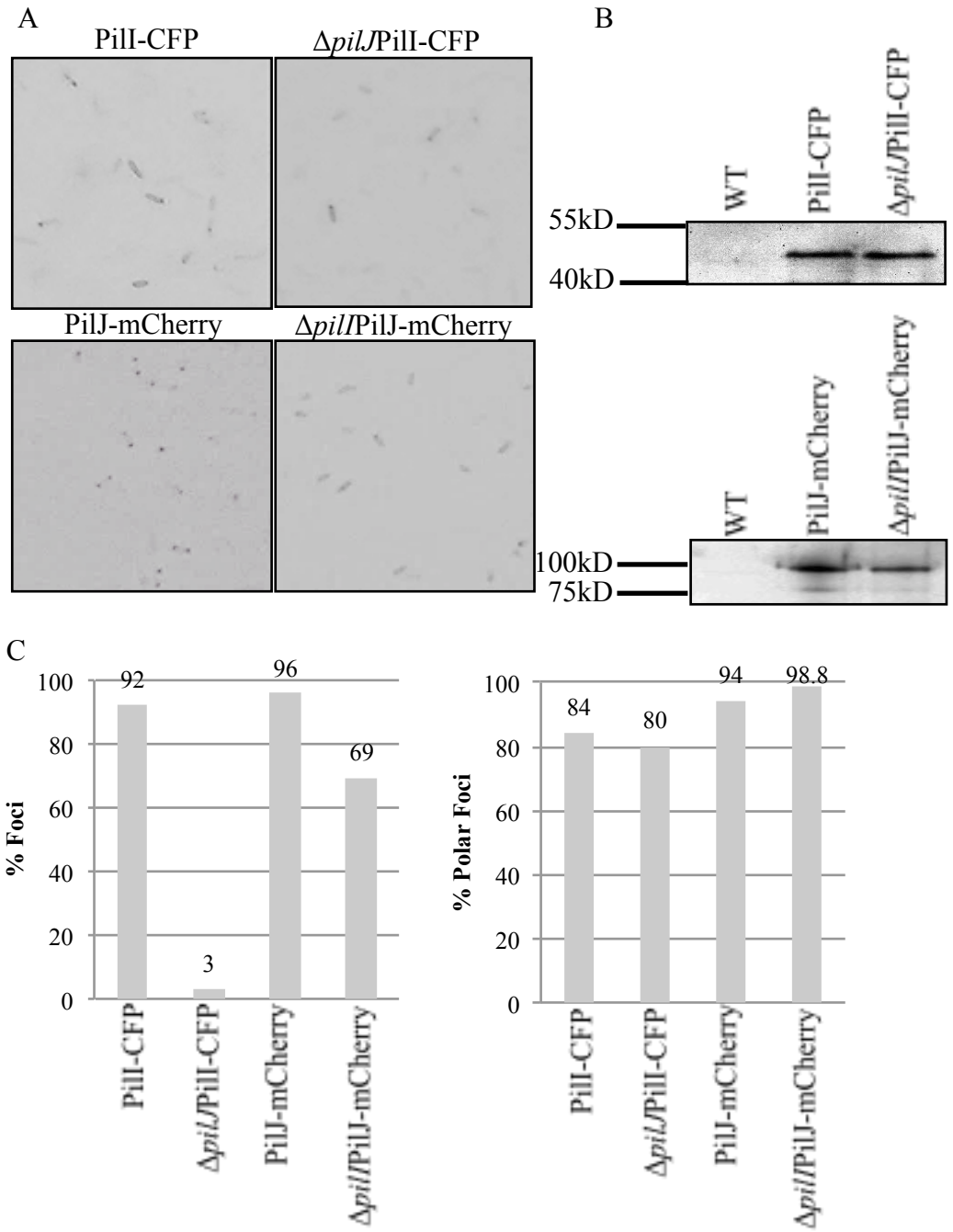



Fig. 3.1. PilJ is required for PilI-CFP foci formation. A) Representative inverted fluorescence microscopy images of PilI-CFP and PilJ-mCherry in the indicated strains. B) Levels of PilI-CFP and PilJ-mCherry as determined by western blotting in the indicated strains. C) Percentage of cells with foci and the percentage of foci that were polarly localized as determined using fluorescence microscopic images of 200 cells for each indicated strain.

cells expressing PilJ-mCherry) (Fig. 3.1C), suggesting the coupling protein may aid in PilJ foci formation. However, PilJ-mCherry was expressed at a slightly lower level in the absence of *pilI*. This could be due to possible polar effects of *pilI* deletion on PilJ as *pilJ* is encoded immediately downstream of *pilI* although they are not predicted to be part of the same operon. Hence in future, it is important to evaluate PilJ-mCherry foci formation under the conditions of equivalent protein levels. To counter the potential polar effects, a partial *pilI* deletion (which results in the synthesis of a non-functional PilI) expressing PilJ-mCherry may be generated. In all strains examined, PilJ and PilI formed polar foci regardless of the presence or absence of interacting partners. These data clearly emphasize the importance of PilJ in the formation of PilI foci.

It was shown previously in *P. aeruginosa* that intracellular localization of a MCP is important for responding to an external signal and transmitting it to the response regulator (20). Hence it is important to test the phenotype of the strains used for PilJ and PilI localization. Δ*pilA and* Δ*cyaB* are the negative controls for twitching motility and intracellular cAMP assay respectively. A twitching motility assay demonstrated that as expected, Δ*pilA* is null for twitching motility because it lacks the pilin monomer (Fig. 3.2A). Δ*pilJ* and Δ*pilI* also show no twitching, which has previously been reported with these deletions (Fig. 3.2A)(6). PilI-CFP and PilJ-mCherry are partially functional, exhibiting approximately 40% and 60% of WT twitching motility, respectively (Fig. 3.2A). For both fusions, partial function is an indication that their intracellular localization is likely physiologically relevant. The reduced twitching motility is likely due to the fusion of the fluorescent protein impacting signal transduction. We cannot say however whether the decrease is due to a direct effect on twitching motility, or because of the altered levels of cAMP (Fig. 3.2B). High levels of cAMP are responsible for upregulation of genes for T4P biogenesis (4, 10). Hence, altering the levels of cAMP can alter gene expression, affecting surface piliation and possibly twitching motility. Interestingly, comparison of

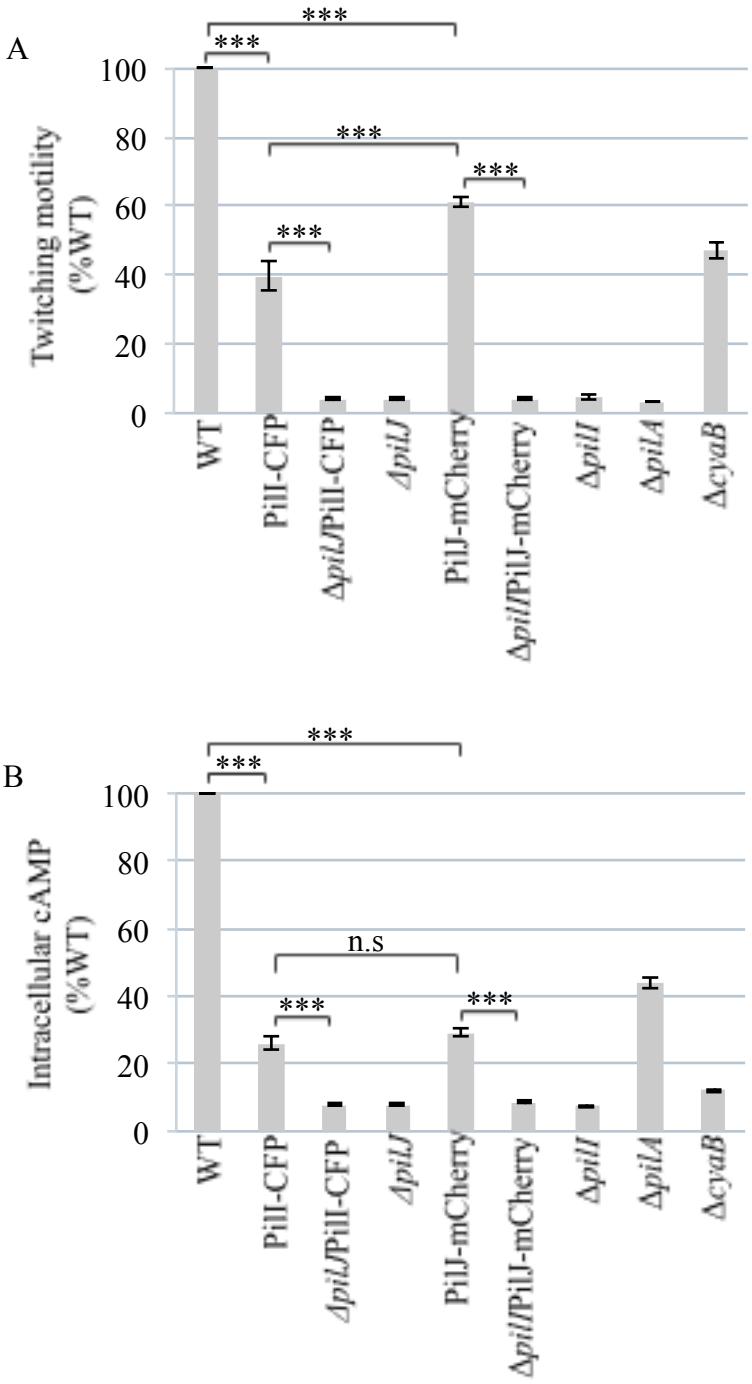



Fig. 3.2. Cells expressing PilI-CFP and PilJ-mCherry exhibit partial function as compared to WT. A) Diameter of the zones of twitching motility for the indicated strains relative to WT. Nine colonies were assayed to determine the average and standard error of the mean. B) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented relative to WT. Three colonies were assayed in triplicate to determine the average and standard error of the mean. Asterisks (***, $p < 0.001$) indicate values significantly different from their isogenic controls as determined by ANOVA followed by the post hoc test TukeyHSD.

intracellular PilI-CFP and PilJ-mCherry reveals similar intracellular cAMP levels, but these strains are significantly different from each other in terms of twitching motility ($p<0.001$) (Fig. 3.2). This difference in twitching motility is therefore likely due to the differential effect of the tags on the function of PilJ and/or PilI or their interaction with partner proteins thus directly affecting regulation of twitching motility.

Previous fluorescent microscopy studies have shown that MCPs are important for the foci formation of the response regulator (RR) of the system. In *E. coli,* deletion of one of the four MCPs, Tsr, reduces CheY-YFP (RR) foci formation (21). It has also been observed with the Wsp system in *P. aeruginosa*, deletion of the sole MCP WspA results in a loss of WspR-YFP (RR) foci formation (20). In contrast, even in the absence of PilJ and ChpA (the HK of Chp system), PilG (one of the two response regulators) maintains its bipolar localization likely through interaction with FimL (27, 34). Hence it appears that PilJ is important for the polar localization of some but not all proteins of the Chp system. Our data indicate that PilJ is required for foci formation of PilI-CFP and by extension we predict that PilJ is involved in the nucleation of the core-signaling complex (PilJ-PilI-ChpA). This is supported by the null phenotypes in both twitching motility and cAMP levels in the Δ*pilJ* strain (Fig. 3.2). Additionally, PilI is important for achieving WT levels of PilJ-mCherry foci formation. This has not been reported previously for the Chp system but it is not entirely surprising for chemoreceptor localization in general. In *R. sphaeroides*, the clustering of membrane spanning chemoreceptor McpG is dependent on chemoreceptor associated cytoplasmic proteins like the coupling proteins $CheW_2$ and $CheW_3$ (35). While the Chp gene cluster also encodes two homologous coupling proteins (PilI and ChpC) it is therefore possible that both independently contribute to PilJ foci formation. It is unlikely however that the other coupling protein (ChpC) would have a large impact on PilJ foci

formation, as this protein appears to be less important than PilI in signal transduction (7, Sharma and Bardy, unpublished). Over all, our data indicate that PilJ is important for polarity of PilI-CFP which is in agreement with previous reports that MCPs generally localize to the cell poles and tether other chemosensory proteins with them (21). Because of this, it is surprising to us that 3% of the PilI-CFP cells formed predominantly polar foci (80%) in the absence of *pilJ* (Fig. 3.1C). More cells must be studied to precisely comment about the polarity of PilI-CFP foci in Δ*pilJ*.

### 3.3.2 The second transmembrane domain is involved in polar localization of PilJ

Having established that PilJ is important for the assembly of PilI foci, we investigated the internal polar localization cue of PilJ. Based on previous studies of WspA, the only known non-polar MCP of *P. aeruginosa*, the C-terminus of the MCP is important for its localization and function (26). Therefore to determine the position of the polar localization cue in PilJ, we generated three C-terminal truncations. These truncations deleted the majority of the cytoplasmic domain (designated as N398), the cytoplasmic domain and the HAMP domain (N346) and the 2$^{nd}$ transmembrane, HAMP domain and the cytoplasmic domain (N306), wherein the numbers refer to the N-terminal amino acids that remain (Fig. 3.3). In order to localize these constructs, the truncations were fused with a C-terminal mCherry fluorescent protein tag.

Localization studies revealed a bi-polar localization pattern for full length PilJ-mCherry, which is in agreement with previous studies (Fig. 3.4A)(14). The C-terminal truncations showed a moderate to extreme reduction in the number of foci formed as compared to full-length PilJ-mCherry (Fig. 3.4B). This reduction in foci formation was likely caused by the absence of the trimer interaction domain (TID). This is part of the signaling domain in the cytoplasmic region of a MCP and is important for formation of trimers-of-dimers as shown in *P. aeruginosa* (MCP WspA) and *E. coli* (MCP Tsr) and for interaction with other chemosensory proteins (26, 36-38).

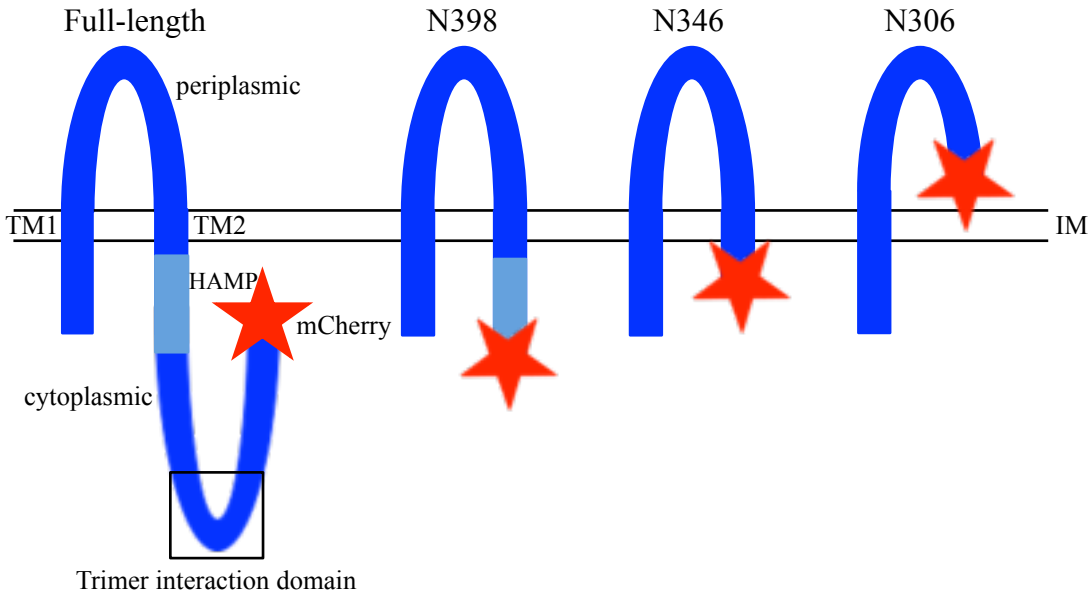



Fig. 3.3. Membrane topology of PilJ-mCherry (full-length) and its truncated derivatives $PilJ_{N398}$-mCherry (N398), $PilJ_{N346}$-mCherry (N346) and $PilJ_{N306}$-mCherry (N306) as predicted by Uniprot.

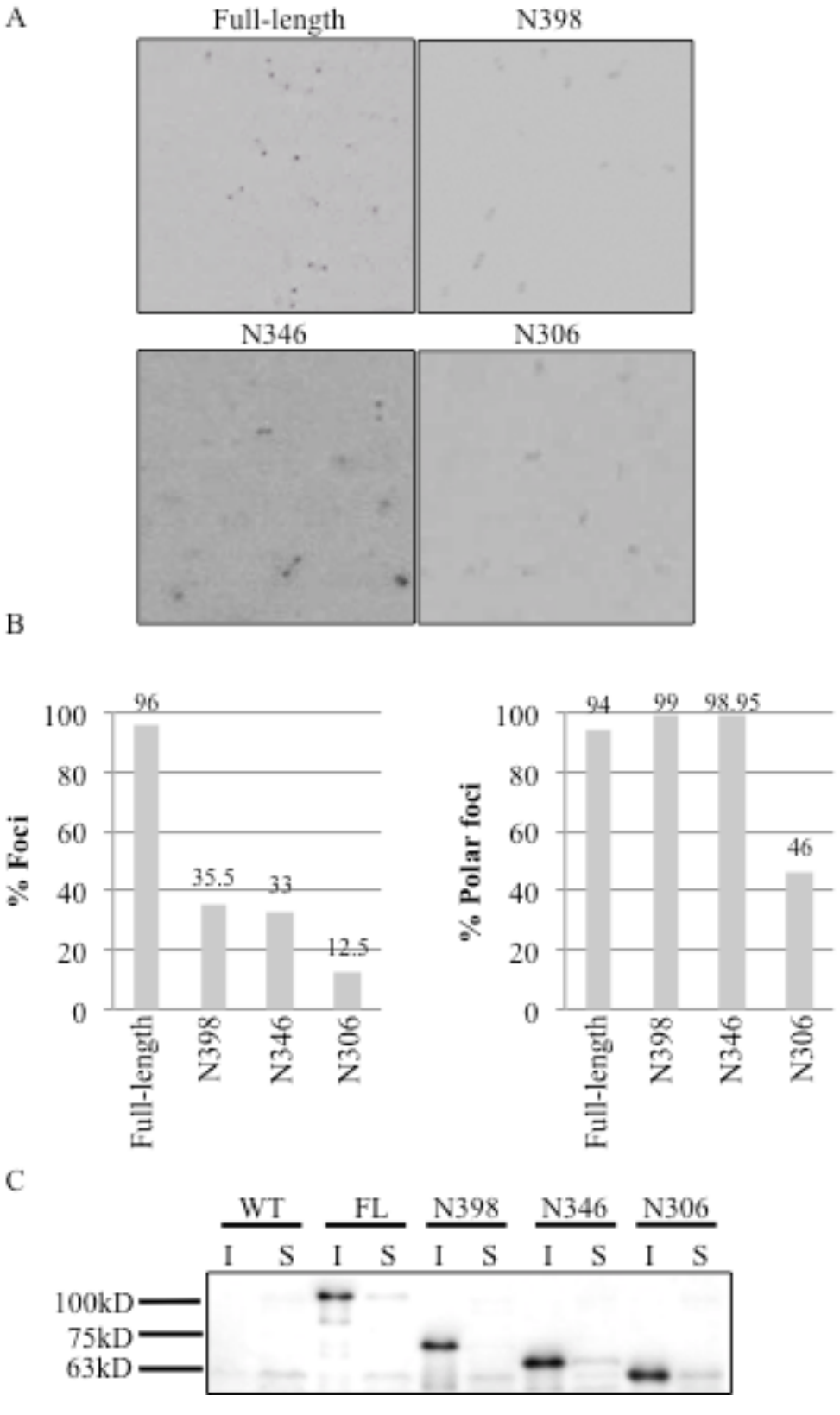

A
Full-length
N398
N346
N306
B
% Foci
100
80
60
40
20
0
96
35.5
33
12.5
Full-length
N398
N346
N306
% Polar foci
94
99
98.95
46
C
WT
FL
N398
N346
N306
I S I S I S I S I S
100kD
75kD
63kD

Fig. 3.4. Identification of PilJ domains involved in foci formation and localization. The trimer interaction domain and the second transmembrane domain are likely involved in WT levels of foci formation and polar localization respectively. A) Inverted fluorescence microscopy images of PilJ-mCherry (full-length) and its truncated derivatives $PilJ_{N398}$-mCherry (N398), $PilJ_{N346}$-mCherry (N346) and $PilJ_{N306}$-mCherry (N306). B) Quantification of foci formation and localization by PilJ truncations. Percentage of cells with foci and percentage of foci that are polar as determined for 200 cells using fluorescence microscopic images of the indicated strains. C) Levels of PilJ-mCherry and its truncated derivatives in the insoluble (I) and soluble (S) cell fractions as determined by western blotting.

Although there was a reduction in foci formation with deletion of the cytoplasmic (N398) and the HAMP domain (N346), the foci that were formed retained polar localization at levels similar to WT (Fig. 3.4B). However, on deletion of the second transmembrane domain (N306), there was a 46% reduction in polarity compared to WT (94%) (Fig. 3.4B). These results suggest that the second transmembrane domain is involved in the polar localization of PilJ. To our knowledge, this is the first report of an MCP wherein a domain other than the cytoplasmic region influences localization. Previously, MCPs in *C. crescentus* and *P. aeruginosa* have been shown to have their localization cues in the cytoplasmic region (26, 39). All truncations were found in the insoluble fraction of cell lysates, which indicates that the truncated PilJ is membrane bound, similar to the full-length protein (Fig. 3.4C).

We also examined the mCherry fusion proteins for function. The PilJ-mCherry (full-length) exhibited partial function as compared to WT, whereas the C-terminus PilJ truncations showed a loss of twitching motility and intracellular cAMP (Fig. 3.5). This is expected since the cytoplasmic region of an MCP also carries the signaling domain, which is the site of interaction with CheA and CheW without which signal transduction is impaired (40, 41). This also corresponds with the earlier results for the *ΔpilI* strain, where deletion of this coupling protein resulted in a loss of twitching motility and significant reduction in intracellular cAMP (Fig. 3.2).

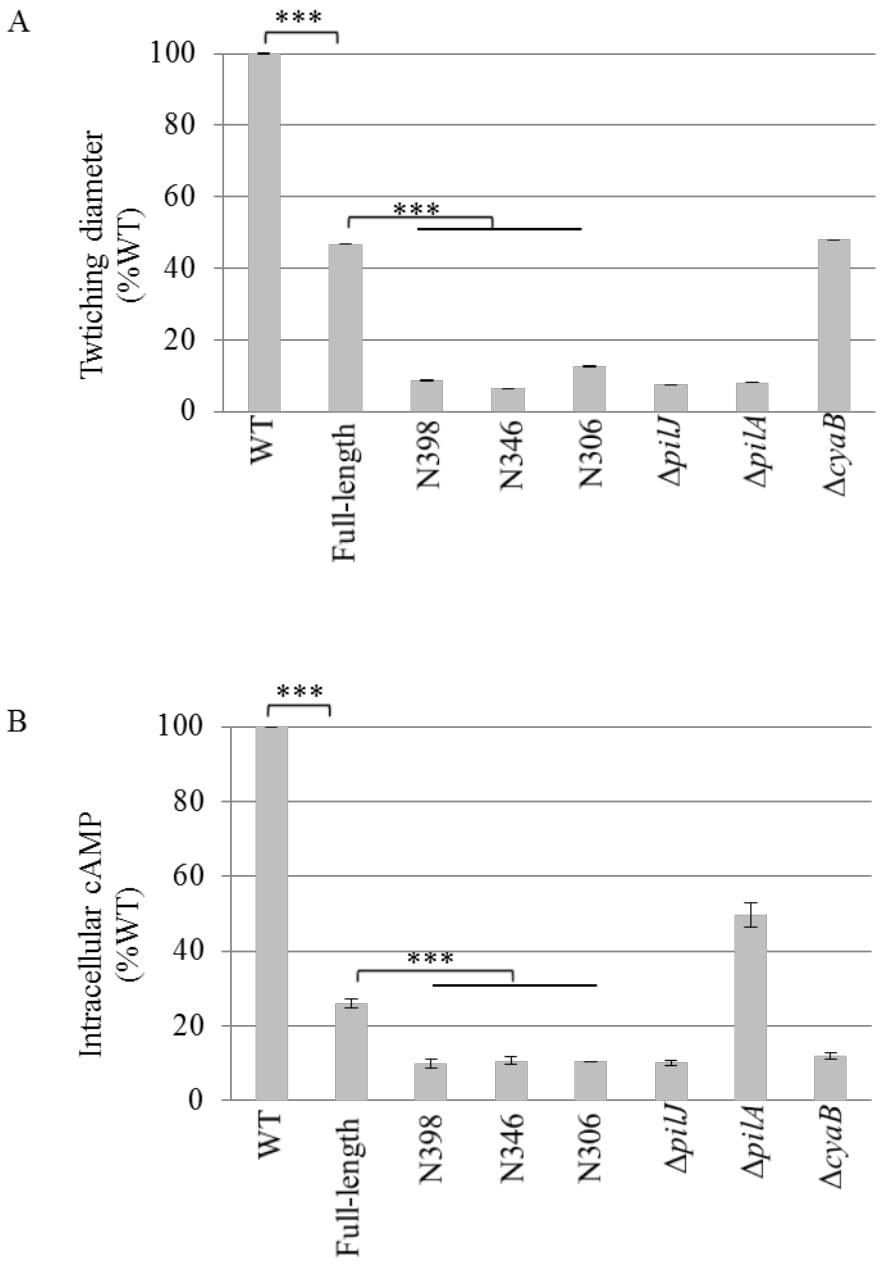



Fig. 3.5. Deletion of the C-terminus of PilJ prevents signal transduction controlling intracellular cAMP levels and twitching motility. The PilJ truncated derivatives $PilJ_{N398}$-mCherry (N398), $PilJ_{N346}$-mCherry (N346) and $PilJ_{N306}$-mCherry (N306) show complete loss of function. A) Diameter of the zones of twitching motility for the indicated strains relative to WT. Nine colonies were assayed to determine the average and standard error of the mean. B) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented relative to WT. Three colonies were assayed in triplicate to determine the average and standard error of the mean. Asterisks (***, $p< 0.001$) indicate values significantly difference from their isogenic controls as determined by ANOVA followed by the post hoc test TukeyHSD.

### 3.3.3 Native amino acid sequence of the periplasmic and transmembrane domains of PilJ is required for signal transduction and foci formation

The mechanosensor model for surface sensing by T4P suggested that PilJ interacts with its potential signal PilA through its periplasmic domain (17). We have previously shown that the periplasmic domain plays an important role in regulation of cAMP levels while regulation of twitching motility is independent of this domain (Fig. 2.1 and 2.3). If the proposed mechanosensor model was correct in assuming that signal transduction resulted from PilA interaction with the periplasmic domain of PilJ, then we expect the levels of cAMP in $pilJ_{\Delta 74\text{-}273}$ and Δ*pilA* to be similar, but they are not (Fig. 2.1D). The increased levels of cAMP in $pilJ_{\Delta 74\text{-}273}$ as compared to Δ*pilJ* and Δ*pilA* suggest that the transmembrane and/or the HAMP domains may be involved in signal transduction. Such non-traditional signal transduction has been previously observed in Tsr in *E. coli* where the transmembrane and the HAMP domains mediate phenol sensing (29). To investigate such possibilities in PilJ, two chimeric proteins were generated by fusion of Tsr and PilJ (Fig. 3.6). TsrJ is a fusion between the transmembrane and periplasmic domains of Tsr with the HAMP and cytoplasmic domains of PilJ. $TsrJ_{\Delta 55\text{-}164}$ lacks the periplasmic domain of TsrJ (Fig 3.6).

His-tagged constructs of all the PilJ mutants and chimeras were generated initially for phenotypic assays. mCherry-tagged constructs were later generated for intracellular localization studies by fluorescence microscopy. All constructs were expressed in a Δ*pilJ* background from an expression vector with appropriate arabinose induction to ensure equivalent protein levels (Fig. 3.7C and 3.8C). All the His-tagged constructs were detected in the insoluble (membrane) fractions by western blot analysis (Fig. 3.7C). Full length PilJ complements the twitching motility phenotype in Δ*pilJ* regardless of the C-terminal tag (Fig. 3.7A and 3.8A). However, over-complementation is observed for cAMP levels with the same constructs (Fig. 3.7B and

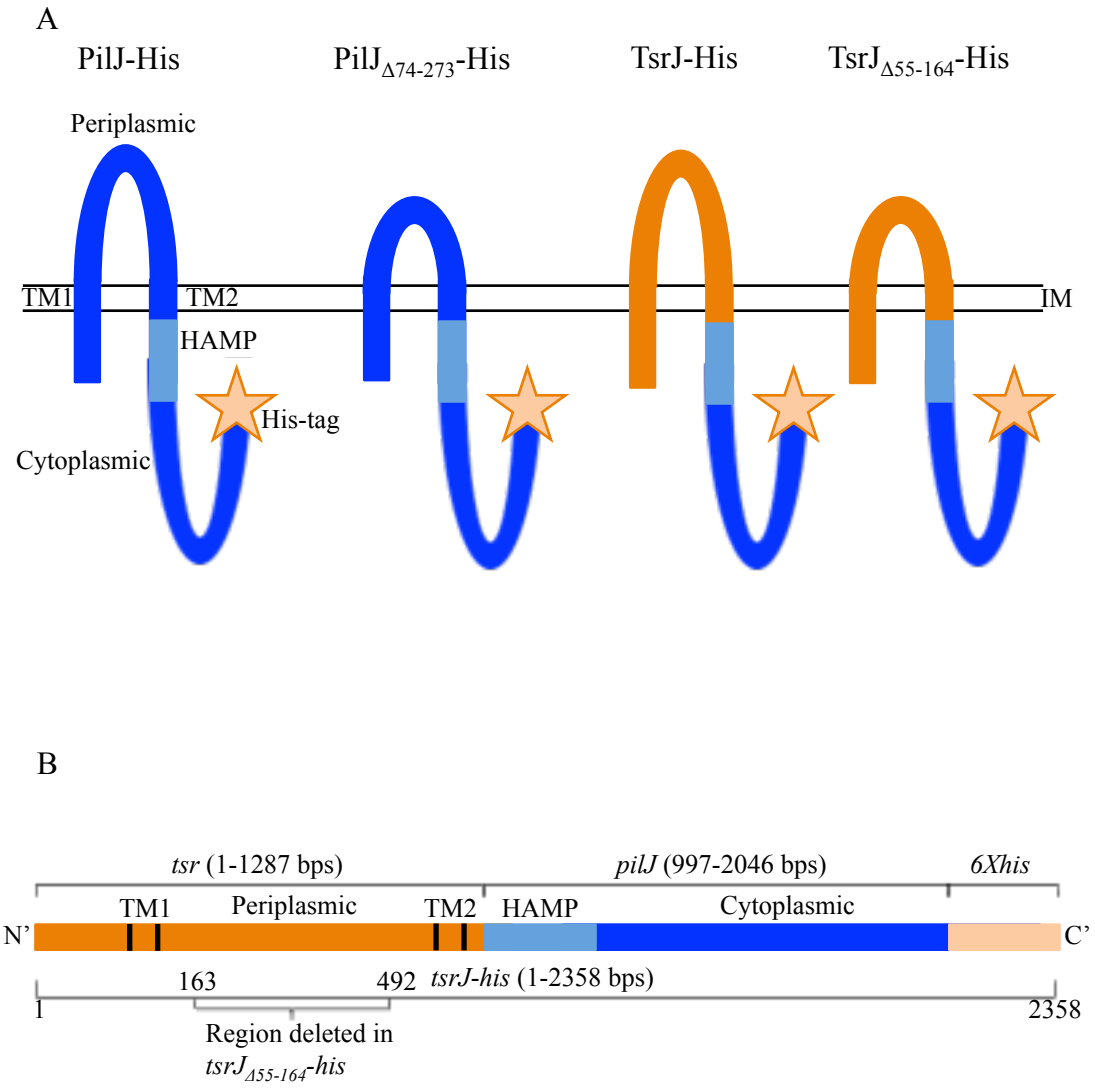
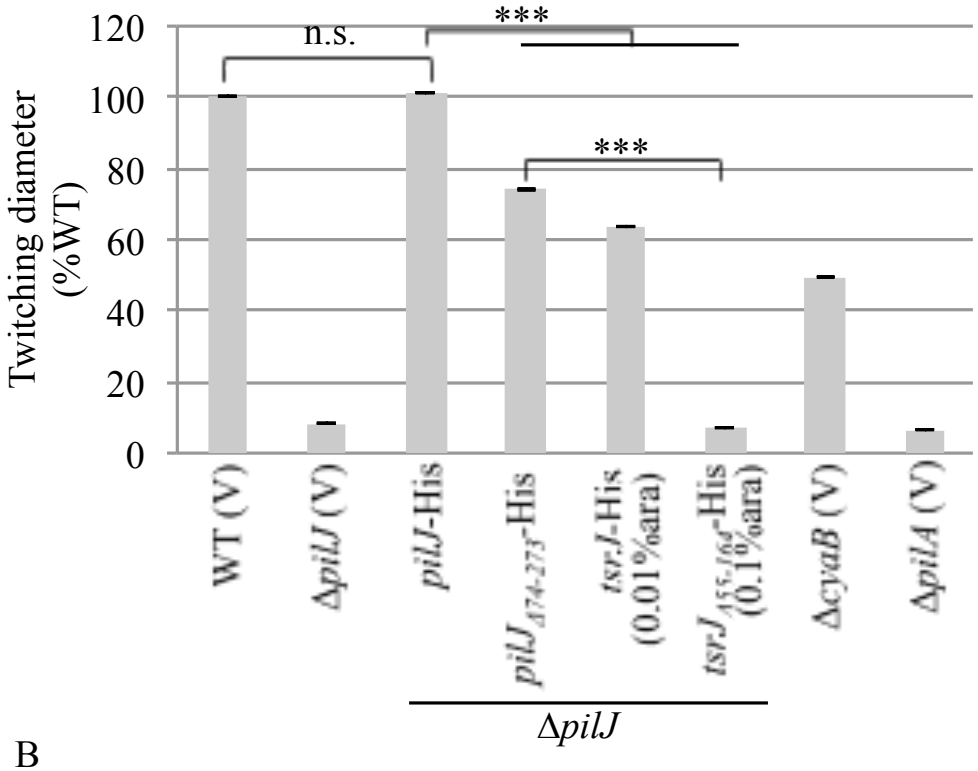
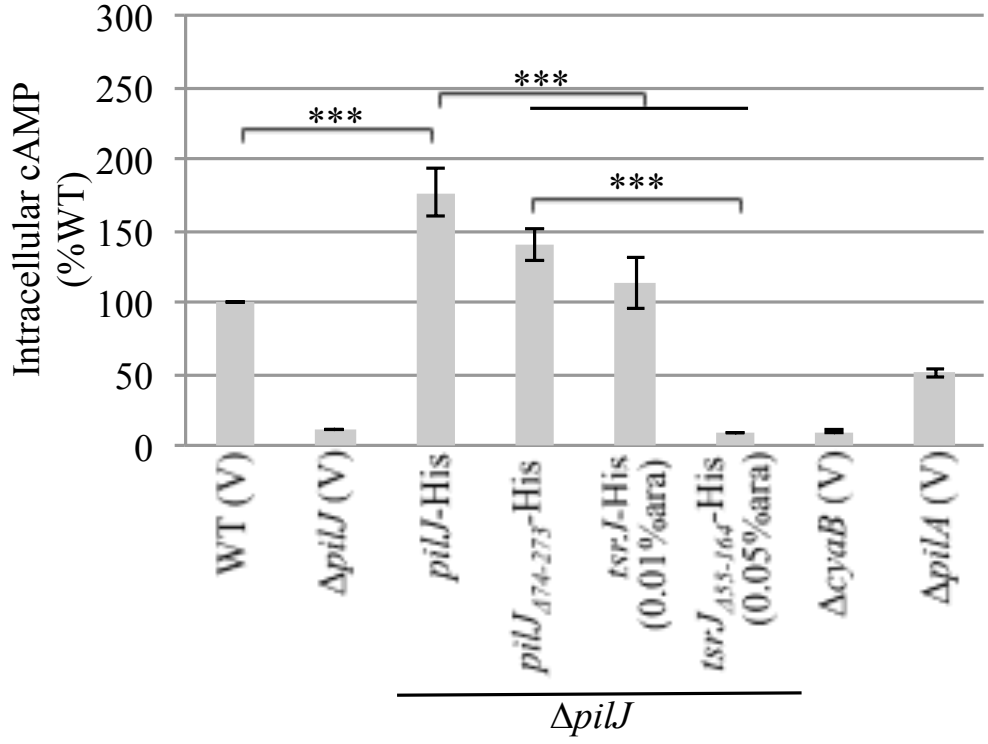



Fig. 3.6. A) Membrane topology of PilJ-His, PilJ$_{\Delta 74-273}$-His, TsrJ-His and TsrJ$_{\Delta 55-164}$-His as predicted by Uniprot. PilJ and Tsr domains are highlighted in blue and orange respectively. B) A schematic of the chimeric construct *tsrJ*-His. A fragment of 163-492 bps was deleted to obtain *tsrJ$_{\Delta 55-164}$*-His. The gene sequences of different domains are colored coded similar to panel A. Image is not drawn to scale.

A


Twitching diameter (%WT)
120
100
80
60
40
20
0
n.s.
***
***
WT (V)
ΔpilJ (V)
pilJ-His
pilJΔ74-273-His
tsrJ (0.01%ara)
tsrJΔ55-164-His (0.1%ara)
ΔcyaB (V)
ΔpilA (V)
ΔpilJ

B


Intracellular cAMP (%WT)
300
250
200
150
100
50
0
***
***
***
WT (V)
ΔpilJ (V)
pilJ-His
pilJΔ74-273-His
tsrJ (0.01%ara)
tsrJΔ55-164-His (0.05%ara)
ΔcyaB (V)
ΔpilA (V)
ΔpilJ

C

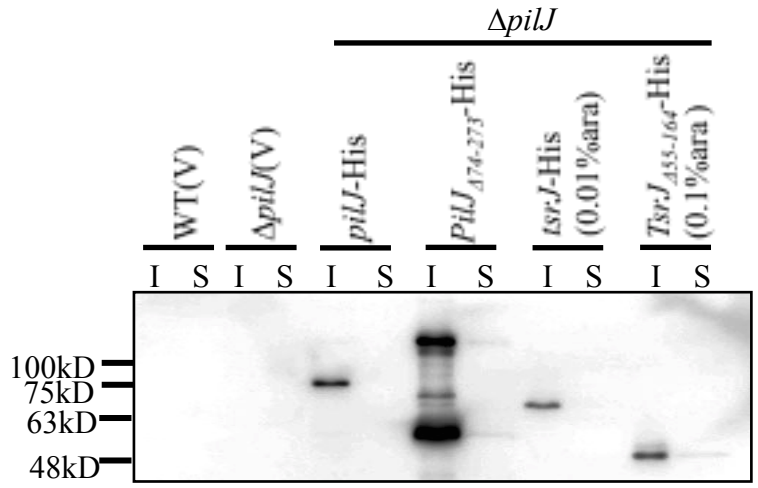
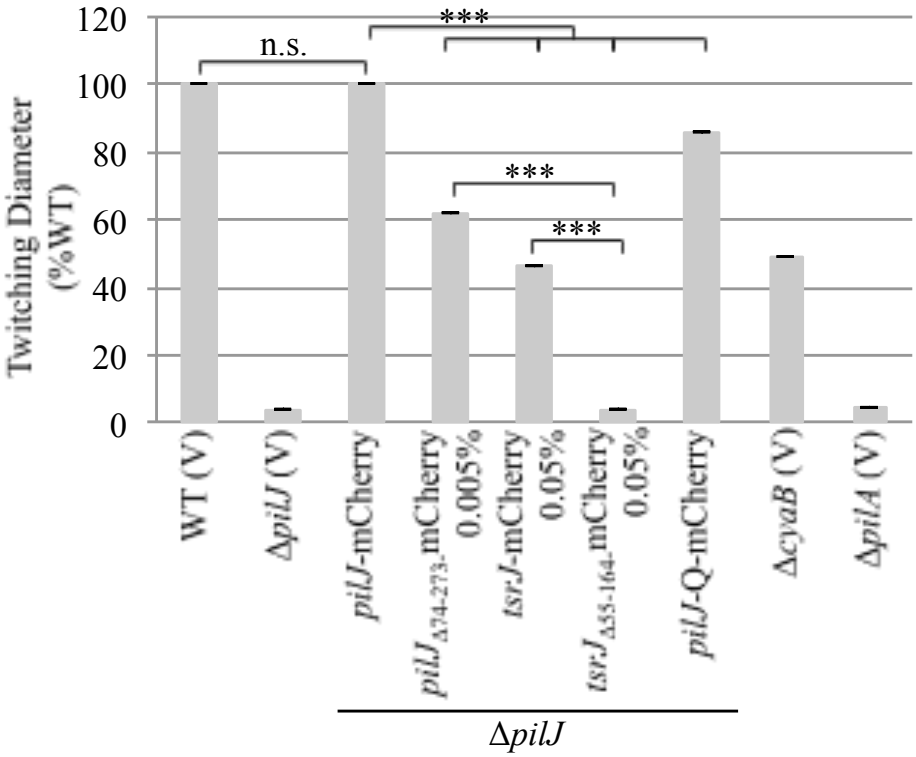
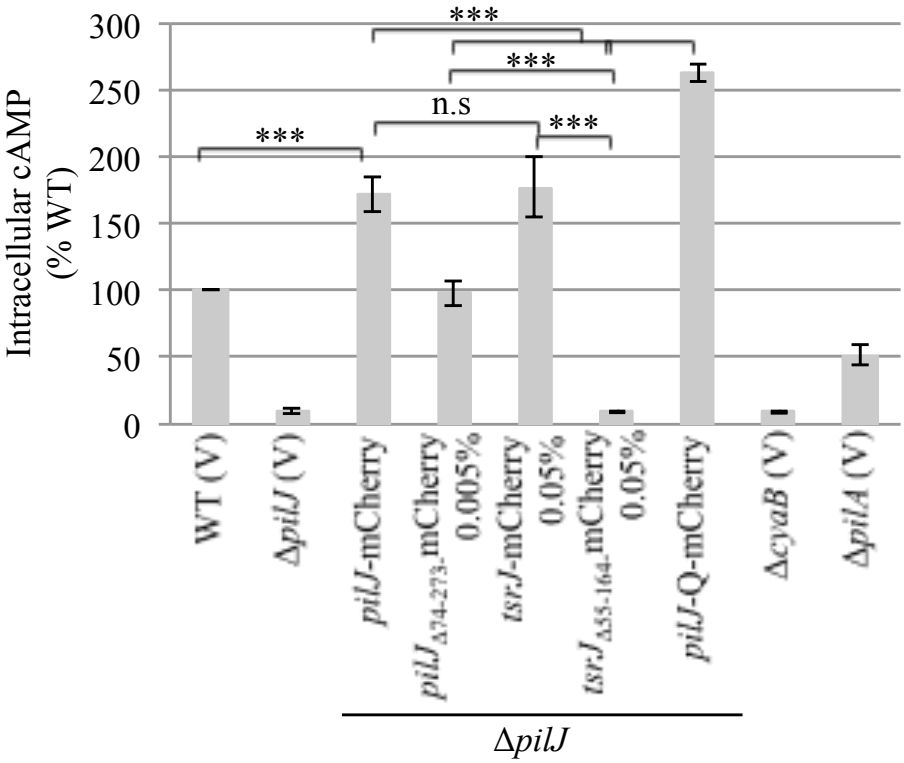



Fig. 3.7. The native amino acid sequence of the N-terminal domains of PilJ is required for WT levels of signal transduction. A) Diameter of the zones of twitching motility for the indicated strains relative to WT. Nine colonies were assayed to determine the average and standard error of the mean. B) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented relative to WT. Three colonies were assayed in triplicate to determine the average and standard error of the mean. Asterisks (***, $p < 0.001$) indicate values significantly difference from their isogenic controls as determined by ANOVA and the post-hoc test TukeyHSD. Non-significant difference is indicated as n.s. C) Levels of PilJ-His and its derivatives in the insoluble (I) and soluble (S) cell fractions as determined by western blotting using the non-reporter strains that were used for twitching motility assays.

A


Twitching Diameter (%WT)
120
100
80
60
40
20
0
n.s.
***
***
***
***
WT (V)
ΔpilJ (V)
pilJ-mCherry
pilJΔ374-273-mCherry 0.005%
tsrJ-mCherry 0.05%
tsrJΔ55-164-mCherry 0.05%
pilJ-Q-mCherry
ΔcyaB (V)
ΔpilA (V)
ΔpilJ

B


Intracellular cAMP (% WT)
300
250
200
150
100
50
0
***
***
n.s
***
***
WT (V)
ΔpilJ (V)
pilJ-mCherry
pilJΔ374-273-mCherry 0.005%
tsrJ-mCherry 0.05%
tsrJΔ55-164-mCherry 0.05%
pilJ-Q-mCherry
ΔcyaB (V)
ΔpilA (V)
ΔpilJ

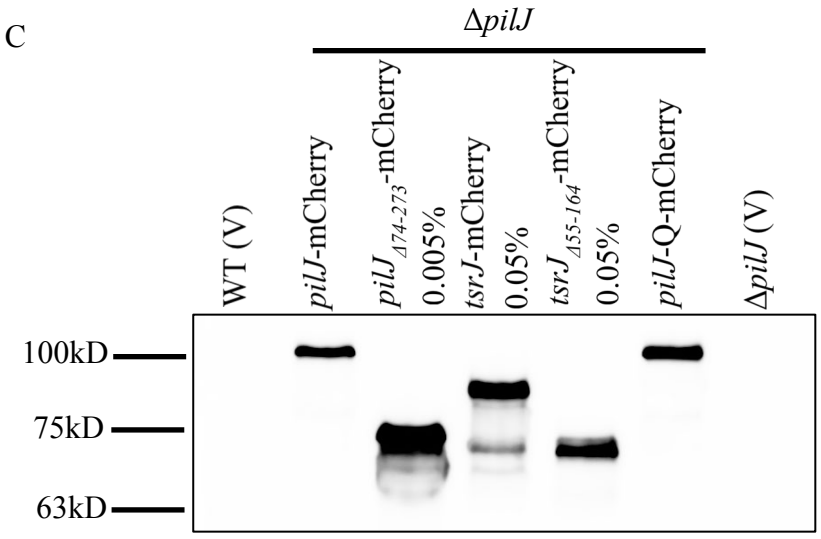



Fig. 3.8. The native amino acid sequence of the N-terminus domains of PilJ is required for WT levels of signal transduction. A) Diameter of the zones of twitching motility for the indicated strains relative to WT. Nine colonies were assayed to determine the average and standard error of the mean. B) Indicated strains were tested for β-Galactosidase activity which is indicative of intracellular levels of cAMP bound to Vfr represented relative to WT. Three colonies were assayed in triplicate to determine the average and standard error of the mean. Asterisks (***, $p < 0.001$) indicate values significantly difference from their isogenic controls as determined by ANOVA followed by the post hoc test TukeyHSD. C) Levels of PilJ-mCherry and its truncated and chimeric derivatives in whole cell lysates as determined by western blotting using the mini-CTX-*lacp1-lacZ* reporter strains that were used for cAMP assays.

3.8B). Plasmid-based expression could potentially result in higher than native protein levels, resulting in an increase in cAMP levels but not twitching motility. The intermediate levels of cAMP in Δ*pilA* have been observed before (Fig. 3.7B and Fig. 2.1D). A null phenotype for twitching motility and cAMP levels observed in Δ*pilJ* has also been previously reported (Fig. 3.7)(4, 6, 7, 14, 42).

#### 3.3.3.1 PilJ vs PilJ$_{\Delta 74\text{-}273}$: The role of the periplasmic domain

Expression of PilJ$_{\Delta 74\text{-}273}$ shows a partial but significant decrease in both the phenotypes tested compared to PilJ full-length (Fig. 3.7 and 3.8). This has been observed before with chromosomal as well as plasmid-based expression of PilJ$_{\Delta 74\text{-}273}$ (Fig 2.1 and 2.3). PilJ$_{\Delta 74\text{-}273}$ has a higher number of total foci as compared to PilJ full-length (Table 3.3). However, the total number of polar foci is the same. This suggests that the decrease in percent polar foci in PilJ$_{\Delta 74\text{-}273}$ is due to the increase in non-polar foci, which corresponds to higher levels of total protein levels as determined by a western blot (Fig. 3.8C, Fig. 3.9B, Table 3.3). It is possible that the superfluous level of protein results in the formation of non-polar foci. We propose that PilJ$_{\Delta 74\text{-}273}$ is localized at the cell poles potentially due to the interactions of the transmembrane domains with the inner membrane pools of PilA or other polar proteins associated with the Chp system such as CyaB (Fig. 3.11). The excess protein that is not accommodated at the poles potentially gets displaced and results in the formation of non-polar foci. This is supported by our previous data, wherein the second transmembrane domain is involved in the polar localization of PilJ (Fig 3.4B). Additionally, the PilJ transmembrane domains have been shown to be required for interaction with PilA (17).

Mislocalization of a MCP has been previously shown to affect their cellular functions (26). While PilJ$_{\Delta 74\text{-}273}$-His and PilJ$_{\Delta 74\text{-}273}$-mCherry are expressed at increased levels compared to PilJ full-length (Fig. 3.7C and 3.8C), the phenotypic consequences are similar to those seen with

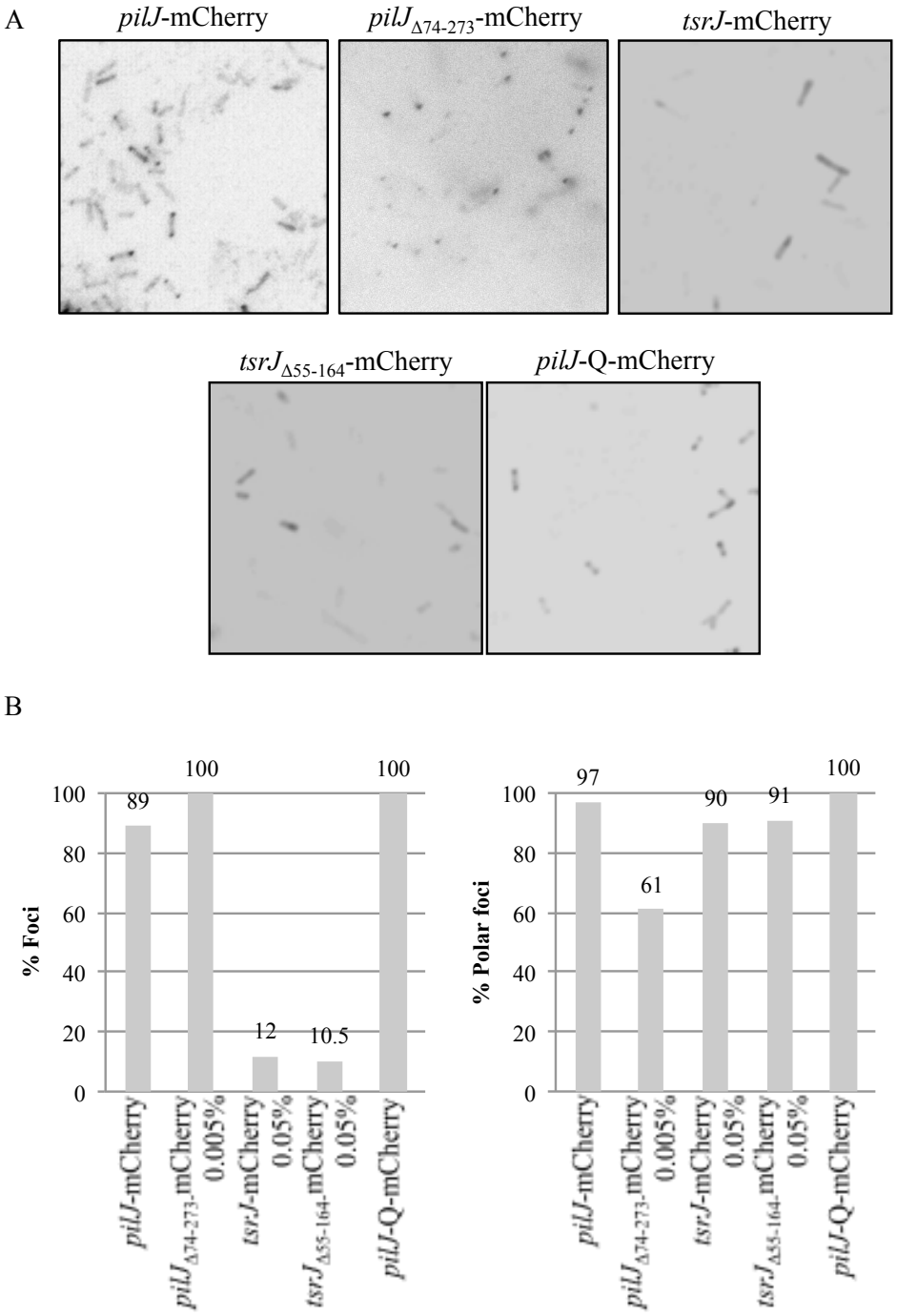



Fig. 3.9. PilJ native amino acid sequence of the N-terminus domains is required to obtain foci formation. The periplasmic domain of PilJ is involved in polar foci formation. A) Inverted fluorescence microscopy images of PilJ-mCherry and its truncated and chimeric derivatives PilJ$_{\Delta 74\text{-}273}$-mCherry, TsrJ-mCherry, TsrJ$_{\Delta 55\text{-}164}$-mCherry and PilJ-Q-mCherry. B) Percentage of cells with foci and percentage of foci that are polar as determined for 200 cells using fluorescence microscopic images of the indicated strains.

Table 3.3. Numerical data of total and polar foci for the PilJ-mCherry full-length, mutants and chimeras.

| | # of cells counted | # of total foci | # of polar foci | % polar foci |
|---|---|---|---|---|
| *pilJ*-mCherry | 200 | 300 | 290 | 97 |
| $pilJ_{\Delta 74\text{-}273}$-mCherry | 200 | 479 | 290 | 61 |
| *tsrJ*-mCherry | 200 | 41 | 37 | 90 |
| $tsrJ_{\Delta 55\text{-}164}$-mCherry | 200 | 35 | 32 | 91 |
| *pilJ*-Q-mCherry | 200 | 304 | 304 | 100 |

the chromosomal constructs that were expressed at a lower level from the native promoter (Fig. 2.1). Thus here the higher protein levels only potentially affect the localization but not function.

**3.3.3.2 TsrJ vs PilJ: The role of the N-terminus**

To determine the importance of the transmembrane and periplasmic domains of PilJ for function and localization, the fusion protein TsrJ was generated. Functional studies were done with both His-tagged and mCherry fusion proteins. TsrJ with either tag (-His/ -mCherry) shows a partial reduction in twitching motility compared to PilJ (Fig. 3.7A and 3.8A), but unexpectedly TsrJ-mCherry does not show any decrease in cAMP levels which is in contrast to the results for TsrJ-His (compare Fig. 3.7B and 3.8B). This discrepancy could be either because of the difference in the tags and their effect on protein folding or interaction with partner proteins in the chemosensory system, or a difference in protein levels between the His-tagged and mCherry fusions. For the cAMP assays, TsrJ-His is expressed at levels slightly lower than PilJ-His, similar to the levels shown in Fig. 3.7C. In contrast, TsrJ-mCherry is present at slightly higher levels than its PilJ-mCherry counterpart (Fig. 3.8C). Given the importance of stoichiometry of chemotaxis proteins for signal transduction, one possibility is that this difference in protein expression is driving the discrepancy in relative cAMP levels between these constructs.

The replacement of the periplasmic and transmembrane domains (PilJ) results in a decrease in foci formation (Fig. 3.9B, compare PilJ with TsrJ). This suggests that the native sequence of these domains is important for foci formation. This decrease in the number of total foci could be because of the loss of potential interactions with associated polar proteins like PilA or CyaB (Fig. 3.11). However, the presence of TsrJ-mCherry in the cell membrane is yet to be confirmed. Since TsrJ-His localizes in the membrane (Fig. 3.7C), TsrJ-mCherry will most likely localize in the membrane and membrane dissociation is probably not the reason for reduced foci formation. Because TsrJ forms very few foci, it is important to assess a larger population to

make an accurate comment about the polarity of this chimeric protein.

#### 3.3.3.3 $TsrJ_{\Delta 55\text{-}164}$ vs. $PilJ_{\Delta 74\text{-}273}$: The role of the transmembrane domains

Upon comparing $TsrJ_{\Delta 55\text{-}164}$ with $PilJ_{\Delta 74\text{-}273}$, it is evident that the native amino acid sequence of transmembrane domains (in combination with the loss of the periplasmic domain) of PilJ is important for signal transduction, as $TsrJ_{\Delta 55\text{-}164}$ is null for both cAMP and twitching motility (Fig. 3.7 and 3.8). It is possible that the loss of twitching motility in $TsrJ_{\Delta 55\text{-}164}$ is due to reduced cAMP levels since cAMP plays an important role in T4P biogenesis (10). The cAMP independent effect of this substitution on twitching motility will be tested in future by restoring the cAMP levels in these genetic backgrounds through the expression of a Chp independent $CyaB_{R412H}$ point mutant. The slightly less protein levels of $TsrJ_{\Delta 55\text{-}164}$–His (similar to the levels shown in Fig 3.7C) do not seem to affect twitching motility and cAMP levels since the $TsrJ_{\Delta 55\text{-}164}$-mCherry was also null for both phenotypes at slightly higher protein levels compared to PilJ-mCherry (Fig. 3.8C).

Compared to PilJ, $TsrJ_{\Delta 55\text{-}164}$ has a deletion of the periplasmic and replacement of the transmembrane domains and shows reduced foci formation (Fig. 3.9B, compare $TsrJ_{\Delta 55\text{-}164}$ with PilJ and $PilJ_{\Delta 74\text{-}273}$). This suggests that the native sequence of the transmembrane domain is crucial in foci formation. Owing to the membrane localization of $TsrJ_{\Delta 55\text{-}164}$–His (Fig. 3.7C), it is very likely that $TsrJ_{\Delta 55\text{-}164}$-mCherry also localizes to cell membrane and this will be ascertained in future. Hence membrane dissociation does not appear to be a reason for reduced foci formation. Large numbers of cells need to be accessed to reach an accurate conclusion about the polarity of this chimeric protein since it forms very few foci.

It is noteworthy that TsrJ and $TsrJ_{\Delta 55\text{-}164}$ have similar reductions in percent foci formation suggesting the periplasmic domain of Tsr doesn't influence localization. Despite the reduced foci formation their signal transduction phenotypes are not the same (Fig. 3.7, 3.8 and 3.9B). $TsrJ_{\Delta 55\text{-}}$

$_{164}$ shows complete loss of function whereas TsrJ has intermediate levels compared to PilJ (Fig. 3.7 and 3.8). We think that despite the reduction in number of foci, the presence of the periplasmic domain allows TsrJ to achieve a conformation that results in stronger activation of the downstream signaling. The reduction in foci formation in the two Tsr-PilJ chimeras suggest that the amino acids in the N-terminus of PilJ are important for foci formation (Fig. 3.9B).

### 3.3.4 Key residues in the trimer interaction domain are not involved in PilJ localization

WspA is a MCP associated with the Wsp chemosensory system of *P. aeruginosa*. It is the sole punctate MCP in *P. aeruginosa*, and it is proposed that reduced clustering drives this localization pattern (26, 43). In a study by O'Connor et. al (2012), a sequence alignment of the trimer interaction domain (TID) of WspA with PctA (a polar MCP of *P. aeruginosa*) highlighted 5 non-conserved amino acid residues in WspA that were changed to match PctA by site-directed mutagenesis. This WspA mutant formed stronger trimer-of-dimers and had an increased likelihood for localizing at the old cell pole. There was an increase in percent polar foci at the old cell pole from 18.5% in wild type WspA to 33.8% in WspA TID mutant (26).

In our search for the polar localization cue of PilJ, concurrently with the C-terminal truncations studies, we assayed these key amino acid residues in the trimer interaction domain of PilJ to determine their role in polar localization. An amino acid sequence alignment of the TID of PilJ, WspA and the polar MCP PctA (Fig. 3.10) revealed that 4 out of the 5 critical amino acids that drove punctate localization of WspA were not conserved in PilJ. These 4 non-conserved residues from PilJ were mutated to match the residues in WspA by site-directed mutagenesis (A523S, S531E, D545T and R553Q) in order to determine the importance of these residues in polar localization. This new PilJ with four point mutations was termed as PilJ-quadruple mutant (PilJ-Q). The quadruple mutant was fused to a mCherry tag at its C-terminus, now called PilJ-Q-mCherry. PilJ-Q-mCherry formed foci and localized to the cell poles like PilJ-

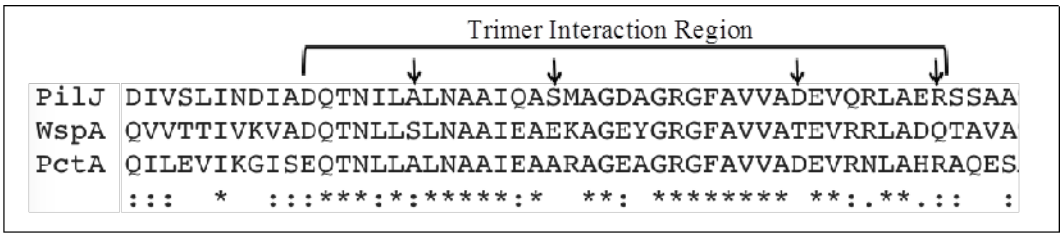



Fig. 3.10. Alignment of amino acid sequences of PilJ, WspA and PctA using ClustalW. Arrows indicate non-conserved residues between PilJ and WspA.

mCherry, which suggests that the 4 amino acids in TID do not affect polar localization (Fig. 3.9B). However PilJ-Q-mCherry showed a distinct increase in cAMP levels compared to PilJ-mCherry (Fig. 3.8B). Such a rise was not observed for twitching motility; instead a slight decrease was seen in twitching for PilJ-Q-mCherry (Fig. 3.8A). Such an inverse relationship between cAMP levels and twitching motility has been previously reported. In a *pilH* mutant despite having elevated cAMP levels, there was a twitching motility defect, which indicated that cAMP levels do not always correlate with twitching motility (7). Future experiments including determination of surface pilin levels will be needed to help decipher the effects of these TID mutations on signal transduction.

### 3.3.5 Model for polar localization of PilJ

MCPs are responsible for determining the intracellular localization of chemosensory systems. Using fluorescence microscopy, we found that PilJ, the sole predicted MCP of the Chp system is required for PilI foci formation (Fig. 3.1C). This suggests that PilJ is required for the nucleation of the core-signaling complex comprising of PilJ-PilI-ChpA (and possibly the second coupling protein ChpC). Having shown the importance of PilJ for the assembly of the Chp system, we propose that PilJ dictates its localization as well. In our quest to determine the polar localization cue for PilJ, we found that the second transmembrane domain of PilJ plays an important role in polarity (Fig. 3.4B). This finding is unique because until now localization cues have only been found the cytoplasmic domains of MCPs (26, 39). Based on our data and the literature, we are able to propose a model that predicts several potential mechanisms for polar localization of PilJ (Fig. 3.11).

Our model includes three possible mechanisms for polar localization of PilJ. The first and the most promising one is through its interaction with inner membrane pools of PilA associated with the T4P complex. Previous studies have indicated that PilJ and PilA monomers interact

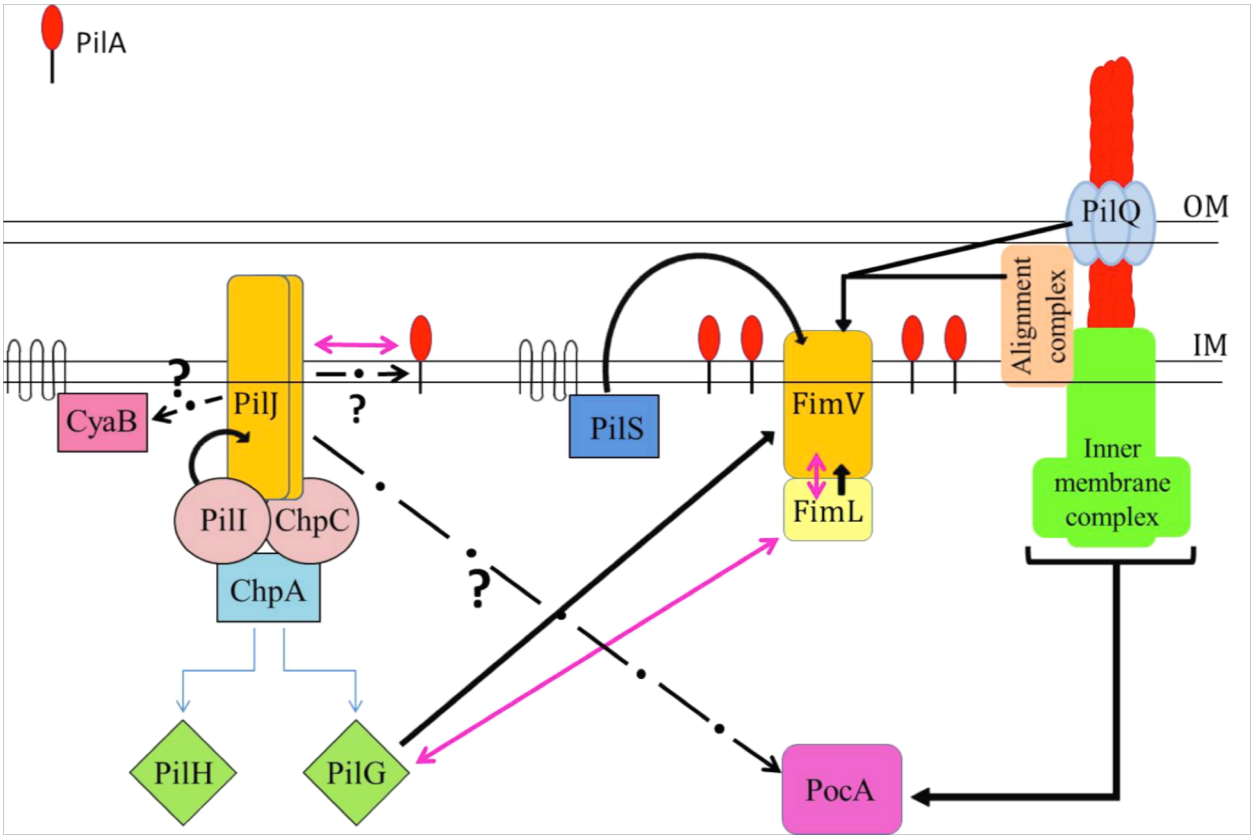



Fig. 3.11. Model depicting dependence of the Chp system, T4P and other related proteins for intracellular localization. The black solid arrows indicate the localization dependence identified through fluorescence microscopy. The arrowhead indicates the protein that dictates the polar localization (i.e. PilG is dependent on FimV). The pink double-sided arrows indicate protein-protein interactions as determined by BACTH or co-immunoprecipitations. The postulated localization dependence of PilJ on related proteins and potential interactions with them are predicted using discontinuous arrows. This model incorporates data from (3, 14, 17, 27, 34, 44-49) and Fig. 3.1.

with each other and PilA potentially acts as a signal for PilJ as a result of T4P retraction. This interaction is lost upon the deletion of the transmembrane domains of PilJ (17). However we find this loss of interaction to be unsurprising since a PilJ transmembrane domain mutant will not localize in the membrane and will lose its proximity to T4P and PilA. Our data with the membrane associated PilJ N306 mutant shows that the second transmembrane domain of PilJ is involved in polarity (Fig. 3.4B and C). Hence we propose that PilJ interacts with the PilA monomers by its second transmembrane domain, and that this interaction helps drive the co-localization of PilJ and T4P at the cell poles. This hypothesis will be tested in future by studying the interaction between PilJ/ TsrJ/ $TsrJ_{\Delta 55\text{-}164}$ with PilA using a BACTH system (Euromedex).

The second possible mechanism driving polar localization of PilJ is based on the mechanosensor model. This model predicts that T4P retraction triggers signal transduction through PilJ/ Chp system (17). This suggests that the proximity of these two protein complexes is essential for efficient signal transduction. Hence, we propose that the localization of PilJ and T4P are either interdependent or controlled by a common mechanism. The Poc complex (PocA-PocB-TonB3) has been found to be essential for the localization of polar motility structures like flagellum and T4P in *P. aeruginosa* (48). The deletion of *pocA* results in non-polar T4P although the exact mechanism behind this remains to be elucidated. Thus, potential PocA dependence is another proposed mechanism for polar localization of PilJ.

Finally, CyaB is a membrane bound polarly localized adenylate cyclase with six predicted transmembrane helices (47, 50). The response regulators of the Chp system modulate the activity of CyaB for cAMP production (7). We have previously shown the importance of the periplasmic domain for the regulation of cAMP levels (28), which is a CyaB dependent phenotype. Thus, there is a possibility that CyaB plays a role in polarity of PilJ.

In summary, these studies have allowed us to discover the importance of PilJ for

localization of the Chp system and the potential internal polar localization cue in PilJ. Initial studies revealed a drastic reduction in PilI-CFP foci formation in the absence of PilJ and thus demonstrate the importance of PilJ for PilI foci formation. We predict that PilJ will also be important for ChpA clustering and thus signal transduction. These data are in accordance to the literature where MCPs localize to the cell poles and dictate the polar localization of other proteins of a chemosensory system (21). Previous studies have shown that the polar localization cue lies in the cytoplasmic region of an MCP (26, 39). Our studies suggest that a transmembrane domain of PilJ plays a role in its polar localization and function. This is a novel mechanism of localization as to our knowledge there are no previous reports of transmembrane domains guiding the localization of a MCP. Both the truncation studies and fusion MCP constructs generated using PilJ and Tsr revealed the importance of the native sequence of the N-terminus (and in particular the second transmembrane domain) for signal transduction and foci formation.

This work is an important step towards identifying the localization cue for PilJ, the MCP of Chp chemosensory system. Future studies will be focused on identifying additional proteins that serve to either directly or indirectly localize PilJ to the poles. Since other proteins that are functionally associated to the Chp system such as the structural and regulatory proteins of T4P and CyaB, are polar (44, 47), it is tempting to propose that PilJ possibly interacts with these proteins potentially through its transmembrane domains that facilitate its polar localization. Our future work will be to investigate potential direct or indirect dependence of PilJ localization on these candidates.

Table. 3.4. List of protein pairs that show independent localization patterns established by fluorescence microscopy. Proteins listed in the left column maintained polar localization in the indicated gene deletion background(s) listed in the right column (i.e. PilJ retained its polar localization in the absence of FimV, FimL ChpA or PilG). This table incorporates data from (27, 34, 44, 47).

| Protein polarly localized | Gene deletion background examined |
|---|---|
| PilJ | *fimV, fimL, chpA, pilG* |
| PilG | *fimL, chpA* |
| FimL | *chpA, pilG, cyaB* |
| CyaB | *fimL* |
| PilS | *pilG* |
| FimV | *pocA* |

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# Chapter four

## Summary

This work has helped to set the stage for identifying and understanding the connection between signal transduction and the localization of PilJ in the Chp chemosensory system in *Pseudomonas aeruginosa*. According to the *E. coli*-based definition of classical signal transduction pathway, the signal is sensed through the periplasmic domain of a MCP and triggers transduction through the system (1). The Chp system has two known outputs and we began to dissect the different domains of PilJ to understand their importance for signal transduction. Our work suggests that the periplasmic domain plays a role in signal transduction for modulating intracellular cAMP levels but not twitching motility (2). We also found that directional twitching motility (DTM) towards phosphotidylethanolamine is independent of PilJ provided sufficient cAMP is present to allow a basal level of twitching (2). These data suggest that the classical signal transduction pathway is not followed for the modulation of twitching motility by the Chp system. This is the first time that a defined domain of PilJ is identified to be involved in a specific output of the Chp chemosensory system.

Because the $PilJ_{\Delta 74\text{-}273}$ mutant showed wild type levels of twitching motility in the presence of sufficient intracellular cAMP, this lead us to speculate that other domains of PilJ may be involved in signal transduction. To investigate the possible involvement of the transmembrane and/or the HAMP domain in signal transduction we generated the Tsr-PilJ fusion constructs. We found that the native sequence of the transmembrane domains is important for signal transduction (Chapter 3). $TsrJ_{\Delta 55\text{-}164}$ where the transmembrane domains of $PilJ_{\Delta 74\text{-}273}$ were replaced with those from Tsr, is null for cAMP levels and twitching motility. This reduction in function also correlated with a loss of foci formation (Chapter 3). The cAMP independent effect of this truncation with transmembrane domain replacement on twitching motility will be tested in future by restoring the cAMP levels using the $CyaB_{R412H}$ point mutant. These findings are in agreement with the literature where the importance of the transmembrane domains for signal

transduction has been established (3). Although the importance of these domains for signal transduction is not unusual, the discovery that the periplasmic domain of PilJ can have a different degree of involvement in the modulation of different outputs of the Chp system is novel.

Concurrently, because the Chp system, CyaB and T4P all localize to the poles (4-7), we wanted to understand if the localization of the Chp system has an effect on its function. Hence we needed to identify the different mechanisms that potentially determine the polar localization of this chemosensory system. MCPs are important for chemosensory cluster formation as well as dictate their intracellular localization (8). Therefore we investigated the importance of PilJ for the localization of the Chp system. Our data clearly demonstrated the importance of PilJ for the clustering of PilI (Chapter 3). This leads us to predict that PilJ is important for the nucleation of the core signaling complex made up of PilJ-PilI-ChpA.

Understanding the importance of PilJ in clustering and its structural domains in signal transduction, we sought to identify the internal polar localization cue(s) of PilJ. Using multiple C-terminal PilJ truncations and fluorescence microscopy, we determined that the 2$^{nd}$ transmembrane domain plays a role in polar localization of PilJ (Chapter 3). This report is unusual as generally the localization cue of MCPs is found in the cytoplasmic domain (9, 10). Since deletion of the 2$^{nd}$ transmembrane domain showed a reduction in polarity and the periplasmic truncation and the cytoplasmic TID point mutations did not affect polarity, we propose that PilJ interacts with other associated polar proteins through its transmembrane domains thereby determining its polar localization.

Taking together the localization and functional studies, we found that the periplasmic and the transmembrane domains of PilJ are important for signal transduction, and the transmembrane domains are also important for foci formation (Chapter 2, 3). Hence it is not surprising that we

also found a polar localization cue for PilJ in its 2nd transmembrane domain (Chapter 3). To understand the polar localization mechanisms of PilJ, future work will include testing the localization dependence of fluorescently tagged PilJ on associated polar proteins such as PilA and CyaB. Loss of PilJ polar foci in any of these deletion strains could indicate a potential mechanism for polar localization. Exploring protein-protein interactions between the protein pairs that show localization dependence using the BACTH system will provide evidence in support. Additionally the Poc complex also potentially plays a role in the polarity of PilJ, and this relationship will be investigated as well. Deletion of *pocA* is shown to have non-polar surface pili (11). If the localization of the Chp system is dependent on T4P, then the Chp system will co-localize with non-polar T4P. Alternatively, if the Chp system is dependent on PocA, then upon deletion of *pocA*, the Chp system will be non-polar but may or may not co-localize with T4P. Localization can be studied by immunofluorescence microscopy using PilA and His-specific antibodies for T4P and His-tagged Chp system proteins respectively. Alternatively, latex spheres conjugated to anti-PilA antibodies could also be used to localize T4P along with fluorescently tagged Chp system proteins. It will be interesting to observe signal transduction in a mislocalized Chp system. Long-term studies will include generation of a mislocalized Chp system and studying its ability for signal transduction. Overall, these experiments will help decipher the polar localization mechanism(s) for PilJ and thus the Chp chemosensory system.

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# CURRICULUM VITAE

Vibhuti H. Jansari

Place of birth: Vadodara, Gujarat. India

## EDUCATION

Ph.D., University of Wisconsin – Milwaukee, August 2017
Major: Biological Sciences – Microbiology
Secondary area of concentration: Molecular Biology

M.S., Maharaja Sayajirao University of Baroda, May 2010
Major: Microbiology

B.S., Maharaja Sayajirao University of Baroda, April 2008
Major: Zoology
Minor: Botany and Chemistry

**DISSERTATION TITLE:** The role of PilJ and its structural domains in the localization and function of the Chp chemosensory system in *Pseudomonas aeruginosa*

## AWARDS AND HONORS

**University of Wisconsin- Milwaukee**

| | |
|---|---|
| 2016 | Graduate Student Travel Award |
| 2016 | Outstanding Oral Presentation Award |
| 2011-2016 | Chancellor's Graduate Student Award |
| 2015 | Ruth Walker Graduate Grant-in-Aid Award, Department of Biological Sciences |

**Council of Scientific and Industrial Research, India**

| | |
|---|---|
| 2009-2010 | Lectureship |
| 2010 | Junior Research Fellowship |

**Indian Council of Medical Research, India**

| | |
|---|---|
| 2010 | Junior Research Fellowship |

## RESEARCH EXPERIENCE

**University of Wisconsin-Milwaukee**

| | |
|---|---|
| 2011-2017 | Graduate Student Researcher |
| 2016 | Undergraduate Research Mentor |

**Maharaja Sayajirao University of Baroda**

| | |
|---|---|
| 2008-2010 | Graduate Student Researcher |

## TEACHING EXPERIENCE

**Graduate Teaching Assistant**

| | |
|---|---|
| 2012-2017 | Foundations of Biological Sciences-II (Bio Sci 152) |
| 2011 | General Survey of Microbiology (Bio Sci 101) |

## CERTIFICATES

2016 The Certificate in Active learning- Kolb's cycle of Experiential Learning. The Center for Excellence in Teaching and Learning- UW-Milwaukee.

## MEMBERSHIPS, CLUBS AND ORGANIZATIONS

2014 American Society of Microbiology

## VOLUNTEER EXPERIENCE

**Badger State Science and Engineering Fair**

2016 Specialty Award Judge
2014-2015 Scientific Process Judge

**University of Wisconsin- Milwaukee**

2016 Panelist, STEM New TA Orientation
2013-2015 Graduate Organization of Biological Sciences

**Maharaja Sayajirao University of Baroda**

2008-2010 Department Student Representative

## CONFERENCES AND SYMPOSIUMS

**Presentations**

February 24, 2017 PilJ: Its role in localization and function of Chp chemosensory system in *Pseudomonas aeruginosa*, University of Wisconsin-Milwaukee. Biological Sciences Colloquium. Milwaukee, WI.

April 22, 2016 A search for the signal sensing domain of PilJ regulating cyclic AMP in *Pseudomonas aeruginosa*. University of Wisconsin-Milwaukee. Biological Sciences Research Symposium. Milwaukee, WI.

**Posters**

September 23-25, 2016 The role of PilJ and its domains in localization and function of the Chp chemosensory system in *Pseudomonas aeruginosa*. 23rd Midwest Microbial Pathogenesis Conference (MMPC). University of Illinois at Urbana-Champaign, IL.

April 19, 2013 Differential roles of the two *cheW* –like genes in the Wsp chemotaxis cluster of *Pseudomonas aeruginosa*. University of Wisconsin-Milwaukee. Biological Sciences Research Symposium. Milwaukee, WI.

## PUBLICATIONS

Jansari VH, Potharla V, Riddell G and Bardy SL. 2016. Twitching motility, directional twitching and regulation of cAMP levels: signal transduction through a single methyl-accepting chemotaxis protein. FEMS Microbiology Letters. Jun; 363 (12): fnw119