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PilJ functions as a MCP and directs assembly of Type IV pili in response to
environmental stimuli

THESIS

Submitted to

The College of Arts and Sciences of the

University of Dayton

In Partial Fulfillment of the Requirements for

The Degree

Masters of Science in Biology

By

Tracy Lynn Collins

UNIVERSITY OF DAYTON

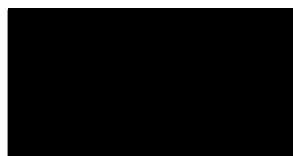
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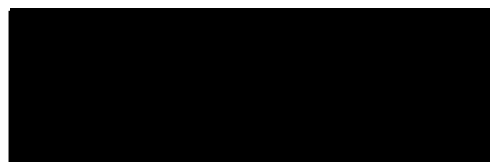
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INTRODUCTION

Pseudomonas aeruginosa is a gram-negative, rod bacterium. This organism is a ubiquitous organism commonly inhabiting soil and aqueous environments, as well as in animal and plant tissues (72). Because it possesses the ability to utilize a wide range of organic compounds as a food source, *P. aeruginosa* can adapt and thrive in nutrient deprived environments. *P. aeruginosa* is an ecologically significant bacterium and is important to the nitrogen cycle, specifically during denitrification and assimilation (73). This bacterium has also proven to be beneficial in bioremediation by degrading specific contaminants such as chlorinated pesticides and crude oil.

P. aeruginosa is not only an ecologically important bacterium, but is classified as an opportunistic pathogen and is responsible for a high percentage of nosocomial infections (16). As an opportunistic pathogen, *P. aeruginosa* exploits the host defenses to initiate infection and has been implicated in urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, gastrointestinal infections, and a variety of other systemic infections. *Pseudomonas aeruginosa* is equipped with a substantial number of virulence factors that provide nutrients for bacterial growth, disrupt the host immune system, and can cause extensive tissue damage.

Immunocompromised patients, such as individuals suffering from cancer or HIV, as well as those with severe burns, are very susceptible to *P. aeruginosa* infections. Additionally, *P. aeruginosa* has been shown to cause lung infections in cystic fibrosis

patients and is a leading cause of mortality among these patients (69). In addition to humans, *P. aeruginosa* is a pathogen of a wide range of animal and plant species, including mice, fruit flies, nematode worms, and mustard plants (13, 21, 58, 67).

Pseudomonas aeruginosa is distinguished by its ability to attach to both abiotic (e.g. medical devices) and biotic (e.g. eye epithelial cells) surfaces. Following attachment to the surface, cells have the potential to colonize and form mature biofilms. Biofilms are encased, microbial colonies attached to a surface and are considered a major contributor to infection. Within these biofilms, bacteria are protected from the effects of antibiotics and attack by the host immune system (10). Mucus blocking the airway passages of cystic fibrosis patients creates a nutrient rich environment ideal for the propagation of bacterial growth and biofilm formation (44). Because of the medical implications of *P. aeruginosa* infections, it is important to understand the mechanisms of biofilms formation and function.

Two cellular components, flagella and Type IV pili, are necessary for the formation of biofilms (56). Flagella and Type IV pili are extracellular filaments that mediate both cell motility and adhesion. During biofilm formation, bacterial cells are propelled towards the surface intended for colonization by the rotation of the flagella in a liquid environment. Once at the surface, the bacterial cell will attach itself to the surface via its Type IV pili, as shown in Figure 1 (56). Through the repeated extension and retraction of the pili, cells translocate across the surface and aggregate into microcolonies. Microcolonies eventually differentiate into mature biofilms (56). The process by which a bacterium translocates across a solid surface through the extension and retraction of pili is known as twitching motility. A similar type of motility called

gliding motility is used by the bacterium *Myxococcus xanthus* and is dependent on Type IV pili (28). Biofilm formation in *M. xanthus* has been shown to require this motility, as well as cell-to-cell signaling.

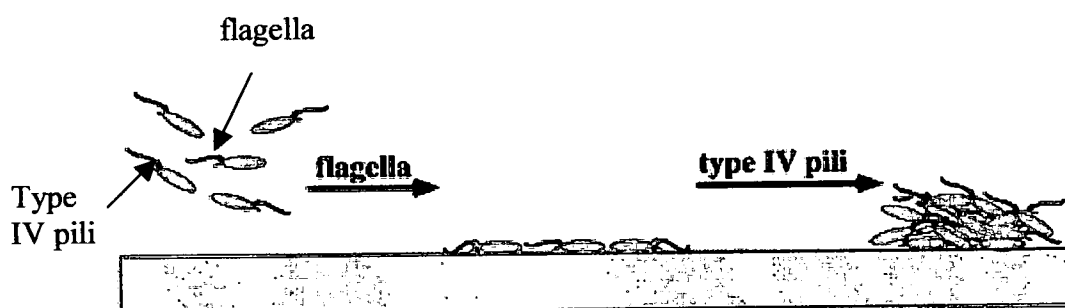


Figure 1. Biofilm formation in *Pseudomonas aeruginosa* (56).

Approximately 40 genes are involved in the biogenesis and function of Type IV pili in *P. aeruginosa* (49). My research has primarily focused on the role that the gene *pilJ* plays in this process. This gene encodes for a putative chemoreceptor protein PilJ. Darzins demonstrated that *pilJ* mutant cells are deficient in twitching motility and do not display functional surface pili (15). Despite the absence of surface pili, the mutant remains sensitive to pili specific phage infection (15). Because *pilJ* mutant cells retain their sensitivity to pili specific phage, we believe that PilJ does not function in pilin production, but rather in the assembly of pili on the surface. The purpose of this study was to determine the mechanism by which PilJ controls pilus assembly and function.

In addition to the function of PilJ in twitching motility, it has yet to be determined whether it affects swarming motility. Swimming and swarming motility are mediated through the rotation of flagella in *Pseudomonas aeruginosa* (38, 59). However, there have been conflicting studies as to whether Type IV pili are necessary for swarming motility. One study using *pilA* mutant cells showed these mutants to be deficient in

swarming motility (38). In another study, *pilA* mutant cells were not defective in their ability to swarm and it was concluded that Type IV pili are not necessary for swarming motility (59). *pilJ* mutant cells are capable of swimming motility similar to wild-type cells (15), but to our knowledge their ability to swarm has not been tested. Because *pilJ* mutant cells do not have functional surface pili, we intend to determine whether PilJ is necessary for swarming motility and, if so, what role it plays in this mechanism.

Genes involved in the biogenesis and function of Type IV pili in *P. aeruginosa* have been shown to have homology to genes encoding for the Che, or chemotaxis, proteins of *Escherichia coli* (15). Chemotaxis is directed movement in response to a chemical attractant. In *E. coli*, Che chemotaxis proteins are necessary for flagella driven movement in response to a chemical gradient. The similarity of the *pil* genes to the Che genes led us to investigate whether chemotaxis is associated with twitching motility in *P. aeruginosa*.

One of the proteins necessary for bacterial chemotaxis is methyl-accepting chemotaxis proteins, or MCPs. MCPs are transmembrane proteins that sense chemicals in the environment and activate a signal transduction pathway within the cell, directing its movement via flagella. As previously stated, *pilJ* encodes for a putative MCP involved in the Type IV pili signal transduction pathway in *P. aeruginosa*. PilJ has been shown to be 26% identical to Tsr, an *E. coli* MCP. In addition, genetic analysis of PilJ has identified regions similar to MCP's structure (15). My investigation focused on confirming PilJ as a true MCP.

The specific ligand(s) responsible for binding to PilJ and turning on the Type IV pili signal transduction pathway in *P. aeruginosa* have not been identified, nor their

effect on extension and retraction of pili. Studies conducted with *M. xanthus* show chemotaxis, towards phosphatidylethanolamine (PE), a common constituent of all cell membranes (32). Also, additional research demonstrated that *P. aeruginosa* exhibits directed movement up gradients of certain species of PE, as well as an increase in cell velocity in uniform concentrations (33). Thus I set out to determine the specific chemical and/or physical stimuli that signal PilJ and how they affect twitching motility in *P. aeruginosa*.

LITERATURE REVIEW

Twitching motility

Pseudomonas aeruginosa possesses the ability to translocate across abiotic and biotic surfaces through a mechanism known as twitching motility. "Twitching motility" was first coined by Lautrop in 1961 (40). When examining *Acinetobacter calcoaceticus*, Lautrop observed that these cells appeared to be moving in a jerking fashion. This movement has also been observed in *Neisseria gonorrhea* and *Myxococcus xanthus*. Twitching motility is almost entirely restricted to Gram-negative bacteria, but has been observed in *Streptococcus sanguis*, a Gram-positive bacterium.

Twitching motility allows cells to rapidly colonize a surface during high nutrient availability and involves cell to cell contact. Furthermore, this type of motility facilitates the colonial behavior involved in biofilm and fruiting body formations (56). Twitching motility occurs on surfaces with low water content as well as hydrated surfaces (8). It is distinct from other forms of motility, such as swimming and swarming, because it is mediated through the repeated extension and retraction of Type IV pili rather than the rotation of flagella (8).

In *P. aeruginosa*, approximately 40 genes have been identified whose products are required for twitching motility (5, 6). Several of these proteins are directly involved

in the biogenesis and assembly of Type IV pili. Type IV pili are 5-7nm in diameter, extending several μm in length and are composed of a single protein subunit, pilin, which is encoded by the *pilA* gene (49). Pilin is arranged in a helical conformation consisting of 5 subunits per turn. The primary structure of pilin is approximately 145-160 amino acids long with a positively charged leader sequence and a highly conserved, hydrophobic amino terminal domain (34). This domain forms the core of the pilus fiber, a coiled-coil of α -helices. Beyond the conserved amino-terminal region, there are also a hydrophilic central and carboxy-terminal domains. These domains surround the core region as a scaffold of β -sheets as shown in Figure 2 (19). Furthermore, the majority of structural and antigenic variations of pilin among species resides in the central and carboxy-terminal domains (68).



Figure 2. Model of the structure of Type IV pilin monomer in *Pseudomonas aeruginosa* (49).

In addition to PilA, minor pilin-like proteins have been identified in *P. aeruginosa* (PilE, PilV, PilW, PilX, FimT, FimU). These pilin-like proteins resemble PilA in that they also possess the hydrophobic amino-terminal α -helical regions (2, 3, 4). The exact function of these proteins is not entirely known; however, they are required for twitching motility and are believed to form the base of the pilus fiber.

During biogenesis and assembly of pili, the PilA subunit is modified by PilD. This protein has dual functions, acting first as a peptidase, cleaving the pilin leader sequence and then as a transmethylase adding a methyl group to the resulting N-terminal residue (43, 66). Following pre-pilin modification, pilin is assembled on a base of the minor pilins through the actions of PilB and PilC (53). PilB is an ATPase while PilC is believed to be required to cap or stabilize pili. The pilus is assembled through a gated pore in the outer membrane, composed of a dodecameric doughnut-shape complex comprised of PilQ subunits (47). Retraction of the pilus is mediated through the action of PilT and PilU, PilB homologs (Figure 3) (25, 29). These ATPases are responsible for the depolymerization of the pilus into its pilin subunits, or retraction.

In *P. aeruginosa*, production of Type IV pili is controlled by a sensor-regulator pair, PilS and PilR (24, 66). PilS, a histidine kinase, acts as the sensor protein autophosphorylating in response to a particular environmental stimulus. PilS is localized to the poles (7). Following autophosphorylation, PilS transfers its phosphate group to PilR, the response regulator. In its phosphorylated form, PilR activates transcription of *pilA* by interacting with RNA polymerase and RpoN (Figure 7). The specific stimulus responsible for turning on this system, however, is unknown.

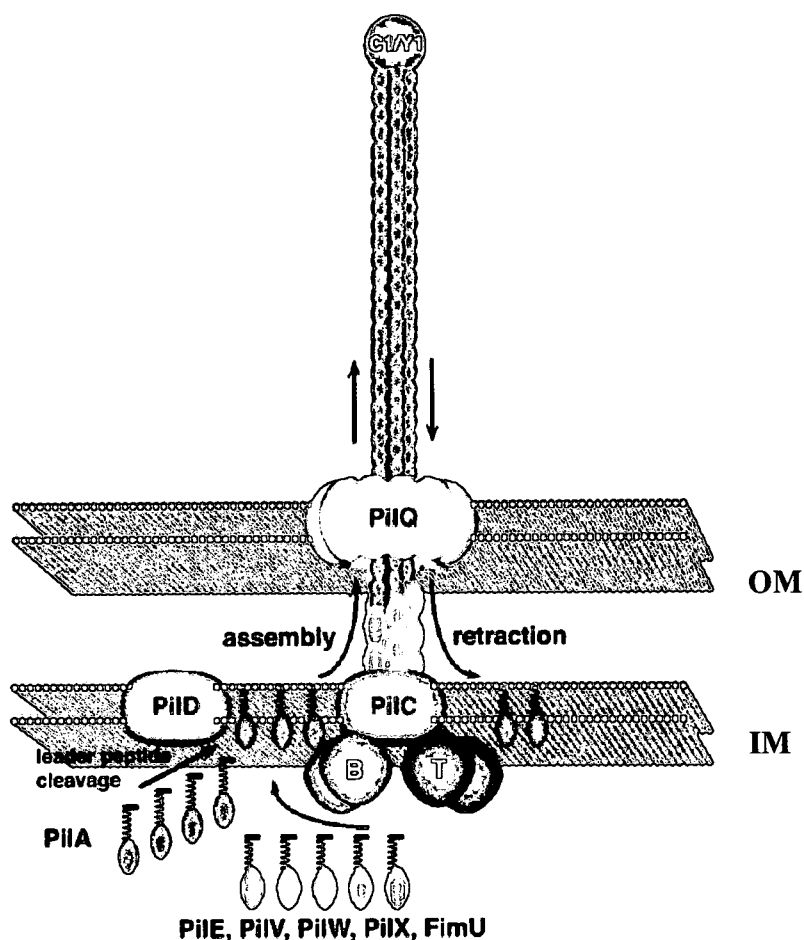


Figure 3. Model of Type IV pili assembly and retraction in *Pseudomonas aeruginosa* (49).

Chemotaxis and MCPs

Pseudomonas aeruginosa is a motile bacterium exhibiting flagella mediated chemotaxis to a wide range of chemical stimuli (22, 31, 35, 51). *P. aeruginosa*'s ability to chemotax towards or away from these chemical stimuli is mediated through chemotactic transducers known as MCPs, or methyl-accepting chemotaxis proteins. Sequence analysis indicates that *P. aeruginosa* MCPs are transmembrane proteins resembling *E. coli* MCPs (18, 65). X-ray crystallography of *E. coli* MCPs has revealed that they are dimers consisting of a periplasmic and cytoplasmic domain (Figure 4) (36,

41, 50). The periplasmic ligand binding domain of a MCP is responsible for detecting chemoeffector levels, while the cytoplasmic region is a signaling domain that undergoes reversible methylation of 4-6 glutamic acid residues (50, 62). Thus, the methylation state of a MCP acts as molecular memory of the chemical environment communicating to the cell whether it is moving up or down the chemical gradient.

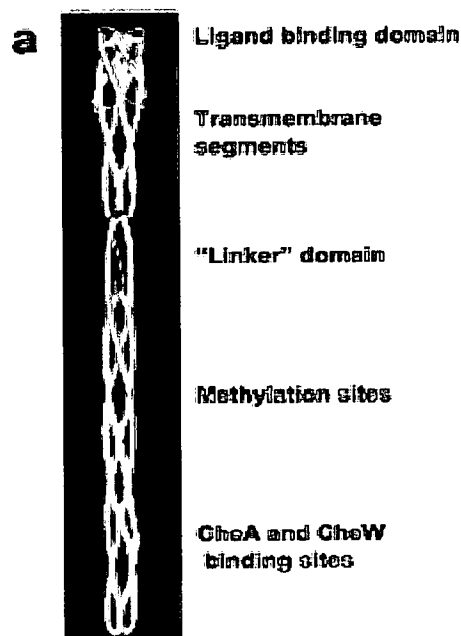


Figure 4. Structural model for the full-length Tsr dimer based on structures of the periplasmic and cytoplasmic domains (41).

MCPs of *E. coli* belong to a "two component" signal transduction pathway directing chemotactic responses. Within this pathway, six additional chemotaxis proteins act in concert with the MCP to direct cell movement (Figure 5). The MCP acts a receptor binding the ligand. Upon binding the ligand, this protein undergoes a conformational

change transducing a signal that controls two interrelated processes; excitation and sensory adaptation. Excitation is the process that controls the direction of cell movement in response to a chemical stimulus (23). During excitation in *Escherichia coli*, the MCP forms a ternary complex with CheA (histidine kinase) and CheW (linker protein). The transduced signal alters CheA activity by stimulating autophosphorylation. The phosphorylated CheA, or CheA-P, then acts as a phosphodonor to the response regulator protein, CheY (23). Depending on its phosphorylation state, CheY controls the rotation of the flagella.

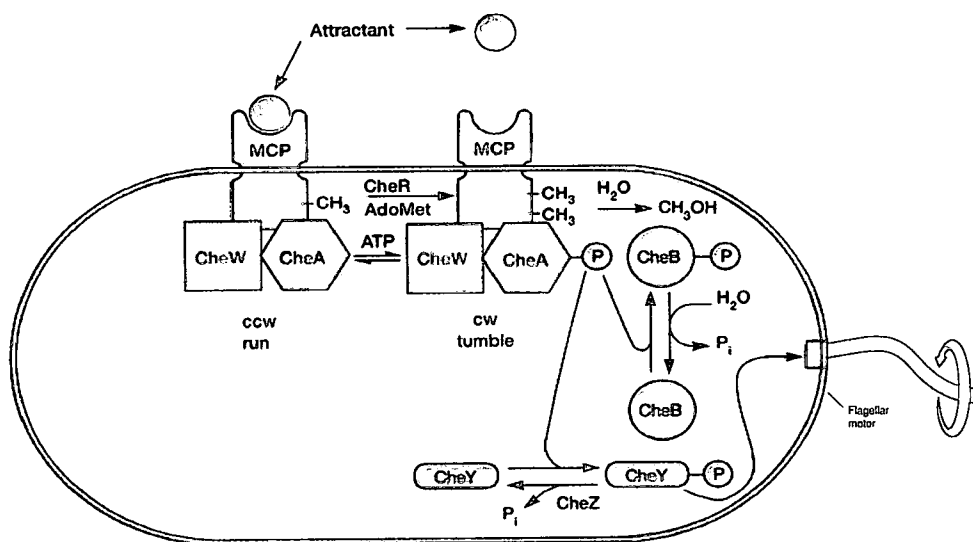


Figure 5. Chemotaxis signal transduction pathway in *Escherichia coli*. (46)

In *E. coli* and *S. enterica*, the flagellum motor has been shown to have a default counterclockwise rotation. During counterclockwise rotation, peritrichous flagella form a bundle sheath causing the cell to swim in a single direction called a “smooth run”. When phosphorylated, CheY interacts with FliM, the flagellum motor. This interaction triggers the flagella to rotate clockwise. Clockwise rotation of the flagellum motor causes the

flagellar bundle to come apart causing the cell to “tumble”, thus changing direction. In the presence of a chemoattractant, a bias is generated such that cells exhibit long runs punctuated by periodic tumbles, thus allowing the cell to change direction in order to migrate in response to a chemical gradient.

Sensory adaptation counteracts the excitation process by allowing the bacteria to adapt to a sustained stimulus. This process is regulated by methylation and demethylation of the MCP. In the presence of a positive stimulus, MCPs are methylated; whereas, in the presence of a negative stimulus or absence of a positive stimulus MCPs are demethylated. In *E. coli*, the methylation state of MCPs is controlled by two proteins, CheR and CheB (63). CheR is a methyltransferase protein using S-adenosylmethionine as a methyl source to methylate glutamate residues in the cytoplasmic region of the MCP (63). CheB is a methylesterase that cleaves the methyl groups from the glutamate residues of the MCP (64). CheB activity is positively controlled by CheA-P which donates a phosphoryl group to CheB following binding of the ligand. Therefore, methylation of the MCP favors the kinase-on signaling state, while demethylation of the MCP favors the kinase-off state.

Compared with the chemotaxis signal transduction system in *E. coli*, there appears to be a high level of diversity among motile bacteria. Most motile bacteria possess multiple homologs of *che*-like genes such as the *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*, and *Caulobacter crescentus*. *Pseudomonas aeruginosa* has *che*-like genes arranged in five clusters (Figure 6). Cluster I, II, III, and V have been shown to be involved in swimming motility chemotaxis and cluster IV is directly related to twitching motility (15, 18, 30, 33, 48).

A comparative analysis of *E. coli* and *P. aeruginosa* has led to a proposed model of the signal transduction pathway controlling twitching motility in *P. aeruginosa* (14). In this model, an environmental signal(s) stimulates the formation of a ternary complex consisting of PilJ (MCP), PilL (CheA homolog), and PilI (CheW homolog) (Figure 7). This complex activates the kinase activity of PilL transferring a phosphate group to the response regulators, PilG and/or PilH (CheY homologs). These response regulators are believed to regulate pili biogenesis and twitching motility. In this model, sensory adaption is controlled by PilK and ChpB. PilK, a CheR homolog, is thought to act as a methyltransferase adding methyl groups to the MCP, while ChpB, a CheB homolog, is believed to be a methylesterase removing these methyl groups (17).

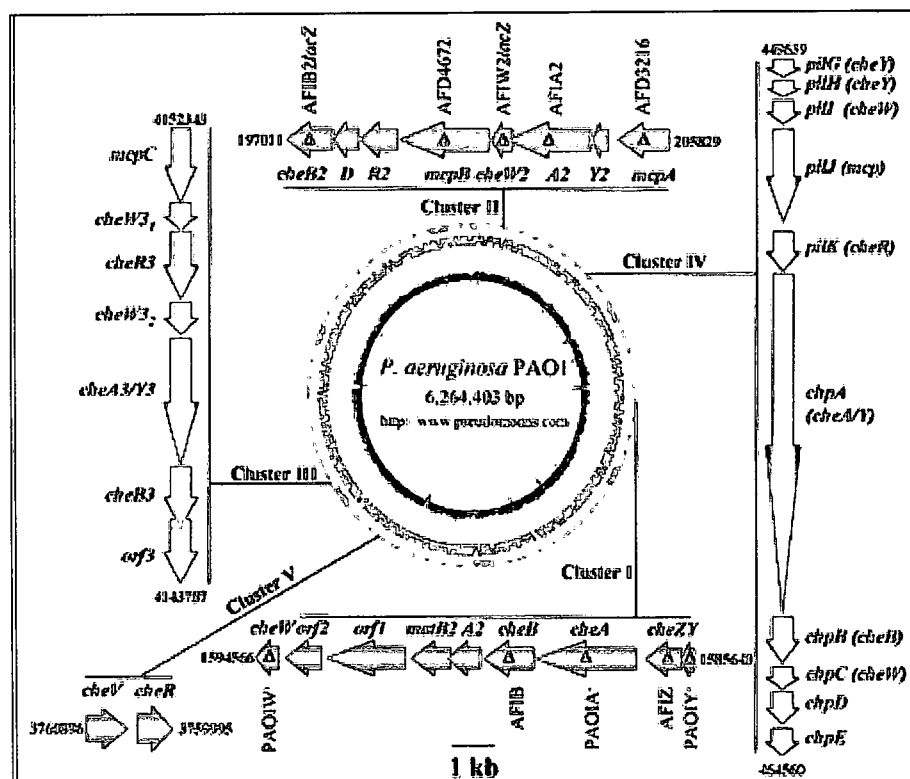


Figure 6. Chemotaxis genes in *Pseudomonas aeruginosa*. Chemotaxis genes are arranged in five gene clusters. Cluster IV genes are associated with twitching motility (18).

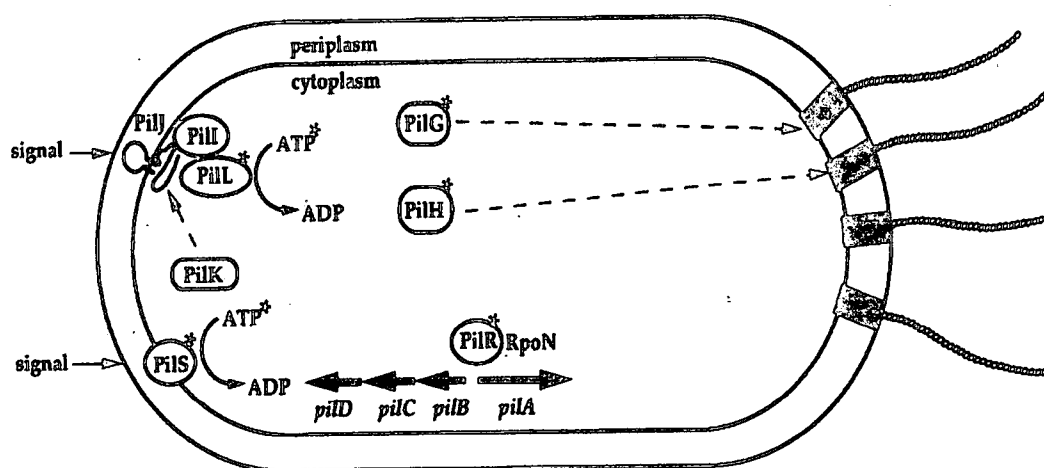


Figure 7. Model of the main regulatory networks governing pilus biogenesis and twitching motility in *Pseudomonas aeruginosa* (14).

Studies have demonstrated there to be diversity among *E. coli*'s methyl-accepting chemotaxis proteins. *E. coli* has five genes encoding the MCPs; Tar, Tap, Trg, Tsr, and Aer. Each of these MCPs binds and detects specific ligands: Tsr (serine), Tar (aspartate/maltose), Tap (dipeptides), Trg (galactose/ribose), and Aer (oxygen). Currently, 26 genes in *P. aeruginosa* have been identified as *E. coli* MCP homolog genes; however, these genes and their protein products have not been as extensively characterized (65).

Previous studies have demonstrated that *P. aeruginosa* exhibits flagella based chemotactic responses to organic compounds, L-amino acids, sugars, thiocyanic, isothiocyanic esters, and inorganic phosphate (12, 31, 51, 52). In addition, MCP methylation assays of *P. aeruginosa* have shown that an approximately 73kDa protein is

labeled with L-[methyl-³H]methionine in response to L-serine, L-arginine, and α -aminoisobutyrate (11). This study demonstrated that chemotaxis towards these amino acids is mediated through MCPs.

Studies using a *P. aeruginosa* mutant defective in chemotaxis to L-serine led to the identification of a MCP transducer gene designated *pctA*. This gene encodes for a 629 amino acid polypeptide with a calculated mass of 68,042 Daltons. PctA also possesses structural features typical of MCPs. These features include two hydrophobic transmembrane domains, a hydrophilic periplasmic region, and a hydrophilic cytoplasmic domain. In addition, PctA has two potential methylation sites within the cytoplasmic domain. Significant identity to enteric MCPs was observed in the highly conserved domain (HCD) of this protein (39).

In *Pseudomonas aeruginosa*, PctA is directly involved in chemotaxis to L-amino acids. PctA has been shown to be specifically necessary for chemotaxis to L-glycine, L-serine, L-threonine, and L-valine. There also appears to be a reduction in chemotaxis to other L-amino acids in a *pctA* mutant (39).

In addition to *pctA*, two other *che*-like genes, *mcpA* and *mcpB*, have been identified in *P. aeruginosa* as genes encoding for MCPs (18). These genes belong to cluster II genes and are involved in swimming/swarming motility. Both *mcpA* and *mcpB* mutants demonstrate a general chemotaxis defect towards amino acids, sugars, organic acids, and aromatic compounds in tryptone and LB soft agar plates. This defect is complemented when *mcpA* and *mcpB* are expressed in *trans* in their respective mutants (18). However, in minimal-medium soft agar plates the *mcpA* mutant is not deficient in chemotaxis and behaves like wild-type cells.

Like PctA, McpA and McpB possess certain structural features resembling *E. coli*-like MCP architecture. McpA contains a HCD, but only one transmembrane domain. McpB also contains a HCD, but, in contrast to McpA, it does not have any transmembrane domains commonly found in MCPs. Additionally, McpA and McpB are the only *P. aeruginosa* MCPs that have C-terminal pentapeptides such as those associated with the high abundance receptors of *E. coli* (18). This structural analysis, along with chemotaxis assays, supports the idea that McpA and McpB play a role in chemotactic signal transduction in *P. aeruginosa*.

Pseudomonas aeruginosa not only possesses *E. coli* MCP homologs necessary for chemotactic responses in swimming and swarming conditions, but also has one required for twitching motility. This homolog, *pilJ*, belongs to the *pilGHIJKL*, cluster IV, chemotactic system (15). Comparative analysis of PilJ with other MCPs revealed that this protein is similar to *E. coli* MCPs and, more specifically, is 26% identical to Tsr (15).

Like enteric MCPs (e.g. Tsr), PilJ consists of two hydrophobic amino acid sequence acting as transmembrane domains. The first transmembrane region is located at the N-terminus of the protein and the second transmembrane region is located near the middle of the protein. In addition, PilJ and Tsr share similar Kyte-Doolittle hydrophilicity-hydrophobicity plots (15). These plots show two large stretches of hydrophobic amino acids, TM1 and TM2. TM1 is 25 amino acids long found near the N-terminus and TM2 is 27 amino acids long found near the middle of the protein.

The strongest degree of identity between PilJ and Tsr is located in the C-terminal domains, especially within the highly conserved domain (HCD). HCDs are found in all MCPs and located near the middle of the cytoplasmic signaling domain (1). Genetic

evidence suggests that CheW directly interacts with this domain and CheA to form a ternary complex during chemotaxis (42). In addition to the HCD, two methylation regions, K1 and R1, are positioned in the cytoplasmic domain of PilJ which is also similar to Tsr.

In contrast to the cytoplasmic domain, PilJ displays little similarity in the periplasmic domain to Tsr (15). Because the periplasmic domain is involved in ligand binding, this implies that Tsr and PilJ bind different ligands. Additional studies have shown that PilJ also does not possess a NIT domain present in several bacterial chemotaxis receptors, such as in *E. coli* receptors (60). NIT domain is a nitrate- and nitrite-sensing domain detected in various receptor components of signal transduction pathways. Even though the exact ligand(s) detected by PilJ has not been determined, comparative analysis of PilJ has revealed that its ligand binding domain is most similar to *E. coli* canonical MCPs (I. Zhulin, personal communication).

As stated above, *pilJ* belongs to the *pilGHIJKL* operon which is under the control of a promoter located upstream of *pilG*. Little is known about the regulation of *pilJ*; however, microarray analysis of *P. aeruginosa* revealed that *pilJ* is quorum sensing (QS) regulated in a negative fashion (70). It has been shown that genes containing a *las* box within the gene, rather than upstream in the promoter, are usually negatively regulated by quorum sensing (70). In analyzing the sequence of the *pilJ* gene, we discovered a putative *las* box, although further tests are needed to confirm the presence of this box (Wagner, personal correspondence).

As previously stated, PilJ varies from other MCPs in *P. aeruginosa* in its function. PilJ has been shown to be necessary for twitching motility, but is not required for

swarming motility. Macroscopic twitching motility assays showed that *pilJ* mutant cells do not exhibit a twitch zone. Also, microscopic observation of these cells revealed that they were non-motile and grew as compact colonies (15). In comparison to the wild-type (PAO1), *pilJ* mutant cells do not display external pili. Despite the absence of surface pili, *pilJ* mutant cells exhibit wild-type sensitivity to some pili specific phage (PO₄, B3, and F116) (15, 71). This sensitivity led us to postulate the presence of tiny, "stubs" of surface pili too short to be sheared off and recovered for immunoblot analysis. The presence of these "stubs" of pili may also account for the existence of certain strains of *P. aeruginosa* (i.e. PAK) in which *pilJ* mutant cells are able to twitch, but demonstrate aberrant twitching motility (71). Interestingly, *E. coli* MCP mutants, unlike *pilJ* mutants, remain motile. Thus *P. aeruginosa*'s PilJ appears to have dual function and is involved not only in chemotaxis, but motility in general.

Presently, the exact location of PilJ within the cells of *P. aeruginosa* has not been determined. Even though PilJ has not been localized, the presence of pili and the pili assembly ATPases (PilB, PilT, and PilU) at the cellular poles suggests that PilJ may also be found in these regions (9). In addition, polar localization of chemoreceptor complexes in *E. coli* has shown that Tsr receptors are located at the polar region of the cells (45). These receptors are organized into groups of three, with extensive interaction between the dimer interfaces. This clustering of receptors, referred to as a "trimer of dimers", is thought to be responsible for the cell's high sensitivity to changes in the chemical environment and wide dynamic range (37).

Furthermore, three dimensional electron microscopic imaging of the polar region of *E. coli* revealed the presence of membrane invaginations. These invaginations

are believed to facilitate protein-protein interaction of chemoreceptors, such as Tsr (41). Also, this three-dimensional network of membranes may serve to stabilize the formation of the CheA and CheW complex with the receptor. Such membrane invaginations, as well as clustering of chemoreceptors, has not been identified in *P. aeruginosa*. However, if future research identifies PilJ at the polar regions of the cell, this may suggest that there is clustering of chemoreceptor molecules and the presence of membrane invaginations in *P. aeruginosa*.

CHAPTER I

PilJ controls the assembly and positioning of Type IV pili in

Pseudomonas aeruginosa

Chapter I

PilJ CONTROLS THE ASSEMBLY AND POSITIONING OF TYPE IV PILI IN

Pseudomonas aeruginosa

Abstract

Pseudomonas aeruginosa has many genes involved in twitching and swarming motility. Previous studies have determined that the gene *pilJ* is necessary for twitching motility, but its role in swarming motility has not been established. Through macroscopic and microscopic twitching motility assays using multiple wild-type strains and their isogenic *pilJ* mutants, we confirmed that *pilJ* is absolutely necessary for twitching motility. Competition assays using GFP-labeled wild-type PAO1 and *pilJ* (FA6) mutant cells, demonstrated that wild-type cells are unable to restore twitching motility in *pilJ* mutant cells or to rescue these cells. In addition, transmission electron microscopy and western blot analysis of wild-type and *pilJ* mutant cells revealed the absence of external pili on mutant cells; however, *in situ* immunofluorescence showed the presence of short pili on the surface of these mutant cells. *In situ* immunofluorescence also revealed that *pilJ* mutant cells possess pilin complexes at both poles, whereas wild-type cells have these complexes at one pole. Furthermore, western blot analysis of whole cell lysates demonstrated that wild-type and mutant cells produce equivalent amounts of pilin. These results suggest that PilJ is not involved in pilin production, but in the assembly of pili on the surface. In terms of swarming motility, *pilJ*

mutant cells swarmed at a faster rate than wild-type cells. Western blot analysis and transmission electron microscopy of flagella revealed that wild-type and *pilJ* mutant cells produce equivalent amounts of flagellin and assemble one polar flagellum on the surface during swarming conditions. Expression of *pilJ* is not necessary for biogenesis of flagella, but plays a role in regulating swarming motility.

METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 1. Media used for each assay is described in each corresponding section.

Twitching motility assays. (i) Macroscopic. LB (1% agar) plates were stab inoculated with a needle to the bottom of the Petri dish with overnight culture of plate grown *P. aeruginosa* cells and incubated for 24 hours at 37°C. LB plates containing 60µg/ml carbenicillin were used for complemented strains containing wild-type *pilJ* on a plasmid.

(ii) Microscopic. Strains were inoculated onto blocks of LB (1% agar) and covered with glass cover slips. Following 5 hour incubation at 37°C, the outermost regions of the motile zone were examined using Olympus BH2-RFC fluorescence microscope at a magnification of 600X.

(iii) Motility rescue. *P. aeruginosa* cells (wild-type, *pilJ* mutant [FA6], *pilA* mutant [PAO-NP], and wild-type PAO1 cells expressing GFP) were grown overnight in LB broth and diluted the following day to an OD_{600nm} of 0.3. Equal numbers of GFP labeled wild-type and non-labeled mutant cells were mixed together. 1µl of the cell mixture was placed on a LB (1% agar) square and covered with a cover slip as described above (ii). Following a 5 hour incubation at 37°C, the twitch zone was observed using an

Olympus BH2-RFC fluorescence microscope and DIC at a magnification of 600X. These were compared to controls in which equal numbers of GFP labeled wild-type PAO1 and non-labeled wild-type PAO1 cells were mixed.

Swarming motility assay. Swarm agar plates were composed of nutrient broth supplemented with 0.5% glucose and solidified with 0.5% agar. Plates were allowed to solidify for 5 hours and were stab inoculated using a needle. Plates were incubated for 24 hours at 37°C.

Isolation of cell protein for immunoblot analysis. (i) Whole cell lysates of pilin and flagellin. Whole cells lysates used to test for pilin were harvested from LB broth or LB (1% agar) plates and solubilized by boiling for 10 min in Laemmli sample buffer. Whole cells lysates used to test for flagellin were harvested from swarm (0.5% agar) plates and solubilized by boiling for 10 min in Laemmli sample buffer.

(ii) Purification of surface pili. Surface pili were isolated as previously described (15). LB (1.5% agar) plates were inoculated and grown overnight at 37°C. The following day, cells were re-suspended in dH₂O. Pili were removed from the surface of the cells by vortexing and cells were removed by centrifugation. Pili present in the supernatant were precipitated by lowering the pH to the isoelectric point of pilin (pH 4.5) and incubated on ice for 2 hours. Pili were recovered by centrifugation at 12,000 x g for 20 min.

Immunoblot analysis of pilin. Equivalent amounts of total cell protein were separated by SDS-PAGE on a 12% Tris-HCl gel. Proteins were transferred to a nitrocellulose membrane by electroblotting. Purified whole cell pilin and surface pilin protein was detected using antipilin antiserum (a gift of Randy Irvin, U of Alberta, Canada) as the primary antibody followed by the secondary antibody (anti-rabbit immunoglobulin G conjugated to alkaline phosphatase) (Promega, Madison, WI).

Immunoblot analysis of flagellin. Equivalent amounts of cell protein were separated by SDS-PAGE on a 12% Tris-HCl gel. Proteins were transferred to a nitrocellulose membrane by electroblotting. Purified flagellin protein was detected using anti-flagellin B antibody as the primary antibody (a gift of Daniel Wozniak, Wake Forest University School of Medicine, Winston-Salem, NC) followed by the secondary antibody (anti-rabbit immunoglobulin G conjugated to alkaline phosphatase) (Promega, Madison, WI).

Transmission electron microscopy. (i) Image analysis of pili. LB (1% agar) plates were inoculated and covered with sterile glass cover slips. Plates were allowed to incubate for 5 hours at 37°C. Following the 5 hour incubation, cover slips were removed and carbon-coated formvar grids (400 mesh) were touched to the surface of twitch zones on LB (1% agar) plates. Each grid was rinsed in dH₂O and negatively stained with 0.5% uranyl acetate for 4 min. Grids were examined using a JEOL JEM-100CXII transmission electron microscope.

(ii) **Image analysis of pili using PO₄ phage.** LB broth was inoculated and incubated overnight at 37°C and 250rpm. The following day, each culture was diluted to an OD_{600nm} of 0.5 in LB broth. A 1:50 dilution of PO₄ phage (10⁹ pfu/ml) was made in LB broth. Diluted PO₄ and *P. aeruginosa* cells were mixed in equal volumes and incubated for 5 min at room temperature to allow for absorption of the phage. Carbon-coated formvar grids (400 mesh) were floated on top of this mixture for 1 min to promote the attachment of cells to the grid. Each grid was rinsed 1X in dH₂O and negatively stained with 0.5% uranyl acetate for 4 min. Grids were examined using a JEOL JEM-100CXII transmission electron microscope.

(iii) **Image analysis of flagella.** Swarm (0.5% agar) plates were stab inoculated with a needle and were incubated for 24 hours at 37°C. Carbon-coated formvar grids (400 mesh) were touched to the surface of the swarm zone on swarm (0.5% agar) plates and rinsed in dH₂O to remove excess cells. Each grid was negatively stained with 0.5% phosphotungstic acid for 50 sec and examined using a JEOL JEM-100CXII transmission electron microscope.

***In situ* immunofluorescence of surface pili.** *P. aeruginosa* cells (wild-type PAO1 and *pilJ* mutant [FA6]) were grown overnight in LB broth at 37°C. The following day, LB (1% agar) plates were inoculated and covered with a sterile glass cover slip. Plates were allowed to incubate for 5 hours at 37°C. Cells were harvested from each plate and fixed with 2.5% formaldehyde for 15 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 for 45 min at room temperature. Pilin protein was detected using antipilin antiserum (a gift of Randy Irvin, U of Alberta, Canada) as the primary

antibody followed by the secondary antibody (Qdot® 525 goat F(ab')₂ anti-rabbit IgG conjugate (H+L) *highly cross-absorbed) (Quantum Dot Corporation, Hayward, CA). Cells were observed using an Olympus BH2-RFC fluorescence microscope and DIC at a magnification of 600X.

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

Stain or plasmid	Relevant genotype or description	Source or reference
PAO1 (AD)	Wild-type, twt+	Darzins 1994
FA6	PAO1 $\Delta pilJ::Tc^R$, twt-	Darzins 1994
FA6 (pUCP18:: <i>pilJ</i>)	Complemented <i>pilJ</i> mutant Cb^R , twt+	This study
PAO1 (pUCP18:: <i>pilJ</i>)	Wild-type overexpressing <i>pilJ</i> Cb^R , twt+	This study
PAO1 (pUC819)	Wild-type vector control Cb^R , twt+	This study
PAO1 (pMRP9.1)	Wild-type pUCP18::GFP plasmid Cb^R , twt+	B. Iglewski
pUCP18	pUCP18 with 1.8Kb stabilizing fragment of pR01614 (Cb^R)	B. Iglewski
pUCP18:: <i>pilJ</i>	pUCP18 with 2.1Kb BamHI and HindIII <i>pilJ</i> fragment	Lab collection
pMRP9.1	pUCP18 with GFP Cb^R	B. Iglewski

RESULTS

Macroscopic and microscopic twitching motility. Macroscopic twitching motility assays were performed using the following strains: *P. aeruginosa* PAO1 wild-type (AD), isogenic *pilJ* mutant (FA6), the complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*), wild-type cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (vector control) to determine their ability to twitch. Twitching motility is noted by the presence of a “halo” surrounding a surface colony. This “halo” represents movement at the interface between the agar and plate. In this assay, wild-type PAO1 (AD) cells displayed a surface colony surrounded by a distinct twitch zone (Figure 8A). In contrast, *pilJ* mutant (FA6) cells exhibited surface growth, but did not display an active twitch zone (Figure 8B). In the complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*), twitching motility was restored to wild-type PAO1 (AD) levels (Figure 8C). Additionally, cells overproducing *pilJ* (PAO1 + pUCP18::*pilJ*) exhibited a reduced twitch zone compared with wild-type PAO1 (AD) cells (Figure 8D).

Twitching motility of wild-type PAO1 (AD) cells was also confirmed microscopically. Using bright field microscopy (600X), rafts of cells were observed twitching away from the point of inoculation (Figure 9A). Additionally, an examination of *pilJ* mutant (FA6) cells revealed that *pilJ* mutant (FA6) cells did not move in rafts of

cells and appeared to be packed against one another, thus confirming the macroscopic assay results (Figure 9B). Cells overexpressing *pilJ* displayed twitching motility, but this movement was not coordinated into rafts as the wild-type cells were and appeared altered (Figure 9C).

Western Blot analysis of pilin in whole cell lysates. Western blot analysis was conducted on whole cell lysates of wild-type PAO1 (AD), *pilJ* mutant (FA6), complemented *pilJ* mutant (FA6+pUCP18::*pilJ*), cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (vector control) using anti-pilin antibodies. Whole cell lysates represented the total complement of pilin (internal and external) produced by cells. Plate grown and broth grown cells were analyzed. A single band was observed at approximately 18kd for both plate and broth grown whole cell lysates (Figures 10A and 10B). This band corresponded to the predicted molecular weight of the pilin monomer in *P. aeruginosa* which is approximately 15.5kd. The intensity of this band appeared to be equivalent in each lane and did not vary between the wild-type PAO1, *pilJ* mutant (FA6), and complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*) cells. In addition, overexpression of *pilJ* did not appear to affect the production of total cell pilin.

Western blot analysis of the presence of surface pili. Western blot analysis was used to analyze the levels of external pili present on wild-type PAO1 (AD), *pilJ* mutant (FA6), complemented *pilJ* mutant (FA6+pUCP18::*pilJ*), cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (vector control) using anti-pilin antibodies. In the lanes containing wild-type PAO1, complemented *pilJ* mutant (FA6+pUCP18::*pilJ*),

overexpressed *pilJ* cells (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (vector control), a single band was detected at approximately 18kd, the close to the predicted molecular weight of the pilin monomer in *P. aeruginosa* (Figure 11). There were no bands observed in the lane containing the *pilJ* mutant (FA6) indicating the absence of external pili.

Transmission electron microscopy of surface pili. Wild-type PAO1, *pilJ* mutant (FA6), and complemented *pilJ* mutant (FA6+pUCP18::*pilJ*) cells, and cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*) were negatively stained with uranyl acetate and examined using transmission electron microscopy. Image analysis of wild-type PAO1 revealed the presence of long filamentous extensions at the poles of the cells that appeared to be pili (Figure 12A). Pili were also observed at the poles of complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*) cells (Figure 12C). In contrast to wild-type PAO1 and the complemented *pilJ* mutant, *pilJ* mutant (FA6) cells did not have pili; however, these cells did possess flagella (Figure 12B). In addition, cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*) did not resemble wild-type PAO1 or the complemented *pilJ* mutant either. Cells overexpressing *pilJ* were twice as long, possessing several long filaments resembling pili which were not segregated at the poles, but instead were located around the periphery of the cell (Figure 12D).

Electron micrographs of negatively stained *pilJ* mutant (FA6) cells did not reveal the presence of pili comparable to wild-type cells: however, if only short “stubs” of pili were present, they might have been obscured by the negative stain. To assess whether shortened pili, or “stubs,” were present we used pili specific phage to decorate or enhance

the filaments. Wild-type PAO1 and *pilJ* mutant (FA6) cells were exposed to PO₄ pili-specific phage and then observed using transmission electron microscopy. These experiments were not conclusive because PO₄ did not attach to wild-type PAO1 pili as was expected. In addition, the majority of *pilJ* mutant (FA6) cells did not appear to have "stubs" of pili with attached PO₄ phage. Circular dark spots were observed at the pole and periphery of a couple of *pilJ* mutant (FA6) cells; however, it could not be determined if these spots were PO₄ phage (data not shown).

***In situ* Immunofluorescence.** *In situ* immunofluorescence was conducted to determine whether *pilJ* mutant (FA6) cells possess shorten pili using anti-pilin antibodies. Wild-type PAO1 cells appeared to have a long string of fluorescent dots extending from a single pole (Figure 13A). These extensions of dots are most likely pili. In contrast, *pilJ* mutant (FA6) cells did not have a fluorescent tail or pili. The majority of *pilJ* mutant (FA6) cells possessed a single fluorescent dot located at both poles. Some of these cells had only a single dot at one pole (Figure 13B).

Competition. When an equal ratio of unlabeled wild-type PAO1 (AD) and GFP-labeled wild-type PAO1 cells were mixed together, approximately an equal number of each type of cell was distributed throughout the rafts (Figures 14A and 14B). However, *pilJ* mutant (FA6) cells were not observed in the rafts located at the edge of the twitch zone when co-inoculated with GFP-labeled wild-type PAO1 cells (Figures 14C and 14D). In parallel experiments using a *pilA* mutant (PAO-NP), similar results were obtained (Figures 14E and 14F).

Swarming motility. Wild-type PAO1 (AD), *pilJ* mutant (FA6), and complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*) cells ability to swarm was determined by inoculating nutrient broth + glucose (0.5% agar) plates. Wild-type PAO1 (AD) and the complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*) cells both formed a swarming pattern characterized by branches or tentacles spreading from the inoculation point (Figures 15A and 15C). *pilJ* mutant (FA6) cells also demonstrated the ability to swarm, but these cells appeared to swarm at a faster rate and had an altered swarming pattern (Figure 15B). This pattern was distinguished by closely packed branches giving the appearance of a "sunburst".

Western blot analysis of flagellin from whole cell lysates. Western blot analysis was used to assess the levels of flagellin in whole cell lysates of wild-type PAO1, *pilJ* mutant (FA6), complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*), cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (control plasmid) cells using anti-flagellin B antibodies. Whole cell lysates represented the total complement of flagellin (internal and external) from cells grown on LB (1.0% agar) and on swarm (0.5% agar) plates. Protein from whole lysates of cells grown on LB (1.0% agar) and on swarm (0.5% agar) plates proteins were separated using SDS-PAGE and blotted. In all cases, a distinct band was observed at approximately 60kd, the predicted molecular weight of *P. aeruginosa* flagellin monomer (Figures 16A and 16B). This band was observed in each lane and did not vary in intensity between cell types. Thus as for pilin, PilJ does not affect production or accumulation of flagellin. To assess whether PilJ affected assembly or location of flagella I used TEM.

Transmission electron microscopy of flagella. Wild-type PAO1 and *pilJ* mutant (FA6) cells were negatively stained with phosphotungstic acid and observed using transmission electron microscopy. Fifty of each type of cell was examined to determine the quantity and location of flagella on the surface of the cell. Image analysis of wild-type PAO1 and *pilJ* mutant (FA6) cells did not show any noticeable difference between the two cell types. Representative cells of wild-type PAO1 and the *pilJ* mutant (FA6) all possessed a single polar flagellum (Figures 17A and 17B).

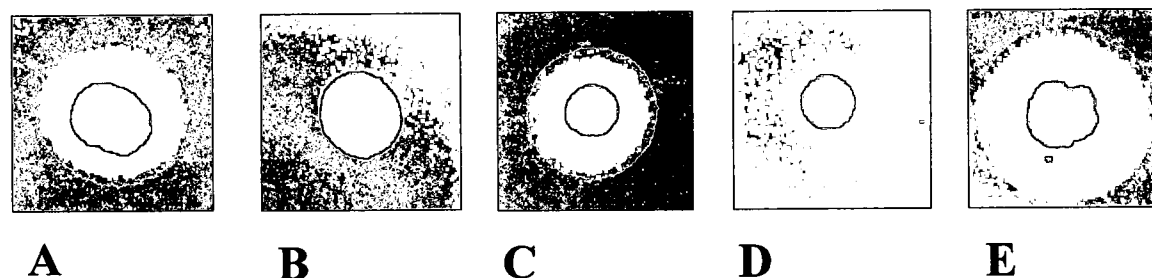


Figure 8. Macroscopic twitching motility. Twitch plates were incubated for 24 hours at 37°C. The smaller white zones represent cells growing on the agar surface. Diffuse zones (twitch zones) represent cells moving at the agar-petri dish interface. (A) PAO1 wild-type (B) FA6 (PAO1 $\Delta pilJ::Tc$) (C) FA6 (pUCP18::*pilJ*) (D) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (E) PAO1 wild-type (pUCP18) vector control.

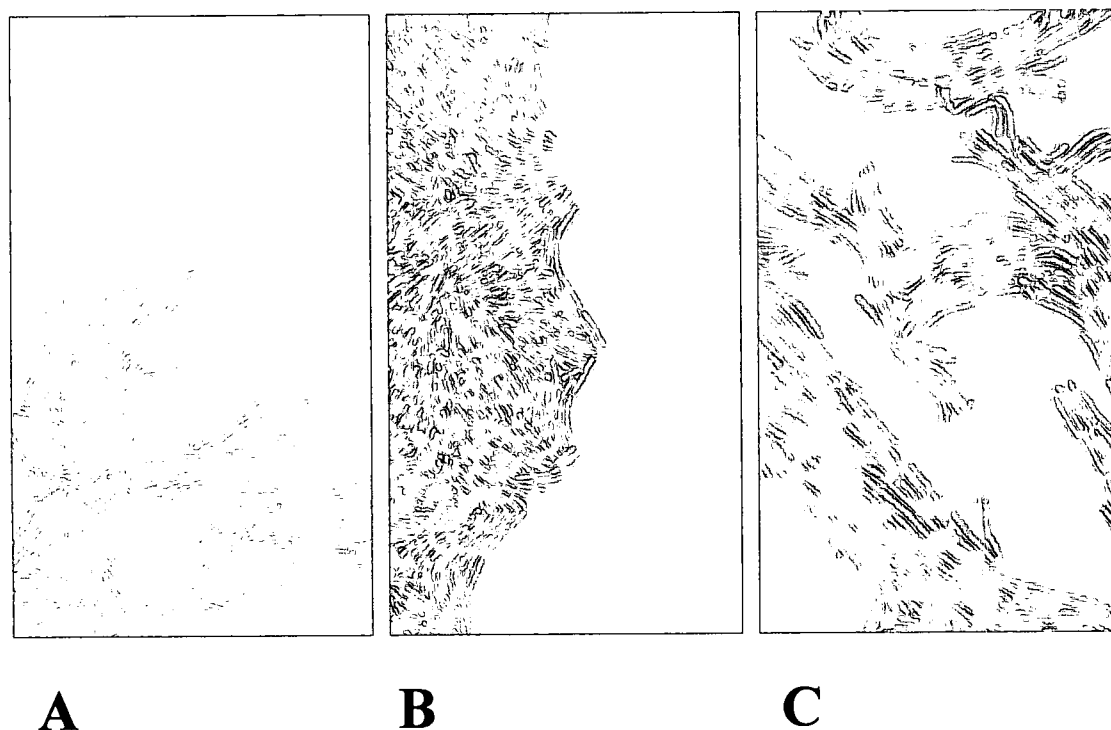


Figure 9. Microscopic twitching motility. Strains were inoculated onto blocks of LB (1% agar) plates and covered with glass coverslips. Following 5 hour incubation at 37°C, the outermost regions of the motile zone were photographed using bright-field microscopy at a magnification of 600X. (A) PAO1 wild-type (B) FA6 (PAO1 $\Delta pilJ::Tc$) (C) $PilJ^{++}$ (PAO1 wild-type + pUCP18::*pilJ*).

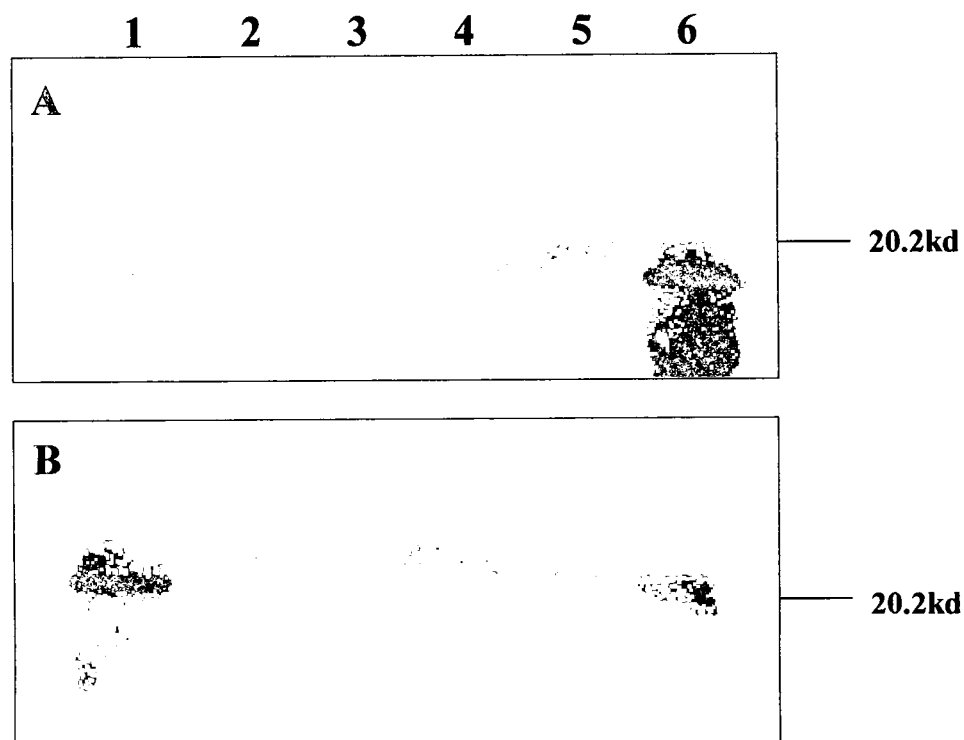


Figure 10. Western blot analysis of total pilin. Cells grown in LB broth (A) or LB agar plates (B) and whole cell suspensions solubilized by boiling for 10 minutes in Laemmli sample buffer prior to SDS-PAGE analysis on a 12% Tris-HCl gel. Bands were detected with *P. aeruginosa* antipilin antibody. Equivalent amounts of cell protein were loaded for each sample. A: (1) PAO1 wild-type (2) FA6 (PAO1 $\Delta pilJ::Tc$) (3) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (4) FA6 (pUCP18::*pilJ*) (5) PAO1 wild-type (pUCP18) vector control (6) pilin. B: (1) pilin (2) PAO1 wild-type (3) FA6 (PAO1 $\Delta pilJ::Tc$) (4) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (5) FA6 (pUCP18::*pilJ*) (6) PAO1 wild-type (pUCP18) vector control

1 2 3 4 5 6

— 20.2kd

Figure 11. Western blot of external pilin. Pili were removed from cells by vortexing and centrifugation. Pili present in the supernatant were then precipitated by lowering the pH to 4.5 and harvested by centrifugation. Precipitated pilin samples were boiled for 10 minutes in Laemmli sample buffer prior to SDS-PAGE analysis on a 12% Tris-HCl gel. Bands were detected with *P. aeruginosa* antipilin antibody. Equivalent amounts of cell protein were loaded for each sample. (1) PAO1 wild-type (2) FA6 (PAO1 $\Delta pilJ::Tc$) (3) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (4) FA6 (pUCP18::*pilJ*) (5) PAO1 wild-type (pUCP18) vector control (6) pilin

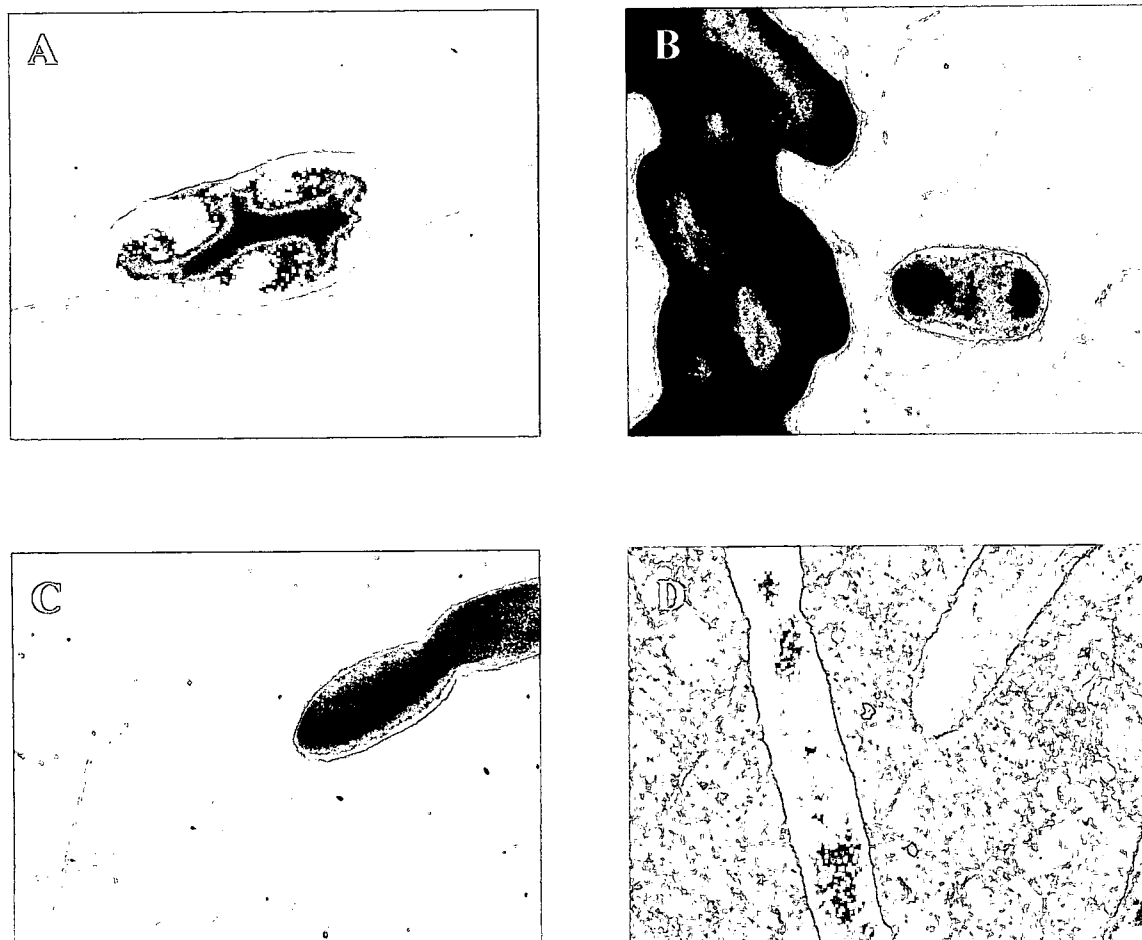


Figure 12. Transmission electron microscopy of surface pili on wild-type PAO1, *pilJ* mutant, complemented *pilJ* mutant, and cells overexpressing *pilJ*. Cells were stained using 0.5% uranyl acetate and photographed at a magnification of 14,000X. (A) PAO1 wild-type (B) FA6 (PAO1 $\Delta pilJ::Tc$) (C) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (D) FA6 (pUCP18::*pilJ*).

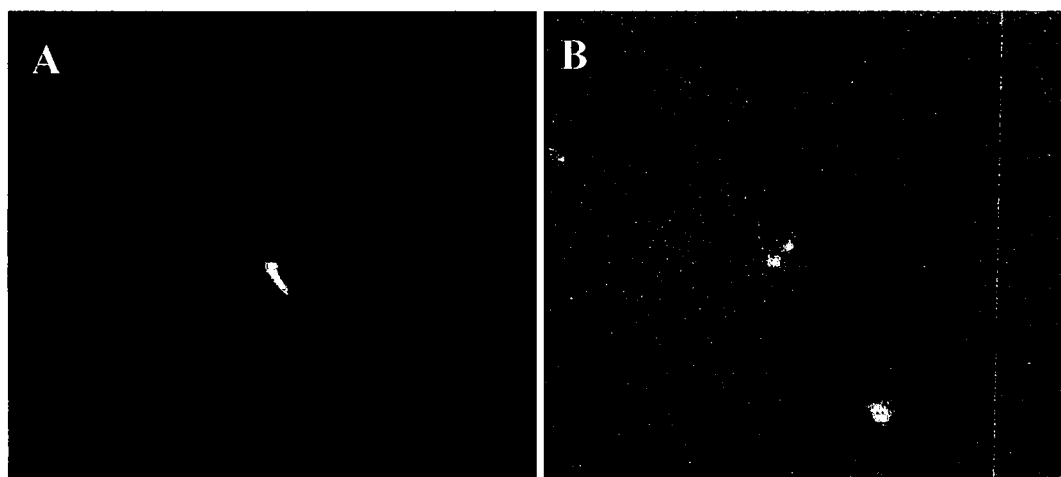


Figure 13. *In situ* immunofluorescence of wild-type PAO1 and *pilJ* mutant (FA6) cells. Cells were fixed in 2.5% formaldehyde. Pilin protein was detected using *P. aeruginosa* antipilin antibodies followed by secondary antibodies conjugated to quantum dots. Cells were visualized using fluorescent microscopy at a magnification of 600X. (A) PAO1 wild-type, and (B) FA6 (PAO1 $\Delta pilJ::Tc$).

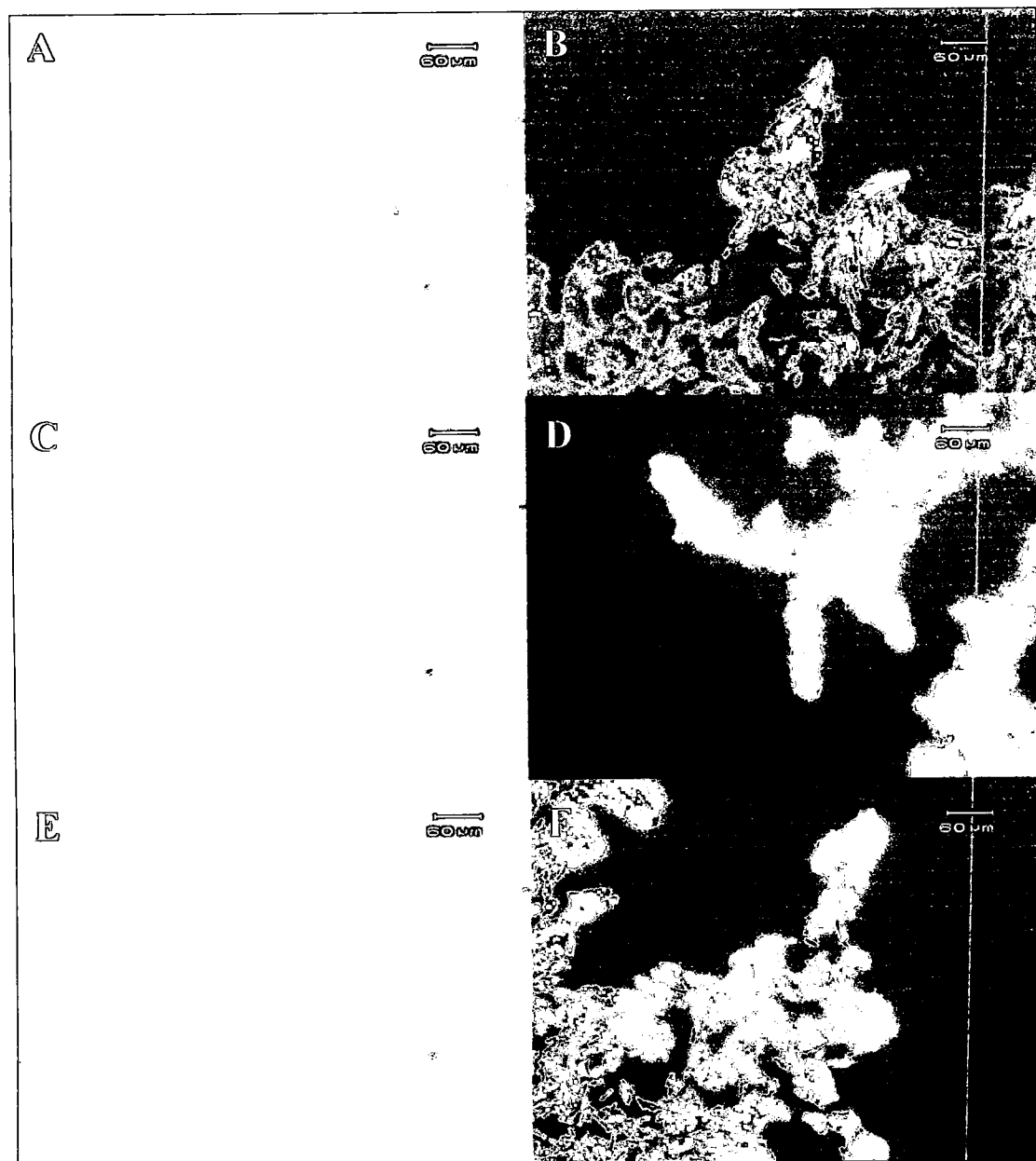


Figure 14. Rescue of *pilJ* and *pilA* mutant cells with GFP-labeled wild-type cells. Equivalent number of GFP-labeled wild-type cells and un-labeled cells were mixed together, spotted on a LB (1% agar) square, and covered with a glass coverslip. Following 5 hour incubation at 37°C, the outermost regions of the motile zone were photographed using bright-field microscopy (BFM) and fluorescence microscopy (FM) at a magnification of 600X. (A) GFP-labeled wild-type PAO1 + un-labeled wild-type PAO1 (BFM) (B) GFP-labeled wild-type PAO1 + un-labeled wild-type PAO1 (FM) (C) GFP-labeled wild-type PAO1 + un-labeled *pilJ* mutant (FA6) (BFM) (D) GFP-labeled wild-type PAO1 + un-labeled *pilJ* mutant (FA6) (FM) (E) GFP-labeled wild-type PAO1 + un-labeled *pilA* mutant (PAO-NP) (BFM) (F) GFP-labeled wild-type PAO1 + un-labeled *pilA* mutant (PAO-NP) (FM).

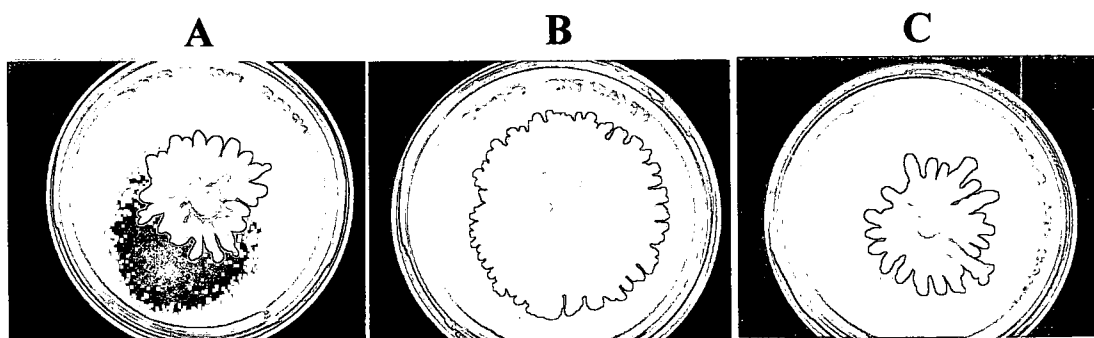


Figure 15. Swarming motility with wild-type PAO1, *pilJ* mutant, and complemented *pilJ* mutant cells. Swarm (0.5% agar) plates were stabbed inoculated and incubated overnight at 37°C. (A) PAO1 wild-type (B) FA6 (PAO1 $\Delta pilJ::Tc$) (C) FA6 (pUCP18::*pilJ*).

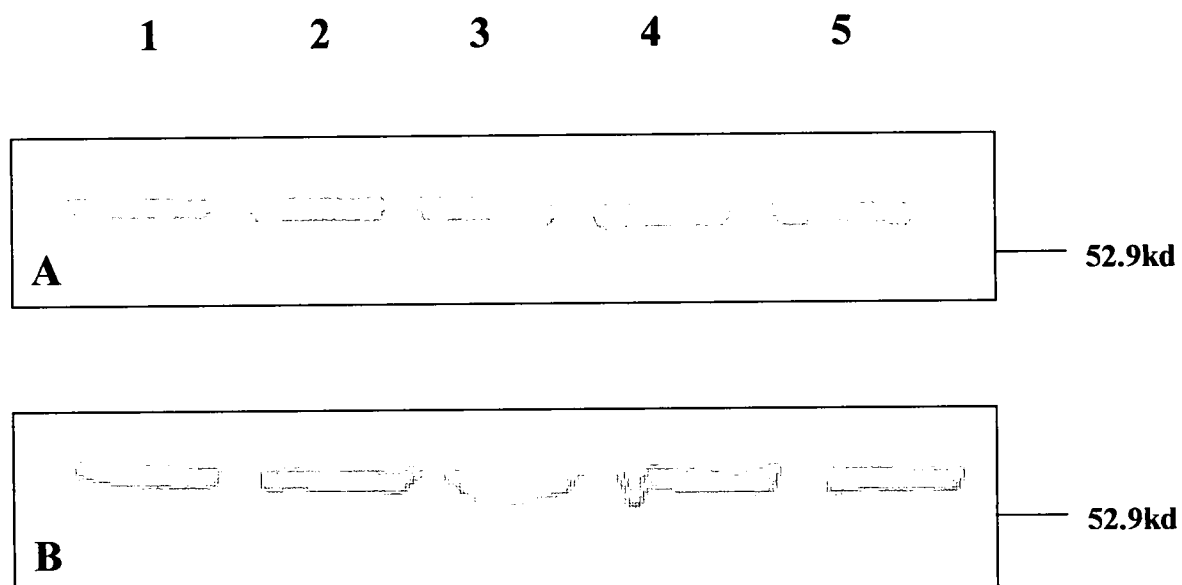


Figure 16. Western blot analysis of total flagellin. Cells were grown on LB (1% agar) (A) or swarm (0.5% agar) (B) plates. Whole cell suspensions were solubilized by boiling 10 min in Laemmli sample buffer prior to SDS-PAGE analysis on a 12% Tris-HCl gel. Bands were detected using *P. aeruginosa* anti-flagellin B antibodies. A and B: (1) PAO1 wild-type (2) FA6 (PAO1 $\Delta pilJ::Tc$) (3) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*) (4) FA6 (pUCP18::*pilJ*) (5) PAO1 (pUCP18) vector control.

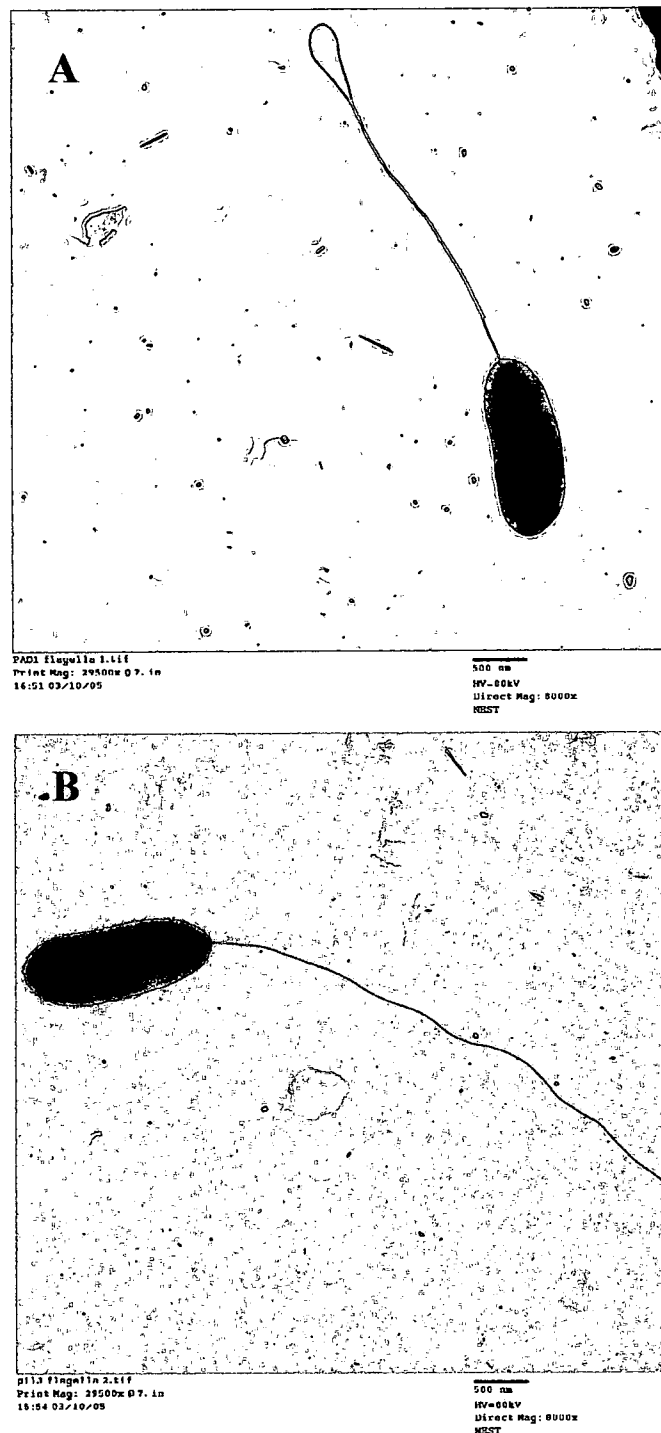


Figure 17. Transmission electron microscopy of flagella on wild-type PAO1 and *pilJ* mutant cells. Cells were negatively stained with 0.5% phosphotungstic acid and observed at a magnification of 8,000X. (A) PAO1 wild-type (B) FA6 (PAO1 $\Delta pilJ::Tc$).

DISCUSSION

Macroscopic and microscopic twitching motility assays were conducted to assess the role of PilJ in twitching motility. Wild-type PAO1 cells demonstrated the ability to twitch, while *pilJ* mutant cells were completely deficient in this function (Figures 8 and 9). These results correspond to previous studies using the same wild-type strain and *pilJ* mutant (15). Furthermore, complementation of the *pilJ* mutant restored twitching motility (Figure 8C). These findings suggest that PilJ is necessary for twitching motility in *Pseudomonas aeruginosa*. However, there is some debate over the role of PilJ. A recent study using a *pilJ* mutant in the PAK strain of *P. aeruginosa* showed that *pilJ* mutant cells were capable of twitching, but this motility was aberrant (71). While those findings do not completely correspond to previous and current studies using *pilJ* mutants, they do suggest that PilJ is necessary for normal twitching motility. Also, this difference may be due to strain variation as there are numerous studies that have shown significant genetic variation between the PAO1 and PAK strains of *P. aeruginosa*.

In order, confirm PilJ's role in twitching motility, additional *pilJ* mutants have been constructed in our lab using multiple wild-type strains of *P. aeruginosa*. These strains include PAO1 (Urs Ochner, laboratory strain), DAP119 (an environmental isolate), and GSU-3 (a clinical isolate from bacterial keratitis). The *pilJ* mutant constructed in each of these strains was entirely twitch deficient (data not shown).

Twitching motility was restored when the *pilJ* mutant was complemented. This further supports the claim that PilJ is necessary for twitching motility.

Transmission electron microscopy of wild-type and *pilJ* mutant cells was used to determine the presence or absence of surface pili on these strains. Wild-type PAO1 (AD) cells possessed external pili located at either pole of the cell (Figure 12A). In contrast, *pilJ* mutant cells did not have any surface pili (Figure 12B). When the *pilJ* mutant was complemented, pili were observed at the poles of the cell as seen in wild-type cells (Figure 12C). These results were also confirmed using western blot analysis of surface pili with antipilin antibodies (Figure 11). Previous studies using wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells yielded the same results (15). The absence of pili on the surface of *pilJ* mutants directly accounts for the inability of these cells to twitch. Additionally, it suggests that PilJ either functions in biogenesis or assembly of pili.

In order to determine whether PilJ is directly involved in the production of pilin, western blot analysis was conducted on whole cell lysates of wild-type PAO1 and *pilJ* mutants using antipilin antibodies. Wild-type PAO1 and *pilJ* mutant (FA6) cells produced equivalent amounts of pilin (Figure 10). These results indicate that PilJ functions in the assembly of pili on the surface of the cell and/or the extension and retraction of pili.

Phage sensitivity experiments of *pilJ* mutants have shown that these cells are sensitive to infection by pili specific phage (15). In contrast to transmission electron microscopy and western blot analysis, sensitivity of *pilJ* mutants to pili specific phage suggests that these cells may have the ability to extend pili under selected circumstances, or have short, "stubs" of pili, too small to be examined or sheared off for western blot

analysis. The presence of short pili would account for *pilJ* mutant cells' sensitivity to pili specific phage. To determine whether *pilJ* mutants possess short pili, cells were treated with pili specific phage (PO₄) and examined under the microscope. These results were inconclusive and PO₄ did not appear to attach to mutant or wild-type cells.

In situ immunofluorescence was conducted using antipilin primary antibodies followed by secondary antibodies conjugated with quantum dots. In this way, I hoped to directly visualize pili. Long strings of fluorescent dots, pilin, were observed extending from a single pole of wild-type PAO1 cells (Figure 13A). In contrast, long strings of pili were not seen on *pilJ* mutant cells, rather single dots were observed at the edge of one or both poles of *pilJ* mutant cells (Figure 13B). This confirms the presence of short "stubs" of pili on the surface of *pilJ* mutant cells. These results also imply that in the absence of PilJ (or the ligand) cells prepare to assemble pili at either pole, but do not extend pili. Type IV pili have never, to my knowledge, been observed to extend from both poles of wild-type cells. Thus it is PilJ that, in response to some environmental signal(s), directs extension of pili at whichever pole is optimal. In the absence of the signal (or in a *pilJ* mutant), the cell directs itself to extend pili from either pole.

Competition assays were conducted by mixing equal number of GFP-labeled wild-type PAO1 cells with *pilJ* mutant (FA6) cells. No *pilJ* mutant cells were observed at the edge of the twitch zone (Figures 14C and 14D). These results indicate that *pilJ* mutant cells cannot be rescued by wild-type PAO1 cells. To determine whether the inability of wild-type cells to rescue *pilJ* mutant cells was due to a lack of functional surface pili or to signal transduction, the experiment was repeated using a *pilA* mutant (PAO-NP) cells which do not produce any pilin or have surface pili. We found that *pilA*

mutant cells were also unable to be rescued by wild-type cells; therefore, it is the lack of pili, and not signal transduction, that is necessary for cells to move with the pack of twitching cells. This finding suggests that cells must be able to attach to other neighboring cells in order to migrate with a group of cells.

PilJ's role in other types of motility was assessed by conducting swimming and swarming assays. As seen in previous studies, swimming motility was unaffected in a *pilJ* mutant (15). Swarming motility assays of wild-type PAO1 and *pilJ* mutant (FA6) cells demonstrated varying phenotypes. *pilJ* mutant (FA6) cells consistently swarmed at a slightly faster rate than wild-type PAO1 cells (Figure 15). To determine whether this increase in swarming was related to flagellin production, western blot analysis of whole cell flagellin was done on wild-type and *pilJ* mutant cells. The amount of flagellin produced by wild-type and *pilJ* mutant cells was equivalent; therefore, PilJ does not control flagellin production (Figure 16).

Although, the mutant and wild-type produced the same amount of flagellin protein, the arrangement and number of flagella on the surface of the cell was examined to see if any difference contributed to this altered phenotype. Swarming behavior in a number of bacteria, e.g. *Proteus*, involve the production of hundreds of peritrichous or lateral flagella. *P. aeruginosa* has been reported to have lateral flagella. Transmission electron microscopy of wild-type PAO1 and *pilJ* mutant (FA6) revealed the presence of single polar flagella on the surface of both types of cells (Figure 17). Because the production of flagella subunits and assembly does not vary between the mutant and wild-type cell, the difference observed in swarming motility is not due to any mechanical variation in strains.

The factors contributing to the differences between wild-type and *pilJ* mutant cells during swarming motility have not been determined. Possibly the signal transduction pathway PilJ belongs to not only controls twitching motility and chemotaxis, but may somewhat negatively regulate swarming. During twitching motility, PilJ might transduce a signal to another MCP, such as PctA, shutting down or reducing swarming motility. In the absence of PilJ, swarming motility is not regulated by PilJ and the cells swarm at a faster rate. Such coordination between swarming and twitching signal transduction pathways makes sense as both forms of motility are used on surfaces, but under different conditions. For instance, cells moving along a non-homogenous surface would exhibit twitching motility along areas with low water content and display swarming motility on more hydrated areas.

In contrast to previous studies using *pilA* mutants, we have determined that Type IV pili are not required for swarming motility (38). In fact, the lack of pili may enhance swarming because cells would not be capable of attaching to each other. Our results are consistent with other studies using *pilA* mutant that were not defective in swarming motility (59).

CHAPTER II

Farnesol is sensed by PilJ and inhibits surface motility in *Pseudomonas aeruginosa*

Chapter II

FARNESOL IS SENSED BY PilJ AND INHIBITS SURFACE MOTILITY IN

Pseudomonas aeruginosa

Abstract

Genetic analysis of *P. aeruginosa* has shown PilJ to be homologous to enteric methyl-accepting chemotaxis proteins (MCPs). *In vivo* ^3H -methylation of *P. aeruginosa* revealed the presence of a distinct band at ~72kd in wild-type, but not in *pilJ* mutant cells. This confirms PilJ's role as a MCP in *P. aeruginosa*. *In vivo* ^3H -methylation of early log and late log wild-type cells revealed a substantially higher level of methylation in early log cells, thus, providing further evidence that PilJ is quorum sensing controlled. *In vivo* ^3H -methylation of wild-type and *pilJ* mutant cells in response to various chemicals was tested in order to determine the ligand(s) PilJ binds. The greatest response was to dioleoyl (C18:0, cis 9) and dilauroyl (C12:0) L- α -phosphatidylethanolamine [PE]. The response to both species of PE was more intense when cells were harvested from plates rather than broth. This suggests that PilJ responds to PE under environmental conditions that require twitching motility. In addition, the level of methylation of wild-type cells increased in response to *trans, trans*-farnesol, the yeast quorum sensing molecule. Furthermore, in the presence of farnesol, the ability of wild-type cells to twitch and swarm was greatly impaired. In contrast, *pilJ* mutant cells were only slightly impaired in their ability to swarm in the presence of farnesol and were able to overcome

the inhibitory effects of this chemical. These results indicate that *trans, trans*-farnesol may act as a ligand of PilJ and negatively regulate twitching and swarming motility in *P. aeruginosa*.

METHODS

***In vivo* ³H-methylation assay.** Cells were grown in MSS medium supplemented with methionine to induce the uptake of the labeled methionine. Cells were harvested and washed 3X in CTX buffer to remove any residual MSS media. Chloramphenicol was added at 200µg/ml to inhibit *de novo* protein synthesis. 10µCi of L-[*methyl*-³H]methionine was added to washed cells and incubated for 30 min at 37°C. Cells were pelleted by centrifugation for 5 min at 12,000 x g. Following centrifugation, cells were solubilized by boiling 10 min in Laemmli sample buffer and proteins separated by SDS-PAGE on a 12% Tris-HCl gel. Following separation, the gels were soaked in Amplify (Amersham Pharmacia, Piscataway, NJ) to enhance labeled proteins and dried for 55 min at 60°C. Hyperfilm was exposed to the dried gel for a period of 3 to 14 days and developed. To test the effects of various chemical stimulants or repellents, cells were exposed to each chemical, respectively, following addition of L-[*methyl*-³H]methionine. The concentration and exposure time varied for each chemical tested (Table 2).

Twitching motility assay in the presence of *trans, trans*-farnesol. LB (1% agar) plates were made with and without 25µM of *trans, trans*-farnesol. Plates were stab inoculated with a needle to the bottom of the Petri dish and incubated for 24 hours at 37°C.

Swarming motility assay in the presence of *trans, trans*-farnesol. Swarm agar was composed of nutrient broth supplemented with 0.5% glucose and solidified with 0.5% agar. Plates were made with and without 25 μ M *trans, trans*-farnesol and allowed to solidified for 5 hours before stab inoculating. Swarm plates were incubated 48 hours at 37°C.

RESULTS

***In vivo* ^3H -methylation of wild-type PAO1 and *pilJ* mutant cells.** Wild-type PAO1 (AD) and *pilJ* mutant (FA6) were labeled with L-[*methyl*- ^3H] methionine. Several methylated proteins were observed in the lanes containing whole cell lysates from both cell types. In the lane containing wild-type PAO1 (AD), a distinct band was observed at approximately 72kd (Figure 18). This band corresponds to the reported molecular weight of PilJ (15). In contrast to the wild-type cells, no detectable methylated proteins were observed at 72kd in *pilJ* mutant (FA6) cells. Coomassie stained gels indicate equivalent levels of protein for wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells.

An additional methylated protein band was observed at approximately 18kd (Figure 18). This band corresponds to the molecular weight of the pilin monomer (~15kd) and was also present in the lane containing the *pilJ* mutant (FA6). However in wild-type PAO1, methylation of this band was relatively more intense than in the mutant. Western blot analysis using antipilin antibodies also confirmed this band to be pilin.

***In vivo* ^3H -methylation of early log vs late log phase wild-type PAO1 cells.** Early log and late log phase wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine. Several proteins were labeled in the lanes containing early and log cells. The methylation pattern was the same between early and late log phase cells; however,

the level of methylation of these proteins was much more intense for early log phase cells (Figure 19).

***In vivo* ^3H -methylation of wild-type PAO1 and *pilJ* mutant cells in response to L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0).** Plate grown wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0). Stimulated and unstimulated cells were examined. In the lanes containing wild-type PAO1 (AD), a distinct methylated band was observed at approximately 72kd, the predicted molecular weight of PilJ. This band was more intense in the stimulated cells than the unstimulated cells (Figure 20).

In the lanes containing the *pilJ* mutant (FA6), a band was also observed at approximately 72kd; however, this band was substantially less distinct and intense in comparison to the wild-type (Figure 20). The intensity of this band did not vary between unstimulated and stimulated *pilJ* mutant (FA6) cells.

***In vivo* ^3H -methylation of plate grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0).** Wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0) for 30 min. Wild-type PAO1 cells were stimulated by PE (C12:0) at various concentrations to determine the concentration of PE that resulted in the maximum methylation of PilJ. The intensity of the PilJ band

was the greatest in the cells stimulated with 20 μ g/ml PE (C12:0) followed by 5 μ g/ml, 30 μ g/ml, and 10 μ g/ml PE (C12:0) (Figure 21).

***In vivo* ^3H -methylation of broth grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0).** Wild-type PAO1 (AD) broth grown cells were labeled with L-[*methyl*- ^3H] methionine and exposed to various concentrations of L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0) for 30 min. In contrast to plate grown cells, the level of methylation of PilJ did not significantly vary between stimulated and unstimulated cells (Figure 22). Interestingly, the level of methylation of PilJ appeared slightly greater in the unstimulated cells than the stimulated cells. These results suggest that PilJ's response to PE may depend on environmental conditions.

***In vivo* ^3H -methylation of plate grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dioleoyl (C18:0, [cis] 9).** Wild-type PAO1 (AD) broth grown cells were labeled with L-[*methyl*- ^3H] methionine and exposed to various concentrations of L- α -phosphatidylethanolamine [PE] dioleoyl (C18:0, [cis] 9). Cells were exposed to this chemical for two different time intervals, 2 min and 30 min. Several methylated bands were observed in each lane (Figure 23). The intensity of these bands was greater when examining stimulated cells rather than unstimulated cells. In addition, the level of methylation was the highest in those cells exposed to PE (C18:0) for 30 min rather than 2 min. The concentration of PE (C18:0) that produced the greatest response varied depending on the time of exposure. Those

cells stimulated by PE (C18:0) for 2 minutes were more methylated at a concentration of 2 μ g/ml. In contrast, those cells stimulated for 30 minutes were relatively more methylated at a concentration of 0.2 μ g/ml than at a concentration of 2 μ g/ml.

Further *in vivo* ^3H -methylation of wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells produced variable results (data not shown). It could not be determined whether the *pilJ* mutant (FA6) varied in its methylation pattern in response to PE (C18:0).

***In vivo* ^3H -methylation of wild-type PAO1 in response to *N*-acetylglucosamine.**

Wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to *N*-acetylglucosamine (NAG) at a concentration of 10mM. Several bands were labeled in the lanes containing stimulated and unstimulated cells. There appeared to be a noticeable increase in the intensity of these bands in the presence of *N*-acetylglucosamine (Figure 24).

Additional *in vivo* ^3H -methylation of wild-type PAO1 (AD) and *pilJ* mutant (FA6) produced variable results. Unusual results were obtained when the *pilJ* mutant (FA6) was treated with NAG. In comparison to unstimulated cells, stimulated cells exhibited an increase in the level of methylation of bands located at approximately 85 and 20kd and a decrease in methylation of a band located at 91kd (data not shown).

***In vivo* ^3H -methylation of wild-type PAO1 in response to chitin.**

Wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to chitin, a polymer of *N*-acetylglucosamine. Cells were stimulated with chitin (1mg/ml or 0.1mg/ml) for either 2 or 5 min. Several labeled protein bands were observed in each lane. The

intensity of these bands did not vary significantly between stimulated and unstimulated cells, except when cells were exposed to 1mg/ml of chitin for 2 min (Figure 25). The level of methylation of cells treated with 1mg/ml of chitin for 2 min was much more intense than the unstimulated cells.

***In vivo* ^3H -methylation of wild-type PAO1 in response to 3-oxo- C_{12} HSL.** Wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to 3-oxo- C_{12} HSL (OdDHL) for 30 min. Cells were stimulated with OdDHL at various concentrations. It was difficult to identify distinct protein bands in each lane. The level of methylation of stimulated cells was slightly greater than unstimulated cells (Figure 26). However, this increase in methylation did not significantly vary over the range of concentrations (0.25nM – 0.5nM) of OdDHL tested.

***In vivo* ^3H -methylation of wild-type PAO1 and *pilJ* mutant in response to *trans*, *trans*-farnesol.** Wild-type PAO1 (AD) cells were labeled with L-[*methyl*- ^3H] methionine and exposed to *trans*, *trans*-farnesol at two concentrations, 10 μM and 25 μM , for 2 or 5 min. Several methylated protein bands were observed in the lanes containing unstimulated and stimulated cells. The intensity of these bands slightly increased with the addition of farnesol (Figure 27). The greatest response to farnesol was observed when farnesol was added at a concentration of 25 μM for 30 min. In contrast, the least response was observed when farnesol was added at a concentration of 10 μM for 30 min. Wild-type PAO1 (AD) reaction to farnesol does not appear to be dependent on exposure time, but rather the concentration of farnesol.

In addition to wild-type PAO1 cells, *pilJ* mutant (FA6) cells were also labeled with L-[methyl-³H] methionine and exposed to *trans, trans*-farnesol. Several methylated protein bands were observed in the lanes containing unstimulated and stimulated cells. The intensity of these methylated proteins, however, did not vary when *pilJ* mutant (FA6) cells were exposed to farnesol for 5 or 30 min (Figure 28).

***In vivo* ³H-methylation of wild-type PAO1 and *pilJ* mutant (FA6) in response to various chemical/physical stimulants.** Wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells were labeled with L-[methyl-³H] methionine and exposed to several physical/chemical stimulants; such as α -aminoisobutyric acid, L-arginine, L-leucine, deferroxamine, 3-oxo-C₄ HSL, PO₄ phage, and antipilin antibodies. The time of exposure and concentration used varied for the different chemical/physical stimulants. These results are summarized in Table 2. No reproducible positive results were obtained using these additional chemical/physical stimulants.

The effects of *trans, trans*-farnesol on twitching and swarming motility. LB (1.0% agar) plates with and without 25 μ M of *trans, trans*-farnesol were inoculated with wild-type PAO1 (AD) cells. Active twitch zones were observed on plates with and without farnesol; however, there was a significant reduction in the size of the twitch zone on plates containing farnesol (Figure 29). *pilJ* mutant (FA6) cells were not tested as they are completely deficient in twitching motility.

The ability of wild-type PAO1 (AD) and *pilJ* mutant (FA6) cells to swarm in the presence of *trans, trans*-farnesol was determined by inoculating swarm (0.5% agar)

plates with and without 25 μ M of *trans, trans*-farnesol. Wild-type PAO1 (AD) cells demonstrated normal swarming pattern when inoculated on plates without farnesol (Figure 30A). In contrast, wild-type PAO1 cells inoculated on plates with farnesol were significantly inhibited in their ability to swarm (Figure 30B).

In comparison to wild-type PAO1 cells, *pilJ* mutant (FA6) cells also demonstrated the ability to swarm, but these cells swarmed at a faster rate (Figure 23C). When inoculated on plates containing farnesol, *pilJ* mutant (FA6) cells were slightly inhibited in their ability to swarm. However after 48 hours, *pilJ* mutant (FA6) cells exposed to farnesol were able to overcome the inhibiting effects of farnesol and were restored in their swarming capacity (Figure 30D).

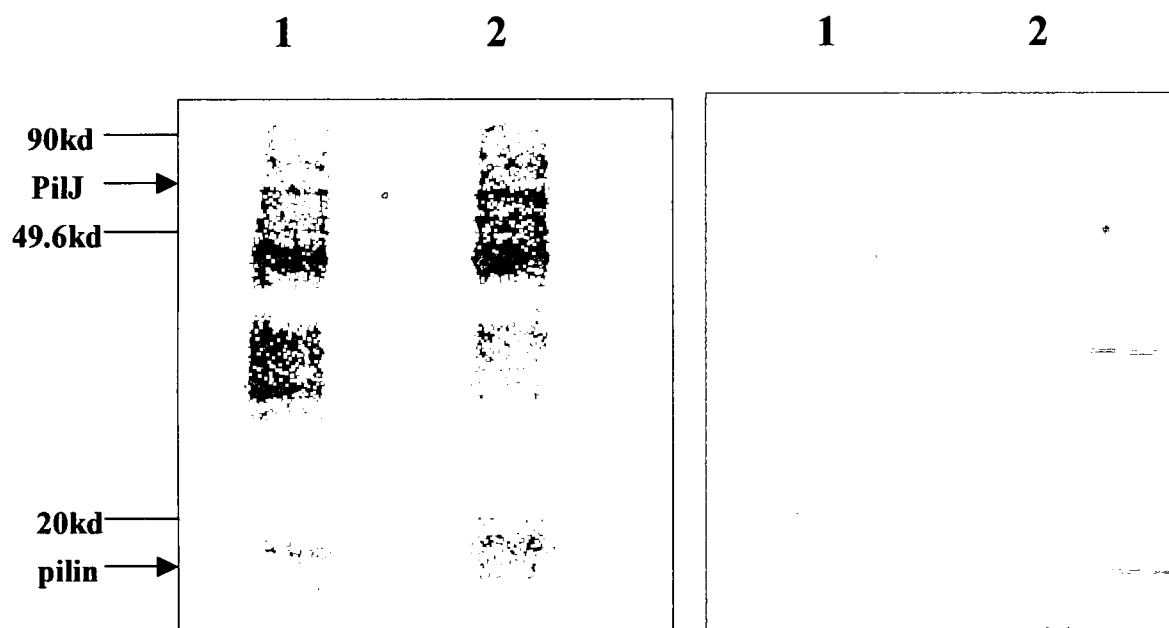


Figure 18. *In vivo* ³H-methylation of wild-type PAO1 and *pilJ* mutant (FA6) cells. The bacteria were labeled with L-[methyl-³H]methionine. The coomassie stained gel appears on the left and the fluorogram appears on the right. (1) PAO1 wild-type, and (2) FA6 (PAO1 Δ *pil J*::Tc).

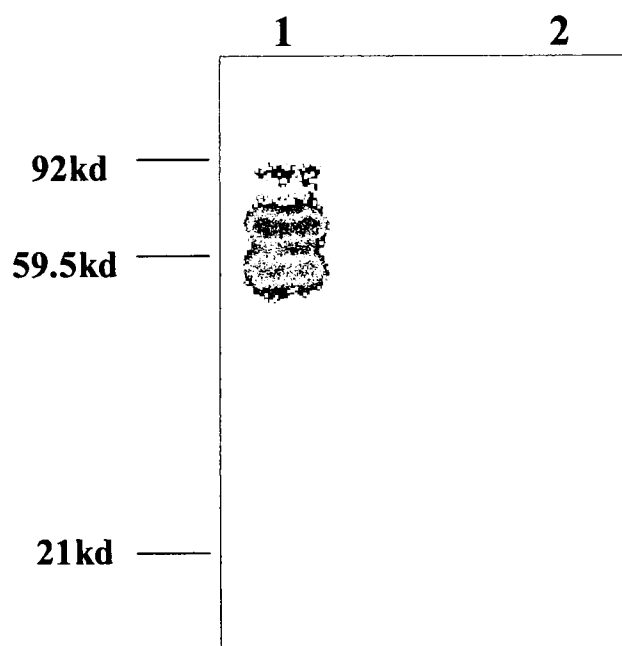


Figure 19. *In vivo* ^3H -methylation of early log vs late log phase wild-type PAO1 cells. The bacteria were labeled with L-[*methyl*- ^3H]methionine. (1) Early log phase PAO1 wild-type, and (2) Late log phase PAO1 wild-type.

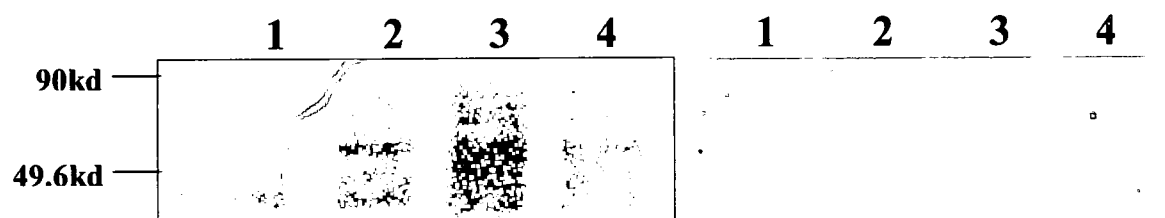


Figure 20. *In vivo* ^3H -methylation of plate grown wild-type PAO1 and *pilJ* mutant cells in response to L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0). Cells were labeled with L-[methyl- ^3H]methionine and exposed to 20 $\mu\text{g}/\text{ml}$ of PE for 30 min. The coomassie stained gel appears on the left and the fluorogram appears on the right. (1) PAO1 wild-type (control, buffer only) (2) PAO1 wild-type exposed to PE; (3) FA6 (PAO1 $\Delta pil J::Tc$) [control, buffer only], and (4) FA6 (PAO1 $\Delta pil J::Tc$) exposed to PE.

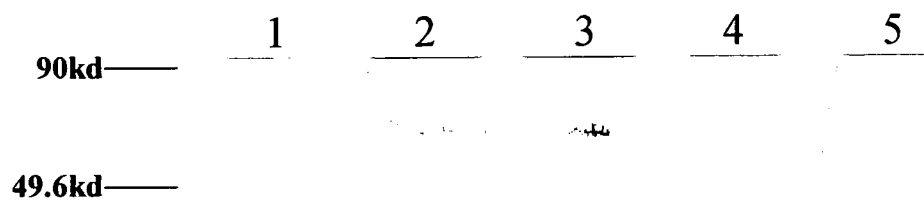


Figure 21. *In vivo* ^3H -methylation of plate grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0). Cells were labeled with L-[methyl- ^3H]methionine and exposed to PE for 30 min. (1) control, buffer only (2) 5 $\mu\text{g/ml}$ PE (3) 20 $\mu\text{g/ml}$ PE (4) 10 $\mu\text{g/ml}$ PE, and (5) 30 $\mu\text{g/ml}$ PE.

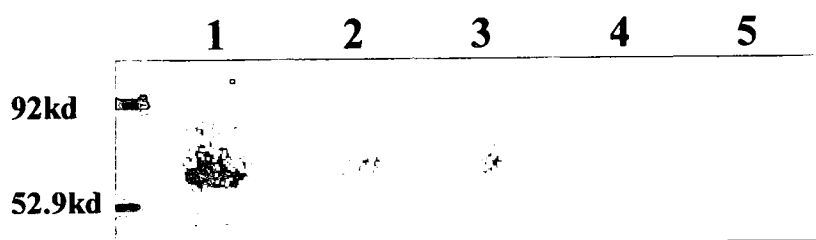


Figure 22. *In vivo* ^3H -methylation of broth grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dilauroyl (C12:0). Cells were labeled with L-[methyl- ^3H]methionine and exposed to PE for 30 min. (1) control, buffer only (2) control, 0.2 μl of chloroform:methanol (3) 2 $\mu\text{g}/\text{ml}$ PE (4) control, 2.0 μl of chloroform:methanol, and (5) 20 $\mu\text{g}/\text{ml}$ PE.

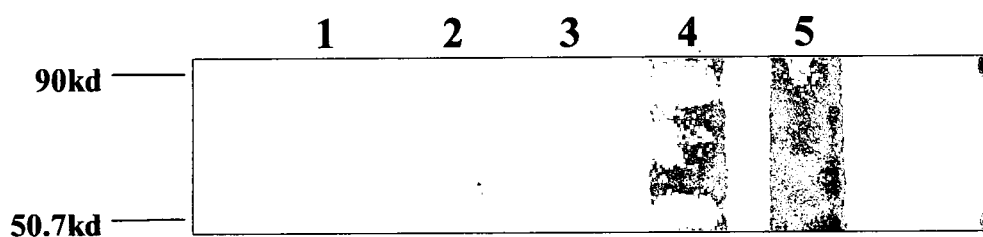


Figure 23. *In vivo* ^3H -methylation of plate grown wild-type PAO1 cells in response to various concentrations of L- α -phosphatidylethanolamine [PE] dioleoyl (C18:0, [cis] 9). Cells were labeled with L-[methyl- ^3H]methionine and exposed to PE. (1) control, buffer only (2) 2 $\mu\text{g/ml}$ PE, 2 min (3) 0.2 $\mu\text{g/ml}$ PE, 2 min (4) 2 $\mu\text{g/ml}$ PE, 30 min, and (5) 0.2 $\mu\text{g/ml}$ PE, 30 min.

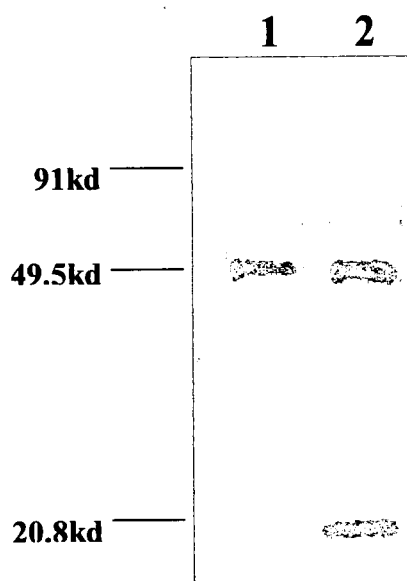


Figure 24. *In vivo* ³H-methylation of wild-type PAO1 in response to *N*-acetylglucosamine. Cells were labeled with L-[methyl-³H]methionine and exposed to *N*-acetylglucosamine (NAG) for 2 min. (1) control, buffer only, and (2) 10mM NAG.

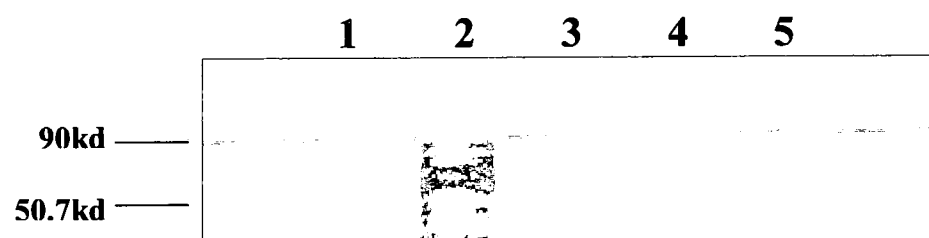


Figure 25. *In vivo* ³H-methylation of wild-type PAO1 in response to chitin. Cells were labeled with L-[methyl-³H]methionine and exposed to chitin. (1) control, buffer only (2) 1mg/ml chitin, 2 min (3) 1mg/ml chitin, 5 min (4) 0.1mg/ml chitin, 2 min, and (5) 0.1mg/ml chitin, 5min.

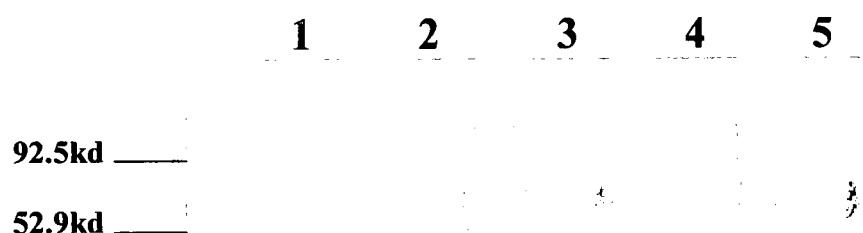


Figure 26. *In vivo* ³H-methylation of wild-type PAO1 in response to 3-oxo-C₁₂ HSL. Cells were labeled with L-[methyl-³H]methionine and exposed to 3-oxo-C₁₂ HSL (OdDHL) for 30 min. (1) control, buffer only (2) 5nm OdDHL (3) 2.5nm OdDHL (4) 0.5nm OdDHL, and (5) 0.25nM OdDHL.

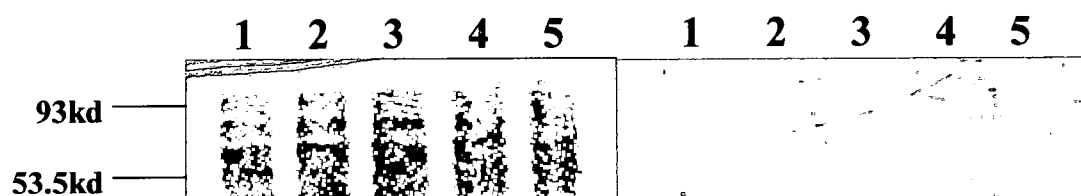


Figure 27. *In vivo* ^3H -methylation of wild-type PAO1 in response to *trans, trans*-farnesol. Cells were labeled with L-[methyl- ^3H]methionine and exposed to farnesol. The coomassie stained gel appears on the left and the fluorogram appears on the right. (1) control, buffer only (2) 10 μM farnesol, 2 min (3) 10 μM farnesol, 30 min (4) 25 μM farnesol, 2 min, and (5) 25 μM farnesol, 30 min.

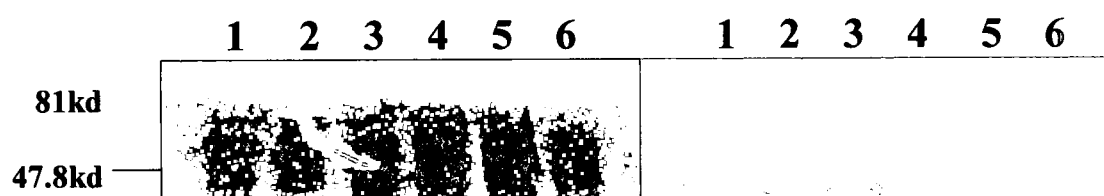


Figure 28. *In vivo* ^3H -methylation of wild-type PAO1 and *pilJ* mutant in response to *trans, trans*-farnesol. Cells were labeled with L-[*methyl*- ^3H]methionine and exposed to $25\mu\text{M}$ of farnesol. The coomassie stained gel appears on the left and the fluorogram appears on the right. (1) PAO1 wild-type (control, ethanol only) (2) PAO1 wild-type (farnesol, 5 min) (3) PAO1 wild-type (farnesol, 30 min) (4) FA6 (PAO1 Δ *pil J*::Tc) (control, ethanol only) (5) FA6 (PAO1 Δ *pil J*::Tc) (farnesol, 5 min), and (6) FA6 (PAO1 Δ *pil J*::Tc) (farnesol, 30 min).

Table 2. *In vivo* ^3H -methylation of wild-type PAO1 and *pilJ* mutant (FA6) in response to various stimuli.

Strain	Stimulant						
	AIB ¹	L-arginine	L-leucine	deferrioxamine	OHHL ²	PO ₄ phage	Anti- <i>pln</i> antibodies
Wild-type PAO1	+ ^a	+ ^b /NC	+ ^b	+ ^c /NC	+ ^d /NC	NC	+ ^e /NC
FA6 (<i>pilJ</i>) mutant	ND	+ ^b /NC	+ ^b /NC	ND	ND	ND	ND

(+) = increase in the level of methylation, NC = no change in the level of methylation, ND = response to this chemical was not determined, ¹ = α -aminoisobutyric acid, ² = 3-oxo-C₄ HSL, ^a = (+) at 10mM and 100mM for 10 min, ^b = 0.1mM and 1.0mM for 2 min and 15 min, ^c = 2.0mM for 2 min, 5 min, and 15 min, ^d = 10 and 100nM for 30 sec, 2 min, 5 min, and 30 min, ^e = 1.6×10^3 for 5 sec, 30 sec, 2 min, and 5 min



Figure 29. Twitching motility of wild-type PAO1 in the presence of *trans, trans*-farnesol. LB (1% agar) plates with and without 25 μ M of farnesol were stab inoculated and incubated for 48 hours at 37°C. (A) PAO1 wild-type, control, and (B) PAO1 wild-type (25 μ M farnesol).

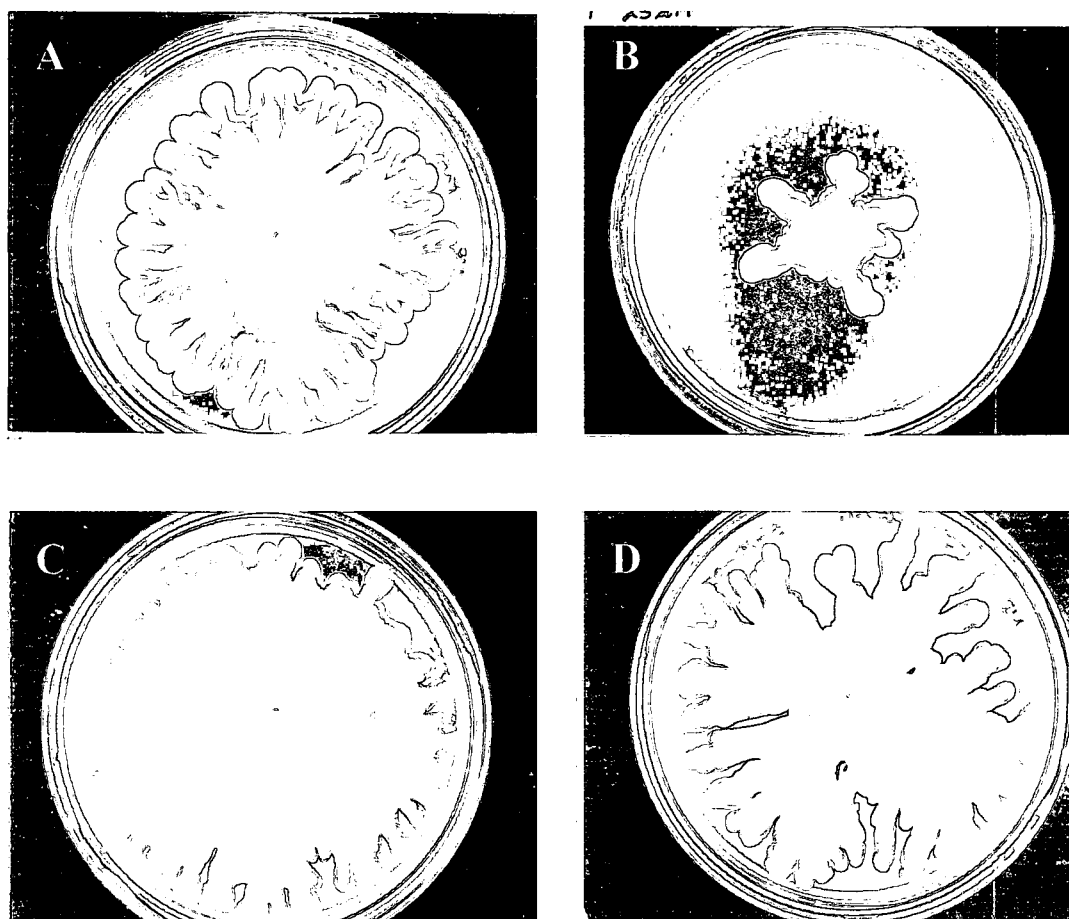


Figure 30. Swarming motility of wild-type PAO1 and *pilJ* mutant cells in the presence of *trans, trans*-farnesol. Swarm (0.5% agar) plates with and without farnesol were stab inoculated and incubated for 48 hours at 37°C. (A) PAO1 wild-type (B) PAO1 wild-type (25μM farnesol) (C) FA6 (PAO1 $\Delta pilJ::Tc$), and (D) FA6 (PAO1 $\Delta pilJ::Tc$) (25μM farnesol).

DISCUSSION

In vivo ^3H -methylation of wild-type PAO1 and *pilJ* mutant (FA6) cells was tested to determine if PilJ functions as a true MCP. With the incorporation of L-[*methyl*- ^3H methionine], a distinct band was observed at approximately 72kd in wild-type PAO1 proteins, but was absent in *pilJ* mutant proteins (Figure 18). The predicted molecular weight of PilJ is 72kd which corresponds to the methylated protein band in wild-type cells (15). Methylation of PilJ protein confirms the presence of functional methylation regions commonly found in MCPs and identified by sequence analysis of PilJ. Previous studies revealed that PilJ is 26% identical to Tsr with the strongest identity to the highly conserved domain, or HCD (15). Within this domain, two regions, K1 and R1, are proposed methylation regions in PilJ. Methylation of PilJ and comparative analysis to other MCPs confirms PilJ's role as an MCP involved in the Type IV pili signal transduction pathway.

An additional methylated protein was identified at approximately 18kd (Figure 18). This protein was observed in wild-type PAO1 and *pilJ* mutant (FA6) cells; however, the overall intensity of this protein band was greater for wild-type than *pilJ* mutant cells. In contrast to the fluorogram, this band appeared to be equivalent between the wild-type and mutant cells in the coomassie stained protein gels. This protein corresponded to the predicted molecular weight of the pilin subunit which was confirmed by western blot

analysis. Prior to pilus assembly, this subunit is modified by PilD which cleaves the leader sequence and then adds a methyl group. An increase in the methylation levels of wild-type pilin, compared to pilin produced by *pilJ* mutant cells suggest that PilJ may affect PilD activity. For example, if during sensory adaptation the methyl groups removed by ChpB are transferred from PilJ to PilD, then in the absence of *pilJ* this transfer would not occur. This would account for the observed decrease in the methylation of pilin subunits in the *pilJ* mutant.

The existence of such a methyl relay between PilJ and PilD has not been verified; however, this is not the only mechanism by which PilJ interacts with PilD. Because PilJ belongs to a signal transduction pathway, it may transduce a signal directly or indirectly activating PilD. In the absence of *pilJ*, no signal would be transduced to PilD and pilin would not be methylated.

Interactions between PilJ and PilD may explain how PilJ's function in assembly of pili on the surface of the cell. Assuming this communication between PilJ and PilD, either through a methyl relay or signal transduction pathway, is necessary for Type IV pili biogenesis, we would expect *pilD* mutants to have the same twitching motility phenotype as *pilJ* mutants. Previous studies using *pilD* mutants have shown that these cells are deficient in twitching motility (54). These findings support the premise that PilJ and PilD interact because the *pilD* mutants examined displayed the same twitching phenotype as *pilJ* mutant cells. However, not all *pilD* mutants are deficient in twitching motility. In frame Tn mutants of *pilD* in the MPAO1 strain of *P. aeruginosa* (Seattle, Tn mutant library) exhibit wild-type twitching. In addition, point mutations eliminating PilD N-methyltransferase activity do not interfere with the ability of the cells to assemble pili

(57). Twitching motility in these *N*-methyltransferase mutants is aberrant, though. It should be noted, however, that this mutation was made in a PAK strain rather than the PAO1 strain that we use. In addition, *pilJ* mutants in PAK strains exhibit aberrant twitching motility (71). Thus, in the PAK strain *pilJ* and *pilD* mutants exhibit the same twitch phenotype.

In vivo ^3H -methylation of proteins from early and late log phase cells were compared to determine at what stage in bacterial growth *P. aeruginosa* incorporates maximal amount of L-[methyl- ^3H methionine]. While the pattern of methylated protein was the same for early log and late log phase cells, the degree of methylation of early log proteins was more than twice the intensity of late log proteins (Figure 19). This decrease in the level of protein methylation in late log phase cells suggests that PilJ is quorum sensing (QS) regulated in a negative fashion. Microarray analysis of *P. aeruginosa* QS regulon has identified 222 genes as being QS repressed (70). Among these genes repressed by QS are several chemotaxis transducers, including *pilJ*. There is a -1.5 fold change in the expression of *pilJ* during early stationary phase. With a decrease in the amount of PilJ present, there would be a corresponding decrease in the amount of label incorporated into this protein by late log cells.

In vivo ^3H -methylation of PilJ in response to a variety of chemicals was assessed in order to determine the specific ligand(s) that PilJ binds. The greatest response was observed when wild-type PAO1 cells were exposed to dilauroyl (C12:0) phosphatidylethanolamine (PE). PE is a lipid found in the cell membrane of most organisms. Previous studies have shown that *P. aeruginosa* exhibits biased movement up concentration gradients of dilauroyl (C12:0) and dioleoyl (C18:0) PE in twitching

conditions (33). Directed movement towards PE has also been observed in *Myxococcus xanthus* (32). Because chemotaxis towards PE is clearly demonstrated in twitching conditions, it is probable that it binds to PilJ turning on the Type IV pili signal transduction pathway.

In vivo ^3H -methylation of wild-type PAO1 cells resulted in an increase in the level of methylation of the 72kd band when cells were exposed to C12:0 or C18:0 PE (Figures 20 and 23). This response was optimal when wild-type cells were stimulated by $20\mu\text{g/ml}$ PE (C12:0) or $0.2\mu\text{g/ml}$ PE (C18:0) for 30 min (Figures 21 and 22). In contrast to wild-type cells, the methylation pattern and intensity of the methylated proteins was the same for *pilJ* mutants cells that had been exposed to PE (C12:0) as unstimulated cells. Because there was no variation between stimulated and unstimulated *pilJ* mutant cells, we can conclude that PE acts as a positive stimulus binding to and promoting the methylation of PilJ in wild-type PAO1 cells. These responses were observed using cells recovered from twitch, LB (1% agar), plates. Interestingly, wild-type cells show little response to PE (C12:0) when grown in broth (Figure 22). These results imply that PilJ's response to PE is dependent on environmental conditions that favor twitching motility.

Additional chemicals examined as possible PilJ ligands were α -aminoisobutyric acid (AIB), L-leucine, L-arginine, 3-oxo-C₁₂ HSL, 3-oxo-C₄ HSL, *N*-acetylglucosamine, chitin, and defferoxamine. AIB (a non-metabolizable amino acid analog), L-leucine, and L-arginine were examined because *P. aeruginosa* exhibits a chemotactic response to these amino acids (12). 3-oxo-C₁₂ HSL and 3-oxo-C₄ HSL, quorum sensing autoinducers, were tested as possible PilJ ligands because quorum sensing has been shown to be directly involved in twitching motility and *pilJ* is QS regulated (20, 70). *N*-

acetylglucosamine (NAG) and chitin were tested because NAG is a component of the bacterial cell wall and chitin, a polymer of NAG, is a component of fungal cell walls. Deferoxamine was also examined as a ligand candidate because it is responsible for chelating Fe and has been shown to promote twitching motility (61). There appeared to be a slight increase in the level of PilJ methylated in wild-type cells in response to NAG, chitin, and 3-oxo-C₁₂ HSL; however, these results could not be replicated (Figures 24, 25, and 26). Also, no reproducible positive results were obtained using the additional chemical stimulants listed above.

In addition to chemical stimuli, PilJ's response to physical stimuli, such as the pili specific phage, PO₄, and antipilin antibodies, was examined. We hypothesized that the binding of phage or antibodies to pili would result in the generation of a physical torque which could transduce a signal to PilJ promoting twitching motility. No reproducible positive results were obtained when cells were exposed to either PO₄ phage or antipilin antibodies.

Finally, *in vivo* ³H-methylation of PilJ in response to *trans, trans*-farnesol was tested. Farnesol is a quorum-sensing molecule of *Candida albicans* that prevents yeast-to-mycelium conversion (27). The ability of PilJ to respond to farnesol was tested because recent studies have found that 3-oxo-C₁₂ AHLs can mimic farnesol in inhibiting the yeast-to-mycelium conversion (26).

In vivo ³H-methylation of wild-type PAO1 showed an increase in the level of methylation of PilJ when *trans, trans*-farnesol was present (Figures 27 and 28). This increase was not observed when *pilJ* mutant cells were exposed to *trans, trans*-farnesol (Figure 28). These results suggest that farnesol acts on *P. aeruginosa* by binding to PilJ.

If PilJ binds farnesol, we would expect to observe a distinct change in cell movement, specifically twitching motility. In order to determine effects of farnesol on cell movement, twitching motility assays were conducted with wild-type PAO1 cells using LB (1%) agar with and without farnesol. In the presence of farnesol, twitching motility was greatly reduced (Figure 29). This finding supports the premise that farnesol binds to PilJ, turning off the Type IV pili signal transduction pathway. In addition, chemotaxis assays conducted by others in our lab using wild-type PAO1 cells have shown cells migrating down gradients of farnesol. Migration of *P. aeruginosa* away from farnesol implicates it as a possible chemical repellent (data not shown).

Swarming motility in wild-type cells was greatly inhibited in the presence of farnesol (Figure 30B). In contrast, *pilJ* mutant cells' initial ability to swarm was only slightly reduced, and after 48 hours these cells were able to swarm as much as *pilJ* mutant cells that had not been exposed to farnesol (Figure 30D). Because *pilJ* mutant cells maintained their ability to swarm in the presence of farnesol, this further confirms that farnesol acts on *P. aeruginosa* primarily through PilJ. The slight decrease in *pilJ* mutant cells swarming motility in the presence of farnesol may indicate that farnesol binds to MCPs other than PilJ.

These findings implicate that farnesol not only functions as a *C. albicans* quorum sensing molecule, but may be used by *C. albicans* to compete with *P. aeruginosa*. Again, farnesol prevents the yeast form of *C. albicans* from being converted to mycelium. Previous studies have shown that *P. aeruginosa* primarily colonizes the mycelium form of *C. albicans* and not yeast (26). In the presence of farnesol, *C. albicans* maintains its yeast form preventing colonization by *P. aeruginosa*. Because *P. aeruginosa* cells are

still able to twitch and swarm in the presence of farnesol, this implies that *C. albicans*' competitive advantage over *P. aeruginosa* is not complete.

Literature Cited

1. Alley, M.R.K., Maddock, J.R., and L. Shapiro. 1992. Polar localization of a bacterial chemoreceptor. *Genes Devel* 6:825-836.
2. Alm, R.A., Hallinan, J.P., Watson, A.A., and J.S. Mattick. 1996. Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of Type IV fimbriae to the GSP protein-secretion systems and *pilY1* encodes a gonococcal PilC homologue. *Mol. Microbiol.* 22:161-173.
3. Alm, R.A., and J.S. Mattick. 1995. Identification of a gene, *pilV*, required for Type IV fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pilin-like leader sequence. *Mol. Microbiol.* 16:485-496.
4. Alm, R.A., and J.S. Mattick. 1996. Identification of two genes with prepilin-like leader sequences involved in Type IV fimbrial biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 178:3809-3817.
5. Alm, R.A., and J.S. Mattick. 1997. Genes involved in the biogenesis and function of Type IV fimbriae in *Pseudomonas aeruginosa*. *Gene.* 192:89-98.
6. Beatson, S.A., Whitchurch, C.B., Sargent, J.L., Levesque, R.C., and J.S. Mattick. 2002. Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J. Bacteriol.* 184:3605-3613.
7. Boyd, J.M. 2000. Localization of the histidine kinase PilS to the poles of *Pseudomonas aeruginosa* and identification of a localization domain. *Mol. Microbiol.* 2000:153-162.
8. Bradley, D.E. 1980. A function of *Pseudomonas aeruginosa* PAO pili: twitching motility. *Can. J. Microbiol.* 26:146-154.
9. Chiang, P., Habash, M., and L.L. Burrows. 2005. Disparate subcellular localization patterns of *Pseudomonas aeruginosa* Type IV pilus ATPases involved in twitching motility. *J. Bacteriol.* 187:829-39.

10. Costerton, J.W. 2001. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol.* 9: 50-52.
11. Craven, R.C., Montie, T.C. 1983. Chemotaxis of *Pseudomonas aeruginosa*: involvement of methylation. *J Bacteriol.* 154:780-786
12. Craven, R.C., and T.C. Montie. 1985. Regulation of *Pseudomonas aeruginosa* chemotaxis by nitrogen source. *J. Bacteriol.* 164:544-549.
13. D'Argenio, D.A., Calfee, M.W., Rainey, P.B., and E.C. Pesci. 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J. Bacteriol.* 184:6481-6489.
14. Darzins, A., and M.A. Russell. 1997. Molecular genetic analysis of Type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system. *Gene* 192:109-115.
15. Darzins, A. 1994. Characterization of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis protein of enterics and the gliding bacterium *Myxococcus xanthus*. *Mol. Microbiol.* 11:137-153.
16. Emori, T.G., and R.P. Gaynes. 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. *Microbiol. Rev.* 6:428-442.
17. Faulke, J.J., Bass, R.B., Butler, S.L., Chervitz, S.A., and M.A. Danielson. 1997. The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. *Annu. Rev. Cell Dev. Biol.* 13: 457-512.
18. Ferrandez A, Hawkins AC, Summerfield DT, Harwood CS. 2002. Cluster II *che* genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. *J Bacteriol.* 184:4374-4383.
19. Forest, K.T. and J.A. Tainer. 1997. Type IV pilus structure: outside to inside and top and bottom. *Gene* 192:165-169.
20. Glessner, A., Smith, R.S., Iglewski, B.H., and J.B. Robinson. 1999. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of twitching motility. *J Bacteriol.* 181:1623-1629.
21. Hahn, H.P. 1997. The Type IV pilus is the major virulence-associated adhesion of *Pseudomonas aeruginosa*-a review. *Gene* 192:99-108.
22. Hamilton, P.B., and G. Sheely. 1971. Chemotactic response to amino acids by *Pseudomonas aeruginosa* in a semisolid nitrate medium. *J. Bacteriol.* 108:596-598.

23. Hess, J.F., Oosawa, K., Kaplan, N., and M.I. Simon. 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* 53:79-87.
24. Hobbs, M., Collie, E.S., Free, P.D., Livingston, S.P., and J.S. Mattick. 1993. PilS and PilR, a two component transcriptional regulatory system controlling expression of type IV fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 7:669-682.
25. Hobbs, M., and J.S. Mattick. 1993. Common components in the assembly of Type IV fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. *Mol. Microbiol.* 10: 233-243.
26. Hogan, D.A., and R. Kolter. 2002. *Pseudomonas-Candida* interactions: An ecological role for virulence factors. *Science* 296:2229-2231.
27. Hornby, J.M., Jensen, E.C., Lisec, A.D., Tasto, J.J., Jahnke, B., Shoemaker, R., Dussault, P., and K.W. Nickerson. 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* 67:2982-2992.
28. Kaiser, D., and C. Crosby. 1983. Cell movement and its coordination in swarms in *Myxococcus xanthus*. *Cell Motil.* 3: 227-245.
29. Kaiser, D. 2000. Bacterial motility: How do pili pull? *Curr. Biol.* 10:R777-780.
30. Kato, J., Nakamura, T., Kuroda, A., and H. Ohtake. 1992. Cloning and characterization of chemotaxis genes in *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* 63:155-161.
31. Kato, J., Ito, A., Nikata, T., and H. Ohtake. 1992. Phosphate taxis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 174:5149-5151.
32. Kearns, D.B., and L.J. Shimkets. 1998. Chemotaxis in a gliding bacterium. *Proc. Natl. Acad. Sci. USA.* 95: 11857-11962.
33. Kearns, D.B., Robinson, J., and L.J. Skimkets. 2001. *Pseudomonas aeruginosa* exhibits directed twitching motility up phosphatidylethanolamine gradients. *J. Bacteriol.* 183:763-767.
34. Keizer, D.W., Slupsky, C.M., Kalisiak, M., Campbell, A.P., Crump, M.P., Sastry, P.A., Hazes, B., Irvin, R.T., and B.D. Sykes. 2001. Structure of a pilin monomer from *Pseudomonas aeruginosa*: implications for the assembly of pili. *J. Biol. Chem.* 276:24186-24193.
35. Kelly-Wintenberg, K., and T.C. Montie. 1994. Chemotaxis to oligopeptides by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 60:363-367.

36. Kim, K.K., Yokota, H., and S.H. Kim. 1999. Four-helical-bundle structure of the cytoplasmic domain of a serine chemotaxis receptor. *Nature* 400:787-792.
37. Kim, S., Wang, W., and K.K. Kim. 2002. Dynamic and clustering model of bacterial chemotaxis receptors: Structural basis for signaling and high sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 99:11611-11615.
38. Kohler, T., Curty, L.K., Barja, F., Van delden, C., and J. Pechere. 200. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* 182:5990-5996.
39. Kuroda, A., Kumano, T., Taguchi, K., Nikata, T., Kato, J., Ohtake, H. 1995. Molecular cloning and characterization of a chemotactic transducer gene in *Pseudomonas aeruginosa*. *J Bacteriol.* 177:7019-7025.
40. Lautrop, H. 1961. *Bacterium anitratum* transferred to the genus *Cytophaga*. *Int. Bull. Bacteriol. Nomencl.* 11:107-8.
41. Lefman, J., Zhang, P., Hirai, T., Weis, R.M., Juliani, J., Bliss, D., Kessel, M., Bos, E., Peters, P., and S. Subramaniam. 2004. Three-dimensional electron microscopic imaging of membrane invaginations in *Escherichia coli* overproducing the chemotaxis Receptor Tsr. *J. Bacteriol.* 186:5052-5061.
42. Lui, J.D., and J.S. Parkinson. 1991. Genetic evidence for interaction between the CheW and Tsr proteins during chemoreceptor signaling by *Escherichia coli*. *J Bacteriol.* 173:4941-4951.
43. Lory, S., and M.S. Strom. 1997. Structure-function relationship of Type IV prepilin peptidase of *Pseudomonas aeruginosa*-a review. *Gene* 192:117-121.
44. Lyczak, J.B., Cannon, C.L., and G.B. Pier. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect.* 2:1051-1060.
45. Maddock, J. R., and L. Shapiro. 1993. Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* 259: 1717-1723.
46. Madigan, M.T., Martinko, J.M, and J. Parker. 1997. *Biology of microorganisms*. Prentice Hall, Upper Saddle River, NJ, pp. 244.
47. Martin, P.R., Hobbs, M., Free, P.D., Jeske, Y., and J.S. Mattick. 1993. Characterization of *pilQ*, a new gene required for the biogenesis of Type IV fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 9:857-868.

48. Masduki, A., Nakamura, J., Ohga, T., and Umezaki, R., Kato, J., and H. Ohtake. 1995. Isolation and characterization of chemotaxis mutants and genes of *Pseudomonas aeruginosa*. J. Bacteriol. 177:948-952.
49. Mattick, J.S. 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. 56:289-314.
50. Milburn, M.V., Prive, G.G., Milligan, D.L., Scott, W.G., Yeh, J., Jancarik, J., Koshland, D.E. Jr, and S.H. Kim. 1991. Three-dimensional structures of the ligand-binding domain of the bacterial aspartate receptor with and without a ligand. Science 254:1342-1347.
51. Moench, T. T., and W.A. Konetzka. 1978. Chemotaxis in *Pseudomonas aeruginosa*. J. Bacteriol. 133:427-429.
52. Moulton, R.C., and T.C. Montie. 1979. Chemotaxis by *Pseudomonas aeruginosa*. J. Bacteriol. 137:427-429.
53. Nunn, D., Bergman, S., and S. Lory. 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. 172:2911-2919.
54. Nunn, D.N., and S. Lory. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. 1991. Proc. Natl. Acad. Sci. USA 88(8):3281-3285.
55. Ohga, T., Masduki, A., Kato, J., Ohtake, H. 1993. Chemotaxis away from thiocyanic and isothiocyanic esters in *Pseudomonas aeruginosa*. FEMS Microbiol Lett. 113:63-66.
56. O'Toole, G.A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30:295-304.
57. Pepe, J.C., and S. Lory. 1998. Amino acid substitutions in PilD, a bifunctional enzyme of *Pseudomonas aeruginosa*. Effect on leader peptidase and N-methyltransferase activities *in vitro* and *in vivo*. J Biol Chem. 273:19120-19129.
58. Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Goumnerov, B.C., and G.W. Lau. 2000. Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. USA 97: 8815-8821.
59. Rashid, M.H., and A. Kornberg. 200. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 97:4885-4890.
60. Shu, C.T., Ulrich, L.E., and I.B. Zhulin. 2003. The NIT domain: a predicted nitrate-responsive module in bacterial sensory receptors. Trends Biochem. Sci. 28:121-124.

61. Singh, P.K., Parsek, M.R., Greenberg, E.P., and M.J. Welsh. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature* 417:552-555.
62. Springer, M.S., Goy, M.F., and J. Adler. 1977. Sensory transduction in *Escherichia coli*: two complementary pathways of information processing that involve methylated proteins. *Proc. Natl. Acad. Sci. U.S.A.* 74:279-284.
63. Springer, W.R., and D.E. Koshland. 1997. Identification of a protein methyltransferase as the *cheR* gene product in the bacterial sensing system. *Proc. Natl. Acad. Sci. U.S.A.* 74:533-537.
64. Stock, J.B., and D.E. Koshland. 1978. A protein methylesterase involved in bacterial sensing. *Proc. Natl. Acad. Sci. U.S.A.* 75: 3659-3663.
65. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E., Lory, S., and M.V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406:959-964.
66. Strom, M.S., and S. Lory. 1993. Structure-function and biogenesis of the Type IV pili. *Annu. Rev. Microbiol.* 47:565-596.
67. Tan, M.W., and F.M. Ausubel. 2000. *Caenorhabditis elegans*: a model genetic host to study *Pseudomonas aeruginosa* pathogenesis. *Curr. Opin. Microbiol.* 3:29-34.
68. Tennet, J.M., and J.S. Mattick. 1994. Type IV fimbriae. In *Fimbriae: Aspects of adhesion, genetics, biogenesis, and vaccines*, ed. P Klemm, pp. 127-146. Boca Raton: CRC.
69. Van delden, C., and B.H. Iglewski. 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Infect. Dis.* 4: 551-560.
70. Wagner, V.E. Bushnell, D., Passador, L., Brooks, A.I., and B.H. Iglewski. 2003. Microarray analysis of the *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185: 2080-2095.
71. Whitchurch, C.B., Leech, A.J., Young, M.D., Kennedy, D., Sargent, J.L., Bertrand, J.J., Semmler, A.B., Mellick, A.S., Martin, P.R., Alm, R.A., Hobbs, M., Beatson, S.A., Huang, B., Nguyen, L., Commolli, J.C., Engel, J.N., Darzins, A., and J.S. Mattick. 2004. Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*. *Mol Microbiol.* 52:873-93.

R002592178

72. Wilson, R., and R.B. Dowling. 1998. Lung Infections. 3. *Pseudomonas aeruginosa* and other related species. *Thorax* 53:213-219.
73. Zumft, W.G., and L.E. Mortenson. 1975. The nitrogen-fixing complex of bacteria. *Biochim Biophys Acta*. 416:1-52.