

2007

PilJ localizes to cell poles and is required for type IV pilus assembly and extension in *Pseudomonas aeruginosa*

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PilJ localizes to cell poles and is required for Type IV pilus
assembly and extension in *Pseudomonas aeruginosa*

DISSERTATION

Submitted to

The College of Arts and Sciences of the

UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Doctor of Philosophy in Biology

by


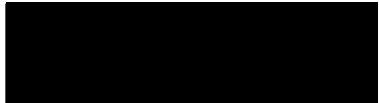



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ABSTRACT

PilJ localizes to cell poles and is required for Type IV pilus assembly and extension in *Pseudomonas aeruginosa*

Paul A. DeLange
University of Dayton, 2007

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Twitching motility allows *Pseudomonas aeruginosa* to move along surfaces by extending and retracting its Type IV pili. PilJ is a protein necessary for this surface associated twitching motility and bears high sequence identity with *Escherichia coli* methyl-accepting chemotaxis proteins (MCP). Here we report that *pilJ* is required for twitching motility in common laboratory strains, as well as clinical and environmental isolates of *P. aeruginosa*. We show that wild type cells extend pili at a single pole, but *pilJ* mutants assemble shortened pili at both poles despite normal levels of pilin accumulation. Many Type IV pilus assembly proteins localize to the cell poles. We used a functional PilJ-YFP fusion protein to determine that PilJ is localized to both poles of the cell regardless of growth conditions. This localization is independent of many other polar proteins required for twitching motility, including PilT, PilU, PilB. Neither does its localization require certain proteins involved in the putative pil chemosensory system, PilI or PilH. Our data suggest a model in which PilJ facilitates switching the active pole for pilus assembly, extension and retraction which allows cells to switch direction without physically turning the cell.

ACKNOWLEDGEMENTS

My special thanks to my advisor, Dr. Jayne Robinson, for allowing an 'old guy' to work in her lab. Her encouragement through long days of PCR, cloning, gel electrophoresis, and microscopy helped me stay on course. I am especially grateful for her help in arranging the tuition assistance with the help of Dr. George Pierce that made this degree possible. I would also like to thank the other members of my committee: Dr. John Rowe, Dr. Robert Kearns, Dr. Panagiotis Tsonis, and Dr. George Pierce for their help and support throughout the entire process.

I want to express sincere appreciation to all those I worked with in our laboratory: Dr. Mary Connolly for her assistance in the lab especially during my first several months; Tracy Collins, Amy Beumer, Michelle Yingling, and Chris Mohler for their enjoyable presence and comradorie in the lab as well as their assistance with experiments and making me feel almost as young as them. Thanks also to Chris Noriega and Vandy Sharma, in "the lab next door", for all of the time they spent talking through molecular techniques and results with me as well as the hours of conversation while waiting through incubation periods.

I am also grateful to Dr. Norman Wendth, my Academic Dean at Kettering College of Medical Arts, for his support in allowing me to complete this degree; my secretary, Neida Rowland, for keeping things in order in our department while I worked

in the lab; and all of my colleagues in the Division of Arts and Sciences at KCMA who supported me in this effort.

Very special thanks is due to my family, Julie, Abby, Hannah, and Matthew, for their patience and willingness to allow me time away from them to work on this degree. I am extremely grateful to Julie, my wife of 25 years, for her constant patience and encouragement for seven years. She always believed I could finish the process and provided the love and support to make it happen. THANKS Julie!

TABLE OF CONTENTS

Abstract	iii
Acknowledgments	iv
List of Figures	vii
List of Tables	ix
Introduction	1
Literature review	4
Twitching motility and Type IV pili (TFP)	4
Chemotaxis and methyl-accepting chemotaxis proteins (MCPs)	18
Methods	32
Results	49
Discussion	90
Appendix	104
References	117
Article Manuscript (Published in Current Microbiology)	145

LIST OF FIGURES

Figure 1.	Macroscopic twitching motility zone.....	6
Figure 2.	Excitation and adaptation in the bacterial chemotaxis pathway	24
Figure 3.	Engineering of the <i>pilJ-yfp</i> fusion.....	41
Figure 4.	PCR of 600 bp of <i>pilK</i> with 5' <i>Xba</i> I and 3' <i>Eco</i> RI sites added	45
Figure 5.	Process used to generate the suicide plasmid pEX19JYK.....	46
Figure 6.	Allelic exchange mutant construction.....	50
Figure 7.	Construction of plasmid pEX <i>pilJ</i>	51
Figure 8.	Construction of plasmid pEXJGm.....	52
Figure 9.	Confirmation of pEXJGm.....	53
Figure 10.	Verification of excision of suicide plasmid	54
Figure 11.	Loss of twitching motility phenotype by <i>pilJ</i> mutants	56
Figure 12.	Macroscopic twitching motility of multiple <i>P. aeruginosa</i> strains.....	57
Figure 13.	Comparison of macroscopic twitching motility assays	58
Figure 14.	Microscopic twitching motility assay of PAO1-AD strains	60
Figure 15.	Microscopic twitching motility assay of multiple <i>P. aeruginosa</i> strains and mutants	61
Figure 16.	Western blot analysis of pilin	64
Figure 17.	<i>In situ</i> immunofluorescence of pili on wild type PAO-1 and <i>pilJ</i> mutant (FA6) cells	67
Figure 18.	Restriction endonuclease digest and schematic representation of plasmid p19yfp	71

Figure 19.	PCR of <i>pilJ</i> and <i>yfp</i> with restriction sites added	72
Figure 20.	Restriction endonuclease digest and schematic representation of plasmid p19JY	73
Figure 21.	Complementation of <i>pilJ</i> mutant by p19JY	74
Figure 22.	Localization of PilJ in numerous strains using p19JY	75
Figure 23.	Localization of PilJ using an in-frame chromosomal fusion	76
Figure 24.	Restriction endonuclease digest of pSB94JY	77
Figure 25.	Complementation of <i>pilJ</i> mutant with pSB94JY	78
Figure 26.	Localization of PilJ using the expression vector pSB94JY	79
Figure 27.	Gel electrophoresis of PCR product of first 600 bp of <i>pilK</i> from pADD698	80
Figure 28.	Restriction endonuclease digest of pCRK	81
Figure 29.	Restriction endonuclease digest of p19JY with <i>HindIII</i> and <i>XbaI</i> to release 2.9 kb <i>pilJ-yfp</i> fusion	82
Figure 30.	Restriction endonuclease digest of suicide plasmid pEX19JYK	83
Figure 31.	Integration and excision events of the suicide vector pEX19JYK in the FA6 chromosome	86
Figure 32.	Complementation of FA6 with in-frame chromosomal fusion demonstration by twitching motility assay	87
Figure 33.	Western blot analysis of YFP constructs	88
Figure 34.	Localization of PilJ using an in-frame chromosomal fusion	89
Figure 35.	Polar switching model for <i>P. aeruginosa</i>	99

LIST OF TABLES

1. Comparison of Che and Che-like proteins of *E. coli*, *M. Xanthus*
and *P. aeruginosa*31
2. Table of strains and plasmids used in this study.....33

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in the environment and is an opportunistic pathogen frequently implicated in nosocomial infections (20). Type IV pili (TFP) aid in the process of adherence to host cells, surfaces necessary for establishing infection (119, 187), and are responsible for a unique form of surface-associated, flagellum-independent motility known as twitching motility, (27, 40, 62, 62, 144). Over 25 Gram-negative bacteria, including *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Moraxella bovis* and enteropathic *Escherichia coli* (EPEC) express TFP (104). At least 40 genes are necessary for TFP biogenesis and function (3, 72, 104)

Twitching motility is the result of the repeated assembly and extension of the TFP: attachment of the pilus tip to the surface, and retraction of the TFP, which pulls the cell toward the point of the attachment (27, 100, 109, 120, 144). The energy required to generate this type of movement is provided by TFP-ATPases, resulting in the assembly and disassembly of the pilus fiber (76, 109, 171). In *P. aeruginosa*, these TFP-ATPases are PilB, which powers the extension of the pilus fiber, and PilT, which is responsible for the disassembly and retraction of the pilus (104, 182). PilU, a paralogue of PilT, is also required for twitching motility, although its exact function remains unclear (179). PilT and PilB have been shown to localize to both poles of the cell in *P. aeruginosa* while PilU localizes only to the piliated pole (32); however, in *P. aeruginosa*, TFP extend at only one pole (27, 47, 162, 171).

The chemosensory systems controlling swimming motility and chemotaxis in *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* have been extensively studied (23, 28). Complex chemosensory systems similar to those required for swimming motility in *E. coli* also control swarming, gliding and twitching motility in other bacteria (9, 104, 184). The *P. aeruginosa* PAO1 genome contains five gene clusters encoding chemotaxis-like phosphorelay signal transduction systems as shown Stover, et. al article (34, 158). Cluster IV contains the genes involved in twitching motility (*pilGHIJK* and *chpA-E*) (38, 183). The Cluster IV genes possess features similar to the chemotactic (*che*) system controlling flagellar rotation in the swimming motility of enteric bacteria such as *E. coli* (38-40) and to the *frz* cluster responsible for twitching motility in *Myxococcus xanthus* (38, 173). Both the *Pseudomonas* and *Myxococcus* chemosensory signal transduction pathway gene clusters are thought to be involved in the biogenesis and extension of TFP.

There is genetic evidence to suggest PilJ is a methyl-accepting chemotaxis protein (MCP). A comparison of the *P. aeruginosa pilJ* gene, from Cluster IV, with the *E. coli* MCP gene *Tsr* showed the two genes to be 26% identical (38). Like most MCP proteins, PilJ contains two stretches of hydrophobic amino acids which function as transmembrane regions, one near the N-terminus of the protein and the other near the middle of the protein, thus separating the protein into an N-terminus periplasmic chemosensory domain and a C-terminus cytoplasmic signaling domain.

Previous studies have demonstrated that in chemosensory signal transduction pathways, such as the Che system of *E. coli*, the MCPs are localized to a polar region of the cell (2, 52, 60, 69, 99, 169). The MCP serves to anchor other chemotaxis proteins,

which also cluster at the poles in *E. coli*, such as CheA, CheY, and CheZ (148). Recently, several proteins involved in twitching motility in *P. aeruginosa* have been shown to cluster at the cell poles. PilS, the sensor-kinase in the PilA biosynthesis pathway that initiates the transcription of the PilA subunit, was shown to localize to both poles of the cell in *P. aeruginosa* (25) as have both ATPases, PilB and PilT (32). In contrast, FimX, (a protein involved in twitching chemotaxis) (70) demonstrates unipolar localization in *P. aeruginosa*. All of these studies were conducted with the aid of functional fluorescent protein fusions.

The localization of MCPs in *E. coli* and pilus assembly proteins in *P. aeruginosa* to the cell poles prompted us to examine PilJ localization. We show that PilJ is localized to both poles regardless of growth conditions. PilJ remains localized to both poles in several mutants including *pilT*, *pilU*, *pilB*, *pilH*, and *pilI*. These findings strengthen the model that cells assemble a complete twitching motility apparatus at both cell poles. In addition, we show that FimX localizes to only one pole in a *pilJ* mutant as well. We also show that when PilJ is absent, cells assemble shortened pili at both poles and are unable to perform twitching motility. These results indicate PilJ is necessary for the cell to designate the leading pole and to fully extend TFP. The simplest model to explain the function of localized PilJ to both poles is that it allows the cell to respond quickly to signals coming from either direction and to avoid the need to turn the cell body on a surface.

LITERATURE REVIEW

Twitching motility and TFP

Among the forms of motility exhibited by bacteria is a form of flagellar-independent surface translocation, referred to as twitching motility. The term was first proposed by Lautrop in 1961 to describe the jerky movement seen in suspensions of *Acinetobacter calcoaceticus* (88). Type IV pili (TFP), typically located at one pole of the cell in *P. aeruginosa*, *Neisseria* and *M. xanthus* are responsible for twitching motility (27, 62, 77). The term 'type IV pili' was proposed by Ottow in 1975 to describe a class of extracellular filaments located at the poles of the bacteria that exhibit twitching motility (118). This form of motility is distinct from swimming and swarming motilities which are mediated by the action of rotating flagella.

Since that first observation and characterization, twitching motility has been observed among the β , γ , and δ subdivisions of the Proteobacteria (63). Twitching motility is found in numerous bacteria including species of *Aeromonas*, *Bacteroides*, *Branhamella*, *Dichelobacter*, *Legionella*, *Moraxella*, *Pasteurella*, *Pseudomonas*, *Synechocystis* and *Vibrio*, and *Escherichia coli* (EPEC) (104) (17, 43, 48, 55, 63, 91, 92, 126, 138). Bacteria capable of this form of motility demonstrate the classic observable twitching motility phenotype, TFP, and the presence of genes involved in the synthesis of TFP. Among the best studied bacteria that exhibit twitching motility are *Pseudomonas*

aeruginosa, *Neisseria gonorrhoeae* and *Myxococcus xanthus*. In *M. xanthus*, type IV pilus mediated motility has often been referred to as "social gliding motility".

P. aeruginosa has been considered the primary model for the genetic and functional analysis of twitching motility. Several factors make this bacterium a good model. They are: the genome has been completely sequenced, it can grow aerobically and is easily cultured, has good host-vector systems for gene cloning, expression and allelic exchange, and has an easily identifiable twitching motility phenotype (3, 137). *P. aeruginosa* is also a model system for studying biofilm formation and some genes affecting twitching motility are also necessary for biofilm formation (116, 181). Some genes necessary for twitching motility, such as *pilT*, *pilU* and *pilJ* have also been shown to be necessary for establishing infection in some instances (33, 36). *P. aeruginosa* is a versatile organism well known for its ability to cause infection in immunocompromised persons such as those with AIDS and those receiving chemotherapy as well as burn victims and cystic fibrosis patients (97). In addition, it can infect a broad range of non-human hosts, including mice, the fruit fly (*Drosophila melanogaster*), the nematode worm (*Caenorhabditis elegans*), and the mustard plant (*Arabidopsis thaliana*) (124).

Twitching motility occurs on a variety of surfaces, both biotic and abiotic, at low water availability which allows *Pseudomonas* to exploit a variety of niches. Swimming and swarming motility are the preferred modes of movement in aqueous environments. The phenotypic description of twitching motility has been quite well characterized over the last 30 years. In certain species of bacteria evidence of twitching motility, or the lack of it, can actually be seen by close examination of the colonial morphology. For example, in *P. aeruginosa*, twitching motility deficient mutants generate smooth convex

colonies on agar plates. Wild-type colonies of *P. aeruginosa* produce flat spreading colonies with a classic rough appearance sometimes described as a fine serrated or “ground glass” edge around the colony (105, 137). Twitching motility can be observed at the interface between agar and plastic or glass (Figure 2). As the bacteria move across this interface, fine twitching zones or halos appear that can reach 2-3 cm in diameter after overnight growth, expanding at rates of up to 1 mm/hr (137). These zones are quite visible but can be more easily seen by staining using Coomassie Blue (107) and are absent in twitching-deficient mutants. The size of the zone is a relative measure of the activity of twitching motility and as such has led to the identification of numerous genes with varying effects on twitching motility.

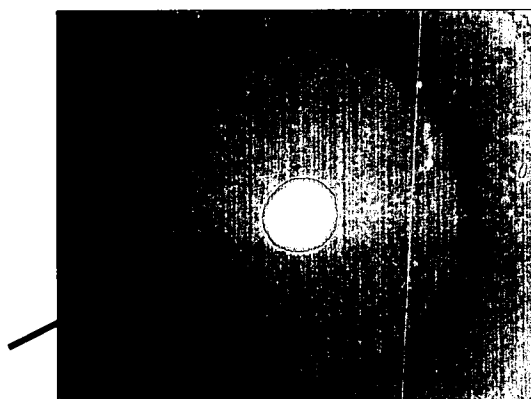


Figure 2. Macroscopic twitching motility zone. The smaller white zone in the center represents cells growing on the agar surface. The diffuse zone around the agar surface growth represents cells moving at the agar-Petri dish interface and is referred to as the twitch zone. The arrow is pointing to the leading edge of this zone. (Personal research results)

Microscopically, twitching motility has been shown to be colonial in nature and generally involves cell-cell contact. With the aid of time-lapse video microscopy at a gel-glass interface, Semmler (137) was able to demonstrate that twitching motility occurs as cells move out from the center of a colony in rafts or spearhead-like clusters of cells, usually 10-50 cells in width. These formations are characteristic for twitching in different bacteria (27, 64) and are also observed in the social gliding motility of *M. xanthus* (171). In each case the cells begin their movement radially outward, following the long axis of the cells, tightly aligned in cell-cell contact. As they continue their movement, the rafts may meander and occasionally individual cells may reverse their direction. Behind the leading rafts of cells, other groups of cells move out in different directions, eventually interconnecting with other cell groups. These different groups of cells will only join other cell groups when in close proximity to each other. When the cells are close enough, they touch with their poles and quickly snap into an aligned position, thus the characteristic jerky "twitching" movement for which this form of motility is named (27, 62, 64, 137). The result of this intricate process is the formation of a lattice-like network of trails of cell groups, often reversing direction but always doing so along the long axis of the cell (137).

For some time there was debate as to whether twitching motility in *P. aeruginosa* and other bacteria was different from social gliding of *M. xanthus*. However, it is now generally considered that the social form of gliding motility is a variation of twitching motility and that the two forms are mechanistically equivalent (150, 171). Henrichsen has provided perhaps the best description of the above process in describing the gliding motility in *Myxococcus*:

“under conditions optimal for gliding, the colonies will be seen as ‘completely flat, rapidly spreading almost invisible swarms’ or as a spreading, rhizoid growth with a honeycomb appearance. Movement takes place mainly in ‘spearheads’ (i.e., spearhead shaped cell aggregates at the edge of the colony), single isolated cells very barely being motile, and the picture is one of a ‘changing dispersed border’ with interlacing bands being continuously rearranged.... The cells are arranged in a loose pattern of interlacing bands of rafts and cells.... Groups of cells resembling spearheads are seen projecting outward. The locomotion, which is principally seen in the groups of cells, i.e., rafts and spearheads, and takes the direction of the longitudinal axis of the bacteria, gives rise to a constantly changing picture, steadily gliding groups of cells uniting or dividing....” (64).

Twitching motility in *P. aeruginosa* could also be described in these same terms. Because of the sometimes broad or ambiguous use of the term gliding motility and the rather precise use of the term twitching motility, it has been suggested that perhaps a new term is appropriate for this form of motility, such as “retractile motility” (104).

Mechanism of twitching motility

In 1980, Bradley devised a clever experiment that proved that the driving force for twitching motility is pilus retraction (27). In this study, Bradley examined the binding of pilus-specific bacteriophages to twitching motility mutants of *P. aeruginosa* using electron microscopy and discovered two types of phage resistant mutants. One type lacked pili, the other was hyperpilated yet both mutants were twitching motility deficient. A comparison of the location of phage in either mutant type and the wild type showed three different patterns. Phage were located on the surface of the cell at the junction of the pili and cell pole in the wild-type cells. In the nonpilated mutants the phage were not found on the cell surface and in the hyperpilated mutants phage were observed at various points along the pili, but not on the cell surface. Based on these

observations Bradley concluded that phage infection and twitching motility were dependent on pilus retraction. In the wild-type cells, the phage attached to the pili and were then carried to the surface of the cell as the pilus retracted as shown in Wall's article (171). Therefore Bradley concluded that this retraction was the mechanism for twitching motility.

It has since been shown that the hyperpilated mutant had small deletions in the *pilT* gene (179, 182), a NTP-binding protein essential for pilus retraction (109, 144, 163, 182). PilT is homologous to another NTP-binding protein (PilB) which is required for pilus assembly (182). A complete discussion of the assembly and disassembly process will be presented later following more details on the structure of the pilin monomer that composes the TFP fiber.

The same mechanism that pulls the phage to the cell surface is the one that pulls the cell forward following attachment to other surfaces. Once the pilus has retracted, it extends and attaches again, repeating the process of retraction, pulling the cell forward again (Figure 6). Since Bradley's proposal in 1980 that twitching motility was the result of pilus retraction, three other studies have confirmed it. One study examined *N. gonorrhoeae* (109), another *M. xanthus* (163) and a third *P. aeruginosa* using fluorescently labeled pili in which pilus retraction was observed directly using fluorescence microscopy at approximately 0.5 $\mu\text{m}/\text{sec}$ (144). Thus, twitching motility is the result of extension of the pilus followed by the attachment of the pilus to a surface followed by the retraction to pull the cell forward. While pili have been shown to pull the cell forward, they do not appear to have the strength to push a cell. Sun and Bourret have provided some helpful diagrams to demonstrate this mechanism (23, 163).

Structure of Type IV Pili

TFP are capable of binding to a variety of surfaces from inert surfaces to bacterial and eukaryotic cells in which case they mediate contact and colonization through pilus retraction (108, 163). This attachment is always at the distal end of the pilus whether attaching to living or non living surfaces (144, 163).

TFP are composed of polymers of the pilin protein, encoded for by the PilA gene (105), which auto-oligomerize to form a fiber approximately 5-7 nm in diameter and 1000-4000 nm in length (46, 81). In the pilin subunits of *P. aeruginosa*, *N. gonorrhoeae*, and *N. meningitidis*, a conserved C- terminal disulfide-bonded loop of 12-17 semiconserved amino acid residues, normally buried within the filament, is exposed at the tip of the pilus (35, 61). When binding to living tissues, this region binds to the carbohydrate moiety of the glycosphingolipids asialo-GM1 and asialo-GM2 on epithelial cells (58, 61, 89). The adhesion is accomplished by the C-terminal loop, PilY1 in *P. aeruginosa*; in *Neisseria* PilC also plays a contributing role (5, 133, 186). Both proteins are associated with the cell outer membrane and the extracellular pili. In the absence of this protein, extracellular pili and twitching motility are absent and thus the bacteria cannot attach. It is likely that these proteins are functioning to cap or stabilize the pili (186). Without this stabilization, the TFP are incapable of functioning properly.

The pilin monomer units are assembled and disassembled using a system of proteins at the base of the pilus to extend or retract the fiber. The crystal structure of *N. gonorrhoeae* MS11 pilin monomer was elucidated in 1995 (121) and that of *P. aeruginosa* PAK in 2000 (61).

Each pilin monomer consists of approximately 145-160 amino acids and consists of a highly conserved and hydrophobic amino-terminal domain in an α -helix arrangement that forms the core of the pilus fiber (61) (Figure 7). The pre-pilin protein is initially produced with a 6 amino acid positively charged leader sequence at the N-terminus (35) which is cleaved before assembly into the pilus fiber. Cleavage depends on the presence of a glycine residue at the -1 position. Once cleaved, the N-terminal residue, generally phenylalanine, is methylated. This process is dependent on glutamate at the +5 position (161). This charged residue plays a major role in proper pili formation as proved by a study in which a glutamate to lysine mutation at position +5 prevented proper pilus formation (98, 122). The result is a methylated phenylalanine at the N-terminal residue of mature pilin monomers (93, 159-161). Both the cleavage and methylation are carried out by the membrane bound bi-functional enzyme, pre-pilin peptidase, PilD.

Although the N-terminus region of the monomer is hydrophobic and highly conserved, the remaining two thirds of the protein is largely hydrophilic and variable from species to species. Near the C-terminal end of this region is the disulfide-bonded loop, mentioned earlier, that appears to act as the proposed binding site for attachment. Hazes paper includes a helpful diagram of the pilin monomer (61). The figure in that paper shows how the majority of the C-terminal 120+ residues forms a four-stranded anti-parallel β -sheet which partially surrounds the C-terminal portion of the α -helix. A slightly different view of the pilin monomer is seen in the article by Keizer (81) and shows another view of a pilin monomer to demonstrate the 3-dimensional structure of the protein and the relationship of the α -helix and the four-stranded anti-parallel β -sheet.

As mentioned previously, the pilus is the result of the oligomerization of the pilin monomers. Careful studies of the *P. aeruginosa* pilus (46, 81, 174) indicate that the pilin subunits assemble into a helical structure consisting of five pilin molecules per turn. The result is a core composed of parallel, overlapping, hydrophobic N-terminal α -helices surrounded by a highly organized arrangement of β -sheets pushed flat against the core. Of particular importance in the assembly of the pilus fiber is the presence of, and the content of, the first 22 amino acid residues in the N-terminus as mentioned previously. It has also been observed that there is some electrostatic complementarity of the pilin monomers (81) that may contribute to proper assembly of the monomers. Specific positive and negatively charged regions of the protein are likely interacting with one another to create what could be described as a "lock and key" mechanism for the assembly of the subunits.

Genes and gene products involved in TFP biogenesis and function

A study of TFP biogenesis and twitching motility in *P. aeruginosa* reveals there are over 40 genes and gene products involved (3, 104). Several minor pilin-like proteins (PilE,V,W,X and FimT and U), all containing hydrophobic amino-terminal helical regions similar to PilA, with the exception of FimT, are thought to localize in the cell membrane and form the base structure of the pilus fiber (67, 118). The structural similarities of these proteins suggest they may also be shared by the type II protein secretion system (4, 5, 130). Twitching motility also requires PilB, a nucleotide binding protein homologous to PilT, but with opposite function. Both proteins are likely associated with the inner membrane and perhaps extend into the periplasmic space (31,

123). Other necessary proteins include the pre-pilin peptidase and methylase PilD, and a multimeric outer membrane protein, PilQ, which forms a gated pore, about 53 Å in diameter, through which the pilus fiber is thought to extend and retract (3, 67, 93, 103, 115, 129, 162). The diameter of the twelve subunit doughnut shaped protein complex formed by PilQ corresponds to roughly the same diameter (52 Å) of the TFP in *P. aeruginosa* ((18, 81).

Four other genes required for TFP biogenesis, twitching motility, and phage sensitivity in *P. aeruginosa*, *pilM*, *N*, *O* and *P*, are located in the same operon as the *pilQ* gene (102). PilM protein seems to be a multimeric complex also and it is thought quite possible that TFP are directed through PilM to the cell pole (102). Both PilN and PilO contain long N-terminal stretches of hydrophobic residues that likely act as inner membrane anchoring domains and whose roles in TFP biogenesis and function are not well understood. PilP is a lipoprotein that appears to stabilize the formation of the PilQ complex (44, 102).

In summary, the assembly/extension of TFP begins as pre-pilin subunits are produced and embedded in the inner membrane, cleaved and methylated. The fiber is then assembled with the aid of the PilB ATPase resulting in the extension through the PilQ pore. Disassembly occurs as the PilT ATPase retracts the pilus fiber inside the cell by disassembly of the pilin subunits using the energy from ATP. It is this disassembly process that results in the retraction and power for twitching motility (81, 185).

Other genes or gene products are involved in the transcriptional regulation and chemosensory pathways that regulate the expression or activity of the TFP/twitching motility system. Among these regulatory proteins is the classical two-component sensor-

regulator pair, PilS-PilR which are required for the transcription of the *PilA* subunit and is dependent on the alternative sigma factor RpoN for transcription (68). Another pair of proteins, the atypical sensor-regulator pair FimS-AlgR, regulate twitching motility and are also dependent on an alternative sigma factor, AlgU (180). Both *fimS* and *algR* mutants lack extracellular pili; however, both are capable of *pilA* expression indicating that the regulation is in the assembly phase. The global carbon metabolism regulator Crc regulates *pilA* expression and thus twitching motility as well (117). Recently Vfr, a homolog of the catabolite repressor protein Crp in *E. coli*, was shown to control the expression of many genes required for TFP biogenesis and twitching motility presumably by binding cAMP and cGMP (15).

Additionally, both *M. xanthus* and *P. aeruginosa* have chemosensory phosphotransfer signal transduction systems (*frz* and *chp*) that control twitching motility. These complex systems are similar to the chemotactic (*che*) system controlling flagellar rotation in swimming motility in other bacteria, as typified by *E. coli* (3, 171-173). The *che* system has three primary proteins in the system: 1) methyl-accepting chemotaxis proteins (MCPs) capable of inducing autophosphorylation of a 2) central histidine kinase (CheA) which is then capable of phosphorylating the 3) response regulator CheY causing the flagellar motor to reverse directions. In addition there are other proteins involved, such as CheW which acts as an adaptor between the MCPs and CheA, CheR and CheB which regulate the methylation of the MCPs, and CheZ which regulates the phosphorylation of CheY.

The *frizzy* (*frz*) chemosensory system of *M. xanthus* was the first such system discovered to control pili-dependent motility (173). Homologs of nearly all the Che

proteins have been discovered in this organism encoded by the *frz* gene cluster. However, as has already been stated, this system is much more complex than the Che system. For example there are three CheY-like domains; two in the FrzZ protein and one in the FrzE protein. It is the *frz* system that controls the frequency of cell reversals and thus the direction and pattern of social gliding motility (173).

In *P. aeruginosa*, it is the *pil/chp* gene cluster that resembles the Che system of *E. coli* and the *frz* of *M. xanthus*. Like *M. xanthus*, the *chp* system of *P. aeruginosa* contains three CheY-like domains; two in the PilG and PilH proteins (probably equivalent to FrzZ) and another in the CheA homolog, PilL/ChpA referred to as ChpA (3, 38, 39). ChpA is a very large and complex protein of almost 2500 amino acids which contains not one, but seven histidine phosphotransfer domains making it one of the most sophisticated signal transduction proteins discovered to date (3). Mutations affecting PilG/PilH (CheY), PilI (CheW), and PilJ (MCP) are all deficient in twitching motility and extracellular pili, yet remain phage sensitive.

The environmental signals controlling twitching motility are poorly understood although there is some evidence that *P. aeruginosa* may be responsive to phosphatidylethanolamine (79) and there is some debate about the effect of quorum-sensing on twitching motility (14, 54). A recent study has revealed that quorum sensing regulation of twitching motility involves nine different quorum sensing transcriptional regulators (170). It does seem true that the signals that one bacterial species may respond to may be different than another depending on the environment. Light has been shown to control motility and TFP production in *Synechocystis* (16). There remains much work to

be done to determine the environmental signals to which many bacteria respond for TFP production and twitching motility.

Roles of type IV pili

In addition to their role in twitching motility, TFP play other roles. TFP are required for bacteriophage infection as clearly demonstrated by Bradley (27). TFP are required for competence for DNA transformation (49, 128, 156). Because the core of the pilus filament is too narrow for DNA to pass through there must be another method by which TFP mediate the transfer of DNA. It has been proposed that DNA may enter directly through the PilQ pore (55). Finally twitching motility/TFP are required for host colonization and pathogenesis. Pathogenic strains of bacteria deficient in PilA production have reduced or no virulence (33) and vaccination using purified pilin has been shown to protect against infection with serologically related strains. Although TFP have been shown to be necessary for virulence, their mere presence is not enough. For example, a *pilT* mutant of *P. aeruginosa*, which is actually hyperpilated, is incapable of infecting corneal tissue and exhibits reduced adherence and cytotoxicity to epithelial cells in culture (33). A 2001 study of virulence of *P. aeruginosa* in *Drosophila* showed that bacteria with mutations in the *pilGHIJKL-chpABCDE* chemosensory gene cluster, which lack twitching motility, were greatly impaired in their ability to kill flies. (36).

These studies point to the complexity of the role of TFP and twitching motility and the need for continued study. It is clear that TFP and twitching motility are valuable elements of many bacteria; that they are complex elements, and that there is still much to be learned about these interesting appendages and the processes they mediate in order to

better understand the pathogenicity and symbiosis of bacteria capable of this form of motility.

Chemotaxis and methyl-accepting chemotaxis proteins (MCPs) in bacteria

Since Leeuwenhoek first observed his moving 'animalcules' in 1683, studies have continued to indicate that most bacteria are motile during at least part of their life cycle (45). In a constantly changing environment in which most bacteria find themselves, they must be able to respond to these changes and one such response is to move. Bacterial motility can be classified as either flagellum-dependent or flagellum-independent. Some bacteria, such as *P. aeruginosa* are able to move using both types of flagella-dependent motility: swimming and swarming, and the flagellum-independent motility known as twitching motility (125). The focus of the previous chapter was to discuss twitching motility which is mediated through the extension, adherence, and retraction of the TFP (109, 144, 163). In this section, the focus is to examine the mechanism and proteins involved in the chemotactic movement of bacteria that allows them to be successful in a rapidly changing environment.

Nearly 200 years after Leeuwenhoek discovered that bacteria were motile, Theodor Engelmann and Wilhelm Pfeffer discovered that bacterial movement was not random and arbitrary but rather directed toward some stimuli and away from others, a response they termed 'chemotaxis' (95). In short, bacterial chemotaxis is the biased movement towards areas of higher concentrations of beneficial chemicals/nutrients (chemoattractants) or away from higher concentrations of toxic/harmful chemicals (chemorepellants).

Although both eukaryotic and prokaryotic organisms utilize sensory signal transduction pathways, the most common sensory pathways in prokaryotic organisms use a histidine-aspartate phosphorelay (HAP) system while most eukaryote sensory pathways

rely on serine, threonine, or tyrosine kinases (168). The most basic HAP systems have at least two components: a dimeric histidine phosphokinase (HPK) and a response regulator (RR) (177) but chemotactic signal transduction systems in bacteria are often much more complicated.

Chemotactic Signal transduction

In the past forty-plus years, researchers have developed a comprehensive understanding of the molecular mechanisms of bacterial chemotaxis. The signaling pathway of this process has been most extensively studied in the enteric bacteria, *Eschericia coli* and *Salmonella enterica* serovar *typhimurium* (10, 29). In simplest terms, bacterial chemotactic signal transduction pathways have three basic elements: (1) SIGNAL RECEPTION: chemoreceptors located in the cytoplasmic membrane detect signals; (2) RELAY: cytoplasmic transduction proteins relay the signal from the membrane receptors to the motor; and (3) ADAPTATION: signal adaptation proteins that serve to desensitize the initial signal input. The process can be divided into two phases: (1) Excitation: the signal reception and relay; (2) Adaptation: the portion of the chemotactic signal transduction process that operates so that the bacterium can constantly monitor its surroundings by allowing the cell to reset itself in order to continue to respond to change.

Methyl-accepting chemotaxis protein (MCP) structure and organization

Bacterial chemotaxis is a complicated process involving numerous proteins and can be summarized as a series of interactions between transducers, chemotaxis (Che)

proteins, and flagellar motor proteins which ultimately control the rotation of the flagellum. The process is initiated by bacterial chemoreceptors located in the cell membrane whose structure may be altered by methylation at specific amino acid residues and are therefore referred to as methyl-accepting chemotaxis proteins (MCPs) (85). This process is accomplished when a ligand binds to the ligand binding domain of the MCP triggering CheR to methylate the cytoplasmic domain. Adaptation occurs as the methylesterase, CheB, removes the methyl group. Once a signal is detected by the sensing domain of the molecule, conformational changes that occur in the MCP cause the signal to be transduced across the membrane. The adaptation process of the MCPs allows the cell to sense changes in concentration of substances in the environment as the cell moves. As a result the cell is responding to changes in concentration. MCPs are also referred to as receptor-transducer proteins or simply transducers. The actual number of chemoreceptor genes in bacteria ranges from 1–60, based on genome sequence analysis, with most bacteria having at least 10 such genes (112).

Structurally the MCP monomers can be subdivided into three major parts: a periplasmic ligand-binding or sensing domain, a transmembrane domain and a cytoplasmic domain. In the well studied MCPs of the enterics, X-ray crystallography indicates that the sensory domain consists of a four-helix bundle, two of which extend into and across the cytoplasmic membrane as the transmembrane domain and two of which form the ligand binding domain (111, 188). The binding of ligands at this sensing region alters interactions between the dimers and the four-helix bundle causing the transduction of the signal through or across the transmembrane region in a manner yet to be fully understood. One possible scenario is a piston model that suggests one or more

transmembrane segments (TMS) may slide vertically with respect to the other TMS (1). While most bacterial chemoreceptors are located within the membrane and contain transmembrane segments, some bacteria have cytoplasmic chemoreceptors lacking the transmembrane domain (157).

The cytoplasmic signaling domain can actually be further divided into three subdomains based on their functions. The portion closest to the membrane consists of the HAMP linker subdomain. The exact structure of this region is unknown but is common to many signal pathways. The term HAMP is derived from its presence in Histidine kinases, Adenyl cyclases, MCPs and Phosphatases (8). Its exact function in the chemoreceptor molecule is still unclear although disruption of this region does cause the loss of signaling (7). It has been suggested it may be involved in the dimer formation (95).

The next portion of the cytoplasmic domain contains the adaptation subdomain which includes the methylation sites: 4-6 glutamate residues that can be reversibly methylated (175). Methylation of these residues is essential to the adaptation process in order to sense changes in concentration of chemicals in the surrounding environment and is regulated by the opposing activities of CheB, a methylesterase, and CheR, a methyltransferase (80). The level of methylation affects the MCP conformation and therefore controls the adaptation to a sensory signal. The methylation/demethylation process allows resetting of the signaling state of the receptor so that it can monitor even slight changes in concentrations, serving as a molecular memory.

The final portion of the cytoplasmic domain contains the actual signaling subdomain with sites for interaction with two other key proteins in the system, CheW and

CheA, that relay the signal to the flagellar motor proteins through phosphotransfer reactions that will be described later. A portion of this region appears to be highly conserved among MCPs, sometimes referred to as the highly conserved domain (HCD) (38). The CheW protein interacts with both the cytoplasmic signaling domain of the MCP and the HPK protein CheA (22). The formation of this quaternary MCP dimer-CheW-CheA complex at the poles of the cells is necessary for signal transduction (99, 101). CheW has no known enzymatic activity and is thought to play a simple scaffold role, although because its structure and function is conserved among many different bacteria (59), it may have a more significant role than just a link between the MCP and CheA.

In recent years it has been demonstrated that these receptor transducers do not function in isolation but rather work in 'teams' to generate stronger signal amplification (6, 50). Typically MCPs form homodimers and then arrange themselves into trimers of dimers (82) and tend to cluster into large groups at the cell poles in many bacteria forming a receptor complex (2, 52, 60, 99). The receptor complex is shown in Wadhams article (168).

Excitation and adaptation

The chemotaxis signal transduction pathway is a complicated one that has two major phases: (1) Excitation which is the sensing and transducing of a signal and (2) Adaptation which is the monitoring and adjusting to changing concentrations of chemicals.

The signal sensed by the chemoreceptor molecule triggers an excitation response that is relayed to the flagellar motor by the CheA (histidine phosphokinase or HPK) and CheY (response regulator or RR). CheA, an autophosphorylating protein utilizing a highly conserved histidine residue, and ATP as its substrate, eventually transfers the phosphate to a conserved aspartate residue of the RR CheY (21, 65). Another RR, CheB, the methylesterase/amidase required for adaptation (65), competes with CheY for the phosphate which will be discussed later. The conformational change in CheY, resulting from its phosphorylation, decreases its affinity for CheA (134, 164) and increases its affinity for the flagellar motor protein FliM (176). The phosphorylated CheY protein interacts directly with the flagellar switch protein FliM inducing clockwise rotation of the flagella causing the cell to tumble and change direction (30, 131, 176). This is the normal default condition present in the absence of an attractant. In the presence of an attractant, autophosphorylation of CheA is decreased resulting in decreased CheY phosphorylation. The unphosphorylated CheY does not interact with the flagella motor and the result is a reversal of the flagellar rotation to the counterclockwise direction so that the bacteria now swim in a smooth manner rather than random tumbling. If the attractant concentration decreases, the CheA is autophosphorylated once again and the pathway causes the flagellar rotation to revert back to the default clockwise rotation and tumbling occurs. The events in the signal transduction pathway for bacterial chemotaxis are shown in Figure 2.

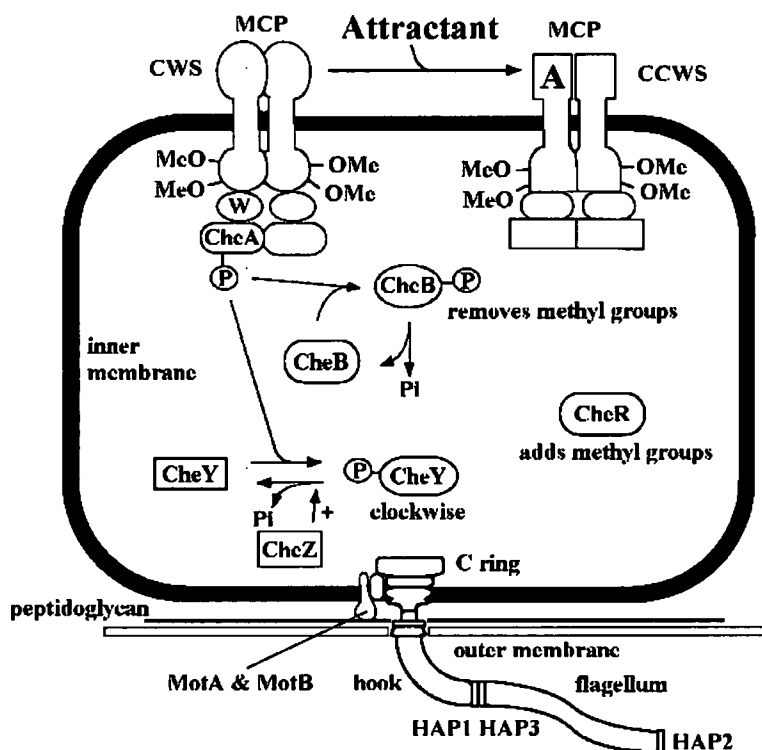


Figure 2. Excitation and adaptation in the bacterial chemotaxis pathway. The MCP at the left depicts the constant or default condition of clockwise flagellar rotation due to autophosphorylation of CheA and the subsequent phosphorelay pathway causing the cell to tumble. The MCP diagram to the right shows the effect of attractant binding to abolish the phosphorylation pathway. CheA autophosphorylation is reduced, as is CheY-P and CheB-P. In this state the MCP is heavily methylated and the lack of CheY-P causes a reversal of the flagellar rotation to a counter clockwise rotation to generate smooth swimming motility. Eventually the saturated methylation of the MCP causes a conformational change and CheA autophosphorylation resumes, CheY-P and CheB-P levels rise and the process is reset so the MCP can once again respond to attractant. (<http://www.bmb.leeds.ac.uk/illingworth/bioc1020/>)

The cell is able to continually monitor the concentration of the stimulant and respond appropriately to it by sensory adaptation, the ability of an organism to reset or clear its signal transduction system to the original stimulus. In this manner the organism is able to reassess the environment to determine if the concentration has changed relative to its new position. This is accomplished by reversible methylation of the MCP cytoplasmic signaling domain via two enzymes: CheR, a methyltransferase (142, 151) and CheB a dual function enzyme with deamidase and methylesterase activity (42, 154). CheR continually adds methyl groups to the MCP at a slow rate. In the default state, high autophosphorylation of CheA, there is sufficient CheB-P to remove methyl groups from the MCP. Attractant binding by MCPs inhibits the autokinase activity of CheA and thus results in lower CheB-P levels. Lower CheB-P levels result in increased methylation of the MCP molecule. Eventually chemoreceptor methylation overrides the conformational changes in the cytoplasmic signaling domain that first caused the reduction of CheA autophosphorylation activity. CheA autokinase activity increases again until CheA reaches its pre-stimulation levels. As a result of the autophosphorylation of CheA, CheY-P levels also rise and flagellar rotation reverts back to clockwise, and random movement. This back and forth phosphorylation is what results in the net migration toward or away from a chemical (*i.e.* directed movement). Fully methylated MCPs cannot respond to an attractant or repellant. However, the increase in phosphorylated CheB causes demethylation of the receptor. In this manner, the receptors (MCPs) are 'reset' so that they can once again respond to attractant. This adaptation/desensitization process allows the cell to continually evaluate the concentration of the attractant (or repellant) and modify its chemotactic response. This process is summarized in Figure 2.

Another protein involved in the adaptation process in *E. coli* and *S. enterica* serovar *typhimurium* is CheZ, a CheY-specific phosphatase (65). As mentioned previously both CheB and CheY compete for the phosphate from CheA. The CheB-P has a much shorter half-life than CheY-P (152). To compensate, CheZ functions specifically to increase dephosphorylation of CheY (65).

Because of the extensive studies over many decades of the chemotaxis systems of *E. coli* and *S. enterica* serovar *typhimurium*, they eventually became the standard for understanding bacterial chemotaxis. However as many more bacterial species outside the enteric world are studied and with the wealth of genomic information so readily available, it is becoming increasingly apparent that although many components of the basic mechanism described earlier are highly conserved in the bacterial world, the variations being discovered are numerous and could become the rule rather than the exception. For example a new CheX protein found in spirochetes (57) is required for chemotaxis, with a possible role in controlling reversal of direction in cell motility. Homologues of *cheX* have been found in other bacteria as well although functional studies have not been done in those bacteria. In *Bacillus subtilis*, three unique proteins, CheC, CheD, and CheV, not found in *E. coli*, are involved in aspects of adaptation (78, 127) and are required in addition to the standard CheR and CheB proteins (83, 84). Another variation of the enteric chemotaxis system occurs in the soil bacterium *Sinorhizobium meliloti*. This bacterium lacks the phosphatase CheZ, but has an additional CheY homologue that acts as a phosphate sink, substituting for CheZ function (56, 149). Finally *Rhodobacter spheroides* contains several complete chemotaxis

pathways, including thirteen MCPs, four CheW, four CheA, six CheY two CheB and three CheR homologues (101).

The Pil chemosensory system

Although there are numerous examples of variation on the enteric chemotaxis theme, there are also some close similarities among bacteria. For example, *P. aeruginosa* contains a gene cluster that codes for products remarkably similar to the chemotaxis proteins of the enterics and the gliding bacterium *M. xanthus*. This cluster is referred to as the *pilGHIJKchp* cluster (38, 40). These proteins are part of a larger system of genes referred to as *pil* genes.

The *pilJ* gene shows substantial similarity to the methyl-accepting chemotaxis proteins (MCPs) of *E. coli* (38, 39, 86). The *pilJ* gene contains two transmembrane regions, a putative periplasmic binding region, cytoplasmic methylation sites, and a highly conserved domain (HCD) (Figure 15) found among most MCPs that likely interacts with the CheW protein (38). It is highly likely that PilI and PilJ proteins interact together with another Pil protein, PilL (sequence identity with CheA) in a manner very similar to the MCPs, CheW and CheA of the enteric system when the MCP is stimulated. This concept is supported by evidence that both *pilJ* and *pilI* mutants share the same phenotype; they are unable to assemble TFP and are incapable of twitching motility (40).

The *pilI* gene product has high sequence identity with the CheW protein of *E. coli* and the FrzA protein of *M. xanthus* involved in their chemotaxis signaling system (106).

The *pilG* and *pilH* genes encode proteins with high sequence identity to the CheY protein of *E. coli* (153, 155). The PilG and PilH proteins are structurally similar to CheY

because they retain many of the residues that make up the hydrophobic core of CheY and have Asp and Lys residues that correspond to the CheY phosphorylation active site (24, 38, 39, 94). However, gene insertion mutations in *pilG* and *pilH* cause different effects on TFP production and function. The *pilG* mutant is unable to assemble TFP and does not display twitching motility while the *pilH* mutant is piliated and has an aberrant form of twitching motility (38, 39). This leads to the possibility that these two proteins, which are similar to CheY, are part of a much more complex chemosensory transduction system in *P. aeruginosa*. Therefore it seems likely that *P. aeruginosa* may be more like the chemosensory signal transduction system of *Sinorhizobium meliloti* with its two CheY proteins (149). *Pseudomonas* does not appear to have a CheZ homolog which is also true of *S. meliloti*.

Located directly downstream from *pilJ* is *pilK*, a gene that encodes a homolog of the chemotactic methyltransferase, CheR and is likely co-transcribed with *pilJ* (37). Further downstream is *chpB* which seems to be a *cheB* homolog.

The genetic analysis of the *pilGHIJK* cluster of *P. aeruginosa*, and the proteins it encodes, originally done by Darzins, demonstrates remarkable similarity to the chemotaxis proteins of the enterics and *M. xanthus*. Based on these similarities, Darzins developed a model to support the concept that these proteins are part of a signal transduction system of *P. aeruginosa* that controls TFP biosynthesis and twitching motility (40).

Two separate signal transduction pathways seem to be required for the biosynthesis of TFP and the resulting twitching motility. The first involves the PilS-PilR sensor regulator pair. PilS is the sensor protein located in the cytoplasmic membrane (26) that

autophosphorylates in the presence of an unidentified stimulus. The phosphate is then transferred to PilR, the response regulator, which interacts with the RNA polymerase and RpoN to activate transcription of *pilA* generating the pilin subunits, PilA.

The rotation of flagella, controlled by the Che system of chemotaxis, in the enterics is an illustration of a two stage motor (Figure 18). In this system, the motor controlling flagellar rotation is either set to a counterclockwise or clockwise direction depending on the presence or absence of stimulants and the transmission of appropriate signals using a series of signal transduction proteins. The Pil model proposed by Darzins seems to represent an analogous two stage motor system for the assembly/extension of TFP and the control of twitching motility. However, rather than controlling the direction of rotation of flagella, the Pil system controls the extension and retraction of TFP. In other words, in the *E.coli* model, the flagellar motor is engaged in stage one when the motor is rotating in clockwise fashion and is engaged in stage two when it reverses to a counterclockwise rotation. In the TFP model, the 'pilus motor' stage one is represented by extension and stage two by retraction. Our study will provide more details on the role of PilJ in the control of this process. In order for twitching motility to occur the pili must be able to do both extension and retraction and that process involves many proteins as described earlier. In spite of some of the differences between the Che system and the Pil system, there is a substantial amount of similarity between the two stage systems they represent.

Although this model seems quite plausible it is based largely on sequence data and analysis of *pil* mutants. Unanswered questions remain: what is the stimulus to which

PilJ and PilS are responding? Exactly how are PilG and PilH interacting if at all with pilus assembly proteins?

Table 1 provides a comparison of some of the major proteins in the chemosensory signal transduction pathways of the enterics, *M. xanthus* and *P. aeruginosa*. Although much is known about the Che system of the enterics, what remains to be learned is how bacteria with multiple chemotactic signal transduction pathways such as *P. aeruginosa* with its five pathways, integrates the various signals. Learning more about this integration can lead to greater understanding of the pathogenesis and symbiosis of these bacteria.

Table 1. Comparison of Che and Che-like proteins of *E. coli*, *M. xanthus* and *P. aeruginosa*.

Model Che system (<i>E. coli</i>)	Function	Frz System (<i>M. xanthus</i>)	Pil system (<i>P. aeruginosa</i>)
MCP (4 MCPs)	Chemoreceptor sensing protein	FrzCD (19)	PilJ (38) + others (26 MCPs)
CheW	Scaffold protein	FrzA (19)	PilI (38)
CheA	Histidine Phosphokinase (HPK) – relay protein	FrzE (19) , FrzZ (167)	ChpA (38)
CheY	Response Regulator (RR) Flagellar motor binding protein		PilG and PilH (38)
CheB	RR – methylesterase – remove CH ₃		ChpB (38)
Che R	Methyltransferase – add CH ₃	FrzF (19)	PilK (38)
CheZ	CheY-specific phosphatase	None identified	None identified

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 2.

Media and growth conditions. LB medium (Difco, Detroit, Michigan) was used for growth of *E. coli* and *P. aeruginosa* strains in most experiments as noted. For standard growth plates, agar (Difco) was added at a concentration of 1.5%. For twitching motility stab assays, the agar concentration was reduced to 1% in a volume of 11 ml per plate. Pseudomonas Isolation Agar (PIA) (Difco) was used in the generation of *pilJ* mutants. Other media used included Minimal Salts plus Succinate (MSS) (113) and Glucose YE (74). The antibiotic concentrations for *E. coli* were as follows: ampicillin, 100 µg/ml; and gentamicin, 15 µg/ml. For *P. aeruginosa* transconjugates, gentamicin and carbenicillin concentrations were 300 µg/ml. For all other growth of *P. aeruginosa* requiring antibiotics, the following concentrations were used: gentamicin, 200 µg/ml and carbenicillin, 60-100 µg/ml. All cultures were incubated at 37° C overnight with shaking (150 rpm) unless otherwise stated.

TABLE 2. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
<u>Strain</u>		
<i>P. aeruginosa</i>		
strains		
PAO1-AD	Wild type, (Twt ⁺); pili-specific phage sensitive	(38)
FA6	<i>pilJ::tc^r</i> derivative of PAO1-AD (Twt ⁻); pili-specific phage sensitive	(38)
PAO1-UO	Wild type (Twt ⁺)	Urs Ochsner
PAO1-UO <i>pilJ</i>	<i>pilJ::lacZ/gm</i> derivative of PAO1-UO (Twt ⁻)	This study
DAP119J	Wild type, Environmental isolate; aggressive twitching motility (Twt ⁺)	George Pierce, GSU
DAP119J <i>pilJ</i>	<i>pilJ::lacZ/gm</i> derivative of DAP119J (Twt ⁻)	This study
GSU-3	Wild type, Clinical isolate from bilateral keratitis (Twt ⁺)	George Pierce, GSU
GSU-3 <i>pilJ</i>	<i>pilJ::lacZ/gm</i> derivative of GSU-3 (Twt ⁻)	This study
mPAO1	Wild type, (Twt ⁺)	(72)
32512	IS <i>phoA</i> insertion in <i>pilJ</i> , at codon 332 of 683, (Twt ⁻)	(72)
31801	IS <i>phoA</i> insertion in <i>pilJ</i> , at codon 309 of 683, (Twt ⁻)	(72)
PAK	Wild type (Twt ⁺)	(32)
R364	Tn5-B21 insertion in <i>pilT</i>	(32)
S34	Tn5-B21 insertion in <i>pilU</i>	(32)
PAK-BΩ	Ω insertion mutant in <i>pilB</i>	(32)
<i>E. coli</i> strains		
DH5α	<i>recA endA1 gyrA96 thi-1 hsdR17 sup44 relA1 lacZ</i>	Gibco
	Δ <i>M15</i>	(Carlsbad, CA)
17-1	<i>RP4 2-Tc::Mu-Km::Tn7 pro r⁺ m⁺ (Tp^r Sm^r)</i>	(143)
Top 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) 80 <i>lacZ</i> M15	Invitrogen
	<i>lacX74 recA1 araΔ139 (ara-leu)7697 galU galK rpsL (Str^r) endA1 nupG</i>	(Carlsbad, CA)
<u>Plasmids</u>		
pUCP18	pUC18 with 1.8 kb stabilizing fragment of pRO1614 (<i>Ap^r</i>)	(178)
pUC <i>pilJ</i>	pUCP18 with 2.1 kb <i>Bam</i> HI- <i>Hind</i> III fragment (<i>pilJ</i>) (<i>Ap^r</i>)	This study
pEX18Ap	<i>Ap^r oriT⁺ sacB⁺</i> , gene replacement vector with MCS from pUC18	(66)
pEX <i>pilJ</i>	pEX18Ap with 2.1 kb <i>Bam</i> HI- <i>Hind</i> III <i>pilJ</i> fragment	This Study
pZ1918Gm	<i>Gm^r</i> , <i>Ap^r</i>	(135)

pEXJgm	pEXpilJ with 4 kb <i>SalI</i> fragment (<i>lacZ-Gm</i> cassette)	This Study
pEYFP	from pZ1918Gm <i>yfp</i> fragment (<i>Ap^r</i>)	Clontech (Mountain View, CA) (178)
pUCP19	pUC19 with 1.8 kb stabilizing fragment of pRO1614 (<i>Ap^r</i>)	
p19YFP	pUCP19 with 0.75 kb <i>Bam</i> H1- <i>Eco</i> R1 fragment of <i>yfp</i>	This study
p19JY	pUCP19 with 2.9 kb <i>Hind</i> III- <i>Eco</i> R1 <i>pilJ-yfp</i> fusion	This study
pBH223	RFP- <i>fimX</i> fusion, (<i>Ap^r</i>)	(70)
pSB94	6.1 kb plasmid containing the pBAD promoter	(13)
pSB94JY	pSB94 with 2.9 kb <i>Hind</i> III- <i>Xba</i> I <i>pilJ-yfp</i> fusion	This study
pCR2.1	TA cloning® vector	Invitrogen, (Carlsbad, CA)
pCRK	TA cloning® vector containing the 600 bp <i>Xba</i> I- <i>Eco</i> RI fragment of <i>pilK</i>	This study
pEX19Ap	<i>Ap^r oriT⁺ sacB⁺</i> , gene replacement vector with MCS from pUC19	(66)
pEX19K	pEX19Ap with the 600bp <i>Xba</i> I- <i>Eco</i> RI fragment of <i>pilK</i>	This study
pEX19JYK	pEX19K with 2.9 kb <i>Hind</i> III- <i>Xba</i> I <i>pilJ-yfp</i> fusion	This study
pADD698	6.2 kb <i>Eco</i> R1 fragment containing <i>pilG</i> , <i>pilH</i> , <i>PilI</i> , <i>PilJ</i> , <i>pilK</i> in pUCP18	(38)

^a Twt, twitching motility

Ap^r = ampicillin resistance in *E. coli*; when selecting for *P. aeruginosa*, carbenicillin was used.

DNA methods. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.) or Promega Corporation (Madison, Wisconsin) and were used as directed by the suppliers. Plasmid DNA was extracted and purified using the QIAprep[®] Spin Miniprep Kit (QIAGEN Sciences, Maryland) as described in the manual. When restriction digests were purified, the QIAquick[®] PCR Purification Kit (QIAGEN Sciences, Maryland) was used. DNA fragments were purified from agarose gels after electrophoretic separation using the QIAEX[®] II Gel Extraction Kit (QIAGEN Sciences, Maryland). PfuTurbo Hotstart DNA polymerase (Stratagene, La Jolla, CA) was used for PCR reactions. All PCR primer oligonucleotides were purchased from Sigma/Genosys (The Woodlands, TX).

Construction of isogenic mutants. The *pilJ* mutant, FA6, was derived from parent strain PAO1 as previously described (38) and obtained from that author. We refer to the parent strain PAO1 as PAO1-AD. We created isogenic *pilJ* mutants in other strains (PAO1-UO, DAP119J and GSU-3) by allelic exchange using a protocol based on the sucrose selection system as described previously (136).

To create *pilJ* isogenic mutants in these wild-type strains (PAO1-UO, DAP119J, and GSU-3), the *pilJ* gene from PAO1-AD, cloned into pUCP18, was digested and released by *Bam*HI and *Hind*III. The *pilJ* gene fragment was subcloned into the suicide vector pEX18Ap to generate the plasmid pEX*pilJ*. The *lacZ/gentamicin* cassette from pZ1918Gm was released with *Sal*I digestion and inserted into the *Xho*I site (base pair 793) of *pilJ* in pEX*pilJ* to disrupt the *pilJ* gene creating pPEXJGm. The recombinant vector (pEXJGm), containing the disrupted *pilJ* gene, was transformed into *E. coli* strain S17-1 for mating with the *P. aeruginosa* strains. Briefly the procedure was as follows.

On day one, PAO1-AD was grown for 48 hours in LB broth at 42°C without shaking (increased temperature improves mating by turning off restriction systems). On day two, *E. coli* S17-1 cells containing the new suicide plasmid pEXJGm were grown overnight in LB broth with 100 µg/ml ampicillin at 37°C with shaking. The following day 2.5 ml of the overnight S17-1 cells was added to 5 ml of LB_{Amp100} and grown for 5-6 hours at 37°C with shaking. In separate tubes, 6 mls of S17-1 and 1.5 ml of the PAO1-AD cells were spun and pelleted. Each pellet was then washed once with M63 media to remove the antibiotics and LB broth. Pellets were resuspended in 200 µl of M63 media and 50 µl of each bacterium was combined in a new tube, mixed and collected by centrifugation at 13,000 x g. After removing 50 µl of the supernatant, the pellet was resuspended and spotted onto a sterile 22 µm filter on an LB agar plate and incubated at 37°C overnight. The following day cells were collected from the filter by mixing and vortexing in a 15 ml conical centrifuge tube containing 1 ml of LB broth. Transconjugates were selected by growing on PIA-gentamicin plates. Gentamicin resistant transconjugates were replica plated onto on LB/Cb₃₀₀/5% sucrose agar and LB/Cb₃₀₀ agar to confirm plasmid integration. Cb resistant colonies were replica plated onto LB-gentamicin-5% sucrose agar and LB-carbenicillin agar. Sucrose resistant/carbenicillin sensitive putative mutants were screened using the standard twitching motility assay and confirmed by restoration of wild type phenotype using the vector containing the wild type *pilJ* gene (pUC*pilJ*).

Twitching Motility assays. Twitching motility was assayed by two methods as previously described (39). (i) **Macroscopic motility assay.** LB (1% agar) plates were stab-inoculated to the bottom of the polystyrene dish. Cultures were incubated 24-48 hours at 37°C. The visible zone of twitching motility between the agar and the Petri dish

interface was measured and photographed. In some cases the twitch zone was stained using coomassie blue as previously described (107). **(ii) Microscopic motility assay.** Twitching motility was assessed microscopically using the slide culture method described by Darzins (39). Strains to be tested in this manner were point inoculated onto the surface of a small square of LB (1% agar), placed on a microscope slide and covered with a cover slip. The slide cultures were incubated at 37°C for five hours in a humid environment. Twitching motility was observed under an Olympus BH2-RFC microscope at a magnification of 600X. The wild-type strain (Twt⁺) was included as a control in both assays. LB plates containing 60-100 µg/ml carbenicillin were used for assays with *pilJ* complemented strains.

Detection of pilin by Western Blot analysis. **(i) Total pilin.** To test for total pilin, cells were harvested from LB broth or LB (1% agar) plates and lysed by boiling for 10 min in Laemmli sample buffer. (This work done by Tracy Collins.) **(ii) Surface pilin.** Pili were isolated by isoelectric point precipitaton as previously described (38). LB (1.5% agar) plates were inoculated and grown overnight at 37°C. The following day, cells were resuspended in dH₂O. Pili were removed from the surface of the cells by vortexing and cells 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. (This work done by Tracy Collins.) **(iii) Cytoplasmic and periplasmic pilin.** In order to evaluate periplasmic and cytoplasmic levels of pilin, periplasmic and cytoplasmic proteins were obtained using a modification of the osmotic shock protocol of Nossal and Heppel (114) and that used for *Pseudomonas* by Jensch and Friche (74). Briefly, cells

were grown overnight in Glucose-YE media. The overnight culture was subcultured into fresh Glucose-YE media and grown to mid-log phase and 1 g wet weight of cells was recovered by centrifugation. Cells were then washed three times in 0.5% NaCl. The cell pellet was resuspended in 0.033M Tris-HCl buffer, pH 7.1 and mixed with an equal volume of 40% sucrose. After a 30 min equilibration with gentle shaking at room temperature, the suspension was centrifuged and the pellet quickly resuspended in ice cold 0.5mM MgCl₂ for 10 min with shaking. The suspension was then centrifuged and the supernatant containing the periplasmic proteins collected. The pelleted cells were resuspended in phosphate buffer and sonicated to rupture cells, releasing cytoplasmic proteins. Equivalent amounts of protein, as determined by the BCA protein assay (Pierce, Rockford, ILL), were separated by SDS-PAGE on a 12% Tris-HCl gel. Proteins were transferred to a nitrocellulose membrane by electroblotting. Pilin was probed using 1:4000 dilution of anti-pilin antiserum (a gift of Randy Irvin, University of Alberta, Canada), followed by the secondary antibody (anti-rabbit immunoglobulin G conjugated to alkaline phosphatase) (Promega, Madison, WI) diluted 1:4000, and detected using Western Blue Stabilized Substrate for alkaline phosphatase (Promega, Madison, WI).

***In situ* immunofluorescence.** Pili were observed using a modified *in situ* immunofluorescence procedure excluding the permeabilization step (41). Briefly, wild-type and *pilJ* mutant (FA6) cells of *P. aeruginosa* were grown overnight in LB broth at 37°C. The following day, cells were subcultured in LB broth and grown to early log phase. Cells were fixed with 3.5% formaldehyde for 15 min at room temperature, washed twice in PBS, and incubated in 0.1% Triton in PBS for 45 min at room temperature. Pilin protein was detected using antipilin antiserum 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 a Nikon Eclipse TS100 microscope at a magnification of 600X. (This work done by Tracy Collins.)

Construction of *pilJ-yfp* fusion in a multicopy plasmid. The *yfp* gene from pEYFP (Clontech, Mountain View, CA) was subcloned into the *Bam*HI-*Eco*R1 site of pUCP19, creating plasmid p19YFP, and transformed into *E. coli* Top 10 cells (Invitrogen, Carlsbad, CA) as described in the manual. This plasmid was used as a control to check YFP synthesis and cellular distribution when not fused to PilJ. A second plasmid, p19JY, was created containing the *pilJ-yfp* fusion. This fusion was created using PCR to add a *Hind*III and *Bam*HI site to the 5' (primer PILJOUT1) and 3' (JREVBAM) end of *pilJ* respectively. The plasmid pUCpilJ was used as a template, and the following oligonucleotide sequences (inserted restriction sites underlined): PILJOUT1, 5'GGCCGGCGCGGAAAGCTTGGGGCCAAAATATGAAGAA3' and JREVBAM, 5'CATTAAGCATTTGGATCCGGCCTGCTCCACGCCCTC3' used as primers. The following PCR conditions were used: initial denaturation at 96°C for 1 min, thirty seven cycles (96°C for 1 min, 83°C for 1 min, 72°C for 6 min), and a final extension of 72°C for 10 min.

In order to ensure the transcription and translation of the single protein fusion, PilJ-YFP, the *pilJ* stop codon was removed and replaced with the *Bam*HI site using the primer JREVBAM during the PCR reaction.

In a separate PCR reaction, a *Bam*HI site was added to the 5' end of the *yfp* gene in order to complement the 3' *Bam*HI site of the *pilJ* gene using the primer

BAMYFPFOR. The plasmid p19YFP was used as the template, and the following oligonucleotide sequence (inserted restriction sites underlined): BAMYFPFOR, 5'ATACGGATCCGTCCGCCACCATGGTGACGAAGGGCGAG3' was used as the forward primer. In addition, three amino acid linkers (shown in italics) from the MCS region of pEYFP were left in the sequence immediately following the *Bam*H1 site and just upstream of the start codon for the *yfp* gene. The pEYFP contained an *Eco*R1 site at the 3' end of the *yfp* fragment which was retained using PCR and the following oligonucleotide sequence (restriction site underlined) as the reverse primer: YFPOUT2, 5'CCGGCGCTCAGTTGGAATTCTAGAGTCGCGGCCGCT3'. PCR conditions were the same as previously described for the PCR of *pilJ*.

The gel-extracted *pilJ* PCR product was digested with *Hind*III and *Bam*H1 restriction endonucleases and the *yfp* PCR product with *Bam*H1 and *Eco*R1. pUCP19 was digested with *Hind*III and *Eco*R1. A three way ligation joined the two PCR products into the pUCP19 vector using the 5' *Hind*III site of *pilJ* and the 3' *Eco*RI site of *yfp* to generate the gene fusion construct p19JY. Figure 3 summarizes the process of engineering the *pilJ-yfp* fusion in pUCP19. The ligation product was transformed into *E. coli* Top 10 cells (Invitrogen) as described in the manual.

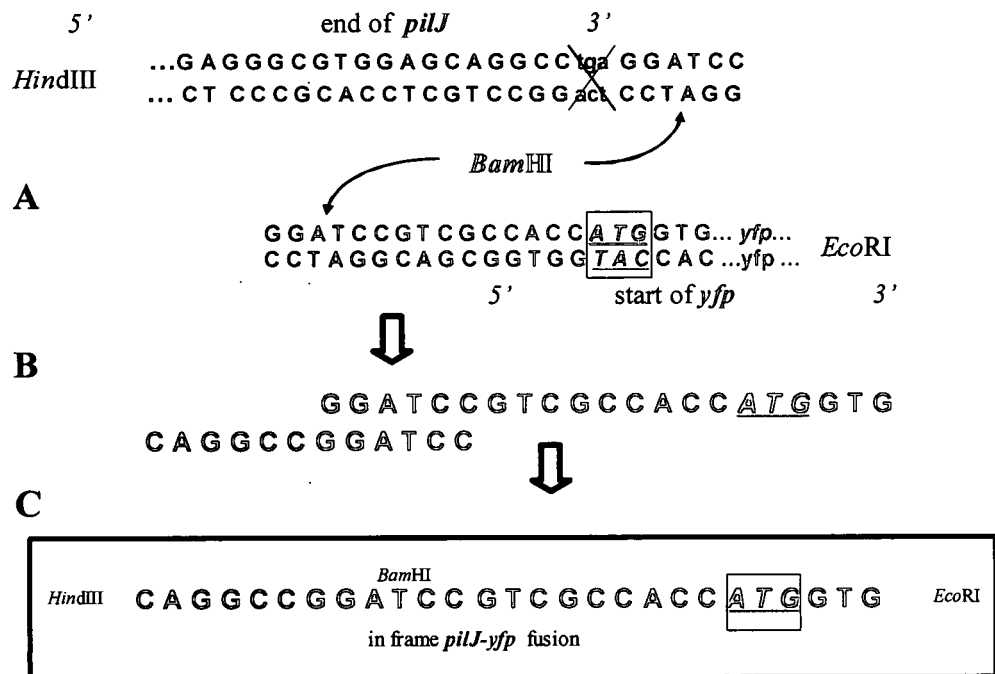


Figure 3. Engineering of the *pilJ*-*yfp* fusion. A) Adding restriction sites for fusion. A *HindIII* site is placed at the 5' end of *pilJ*. A *BamHI* site is inserted in place of the stop codon for *pilJ*. Another *BamHI* site is inserted into the 5' region of the MCS of the pEYFP plasmid to ensure the fusion is in frame. The existing *EcoRI* site at the 3' end of the *yfp* gene is retained. B) *BamHI* sites of the two genes line up and ligate together. C) Completed fusion contains a *HindIII* site at the 5' end, a joining *BamHI* sequence with three linker amino acids between the two genes and a 3' *EcoRI* site. The fusion can be ligated into the *HindIII* – *EcoRI* sites of pUCP19. ATG in the small box represents the start sequence for the *yfp* gene.

Plasmid constructs were extracted from the *E. coli* cells, confirmed by restriction digests, and transformed into *P. aeruginosa* using electroporation (146). Functional PilJ activity of the fusion construct was confirmed by complementation of *pilJ* mutants with p19JY to restore wild-type twitching motility phenotype. Cells containing the *yfp* constructs (p19YFP and p19JY) were grown on LB agar or broth supplemented with carbenicillin. Wet mounts were made from these cultures and examined to determine if the *yfp* was functional, as well as to determine localization of PilJ, using a Nikon Eclipse TS100 microscope at a magnification of 600X. In addition to the localization of PilJ in FA6, PilJ localization was also determined in *pilH* and *pilI* mutants of PAO1-AD as well as in *pilT*, *pilU*, and *pilB* mutants of the PAK strain. This was accomplished by the electroporation of the p19JY plasmid into the various strains. Cells grown in LB broth or slide cultures were examined to determine the effect of active twitching on localization of the PilJ protein.

In another experiment, we examined the location of the FimX protein in the FA6 strain using plasmid pBH223, containing a RFP-FimX fusion (70), to determine any possible relationship between PilJ and FimX. The plasmid pBH223 was electroporated into FA6 and cells were later examined for the localization of FimX using a Nikon Eclipse TS100 microscope at a magnification of 600X.

Inserting *pilJ-yfp* into the expression vector pSB94. In order to control the level of expression, the *pilJ-yfp* fusion was subcloned into an expression vector containing the pBAD system with an arabinose promoter (13). The plasmid map for pSB94 is shown in Appendix Figure F. In order to subclone the *pilJ-yfp* fusion into pSB94, p19JY and pSB94 were double digested with *HindIII* and *XbaI*. The digest of

p19JY released the *pilJ-yfp* fusion. For a 50 µl digest the following volumes were used: 28 µl water, 5 µl of 10x BSA, 5 µl of 10x Promega buffer E, 10 µl of pSB94 or p19JY, and 1 µl each of *HindIII* and *XbaI*. The digest was incubated at 37°C for 1 hour and inactivated for 20 minutes at 70°C before being separated on a 0.7% agarose gel containing 5 µl of ethidium bromide (1 mg/ml) for 1 hour at 70 volts. After gel extraction of the fusion, the fusion and pSB94 were ligated using 4 µl of Ligafast 2x buffer (Promega, Madison, WI), 2 µl of the vector digest, 1 µl of the extracted fusion and 1 µl of T4 DNA ligase (Promega, Madison, WI). The ligation reaction was incubated at room temperature overnight followed by transformation of the entire ligation reaction into Top 10 cells as described earlier, using LBgm₂₀ plates. The following day, colonies were subcultured and plasmid DNA extracted, followed by restriction digests using *HindIII* and *XbaI* as previously described to confirm the construction of the new plasmid pSB94JY. The new plasmid was electroporated into FA6 cells as described earlier.

The functionality of the construct was confirmed by examining cells for twitching motility and observation of fluorescence using a Nikon Eclipse TS100 microscope at a magnification of 600X with a FITC filter. Expression was tested by the addition of 0.2% L-arabinose to induce expression or 0.5% - 2% fucose and 0.5% - 2% glucose as inhibitors of expression. Cells were examined at those concentrations for twitching motility on MSS agar containing 0.5% casamino acids supplemented with gentamicin at a concentration of 50 µg/ml. To examine cells for localization of PilJ, 100 µl of overnight growth in LBgm₅₀ was inoculated into 10 ml of the same, incubated at 30°C while shaking, and grown to an optical density of 0.2 at 590nm before being induced or inhibited by arabinose and glucose respectively. After the addition of the inducer or

inhibitor, cells were grown about 1 additional hour as before to an optical density of 0.4 – 0.5. Cells were examined using a Nikon Eclipse TS100 microscope at a magnification of 600X.

Construction of an in-frame chromosomal fusion of *pilJ-yfp* to determine localization of PilJ under native expression levels. In order to optimize homologous recombination of the *pilJ-yfp* fusion, the 600 base pair region immediately downstream of *pilJ* was added to the 3' end of the *pilJ-yfp* fusion previously generated. This 600 base pair fragment containing a portion of *pilK* was subcloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA). This was done using the PCR and the plasmid pADD698 as the source DNA to add an *Xba*I and *Eco*RI site to the 5' and 3' end of the *pilK* fragment respectively to create the plasmid pCRK. The following primers were used to amplify the 600 bp fragment: XPILKFOR2 (forward; 5'ATAATCTAGAAAGCATAGGCGCGGCG3') and PILKEREV3 (reverse; 5'AATAGAATTCCTTGCGCGCCGGATAG3'). This process is shown in Figure 4. PCR condition were as follows: initial denaturation at 98°C for 10 minutes, 29 cycles (94°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute) and final extension of 72°C for 10 minutes. The *pilK* fragment was then subcloned into suicide vector pEX19Ap using the *Xba*I and *Eco*RI sites to create pEX19K. The original *pilJ-yfp* fusion previously generated in p19JY was then subcloned into the pEX19K plasmid using the *Hind*III and *Xba*I sites to generate pEX19JYK in *E. coli* Top 10 cells. This process is summarized in Figure 5.

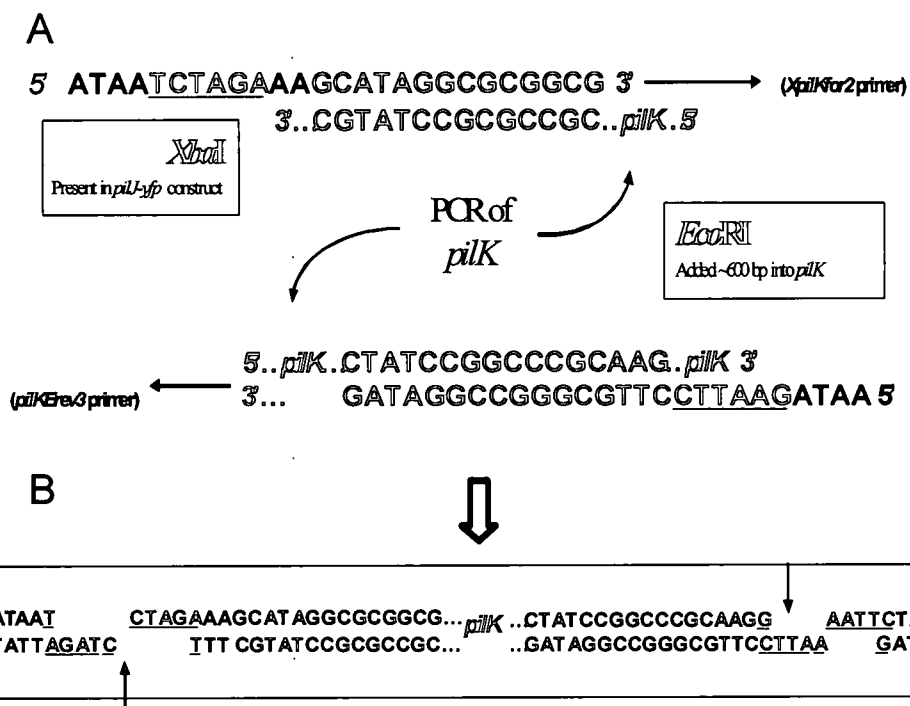


Figure 4. PCR of 600 bp of *pilK* with 5' *XbaI* and 3' *EcoRI* sites added. A. Primers used to generate 600 bp sequence of *pilK*. **B.** Result of PCR is sequence of *pilK* with 5' *XbaI* site and 3' *EcoRI* site for ligating into plasmid. This 600 bp sequence contains an intergenic sequence prior to the start codon for *pilK*.

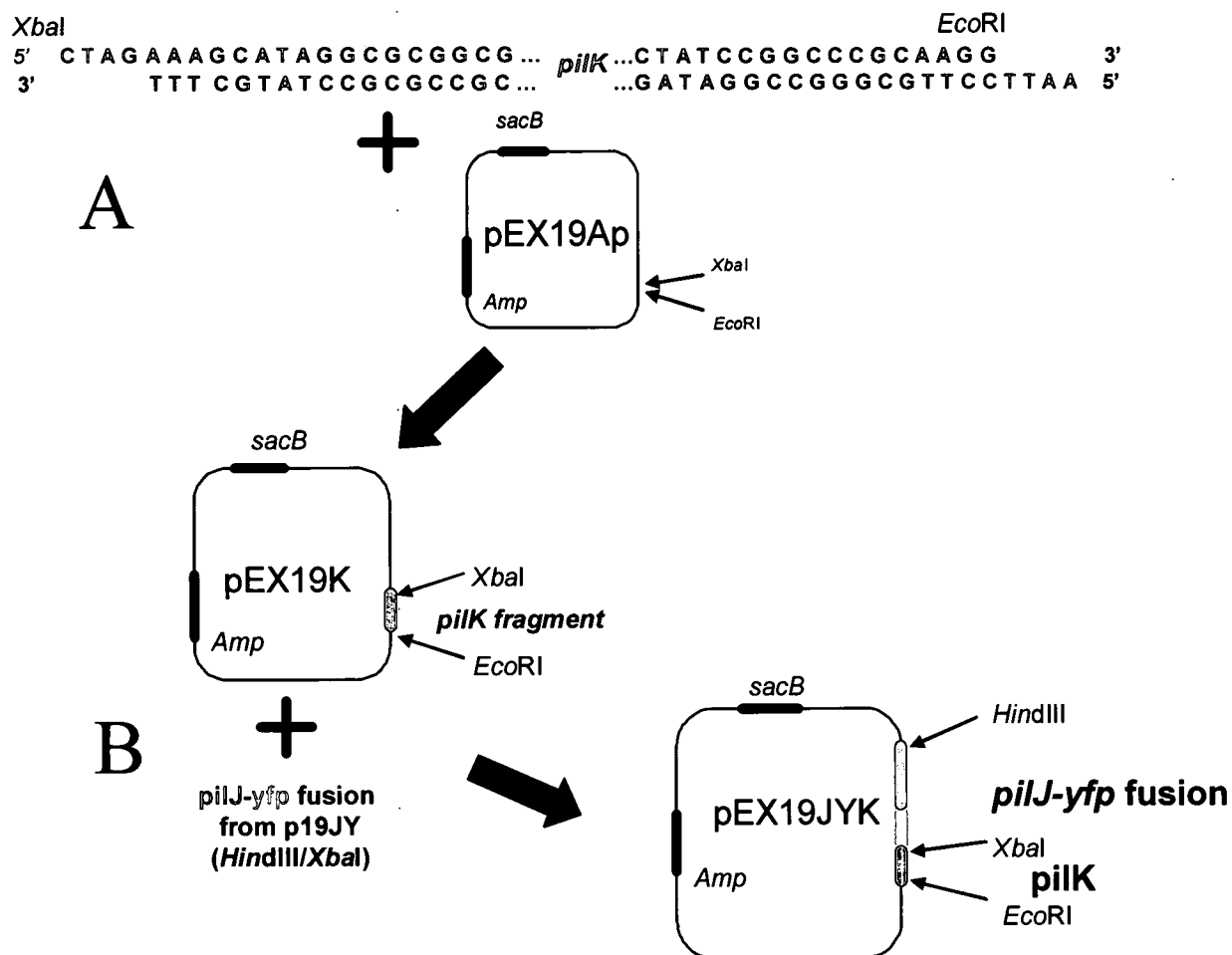


Figure 5. Process used to generate the suicide plasmid pEX19JYK. **A.** Subcloning of 600 bp fragment of *pilK* into *XbaI* and *EcoRI* sites of pEX19Ap yields pEX19K. **B.** Subcloning of *pilJ-yfp* fusion from p19JY into *HindIII* and *EcoRI* sites of pEX19K yields pEX19JYK.

The recombinant suicide vector, pEX19JYK, was transformed into *E. coli* S17-1 for mating with the *P. aeruginosa pilJ* mutant, FA6. This allelic exchange of the mutant *pilJ* gene of FA6 with the pEX19JYK was accomplished using the protocol based on the sucrose selection system as previously described (136). Briefly the procedure was as follows. On day one, FA6 was grown for 48 hours in LB broth at 42°C without shaking (increased temperature improves mating by turning off restriction systems). On day two, *E. coli* S17-1 cells containing the new suicide plasmid pEX19JYK were grown overnight in LB broth with 100 µg/ml ampicillin at 37°C with shaking. The following day 2.5 ml of the overnight S17-1 cells was added to 5 ml of LBamp₁₀₀ and grown for 5-6 hours at 37°C with shaking. In separate tubes, 6 mls of S17-1 and 1.5 ml of the FA6 cells were spun and pelleted. Each pellet was then washed once with M63 media to remove the antibiotics and LB broth. Pellets were resuspended in 200 µl of M63 media and 50 µl of each bacteria was combined in a new tube, mixed and collected by centrifugation at 13,000 x g. After removing 50 µl of the supernatant, the pellet was resuspended and spotted onto a sterile 22 µm filter on an LB agar plate and incubated at 37°C overnight. The following day cells were collected from the filter by mixing and vortexing in a 15 ml conical centrifuge tube containing 1 ml of LB broth. 200 µl of the suspension was spread on the surface of a PIACb₅₀₀ agar plate and incubated for 1-2 days at 37°C. Colonies were replica plated onto LB/Cb₃₀₀ and LB/Cb₃₀₀/5% sucrose agar plates and grown overnight to check for integration of the plasmid. Cells growing on the LB/Cb₃₀₀ were replica plated onto LB/Cb₃₀₀ and LB/5% sucrose agar plates and grown overnight. Cells growing on the LB/5% sucrose agar plates should contain the recombinant cells with the chromosomal *pilJ-yfp* fusion instead of the mutant *pilJ* gene of FA6. Recombination of

the *pilJ-yfp* fusion in the FA6 chromosome was confirmed by loss of sucrose sensitivity, demonstration of restored twitching motility in the FA6 mutant to wild-type levels and Western blot analysis.

Localization of PilJ. Cells containing p19YFP or p19JY were grown in LB broth to log phase and examined using fluorescence microscopy. Cells containing the in-frame chromosomal fusion were inoculated into LB supplemented with 0.4% NaCl, to enhance the quantum yield of YFP, and grown to log phase or 24 hours at 37°C. Cells were observed using a Nikon eclipse TS100 or Olympus BX51 microscope at a magnification of 600X.

Western blot analysis of *pilJ-yfp* fusions. Cells grown in LB broth either overnight or subcultured and grown to an optical density of 0.6 at 590 nm were harvested and lysed by boiling for 10 min in Laemmli sample buffer. Proteins were separated by SDS-PAGE on a 12% Tris-HCl gel. Proteins were transferred to a nitrocellulose membrane by electroblotting. YFP was probed using a 1:7,000 dilution of an anti-YFP antibody, referred to as A.V. monoclonal (JL-8) antibody (BD Biosciences Clontech, Palo Alto, CA), followed by the secondary antibody (anti-mouse immunoglobulin G conjugated to alkaline phosphatase; Promega, Madison, WI) diluted 1:5000, and detected using Western Blue Stabilized Substrate for alkaline phosphatase (Promega, Madison, WI). For increased sensitivity we used chemiluminescent detection: YFP was probed using a 1:50,000 dilution of the anti-YFP antibody, followed by anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody (Pierce, Rockford, IL) diluted 1:3000 and detected with SuperSignal® West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL) as described in the manual.

RESULTS

Allelic exchange construction of *pilJ* mutants. The allelic exchange method is diagrammed in Figure 6. The gene *pilJ* was successfully inserted into the suicide vector, pEX18Ap, generating the pEX*pilJ* plasmid of approximately 8 kb as demonstrated by restriction digests and electrophoresis as shown in Figure 7. In the next step the *pilJ* gene in pEX*pilJ* was disrupted by the addition of a *lacZ/gentamicin* cassette yielding a large plasmid of approximately 12 kb, referred to as pEXJgm (Figures 8 and 9). This construct was then transformed into *E. coli* S17-1 and mated with the *P. aeruginosa* strains. The resulting transconjugates were plated on PIA/Gm agar to select for plasmid integration. These colonies were replica plated on LB/gm/5% sucrose and LB carbenicillin to counterselect for chromosomal insertion. The results of that counterselection are shown in Figure 10. Growth on the Gm/sucrose plates demonstrated the incorporation of the mutated *pilJ* gene, containing the gentamicin cassette, into the chromosome and loss of the suicide plasmid containing the *sacB* gene. The loss of the pEX18Ap vector that transported the disrupted *pilJ* gene was also demonstrated by the new colony's sensitivity to carbenicillin on the replica plate.

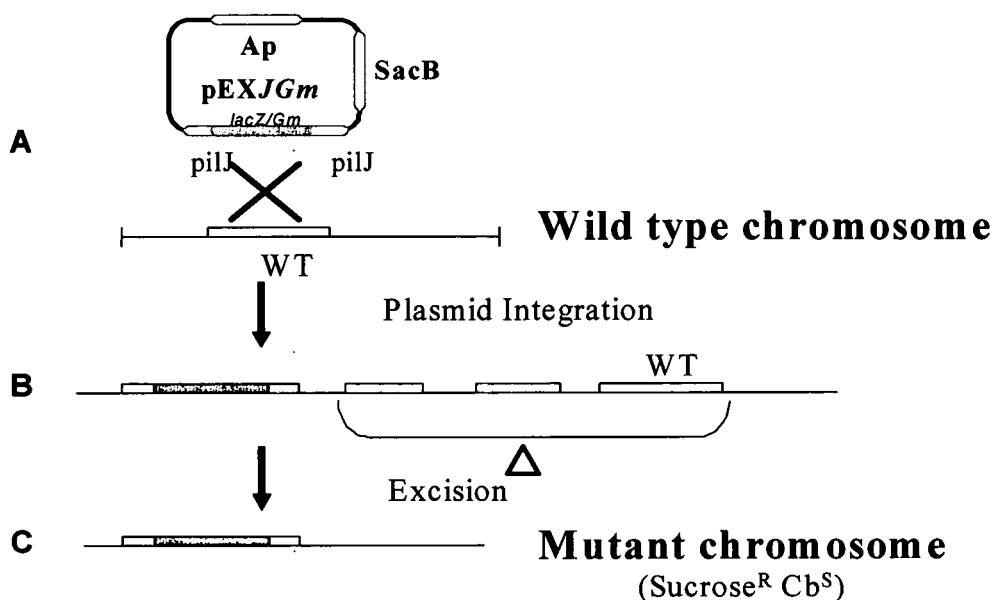


Figure 6. Allelic exchange mutant construction. A. Mating: an *E. coli* strain (S-17) carrying the suicide plasmid pEXJGm (containing the disrupted *pilJ* gene) was mated with a strain of *P. aeruginosa*. B. Plasmid integration: the plasmid carrying *pilJ* gene disrupted by the Gm cassette was incorporated into the chromosome in the same area as the wild-type gene. Cells carrying the suicide vector cannot grow in the presence of sucrose but carry the resistance gene to the antibiotic(s) on the plasmid. C. Mutant gene expressed; cells carrying the new mutated gene lack wild-type phenotype and characteristics originally carried on the excised suicide plasmid.

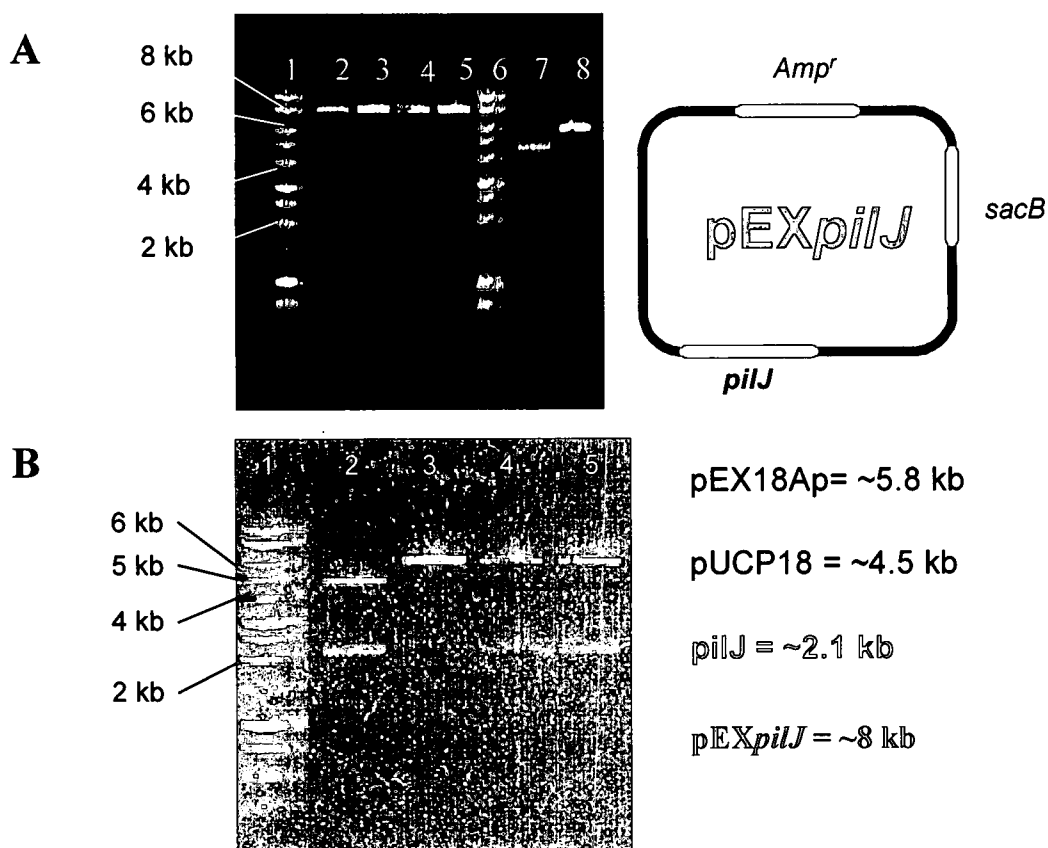


Figure 7. Construction of plasmid pEXpilJ. A) The insertion of *pilJ* into the suicide vector pEX18Ap yields plasmid pEXpilJ. Ln 1: markers; Ln 2-5 pEXpilJ linearized (*Hind*III digest); Ln 6: markers; Ln 7: pUC*pilJ* (*Hind*III, *Bam*HI digest); Lane 8: Vector pEX18Ap *Hind*III digest. B) Digest of pEXpilJ. Ln 1: markers; Ln 2: pUC*pilJ* digest with *Hind*III and *Bam*HI; Ln 3: pEX18Ap digest with *Hind*III; Ln 4-5: pEXpilJ digest with *Hind*III and *Bam*HI.

Disruption of *pilJ* with *lacZ/Gm* cassette

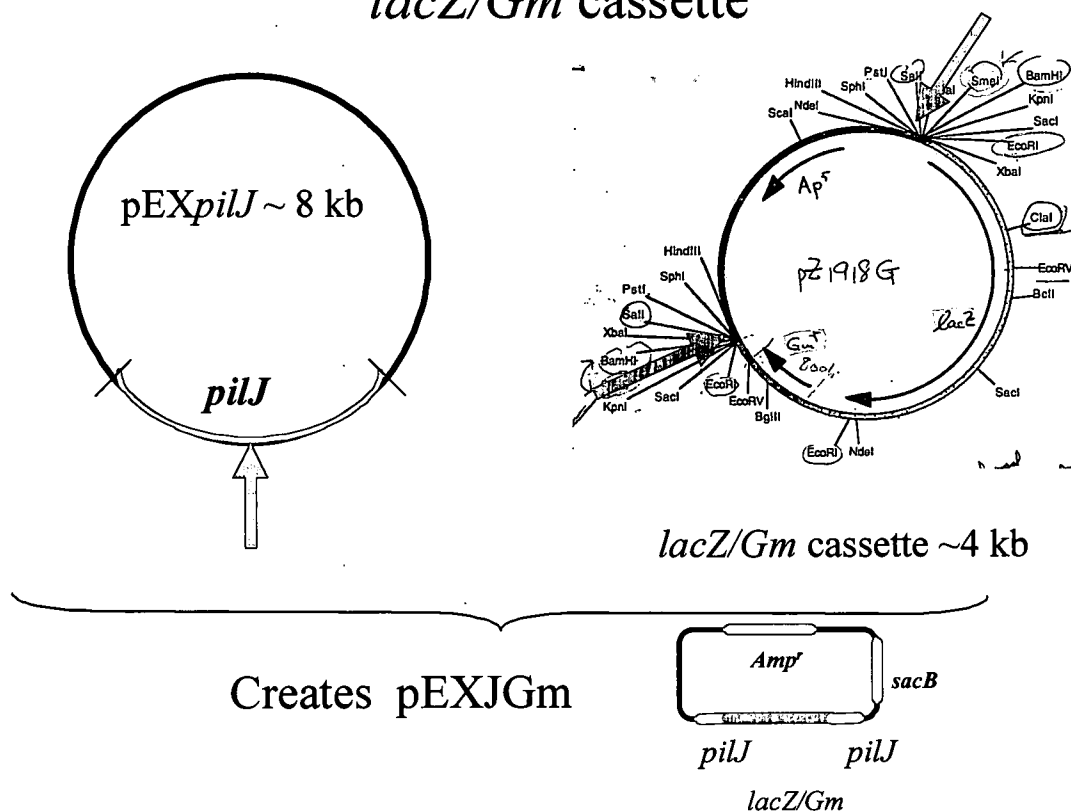


Figure 8. Construction of plasmid pEXJGm. *pEXpilJ* is cut with *XhoI* within *pilJ*. The plasmid *pZ1918G* is cut with *SalI* to release the *lacZ/Gm* cassette. The overlapping bases of these digests allow for the ligation of the *lacZ/Gm* cassette into *pEXpilJ* disrupting *pilJ* and creating the new plasmid *pEXJGm* still carrying the ampicillin resistance marker and the *sacB* gene.

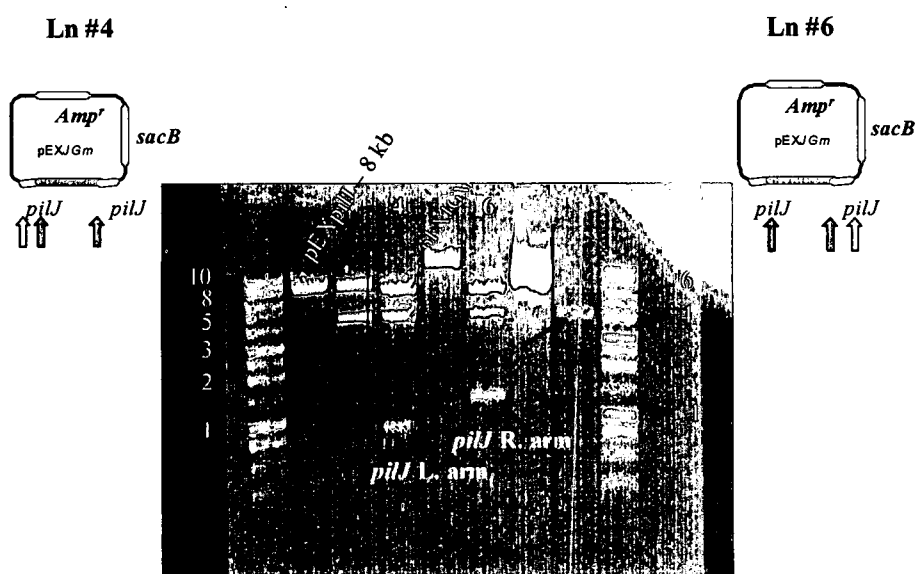


Figure 9. Confirmation of pEXJGm. The pEX*pilJ* construct was digested with *XhoI* and the *SaII* digested *lacZGm* cassette was ligated into the *XhoI* sites of pEX*PilJ* to generate pEXJGm. Lane 1: Markers; Lane 2: pEX*pilJ* linearized with *XhoI*; Lane 3: pEXJGm digested with *XbaI* released *lacZGm* fragment (4 kb); Lane 4: pEXJGm digested with *BamHI* released left arm of disrupted *pilJ* (~750 kb) and remainder of *pilJ* fused with the *lacZGm* cassette; Lane 5: pEXJGm linearized with *HindIII* (~12 kb); Lane 6: pEXJGm digested with *HindIII* and *XbaI* released right arm of disrupted *pilJ* (~1.3 kb) and remainder of *pilJ* fused with the *lacZGm* cassette; Lane 7: pEXJGm uncut; Lane 8: the *lacZGm* fragment digested with *SaII* and extracted from a gel (4 kb); Lane 9: Markers. Samples run on 0.7% agarose gel with ethidium bromide (5 μ l of 1 mg/ml).

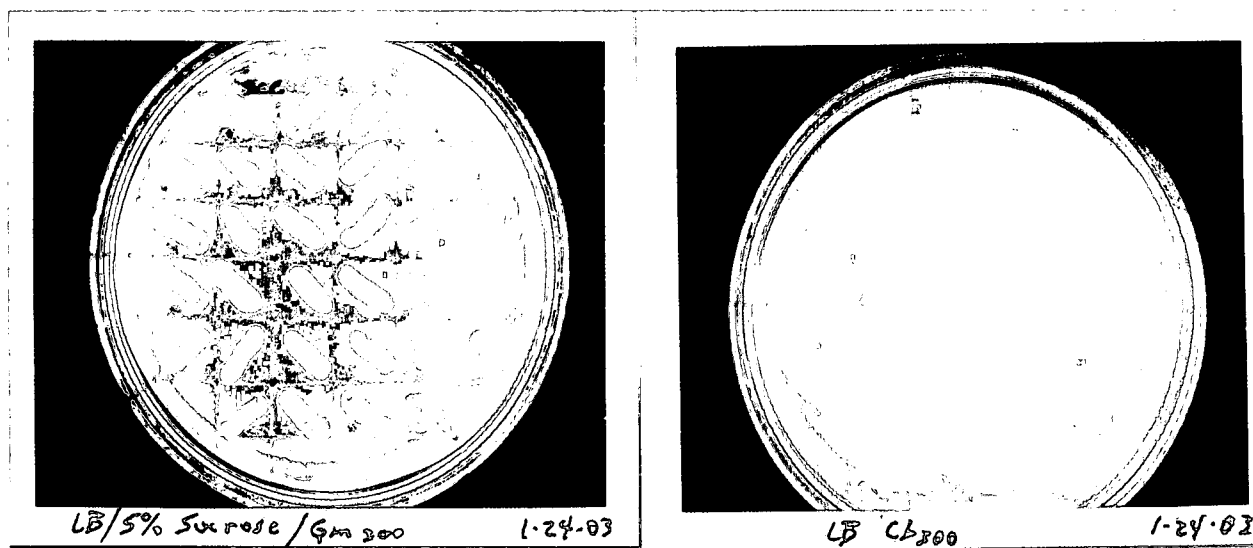


Figure 10. Verification of excision of suicide plasmid. **A.** Cells inoculated on LBgm₃₀₀/5% sucrose. Cells are capable of growth due to incorporation of the gentamicin cassette which was part of the disruption cassette in the *pilJ* gene, indicating the disrupted *pilJ* gene was present. Cells are also growing in the presence of sucrose indicating the suicide vector containing the *sacB* gene was excised. **B.** Same cells, replica plated onto LB/Cb 300 µg/ml, cannot grow due to the loss of the ampicillin resistance gene excised with the suicide vector.

***pilJ* mutants are incapable of twitching motility.** Macroscopic twitching motility assays were performed using five different wild-type strains of *P. aeruginosa*, PAO1-AD, PAO1-UO, mPAO-1, DAP119J, and GSU-3 to determine whether there was any difference between the standard laboratory strains (PAO1-AD, PAO1-UO and mPAO-1) and clinical (GSU-3) or environmental (DAP119J) isolates. For PAO1-AD, PAO1-UO, GSU-3 and DAP119J, the corresponding isogenic *pilJ* mutant was tested whereas for mPAO-1, two different transposon mutants from the Seattle Transposon Library, 31801 and 32512, were tested, as was the complemented mutant strain, and the isogenic mutant plus vector (pUCP18) alone. Twitching motility in the macroscopic assay is noted by the presence of a "halo" or twitch zone surrounding a surface colony. This twitch zone represents movement of cells at the interface between the agar and plate. Figure 11 shows the twitching motility deficiency of the first *pilJ* mutants in PAO1-UO and DAP119J. The composite results of the assay for all strains tested are shown in Figure 28. In all cases the wild-type strains displayed a surface colony surrounded by a distinct twitch zone (Figure 12, row 1). In contrast, the *pilJ* mutant strains exhibited surface growth, but lacked a twitch zone (Figure 12, row 2). In the complemented *pilJ* mutants, twitching motility was restored. (Figure 12, row 3). Using the method described by McMichael (107) to detect twitch zones by staining, we observed no difference between the two twitch motility assays as shown in Figure 13.

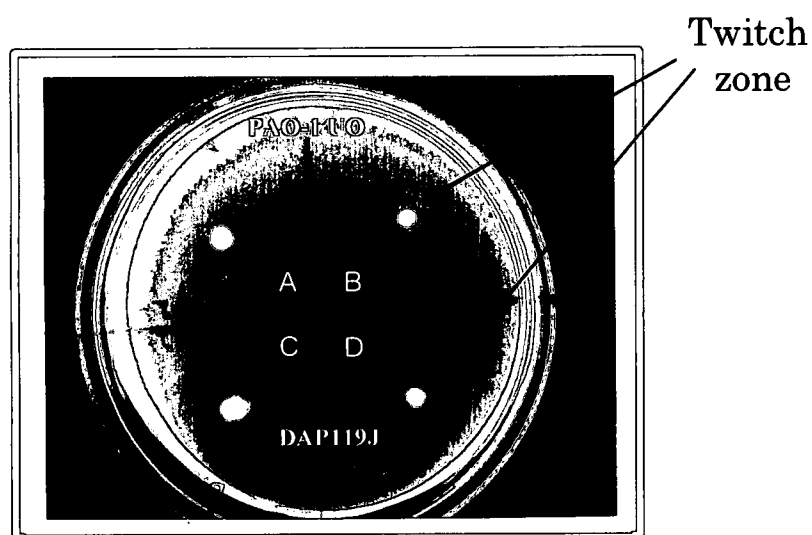


Figure 11. Twitching motility phenotype of *pilJ* mutants. Cells were stab inoculated onto 1% LB plates and incubated for 48 hours at 37°C. Twitching motility is seen as halos of growth around the spot inoculation. **A.** PAO1-UO *pilJ* mutant (PAO1-UO::*pilJGm*), **B.** PAO1-UO wild-type, **C.** DAP119J *pilJ* mutant, (DAP119J::*PilJGm*), **D.** DAP119J wild-type.

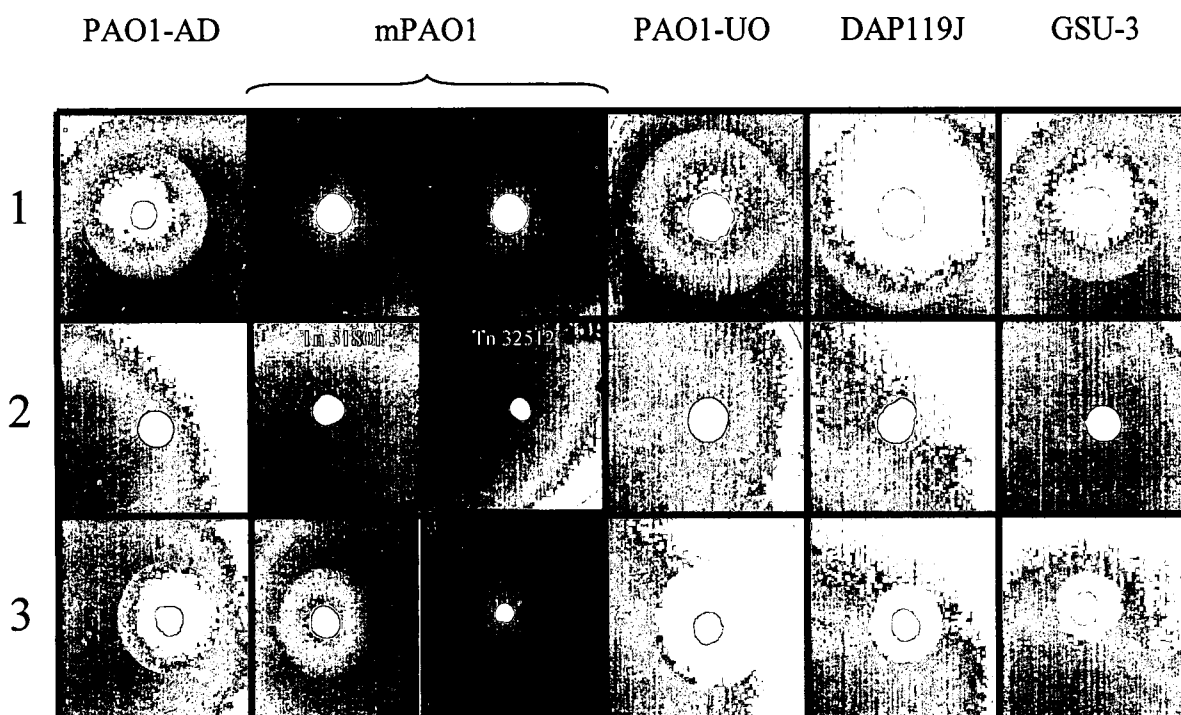


Figure 12. Macroscopic twitching motility of various wild-type strains of *P. aeruginosa* and *pilJ* mutants. 1% LB agar plates were stab inoculated and incubated for 48 hours at 37°C. The smaller white zones represent cells growing on the agar surface. Diffuse zones (twitch zones) represent cells moving at the agar-petri dish interface. Row 1: wild-type; Row 2: *pilJ* mutants; Row 3: complemented *pilJ* mutants, complemented with pUCP18::*pilJ*. Addition of control vector, pUCP18, to *pilJ* mutants had no effect on twitching motility (data not shown).

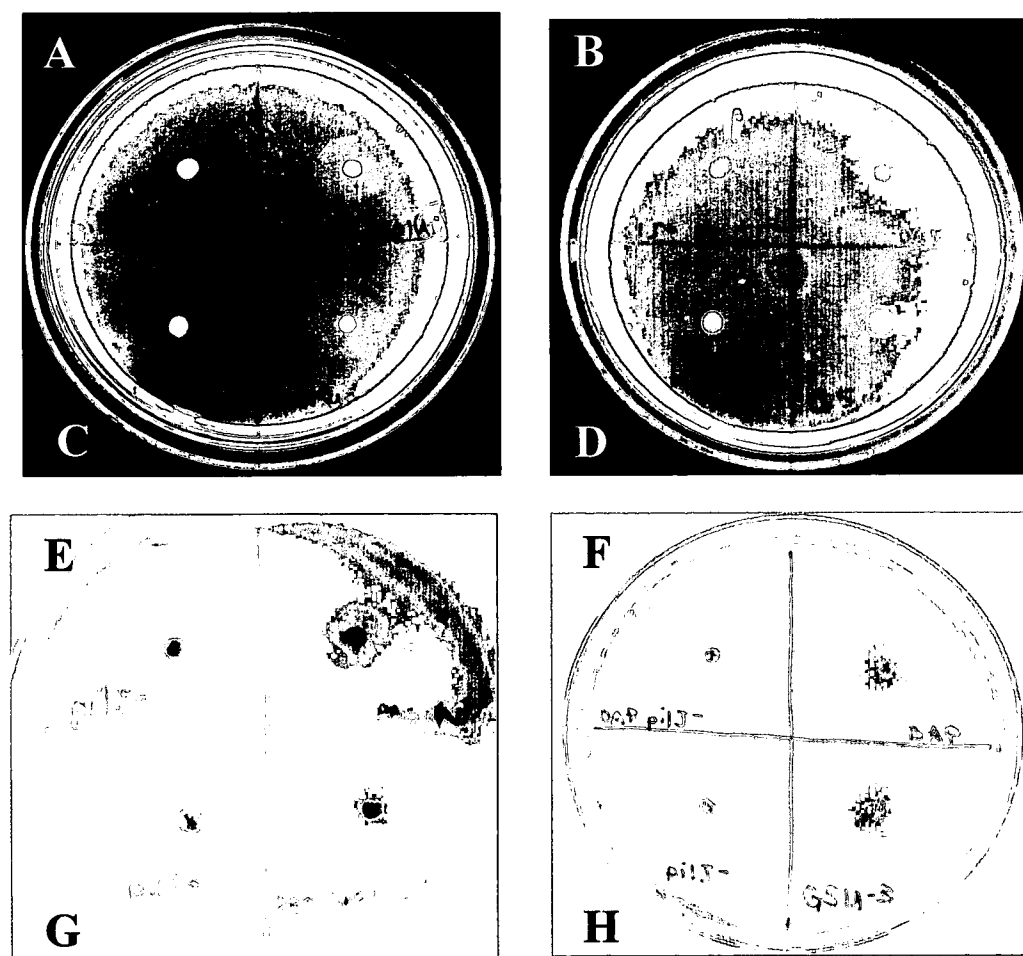


Figure 13. Comparison of macroscopic twitching motility assays. A-D) Standard twitch assay. A) FA6 and PAO1-AD wild-type; B) DAP119J *pilJ* mutant and wild-type; C) PAO1-UO *pilJ* mutant and wild-type; D) GSU-3 *pilJ* mutant and wild-type. E-H) Coomassie blue stained twitching motility assay. E) FA6 and PAO1-AD wild-type; F) DAP119J *pilJ* mutant and wild-type; G) PAO1-UO *pilJ* mutant and wild-type; H) GSU-3 *pilJ* mutant and wild-type.

Twitching motility phenotypes were confirmed microscopically. Using phase contrast microscopy (600X), rafts of wild-type PAO1-AD cells were observed twitching away from the point of inoculation as in Figure 14. An examination of the *pilJ* mutant strain, FA6, revealed that it was unable to move in rafts of cells and appeared to be packed against one another (Figure 14). Figure 14 also shows complementation of the mutant to wild-type phenotype in the microscopic twitch motility assay. Similar results were obtained with all other wild-type isolates, independent of their origin, and their corresponding *pilJ* mutants indicating the consistency of PilJ's role in twitching motility. Thus, FA6 is representative of all *pilJ* mutants we created. Composite results of the microscopic twitching motility assays for other strains are shown in Figure 15.

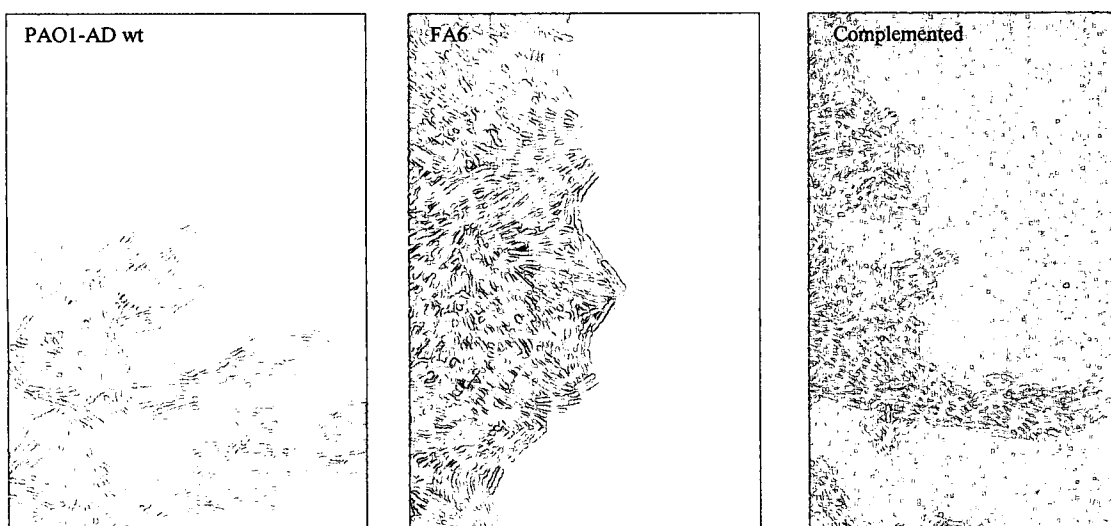


Figure 14. Microscopic twitching motility assay of PAO1-AD strains. Strains were inoculated onto blocks of LB (1% agar) plates and covered with glass coverslips. Following 5 hour incubation at 37°C, the outermost regions of the motile zone were photographed using bright-field microscopy at a magnification of 600X. PAO1 wild-type, *pilJ* mutant, FA6 (PAO1*pilJ*::Tc), and complemented *pilJ* mutant, FA6 + pUCP18::*pilJ*.

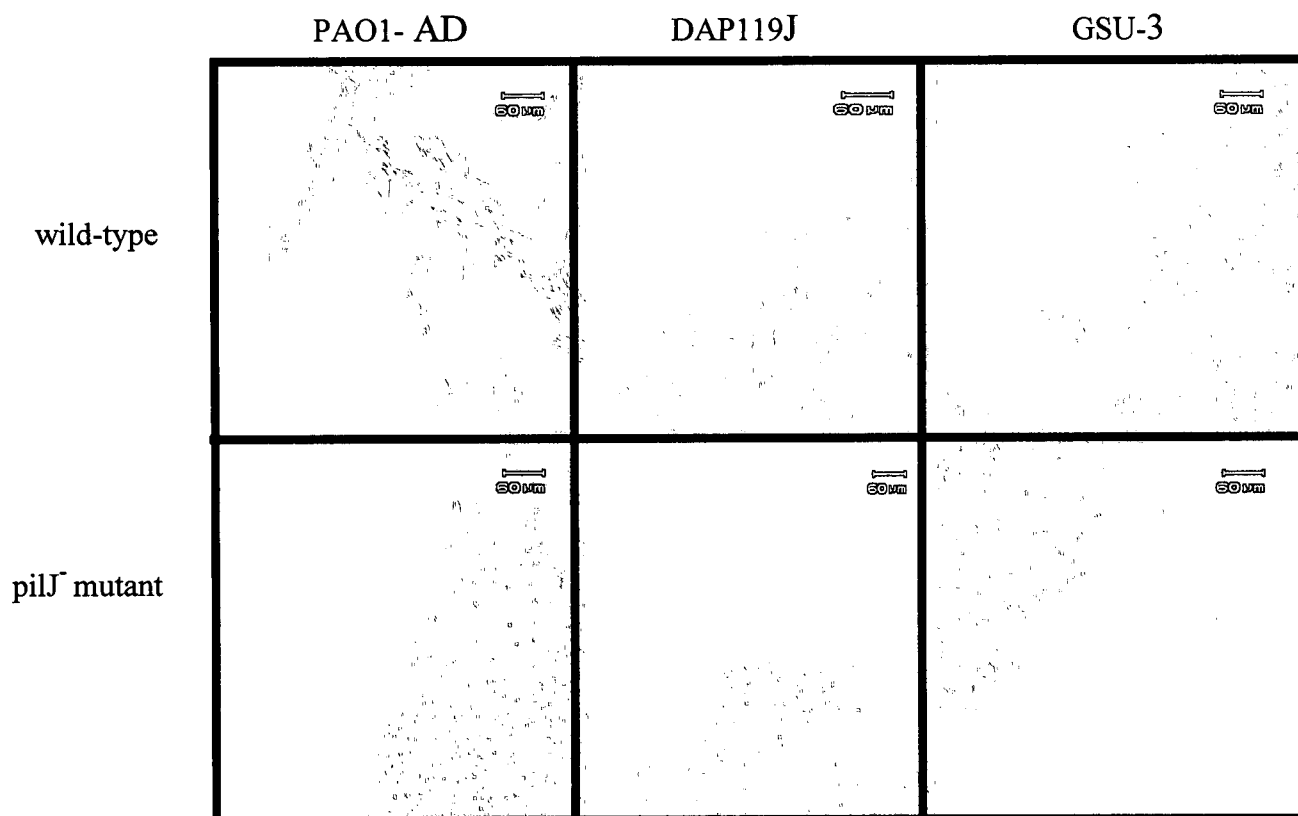


Figure 15. Microscopic twitching motility assay of multiple *P. aeruginosa* wild-type strains and mutants. Strains were inoculated onto blocks of LB (1% agar) plates and covered with glass coverslips. Following 5 hr incubation at 37°C, the outermost regions of the motile zone were photographed using bright-field microscopy at a magnification of 600X.

External pilin levels are nearly undetectable in *pilJ* mutants. We used Western blot analysis to compare pilin levels of wild-type and *pilJ* mutant cells. Whole cell lysates of wild-type PAO1-AD, FA6, complemented *pilJ* mutant (FA6+pUCP18::*pilJ*), cells overexpressing *pilJ* (PAO1 + pUCP18::*pilJ*), and PAO1 + pUCP18 (vector control) were probed using anti-pilin antibodies. Whole cell lysates represented the total complement of pilin (internal plus external) produced by cells. Plate-grown and broth-grown cells were analyzed. A single band was observed at approximately 18kDa for both plate and broth grown whole cell lysates (Figure 16), which corresponds to the predicted molecular weight of the pilin monomer in *P. aeruginosa*. The intensity of this band did not vary substantially among the wild-type PAO1-AD, *pilJ* mutant (FA6), and complemented *pilJ* mutant (FA6 + pUCP18::*pilJ*) cells. Overexpression of *pilJ* did not appear to affect the production of total cell pilin. Similar results were obtained for the PAO1-UO strains (data not shown).

Having established that total pilin production is unaffected in *pilJ* mutant cells, we examined the levels of external pilin present on the wild-type and *pilJ* mutant cells by Western blot analysis. A single band was detected corresponding to the predicted molecular weight of the wild-type pilin monomer in all strains with the exception of FA6 (Figure 16). There were no bands observed in the lane containing the *pilJ* mutant (FA6) indicating the absence of external pili. Overexpression of *pilJ* in wild-type background had no effect on external pilin levels. Similar results were obtained for the PAO1-UO strains (data not shown). (This work done by Tracy Collins, unpublished.)

The lack of detectable surface pilin on *pilJ* mutant cells explained the inability of those cells to actively twitch; however, they remain sensitive to pili specific phage (38).

These results prompted us to explore the role of PilJ in TFP assembly/extension. We considered two possibilities. The first possibility was that PilJ may be necessary for the movement of pilin subunits across the inner cell membrane only. In this case, if PilJ were absent, the pilin would be unable to move across the inner cell membrane for assembly into pili resulting in reduced levels of periplasmic pilin compared with that of the wild-type cells. The second possibility was that PilJ is not required for the movement of pilin subunits across the inner cell membrane, but instead controls the movement across the outer membrane. In this case, if PilJ were absent, pilin would accumulate in the periplasm of the mutant cells to a greater extent than in wild-type cells. To this end we compared the periplasmic and cytoplasmic levels of pilin by Western blot analysis. Results of the Western blots indicated there were no substantial differences in levels of periplasmic or cytoplasmic pilin between wild-type and *pilJ* mutant cells (Figure 16), suggesting that PilJ is not involved with the movement of pilin across the inner membrane.

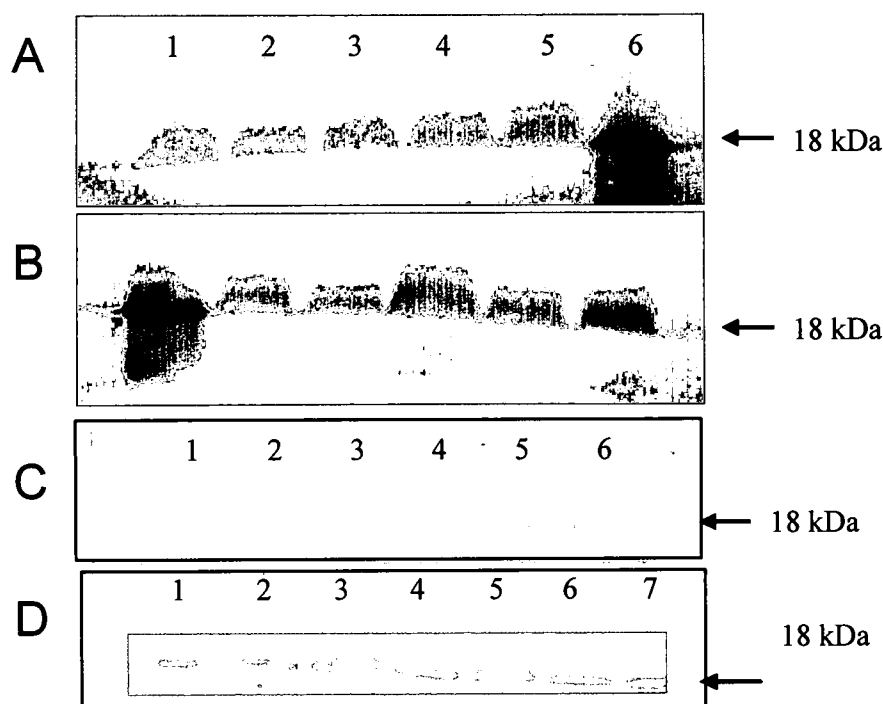


Figure 16. Western blot analysis of pilin A,B: **Western Blot of total pilin detected with *P. aeruginosa* antipilin antibody.** Whole cell lysates of cells grown in (A) LB broth (1) PAO1 wt, (2) FA6 (PAO1*pilJ*::Tc), (3) PilJ++ (PAO1 wild-type + pUCP18::*pilJ*), (4) Complemented FA6 (pUCP18::*pilJ*), (5) PAO1 wt + (pUCP18) vector control, (6) pilin. (B) **LB agar plates** (1) pilin, (2) PAO1 wt, (3) FA6 (PAO1*pilJ*::Tc), (4) PilJ++ (PAO1 wt + pUCP18::*pilJ*), (5) Complemented FA6 (pUCP18::*pilJ*), (6) PAO1 wt + (pUCP18) vector control. **C: Western blot of external pilin probed with antipilin antibody.** Equivalent amounts of cell protein were loaded for each sample. (1) PAO1 wt, (2) FA6 (PAO1*pilJ*::Tc), (3) PilJ++ (PAO1 wt + pUCP18::*pilJ*), (4) Complemented FA6 (pUCP18::*pilJ*), (5) PAO1 wt + (pUCP18) vector control, (6) pilin. **D. Western blot of periplasmic, cytoplasmic, and total pilin probed with antipilin antibody,** (1) wt periplasmic pilin, (2) *pilJ* mutant periplasmic pilin, (3) wt cytoplasmic pilin, (4) *pilJ* mutant cytoplasmic pilin, (5) wt total pilin, (6) *pilJ* mutant total pilin, and (7) pilin control. (A-C done by Tracy Collins.)

Direct analysis of orientation and extension of surface pili. We were unable to detect pilin recovered from the surface of *pilJ* mutant cells by Western blot; however, pilin levels in the periplasm of the mutant were equivalent to those in wild-type cells. This suggested that PilJ may be controlling pilin movement across the outer membrane which could result in either no external pili or incompletely assembled, short, pili that were too short to shear from the surface of the cell. To determine if PilJ was controlling pilin movement across the outer membrane, and therefore assembly and extension of pili, we examined cells for the presence of TFP on the surface of the cells using immunofluorescence. The fact that the *pilJ* mutants remain sensitive to pilus specific phage and were unable to twitch suggested that there was perhaps some small amount of pilin present on the surface that may not be detected by the Western blot analysis. Using anti-PilA antibodies, cells recovered from twitch plates or broth were examined using fluorescence microscopy for evidence of pili. As shown in Figure 17, wild-type PAO1 cells typically possessed pili extending from a single pole. On very rare occasions we observed wild-type cells with pili at both poles, although the pili were only fully extended at a single pole. In contrast, none of the *pilJ* mutant cells possessed long pili like those observed on the wild-type cells. Instead the mutant cells were found to have only single fluorescent dots or shortened pili. Surprisingly these shortened pili, or stubs, were frequently present at both poles of the mutant cells (Figure 17) in stark contrast to wild-type cells which typically had pili extending from only a single pole. Although the percentage of cells that were labeled was low, this percentage remained constant regardless of the level of fixative or amount of primary or secondary antibody and was

similar between wild type (25%) and *pilJ* mutant cells (19%). The low percentage of labeled cells may be attributed to the possibility that only a small number of cells have extended pili at any time, or that a large number of the fragile pili may be broken off during the protocol itself despite all efforts to avoid shearing. (This work done by Tracy Collins in our lab.)

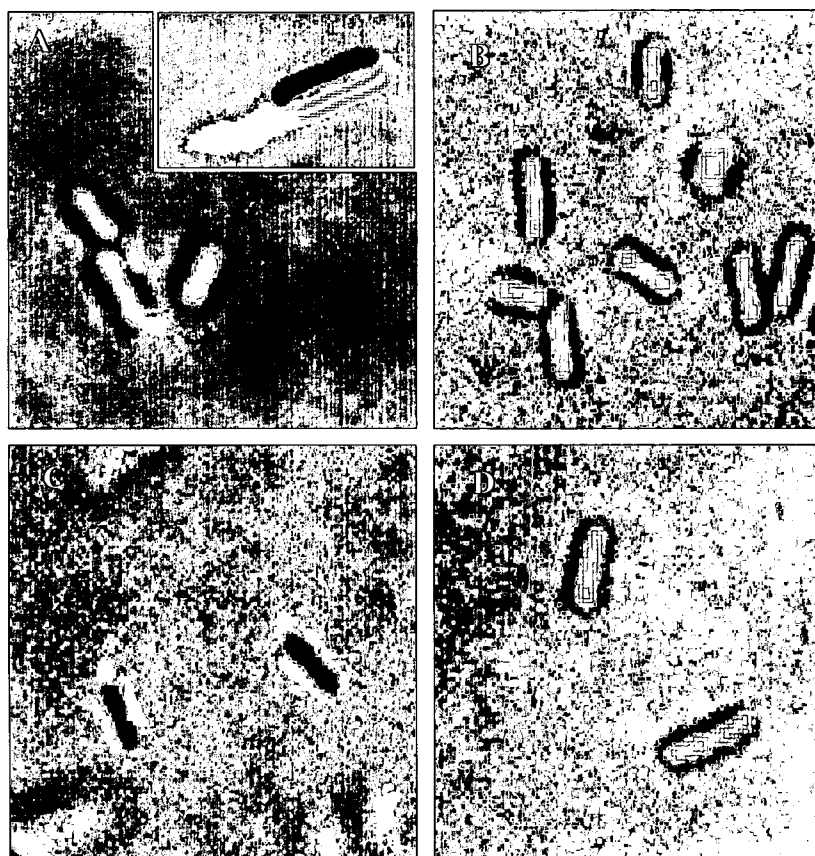


Figure 17. *In situ* immunofluorescence of pili in wild-type PAO1 and *pilJ* mutant (FA6) cells. Cells were fixed in 2.5% formaldehyde. Pili were detected using *P. aeruginosa* anti-PilA antibodies followed by secondary antibodies conjugated to quantum dots. Cells were observed using fluorescence microscopy at a magnification of 600X. (A) PAO1 wild-type; (B) Control PAO1 wild-type with secondary antibody only; (C) FA6 (PAO1*pilJ*::Tc); (D) Control FA6 (PAO1*pilJ*::Tc) with secondary antibody only. (This work done by Tracy Collins.)

PilJ is localized to both poles of the cell. MCPs have been shown to localize to the cell poles (2, 52, 60, 69, 99, 169). Because of the genetic similarities between PilJ and *E. coli* MCPs, we sought to determine the localization pattern of PilJ. In order to study the intracellular location of PilJ, we created a PilJ-YFP fluorescent protein fusion in a multicopy plasmid derived from pEX19Ap and referred to the new plasmid as p19JY. The first step was to subclone the *yfp* gene into pUCP19 to generate p19yfp as shown in Figure 18. As described earlier, restriction sites were added to the two genes using the PCR. Results of the PCR are shown in Figure 19. The PCR products were then ligated into pUCP19 as described generating the gene fusion plasmid, p19JY. Restriction digest analysis of p19JY is shown in Figure 20.

This plasmid was able to complement the *pilJ* mutant, FA6, by restoring twitching motility (Figure 21). In addition, we created a plasmid containing the *yfp* gene alone, plasmid p19YFP. When p19YFP was introduced into *E. coli* and FA6, cells fluoresced uniformly at 527 nm indicating that YFP itself was not localized to any particular location in either species (Figure 22A and B).

Having established that *pilJ* is functional in the p19JY construct and restores the wild-type twitching phenotype in FA6, we examined cells containing the *pilJ-yfp* fusion (p19JY) using fluorescence microscopy to determine the subcellular location of the PilJ protein in wild-type strains PAO1-AD, PAO1-UO, and *pilJ* mutants FA6, and the UO *pilJ::Gm* mutant. In contrast to cells containing only the *yfp* gene, *P. aeruginosa* FA6 cells containing p19JY fluoresced at polar regions of the cells grown to early log phase (Figure 22D). Surprisingly, this localization pattern was also observed in the *E. coli* carrying this plasmid construct (Figure 22C). In both wild-type strains tested, the PilJ-

YFP fusion construct was localized to both ends of the poles. PilJ remained localized to both poles in the PAO1-AD *pilH* and *pilI* mutants (Figure 22E and F) as well as in PAK mutants, *pilT*, *pilU* and *pilB* (Figure 22G, H, and I). While localization remained at the poles in all cases, the intensity of the fluorescence appeared much brighter in the *pilU* mutant (Figure 22H). The amount of fusion present between the poles was less in the *pilT*, *pilU* and *pilB* mutants. When the fusion was overexpressed (cells grown overnight) the fusion was also observed between the poles of the FA6 cells, at times visible in a loosely organized spiral pattern (Figure 23).

Because we observed some PilJ-YFP between the poles in cells containing the multi-copy plasmid born *pilJ-yfp* fusion in *P. aeruginosa* FA6 cells we inserted the fusion into an expression vector containing the pBAD system with an inducible arabinose promoter. The first step was to insert the *pilJ-yfp* fusion into the pSB94 expression vector. Results of the electrophoretic confirmation of that step are shown in Figure 24. Complementation of the FA6 cells with pSB94JY did restore twitching motility demonstrating that the *pilJ* gene in the fusion was functional as shown in Figure 25. However, because these twitch plates containing no inducer (arabinose) and yet still showed complementation, it appeared that the arabinose promoter was leaky, induced even in the absence of inducer. A closer look at the literature and source of the plasmid from Bardy (13) indicated that there was indeed some expression with this plasmid in the absence of inducer as demonstrated by Western blot analysis. Since fucose and glucose are inhibitors of arabinose induction, we tested these in an attempt to turn off or decrease expression. Results of the twitching motility assay of cells containing the expression plasmid with arabinose, fucose and glucose were inconsistent. We were unable to affect

expression (degree of twitching motility) in any meaningful manner (results not shown). While we were able to see general localization of the fluorescent fusion at both poles using the pSB94JY construct (Figure 26), we also observed scattered fluorescence between the poles and were unable to detect any substantial change in fluorescence of this vector using either of the inducers or inhibitors. The most significant difficulty was the inability to stop or control expression with this vector.

Because we observed some PilJ-YFP between the poles in cells containing the multi-copy plasmid born *pilJ-yfp* fusion and the expression vector construct pSB94JY in *P. aeruginosa* FA6 cells and were unable to control the expression using the pSB94 expression vector, we created an in-frame chromosomal fusion of *pilJ-yfp*, replacing the mutant *pilJ* gene, to examine localization under native expression levels. The first step in that process was to generate a *pilK* fragment and join it to the 3' end of the *pilJ-yfp* fusion to optimize homologous recombination. Figure 27 shows the results of the PCR to generate the 600 bp fragment of *pilK*. Once generated, the *pilK* fragment was subcloned into the PCR vector, pCR2.1 to generate the new plasmid pCRK and confirmed by restriction digest and gel electrophoresis as shown in Figure 28. The next step in the construction of the plasmid for use in the allelic exchange was to extract the *pilJ-yfp* fragment from p19JY using *HindIII* and *XbaI*. Results of that digest are shown in the gel in Figure 29. The suicide plasmid pEX19Ap was also linearized with *HindIII* and *EcoRI*. Initially the *pilK* fragment was subcloned into pEX19Ap generating pEX19K (Figure 29). Finally, the *pilJ-yfp* fusion was subsequently subcloned into the *HindIII* and *XbaI* sites of pEX19K generating the new suicide vector pEX19JYK as shown in Figure 30.

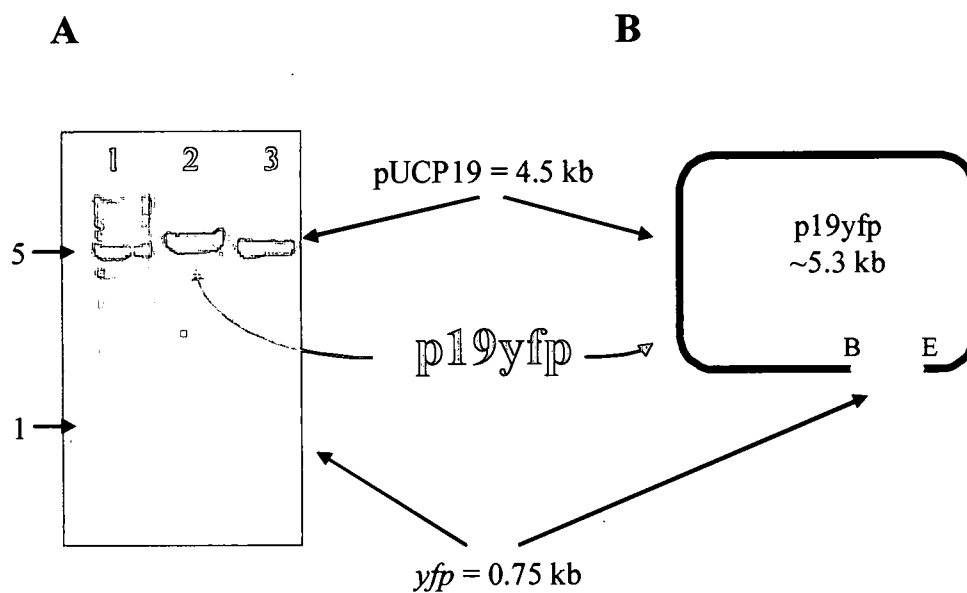


Figure 18. Restriction endonuclease digest and schematic representation of plasmid p19yfp. A) restriction digest of p19yfp. Ln 1: markers; Ln 2: p19yfp digested with *Bam*HI alone; Ln 3: p19yfp digested with *Bam*HI and *Eco*RI. B) Representation of p19yfp showing restriction sites used in the construction; B = *Bam*HI, E = *Eco*RI.

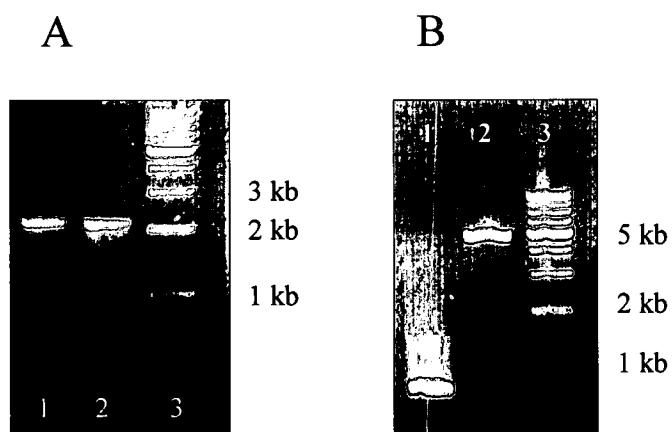


Figure 19. PCR results of *pilJ* and *yfp* with restriction sites added. A) PCR product of *pilJ* (2.1 kb) with the addition of a 5' end *HindIII* site and a 3' *BamHI* site in place of the stop codon for *pilJ*. Ln 1-2: *pilJ* PCR product; Ln 3: markers. B) PCR product of *yfp* (0.75 kb) with a new 5' *BamHI* site and the existing *EcoRI* site at the 3' end of the gene. Ln 1: *yfp* PCR product; Ln 2: *BamHI* and *EcoRI* digest of pUCP19; Ln 3: Markers.

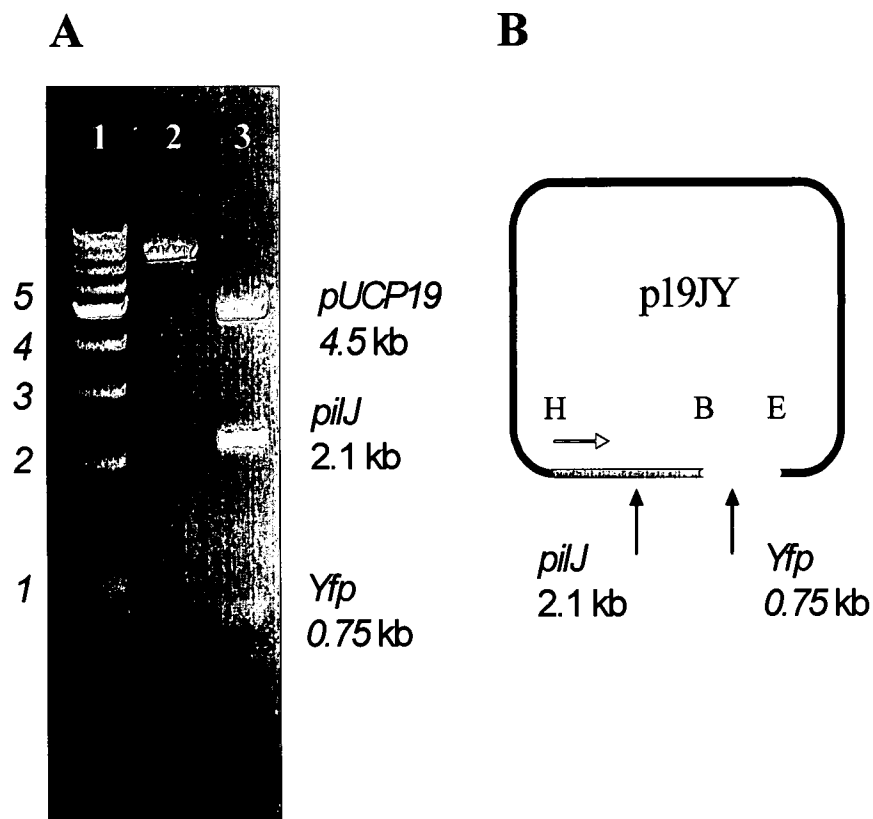


Figure 20. Restriction endonuclease digest and schematic representation of plasmid p19JY. A) Restriction digest of p19JY. Ln 1: markers; Ln 2: p19JY digested with *Hind*III only; Ln 3: p19JY digested with *Hind*III, *Bam*HI, and *Eco*RI to release all elements of the construct. B) Representation of p19JY showing restriction sites used; H = *Hind*III; B = *Bam*HI; E = *Eco*RI. Red arrow represents direction of transcription.

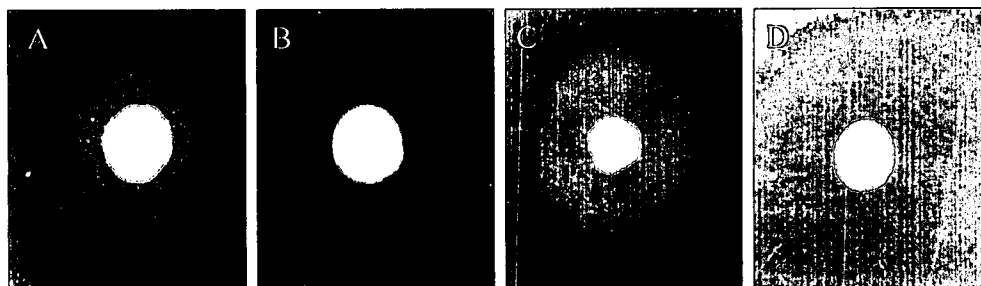


Figure 21. Complementation of *pilJ* mutant by p19JY. 1% LB agar plates were stab inoculated and incubated for 48 hours at 37°C. The smaller white zones represent cells growing on the agar surface. Diffuse zones (twitch zones) represent cells moving at the agar-petri dish interface. A) PAO1 wild-type, B) *pilJ* mutant, FA6 (PAO1*pilJ*::Tc), C) Complemented *pilJ* mutant, FA6 (p19JY), and D) FA6 with vector control, pUCP19.

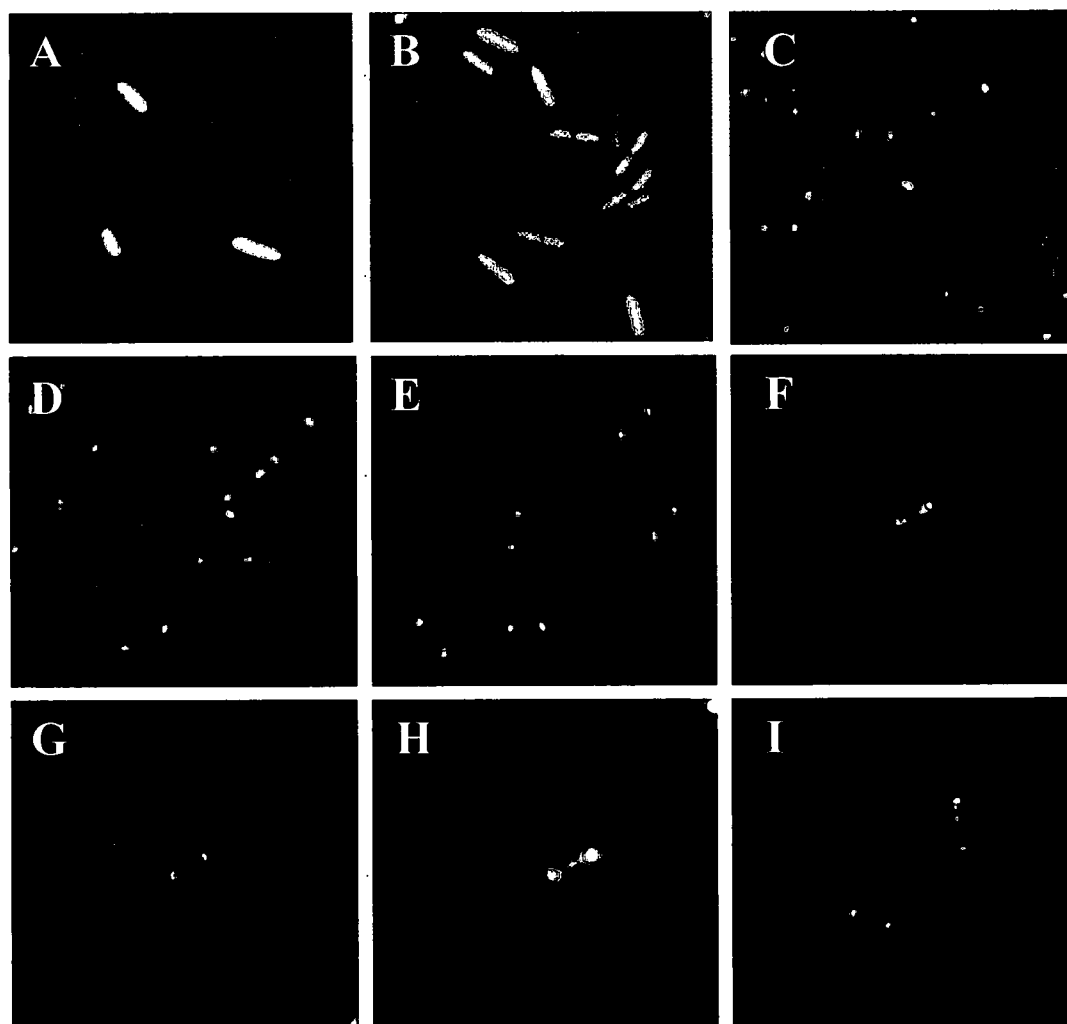


Figure 22. Localization of PilJ in numerous strains using p19JY. Plasmids containing either the *yfp* gene alone (p19yfp) or the fluorescent gene fusion *pilJyfp* (p19JY) were electroporated into *E. coli* Top 10 wild-type cells or *P. aeruginosa* mutant cells. Cells were inoculated into LB_{amp100} broth, grown to log phase and examined using fluorescence microscopy. A) Plasmid p19yfp in *E. coli*; B) Plasmid p19yfp in *P. aeruginosa* FA6 (*pilJ* mutant); C) Plasmid p19JY in *E. coli*; D) Plasmid p19JY in FA6; E) Plasmid p19JY in *pilH* mutant cells; F) Plasmid p19JY in *pilI* mutant cells; G) Plasmid p19JY in PAK *pilT* mutant cells; H) Plasmid p19JY in PAK *pilU* mutant cells; I) Plasmid p19JY in PAK *pilB* mutant cells.

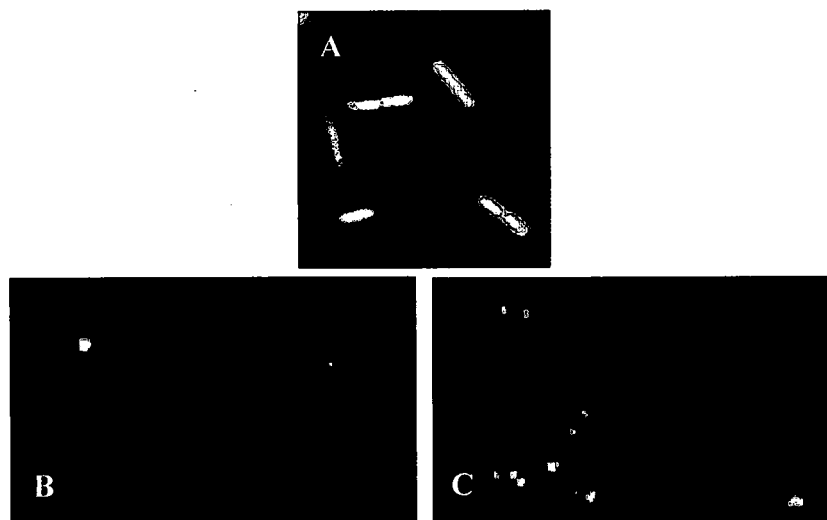


Figure 23. Overexpression of *pilJ* by the multi-copy plasmid p19JY. (A) FA6 with p19YFP, (B) FA6 with multi-copy plasmid born p19JY (log phase), (C) FA6 with multi-copy plasmid born p19JY, (24 hour growth),

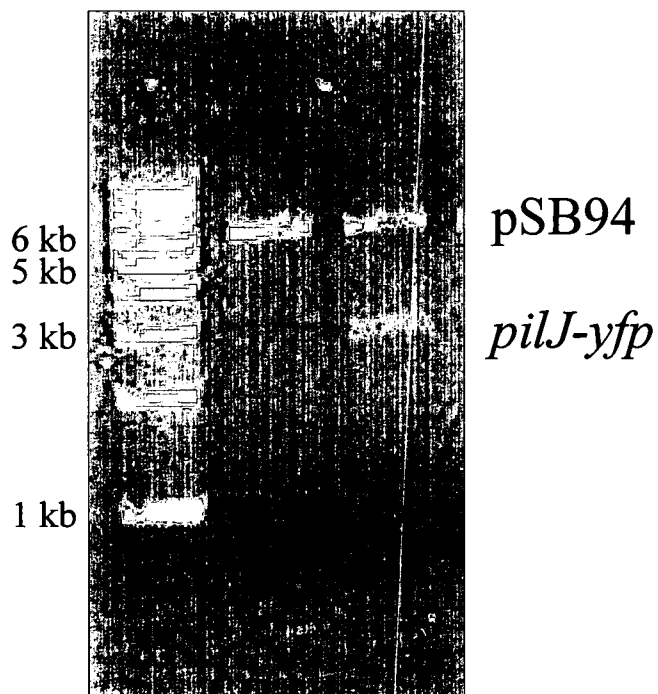


Figure 24. Restriction endonuclease digest of pSB94JY. Double digest of pSB94JY with HindIII and XbaI to release *pilJ-yfp* fusion. Lane 1 = 1 kb marker; Lane 2 = pSB94 digest with HindIII and XbaI; Lane 3 = pSB94JY digested with HindIII and XbaI. pSB94 = 6.1 kb and *pilJ-yfp* fusion = 2.9 kb.

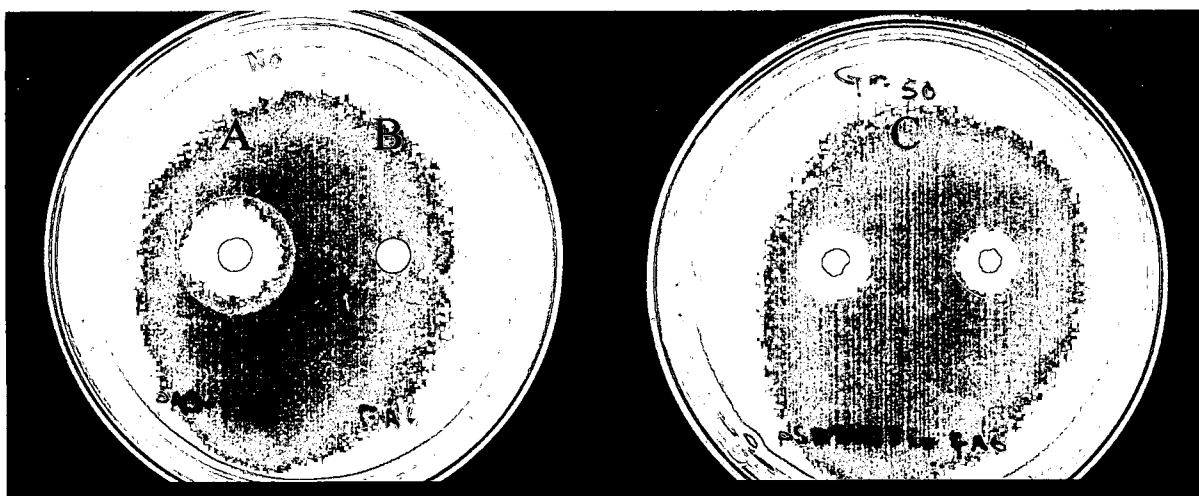


Figure 25. Complementation of *pilJ* mutant with pSB94JY. A. PAO1-AD (wt); B. FA6. A and B inoculated onto 1% twitch plates containing M63 media and no antibiotic. C. FA6 complemented with pSB94JY on 1% twitch plate containing M63 media, 50 μ g/ml gentamicin, no arabinose.

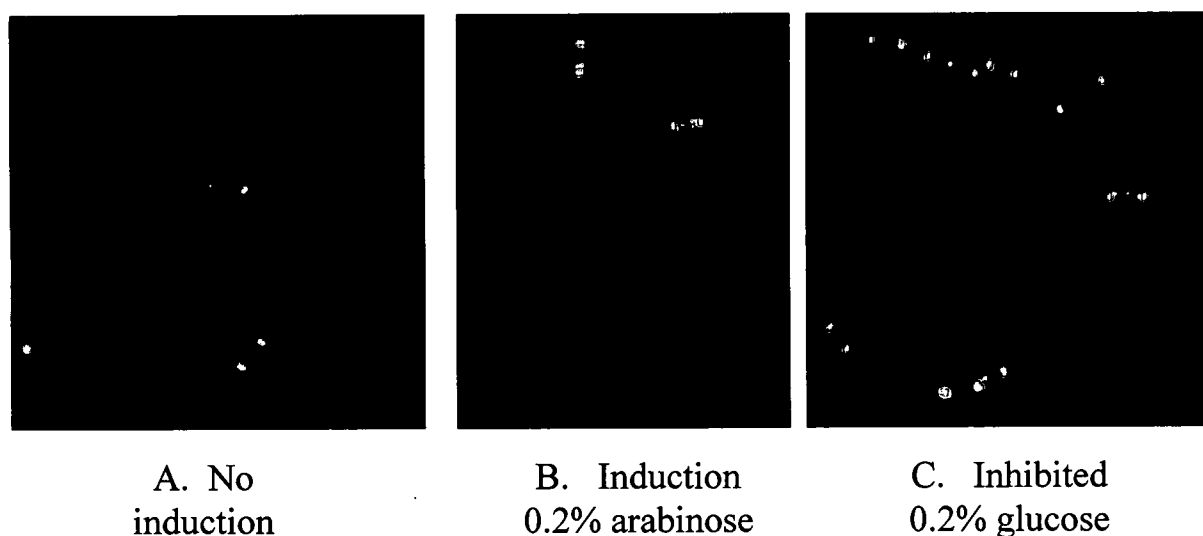


Figure 26. Localization of PilJ using the expression vector pSB94JY. A. pSB94JY in FA6 with no induction (arabinose); B. pSB94JY in FA6 with induction by 0.2% arabinose; C. pSB94JY in FA6 with inhibitor, 0.2% glucose. 100 μ l of overnight growth in LBGm₅₀ was inoculated into 10 ml of the same, incubated at 30°C while shaking and grown to an optical density of 0.2 before being induced or inhibited by the arabinose and glucose respectively. After the addition of the inducer or inhibitor, cells were grown about 1 hour as before to an optical density of 0.4 – 0.5. Cells were examined using a Nikon Eclipse TS100 microscope at a magnification of 600X.

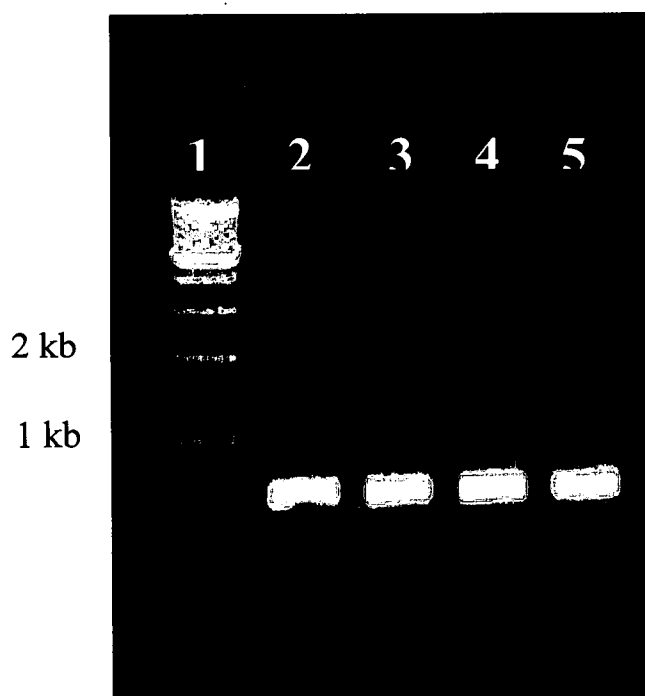


Figure 27. Gel electrophoresis of PCR product of first 600 bp of *pilK* from pADD698. PCR conditions were as follows: initial denaturation at 98 °C for 10 minutes, 29 cycles (94 °C for 1 minute, 63 °C for 1 minute, 72 °C for 1 minute) and final extension of 72 °C for 10 minutes. 10 µl of the PCR solution was added to a 0.7% agarose gel (50 ml) with 5 µl ethidium bromide (1 mg/ml) and run at 70 volts for 1 hour. Lane 1 = 1 kb marker; lanes 2-5 = PCR product of 600 bp of *pilK*.

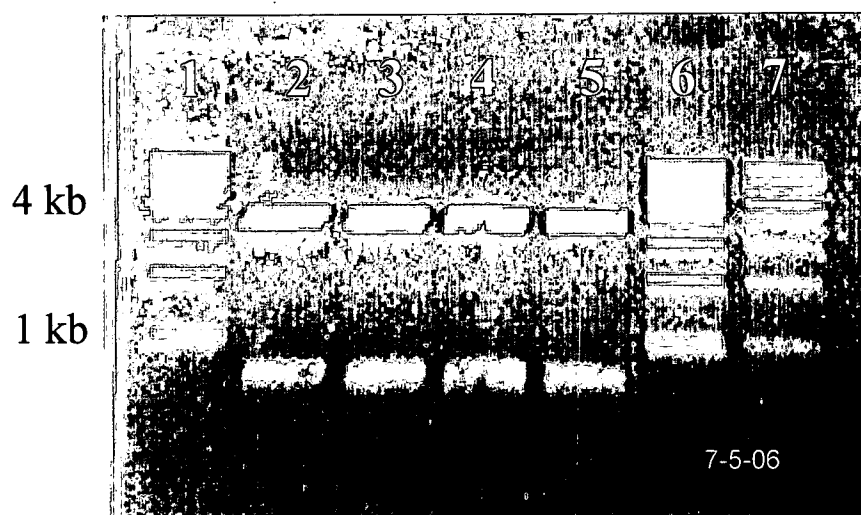


Figure 28. Restriction endonuclease digest of pCRK. pCRK double digested with *Xba*I and *Eco*RI results in 3.9 kb pCR2.1 vector and 600 bp *pilK* fragment. Digest product run on 0.7% agarose gel (50 ml) with 5 ul ethidium bromide (1 mg/ml) at 70 volts for 1 hour. Lane 1 = 1 kb Marker; lanes 2-5 = pCRK double digest; lanes 6, 7 = 1 kb digest.

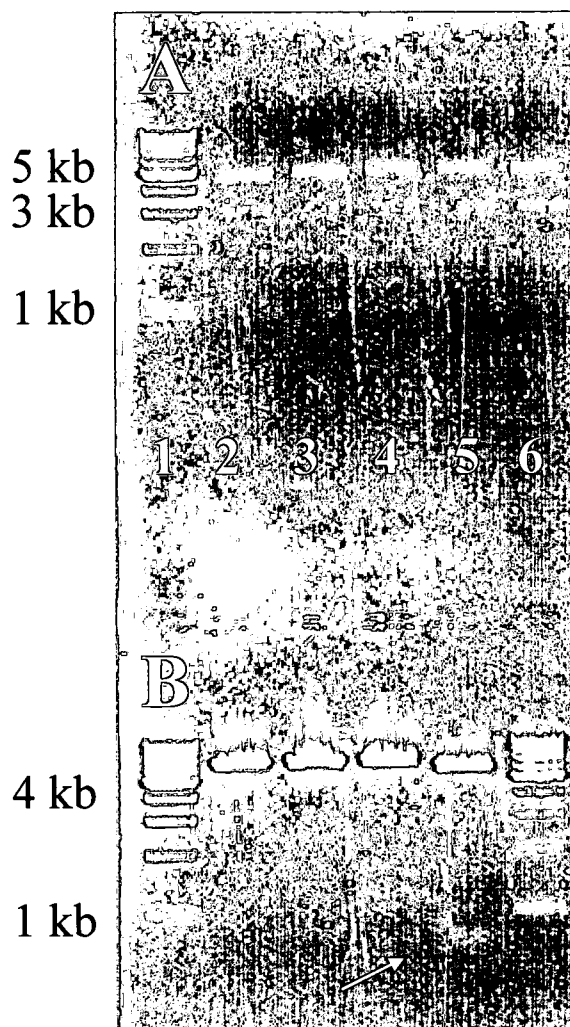


Figure 29. A. Restriction endonuclease digest of p19JY with *Hind*III and *Xba*I to release 2.9 kb *pilJ-yfp* fusion. pUCP19 size = 4.7 kb. Lane 1 = 1 kb marker; Lanes 2-5 = p19JY double digested with *Hind*III and *Xba*I. **B. Confirmation of pEX19K by digestion with *Hind*III and *Eco*RI yields 5.8 kb pEX19Ap and 600 bp fragment of *pilK*.** Lane 1 = 1 kb marker; Lane 2 = pEX19K *Hind*III; lane 3 = pEX19K *Xba*I; Lane 4 = pEX19K *Hind*III and *Xba*I. Digests were run on 0.7% agarose gel (50 ml) with 5 ul ethidium bromide (1 mg/ml) at 70 volts for 1 hour.



Figure 30. Restriction endonuclease digest and schematic representation of suicide plasmid pEX19JYK. Lane 1 = 1 kb marker; Lane 2 = pEX19Ap *Hind*III (5.8 kb); Lane 3 = pEX19K *Hind*III (5.8 + 0.6 kb); Lane 4 = pEX19JYK *Hind*III (5.8 + 0.6 + 2.9); Lane 5 = pEX19JYK *Hind*III/*Xba*I (6.4 + 2.9); Lane 6 = pEX19JYK *Hind*III/*Eco*RI (5.8 + 2.9 + 0.6); Lane 7 = 1 kb marker. 5' fragment of *pilK* = 0.6 kb; *pilJ-yfp* fusion = 2.9 kb. Digests were run on 0.7% agarose gel (50 ml) with 5 μ l ethidium bromide (1 mg/ml) at 70 volts for 1 hour.

Several steps were performed to verify that the fusion was present and functional. Evidence of the integration and excision of the suicide plasmid in FA6 is shown in Figure 31. Replacement of the mutant *pilJ* gene by the in-frame chromosomal *pilJ-yfp* fusion in the FA6 background resulted in complete restoration of twitching motility indistinguishable from that of wild-type cells (Figure 32). Levels of PilJ-YFP were not detectable by Western blot analysis, using either colorimetric (Figure 33) or chemilluminiscent detection (data not shown), until after 22-24 hours of growth for the in-frame chromosomal fusion while easily detectable in log phase cells containing the multi-copy plasmid born fusion. Cells carrying the original YFP plasmid, pEYFP, in *E. coli* generated a band at approximately 32 kDa corresponding to the molecular weight of the yellow fluorescent protein. Similarly *P. aeruginosa* cells containing the YFP in the pUCP19 vector, p19YFP, also produced a product of the same expected size. The Western blot analysis demonstrated that the mutli-copy plasmid does overexpress the gene as seen by comparing the bands in lanes 2 and 4 of Figure 33. In order to verify that the fluorescence we would see microscopically was the result of the intact PilJ-YFP fusion, and not YFP alone, it was important to show that the fusion was translated as a single functional product. Western blot analysis verified that the fusion was the combined size of the two fusion elements, PilJ and YFP. The complementation experiments proved that the PilJ portion was functional and fluorescence microscopy would reveal that the YFP was functional. Evidence that the PilJ-YFP fusion remained intact is seen in lane 3 and 7. Lane three contains product from the log phase cells expressing the p19JY. In lane 7 is the product from the in-frame chromosomal fusion. Both products are near the expected size (103 kDa) for the fusion product. This analysis

indicates that native expression levels of *pilJ* are lower than the overexpressed levels seen from plasmid production.

Epifluorescence microscopy revealed that PilJ was restricted to the poles of the cells containing the in-frame chromosomal *pilJ-yfp* fusion (Figure 34). Localization of PilJ using this construct was visible when cells were grown in LB with 0.4% NaCl and, consistent with the Western blot analysis, was most pronounced at 22-24 hours incubation. Since the Western blot analysis clearly showed that the fusion remained intact within the cells, the PilJ-YFP observed between the poles in the cells containing the mutli-copy plasmid born fusion is likely the result of higher levels of expression, and not free YFP resulting from proteolytic degradation of the fusion.

Examination of a RFP-FimX protein fusion indicated that FimX remains localized to only one pole in both the PAO1-AD wild-type as previously reported (70) and FA6 (data not shown). We were unable to determine if this localization is associated with the same pole in each case or if it is associated with the pole containing the flagellum. Previous studies experienced similar difficulties (70).

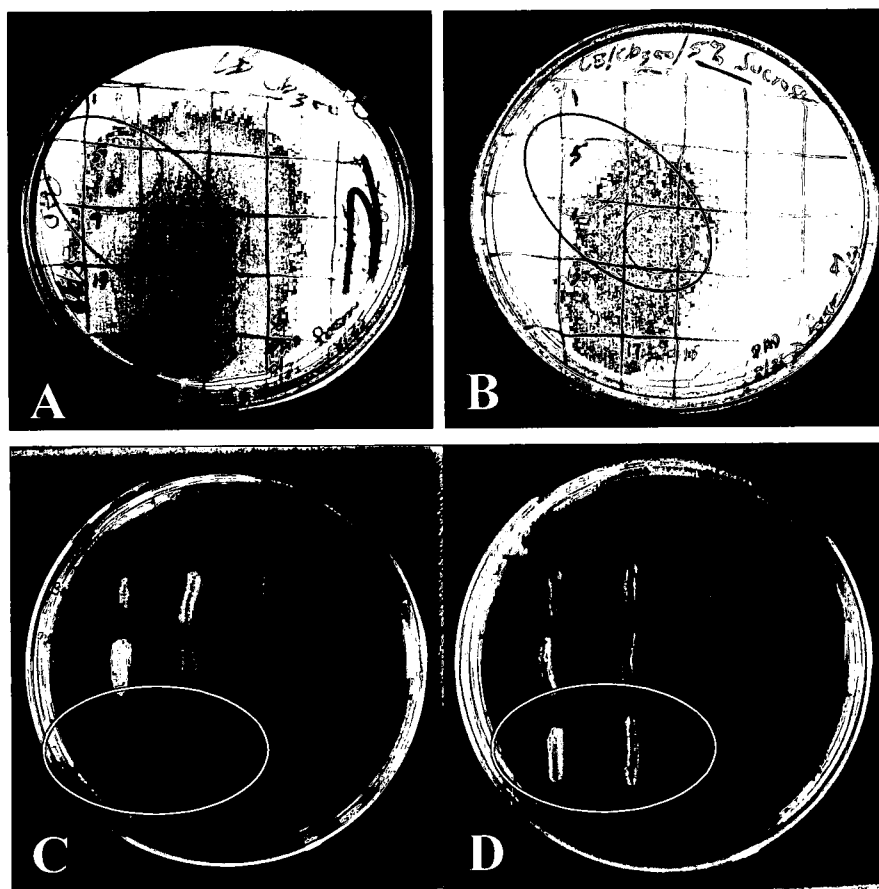


Figure 31. Integration and excision events of the suicide vector pEX19JYK in the FA6 chromosome. A and B) Replica plating of transconjugates from PIA Cb₅₀₀ agar plates and integration of the plasmid; **A)** growth of the transconjugates on LBCb₃₀₀ indicates antibiotic resistance from integrated plasmid; **B)** lack of growth on LBCb₃₀₀ with 5% sucrose indicates integration of *sacB* gene and lethal affect of sucrose. **C and D)** Replica plating of colonies from LBCb₃₀₀ (**A**) and excision of suicide plasmid from chromosome; **C)** inability of transconjugates to grow on LBCb₃₀₀ indicates loss of antibiotic marker; **D)** growth on LB with 5% sucrose indicates loss of *sacB* gene and null affect of sucrose on growth.

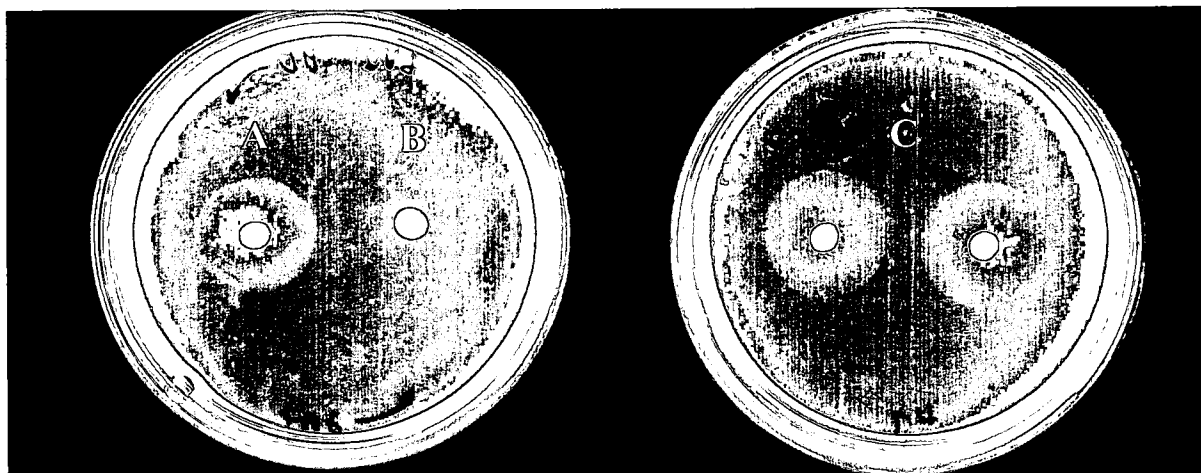


Figure 32. Complementation of FA6 with in-frame chromosomal fusion demonstrated by twitching motility assay. A. PAO1-AD; B. FA6; C. FA6 containing the in-frame chromosomal fusion from pEX19JYK. Cells were inoculated with a needle onto 1% twitch plates and incubated at 37°C for 48 hours.

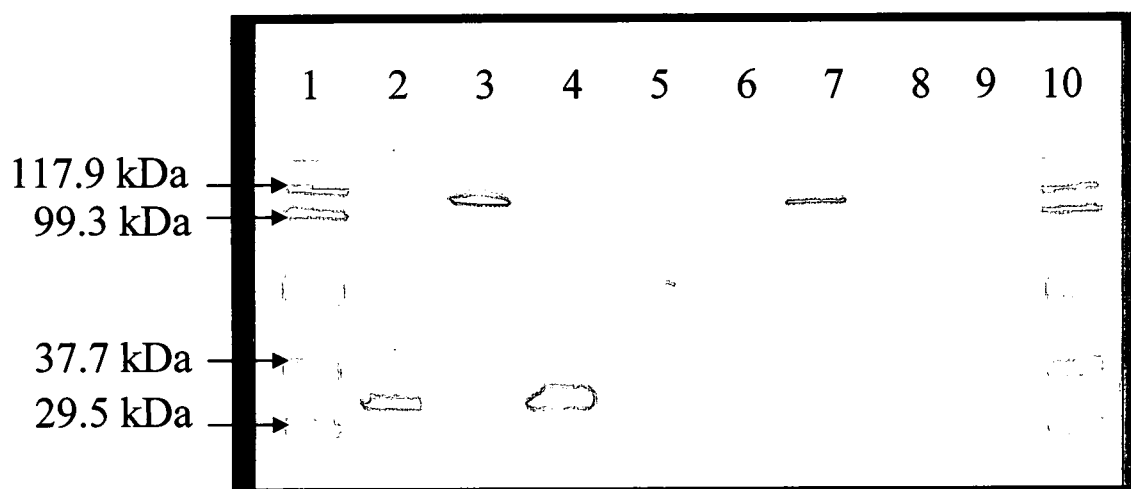


Figure 33. Western blot analysis of YFP and fusion proteins. Lane 1: Broad range markers; Lanes 2-5 are from log phase growth. Ln 2: *E. coli* with pEYFP; Ln 3: FA6 with p19JY; Ln 4: FA6 with p19YFP; Ln 5: FA6 chromosomal fusion *pilJ-yfp*; Ln 6: blank; Ln 7: Over night growth of FA6 chromosomal fusion *pilJ-yfp*; Ln 8: Blank; Ln 9: Control, FA6 log phase growth; Ln 10: Broad range markers. YFP = 32 kDa, PilJ = 71 kDa, PilJ-YFP = 103 kDa.

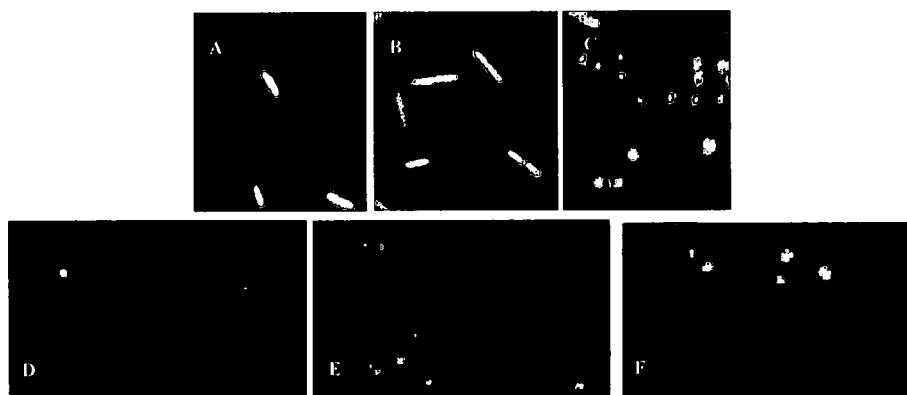


Figure 34. Localization of PilJ using an in-frame chromosomal fusion. (A) *E. coli* with p19YFP, (B) FA6 with p19YFP, (C) *E. coli* with multi-copy plasmid born p19JY, (D) FA6 with multi-copy plasmid born p19JY (log phase), (E) FA6 with multi-copy plasmid born p19JY, (24 hour growth), (F) FA6 with in-frame chromosomal fusion *pilJ-yfp*, (24 hour growth).

DISCUSSION

Previous studies that examined the role of *pilJ* in twitching motility have produced conflicting results. Darzins found the *pilJ* mutant of PAO1 to be completely deficient in twitching motility (38) while Whitchurch et al., (183) observed reduced and aberrant twitching for the *pilJ* mutant of the PAK strain. These differences may be attributable to well documented strain variations between PAO1 and PAK. To resolve this discrepancy and determine the role of PilJ in twitching motility, we constructed *pilJ* mutants in several different wild-type backgrounds. In our study we show that PilJ is universally necessary for twitching motility in a wide variety of *P. aeruginosa* wild-type strains including three common laboratory strains, PAO1-AD, PAO1-UO, and mPAO1, as well as an environmental isolate (DAP119J), and a clinical isolate taken from a case of bilateral keratitis (GSU-3). In addition we also verified the need for *pilJ* for twitching motility in two transposon mutants. All *pilJ* mutants we generated were incapable of twitching motility under the conditions employed in this study. Clearly PilJ plays a key role in twitching motility.

Darzins and Whitchurch, et al. have shown total pilin production in wild-type and *pilJ* mutant cells to be equivalent while external pilin levels were nearly undetectable in the mutants (38, 183). Yet the mutant cells remained sensitive to pilus specific phage. We had similar results (unpublished data from our lab; Tracy Collins). Sensitivity of the *pilJ* mutant cells to pilus specific phage could be explained by the presence of small stubs

of external pili present on the mutant cells; pili long enough to bind phage, but too short to be sheared and collected for Western blot analysis or to initiate twitching motility.

Our IMF results (Tracy Collins work) indicate that the reason *pilJ* mutants are incapable of twitching motility is that PilJ is necessary for the full assembly/extension of TFP. Without PilJ, cells appear to abort the assembly/extension process of TFP resulting in the shortened pili often at both poles. While a defect in TFP retraction could also explain lack of twitching motility observed with *pilJ* mutants, such a defect would be incompatible with the retention of phage sensitivity. Further, if PilJ controls TFP retraction *pilJ* mutant cells would be expected to have a higher percentage of cells with surface pili present compared with wild-type cells, since once pili are extended the cell would be unable to retract them. We observed a slightly lower percentage of mutant cells with surface pili using IMF.

The signal transduction system, including the operon *pilGHJK*, that controls twitching motility in *P. aeruginosa* is similar to the chemosensory systems (*che* genes) controlling flagellar rotation in *E. coli*. Therefore, by controlling the assembly/extension of TFP, PilJ is functioning similarly to the MCPs of the enteric bacteria that initiate signaling that controls the direction of flagellar rotation. How PilJ works with the other proteins necessary for twitching motility is under investigation in our laboratory.

In the *E. coli* model the flagella motor has two stages: counter clockwise and clockwise. The direction of the rotation is determined by the state of phosphorylation of CheY. Since *E. coli* only has one CheY protein and it is either phosphorylated or not, the motor is referred to as a two stage motor. In other bacteria such as *M. xanthus* and *Sinorhizobium meliloti*, there are 2-3 CheY homologs and the flagella may either be

rotating one direction or the other or they may actually be stationary, thus sometimes referred to as a 3 stage motor. Because *P. aeruginosa* has 3 CheY like proteins: PilG, PilH and ChpA, and the pili may be extended, retracted or partially extended, the pil system may control a 3 stage motor for TFP assembly/extension. Also of interest is the lack of CheZ, a CheY phosphatase, in either the *S. meliloti* or *P. aeruginosa*, further supporting the idea that the pil system for TFP assembly/extension is a 3 stage motor. The similarities between *P. aeruginosa*, *S. meliloti* and *M. xanthus* suggest the model is similar to the Che system of *E. coli* but considerably more complex with much more to be learned.

PilJ has been identified as a putative methyl-accepting chemotaxis protein on the basis of its sequence identity to other MCPs (38). Darzin's analysis revealed that PilJ has a periplasmic sensing domain, two transmembrane regions, methylation sites and a highly conserved cytoplasmic signaling domain. In our lab we have shown, using *in vivo* methylation experiments, that a protein of the correct molecular weight of PilJ is methylated in wildtype cells, but is not present in the *pilJ* mutant; (unpublished data by Tracy Collins). Methylation is a signature of MCPs. This result corroborates that PilJ is a functioning MCP as proposed by Darzins (38) and is further support for the bipolar location of PilJ.

Knowing that other MCPs have been shown to localize to the poles of the cell (2, 51, 52, 69, 99, 169), we studied the localization pattern of PilJ using a YFP fusion. We were able to demonstrate that PilJ localizes to both poles of the cell. Localization was independent of the growth conditions, as we saw the same pattern of localization regardless of whether the cells were grown on agar plates, in broth, or slide cultures,

interestingly even under some conditions where twitching motility does not occur. Other proteins involved in TFP biosynthesis and twitching motility, such as the ATPases PilB and PilT, have also been shown to localize to both poles in *P. aeruginosa* (32). PilS, the sensor-kinase in the PilA biosynthesis pathway that initiates the transcription of the PilA subunit, localizes to both poles of the cell in *P. aeruginosa* (25). Because TFP pili are found at the poles of *P. aeruginosa*, it makes sense that these proteins involved in the assembly/extension of TFP co-localize to the poles to carry out the process. With the discovery that PilJ localizes to the poles of the cell, the concept of polar compartmentalization of a complete twitching motility apparatus at both poles is strengthened. Numerous proteins have already been shown to localize to one or both poles of bacteria (73, 96, 139). By having the complete assembly/disassembly apparatus at both poles, along with a MCP required for extension of the TFP, the cell is now capable of quick cell reversals to respond to changing environmental conditions without having to physically reorient the cell. Instead the cell can simply change which pole is the leading one in terms of directional movement by extending pili at one pole and retracting at the other.

The observation that even in the *E. coli* strain used in the cloning process, the PilJ-YFP fusion demonstrated bipolar localization in the same manner as in *P. aeruginosa* implies that the localization may be an intrinsic property of the common and highly conserved sequences shared by PilJ and *E. coli* MCPs rather than a specific function of the host bacteria. This pattern of localization in *E. coli* was different than that of the PilS-GFP fusion used in a previous study (25). In that study, the fusion did not demonstrate any localization in the *E. coli* strain. The structure of PilJ may have enough

sequence identity with MCPs of other bacteria to behave similarly in both species. This further supports the putative identity of PilJ as a MCP in *P. aeruginosa* as proposed by Darzins (38).

During the course of the study, we also observed that when PilJ was overexpressed using a multi-copy plasmid born fusion, PilJ was visible in the cytoplasm of the cell. The fusion protein pattern was spiral shaped suggesting to us that perhaps when PilJ is produced it travels along a cytoskeleton framework to arrive at the pole. When too much PilJ is produced to remain localized at the poles it accumulates along these tracks and is visible in the cytoplasm. The idea of some type of cytoskeleton in prokaryotes is not novel and was first demonstrated (75) in *Bacillus subtilis* in 2001. Since that time other studies have provided further evidence of bacterial cytoskeletons (11, 12, 141, 141, 166)

In addition to determining the localization of PilJ in *P. aeruginosa* PAO1 strains, we were also curious about the localization pattern in mutants of other key proteins involved in TFP assembly/disassembly. Since PilJ localization was unchanged in *pilT*, *pilU*, *pilB*, and *pilI* mutant backgrounds, our results indicated that none of the proteins, PilT, PilU, PilB of PAK or PilI of PAO1-AD, are acting as an anchor for PilJ localization. PilJ remained localized at both poles demonstrating that its localization is independent of these other twitching motility proteins. However, we did observe a difference in the intensity of the fluorescence in some mutant backgrounds. For example, the fluorescence appeared brighter in the *pilU* mutant background. A possible explanation for this may be a necessary stoichiometry or clustering required between some of these proteins that is enhanced or diminished in the various mutant backgrounds.

For example in the *E. coli* Che system there is a definite ratio of CheA:CheW:MCP in order for polar clustering to occur (90). It is also true that in the *E. coli* system all three are required for polar clustering. Our findings may imply that the Pil system is not functioning with the same type of interactions.

The necessity of two other proteins, PilH and Pili, for PilJ localization was also considered. Pili has a high sequence identity with the CheW protein in the Che system and PilH has high sequence identity with CheY. Previous studies have shown that CheW is dependent on the MCP for anchoring and localization (99, 145, 148). When we placed the PilJ-YFP fusion into these mutant backgrounds, its localization pattern was unchanged; thus we did establish that PilJ does not require either of these two proteins for its own localization. This finding is in contrast to results in *E. coli*, where MCPs require the CheW protein for polar localization. In the absence of CheW, the MCPs are randomly distributed around the inner membrane of the cell (99). Our results regarding the polar localization of the putative MCP, PilJ, in the absence of Pili (CheW homolog) suggests that the differences between the Che system of the enterics and the Pil system of *P. aeruginosa* may be greater and/or more complex than originally suspected. More research is required to determine the specific anchor that allows PilJ to localize as it does to the cell poles and the unique relationship between PilJ (MCP) and Pili (CheW). It may be that PilJ itself is the anchor, however PilT has been shown to accumulate at the cell poles in the absence of PilJ (Poney Chiang, personal communication). Further study is needed to determine the actual functional similarities of Pili to CheW. Because we did not have a *chpA* mutant, we did not examine the relationship between PilJ localization and the CheA homolog, ChpA.

The results of this study also point out some interesting and perhaps significant similarities between the TFP mediated gliding motility of the TFP mediated twitching motility of *P. aeruginosa*. Kaiser observed TFP located at the pole of the cell in *M. xanthus* were responsible for gliding motility (77). Further studies showed that the frequency of cell reversals during gliding motility in *M. xanthus* were controlled by the *frz* gene system (19). In the Frz system, the FrzCD protein is the MCP homolog, FrzE and FrzZ have CheY-like domains and FrzE also has a CheA-like histidine autokinase function. Sun (163) and Sogaard-Anderson (147) proposed that rapid cell reversals were likely to be the result of switching active pili bundles from one pole to the other, a phenomenon referred to as polar switching. Similarly, *P. aeruginosa* uses its polar TFP to mediate twitching motility, which requires PilJ, a protein that is part of a chemosensory system similar to the Che system of *E. coli* and the *frz* system of *M. xanthus*. Finally twitching cells of *P. aeruginosa* are also capable of reversing the direction of twitching motility (137). Our study is the first to implicate a MCP is involved in possible polar switching.

Recently Sogaard-Andersen (147) suggested three possible scenarios for the polar switching seen in *M. xanthus*. One scenario is that TFP biogenesis proteins are actually transferred from one pole to the other. Another possible explanation is that TFP biogenesis proteins are degraded at one pole and *de novo* synthesis of the TFP biogenesis proteins are assembled at the opposite pole. A third possibility is that the TFP biogenesis proteins are constitutively present at both poles, but the TFP are only assembled/extended at one pole at any given time (147). In a recent study (110) it was demonstrated that the cell reversals seen in *M. xanthus* were the result of a key motility protein, FrzS. FrzS was

shown to oscillate from one pole to the other as the cell reversed direction and that the pole containing the complex of FrzS became the leading pole, having associated with it the extended TFP.

Based on our results and the similarities with TFP mediated gliding motility of *M. xanthus*, we propose the following model for polar switching in *P. aeruginosa* as shown in Figure 35. Certain TFP proteins are found at both poles (PilS, PilB, PilT, PilJ) but may only function under the direct control of a protein that may oscillate between the poles to establish the leading pole and the need for extension of TFP. All of this may function under the regulation of the sensing protein, PilJ found at both poles. The proposed model highlights the similarities between the pil chemosensory system of *P. aeruginosa* as suggested by Darzins (40), the Che system in the enterics and the Frz system of *Myxococcus* (110).

Recently another protein required for twitching motility, FimX, was discovered to localize to just one pole in *P. aeruginosa* (70). FimX appears to be a signal transduction protein connecting environmental signals to control of twitching motility although the exact mechanism is unknown. Our model suggests that perhaps FimX is working together with PilJ to coordinate the full extension of pili from a single pole in response to an environmental signal that remains unknown at this time. Since FimX has been demonstrated to localize to only one pole, perhaps FimX functions in a manner very similar to the FrzS protein of *M. Xanthus*, oscillating between poles to signal which pole is to become the dedicated one for assembly and extension of TFP in concert with the signaling response from PilJ. One can easily imagine a scenario similar to the one demonstrated by Mignot (110) may be occurring in *P. aeruginosa*. In our study we found

that the RFP-FimX fusion remained localized to a single pole in both PAO1-AD and the FA6 cells suggesting that FimX does not rely on PilJ for its localization. We did not attempt to discover for either PilJ or FimX what protein(s) these molecules require for their own localization. Nor were we able to establish a specific relationship between PilJ and FimX as we had hoped.

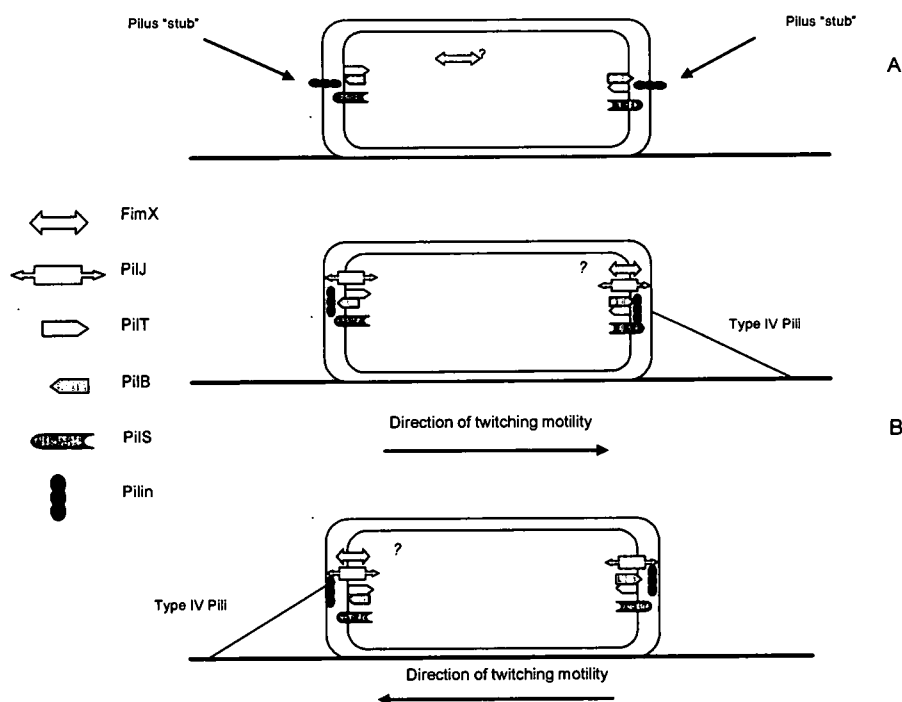


Figure 35. Polar switching model for *P. aeruginosa*. Based on the bipolar location of several key proteins involved in TFP assembly/disassembly and twitching motility, *P. aeruginosa* should be capable of rapidly reversing the direction of twitching motility. (A) In the absence of PilJ (or signal detection) *P. aeruginosa* cells are unable to fully assemble/extend pili and instead produce short "stubs" of pili at both poles of the cell. (B) In wild-type cells where PilJ is functional, pili are fully assembled/extended in response to some signal(s) along with possible assistance from FimX. When PilJ senses a change in the signal and the cell needs to reverse direction, the pili at one end quickly retract via PilT and the pilin subunits at the opposite end are assembled/extended with the aid of PilB under the direction of PilJ with putative assistance from FimX or unidentified protein. As a result *P. aeruginosa* uses key proteins necessary for twitching motility, localized to the poles of the cell, to quickly reverse the direction of polar twitching motility.

An important experiment that may provide further support to our model would examine the relationship of the FimX protein to the leading pole. To do this an experiment would have to be designed to demonstrate that FimX is always located at the same pole of the cell as the TFP. One method of accomplishing this would be to set up an experiment similar to Skerker and Berg (144) in which the TFP of cells containing the RFP-FimX fusion are labeled with a fluorescent dye. The objective would be to observe both the TFP and the FimX protein at the same pole of the cell. Although the previous experiments have demonstrated the unipolar location of FimX, there is no evidence linking the FimX protein to the pole in which TFP are extending and retracting.

Additionally, it would be valuable to know whether FimX may be oscillating between poles (110). This would involve labeling the FimX protein and observing its movement as cells reverse direction. Mignot demonstrated that FrzS clearly oscillates between poles during cell reversals, always moving to the leading pole and was involved in pilus assembly. We believe it is possible that FimX may be functioning in a manner very similar to the FrzS protein of *M. xanthus* as proposed in our model (Figure 35) and that perhaps it is the complex of PilJ and FimX at the leading pole of the cell that is responsible for the initiation of TFP of *P. aeruginosa*. In this model, once PilJ, acting as an MCP, senses a stimulant it may send a signal to FimX or through conformational change now form a complex with FimX to interact at the pole where the attractant is sensed and initiate TFP at that end. If the signal sensed is a repellent, it may signal the FimX to move to the other end to interact with the PilJ there and initiate TFP at the opposite pole. Because the stimulant(s) for PilJ has yet to be determined, these experiments are not feasible.

We are able to conclude from this study that PilJ is an essential protein involved in the full assembly/extension of TFP, is necessary for twitching motility in numerous strains of *P. aeruginosa*, and that it localizes to the cell poles as other MCPs. Finally, we propose that PilJ is a key protein in signaling for the putative polar switching of *P. aeruginosa* and that it may be interacting with FimX or some as yet unidentified protein to establish the leading pole required for full TFP extension and directional twitching motility.

A number of experiments could be done to learn more about the characteristics and role of PilJ in *P. aeruginosa*. Our study showed evidence that PilJ does not require a CheW or CheA homolog for anchoring at the pole. More experiments could be done to determine what protein(s) anchor and localize PilJ to the poles of the cell. Virulence studies with the *pilJ* mutants would also be of interest. Previous studies showed that *pilT* and *PilU* mutants (twitch motility negative; TFP positive) were less cytotoxic than the wild-type cells but more cytotoxic than *pilA* mutants suggesting that TFP add to the virulence of *P. aeruginosa*, but that the mere presence of TFP is not enough to establish wild-type levels of virulence (33). The ability to move by twitching motility is required. Similar experiments using *pilJ* mutants would provide additional support. One would expect *pilJ* mutants to be less virulent than the wild type cells since these *pilJ* mutant cells lack fully assembled/extended TFP and cannot twitch. Continued experiments to determine the stimulant(s) that trigger PilJ are important. In our lab we have recently shown that PilJ is methylated in response to farnesol, a quorum sensing molecule of *Candida albicans* (unpublished data) suggesting that this may be a stimulant for PilJ.

The capacity for *P. aeruginosa* to move via three distinct modes: swimming under high water concentrations, swarming under intermediate water concentrations, and twitching motility under low water concentrations and on surfaces affords it a great ability to survive and flourish in a multitude of environments. As was previously stated, swimming and swarming motility is flagella dependent and twitching motility requires TFP. Like many bacterial pathogens, *P. aeruginosa* uses its TFP to bind to glycosphingolipids of epithelial cell membranes (87, 140), although these receptors are not present in large amounts on normal epithelial cells. However, they are much more abundant on cell surfaces that express the mutant cystic fibrosis (CF) transmembrane channel regulator (71) and may therefore help to explain the increased *P. aeruginosa* infections among CF patients. These TFP are important bacterial adhesions important in pulmonary, burn and corneal infections (33, 132, 165, 189). In these studies, cells lacking TFP and those lacking functional TFP (twitching motility deficient) were attenuated suggesting that adherence is not the only virulence factor associated with TFP. Functional TFP capable of initiating twitching motility are necessary for full virulence in these models. Other studies have shown the versatility of TFP of *P. aeruginosa* to bind to abiotic surfaces also and has implications for its ability to form biofilms on these materials resulting in a number of issues for pipelines and medical devices (53)

This study provides evidence that PilJ may have a rather unique function among MCPs. In addition to a sensing function, PilJ also controls twitching motility by initiating TFP assembly/extension in a manner reminiscent of the way the MCP of the Che system regulates the direction of flagellar rotation. Although the specific stimulant triggering PilJ's role as a MCP is still unidentified, one can easily imagine how PilJ could

respond to a stimulus in order to initiate the assembly/extension of its TFP in order to move toward or away from it. Thus, PilJ may be sensing its environment as well as responding to it by causing the cell to move toward or away from the stimulant via twitching motility to help the cell survive in the complicated world of 'simple' prokaryotic organisms called bacteria.

Acknowledgements

We thank Herbert Schweizer, Mike Jacobs, Urs Ochsner, Al Darzins, Lori Burrows, and John Mattick for generously providing strains. We also thank Randy Irvin for his generous gift of anti-PilA antibodies.

This work was supported by NIH grant # GM61318-01.

APPENDIX

Figure A. Maps of plasmids pUC18 and pUC19.....	105
Figure B. Map of plasmid pEX18Ap.....	106
Figure C. Map of plasmid pZ1918Gm.....	107
Figure D. Restriction enzyme sites in pEXJGm.....	108
Figure E. Map of plasmid pEYFP.....	109
Figure F. Map of plasmid pSB94.....	110
Figure G. Orientation of <i>pilJ</i> on the <i>P. aeruginosa</i> chromosome.....	111
Figure H. Stages of allelic exchange process.....	112
Protocol: Electroporation of <i>Pseudomonas aeruginosa</i> competent cells	113
Protocol: Creation of chemically competent cells of <i>E. coli</i>	115
Protocol: Transformation of chemically competent One Shot® Top Ten (Invitrogen) cells	116

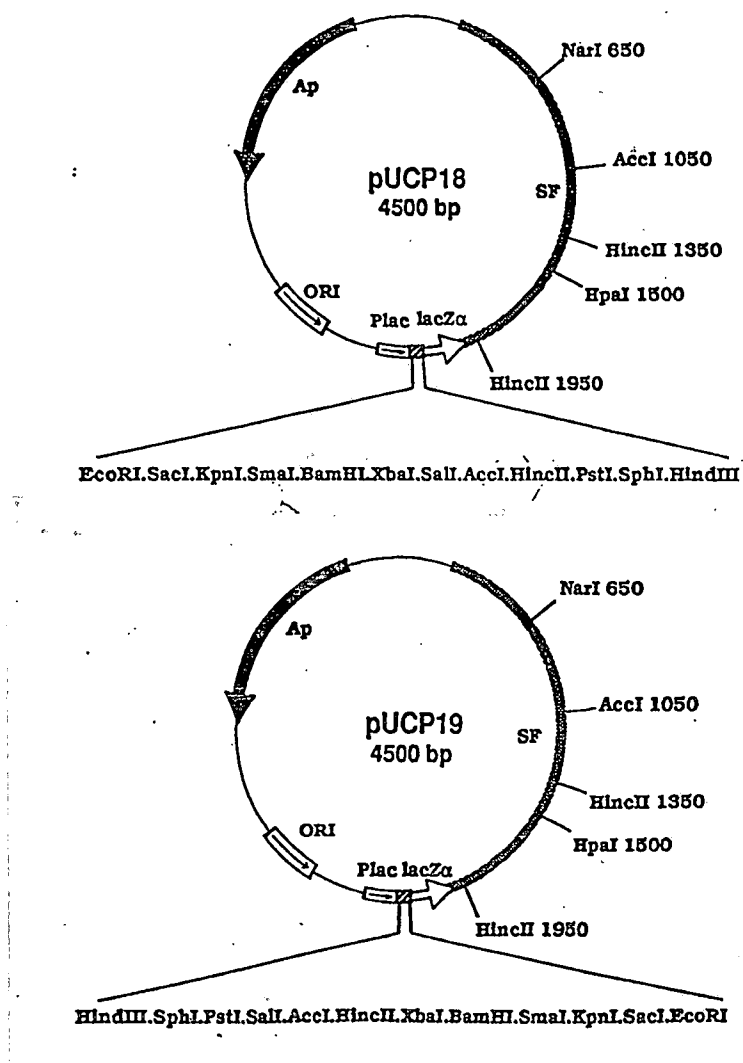


Figure A. Plasmids pUCP18 and pUCP19 including MCS region.

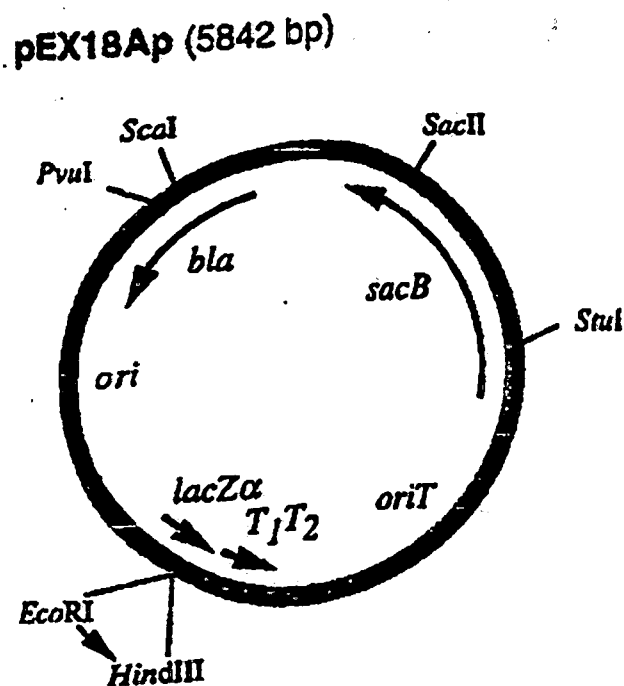


Figure B. Map of the suicide plasmid, pEX18Ap. The plasmid contains an ampicillin marker and the *sacB* gene. The MCS region includes the restriction sites in the following order: EcoRI–SacI–KpnI–SmaI–BamHI–XbaI–SalI–PstI–SphI–HindIII (this is the same order as in the pUCP18 vector). In the corresponding pEX19Ap the order in the sites of the MCS is reversed. The sequence of the pEX18Ap was deposited in GenBank with the Accession Number AF004910.

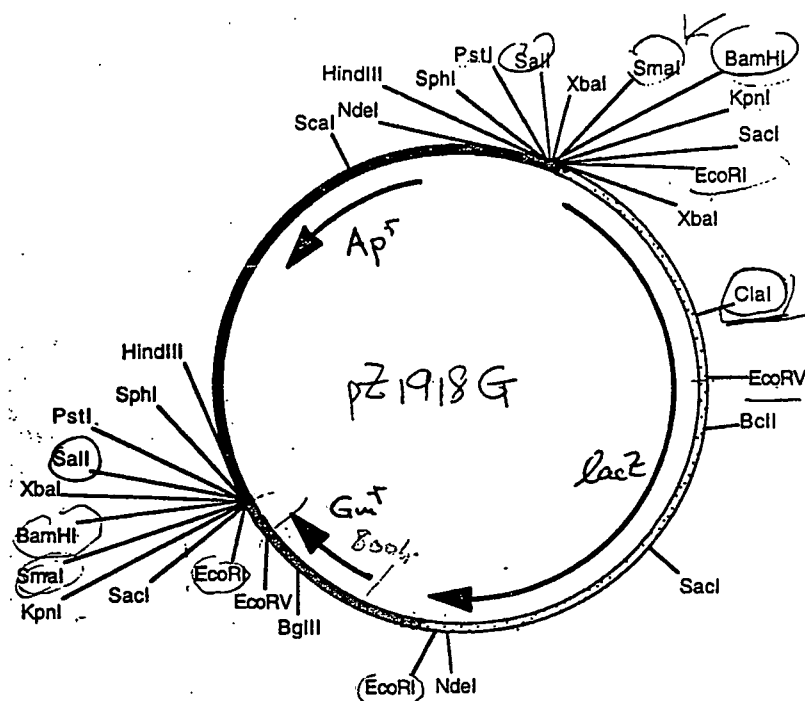


Figure C. Map of plasmid pZ1918Gm. The plasmid contains the *lacZ*/gentamicin cassette and MCS regions on either side of the cassette.

pEYFP

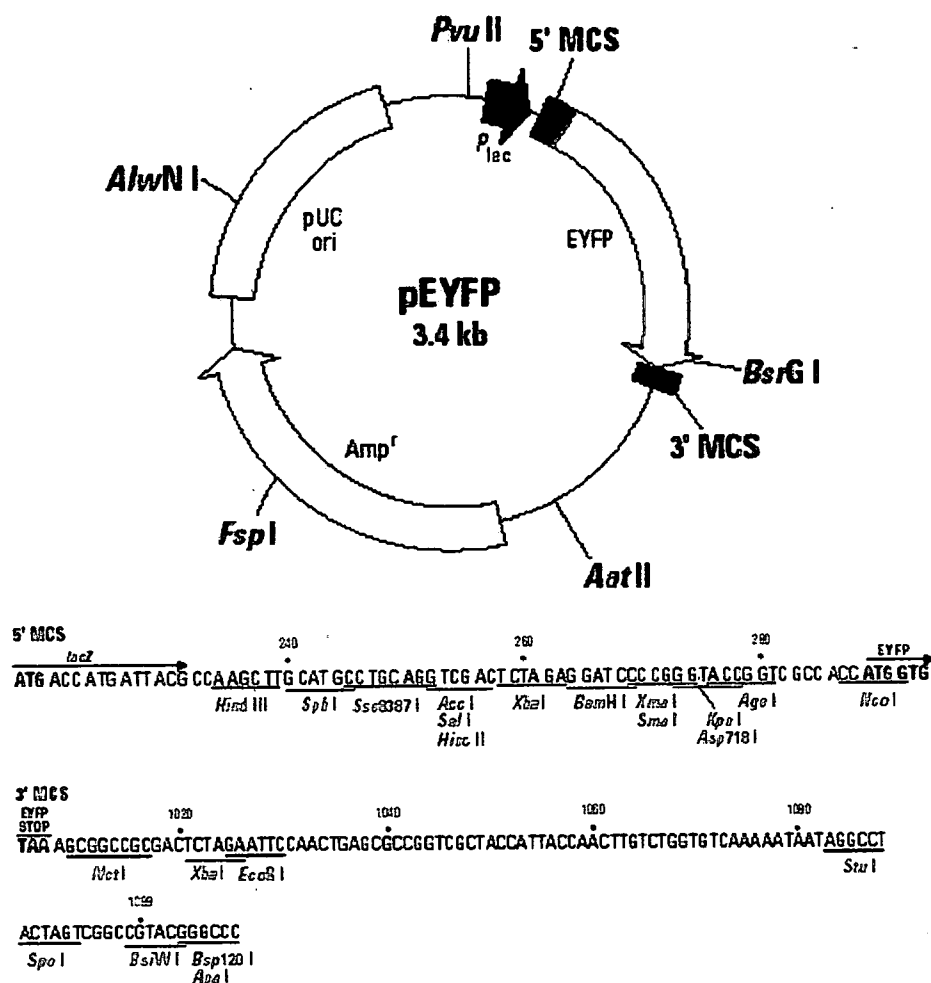


Figure E. Map and MCS region for pEYFP.

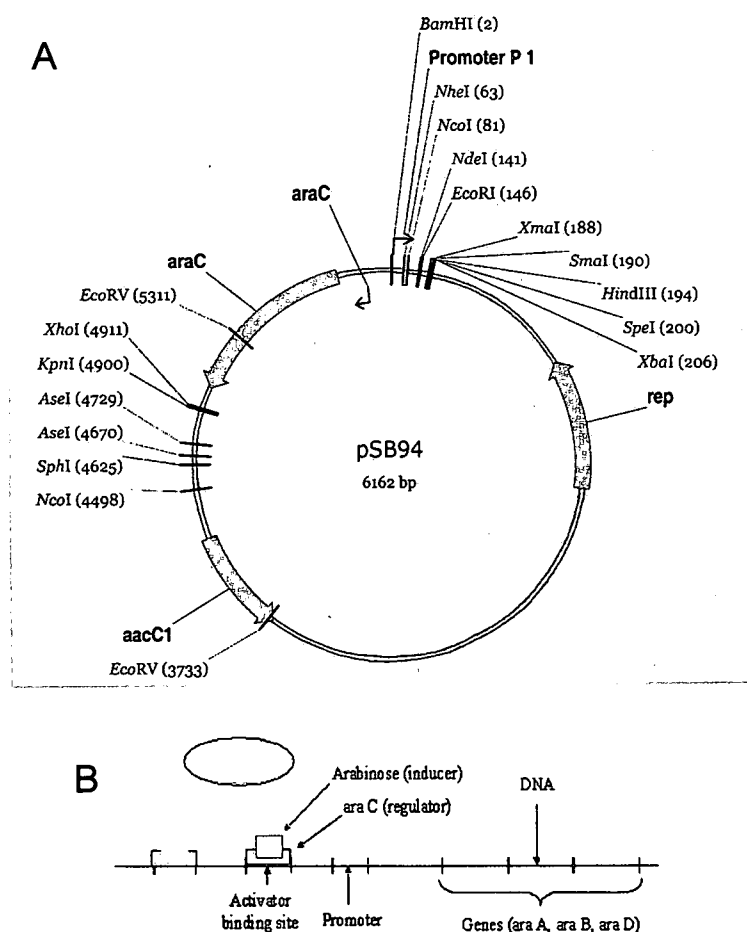
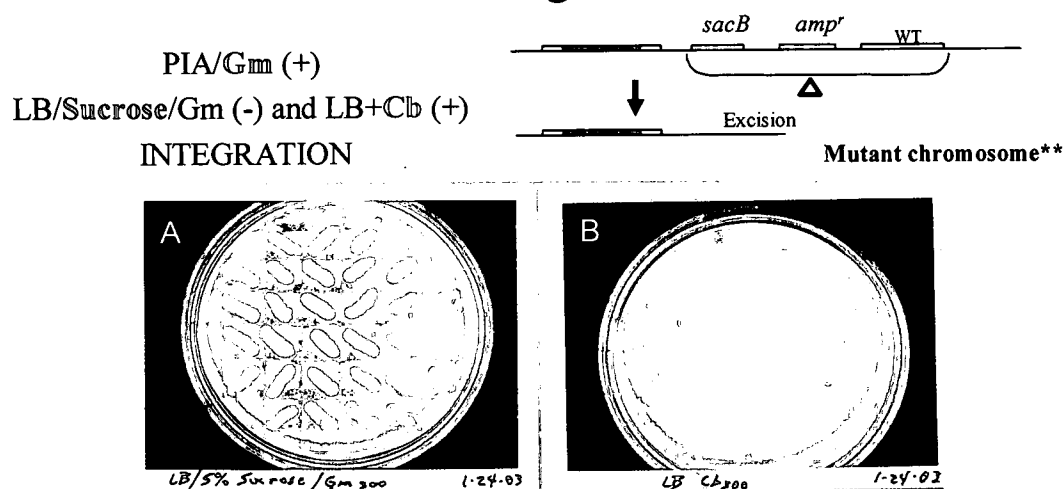


Figure F. A) Map of expression plasmid pSB94. B) Representation of the arabinose operon in this vector. Red oval represents RNA polymerase. RNA polymerase cannot function unless arabinose is present and binds to the araC regulator protein.

Result of mating Plasmid integration/excision



Replica plate Cb^R on
LB/5% sucrose/Gm and LB+Cb

EXCISION:
Mutant cells – Sucrose^R Gm^R and Cb^S**

Figure H. Stages of allelic exchange process. After conjugation, transconjugates are plated on PIA containing gentamicin. Colonies growing on PIA are replica plated onto LB/5% sucrose/Gm plates and LB/Cb. Cb^R colonies indicate integration of the plasmid and are replica plated onto LB/5% sucrose/Gm plates and LB/Cb again. Colonies that are sucrose^R, gentamicin^R and Cb^S likely contain the mutant chromosome and should be screened for the appropriate phenotype.

Protocol for Electroporation of *Pseudomonas aeruginosa*

A. PREPARATION OF CELLS

1. Inoculate 10 ml of LB with *P. aeruginosa* and grow overnight at 37°C.
2. The following day, subculture 1 ml of overnight culture into 30 ml of fresh (pre-warmed) LB in a 125 ml flask. Incubate at 37 °C until early log phase ($OD_{590nm} = 0.3-0.5$).
3. Centrifuge at 10,000g for 10 minutes and discard supernatant. re-suspend in 30 ml of 300 mM sterile sucrose at room temperature.
4. Spin cells down (10 minutes) and discard supernatant. Re-suspend in 6 ml of 300 mM sucrose.
5. Spin cells down at 10,000g for 5 minutes and discard supernatant. Re-suspend in 150 μ l of 300 mM sucrose.
6. Aliquot into 4-5 microcentrifuge tubes (40 μ l each); Freeze at -70 °C.

B. ELECTROPORATION

1. Connect the Pulse Controller to the Gene Pulser. Connect the Cuvette Chamber to the Pulse Controller in this order.
2. Do NOT set Capacitance. Set resistance to 186 Ω . Set controls to 2.5 kV/resistance.

3. Press Set Volts and adjust to 0.8 kV.
4. Add 40 μ l cells to a microcentrifuge tube and mix with 5 μ l of DNA solution (the DNA should be in a low conductivity buffer or water).
5. Immediately transfer to a 0.2 cm electroporation cuvette. Shake sample to bottom of cuvette and dry sides with tissue. Place in white cuvette holder and slide into the chamber making sure contact is made with the electrodes.
6. Press the charging/pulse button. Wait for red light to go out before opening cuvette holder. Immediately add 1 ml of LB, mix well and transfer to 2 ml of LB in a large test tube. Shake at 37°C for 2 hours before plating on selective media (antibiotic).

Protocol for Creation of Chemically Competent Cells

(*E. coli*)

1. Inoculate 5 ml of LB with DH5 α and grow overnight at 37°C with shaking.
2. Next day, add 1 ml of overnight culture to 80-100 ml of LB in 250 ml flask and grow for 3 hours with shaking at 37°C. Check OD₆₀₀ = 0.2 (if curious).
3. Place on ice for 15-20 minutes.
4. Pour LB into 40-45 ml centrifuge tubes (balance tubes).
5. Place tubes in big centrifuge. Rpm – 8, Time 15 minutes, temp 5°C.
6. Pour off supernatant and add 20 ml of 100 ml CaCl₂. Break up pellet: vortex, pipet up and down, etc.
7. Let tube sit on ice for 30 minutes.
8. Re-spin tube for 15 minutes.
9. Pour off liquid.
10. Re-suspend pellet in 1 ml CaCl₂ with 10% glycerol.
11. Aliquot 200 μ l of cell solution into microcentrifuge tubes (~5 per large tube).
12. Place cells in refrigerator overnight.
13. Store cells at -70°C.

Transformation Protocol for Chemically Competent

One Shot® Top Ten (Invitrogen) Cells

1. Thaw, on ice, one 50 µl vial of One Shot® Top Ten cells for each two ligation reactions. (25 µl per reaction.)
2. Split cell suspension into two chilled tubes of 25 µl each for transformation process.
3. Pipet ligation reaction directly into the vial of 25 µl of competent cells and mix by tapping gently. DO NOT MIX BY PIPETTING UP AND DOWN.
4. Incubate the vial(s) on ice for 30 minutes.
5. Incubate for exactly 30 seconds in a 42°C water bath. Do not mix or shake.
6. Remove vials from the water bath and place them on ice.
7. Add 125 µl pre-warmed S.O.C. medium to each vial. S.O.C. is rich medium; sterile technique must be practice to avoid contamination.
8. Place the vial(s) on the side in 37°C incubator and shake at 225 rpm for exactly one hour.
9. Spread all the liquid from the transformation vial on separate LB agar plates containing the appropriate antibiotic.
10. Invert the plate(s) and incubate at 37 °C overnight.
11. Select colonies and analyze by plasmid isolation, PCR, or sequencing.

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**PilJ localizes to cell poles and is required for type IV pilus extension in
*Pseudomonas aeruginosa***

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Running title: PilJ localizes to cell poles

MANUSCRIPT ID CM-07-02-0111.R1

This work was supported by NIH grant # GM61318-01.

Twitching motility allows *Pseudomonas aeruginosa* to respond to stimuli by extending and retracting its type IV pili (TFP). PilJ is a protein necessary for this surface associated twitching motility and bears high sequence identity with *Escherichia coli* methyl-accepting chemotaxis proteins (MCP). Here we report that while wild-type *P. aeruginosa* PAO1 cells have extended pili at a single pole, *pilJ* mutant cells had shortened pili often at both poles despite normal levels of pilin accumulation, suggesting that PilJ is required for full TFP assembly/extension. Using yellow fluorescent protein fusions (*pilJ-yfp*), both plasmid born and in-frame chromosomal constructs, we determined that PilJ localizes to both poles of the cell. Overexpression of *pilJ-yfp* resulted in the protein accumulating between the poles.

Pseudomonas aeruginosa is a common Gram-negative bacterium capable of a unique form of surface-associated flagellar-independent motility known as twitching motility (9, 12). This form of motility is mediated by type IV pili (TFP) which extend and retract to pull the cell forward (4, 16, 19).

There are five gene clusters encoding chemotaxis-like phosphorelay signal transduction systems (6, 23) in the *P. aeruginosa* genome. Cluster IV contains genes involved in twitching motility (*pilGHIJK* and *ChpA-E*) (7, 28). These genes possess features similar to the chemotactic (*che*) system controlling flagellar rotation in swimming motility of enteric bacteria such as *Escherichia coli* (7, 8, 9) and to the *frz* cluster responsible for twitching motility in *Myxococcus xanthus* (7, 27). It has been reported that *pilJ* mutant strains are defective in twitching motility (7, 28) but the role PilJ plays in the mechanism has not yet been determined.

There is genetic evidence to suggest that *pilJ* encodes a transmembrane methyl-accepting chemotaxis protein (MCP) (7). Previous studies have demonstrated that MCPs are localized to the polar regions of the cell (1, 14) and serve to anchor other chemotaxis proteins such as CheA, CheY, and CheZ in *E. coli* cells (22). Recently, several proteins necessary for twitching motility in *P. aeruginosa* have been shown to cluster at one or both cell poles: PilS, PilB and PilT cluster at both poles while PilU and FimX appear restricted to a single pole (3, 5, 13). All of the studies with Pil proteins were conducted with the aid of functional fluorescent protein fusions.

In the present study we show that *pilJ* mutant cells are unable to fully assemble/extend TFP. Instead they had shortened pili often at both poles which may explain why these cells are unable to perform twitching motility. In addition, we provide evidence that PilJ is localized to both poles of the cell at native expression levels using an in-frame chromosomal PilJ-YFP fusion. With increased expression, using a multicopy plasmid, PilJ was observed throughout the cells, at times visible in a loosely organized spiral pattern.

Materials and Methods

Media and growth conditions. LB medium (Difco, Detroit, Michigan) was used for growth of *E. coli* and *P. aeruginosa* strains as noted. Broth cultures were grown in LB medium containing appropriate antibiotics unless otherwise stated. For standard growth plates, agar was added at a concentration of 1.5%. For twitching motility stab assays, the agar concentration was reduced to 1% in a volume of 11 ml per plate. The following antibiotics were used: ampicillin was used at 100 µg/ml for *E. coli*; carbinicillin was used at 60-100 µg/ml for *P. aeruginosa* for normal growth conditions and 300 µg/ml for

selection steps in the allelic exchange protocol for matagenesis. *Pseudomonas* Isolation Agar (PIA) (Difco) was used for the allelic exchange protocol. All cultures were incubated at 37° C overnight unless otherwise stated.

DNA methods. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA) or Promega Corporation (Madison, WI) and were used as directed by the suppliers. Plasmid DNA was extracted and purified using the QIAprep® Spin Miniprep Kit as described in the manual. When restriction digests were purified, the QIAquick® PCR Purification Kit was used. DNA fragments were purified from agarose gels after electrophoretic separation using the QIAEX® II Gel Extraction Kit (all QIA kits were purchased from QIAGEN Sciences, MD). PfuTurbo Hotstart DNA polymerase (Stratagene, La Jolla, CA) was used for most PCR reactions. For the PCR reaction to generate a small fragment of *pilK*, the PCR Super Mix (Invitrogen, Carlsbad, CA) was used. Oligonucleotides were purchased from Sigma/Genosys (The Woodlands, TX).

Twitching motility assays. Twitching motility was assayed by the method previously described (8). In some cases, the twitch zone was stained using coomassie blue (15).

***In situ* immunofluorescence (IMF).** Surface pili were observed using a modified *in situ* immunofluorescence procedure excluding the permeabilization step (10). Briefly, early log phase wild-type and *pilJ* mutant (FA6) cells were fixed with 3.5% formaldehyde for 15 min at room temperature, washed twice in PBS, and incubated in 0.1% Triton in PBS for 45 min at room temperature. Pili were detected using anti-PilA antibody (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). Controls in which only the secondary antibody was applied to the cells were used to check for non-specific binding of secondary antibody. Cells were observed using a Nikon Eclipse TS100 microscope at a magnification of 600X.

Construction of the multi-copy plasmid *pilJ-yfp* fusion. The *yfp* gene from pEYFP (Clontech, Mountain View, CA) was subcloned into the *Bam*HI-*Eco*R1 sites of pUCP19, creating plasmid p19YFP, and transformed into *E. coli* Top 10 cells (Invitrogen, Carlsbad, CA) as described in the manual. This plasmid was used as a control to check YFP synthesis and cellular distribution when not fused to PilJ. A second plasmid, p19JY, was created containing the *pilJ-yfp* fusion. This fusion was created using PCR to add *Hind*III and *Bam*HI sites to the 5' and 3' end of *pilJ* respectively. In order to ensure the transcription and translation of the single protein fusion, PilJ-YFP, the *pilJ* stop codon was removed and replaced with the *Bam*HI site during the PCR reaction. In a separate PCR reaction, a *Bam*HI site was added to the 5' end of the *yfp* gene in order to complement the 3' *Bam*HI site of the *pilJ* gene. A three way ligation joined the two PCR products into the pUCP19 vector using the 5' *Hind*III site of *pilJ* and the 3' *Eco*RI site of *yfp* to generate the gene fusion construct p19JY. The ligation product was transformed into *E. coli* Top 10 cells (Invitrogen, Carlsbad, CA) as described in the manual.

All plasmid constructs were extracted from the *E. coli* cells, confirmed by restriction digests, and transformed into FA6 by electroporation (20). Functional PilJ activity of the fusion construct was confirmed by complementation of FA6 with p19JY to restore twitching motility phenotype. Cells containing the *yfp* constructs (p19YFP and p19JY) were grown on LB agar or broth supplemented with carbenicillin.

Construction of the in-frame chromosomal *pilJ-yfp* fusion. In order to optimize homologous recombination and thus integration of the *pilJ-yfp* fusion into the chromosome, the 600 base pair region immediately downstream of *pilJ* was added to the 3' end of the *pilJ-yfp* fusion previously generated. This 600 base pair fragment containing a portion of *pilK* was first subcloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA). This was done using PCR and the plasmid pADD698 as the source DNA to add an *Xba*I and *Eco*RI site to the 5' and 3' end of the *pilK* fragment respectively to create the plasmid pCRK. The *pilK* fragment was then subcloned into suicide vector pEX19Ap using the *Xba*I and *Eco*RI sites to create pEX19K. The original *pilJ-yfp* fusion previously generated in p19JY was then subcloned into the pEX19K plasmid using the *Hind*III and *Xba*I sites to generate pEX19JYK in *E. coli* Top 10 cells. The recombinant suicide vector, pEX19JYK, was transformed into *E. coli* S17-1 for mating with the *P. aeruginosa pilJ* mutant, FA6. This allelic exchange of the mutant *pilJ* gene of FA6 with the pEX19JYK was based on the sucrose selection system previously described (18). Recombination of the *pilJ-yfp* fusion in the chromosome was confirmed by loss of sucrose sensitivity, demonstration of restored twitching motility in the FA6 mutant to wild-type levels and Western blot analysis.

Localization of PilJ. Cells containing p19YFP or p19JY were grown in LB broth to log phase and examined using fluorescence microscopy. Cells containing the in-frame chromosomal fusion were inoculated into LB supplemented with 0.4% NaCl and grown to log phase or 24 hours at 37°C. Cells were observed using a Nikon eclipse TS100 or Olympus BX51 microscope at a magnification of 600X.

Western blot analysis of *pilJ-yfp* fusions. Cells grown in LB broth, either overnight or subcultured and grown to an optical density of 0.6 at 590 nm, were harvested and lysed by boiling for 10 min in Laemmli sample buffer. Proteins were separated using SDS-PAGE precast Tris-HCl gels (12% acrylamide as specified by Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane by electroblotting. YFP was probed using a 1:7000 dilution of an anti-YFP antibody, referred to as A.V. monoclonal (JL-8) antibody (BD Biosciences Clontech, Palo Alto, CA), followed by the secondary antibody (anti-mouse immunoglobulin G conjugated to alkaline phosphatase) (Promega, Madison, WI) diluted 1:5000, and detected using Western Blue Stabilized Substrate for alkaline phosphatase (Promega, Madison, WI). For increased sensitivity we used chemiluminescent detection. YFP was probed using a 1:50,000 dilution of the anti-YFP antibody, followed by anti-mouse horse radish peroxidase (HRP) conjugated secondary antibodies (Pierce, Rockford, IL) diluted 1:3000 and detected with SuperSignal® West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL) as described in the manual.

Results

Direct analysis of surface pili orientation and extension. Previous studies that examined the role of *pilJ* in twitching motility have produced conflicting results. Darzins found the *pilJ* mutant of PAO1 to be completely deficient in twitching motility (7) while Whitchurch et al., (28) observed reduced and aberrant twitching for the *pilJ* mutant of the PAK strain. These differences may be attributable to well documented strain variations between PAO1 and PAK. To resolve this discrepancy and determine the role of PilJ in twitching motility, we constructed *pilJ* mutants in three different wild-type backgrounds. One was a typical laboratory strain from Urs Ochsner (PAO1-UO), another a clinical

isolate (GSU-3) and the third was an environmental strain (DAP119J) shown to be very aggressive at twitching motility. In addition we examined two *pilJ* transposon mutants (Seattle Transposon Library, Department of Medicine, University of Washington Genome Center, Seattle, WA) for twitching motility. All *pilJ* mutants tested were completely deficient in twitching motility (data not shown). Previous studies have shown total pilin production in wild-type and *pilJ* mutant cells to be equivalent while external pilin levels were nearly undetectable in the mutants (7, 28), yet the mutant cells remained sensitive to pilus specific phage. We tested the *pilJ* mutants we constructed for total and external pilin, as well as sensitivity to pili specific phage, with similar results (data not shown). Sensitivity of the *pilJ* mutant cells to pilus specific phage could be explained by the presence of small stubs of external pili present on the mutant cells; pili long enough for phage to bind to, but too short to be sheared and collected for Western blot analysis or to initiate twitching motility. To determine if this was true, we used IMF to compare the length of surface pili on wild-type cells with those on *pilJ* mutant cells.

Using anti-PilA antibodies, cells recovered from twitch plates or broth were examined using immunofluorescence microscopy. As shown in Figure 1A, wild-type PAO1 cells typically possessed long pili extending from a single pole. In contrast, none of the *pilJ* mutant cells possessed long pili like those observed on the wild-type cells (Fig. 1A insert). Instead the mutant cells were found to have shortened pili frequently present at both poles (Fig. 1C). Although the percentage of cells that were labeled was low, this percentage remained constant regardless of the level of fixative or amount of primary or secondary antibody and was similar between wild type (25%) and *pilJ* mutant cells (19%). The low percentage of labeled cells may indicate that only a small number of

cells have pili extended at any time, or that pili may have been broken off during the protocol itself despite all efforts to avoid shearing. Controls, treated with the secondary antibody only, were free of any non-specific binding as indicated by lack of fluorescence in the micrographs (Fig. 1B and 1D).

Localization of PilJ. The plasmid born PilJ-YFP fusion complemented the *pilJ* mutant, FA6, evidenced by the restoration of twitching motility in complemented cells (data not shown). Importantly, Western blot analysis confirmed that PilJ-YFP fusions of the plasmid born, and in-frame chromosomal constructs remained intact as evidenced by single bands at the predicted molecular weight of approximately 103 kDa for the fusion (Fig. 2A).

When the plasmid bearing only *yfp* (p19YFP) was introduced into either *E. coli* or FA6 strains, fluorescence was uniformly distributed throughout the cells indicating that YFP itself was not localized in either bacterium (Fig. 3A and 3B). When the fusion plasmid, p19JY was introduced into the *E. coli* cells, PilJ was localized to both poles (Fig. 3C). While *E. coli* does not produce TFP, it seems reasonable that PilJ may be localizing to the poles of *E. coli* due to the homology between PilJ and other *E. coli* MCPs. This homology may be responsible for common interactions with membrane bound anchor proteins that locate MCPs to the poles in both *E. coli* and *P. aeruginosa*. Similarly, FA6 containing the multi-copy plasmid born *pilJ-yfp* fusion construct, p19JY, fluoresced only at polar regions of the cell when observed under log phase growth conditions (Fig. 3D). Under stationary growth phase conditions with the same cells, the fusion was also observed between the poles of the cells, at times visible in a loosely organized spiral pattern (Fig. 3E).

Because we observed some PilJ-YFP between the poles in cells containing the multi-copy plasmid born *pilJ-yfp* fusion in *P. aeruginosa* FA6 cells, we created an in-frame chromosomal fusion of *pilJ-yfp*, replacing the mutant *pilJ* gene, to examine localization under native expression levels. Levels of PilJ-YFP were not detectable by Western blot analysis, using either colorimetric or chemiluminescent detection, until after 22-24 hours of growth for the in-frame chromosomal fusion while easily detectable in log phase cells containing the plasmid born fusion indicating native expression is substantially lower (Fig. 2A). Replacement of the mutant *pilJ* gene by the in-frame chromosomal *pilJ-yfp* fusion in the FA6 background resulted in complete restoration of twitching motility indistinguishable from that of wild-type cells (Fig. 2B). Epifluorescence microscopy revealed that PilJ was restricted to the poles of the cells containing the in-frame chromosomal *pilJ-yfp* fusion (Fig. 3F). Localization of PilJ using this construct was visible when cells were grown in LB with 0.4% NaCl and, consistent with the Western blot analysis was most pronounced at 22-24 hours incubation. Since the Western blot analysis clearly showed that the fusion remained intact within the cells, the PilJ-YFP observed between the poles in the cells containing the multi-copy plasmid born fusion is likely the result of higher levels of expression, and not free YFP resulting from proteolytic degradation of the fusion.

Discussion

Our results indicate that the reason *pilJ* mutants are incapable of twitching motility is that PilJ is necessary for the full assembly/extension of TFP. Without PilJ, cells appear to abort the assembly/extension process of TFP resulting in the shortened pili often at both poles. While a defect in TFP retraction could also explain lack of twitching motility

observed with *pilJ* mutants, such a defect would be incompatible with the retention of phage sensitivity. Further, if PilJ controls TFP retraction *pilJ* mutant cells would be expected to have a higher percentage of cells with surface pili present compared with wild-type cells. We observed a slightly lower percentage of mutant cells with surface pili using IMF. The signal transduction system, including the gene cluster *pilGHIJK*, that controls twitching motility in *P. aeruginosa* is similar to the chemosensory systems (*che* genes) controlling flagellar rotation in *E. coli*. Therefore, by controlling the assembly/extension of TFP, PilJ is functioning similarly to the MCPs of the enteric bacteria that initiate signaling that controls the direction of flagellar rotation. How PilJ works with the other proteins necessary for twitching motility is under investigation in our laboratory.

The second goal of this study was to determine the localization pattern of PilJ. Based on homology from earlier studies, it was assumed that PilJ is a membrane MCP (7). Therefore, one would expect it to localize to the cell poles as other membrane MCPs do (1, 11, 14, 25, 26). We were able to demonstrate that PilJ localizes to both poles of the cell, consistent with the findings for other MCPs. When expressed at native levels using the in-frame chromosomal fusion, PilJ-YFP was only seen at the poles. However, when *pilJ* was overexpressed, using a multi-copy plasmid born fusion, PilJ was not limited to the cell poles. With increased expression, the fusion was observed throughout the cells, at times visible in a loosely organized spiral pattern. Since the fusion remained intact, as proven by Western blot analysis, this pattern may indicate that the PilJ protein is transported to the cell poles via a bacterial cytoskeleton. Mignot et al., (17) showed

that transport of a gliding motility protein between the cell poles of *Myxococcus* involves a cytoskeleton.

Other proteins involved in TFP biosynthesis and twitching motility, such as the PilS and ATPases PilB and PilT, localize to both poles in *P. aeruginosa* (3, 5). With our discovery that PilJ also localizes to both poles of the cell, the concept of polar compartmentalization of the twitching motility apparatus at both poles is strengthened (14, 21). By having an assembly/disassembly apparatus at both poles, including PilJ, which we have shown is required for extension of the TFP, the cell would be capable of quick cell reversals in response to changing environmental conditions without having to physically reorient the cell on a surface. Likewise, there would be no need to transport proteins from one pole to the other, although this has been shown to be the case for FrzS in *Myxococcus* (17). Instead the cell can simply change the leading pole of the cell in terms of directional movement by retracting the TFP from one end and extending it from the other end as proposed by Sun, et al (24). This has been referred to as polar switching and has been demonstrated in *M. xanthus* (2, 21). PilJ may sense an as yet unidentified stimulus that directs the cell to extend pili at the appropriate pole. In the absence of PilJ, or the stimulus, the default position is to partially assemble pili at both poles which would allow the cell to respond to signals originating from either direction. In *P. aeruginosa*, PilJ is an essential protein in the initiation of TFP extension/assembly and as a putative MCP, is likely involved in establishing the direction of twitching movement by sensing the environment and responding accordingly.

ACKNOWLEDGEMENTS

We would like to thank Ellen Quardokus for her technical assistance and valuable discussions. This work was supported by NIH grant # GM61318-01.

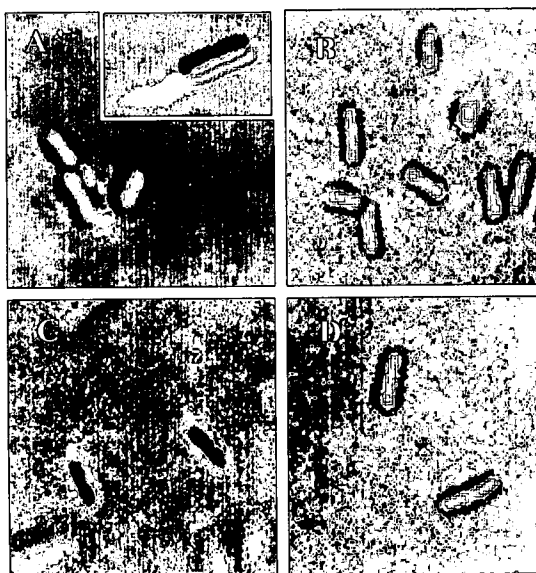


Fig. 1. *In situ* immunofluorescence of pili in wild-type PAO1 and *pilJ* mutant (FA6) cells. Cells were fixed in 2.5% formaldehyde. Pili were detected using *P. aeruginosa* anti-PilA antibodies followed by secondary antibodies conjugated to quantum dots. Cells were observed using fluorescence microscopy at a magnification of 600X. (A) PAO1 wild-type; (B) Control PAO1 wild-type with secondary antibody only; (C) FA6 (PAO1*pilJ*::Tc); (D) Control FA6 (PAO1*pilJ*::Tc) with secondary antibody only.

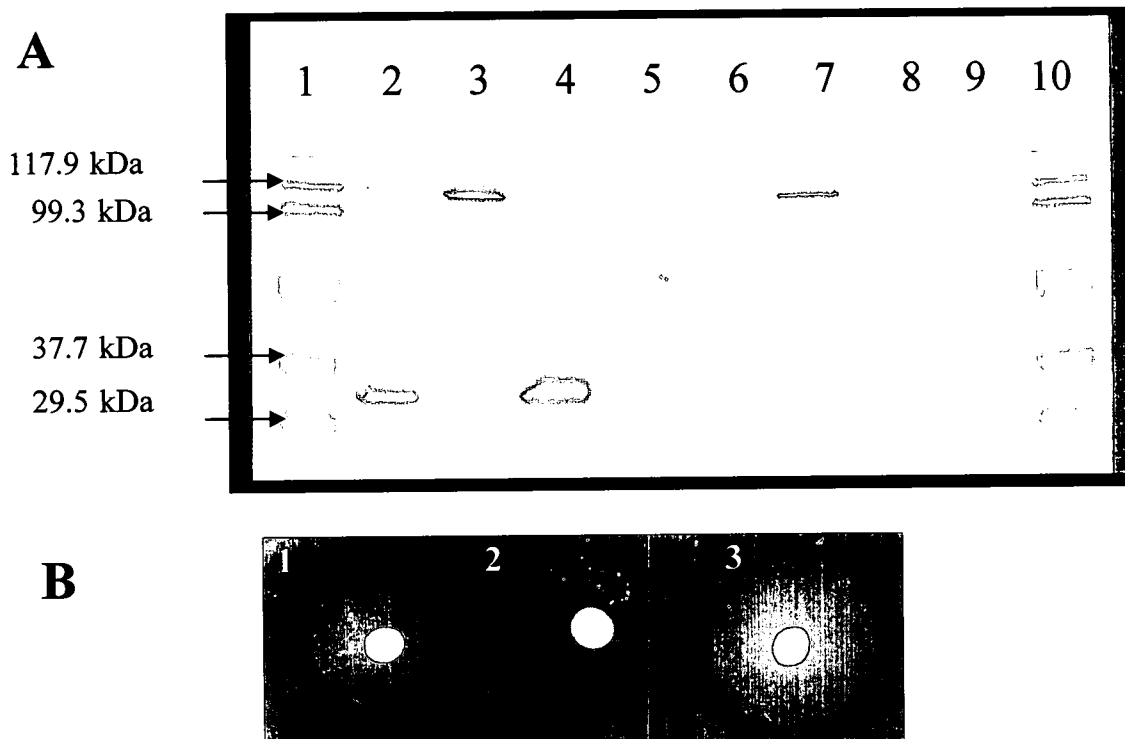


Fig. 2. Western blot of YFP constructs and complementation by the in-frame chromosomal fusion. **(A)** Western blot. Cells were grown to log phase (lanes 2-5 and 9) or overnight (lane 7). Log phase cells were standardized to an OD_{590} of 0.61. Overnight growth was not adjusted. 10 μ l of each sample was loaded in the wells. Membrane was probed with anti-YFP antibody. Lane 1: Broad range markers; Ln 2: *E. coli* with pEYFP; Ln 3: FA6 with multicopy plasmid born p19JY; Ln 4: FA6 with p19YFP; Ln 5: FA6 with in-frame chromosomal fusion *pilJ-yfp*; Ln 6: blank; Ln 7: Overnight growth of FA6 with in-frame chromosomal fusion *pilJ-yfp*; Ln 8: Blank; Ln 9: Control, FA6 log phase growth; Ln 10: Broad range markers. YFP = 32 kDa, PilJ = 71 kDa, PilJ-YFP = 103 kDa. **(B)** Complementation by the in-frame chromosomal fusion using twitching motility assay. 1% LB agar plates were stab inoculated and incubated for 48 hours at 37°C. Diffuse zones (twitch zones) represent cells moving at the agar-petri dish interface. 1) PAO1-AD (wt); 2) FA6 (*pilJ*-); and 3) FA6 with chromosomal fusion *pilJ-yfp*.

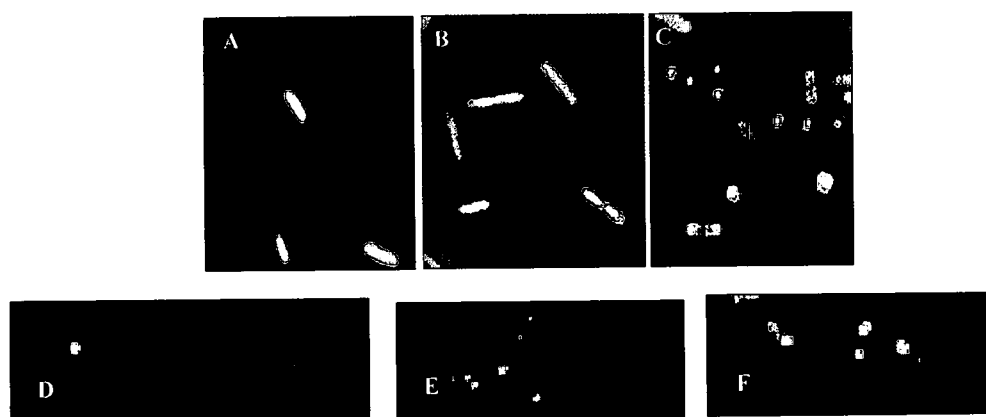


Fig. 3. Localization of PilJ. (A) *E. coli* with p19YFP, (B) FA6 with p19YFP, (C) *E. coli* with multi-copy plasmid born p19JY, (D) FA6 with multi-copy plasmid born p19JY (log phase), (E) FA6 with multi-copy plasmid born p19JY, (24 hour growth), (F) FA6 with in-frame chromosomal fusion *pilJ-yfp*, (24 hour growth)

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R702033648