THE NOVEL *PSEUDOMONAS AERUGINOSA* TYPE IV PILIN ACCESSORY GENES *TFPY* AND *TFPZ* AFFECT PILUS ASSEMBLY DYNAMICS

By

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TITLE: The novel *Pseudomonas aeruginosa* type IV pilin accessory genes *tfp* and *tfpZ* affect pilus assembly dynamics

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ABSTRACT

Pseudomonas aeruginosa uses type IV pili (T4P) to colonize various materials and for surface-associated twitching motility. We previously identified five phylogenetically-distinct alleles of *pilA* in *P. aeruginosa,* four of which occur in genetic cassettes with specific accessory genes (Kus et al., Microbiology 150:1315-1326, 2004). Each of the five pilin alleles, with and without its associated pilin accessory gene, was used to complement a group II PAO1 *pilA* mutant. Expression of group I or IV *pilA* genes restored twitching motility to the same extent as the PAO1 group II pilin. In contrast, complementation with group III or group V *pilA* genes resulted in poor twitching that increased significantly when the cognate *tfpY* or *tfpZ* accessory genes were co-introduced. The enhanced motility was linked to an increase in recoverable surface pili, and not to alterations in total pilin pools. Expression of the pilin genes, with or without accessory genes, in a PAO1 *pilA-pilT* double mutant background resulted in expression of large amounts of surface pili, suggesting that the accessory proteins function to modulate pilin retraction dynamics. Reduction of twitching motility and surface piliation was also observed a *tfpY* knockout mutant of group III strain PA14, confirming that the accessory proteins enhance pilus assembly on the cell surface. The accessory proteins are specific for their cognate pilins; a $PilA_V$-TfpY chimera produced few surface pili, resembling the phenotype of PAO1 complemented with $pilA_V$ alone. The linkage between specific pilin and accessory genes may be evolutionarily conserved because the accessory proteins antagonize pilus retraction, increasing pilus expression on the cell surface and thereby enhancing function.

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ATTRIBUTIONS

Mass spectrometry pilin sample preparation and analysis was done by Julianne Kus, with the technical assistance of John Kelly and Luc Tessier. Cloning of pilins and accessory genes into pBADGr was done by Julianne Kus. Antibody preparation was done by Patrick Yip.

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1.0. INTRODUCTION AND BACKGROUND

1.1. Pseudomonas aeruginosa and Type IV Pili

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen responsible for hospital acquired infections in patients with burn wounds and indwelling medical devices, in addition to its role as a major pathogen in Cystic Fibrosis (CF) patients (26). *P. aeruginosa* infections are a challenge to treat due to the ability of this organism to form highly structured bacterial communities, termed biofilms, which protect the cells from both the host immune response and antimicrobial drugs (17) Patients with CF suffer from chronic lung infections because they lack the ability to clear bacteria from their lungs, which is why *P. aeruginosa* remains the leading cause of mortality for these individuals (26).

Attachment of bacterial cells to a surface is mediated by polar protein appendages named type IV pili (T4P) (32). *P. aeruginosa* T4P have the ability to adhere to both biotic and abiotic surfaces, explaining the diversity of infections caused by this pathogen (25). In addition, T4P are involved in other functions such as flagella-independent surface motility (7).

1.2. Twitching Motility

Twitching motility is the term used to describe the form of surface motility in which bacteria use T4P to move themselves across a surface (51). Twitching occurs through rapid extension and retraction of the pilus, the tip of which adheres to a surface and causes the cell bodies to be translocated when the pilus retracts (53). The mechanism of pili extension and retraction involves three proteins; PilB, PilT and PilU. PilB is involved in assembly of pilin subunits at the base of the pilus, allowing rapid extension of the pili, and PilT is involved in disassembly, allowing retraction (44, 62).

PilU is thought to be involved in retraction since like PilT mutants, PilU null mutants are unable to retract their pili and lack twitching motility; however, PilU mutants are susceptible to the pilus-specific bacteriophage whereas PilT mutants are not (61).

1.3. Diversity of T4P

T4P are found in many Gram-negative bacteria in addition to *P. aeruginosa*, including *Neisseria* spp. and *Vibrio cholerae.* Surface attachment, phage sensitivity and twitching motility are common functions of the T4P of these bacteria. *N. gonorrhoeae* T4P are involved in an additional function, transformation competence, requiring pilus retraction for DNA uptake (66).

There are two subclasses of T4P, type IVa and type IVb. *P. aeruginosa*, *N. gonorrhoeae* and *Myxococcus xanthus* have type IVa pili, whereas *Vibrio cholerae, Salmonella typhi* and enteropathogenic *Escherichia coli* have type IVb pili. The main differences between these two subclasses are the N-terminal leader sequences (6 amino acids vs. 25 amino acids), the post-processing N-terminus residue (Phe for IVa and Met, Leu or Val for IVb) and the length of the mature protein, which is shorter in the type IVa subclass (18). Additional differences are reflected in the structure and folding of the C-terminal regions (20, 28).

1.4. T4P: Structure and Assembly

1.4.1. Structure of T4P

T4P are comprised of thousands of pilin monomers held together by a combination of hydrophobic and ionic interactions. Crystallographic structures of *P. aeruginosa* PAK reveal an elongated N-terminal a-helix and a C-terminal globular head region (Figure 1A), consistent with the structure of *N. gonorrhoeae* GC pilin from strain MS11 (Figure 1B), which was determined previously (20, 45).

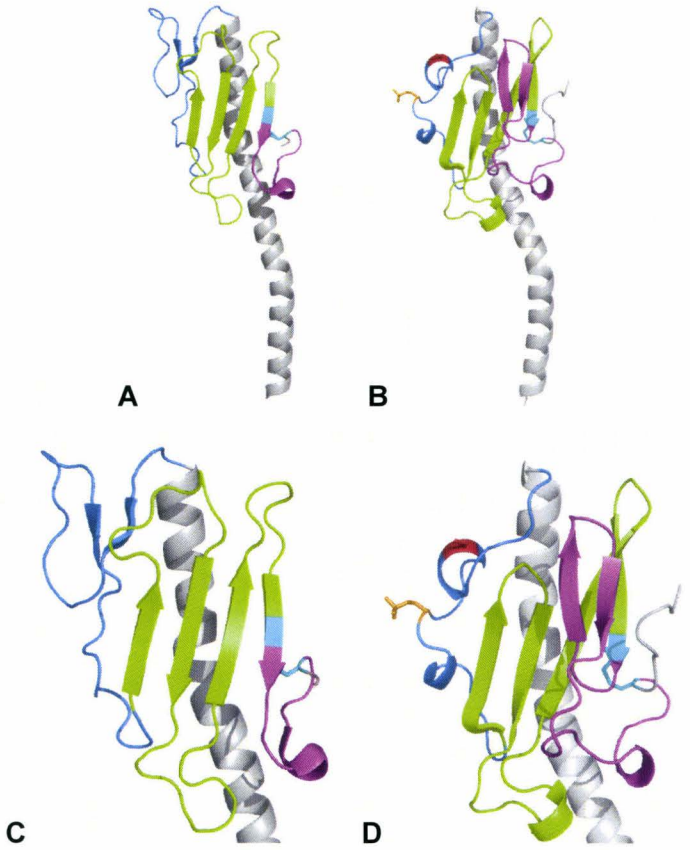


Figure 1. Structural comparison of type IV pilins.

Structures of A) Pseudomonas aeruginosa PAK pilin, PilA; B) Neisseria gonorrhoeae GC pilin, PilE, from strain MS11, showing the positions of post-translational modifications. Ser63 (red) is O-glycosylated and Ser68 (orange) is phosphorylated; C) N-terminally truncated PAK pilin; D) N-terminally truncated GC pilin. In each of the structures the N-terminal α-helix is coloured in grey, the $\alpha\beta$-loop is coloured in dark blue, the disulfide loop is coloured in magenta and the cysteines forming the bond for the disulfide loop are coloured in light blue. These images were created in PyMOL (DeLano Scientific LLC).

The N-terminus consists of mainly hydrophobic residues that form an α-helix, approximately 52 residues in length. The α-helix forms a slight s-shaped curve that can

be seen in the structure, resulting from a Pro at residues 22 and 42 (Gly occurs at position 42 in GC pilin). This curve is thought to be important for proper packing of the pilin subunits into the pilus fibre and its resulting flexibility (18).

The globular C-terminal region of T4P has three distinct features: an anti-parallel β-sheet, an αβ-loop and a disulfide-bonded loop. The overall architecture of the β-sheets in the head region is shared in all type IV pili, however the way in which they fold varies (42). PAK and GC pilins both have a four-stranded antiparallel β-sheet (Figure 1a/d and 1b/c). The αβ-loop in the PAK pilin forms a minor β-sheet with the C-terminal portion of the α-helix (28). This loop (in all T4P) is also predicted to be involved in subunit-subunit interactions (18). At the C-terminal end of the pilin, a disulfide bond is formed from two cysteine residues creating a disulfide loop. This is the most structurally variable region in all type IV pili, where the number of residues ranges between 12 to 31 in *P. aeruginosa*. The 29-residue disulfide loop of GC pilins forms a β-hairpin turn, whereas the disulfide loop of PAK pilin forms a short loop since it is only 12 residues in length (28, 45).

Formation of the disulfide loop is crucial to formation of the pilus fibre. Data from our lab shows that PilA is degraded if either of the two Cys residues involved in forming the disulfide loop is mutated (Harvey *et al.*, in revision). Another key feature of the PilA disulfide loop is that it is thought to contain the receptor-binding site that is responsible for adherence to surfaces (32). Monoclonal antibody binding studies have shown that the attachment site is exposed only at the pilus tip. Along the length of the pilus fibre, the disulfide loop is involved in subunit-subunit interactions, blocking the attachment sites (18, 37).

1.4.2. Assembly of the Pilus Fibre

The pilin monomers assemble in a helical fashion with their hydrophobic N-termini within the core of the filament and the globular C-termini exposed along the surface. Although the structure of the fibre is acknowledged to be helical, there has been controversy about the assembly of the pilus fibre in terms of subunits per helical turn, the number of starting points for individual subunits and the handedness of the helix (8). Recent work, however, has shown that the pilins form a three-start left-handed helix bundle with 3.6 subunits per turn in the pilus fibre (19). The loops formed between the β-strands of one subunit are predicted to interact with multiple sites; the loops between β-strands of subunits above and below, the minor β-strands within the αβ-loop in the PAK pilin, and the disulfide loop of a neighbouring subunit (18). This arrangement causes the C-termini to be exposed on the surface, with the disulfide loop exposed only at the tip of the pilus (19, 37).

1.5. Five Groups of P. aeruginosa Pilins

Recent studies of T4P in *P. aeruginosa* revealed five phylogenetically distinct groups of pilin genes, four of which have unique accessory genes immediately downstream of the pilin gene (36). This study identified novel pilin accessory genes in three of the five groups and thus was the basis for a new nomenclature system where *P. aeruginosa* pilins were grouped according to the sequence of their disulfide loop and accessory genes. Strains with group II pilins include the common PAK and PAO1 lab strains, as well as strains that have been isolated from Cystic Fibrosis patients. This is the only group in which there is not an accessory gene immediately downstream of the pilin gene (36).

Group I strains have an accessory gene, *pilO*/*tfpO*, the product of which glycosylates the pilin with an O-antigen unit of the lipopolysaccharide of the background

strain (12). Castric first identified the differences between group I and group II strains based on DNA sequence analysis (13). Group I pilins are longer than those of group II and have a 17 amino acid disulfide loop, whereas group II pilins have a 12 amino acid disulfide loop (36).

Strains with group III, IV and V pilins contain novel accessory genes immediately downstream of *pilA*. Strains G7 (55) and PA14 (15) were previously identified, however their accessory genes were annotated only as "ORF1" (open reading frame 1). In the 2004 study by Kus and colleagues (36), this gene was renamed *tfpY* (**t**ype **f**our **p**ilin gene **Y**), and assigned to the group III pilins. Group IV and V pilins had not been previously identified and their accessory genes were named *tfpW, tfpX* and *tfpZ* (respectively). Group III pilins have a 31 amino acid disulfide loop, the largest reported for *P. aeruginosa*, the group IV pilin has a 23 amino acid disulfide loop, and group V pilins have a 29 amino acid disulfide loop. The residues are more conserved at the distal end of the loop, suggesting insertions or deletions in the proximal end (36).

Homologues of TfpY and TfpZ were identified through a BLASTp search of Genbank database organisms (Table 1). Most of the accessory gene homologues found in this search were identified through genome sequencing efforts and not associated with experimentally acquired functional data, therefore our current understanding of the putative function of these proteins is still unknown. Of all homologues found, only two have been reported in the literature; *Eikenella corrodens* and *Dichelobacter nodosus* both contain accessory genes that have a type IV pilin gene immediately upstream. The accessory gene product PilB (31% protein sequence identity to TfpZ) from *E. corrodens* was shown to be required for twitching motility. The authors noted that this protein is likely involved in pilus assembly, however they did not further investigate its function (56). On the other hand, *D. nodosus* accessory gene product FimB (32% protein

sequence identity to TfpZ) was found not to be required for pili biogenesis; again, no further experiments were conducted (35).

1.6. Objectives

P. aeruginosa uses its T4P to initiate colonization of a surface in order to establish an infection. Alterations of the pilin adhesive domain markedly decreases cellular adherence (23), making T4P an essential virulence factor for the organism. Identification of novel pilin accessory proteins may provide new ways to disrupt the virulence of the organism. Since the limited literature on homologues of the newly identified pilin accessory proteins reported contradictory results with respect to the phenotypes for mutants lacking these proteins, their specific functions remained unknown. The aim of this research was to investigate the function of the type IV pilin accessory proteins and the basis for their strict association with their cognate pilins, as well as to shed light on why they are present in the group III, IV and V strains, but not the group I and II strains. Based on preliminary results obtained, we hypothesize that the novel pilin accessory proteins TfpY and TfpZ are involved in type IV pilus retraction dynamics.

Table 1. Homologues of TfpY and TfpZ identified through a blastp search. The disulfide loop (DSL) length was calculated by counting the number of residues in between and including the two cycteine residues at the C-terminal end of the PilA protein.

A) Homologues to TfpY from strain PA14

Organism	Protein Name	Accession Number	Protein Identity	E Value	Upstream Pilin Gene	DSL	Published Information
Saccharophagus degradans	Hypothetical Sde_0870	ABD80132	37%	2.00^{-37}	No	29	N/A
Colwellia psychrerythraea 34H	Hypothetical protein	AAZ25334	36%	5.00^{-9}	Yes, TFP: YP_271092	7	N/A
Verminephrobacter eiseniae	Fimbrial assembly	EAT74369	36%	1.00^{-16}	No	18	N/A
Polaromonas naphthalenivorans CJ2	4-hydroxybenzoate polyprenyl transferase	EAQ18183	44%	3.00^{-12}	No	18	N/A
Polaromonas sp. JS666	FimB	ABE46553	40%	2.00^{-12}	No	18	N/A
Acidovorax sp. JS42	FimB	EAT5557	36%	3.00^{-12}	Yes, Fimbrial pilin	17	N/A
Dichelobacter nodosus	FimB	CAA36663	32%	4.00^{-18}	Yes, FimA	14	Not required for pilin biogenesis (35)
Neisseria meningitidis Z2491	Fimbrial assembly	CAB84923	30%	1.00^{-9}	No	37	N/A
Neisseria meningitidis MC58	FimB	AAF41843	29%	7.00^{-10}	No	37	N/A
Neisseria gonorrhoeae FA	FimB	AAW89730	30%	8.00^{-13}	No	35	N/A

B) Homologues to TfpZ from strain 0594

Organism	Protein Name	Accession Number	Protein Identity	E Value	Upstream Pilin Gene	Disulfide loop length	Information
Polaromonas sp. JS666	FimB	ABE46553	32%	2.00^{-21}	No	18	N/A
Eikenella corrodens	PilB	AAC28470	31%	5.00^{-20}	Yes, PilA1	18	Required for twitching (56)
Dichelobacter nodosus	FimB	CAA36663	32%	4.00^{-18}	Yes, FimA	14	Not required for pilin biogenesis (35)
Verminephrobacter eiseniae EF01-2	Fimbrial assembly	EAT74369	31%	1.00^{-16}	No	18	N/A
Neisseria meningitidis MC58	FimB	AAF41843	29%	1.00^{-13}	No	37	N/A
Neisseria meningitidis Z2491	Fimbrial assembly	CAB84923	29%	1.00^{-10}	No	37	N/A
Neisseria gonorrhoeae FA	FimB	AAW89730	30%	8.00^{-13}	No	35	N/A
Acidovorax sp. JS42	FimB	EAT55577	27%	4.00^{-10}	Yes, Fimbrial pilin	17	N/A
Colwellia psychrerythraea 34H	Hypothetical protein	AAZ25334	34%	5.00^{-9}	Yes, TFP: YP_271092	7	N/A
Saccharophagus degradans 2-40	Response regulator	ABD80132	29%	2.00^{-8}	No	29	N/A

2.0 MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and genetic constructs used for this study are listed in Table 2. Bacteria were maintained as glycerol stocks at -80°C and routinely grown on Luria-Bertani plates supplemented where indicated with L-arabinose and antibiotics. Antibiotic concentrations for *Escherichia coli* or *P. aeruginosa* were: 15 or 30 mg/L gentamicin; 100 mg/L ampicillin (*E. coli* only), 200 mg/L carbenicillin (*P. aeruginosa* only) and 75 mg/L piperacillin (*P. aeruginosa* only) unless otherwise noted.

2.2 Genetic manipulations

For pilin complementation studies, *pilA* genes, alone or with their cognate accessory genes, were amplified by PCR from chromosomal DNA of representative group I, II, III, IV and V strains as indicated in Table 2. Primers included restriction sites *EcoRI* or *HindIII* for cloning; sequences and names are listed in Table 3A. Combinations of primers were used to amplify pilin gene from template DNA, prepared using Instagene (Bio-Rad), as following; $pilA_I$ (forward $pilA_I$ and reverse $pilA_I$), $pilA_I$ +*tfpO* (forward $pilA_I$ and reverse $tRNA^{Thr}$), $pilA_{II}$ (forward $pilA_{II}$, $pilA_{III}$, $pilA_V$ and reverse $tRNA^{Thr}$), $pilA_{III}$ (forward $pilA_{II}$, $pilA_{III}$, $pilA_V$ and reverse $pilA_{III}$), $pilA_{III}$ + *tfpY* (forward $pilA_{II}$, $pilA_{III}$, $pilA_V$ and reverse $tRNA^{Thr}$), $pilA_{IV}$ (forward $pilA_{IV}$ and reverse $pilA_{IV}$), $pilA_{IV}$ + *tfpW* (forward $pilA_{IV}$ and reverse *tfpW*), $pilA_{IV}$ +*tfpW* + *tfpX* (forward $pilA_{IV}$ and reverse $tRNA^{Thr}$), $pilA_V$ (forward $pilA_{II}$, $pilA_{III}$, $pilA_V$ and reverse $pilA_V$) and $pilA_V$ + *tfpZ* (forward $pilA_{II}$, $pilA_{III}$, $pilA_V$ and reverse $tRNA^{Thr}$). PCR products were purified using QIAquick gel extraction spin columns (Qiagen), digested with the appropriate enzymes and ligated into the previously linearized pBADGr vector, a modified version of pMLBAD (38) wherein the *dhfr* gene encoding trimethoprim resistance was replaced with the *aacC1* gene from pUCGm (50),

placing the genes under L-arabinose control. Constructs were transformed into *E. coli* DH5α by incubating on ice for 30 min, heat shocking at 42°C for 45 s, put back on ice for 2 min, incubating on a shaker at 37°C for 3 hours, plating on LB agar plates containing 15 mg/L and grown overnight at 37°C. Colonies were patched, grown overnight at 37°C and plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen). After confirming the fidelity of the constructs by DNA sequence analysis, they were introduced into a *pilA* mutant of PAO1 (33) via electroporation, and transformants selected by plating on LB plates containing 30 mg/L gentamicin. The QuikChange mutagenesis kit (Stratagene) was used as directed by the manufacturer to generate premature stop codons within the cloned accessory genes where indicated in Table 2.

2.3 Twitching motility assays

Assessment of twitching motility was performed using an agar subsurface assay as described previously (51); briefly, colonies were stab inoculated through the agar to the bottom of the Petri dish and grown for 48 hours at 37°C. The resulting zones of twitching motility visualized by carefully removing the agar and staining the bacteria adhering to the polystyrene Petri dish with 1% (w/v) crystal violet for 10 min at room temperature, followed by a brief rinse with tap water to remove unbound dye. ImageJ software (NIH) was used to measure and calculate average areas of the resulting twitching zones to acquire quantitative comparative data.

2.4 Analysis of sheared surface proteins by SDS-PAGE

Cell surface appendages (flagella and pili) were isolated using the methods of Castric (11) with modifications. Bacteria were streaked in a grid pattern on LB agar plates containing 30 mg/L gentamicin and 0.2% L-arabinose (two plates per sample) and incubated overnight at 37°C. The bacteria were gently scraped from the agar surface

using a sterile coverslip and resuspended in 2 mL sterile phosphate-buffered saline (PBS, pH 7.4) per sample, and surface proteins were sheared by vigorous vortexing for 30 s. The suspension was transferred to 2 x 1.5 mL microcentrifuge tubes and centrifuged for 5 min at maximum speed to pellet the cells, which were retained for whole cell lysate Western immunoblots (below). The supernatant was transferred to a new tube and centrifuged for an additional 25 min at maximum speed at room temperature to remove remaining cells. To precipitate the sheared surface proteins, 1/10 volume each of 5 M NaCl and 30% polyethylene glycol (MW range 8000) was added to the supernatant and the samples incubated on ice for 60 min. Samples were centrifuged at maximum speed in a microcentrifuge for 25 min at 4°C. After discarding the supernatant, the resulting pellets were resuspended in 1x SDS-PAGE loading dye (125 mM Tris, pH 6.8; 2% (w/v) 2-mercaptoethanol; 20% (v/v) glycerol; 0.001% (w/v) bromophenol blue; 4% (w/v) SDS), boiled for 5 minutes and resolved on a 15% SDS-PAGE with a pre-stained Benchmark Protein Ladder (Invitrogen). The proteins were visualized using Coomassie Blue dye.

2.5 Pilin antibody affinity purification

Pilin from group III strain Pa141123 and group V strain Pa110594 was isolated by the methods of Castric (11) as described above. Pellets were resuspended in PBS to a final concentration of 1 mg/mL, and 5 mL of each was sent to SickKids Hospital (Toronto, ON) for generation of rabbit polyclonal antibodies. Pilin antiserum was affinity purified against sheared surface pilin, from the same strain that was used to generate antibodies, modified from Salamitou (49). Briefly, pilin pellets (isolated as described above (11)) from group III strain Pa141123 and group V strain Pa110594 were resuspended in 1x SDS-PAGE loading dye, boiled for 5 minutes and resolved on a 15% SDS-PAGE with a pre-stained Benchmark Protein Ladder (Invitrogen). After separation,

proteins were transferred for 55 minutes at 225 mA to nitrocellulose, the membrane was stained with 0.1% Ponceau S to visualize proteins and the pilin band was excised. The nitrocellulose band was incubated for 1 h at room temperature in PBS containing 5% dried skim milk, incubated overnight at 4°C on a rocking platform with 4 mL of crude pilin antiserum corresponding to the same pilin group, then washed three times in PBS. The nitrocellulose band was cut into pieces and incubated in 1.05 mL of 0.2M HCl-glycine (pH 2.2) for 15 minutes to elute the antibodies, then neutralized in 450 μl of 1M K_2HPO_4. Purified antiserum was dialyzed overnight at 4°C against PBS using a 3mL Slide-A-Lyzer dialysis cassette (Pierce). Dialyzed serum was removed from cassette and added to 50% sterile filtered glycerol in a 1:1 ratio.

2.6 Western immunoblot analysis of pilins in whole cell lysates

After vortexing to remove surface proteins, the harvested cell pellet was resuspended in sterile PBS to a final optical density of 0.6 at 600 nm. A 200 μl aliquot of the cell suspension was transferred to a 1.5 ml microcentrofuge tube and the cells harvested by centrifugation at maximum speed for 5 min. The supernatant was removed and the cell pellet was resuspended in 150 μl 1X SDS-PAGE sample buffer, boiled for 5 min and 8 μl per sample was separated on a 15% SDS-PAGE minigel as above. After separation, the proteins were transferred for 55 minutes at 225mA to nitrocellulose for Western immunoblot analysis with affinity purified rabbit polyclonal antibodies raised against the PilA proteins from strains Pa141123 (group III) or Pa110594 (group V) (36). Blots were blocked with 5% skim milk in PBS overnight at 4°C, then incubated with a 1/5000 dilution of primary antibody for 60 min at room temperature, washed three times for 10 minutes with PBS and incubated with a 1/5000 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody for 60 min at room temperature. After

washing three times for 10 minutes, blots were developed with BCIP-NBT (Sigma) as per the manufacturer's instructions.

2.7 Mass spectrometry analysis

For mass spectrometry of group III and V pilins, pili were isolated using NaCl/PEG precipitation of sheared surface proteins as reported previously (57). Sixty Luria agar plates were used for each sample. Precipitated proteins were resuspended in 2 ml 50 mM ammonium bicarbonate buffer, pH 8.5, and stored at -20°C. Because the PEG was found to affect the mass spectrometry results, it was removed by adding 9 volumes of ice cold acetone to the resuspended pilins, vortexing 1 min and incubating at -20°C for 30 min. The proteins were collected by centrifugation for 20 min, 4°C at maximum speed in a microfuge. The acetone was removed and the pellet dried for 10 min using a speed vac. The dry pellet was resuspended in 10 µl formic acid, and once in solution, 90 µl of hexafluoro-2-propanol was added to completely solubilize the protein. An intact mass spectrum was obtained using a Q-TOF 2-hybrid quadrupole time of flight mass spectrometer (Waters). The pilin solution was infused at 1 µl /min into the electrospray ionization source. Protein mass spectra were recorded in the range of m/z 800 to 3000. The protein molecular weight profile was generated from the spectra using MaxEnt software (Waters).

2.8 Generation of a chimeric pilin gene cassette

The hybrid group V pilin-group III accessory gene construct was created using splicing by overlap extension (SOE) PCR (30). The *pilA*$_{III}$-*tfpY* and *pilA*$_{V}$-*tfpZ* constructs in pBADGr were used as templates to SOE *pilA*$_{V}$ to *tfpY*, allowing for the amplification of the MCS from pBADGr with the genes for subsequent cloning. PCR primers, forward pBADGr and reverse Pa281457 *pilA* + PA14 *tfpY* tail, were designed to amplify *pilA*$_{V}$

from *P. aeruginosa* Pa281457, leaving a 3' overhang complementary to the 5' end of *P. aeruginosa* PA14 *tfpY*; primers were cartridge-purified (Table 3B). PCR primers, forward PA14 *tfpY* + Pa281457 *pilA* tail and reverse pBADGr, were designed to amplify *tfpY* from *P. aeruginosa* PA14, leaving a 5' overhang complementary to the 3' end of *P. aeruginosa* Pa281457 *pilA*; primers were cartridge-purified. Total volumes were 50 μl consisting of 2 μl template DNA, 1 μl of SOE primer (0.125 ηM forward and 0.155 ηM reverse), 1 μl of pBADGr primer (0.186 ηM forward and 0.233 ηM reverse), 5 μl 10X PCR buffer, 10 μl Q solution (Qiagen), 0.5 μl 100 mM dNTPs and 1 μl Hot Star*Taq*. PCR consisted of a 15 min denaturation at 95°C, followed by 30 cycles of 45 s at 95°C 30s at 55°C and 2 min at 72°C, with a final extension of 7 min at 72°C, and ending at 4°C. Primers were synthesized by Mobix, Hamilton, ON.

PCR products were separated in a 1% agarose gel and purified using QIAquick Gel Purification Kit following the manufacturer's instructions (Qiagen), DNA was resuspended in 50 μl dH_2O. To SOE the two PCR products together, a PCR mixture consisting of 48 μl total containing 0.25 μl each of the two purified PCR products, 5 μl 10X PCR buffer, 10 μl Q solution (Qiagen), 0.5 μl 100mM dNTPs and 1 μl Hot Star*Taq* was made. One μl each of forward and reverse pBADGr primers was added to the PCR mixture after the third cycle of denaturation-annealing-extension to bring the total volume to 50 μl. PCR consisted of a 15 min denaturation at 95°C, followed by 3 cycles of 1 min at 95°C, 1 min at 50°C and 2.5 min at 72°C, addition of the primers, 27 more cycles of the same temperature/time, with a final extension of 7 min at 72°C, and ending at 4°C. The SOE product, *pilA*$_Y$*tfpY*, was gel purified (Qiagen) and DNA was resuspended in 30 μl dH_2O. The pBADGr vector and SOE product were each digested with *EcoRI* and *HindIII*, column purified (Qiagen) and ligated using standard methods. Constructs were transformed into *E. coli* DH5α as mentioned above. Colonies were patched, grown

overnight at 37°C and plasmids were isolated using QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen). After the sequence was confirmed using primers AY in pBADGr and SOE sequence in *pilA*$_V$, the construct was introduced into a *pilA* mutant of PAO1 (33) via electroporation, and transformants selected by plating on LB plates containing 30 mg/L gentamicin.

A 6xHis tag was added to the C-terminal end of TfpY in the chimera construct by PCR amplification using primers forward *pilA*$_V$ TOPO and reverse *tfpY* 6X-His TOPO (Table 3B). Total volumes were 50 μl consisting of 2 μl template DNA isolated using Instagene Matrix (BioRad), 1 μl of 0.064 ηM forward *pilA*$_V$ TOPO, 1 μl 0.079 ηM of reverse *tfpY* 6X-His TOPO, 5 μl 10X PCR buffer, 10 μl Q solution (Qiagen), 0.75 μl 100 mM dNTPs and 1 μl Hot Star*Taq*. PCR consisted of a 15 min denaturation at 95°C, followed by 30 cycles of 45 s at 95°C 45 s at 55°C and 3.5 min at 72°C, with a final extension of 7 min at 72°C, and ending at 4°C. The PCR product was ligated into pCR 2.1-TOPO vector (Invitrogen) using 4 μl of DNA, 1 μl salt solution and 1μl TOPO vector, then incubated at room temperature for 8 minutes and put on ice to stop the reaction. The TOPO vector containing the *pilA*$_V$*tfpY* SOE-6xHis construct was transformed into *E. coli* TOP10 cells (Invitrogen) by incubation on ice for 10 min, heat shocked at 42°C for 30 s, put back on ice for 2 min, incubated at 37°C for 1 h and plated on Amp100 containing 500 mg of X-Gal (OmniPur, EMD). Plasmids were isolated using QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen) and sequenced using M13 forward and M13 reverse (Invitrogen). The TOPO vector containing the *pilA*$_V$*tfpY* SOE-6xHis and the pBADGr vector were digested with *HindIII* and *BamHI* and ligated together using standard methods. The ligation was transformed into *E. coli* DH5α and selected for as described above for *E. coli* DH5α. Plasmids were isolated as

described above, introduced into a *pilA* mutant of PAO1 (33) via electroporation, and transformants selected by plating on LB plates containing 30 mg/L gentamicin.

2.9 Generation of a *tfpY* knockout mutant in PA14

A *tfpY* knockout mutant was created as described previously (57). Briefly, *tfpY* and flanking DNA was amplified by PCR using a forward primer (forward *pilB*, has an *EcoRI* restriction) in the upstream gene *pilB* and a reverse primer (reverse *nadC*, has an *XbaI* restriction site) in the downstream gene *nadC* (Table 3C). Total volumes were 50 μl consisting of 2 μl template DNA isolated using Instagene Matrix (BioRad), 1 μl of 0.17 ηM forward *pilB*, 1 μl of 0.11 ηM reverse *nadC*, 5 μl 10X PCR buffer, 10 μl Q solution (Qiagen), 0.75 μl 100mM dNTPs and 1 μl Hot Star*Taq*. PCR consisted of a 15 min denaturation at 95°C, followed by 30 cycles of 1 min at 95°C 1 min at 63°C and 5 min at 72°C, with a final extension of 7 min at 72°C, and ending at 4°C.

The resulting PCR product was cloned into a suicide plasmid, pEX18Ap (29), and a *SmaI* site was introduced into *tfpY* at nucleotide 115 by changing T117 to G by site-directed mutagenesis using forward *tfpY* 117T to G Sma1 and reverse *tfpY* 117T to G Sma1 (Quikchange, Stratagene). Total volumes were 50 μl consisting of 1 μl template DNA isolated using Instagene Matrix (BioRad), 1 μl of 5.9 ρM forward *tfpY* 117T to G *Sma1*, 1 μl of 3.6 ρM reverse *tfpY* 117T to G *Sma1*, 5μl 10X pfu buffer (Stratagene), 0.75 μl 100mM dNTPs and 1 μl pfu Turbo (Stratagene). PCR consisted of a 1 min denaturation at 95°C, followed by 18 cycles of 50 s at 95°C 50 s at 55°C and 30 min at 68°C, with a final extension of 7 min at 68°C, and ending at 4°C. After completion of PCR reaction, 1 μl of *DpnI* (Stratagene) was added, incubated in a 37°C water bath for 3 hours and the plasmid was transformed into *E. coli* DH5α as described above.

An FRT-flanked gentamicin resistance cassette was released from pPS856 (29) using *SmaI* and cloned into the suicide construct at the newly generated site using standard ligation methods. The resulting knockout construct was introduced into *E. coli* SM10 by electroporation. The knockout construct was then transferred to *P. aeruginosa* PA14 by biparental mating as described previously (9). After counter-selection of *E. coli* SM10 on *Pseudomonas* isolation agar containing 30 mg/L gentamicin, exconjugants were replica plated onto LB agar containing either 30 mg/L of gentamicin or 75 mg/L of piperacillin. Colonies growing on only gentamicin were transformed with pFLP2 to excise the resistance cassette (29). Transformants were plated on 5% sucrose plates to counterselect the pFLP2 plasmid, and then replica plated onto LB agar containing either 30 mg/L of gentamicin, 200 mg/L of carbenicillin or no antibiotic. The genotype of colonies growing only on LB agar were verified by PCR and DNA sequencing (Mobix Lab) to confirm the disruption of *tfpY* by a single FRT site using sequencing primers AY in pBADGr and AY1 in *pilA* (Table 3E).

2.10 Generation of *pilT* and *tfpY-pilT* double mutants in PA14

A *pilT* knockout construct generated by amplifying *pilT* and flanking DNA from the PAO1 chromosome using primers pilT1 and pilT2 (Table 3C) and cloning the product into *BamHI* and *HindIII* sites of pEX18Ap (29). A Gm-FRT cassette released from pPS856 (29) with SmaI was cloned into an NruI site within *pilT*. The resulting knockout construct was introduced into *E. coli* SM10 by electroporation, then transferred to *P. aeruginosa* PA14 wild-type and PA14 *tfpY*::FRT by biparental mating as described previously (9). The Gm cassette was removed by introduction of the pFLP2 plasmid followed by curing of the plasmid by sucrose counterselection (29). Genotypes of the PA14 *pilT* knockout and PA14 *tfpY-pilT* double knockout mutants were verified by PCR and DNA sequencing to confirm the disruption of *pilT*.

2.11 Insertion of a *phoA/lacZα* cassette into the C- and N-terminus of TfpY

ScaI sites were generated within the *pilA$_{III}$tfpY* construct at nucleotide 38 in the 5' end of *tfpY,* and in a separate construct at nucleotide 416 in the 3' end of *tfpY* by SOE PCR methods (30) with modifications; the 3' overhangs contained the *ScaI* restriction site, but the rest of the sequence was complementary to the template DNA. For generation of the 5' *ScaI* site, PCR primers forward AY TOPO and reverse Y N-terminal *ScaI* were designed to amplify *pilA$_{III}$* and the 5' end of *tfpY*, generating a *ScaI* site at nucleotide 38; primers were cartridge-purified (Table 3D). PCR primers forward Y N-terminal *ScaI* and reverse AY TOPO were designed to amplify *tfpY*, generating a *ScaI* site at nucleotide 38; primers were cartridge-purified (Table 3D). Two separate PCR reactions had a total volume of 50 μl consisting of 2 μl template DNA, 1 μl of Y N-terminal *ScaI* (0.045 ηM forward and 0.044 ηM reverse), 1 μl of AY TOPO primer (0.044 ηM forward and 0.053 ηM reverse), 5 μl 10X PCR buffer, 10 μl Q solution (Qiagen), 0.5 μl 100 mM dNTPs and 1 μl Hot Star*Taq*. PCR consisted of a 15 min denaturation at 95°C, followed by 30 cycles of 45 s at 95°C 30s at 55°C and 2.5 min at 72°C, with a final extension of 7 min at 72°C, and ending at 4°C. For generation of the *ScaI* site at the 3' end of *tfpY*, PCR primers forward Y C-terminal *ScaI* (0.044 ηM) and reverse AY TOPO (0.079 ηM) were designed to amplify the 3' end of *tfpY*, generating a *ScaI* site at nucleotide 416; primers were cartridge-purified (Table 3D). PCR primers, forward AY TOPO (0.064 ηM) and reverse Y C-terminal *ScaI* (0.053 ηM), were designed to amplify *pilA$_{III}$* and the 3' end of *tfpY*, generating a *ScaI* site at nucleotide 416; primers were cartridge-purified (Table 3D). PCR parameters and volumes were the same as the N-terminal *ScaI* reaction.

PCR products were separated, purified and SOEn together as described previously to SOE *pilA$_V$* to *tfpY*, using the same PCR volumes and parameters. The newly generated

pilA$_{III}$tfpY-N-terminal *ScaI* and *pilA$_{III}$tfpY*-C-terminal *ScaI* products were verified on a 1 % agarose gel for single bands, ligated into pCR 2.1-TOPO vector (Invitrogen) and transformed into *E. coli* TOP10 cells (Invitrogen) as previously described for TOPO ligation. Plasmids were isolated using QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen) and sequenced using M13 forward and M13 reverse (Invitrogen). The TOPO vector containing either the *pilA$_{III}$tfpY*-N-terminal *ScaI* or *pilA$_{III}$tfpY*-C-terminal *ScaI* and the pBADGr vector were digested with *HindIII* and *BamHI*, ligated together using standard methods, transformed into *E. coli* DH5α and selected for as described above for *E. coli* DH5α.

2.12 Generation of an N-terminal 6xHis tagged TfpY

A 6xHis tag was added to the N-terminus of TfpY by a combination of site-directed mutagenesis (Quikchange, Stratagene) and modified SOE PCR (30). Since the fifth residue of TfpY is histidine, PCR primers were designed to add additional histidines following this residue. Initial attempts to insert 6 histidine codons simultaneously by site-directed mutagenesis were not successful, therefore the codons were added in sequential reactions. PCR primers forward Y-3xHis and reverse Y-3xHis were used to add two His residues to the existing His in the *pilA$_{III}$tfpY* construct using site-directed mutagenesis (Quikchange, Stratagene). After the addition of the two His residues, PCR primers forward Y-4xHis and reverse Y-4xHis were used to add a fourth His residue to the *pilA$_{III}$tfpY*-3xHis construct. For both PCR reactions, a total of 50 μl consisting of 1 μl template DNA (*pilA$_{III}$tfpY* or *pilA$_{III}$tfpY*-3xHis), 1 μl of forward primer (8.99 ρM forward Y-3xHis or 7.0 ρM forward Y-4xHis), 1 μl of reverse primer (6.5 ρM reverse Y-3xHis or 8.0 ρM reverse Y-4xHis), 5 μl 10X pfu buffer (Stratagene), 0.75 μl 100mM dNTPs and 1 μl pfu Turbo (Stratagene) (Table 3G). PCR consisted of a 1 min denaturation at 95°C, followed by 18 cycles of 50 s at 95°C 50 s at 55°C and 30 min at 68°C, with a final

extension of 7 min at 68°C, and ending at 4°C. After completion of PCR reaction, 1 μl of *dpnI* (Stratagene) was added, incubated in a 37°C water bath for 3 hours and the vector was transformed into *E. coli* DH5α as described previously. Two additional His residues were added to the *pilA$_{III}$tfpY*-4xHis template using the same SOE PCR methods as described for the generation of *ScaI* sites in TfpY. PCR primers, forward AY TOPO (0.064 ηM) and reverse Y-6xHis (0.043 ηM), were designed to amplify *pilA$_{III}$* and the 5' end of *tfpY*, adding two His residues to the existing four His residues; primers were cartridge-purified (Table 3G). PCR primers, forward Y-6xHis (0.041 ηM) and reverse AY TOPO (0.079 ηM), were designed to amplify *tfpY*, adding two His residues to the existing four His residues; primers were cartridge-purified (Table 3G). PCR parameters and volumes were the same as the N-terminal *ScaI* reaction.

PCR products were separated, purified and SOEn together as described previously, using the same PCR solution volumes and parameters. The newly generated *pilA$_{III}$tfpY*-6xHis PCR product was verified on a 1 % agarose gel for a single band, ligated into pCR 2.1-TOPO vector (Invitrogen) and transformed into *E. coli* TOP10 cells (Invitrogen) as previously described for TOPO ligation. Plasmids were isolated using QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen) and sequenced using M13 forward and M13 reverse (Invitrogen). The TOPO vector containing *pilA$_{III}$tfpY*-6xHis and pBADGr were digested with *HindIII* and *BamHI*, ligated together using standard methods, transformed into *E. coli* DH5α and selected for as described above for *E. coli* DH5α. Plasmids were isolated as described above, introduced into a *pilA* mutant of PAO1 (33) via electroporation, and transformants selected by plating on LB plates containing 30 mg/L gentamicin.

2.13 Expression of TfpY in pET101/D-TOPO

The 3' half of *tfpY* (nucleotides 306-705) was cloned into the pET101/D-TOPO expression vector by TOPO directional cloning (Invitrogen). PCR primers, forward Y pET expression TOPO and reverse Y pET expression TOPO (Table 3F), were used to amplify the 3' half of *tfpY* for directional cloning; forward primer includes CACC on the 5' end and reverse primer includes an A on the 5' end, allowing for directional cloning of the PCR product into the linearized vector due to the complementary bases and topoisomerase (Invitrogen). The PCR product was verified on a 1 % agarose gel for a single band, ligated into pET101/D-TOPO vector (Invitrogen) and transformed into *E. coli* TOP10 cells (Invitrogen) as previously described for TOPO ligation. Plasmids were isolated using QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen) and sequenced using a T7 promoter sequencing primer (Mobix Lab, Table 3E). The expression construct and control plasmid (pET101/D-*lacZ*) were transformed into BL21 Star (DE3) One Shot cells (Invitrogen). To determine the best expression conditions, pilot expressions were performed with the following variations for the pET101/D-TOPO + *tfpY* construct: 27°C induced with 0.75 mM IPTG or 1.0 mM IPTG for 4 hr, 20 hr, or 28 hr, or at 37°C induced with 0.5 mM IPTG, 0.75 mM IPTG or 1.0 mM IPTG for either 4 hr or 20 hr. For all conditions, 5 mL of LB + 100 μg/mL ampicillin was inoculated with a single colony, grown at 37°C shaking at 225 rpm to an OD of 0.6, then inoculated with the appropriate amount of IPTG and grown shaking at the various temperatures and times. The control expression plasmid was tested with the same variations as the *tfpY* expression construct. After 4, 20 and 28 hours, 500 μl each sample was centrifuged at maximum speed for 30 s to pellet the cells. The supernatent was removed, cells were resuspended in 80 μl of 1X SDS-PAGE loading buffer and 10 μl of each sample was separated on a 15% SDS-PAGE. Samples that appeared to

have protein expression were re-run through a 15% SDS-PAGE, transferred to nitrocellulose membrane, incubated with a 1:3000 dilution of monoclonal αHis-penta and developed as described previously.

A scale-up protein expression and purification was performed using the conditions that showed the best expression in the pilot experiment. For expression, 45 mL of LB + 100 μg/mL ampicillin was inoculated with 5 mL of overnight culture, grown to OD 0.6, induced with 0.75 mM IPTG and grown shaking at 225 rpm for 4 hours at 37°C. After 4 hours of induction, cultures were centrifuged at 3000 g at 4°C for 30 min, supernatent was discarded and the pellet was resuspended in 2mL of 50 mM Tris lysis buffer (pH 8.0). Cells were lysed by French pressing twice at 800 psi (Thermo electron Corp). Lysed cells were centrifuged at 10000 rpm at 4°C for 130 mins to pellet cell debris. The supernatent was added to 100 μl of prepared Nickel-NTA resin (Qiagen) and the mixture was incubated at 4°C overnight on a Nutator (Clay Adams) to bind protein containing a histidine tag to the Ni-NTA beads; the pellet was resuspended in 100 μl of 1X SDS-PAGE loading buffer for later use. To prepare resin, 1 mL of 50 mM Tris lysis buffer (pH 8.0) was added to 100 μl of resin in a microcentrifuge tube, centrifuged for 5 min at 4500 rpm and the supernatant discarded; this was repeated three times. For protein purification, the protein-resin mixture was centrifuged at 4500 rpm for 5 minutes to pellet the beads bound to protein and the supernatent was removed and added in a 1:1 ratio to 2X SDS-PAGE loading buffer. The resin was washed three times with 50mM Tris wash buffer (pH 8.0 + 25 mM Imidazole) by centrifugation at 4500 rpm for 5 min at 4°C and the supernatant from each wash was added in a 1:1 ratio to 2X SDS-PAGE loading buffer. Protein was eluted from the beads by incubating resin with 50 μl of Tris elution buffer (pH 8.0 + 400 mM Imidazole) for 10 min at room temperature on a Nutator (Clay Adams), centrifuging at 4°C for 10 min at 4500 rpm and storing the

elution buffer containing the protein; this was repeated. The beads were added to 2X SDS-PAGE loading buffer in a 1:1 ratio and 20 μl of each elution was added to 20 μl of 2X SDS-PAGE loading buffer. The samples from each step of the purification were separated on a 15% SDS-PAGE, transferred to nitrocellulose membrane, incubated with a 1:500 dilution of monoclonal αHis-penta and developed as described previously.

Table 2. Strains and plasmids.

Strain or Plasmid	Relevant Characteristics	Source
E.coli		
SM10	*thi-1 thr leu tonA lacY supE recARP42-2-Tc^R::Mu, Km^R*	(60)
DH5α	F- *φ80dlacZM15 (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r k-, m k+) phoA supE44 thi-1 gyrA96 relA1 λ-*	Invitrogen
DH5α + *pilA$_{III}$tfpY* + N-terminal *phoA/LacZα*	DH5α complemented with PA14 *pilA$_{III}$* and *tfpY* genes in pBAGDr; contains a *phoA/LacZα* cassette in an N-terminal *ScaI* site at nucleotide 38 in *tfpY*	This study
DH5α + *pilA$_{III}$tfpY* + C-terminal *phoA/LacZα*	DH5α complemented with PA14 *pilA$_{III}$* and *tfpY* genes in pBAGDr; contains a *phoA/LacZα* cassette in a C-terminal *ScaI* site at nucleotide 416 in *tfpY*	This study
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen

BL21 Star (DE3) One Shot	F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3)	Invitrogen
BL21 Star (DE3) One Shot + pET101/D-TOPO + *tfpY*	The C-terminal half (nucleotides 305-705) of *tfpY* cloned into the TOPO site of pET101/D-TOPO	This study
P. aeruginosa		
1244	Group I T4P, used as a source for group I genes	(14)
PAO1	Group II T4P, used as a source for group III genes	(33)
PA14	Group III TFP, used as a source for group III genes	(47)
Pa5196	Group IV T4P, used as a source for group IV genes	(36)
Pa281457	Group V strain, used as source of group V genes	(36)
Pa141123	Group III strain, used as source of pilin for mass spectrometry and to generate polyclonal antisera	(36)
Pa110594	Group V strain, used as source of pilin to generate polyclonal antisera	(36)
Pa5235	Group V strain, used as source of pilin for mass spectrometry	(36)
PAO1 NP	Transposon mutant 30458, Tn5 *ISphoA*/hah insertion in *pilA* at nucleotide 165; has **no** **p**ili (abbreviated NP)	(33)
PAO1 NP + $pilA_{II}$	The NP mutant complemented with the PAO1 $pilA_{II}$ gene in pBADGr	This study
PAO1 NP + $pilA_{I}$	The NP mutant complemented with the strain 1244 $pilA_{I}$ in pBADGr	This study
PAO1 NP + $pilA_{I}$-*tfpO*	The NP mutant complemented with the strain 1244 $pilA_{I}$ and *tfpO* genes in pBADGr	This study
PAO1 NP + $pilA_{IV}$	The NP mutant complemented with the strain Pa5196	This study

	pilA$_{IV}$ gene in pBADGr	
PAO1 NP + *pilA*$_{IV}$-*tfpX*	The NP mutant complemented with the Pa5196*pilA*$_{IV}$ and *tfpX* genes in pBADGr	This study
PAO1 NP + *pilA*$_{III}$	The NP mutant complemented with the strain PA14 *pilA*$_{III}$ gene in pBADGr	This study
PAO1 NP + *pilA*$_{III}$-*tfpY*	The NP mutant complemented with the strain PA14 *pilA*$_{III}$ and *tfpY* genes in pBADGr	This study
PAO1 NP + *pilA*$_{III}$-*tfpY*#	The NP mutant complemented with the strain PA14 *pilA*$_{III}$ and *tfpY* genes in pBADGr; a pre-mature stop codon at Leu59 in TfpY denoted as #	This study
PAO1 NP + *pilA*$_{V}$	The NP mutant complemented with the strain Pa281457 *pilA*$_{V}$ gene in pBADGr	This study
PAO1 NP + *pilA*$_{V}$-*tfpZ*	The NP mutant complemented with the strain Pa281457 *pilA*$_{V}$ and *tfpZ* genes in pBADGr	This study
PAO1 NP + *pilA*$_{V}$-*tfpZ*#	The NP mutant complemented with the strain Pa281457 *pilA*$_{V}$ and *tfpZ* genes in pBADGr; a premature stop codon at Leu58 in *tfpZ* denoted as #	This study
PAO1 NP *pilT::FRT*	The NP mutant with an FRT disruption in the *pilT* gene	Harvey et al., in revision
PAO1 NP *pilT*::FRT + *pilA*$_{III}$	The NP-*pilT* double mutant complemented with the strain PA14 *pilA*$_{III}$ gene in pBADGr	This study
PAO1 NP *pilT*::FRT + *pilA*$_{III}$-*tfpY*	The NP-*pilT* double mutant complemented with the strain PA14 *pilA*$_{III}$ and *tfpY* genes in pBADGr	This study
PAO1 NP *pilT*::FRT + *pilA*$_{III}$-*tfpY*#	The NP-*pilT* double mutant complemented with the strain PA14 *pilA*$_{III}$ and *tfpY* genes in pBADGr; a pre-mature stop codon at Leu59 in *tfpY* denoted as #	This study
PAO1 NP *pilT*::FRT +	The NP-*pilT* double mutant complemented with the	This study

pilA$_V$	strain Pa281457 *pilA$_V$* gene in pBADGr	
PAO1 NP pilT::FRT + *pilA$_V$-tfpZ*	The NP-*pilT* double mutant complemented with with the strain Pa281457 *pilA$_V$* and *tfpZ* genes in pBADGr	This study
PAO1 NP pilT::FRT + *pilA$_V$-tfpZ#*	The NP-pilT double mutant complemented with the strain Pa281457 *pilA$_V$* and *tfpZ* genes in pBADGr; a premature stop codon at Leu58 in *tfpZ* denoted as #	This study
PAO1 NP + *pilA$_V$tfpY*	The NP strain complemented with the strain Pa281457 *pilA$_V$* gene and the *tfpY* gene from group III strain PA14 in pBADGr	This study
PAO1 NP + *pilA$_V$tfpY*-6xHis	The NP strain complemented with the strain Pa281457 *pilA$_V$* gene and the *tfpY* gene from group III strain in pBADGr; a 6xHis tag was added to the C-terminus of *tfpY*	This study
PAO1 NP + *pilA$_{III}$tfpY*-4xHis	The NP strain complemented with the strain PA14 *pilA$_{III}$* and *tfpY* genes in pBADGr; includes a 4xHis tag at the N-terminus of TfpY	This study
PAO1 NP + *pilA$_{III}$tfpY*-6xHis	The NP strain complemented with the strain PA14 *pilA$_{III}$* and *tfpY* genes in pBADGr; includes a 6xHis tag at the N-terminus of TfpY	This study
PAO1 NP + *pilA$_{III}$tfpY*-221# + 6xHis	The NP strain complemented with the strain PA14 *pilA$_{III}$* and *tfpY* genes in pBADGr; includes a 6xHis tag at the N-terminus of TfpY and a premature stop codon (#) at Ala 221	This study
PAO1 NP + *pilA$_{III}$tfpY*-4xHis + weakened transcriptional terminator	The NP strain complemented with the strain PA14 *pilA$_{III}$* and *tfpY* genes in pBADGr; includes a 4xHis tag at the N-terminus of TfpY and a weakened transcriptional terminator between *pilA$_{III}$* and *tfpY* by changing GCCCCTC to CGGGCAG	This study

PA14 + pBADGr	The PA14 strain carrying the pBADGr vector	This study
PA14 + $pilA_{III}$	The PA14 strain complemented with the PA14 $pilA_{III}$ gene in pBADGr	This study
PA14 + $pilA_{III}$-*tfpY*	The PA14 strain complemented with the PA14 $pilA_{III}$ and *tfpY* genes in pBADGr	This study
PA14 *tfpY*::FRT	The PA14 strain with an FRT disruption in the *tfpY* gene	This study
PA14 *pilT*::FRT	The PA14 strain with an FRT disruption in the *pilT* gene	This study
PA14 *tfpY*::FRT *pilT*::FRT	The PA14 strain with FRT disruptions in the *tfpY* and pilT genes	This study
PA14 *tfpY*::FRT + pBADGr	The PA14 strain carrying the pBADGr vector	This study
PA14 *tfpY*::FRT + $pilA_{III}$	The PA14 *tfpY* mutant complemented with the PA14 $pilA_{III}$ gene cloned into pBADGr as above	This study
PA14 *tfpY*::FRT + $pilA_{III}$-*tfpY*	The PA14 *tfpY* mutant complemented with the PA14 $pilA_{III}$ and *tfpY* genes cloned into pBADGr	This study
Plasmids		
pEX18Ap	Ap^R; *oriT*+ *sacB*+	(29)
pEX18Ap + *PA14pilB-pilA-tfpY-nadC*	3' end of *pilB, pilA, tfpY,* 5'end of *nadC* amplified from PA14 chromosomal DNA, digested with EcoRI and XbaI and ligated into the vector's EcoRI and XbaI sites	This study
pEX18Ap + PA14 *pilB-pilAtfpYnadC* –SmaI	SDM of base T117G to create a SmaI site in *tfpY*	This study
pEX18Ap + PA14	Gm-FRT insertion in SmaI site of *tfpY*	This study

pilBpilAtfpYnadC – GmFRT		
pEX18Ap + *pilT::GmFRT*	Gm-FRT insertion in NruI site within *pilT*	Harvey et al., in revision
pPS856	Source of the FRT-flanked gentamicin resistance cassette (*aacC1*) used to disrupt *tfpY* and *pilT*	(29)
pFLP2	Source of the Flp recombinase used to excise the FRT-flanked gentamicin resistance cassette following mutagenesis	(29)
pBADGr	*ori, araC-P_{BAD} dhfr::Gm^r mob^+*	Harvey et al., in revision
pBADGr + *$pilA_I$*	*$pilA_I$* from strain 1244 cloned into the EcoRI and HindIII sites as indicated in the methods	This study
pBADGr + *$pilA_{II}$*	*$pilA_{II}$* from strain PAO1 cloned as above	This study
pBADGr + $pilA_{IV}$	*$pilA_{IV}$* from strain Pa5196 cloned as above	This study
pBADGr + *$pilA_{III}$*	*$pilA_{III}$* from strain PA14 cloned as above	This study
pBADGr + *$pilA_{III}$-tfpY*	*$pilA_{III}$* and *tfpY* from PA14 cloned as above	This study
pBADGr + *$pilA_{III}$-tfpY#*	*$pilA_{III}$* and *tfpY* from PA14 cloned as above; with a pre-mature stop codon at Leu59, denoted as #	This study
pBADGr + *$pilA_{III}$-tfpY*-N-terminal *ScaI*	*$pilA_{III}$* and *tfpY* from PA14; contains an N-terminal *ScaI* site inserted at nucleotide 38 and cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	This study
pBADGr + *$pilA_{III}$-tfpY*-C-terminal *ScaI*	*$pilA_{III}$* and *tfpY* from PA14; contains a C-terminal *ScaI* site inserted at nucleotide 416 and cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	This study

pBADGr + *pilA*$_{III}$-*tfpY*-4xHis	*pilA*$_{III}$ and *tfpY* from PA14; contains a 4xHis tag at the N-terminus of TfpY cloned into the *EcoRI* and *HindIII* sites of pBADGr	This study
pBADGr + *pilA*$_{III}$-*tfpY*-4xHis + weakened transcriptional terminator	*pilA*$_{III}$ and *tfpY* from PA14; contains a 4xHis tag at the N-terminus of TfpY and mutations in the transcriptional terminator of nucleotides GCCCCTC to CGGGCAG cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	This study
pBADGr + *pilA*$_{III}$-*tfpY*-6xHis	*pilA*$_{III}$ and *tfpY* from PA14; contains a 6xHis tag at the N-terminus of TfpY cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	This study
pBADGr + *pilA*$_{III}$-*tfpY*-6xHis + 221#	*pilA*$_{III}$ and *tfpY* from PA14; contains a 6xHis tag at the N-terminus of TfpY and a pre-mature stop codon (#) at Ala 221 cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	
pBADGr + *pilA*$_{V}$	*pilA*$_{V}$ from Pa281457 cloned as above	This study
pBADGr + *pilA*$_{V}$-*tfpZ*	*pilA*$_{V}$ and *tfpZ* from Pa281457 cloned as above	This study
pBADGr + *pilA*$_{V}$-*tfpZ*#	*pilA*$_{V}$ and *tfpZ* from Pa281457 cloned as above; with a pre-mature stop codon at Leu58, denoted as #	This study
pBADGr + *pilA*$_{V}$-*tfpY*	*pilA*$_{V}$ from strain Pa281457 and *tfpY* from group III strain PA14 cloned together by SOE PCR (30) into the *EcoRI* and *HindIII* sites of pBADGr	This study
pBADGr + *pilA*$_{V}$-*tfpY*-6xHis	*pilA*$_{V}$ from strain Pa281457 and *tfpY* from group III strain PA14 cloned into the *XbaI* and *HindIII* sites of pBADGr; contains a 6xHis tag at the C-terminus of TfpY added by PCR amplification as indicated in the methods	This study
pMA632	Bla^{R}, R6Kori, *phoA* + *lacZα*	(1)

pCR® 2.1-TOPO®	Am^R, Km^R, pUC ori, f1 ori, LacZ α multilple cloning site, M13 priming site (not sure how much info to put here)	Invitrogen
pCR® 2.1-TOPO® + *pilA*$_V$-*tfpY*-6xHis	*pilA*$_V$ from strain Pa281457 and *tfpY* from group III strain PA14 cloned into the *XbaI* and *HindIII* sites of pBADGr; contains a 6xHis tag at the C-terminus of TfpY added by PCR amplification as indicated in the methods	This study
pCR® 2.1-TOPO® + *pilA*$_{III}$-*tfpY*-N-terminal *ScaI*	*pilA*$_{III}$ and *tfpY* from PA14; contains an N-terminal *ScaI* site inserted at nucleotide 38 by SOE PCR (30) as indicated in the methods	This study
pCR® 2.1-TOPO® + *pilA*$_{III}$-*tfpY*-C-terminal *ScaI*	*pilA*$_{III}$ and *tfpY* from PA14; contains a C-terminal *ScaI* site inserted at nucleotide 416 inserted by SOE PCR (30) as indicated in the methods	This study
pCR® 2.1-TOPO® + *pilA*$_{III}$-*tfpY*-4xHis + weakened transcriptional terminator	*pilA*$_{III}$ and *tfpY* from PA14; contains a 4xHis tag at the N-terminus of TfpY and mutations in the transcriptional terminator of nucleotides GCCCCTC to CGGGCAG inserted by SOE PCR (30) as indicated in the methods	This study
pCR® 2.1-TOPO® + *pilA*$_{III}$-*tfpY*-6xHis	*pilA*$_{III}$ and *tfpY* from PA14; contains a 6xHis tag at the N-terminus of TfpY inserted by SOE PCR (30) as indicated in the methods	This study
pCR® 2.1-TOPO® + *pilA*$_{III}$-*tfpY*-6xHis + 221#	*pilA*$_{III}$ and *tfpY* from PA14; contains a 6xHis tag at the N-terminus of TfpY and a pre-mature stop codon (#) at Ala 221 cloned into the *XbaI* and *HindIII* sites of pBADGr as indicated in the methods	This study
pET101/D-TOPO®	Am^R, T7 promoter/terminator, pBR322 origin, 6xHis, V5 epitope	Invitrogen
pET101/D-TOPO® +	Nucleotides 306-705 from *tfpY* (C-terminal periplasmic	This study

tfpY	region) cloned into the TOPO directional cloning site

Table 3. List of primers. All primers were synthesized by Mobix Lab unless otherwise indicated

A) Primers for cloning group III, IV and V pilins into pBADGr with and without their accessory proteins. Primers included restriction sites *EcoRI* or *HindIII* for cloning.

Primer	Sequence
forward $pilA_I$	5' AAGAATTCATGAAAGCTCAGAAGGGT
forward $pilA_{II}$, $pilA_{III}$, $pilA_V$	5' AAGAATTCATGAAAGCTCAAAAAGGC
forward $pilA_{IV}$	5' AAGAATTCATGAAAGCGCAAAAAGGC
reverse $pilA_I$	5' AGAAGCTTCAAAACAACTCAAAAAACC
reverse $pilA_{III}$	5' AGAAGCTTGCCATCCTCCTGCTATTC
reverse $pilA_{IV}$	5' AGAAGCTTAAAAAGAGACAAGCCCCGCA
reverse *tfpW*	5' AGAAGCTTATACTGGAAAAAAGAAGATG
reverse $pilA_V$	5' AGAAGCTTCTCACAACTTTCCGTCTTTT
reverse $tRNA^{Thr}$	5' AAAAAGCTTCGAATGAGCTGCTCTACCGACAGAGCT

B) Primers used to make SOE $pilA_V tfpY$ chimera and 6X-His tagged chimera.

Primer	Sequence
forward pBADGr	5' TCTCTACTGTTTCTCCATACCCG
reverse pBADGr	5' CGGCATGGGGTCAGGTGGGA
forward PA14 *tfpY* +	5' AGTTTGCTCCTGCTCAGTGCCGCTAATAGGCTAATGCTGAAA

Pa281457 *pilA* tail	AGAGCCCCTC
reverse Pa281457 *pilA* +	5' GAGGGGCTCTTTTCAGCATTAGCCTATTAGCGGCACTGAGCA
PA14 *tfpY* tail	GGAGCAAACT
forward $pilA_V$ TOPO	5' AGGAGGAGACTGATGAAAGCTCAAAAAGGCTTTACCTTGAT
	CGAGCTGA
reverse *tfpY* 6X-His	5' TCAGTGGTGGTGGTGGTGGTGAGTCGCAGCATCACTCTTG
TOPO	

C) Primers used to make knockouts. Primers included restriction sites *EcoRI*, *XbaI*, *BamHI* and *HindIII* for cloning.

Primer	Sequence
forward *pilB*	5' AAAGAATTCGGCTGGATCGGAGATGCCGACGAACAG
reverse *nadC*	5' AAATCTAGAACCGCCACCCGCAGCCAGCACTACG
forward *tfpY* 117T	5' GGCAATCATCTGTTTGTGGTGGTATCCCGGGGCTTTGTTCGAAATAG
to G Sma1	CAGGAGGATGGCA
reverse *tfpY* 117T	5' TGCCATCCTCCTGCTATTTCGAACAAAGCCCCGGGATACCACCACAA
to G Sma1	ACAGATGATTGCC
pilT1	5' GGATCCGGTGTTTTCCTTGTCCGA
pilT2	5' **AAGCTT**GAATCCTAGACGCAGTTCC

D) Primers for Topology.

Primer	Sequence
forward Y N-terminal	5'
ScaI	TGCATTTCGCTGGAAGTCGTTGGAGAGTACTTTCTATCCATCTACT
	CATTAGTCT
reverse Y N-terminal	5' AGACTAATGAGTAGATGGATAGAAAGTACTCTCCAACGACTTCCA
ScaI	GCGAAATGCA

forward Y C-terminal *ScaI*	5' TCTTCTGAAGAGTTGATGAGGTTTAAAGTACTTTCTCCTGAGTATTTTTATATCGACTTG
reverse Y C-terminal *ScaI*	5' CAAGTCGATATAAAAATACTCAGGAGAAAGTACTTTAAACCTCATCAACTCTTCAGAAGA
forward AY TOPO	5' AGGAGGAGAATAATGAAAGCTCAGAAAGGC
reverse AY TOPO	5' TCAAGTCGCAGCATCACTCTTGCGGA

E) Sequencing Primers

Primer	Sequence
AY knockout (*pilB*)	5' CTGTACCAGATAGGTTACCAGCGA
AY in pBADGr	5' CGGCAGAAAAGGGCGTCACAC
AY1 in *pilA*	5' GCGAATTGCAGAGGCGACTGGT
AY2 in *tfpY*	5' TGCGCATCGTTGCCGCTGTCG
SOE sequence in $pilA_V$	5' TGCCGACTGCCGGTAACACTC
M13 forward (Invitrogen)	5' GTAAAACGACGGCCAG
M13 reverse (Invitrogen)	5' CAGGAAACAGCTATGAC
T7 promoter	5' TAATACGACTCACTATAGGG

F) Primers for making TfpY expression construct.

Primer	Sequence
forward Y pET expression TOPO	5' CACCATGGCGAGGCCGCTTGCTCTTGTTCA
reverse Y pET expression TOPO	5' AGTCGCAGCATCACTCTTGCGGAAGTCAAAGAA

G) Primers for extra/future work.

Primer	Sequence
forward Y-HisX3	5' GGGAGAGTGGTTATAGTGCATCACCACTTCGCTGGAAGTCGTTG GAGA
reverse Y-HisX3	5' TCTCCAACGACTTCCAGCGAAGTGGTGATGCACTATAACCACTCT CCC
forward Y-HisX4	5' GGGAGAGTGGTTATAGTGCATCACCACCACTTCGCTGGAAGTCGT TGGAGA
reverse Y-HisX4	5' TCTCCAACGACTTCCAGCGAAGTGGTGGTGATGCACTATAACCAC TCTCCC
forward Y-HisX6	5' AGAGTGGTTATAGTGCATCACCACCACCACCACTTCGCTGGAAGT CGTTGGAGAGCCTTT
reverse Y-HisX6	5' AAAGGCTCTCCAACGACTTCCAGCGAAGTGGTGGTGGTGGTGATG CACTATAACCACTCT
forward Y-terminator mutation	5' CTAATAGGCTAATGCTGAAAAGACGGGCAGCTAAGAGGGGCTTTT TTTATG
reverse Y-terminator mutation	5' CATAAAAAAAGCCCCTCTTAGCTGCCCGTCTTTTCAGCATTAGCCT ATTAG
forward Y-221#	5' GAACGATCTGTTTCGATAAATGACGCCGTTTTTTCTTTGACTT
reverse Y-221#	5' AAGTCAAAGAAAAAACGGCGTCATTTATCGAAACAGATCGTTC
forward AY TOPO	5' AGGAGGAGAATAATGAAAGCTCAGAAAGGC
reverse AY TOPO	5' TCAAGTCGCAGCATCACTCTTGCGGA

3.0 RESULTS

3.1 Expression of *P. aeruginosa pilA* alleles in a PAO1 *pilA* mutant background

To examine potential functional differences among the five phylogenetically distinct *pilA* alleles in *P. aeruginosa*, as well as to test possible contributions of the accessory genes to pilus function, we expressed the genes in a single genetic background. PAO1 is the most widely used and well-characterized laboratory strain of *P. aeruginosa*, therefore we used a PAO1 *pilA* mutant (designated PAO1 NP, for no pili) as a host. As described in the Methods, representative *pilA* genes from each group, either alone or in combination with their respective accessory genes, were cloned into pBADGr, an L-arabinose-inducible broad host-range plasmid, and introduced into the NP strain. The level of L-arabinose supplementation required to restore wild type twitching motility using the homologous group II PAO1 *pilA* gene ($PilA_{II}$) was determined to be 0.2% (w/v; Figure 2) and this concentration was used for complementation experiments.

3.2 Complementation of twitching motility by *P. aeruginosa pilA* and accessory genes

The ability of each of the five pilin alleles (designated with subscripts) to restore twitching motility to the PAO1 NP strain was tested. The $pilA_I$ and $pilA_{II}$ genes restored twitching motility to wild type levels while $pilA_{III}$ and $pilA_V$ only partially restored motility (Figure 2). $pilA_{IV}$ was unable to restore motility to the NP strain when 0.2% L-arabinose was used to induce expression, but did restore motility to the same extent as the cognate PAO1 gene when 0.01% L-arabinose was used. When the *pilA* genes were introduced with their cognate accessory genes, twitching motility was altered. Provision of $pilA_I$ together with *tfpO* caused a slight decrease in twitching motility compared with

pilA$_I$ alone (Figure 2); similar results were reported previously by Smedley and colleagues (54). Co-introduction of *pilA*$_{III}$ with *tfpY*, *pilA*$_{IV}$ with *tfpX*, or *pilA*$_V$ with *tfpZ* increased twitching motility compared to complementation with the *pilA* genes alone (Figure 2). These data suggest that the pilin accessory genes *tfpX*, *tfpY* and *tfpZ* affect pilus function. Because the group IV pilin behaved aberrantly with respect to the response to arabinose induction compared with pilins of the other 4 groups, further studies focused on the group III and V pilins.

3.3 Mass spectrometry analysis of PilA$_{III}$ and PilA$_V$

The enhancement of twitching motility in the PAO1 background in the presence of TfpY and TfpZ implied that the accessory proteins could play a role in pilin modification, assembly or function. We noted previously that the group III and group V pilins migrate more rapidly on SDS-PAGE gels than would be expected based on their predicted masses. To determine whether these proteins were post-translationally modified, pilins purified from the wild type strains 87141123 (group III) and 5235 (group V) were analyzed by mass spectrometry; these strains were selected because they are representative of their respective groups (36) and produce substantial amounts of surface pili which facilitated preparation of material for analysis. Figure 3 shows the intact mass spectra for these pilins; both have a mass corresponding to that predicted from their amino acid sequences, showing that they are not post-translationally modified. Therefore, unlike the TfpO or TfpW accessory proteins, the function of the TfpY and TfpZ proteins does not appear to be modification of their cognate pilins.

3.4 TfpY and TfpZ increase levels of recoverable surface pili

Motility of recombinant strains expressing group III and V pilins was increased in the presence of the cognate accessory genes; to confirm this result, the

complementation constructs containing *pilA*$_{III}$-*tfpY* and *pilA*$_{V}$-*tfpZ* were mutagenized to introduce a premature stop codon into each of the accessory genes. Twitching motility of the PAO1 NP mutant complemented with the pilin genes only, the pilin and accessory genes, or the pilin and mutant accessory genes (denoted with a # sign) was tested. Inactivation of *tfpY* and *tfpZ* caused a decrease in twitching motility to levels conferred by complementation with *pilA*$_{III}$ or *pilA*$_{V}$ alone, confirming that the accessory gene products are responsible for the observed increases in motility (Figure 4).

TfpY and TfpZ could enhance twitching motility via increased assembly of pili on the cell surface, or by modulating total cellular pilin levels. To distinguish between these possibilities, we compared the levels of sheared surface pili and whole cell pilin levels among the recombinant strains. Figure 5 shows that each of the complemented strains has similar levels of pilins in whole cell lysates, but strains lacking the accessory genes (either by deletion or disruption via premature stop codon) had fewer recoverable pili on the cell surface. This result suggests that the impaired twitching motility of strains lacking functional pilin accessory proteins is due to decreased surface pili levels.

3.5 Conservation of other components of the T4P system among *P. aeruginosa* strains with group I-IV pilin alleles

The inability of group III and V pilins to restore twitching motility to wild type levels in the PAO1 background was unexpected, since these genes were sourced directly from other *P. aeruginosa* strains, and this organism has been previously been used to express heterologous pilins from other bacteria (22, 31, 58). Since reduced twitching motility in the group II background strain PAO1 appeared to arise from decreased surface pili and therefore potential assembly defects, we asked whether other components involved in pilus assembly were conserved among *P. aeruginosa* strains. We first analyzed the currently available genome sequences of *P. aeruginosa* for the

characteristic accessory genes associated with each pilin allele, in order to determine the pilin type of each strain (36). There are two genomes each of strains with group I (LES, 2192), group II (PAO1, PACS2) and group III (PA14, C3719) pilins, and one group IV pilin strain (PA7) currently available. No group V pilin gene-containing strain has yet been sequenced. After identifying the pilin allele, we performed BLASTp or tBLASTn searches using the amino acid sequences of pilus assembly proteins from PAO1 as *in silico* probes for each of the other genomes. A summary of this analysis is shown in Table 4. The major components of the *P. aeruginosa* T4P system, including the prepilin peptidase PilD, the membrane protein PilC, the motor proteins PilB, PilT and PilU and the assembly factors PilM, PilN, PilO, PilP, PilQ, PilF and FimV are highly conserved (greater than 90% amino acid similarity). Interestingly, the minor pilins FimT, FimU, PilV, PilW, PilX, PilY1, PilY2 and PilE are essentially identical between strains carrying group I and group II pilin genes, while these gene products are less similar to the PAO1 lab strain in PA7, a strain carrying a group IV pilin, and even further divergent from PAO1 in strains that have group III pilins (Table 4). However, the predicted minor pilin gene products in the two group III strains sequenced to date (PA14 and C3719) are identical.

3.6 Expression of heterologous pilins in a retraction-deficient background

In *Neisseria*, it has been shown previously that decreased surface piliation in some mutants can arise from alterations in the dynamics of pilus retraction mediated by the PilT retraction ATPase. This phenotype can be distinguished from one in which there is a complete inability to assemble pili by inactivating PilT and testing for the restoration of surface piliation (10, 64, 65). To determine if the decrease in surface piliation seen in recombinant *P. aeruginosa* strains expressing group III or group V pilins was due to altered retraction dynamics, we introduced the constructs into a PAO1 *pilA-pilT* double mutant and examined surface pilus expression (Figure 6). In this

background, all of the strains expressed equally high levels of surface pili, showing that the observed reduction in motility and surface piliation in the *pilT*-replete background is due to altered pilus retraction dynamics. The pilins isolated from the double mutant strains were identical in mass regardless of the presence of the accessory proteins, providing additional evidence that the accessory proteins do not post-translationally modify their associated pilins in either the native or recombinant backgrounds.

3.7 Analysis of a *tfpY* knockout mutant in PA14

Since the above analyses examined the function of the accessory genes in recombinant strains, it was important to examine their role in the native background. A *tfpY* knockout mutant of group III strain PA14 was analyzed for twitching motility, surface pili and whole cell pilin levels. Phenotypes of the knockout mutant recapitulated those observed in the recombinant strains; twitching motility was decreased and the level of recoverable surface pili was reduced compared with the wild type, but the amount of pilin in the whole cell lysates was similar to the PA14 wild-type strain (Figure 7E). Complementation of the *tfpY* mutant with the $pilA_{III}tfpY$ construct restored surface pilin levels to that of the wild type complemented with the same construct. Interestingly, complementation of the *tfpY* mutant with $pilA_{III}$ expressed from the arabinose-inducible promoter increased motility compared with the *tfpY* mutant that has only a single chromosomal copy of *pilA* (Figure 7), though not to wild type levels, even though the amount of recovered surface pilin was similar. These results show that over-expression of $PilA_{III}$ in either the recombinant or mutant strains lacking TfpY is not sufficient to restore motility to wild type levels, and that TfpY is required for normal twitching motility and surface piliation in PA14. To ascertain whether pilus retraction dynamics were affected in the PA14 *tfpY* mutant, a *tfpY-pilT* double mutant was generated. The double mutant expressed large amounts of recoverable surface pili, similar to a PA14 *pilT* single

mutant (Figure 7D, lanes 1 and 2) and substantially more than the *tfpY* single mutant (Figure 7D, lane 4), confirming that TfpY antagonizes PilT-mediated pilus retraction in the PA14 background.

3.8 The accessory proteins are specific for their cognate pilins

In all cases that we have examined to date ((36) and L.L. Burrows, unpublished data), the accessory genes are invariantly associated with a specific pilin allele (ie. TfpX with $PilA_{IV}$, TfpY with $PilA_{III}$ and TfpZ with $PilA_{V}$). To determine whether the accessory proteins are functionally interchangeable, a chimera containing the pilin gene from the group V strain Pa281457 and the accessory gene *tfpY* from the group III strain PA14 was examined for its ability to complement twitching motility and surface piliation in the PAO1 NP background. Although the PAO1 NP + *pilA*$_V$-*tfpY* strain was able to twitch, the size of the twitching zone was similar to that of the strain complemented with *pilA*$_V$ alone (Figure 8). Although the chimera expressed similar levels of pilin in whole cell lysates to strains expressing *pilA*$_V$ alone or with *tfpZ*, the level of recoverable surface pili was similar to the strain lacking the accessory protein (Figure 8). These data suggest that the group III accessory protein TfpY is incompatible with the group V pilin, therefore the accessory proteins are specific for their cognate pilins.

A 6xHis tag was added to C-terminal end of TfpY by PCR amplification and cloning in to pBADGr (see Methods) to test whether or not the phenotype we observed from the SOE recombinant strain was due to the inability of TfpY to complement the function of TfpZ, or if TfpY is not being correctly expressed because it is not in its native operon. Twitching motility was tested and showed no change from the untagged SOE recombinant strain; however, TfpY was not detected in blots of whole cell lysates using an α-His antibody in PAO1 NP at concentrations as high as 1:500. This may be a result of the low amount of *tfpY* expression in the cell, due to a predicted transcriptional

terminator located between the highly expressed *pilA* gene and the *tfpY* gene. Since *tfpY* expression is under the control of the *pilA* promoter, it is likely expressed at lower levels compared to *pilA* due to the presence of the transcriptional terminator. As an alternative, whole cell lysates of *E. coli* strain DH5α containing the SOE TfpY-6X-His were probed with the α-His antibody at the same concentrations to see if the protein could be detected in *E. coli*. Very faint bands corresponding to the appropriate size of TfpY could be detected and were absent in the negative control; a His-tagged protein included as a positive control was readily detected. The putative TfpY band was too faint for adequate electronic reproduction. These data suggest that TfpY is expressed from the SOE construct but the amount of protein is exceedingly low.

Table 4. Conservation of type IV pilus assembly proteins among *P. aeruginosa* strains

	Pseudomonas aeruginosa strain							Reference
	2192	LES	PAO1	PACS2	PA14	C3719	PA7	
pilA allele	Group I	Group I	Group II	Group II	Group III	Group III	Group IV	This study
Accession number	AAKW00000000	Sanger	AE004091	AAQW00000000	CP000438	AAKV00000000	AAQE00000000	
Accessory protein:[a]								
TfpO	+	+	-	-	-	-	-	(11)
TfpWX	-	-	-	-	-	-	+	(36)
TfpY	-	-	-	-	+	+	-	(36)
TfpZ	-	-	-	-	-	-	-	(36)
Assembly protein[b]								
PilA	53	60	100	75	45	45	47	(36)
PilB	94	94	100	98	96	96	94	(44)
PilC	93	93	100	98	94	95	94	(44)
PilD	99	99	100	99	98	99	97	(44)
PilT	100	100	100	100	100	100	100	(16)
PilU	100	100	100	100	100	100	100	(61)
PilM	100	100	100	100	100	100	100	(41)
PilN	100	100	100	100	96	95	100	(41)
PilO	100	100	100	100	97	97	99	(41)

PilP	100	100	100	100	96	96	99	(41)
PilQ	98	98	100	99	96	97	98	(41)
PilF	99	99	100	99	99	99	97	(59)
FimV	100	99	100	99	98	99	94	(52)
PilZ	100	100	100	100	100	100	100	(5)
FimT	99	99	100	99	49	49	81	(2)
FimU	100	100	100	100	65	65	94	(2)
PilV	100	100	100	100	75	75	93	(3)
PilW	98	100	100	99	68	68	93	(4)
PilX	100	100	100	100	75	75	94	(4)
PilY1	95	95	100	95	69	69	94	(4)
PilY2	100	99	100	100	51	51	88	(4)
PilE	100	100	100	100	61	61	97	(4)

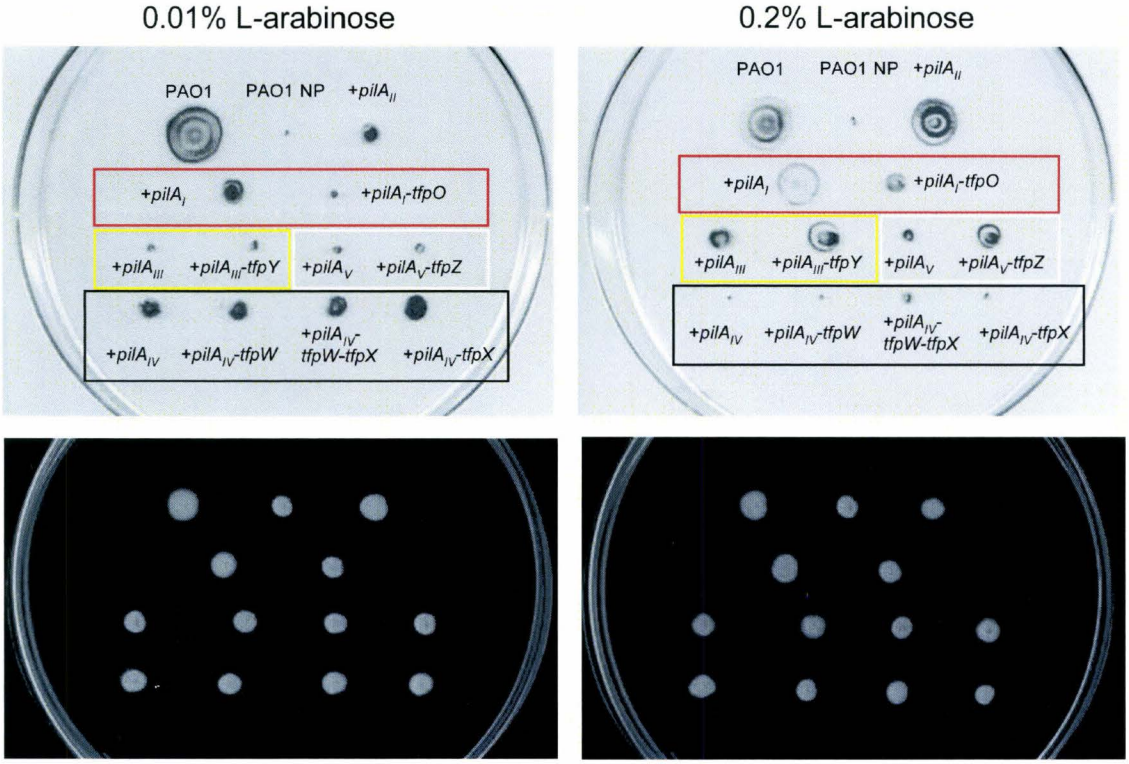


Figure 2. Representative twitching motility of recombinant *P. aeruginosa* PAO1 NP strains.

A group II strain PAO1 NP (no pili) mutant was transformed with constructs expressing genes from group I strain 1244 (accessory gene *tfpO*), boxed in red; group III strain PA14 (accessory gene *tfpY*), boxed in yellow; group IV strain Pa5196 (accessory genes *tfpW* and *tfpX*), boxed in black; and group V strain Pa281457 (accessory gene *tfpZ*), boxed in white. The PAO1 wild type and PAO1 NP mutant, both carrying the vector pBADGr, are positive and negative controls. Twitching motility was tested at two different arabinose concentrations, 0.01% and 0.2%; the cognate PAO1 pilin gene ($pilA_{II}$) restores wild type motility at 0.2% arabinose. The group I pilin gene complements the group II mutant to a wild type levels at 0.2% arabinose, but co-expression of the *tfpO* accessory gene at either arabinose concentration reduces motility. The group III and group V pilin genes do not complement well at either arabinose concentration tested,

although motility is increased upon co-expression of their cognate accessory genes *tfpY* or *tfpZ*, respectively, with 0.2% arabinose. The group IV pilin gene behaves aberrantly in that it complements motility of PAO1 NP to the same extent as the cognate PAO1 pilin gene at 0.01% arabinose but not at 0.2%; this effect is independent of the *tfpW* and *tfpX* accessory genes. Below the twitching plates are the growth controls showing that none of the observed differences in motility are due to growth defects.

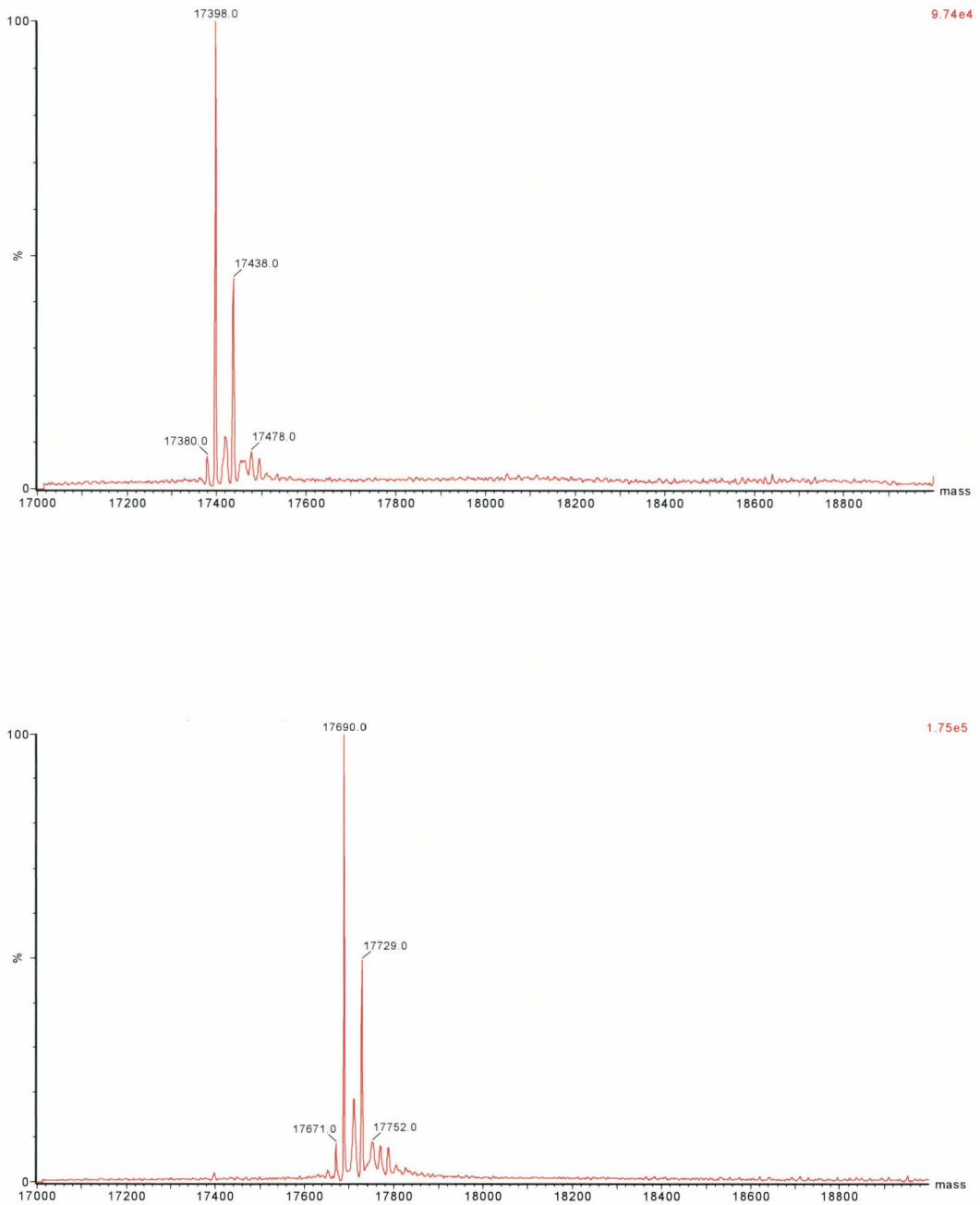
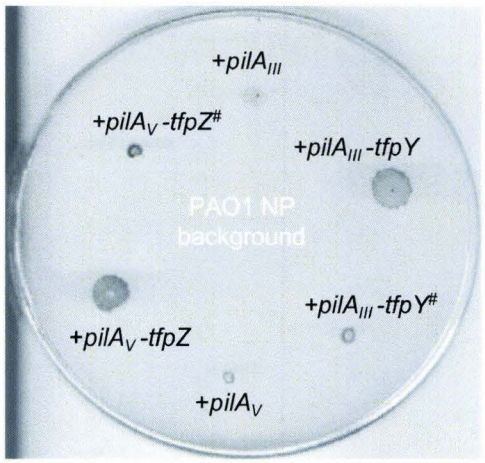


Figure 3. Intact mass spectra of group III and group V pilins.

The observed masses are identical to those predicted from the amino acid sequence from group III (top) and group V (bottom) pilins, showing that the pilins are not post-translationally modified.

A.



B.

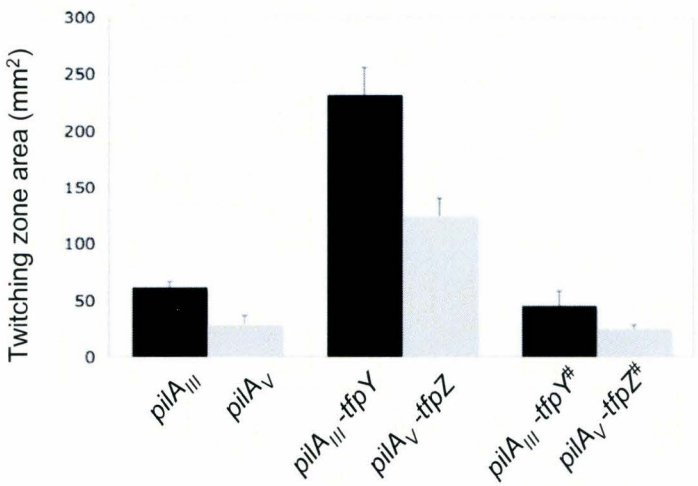


Figure 4. Comparison of twitching zones in recombinant strains.

A) Twitching zones on 0.2% arabinose plates of the PAO1 NP strain expressing the indicated genes from strains PA14 (group III) and Pa281457 (group V). The genes marked with pound (#) signs contain a premature stop codon, introduced by site-directed mutagenesis as outlined in the Methods. B) Quantitation of twitching zone areas (mm^2), average of 12 individual zones for each sample.

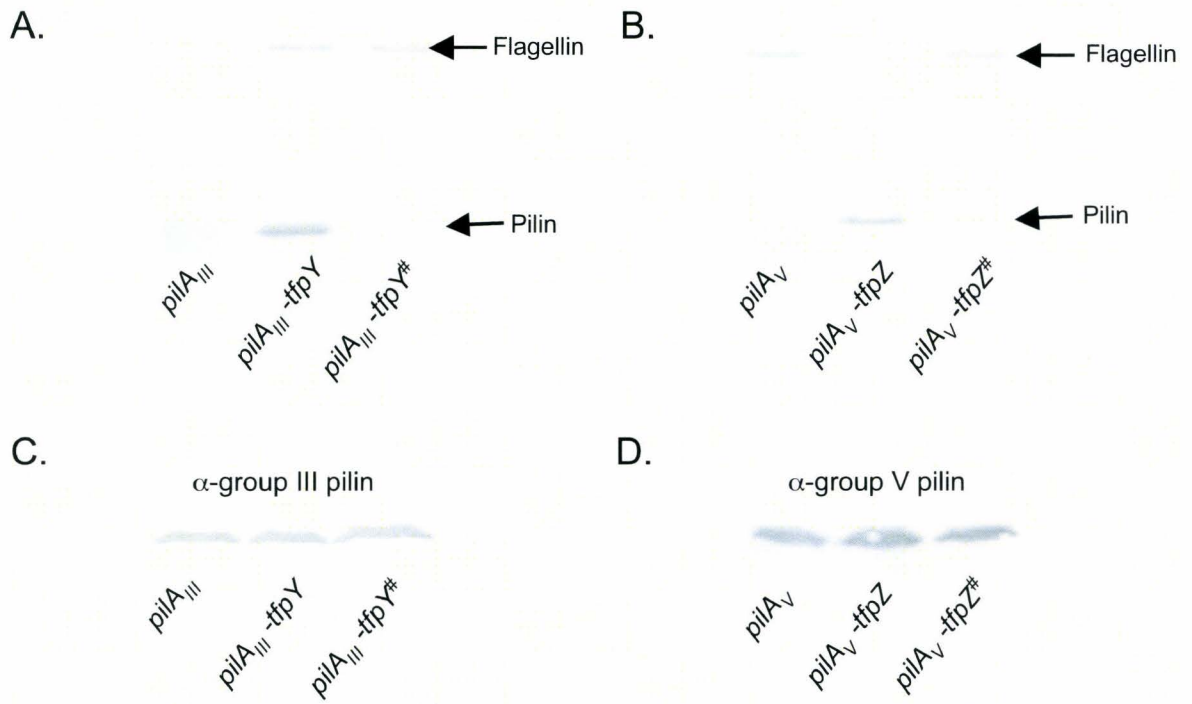


Figure 5. Loss of accessory proteins reduces surface piliation but not whole cell pilin pools.

Lack of the pilin accessory genes reduces surface piliation in PAO1 recombinant strains expressing group III PA14 (A) and group V Pa281457 (B) genes. Pilins are ~15 kDa and flagellins used as a loading control, are ~50 kDa. Western blots of whole cell lysates from PAO1 recombinant strains using α group III pilin (C) and α group V pilin (D) sera show that lack of surface piliation on some strains is not due to degradation of the pilins in whole cell pools.

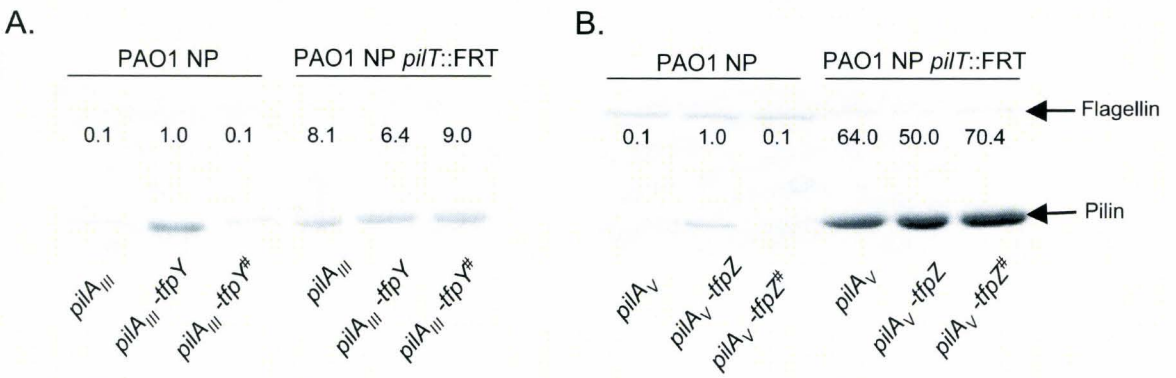


Figure 6. Recovery of surface pili in a *pilT* mutant background.

Expression of the pilin gene cassettes from PA14 (group III; A) and Pa281457 (group V; B) strains in a PAO1 NP *pilT* double mutant resulted in the expression of large amounts of surface pili regardless of the presence of the pilin accessory gene (pound sign denotes a premature stop codon). Pilin levels were normalized to flagellin levels using Scion Image densitometry. Values of 0.98, 8.51, 1.04, 6.92, 5.41 and 7.67 for group III recombinant strains (as indicated in the figure respectively) and 0.32, 1.08, 0.45, 6.92, 5.4 and 7.6 for group V recombinant strains (as indicated in the figure respectively) were obtained representing the amount of pixels measured for the density of the band. Note that the samples prepared from the PAO1 NP *pilT* background were diluted 10-fold compared to those from the PAO1 NP background due to the large amounts of pilin present in these samples.

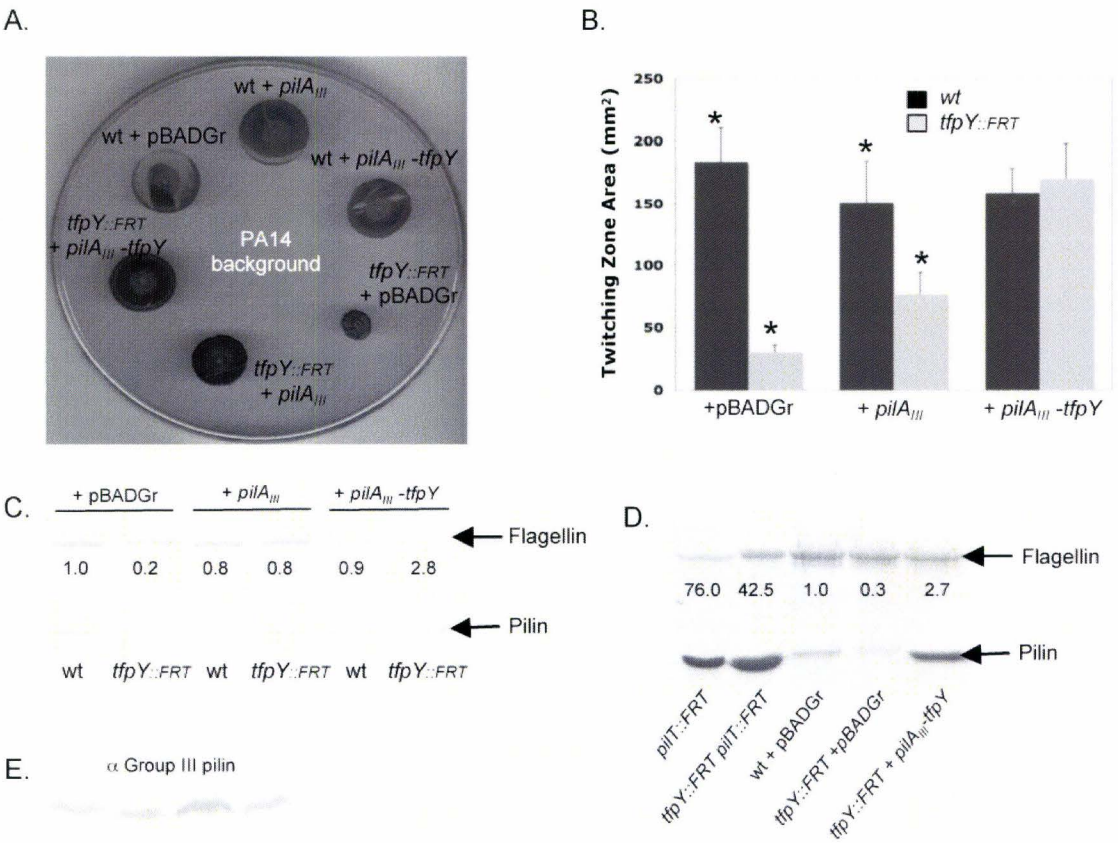


Figure 7. Surface piliation and twitching is reduced in a PA14 *tfpY* knockout.

A) Representative plate showing that twitching motility is reduced in a *tfpY* mutant compared with the wild type (wt), but can be complemented back to wild-type levels in the presence of $pilA_{III}$-*tfpY*. B) Average area in mm^2 of a minimum of 6 twitching zones per strain. Asterisks denote significant differences ($p \leq 0.05$, Student's T-test) between the motility of wild type and mutant strains. C) SDS-PAGE gel of sheared surface proteins from the PA14 wild type and PA14 *tfpY*:FRT mutant, complemented with pBADGr, $pilA_{III}$ or $pilA_{III}$-*tfpY*. In absence of TfpY surface piliation is reduced; complementation of the *tfpY*::FRT mutant with $pilA_{III}$ alone results in increased surface

piliation though motility does not return to wild type levels as shown in panels A and B. Pilin levels were normalized to flagellin levels using Scion Image densitometry. Values of 0.50, 0.12, 0.41, 0.42, 0.42 and 1.4 (as indicated in the figure respectively) were obtained representing the amount of pixels measured for the density of the band. D) SDS-PAGE gel of sheared surface proteins. Although surface piliation of a *tfpY* mutant is low, it is recovered in the *tfpY-pilT* double mutant to the same extent as a *pilT* single mutant. Pilin levels were normalized to flagellin levels using Scion Image densitometry. Values of 5.78, 4.24, 0.50, 0.15 and 1.35 (as indicated in the figure respectively) were obtained representing the amount of pixels measured for the density of the band. E) Whole cell lysates probed with anti-group III pilin antisera show that the reduction of surface piliation in the *tfpY*::FRT strain is not due to degradation of the pilin. Note that the *pilT* and *tfpY-pilT* mutant samples have been diluted 5 fold relative to the other samples due to the large amount of surface pili expressed by these strains.

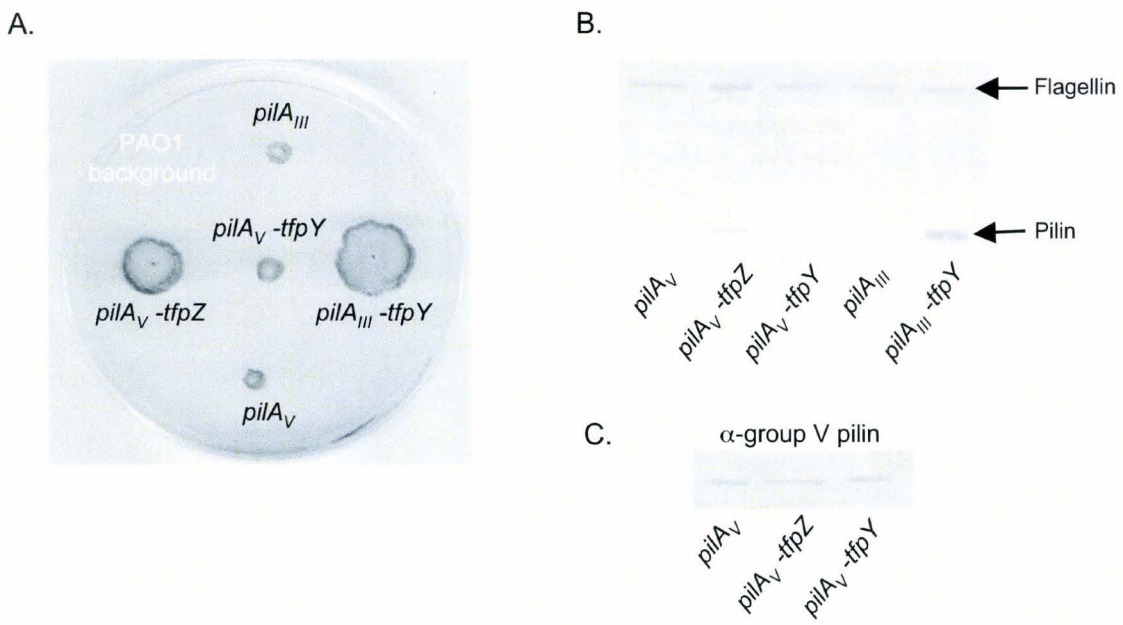


Figure 8. Complementation of PAO1 NP with a group V pilin-group III accessory gene chimera.

The group III PA14 accessory protein TfpY can not replace the function of the group V Pa281457 accessory protein TfpZ. A) Twitching motility in the PAO1 NP recombinant strain is similar to the PAO1 NP recombinant strains lacking a functional accessory protein. B) SDS-PAGE showing that surface piliation is reduced in the strain expressing the chimera compared with that expressing the original *pilA$_V$-tfpZ* cassette. The first two lanes are the group V recombinant strains with and without accessory gene, the third lane is the recombinant strain expressing the chimera and the last two lanes are the group III recombinant strains with and without accessory gene. Whole cell lysates probed with anti-group V pilin antisera show that the reduction of surface piliation in the chimeric strain is not due to degradation of the pilin. The flagellin (~50 kDa) serves as a loading control.

4.0 RESULTS IN PROGRESS FOR FUTURE DIRECTIONS

4.1 Determination of TfpY topology

Hydropathic profiling of the accessory proteins predicted a cytoplasmic N-terminus, three membrane spanning segments and a large C-terminal periplasmic domain (21). If this model is accurate, this C-terminal domain may be the site of interaction between accessory proteins and PilA, which is similarly oriented in the inner membrane. To experimentally confirm the predicted topology, *ScaI* sites were introduced into the C- and N-terminal regions of TfpY in order to be able to insert a *phoA-lacZ*α dual reporter cassette from pPS632 (1). Attempts to introduce *ScaI* sites into the C- and N-terminal regions of TfpY by site-directed mutagenesis were unsuccessful. As an alternative, modified SOE PCR (30) was used to generate the *ScaI* sites as indicated in the methods. PCR products were cloned into the pCR 2.1 TOPO vector (Invitrogen) and subcloned into pBADGr. Attempts to introduce the *phoA-lacZ*α dual reporter cassette have so far been unsuccessful. Future work will involve cloning this cassette into the *ScaI* sites and screening colonies on dual indicator plates containing 6-Chloro-3-indolyl-β-D-galactopyranoside (Red-Gal) and 5-Bromo-4-chloro-3-indoxyl phosphate (X-phos) to detect if the reporter fusions are in the cytoplasmic (Red-Gal positive) or periplasmic (X-phos positive) region.

4.2 Modification of the predicted transcriptional terminator preceding *tfpY*

A number of different constructs have been generated by modified SOE PCR methods (30), and cloned into pBADGr for future studies. We hypothesize the low expression of TfpY from the SOE *pilA*$_V$*tfpY*-6xHis and *pilA*$_{III}$*tfpY*-4xHis strains is due to a predicted transcriptional terminator located between the highly expressed *pilA* gene and the *tfpY* gene. In an attempt to increase the amount of *tfpY* expression, the

transcriptional terminator in the *pilA*$_{III}$*tfpY*-4xHis construct was weakened by changing the 6 of the 7 bases involved in forming the terminator (Figure 9). This new construct has been subcloned from pCR 2.1 TOPO into pBADGR, transformed into PAO1 NP and will be tested for its ability to complement twitching motility compared with the original construct. In addition to the terminator mutation, two histidine codons have been added to the *pilA*$_{III}$*tfpY*-4xHis construct to introduce a 6xHis tag. This construct is different from the SOE 6xHis construct because the 6xHis tag is in the N-terminus of TfpY. The addition of two histidines and location of the tag may strengthen the signal for antibody detection; this will be tested in the future.

4.3 Expression of TfpY for generation of antisera

The detection of TfpY through use of specific antibodies will be important for future experiments. Currently, we have no means of detecting TfpY through western blotting, since detection of a C-terminal 6xHis tagged TfpY and an N-terminal 4xHis using an αHis-penta and αHis-tetra (recognizes 5-6 and 4-6 histidines, respectively) antibodies at various concentrations has been unsuccessful. Since the detection of TfpY is not feasible at the moment, nucleotides 306-705 of *tfpY*, including only the predicted periplasmic domain, have been cloned into the pET101/D-TOPO expression construct and will be used to express protein for antibody production. In order to determine the best expression conditions, a pilot expression study was performed in 5 mL of LB + 100 μg/mL ampicillin; the temperature, expression time and amount of IPTG were altered. After separation of the proteins on SDS-PAGE and staining with Coomassie, a band corresponding to 17.6 kDa (C-terminal TfpY + V5 Epitope + 6xHis) could not be clearly distinguished from other proteins in the gel. The samples were probed with an α-His-penta antibody. The 6xHis tagged TfpY fragment was detected at the appropriate size, and the pilot expression data showed that the best expression conditions are at 37°C for

4 h using between 0.5 and 0.75mM IPTG (Figure 10). Expression was repeated using 50 mL of LB + 100 μg/mL ampicillin, and the protein was purified using Ni-NTA resin. Aliquots from each purification step were retained; proteins were separated on SDS-PAGE, transferred to nitrocellulose and probed with the αHis-penta antibody. Western blotting showed that a portion of the target protein remained in the pellet (lysed cells) after French press and that the amount of protein released from the resin is minimal, as shown by the faint band in the first elution (Figure 11). The majority of protein was retained on the resin, even after two elutions using 400 mM Imidazole. The conditions used for the scale up expression of TfpY are clearly not optimal and will need to be further modified in order to express enough protein for antibody generation.

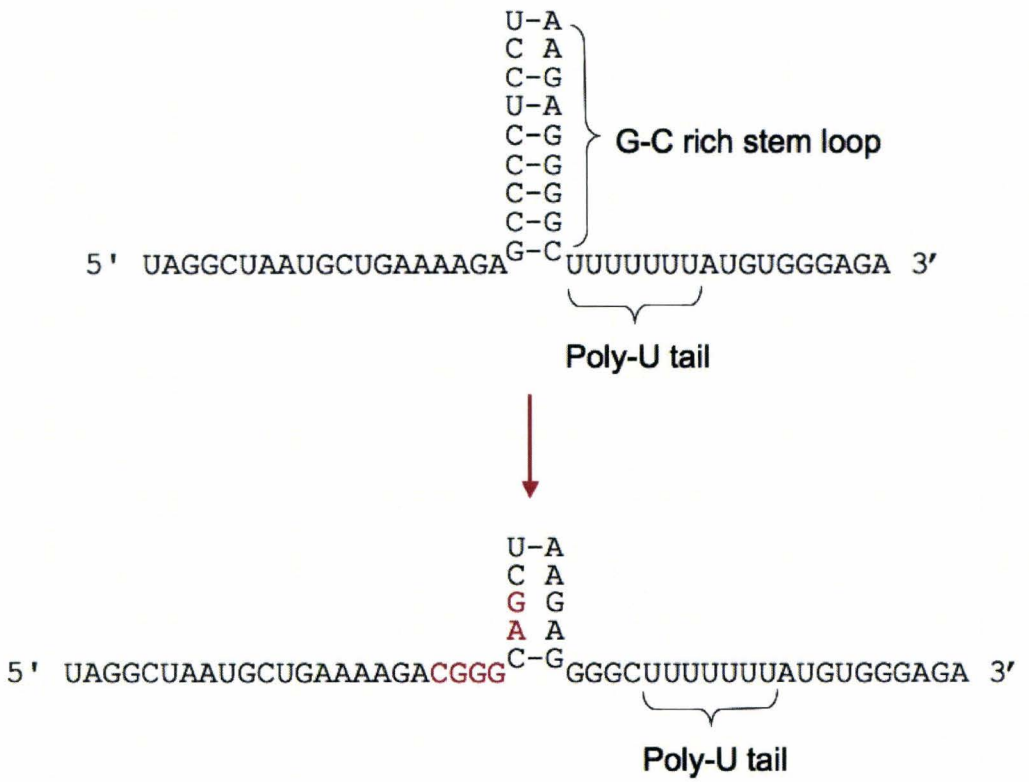


Figure 9. Modifications of the predicted transcriptional terminator.

Bases involved in forming the predicted transcriptional terminator preceding *tfpY*, in the G-C stem loop, have been modified to weaken the terminator; modified bases are shown in red. The sequence shown represents the entire region between the 3' end of $pilA_{III}$ and the 5' end of *tfpY*.

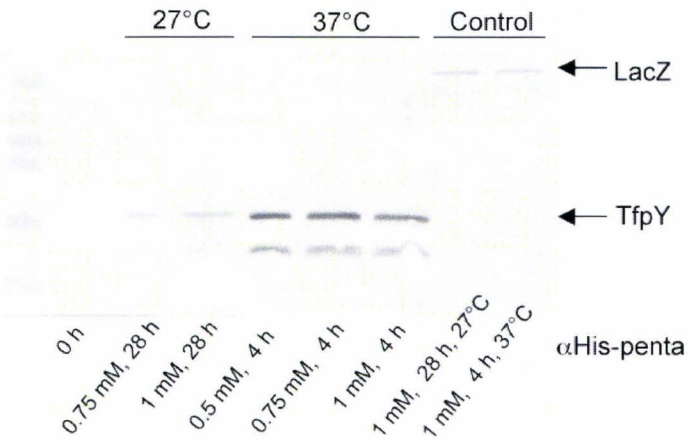


Figure 10. Pilot expression of TfpY at various conditions.

Western blots of TfpY expression in the pET101/D-TOPO (Invitrogen) vector at 27°C for 28 hours shows minimal expression using both 0.75 and 1mM IPTG. Expression is increased at 37°C when induced with 0.5, 0.75 or 1mM IPTG for 4 hours, however the optimal amount of IPTG appears to be between 0.5 and 0.75mM. LacZ expression (control) was conducted under the same conditions.

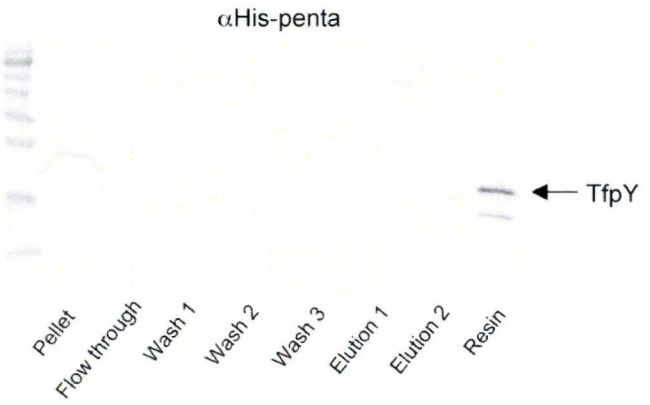


Figure 11. Expression and purification of TfpY yields minimal protein.

Western blots of each step in the purification of TfpY shows the most protein remains in the pellet and bound to the Ni-NTA resin, and elution of TfpY using 400mM Imidazole yields minimal protein.

5.0 DISCUSSION

In our previous study of *P. aeruginosa* pilin diversity, we defined three new pilin alleles that were found in ~25% of the genetically distinct environmental, clinical and cystic fibrosis samples that were tested (36). Strain PA14, which expresses group III pilins, was recently shown by another group to be the most common clonal strain in the *P. aeruginosa* population, representing 7% of 244 strains tested from a wide range of environments (63). The striking linkage between the group III and V pilin genes and their accessory genes *tfpY* and *tfpZ,* respectively, in strains of different genotypes could be fortuitous due to their proximity in the chromosome, or indicative of a functional relationship. My work has revealed that the accessory proteins modulate pilus assembly dynamics, as loss of these proteins decreased surface pili and twitching motility, but did not affect pilin assembly *per se* or alter the whole cell pilin pools compared with wild type strains. These observations, together with the data showing that TfpY is not compatible

with the group V pilin $PilA_V$, lead us to conclude that there is a functional explanation for the observed linkage between specific pilins and their cognate accessory proteins.

To our knowledge, only two type IV pilin accessory genes of this type have been reported in the literature. The accessory gene product PilB from *E. corrodens* (31% protein sequence identity to TfpZ, concentrated in the predicted N-terminal transmembrane regions) was shown to be required for twitching motility and possibly pilus assembly, however its specific function is unknown (56). On the other hand, *D. nodosus* accessory gene product FimB (32% protein sequence identity to TfpZ) was found not to be required for pili biogenesis; again, its function is unknown (35). Although these reports seem contradictory, our findings are consistent with both; for example, loss of the TfpY accessory protein causes decreased twitching motility in both native (PA14) and recombinant (PAO1) backgrounds, but does not completely abrogate pilus assembly.

When the pilins from groups III and V were expressed in a PAO1 background, we were initially surprised to find that they were unable to complement twitching motility and surface piliation to levels seen with the homologous control, since there have been previous reports of successful expression of heterologous type IV pilins in *P. aeruginosa* (22, 31, 58, 64). We showed that the group III and V pilins were stably expressed in the absence of their associated accessory proteins, and that the observed motility defects were occurring at the level of pilus assembly/disassembly. This hypothesis was supported by the recovery of large amounts of surface pili upon expression of the group III and V pilins both with and without their cognate accessory proteins in a *pilA-pilT* double mutant, showing that a) the T4P assembly machinery readily accommodates pilins of diverse sequence with similar efficiency, and b) the reduced surface piliation and motility in accessory protein-deficient strains could not be attributed to assembly defects *per se*. The high conservation among components of the T4P assembly system

in *P. aeruginosa* strains expressing various *pilA* alleles (Table 4) is consistent with the concept that most components of the system are insensitive to differences in pilin primary sequence. Although type IV pilin sequences can be quite divergent, comparison of the structures of those solved to date shows that they have similar architecture (6, 20, 24, 28, 34, 45, 48), supporting the idea that they are likely to be functionally compatible with conserved components of the T4P machinery.

The extent of twitching motility observed, as well as the amount of recoverable surface pili on a specific strain, is determined not only by the ability to assemble a pilus, but also by the crucial balance between rates of pilus extension versus retraction (64-66). Since the process of pilus assembly appeared to be independent of both pilin sequence and the presence/absence of the accessory proteins, the substantial decrease in surface piliation observed in the absence of the accessory proteins suggests that perhaps the *rate* of assembly of heterologous pilins may be slower than that of homologous pilins, resulting in a net increase in PilT-mediated retraction. This would account for fewer recoverable surface pili and a decrease in twitching motility due to less time allotted for the pili to bind the surface. Since PilT is identical in all *P. aeruginosa* strains examined to date (Table 4), it is unlikely to be directly involved in pilin recognition. Although the rate of pilus retraction has been estimated using optical techniques to be approximately 1000-1500 subunits per second (39, 43, 53), it has not yet been possible to measure the rate of pilus extension due to the inherent flexibility and therefore non-linearity of the fibres.

Of the factors involved in type IV pilus assembly that have been identified to date, only the minor pilins exhibit notable differences in sequence between strains with unrelated pilin alleles (Table 4). Interestingly, group I strains 2192 and LES have minor pilins that are 95-100% identical at the amino acid level to those of group II strain PAO1 (Table 4), and the $pilA_I$ gene from strain 1244 was able to complement PAO1 NP to the

same extent as its cognate group II pilin. Similarly, the minor pilins of a group IV strain are similar (88-97%) to those of PAO1, with the exception of FimT (81%), and the *pilA*$_{IV}$ gene restored motility of PAO1 NP to levels commensurate with the homologous PAO1 pilin gene (albeit only at lower arabinose concentrations, for reasons not yet clear). In contrast, the minor pilins from the group III strains PA14 and C3719 (no group V genome is yet available) have substantially less similarity to those of PAO1 (ranging from 49-75%; Table 4), but are identical to one another. The minor pilins are important for the control of pilus assembly, although their specific functions are not yet understood (2-4, 65). We speculated that reduced compatibility between horizontally-acquired heterologous major pilins and the host strain's set of minor pilins may slow the rate of pilus assembly, and thereby cause the observed net retraction of heterologous pili mediated by PilT. However, because the inactivation of *tfpY* in PA14 also results in altered retraction dynamics, it is less likely that the group III and V pilins are unable to complement the group II pilin strain due to differences in the minor pilins between strains. The only other clear differences in proteins of the type IV pilus machinery, other than the accessory proteins, are the pilins themselves. The group III and V pilins have disulfide loops that are more than twice the size of the disulfide loops from group I and II pilins, and the overall size of group III and V pilins are larger than those of group I, II and IV (36). Such differences could result in a reduced rate of assembly compared with other pilins, and they could therefore be more likely to be retracted in the absence of their cognate accessory protein. Regardless of the underlying mechanism, the accessory proteins characterized in this study appear to antagonize such retraction events.

Our examination of a chimeric *pilA*$_{V}$-*tfpY* construct showed that the accessory proteins are specific for their cognate pilins. TfpY and TfpZ are both predicted to have a short cytoplasmic N-terminus, three transmembrane domains and a periplasmic C-terminus consisting of ~132 (TfpY) and ~153 (TfpZ) residues. This orientation

resembles that of the pilins, which are retained in the inner membrane prior to assembly by their hydrophobic N-termini, with their C-terminal domains in the periplasm (Figure 12). The amino acid sequence similarity between TfpY and TfpZ is ~50%, restricted mainly to the membrane-spanning domains. Therefore, the unique sequences of the C-termini are likely to dictate pilin specificity, particularly since it is the C-termini of the pilins that are most variable (46). Future studies involving the insertion of a *phoA/lacZα* cassette into the ScaI sites generated in the *pilA$_{III}$tfpY* construct, and selection of colonies on a dual indicator plate with X-Phos or Red-Gal, will determine the predicted topology of TfpY (1). Confirmation that the C-terminal domain of TfpY is within the periplasm will support our results showing that the accessory proteins are specific for their associated pilin, because of the similar orientation of pilin proteins in the inner membrane. In addition, confirmation of topology will support our idea that the C-terminal domain of TfpY is the functional region of the protein.

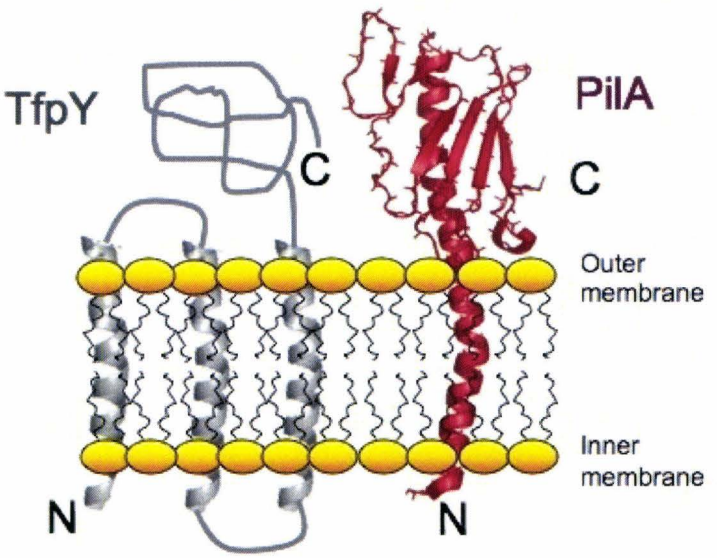


Figure 12. Cartoon representing the orientation of TfpY and PilA within the membrane.

PilA is oriented with its N-terminal in the membrane before it is assembled into the pilus. TfpY is predicted to have three membrane spanning domains with a large C-terminal periplasmic domain. The C-terminal of both proteins lies within the periplasm, and contains the most variable differences in sequence between homologues.

We attempted to show that TfpY is expressed by the chimera via tagging the protein with a 6xHis tag and probing with an α-His penta antibody. Although a very faint band was observed upon expression in *E. coli* DH5α, the putative TfpY band was too weak for adequate electronic reproduction. Since the predicted transcriptional terminator between *pilA* and *tfpY* is likely the cause of the low expression levels, a construct has been designed in which the terminator has been disrupted, by changing the bases involved in formation of the terminator (Figure 9). PilA is expressed at a very high level because thousands of pilin subunits are required for pilus assembly (42). Since *pilA* and *tfpY* are co-transcribed in an operon, the putative transcriptional terminator is important to control the amount of TfpY expression. Because TfpY has three predicted transmembrane domains, it would likely be detrimental to the cell to express as much TfpY as PilA. Future studies will involve expression of a disrupted terminator construct, probing with αHis antisera to determine if detection levels increase, and creating slight variations of the construct if needed to see which one provides the best level of detection without causing toxicity to the cell. In addition, expression of the C-terminal domain of TfpY in the pET101/D-TOPO construct generated in this study will be pursued in order to generate sufficient protein for antisera production. Antibodies generated against the C-terminal domain of TfpY will be necessary for future experiments with any of the constructs where manipulations to TfpY have been made; for example, the premature truncation construct $pilA_{III}tfpY$-221# and the SOE $pilA_{V}tfpY$ construct. To further investigate the putative functional domain of TfpY, we will test

whether or not truncation of TfpY at Ala221 impacts on its ability to enhance surface piliation. The results of this experiment will assist in defining the minimal functional domain of TfpY.

Antibodies that recognize TfpY will also be very important in future studies aimed at studies of $PilA_{III}$ and TfpY interactions. The data we have obtained to date strongly suggests that the accessory proteins interact with their cognate pilins: surface piliation and twitching motility are reduced in absence of the accessory proteins; TfpY cannot complement the function of TfpZ; and the predicted topology suggests that the C-terminal variable domains of both proteins lie within the periplasm before the pili are assembled. With antisera to both proteins in hand, we will perform co-immunoprecipitation experiments to investigate the interaction between these two proteins. It is important to have antibodies that strongly recognize TfpY, because TfpY will be used to co-precipitate PilA. The $PilA_{III}$ antibodies can be used to confirm that PilA was co-precipitated with TfpY, but using these antibodies to pull out TfpY will likely yield inconclusive results due to the expression level differences between PilA and TfpY. Knowing which proteins interact within the type IV pilus assembly machinery will provide a more thorough understanding of this virulence mechanism used by *P. aeruginosa.*

As antigenic surface structures exposed to the environment, type IV pili are subject to evolutionary selection for variation, which can occur by point mutation, by intragenic recombination (in *Neisseria*), by post-translational modifications and by horizontal gene transfer between species (8, 27, 36, 40, 46). The observed linkage between pilin and accessory genes in *P. aeruginosa* appears to be a consequence of evolutionary selection for improved function of T4P. The *tfpO* gene in the group I pilin cassette enhances virulence by post-translational glycosylation of its specific pilin substrate, and functions in any genetic background expressing a suitable O antigen glycan (11, 54). The TfpY and TfpZ proteins characterized here appear to have been

retained in the pilin cassette due to their ability to antagonize disassembly of their cognate pilins, thereby increasing surface piliation and motility, and likely improving the chances that their host strain could successfully colonize a desired environmental niche. Identification of these novel accessory proteins combined with a better understanding of their function will provide a broader perspective of virulence genes in type IV pilus system. In addition, the function of these proteins likely reflects the functions of their homologues in other bacteria with type IV pilus machinery such as *E. corrodens* and *D. nodosus*, or at least the role these proteins had at one point in time. Since homologues of TfpY and TfpZ have been identified in bacteria where no literature is available (Table 1), we can provide a basis for the function of these homologues in other systems. We have added additional proteins to the already complicated type IV pilus system, and have made a lot of progress in identifying their function; we know that TfpY and TfpZ are involved in type IV pilin retraction dynamics, because we observed altered pilus function in their absence and could restore surface piliation in absence of the retraction protein PilT, and we know that the accessory proteins are specific for their cognate pilin.

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