ROLE OF PILC IN PSEUDOMONAS AERUGINOSA TYPE IV PILUS FUNCTION

ROLE OF THE *PSEUDOMONAS AERUGINOSA* INNER MEMBRANE PROTEIN PILC IN TYPE IV PILUS FUNCTION

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Abstract:

Type 4 pili (T4P) are fibrous appendages found on the surfaces of a wide range of bacteria. They are used for adherence to biotic and abiotic surfaces, twitching motility, and biofilm formation. Despite their ubiquitous distribution, identifying the core components required for T4P expression has been difficult due to conflicting data about the functions of orthologous components from the most common model organisms, *Neisseria* and *Pseudomonas*. By inactivating the retraction component of pilus function, genes essential for T4P assembly versus disassembly were discriminated in *P. aeruginosa*. In contradiction to data from the *Neisseria* system, we found that components of the inner membrane sub-complex consisting of PilN/O/P are not essential for surface pilus expression, while the highly conserved inner membrane protein, PilC is essential. The current model of T4P biogenesis suggests that PilC coordinates the activity of cytoplasmic extension (PilB) and retraction (PilT) ATPases via their interaction with its two large cytoplasmic domains. Hydrolysis of ATP by PilB or PilT is proposed to induce domain movements in PilC, resulting in the addition or removal of single pilin subunits from the base of the pilus. Using *in vitro* co-affinity purification we showed that PilB is a potential interaction partner of the N-terminal cytoplasmic domain of PilC. Also, mutagenesis of the C-terminal cytoplasmic domain of PilC produced mutant proteins with a reduced capacity to support twitching motility, suggesting impairment of PilC-PilT interactions. The indispensability of PilC and its potential interactions with the ATPases PilB and

PilT suggest that it is a core element required for function of the T4P system of *P. aeruginosa*.

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List of abbreviations and symbols

ATP	Adenosine-5'-triphosphate
BACTH	Bacterial Adenylate Cyclase Two Hybrid
°C	degree(s) centigrade
GSP	General secretory pathway
h(s)	Hour(s)
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiagalactopyranoside
L	Liter(s)
LB	Lauria-Bertani broth
μg	Microgram(s)
μl	Microliter(s)
ml	Milliliter(s)
min	Minute(s)
ms	Millisecond(s)
ng	Nanogram(s)
nm	Nanometre(s)
NP	*pilA* mutant
PAO1	O1 strain of *Pseudomonas aeruginosa*
PAK	K strain of *Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PIA	Pseudomonas isolation agar
POPE	palmitoyloleoylphosphatidylethanolamine
OM	Outer membrane
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
s	Second(s)
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T2S	Type II secretion
T4P	Type IV pili
TM	Transmembrane
V	Volt(s)
WT	Wild type

Declaration of Academic Achievement and Attributions:

Kevin Kemp was under the supervision of H.K.Takhar as a 4th year thesis student. His project included protein expression and solubility trials and site directed mutagenesis of the C-terminal cytoplasmic domain of PilC.

“Let me tell you the secret that has led me to my goal.
My strength lies solely in my tenacity”

Louis Pasteur (1822-1895)

CHAPTER 1- INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, gram-negative pathogen that inhabits biotic and abiotic surfaces including water, soil, medical equipment, plants, and animals (Hardalo et al., 1997; Giltner et al., 2006*).* The virulence of *P. aeruginosa* can be attributed to its inherent resistance to a number of antibiotics, and to the formation of biofilms, which act as protective mechanisms for the bacteria during infection (Prithiviraj et al., 2005, O'Toole et al., 1998). *P. aeruginosa* uses polar protein appendages called Type IV pili (T4P) for attachment to surfaces and for a form of surface translocation called twitching motility (Burrows et al., 2005). T4P are important virulence factors and without them, bacteria are impaired in host colonization (Hahn et al., 1997). Due to the many significant roles in which T4P are involved, it is important to identify the core essential components necessary for their biogenesis.

1.1 Type IV Pili

T4P are long, thin hair-like fibers formed of thousands of subunits arranged in a helical conformation (Forest et al., 1997). They can be up to several microns long, and are typically found at the poles of many gram-negative and gram-positive pathogens (Pelicic, 2008). T4P provide many functions for bacteria such as adherence to surfaces, cell-cell aggregation, DNA uptake, and a unique form of surface-associated locomotion called twitching motility (Mattick et al., 2000, Craig et al, 2005; Bradley et al., 1972). Twitching motility is a phenomenon described by Bradley, who proposed that the force generated by

retraction of the pilus provided the energy necessary for the flagellar-independent form of translocation (Bradley et al., 1972). Twitching motility was found to arise from repeated rounds of pilus extension, adherence to a surface, and subsequent retraction that pulls the cell body forward, where the pili act as grappling hooks (Skerker and Berg, 2001). T4P are mainly comprised of the major pilin subunit PilA, however, recent evidence supports the incorporation of the minor pilins PilE, PilV, PilW, PilX, and FimU into the pilus (Strom et al., 1993, Giltner et al., 2010). Studies conducted in *Neisseria gonorrhoeae* used laser tweezers to measure the retractile force of a single pilus, and found it to exceed 100 pN, making the T4P motor one of the strongest known (Merz et al., 2000, Maier et al., 2002). Although T4P are proposed to be involved in many important roles in *P. aeruginosa*, the understanding of the specifics of their assembly and function is limited. Their role in virulence makes them a potential target for intervention. Therefore, it is useful to identify vulnerable components of the biogenesis machinery.

1.2 Type IV Pilus Biogenesis Machinery

In *P. aeruginosa,* more than 1% of gene products (~60 genes) are involved in biogenesis and function of T4P (Mattick et al., 2002). The assembly system is complex and spans both the inner and outer membrane **(Figure 1)**. The current model of pilus biogenesis suggests that assembly occurs at the inner membrane. The major pilin subunit has a conserved N-terminal leader sequence which is cleaved and methylated by the bi-functional enzyme PilD (Strom et al., 1993).

Once cleaved, the pilin subunits are recruited to the growing pilus by hydrophobic and charge interactions with preceding subunits, ensuring they are added into the growing filament in the proper orientation (Li et al., 2008).

In *P. aeruginosa* there are three cytoplasmic, hexameric ATPases that are required to polymerize (PilB) and depolymerize (PilT) the pilus (Nunn et al., 1990). The specific function of the PilT paralogue PilU is still unclear, but mutant phenotypes suggest that it plays a role in retraction (Whitchurch et al., 1994; Chiang et al., 2005). Transposon insertions in the ATPase genes yielded mutants that did not twitch and had either a non-piliated (PilB), or hyperpiliated (PilT and PilU) phenotype (Nunn et al., 1990; Whitchurch et al., 1991).

As the pilus is assembled, it must exit through the outer membrane. In *P. aeruginosa* a lipoprotein tethered to the inner leaflet of the outer membrane, PilF—also known as the pilotin—aids in localization and multimerization of the outer membrane secretin, PilQ (Koo et al., 2008). Transmission Electron Microscopy studies in *Neisseria* showed that PilQ is dodecameric (Collins et al, 2001, 2003). The oligomer is extremely stable, and resistant to heat and treatment with detergents (Tonjum et al., 1998).

The ATPases required for (de)/polymerization of the pilus are located in the cytoplasm, while the pilin subunits are embedded in the inner membrane, with their C-termini in the periplasm. Therefore, an inner membrane protein(s) may be required to connect these components. The highly conserved inner membrane platform protein, PilC, has been suggested to perform this function (Py et al.,

2001). Conversely, in *N. meningitidis* the less-conserved inner membrane sub-complex proteins PilM/N/O/P have been proposed to act as the protein scaffold connecting the ATPases and the pilin subunits, while the PilC orthologue was considered dispensable for pilus assembly (Carbonelle et al, 2006). Because of this conflicting data, this study focused on determining which inner-membrane protein(s) are essential for T4P biogenesis in *P. aeruginosa*.

1.3 Type II Secretion System

The T2S system is multi-protein complex that spans both the inner and outer membrane **(Figure 1)**. Based on a high degree of structural and functional similarity between components of the T2S and T4P systems, a common evolutionary origin has been suggested (Pugsley, 1993). Also known as the general secretory pathway (GSP), the T2S system is widely distributed among gram-negative bacteria including *P. aeruginosa* (Cianciotto et al., 2005). It allows bacteria to deliver virulence factors such as proteases, hydrolases, and toxins into the extra cellular environment via the pseudopilus (Filloux et al., 1998, Nunn et al, 1999). Interestingly, homologues of PilC and PilM/N/O/P are found in the T2S system, and have been suggested to perform central roles in secretion. Therefore, examining the functions and interaction partners of the T2S system inner membrane proteins could provide insight, and aid in elucidating the roles of their T4P homologues.

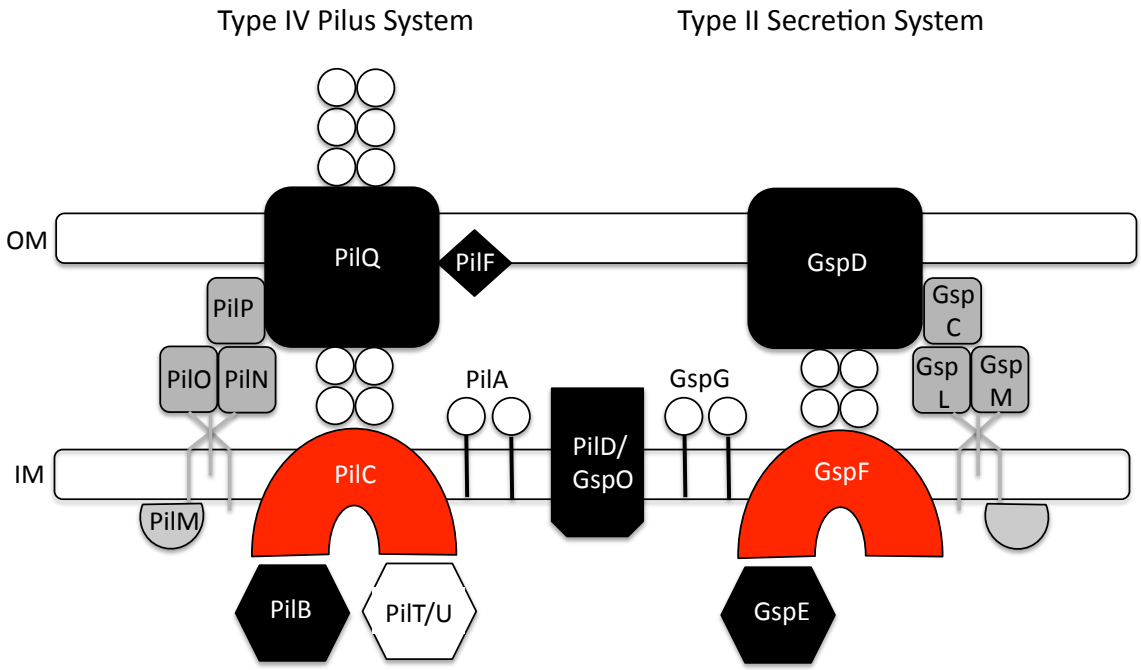

Figure 1: **Conserved architecture of the Type IV Pilus and Type II Secretion systems**. The Type IV pilus (T4P) and Type II Secretion (T2S) systems share homologous proteins and conserved architecture. Proteins colored in black share the most sequence and structural similarity, followed by proteins colored in grey then white. Highlighted in red is the putative platform protein central to both T4P and T2S systems. The pre-pilin peptidase PilD/GspO processes the major and minor pilins of T4P system and pseudopilins of T2S system. Both the T2S and T4P system contain inner membrane sub-complex proteins depicted above in grey. This sub-complex has been proposed to link the cytoplasmic and periplasmic compartments of the cell. The T4P contains three ATPases PilB/PilT/PilU for polymerization and depolymerization of the pilus; whereas the T2S system has only one polymerization ATPase (homologous to PilB). Both systems also contain a secretin in the outer membrane, which allows the secretion of the T4P and exoproteins.

1.4 PilM/N/O/P inner membrane protein sub-complex

The inner membrane sub-complex components of the T2S and T4P systems have been shown to exhibit sequence variability, however, their general architecture is conserved **(Figure 1)** (Ayers et al., 2010). The inner membrane sub-complex proteins of the T4P system of *P. aeruginosa*, PilM/N/O/P, are encoded on a polycistronic operon with the outer membrane secretin PilQ. Bioinformatics analyses predict that PilM is a cytoplasmic protein with an actin-like fold (Martin *et al.*, 1995). PilN and PilO are type II membrane proteins that have domains similar to the periplasmic region of GspL and GspM, respectively. PilP is a periplasmic lipoprotein, and although there is no T2S homologue identified, Tammam *et al.* have shown that in *P. aeruginosa* PilP forms a stable heterotrimer with PilN/PilO *in vitro*, suggesting that PilP is functionally equivalent to the T2S component, GspC (Tammam et al., 2011).

Orthologues of PilM/N/O/P in the T2S system have been shown to form an inner membrane sub-complex required for protein secretion (Possot et al., 2000, Lybarger et al., 2009). Evidence for a sub-complex is also supported by a comprehensive study in *P. aeruginosa*. Immunoblot analyses of *pilM/N/O/P* mutants revealed that loss of any single protein reduced the levels of other components, suggesting the proteins act as a functional unit (Ayers et al., 2009). Also, Ayers and colleagues determined that PilM/N/O/P co-localize to the inner-membrane fraction of whole cell lysates (Ayers et al., 2009).

1.4.1 Essentiality of the inner membrane sub-complex

Mutagenesis studies in retraction-deficient strains of *N. meningitidis* (which report on pilus assembly only, rather than pilus function) showed that products of the *pilM/N/O/P* operon were essential for surface pilus expression (Carbonelle et al., 2006). Studies conducted in *Myxococcus* and *Thermus* also propose that these inner membrane proteins are required for pilus biogenesis (Nudleman et al., 2006, Rumszauer et al., 2006, Karrupiah et al., 2011). Mutants of *pilM/N/O/P* in *P. aeruginosa* resulted in a complete loss of surface exposed pili and the ability to twitch, however, the results of these mutations in a retraction-deficient strains were not reported (Ayers et al., 2009). Therefore, generating sub-complex mutants in a *pilT* mutant background would aid in further defining the role of the proteins in *P. aeruginosa* T4P biogenesis.

1.4.2 Interaction partners of the inner membrane sub-complex

Other than evidence that the inner membrane sub-complex proteins interact, few studies have established other potential interaction partners in the T4P system. Studies of the T2S system of *Vibrio cholerae* suggest that the ATPase GspE interacts with GspL, the structural equivalent of the T4P sub-complex proteins PilM and PilN (Gray et al., 2010, Sampaleanu et al., 2009). Studies in the T2S system of *P. aeruginosa* showed that the stability of the ATPase GspE requires the cytoplasmic portion of GspL, and the putative platform protein GspF (Ball et al., 1999, Arts et al., 2007). Yeast two-hybrid studies in

Erwinia chrysanthemi also identified a heteromeric complex consisting of GspE/F/L/M, and proposed that the entire complex is required for pseudopilus assembly and anchoring to the inner membrane (Py et al., 2001). A recent publication on the T4P system of *N. meningitidis* used the bacterial two-hybrid (BACTH) system to identify novel interactions between the major pilin subunit and the PilN and PilO orthologues (Georgiadou et al., 2012). Although the authors acknowledged the BACTH provides a brief "snapshot" of the dynamic workings of the T4P machinery, they suggested that observation of this interaction solidifies the sub-complex as the link between the cytoplasm and periplasm (Georgiadou et al., 2012). In order to characterize the inner membrane sub-complex proteins as the scaffold for pilus assembly, interactions with the ATPases/ major structural subunit must also be established in the T4P system of *P. aeruginosa*.

1.5 The Putative Platform Protein PilC

Both the T4P and T2S systems contain the highly-conserved polytopic inner-membrane protein PilC (GspF) **(Figure 1)**. Its broad distribution and high level of conservation led to the suggestion that this protein plays an essential role as a platform for pilus/pseudopilus assembly (Nunn *et al.*, 1990; Hobbs *et al.*, 1993; Tonjum *et al.*, 1995).

1.5.1 Essentiality of PilC:

In *P. aeruginosa* PilC was found to be essential for surface pilus expression and twitching motility (Lory et al., 1993). Similarly, a study of the T2S system in *P. aeruginosa* found that its PilC homologue, GspF, is indispensible for secretion (Arts et al., 2007). In contrast, studies in *Neisseria spp*. suggested that the PilC orthologue, PilG, is not essential for pilus assembly (Carbonnelle et al., 2006). Although PilG mutants in *Neisseria* are devoid of surface exposed pili, Carbonnelle and colleagues found that mutation of *pilG* in a retraction deficient background resulted in wild type surface piliation and adhesive properties (Tønjum et al., 1995, Carbonnelle et al., 2006). Due to the discrepancy in the literature supporting the dispensability of PilC in pilus assembly, and its high degree of conservation in the T4P and T2S systems, it is important to clarify the role of PilC in *P. aeruginosa.*

1.5.2 Topology of PilC:

Further fueling controversy, the topology of PilC proteins has also been debated. Bioinformatics predictions have suggested for both three and four transmembrane (TM) domains exist for PilC. These differences, which would affect exposure of specific domains to the cytoplasm versus the periplasm, alter the range of potential interaction partners for PilC. Bioinformatics and topology studies of the PilC homologue BfpE—of the bundle-forming pilus system in *Escherichia coli*—suggested a four-TM topology, with an extra TM in the central

part of the protein (Blank et al., 2001). This data supports a configuration having a large periplasmic domain, which allows for potential interaction with periplasmic components. In *N. meningitidis,* a four- TM topology was also suggested based on hydropathy predictions, although results were not conclusive (Collins et al., 2007).

In contrast, fusion protein studies conducted by Thomas et al. and Arts and coworkers have provided evidence for a three-TM model, with the majority of the protein in the cytoplasm; the three-TM model predicts PilC to have two large cytoplasmic domains and two small periplasmic regions (Thomas et al., 1997; Arts et al., 2007). Although fusion studies have be not been conducted in *P. aeruginosa,* based on the results of hydropathy prediction programs such as TMHMM (http://www.cbs.dtuc.dk/servies/TMHMM) and the high levels of sequence similarity with proteins for which topology has been experimentally determined, we predict that PilC has three transmembrane domains, and two large cytoplasmic domains referred to the N-terminal and C-terminal domains **(Figure 2a)**. Comparisons of the two predicted cytoplasmic domains of T2S and T4P homologues showed that they have considerable primary sequence similarity (Abendroth et al., 2009). In *P. aeruginosa* there is 21% amino acid sequence identity (34% similarity) between the two cytoplasmic domains, the highest value noted by Abendroth et al. **(Figure 2c)** (Abendroth et al., 2009).

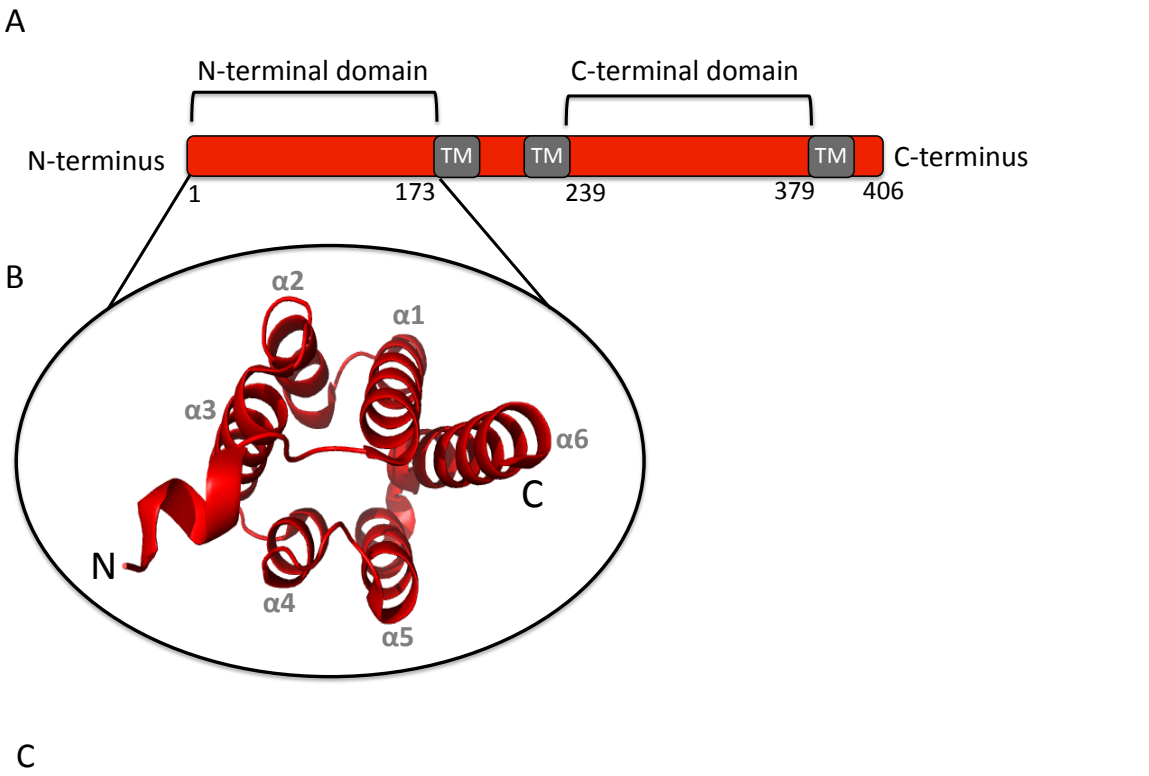

Figure 2A. Schematic diagram predicting cytoplasmic domains of PilC. The PilC protein is 406 amino acids and has two predicted cytoplasmic domains: the N-terminal domain (amino acids 1-173) the C-terminal domain (amino acids 239-379), and three predicted transmembrane domains (TM).

Figure 2B. **Crystal structure of the N-terminal domain of PilC in *Thermus thermophilus*.** One of the two monomers of six alpha helices of the first cytoplasmic domain of PilC forms a dimer in the unit cell of the crystal. There is no structural information for the C-terminal domain. Adapted from Karrupiah et al., 2010.

Figure 2C. Sequence alignment of the N and C-terminal domains of *Pseudomonas aeruginosa* PilC. Identical amino acids are shaded in black (21%) and similar amino acids are shaded in grey (34%). (Geneious alignment)

1.5.3 Structural Studies of PilC

The first low-resolution three-dimensional electron microscopy reconstruction of the PilC orthologue in *N. meningitidis* revealed a tetrameric molecule, which spans the inner membrane (Collins et al., 2007). The authors suggested that this protein could serve as a connection between the cytoplasm and periplasm in T4P and T2S systems (Collins et al., 2007). Abendroth and colleagues published a crystal structure of the N-terminal domain from the *V. cholerae* T2S system homologue, EpsF (Abendroth et al., 2009). The structure comprises two bundles of six alpha helices that form a dimer in the crystal. More recently, the crystal structure of an N-terminal fragment of PilC from *Thermus thermophilus* confirmed an architecture consisting of two bundles of six alpha helices; the authors also proposed that the native conformation of PilC might be a dimer of dimers **(Figure 2b)** (Karrupiah et al, 2010).

1.5.4 Interaction Partners of PilC

Evidence that PilC interacts directly with the ATPases would support its proposed role in the propagation of a conformational change mediating T4P assembly/disassembly. A review of the literature provides abundant indirect evidence of interactions between the putative platform protein and other components, particularly the polymerization ATPase PilB. Yeast two-hybrid studies conducted in T2S system of *E. chrysanthemi* and the T4P system of *E. coli* revealed interactions between the orthologues of PilC, specifically the N-

terminal domain and the assembly ATPase PilB (Py et al., 2001, Crowther et al., 2004). In *E. coli,* deletion of the platform protein destabilizes 12 of the 14 identified pilus proteins, an effect suggesting that it plays a central role in the stability of the system (Ramer et al., 2002). Studies in the T2S system of *P. aeruginosa* demonstrated that the putative platform protein stabilizes the assembly ATPase (Arts *et al.*, 2007). It was also reported in *P. aeruginosa* that the absence of PilC delocalizes the normally bipolar PilB protein (Chiang et al., 2005).

Only one study to date suggested an indirect interaction between the retraction ATPase BfpF (PilT) and BfpE (PilC) (Crowther et al., 2004). To support a role for BfpE in pilus retraction, Crowther and colleagues mutagenized the highly conserved residues D202, R203, P206, and W207 in the second cytoplasmic domain of BfpE, resulting in an auto aggregated or BfpF mutant-like phenotype (Crowther et al., 2004). Interestingly, these residues are also found in the C-terminal domain of PilC in *P. aeruginosa*. A corrigendum was recently published refuting the results obtained by site-directed mutagenesis (Milgotina et al., 2011). However, additional studies including over-expression of the second cytoplasmic domain of BfpE *in trans* produced dominant negative effects, and supported the original hypothesis that this domain potentially interacts with BfpF (Milgotina et al., 2011).

1.6 Significant Findings

Due to the discrepancy in the literature regarding the role of inner membrane sub-complexes, which exhibit a high degree of sequence and/or functional conservation among the T4P and T2S systems, a detailed examination of their dispensability in the T4P system of *P. aeruginosa* was proposed. Mutants of inner-membrane sub-complex consisting of PilN/O/P, and the putative platform protein PilC were generated in a retraction-deficient background. Disruption of these genes in combination with *pilT* (preventing pilus disassembly) allowed us to trap any assembled pili on the cell surface. Interestingly, the PilN/O/P-PilT double mutants were each able to assemble surface pili, suggesting that the inner-membrane sub-complex is not essential for T4P assembly in *P. aeruginosa*. In contrast, mutation of *pilC* alone, and in combination with *pilT,* produced non-piliated strains. These results confirm that PilC is an essential inner-membrane protein for T4P biogenesis in *P. aeruginosa.*

A discrepancy in the reported start site of PilC in the two major laboratory strains PAK and PAO1 was studied in *P. aeruginosa*. Using immunoblot analyses we determined the first start site is used preferentially, while the latter is likely the result of a misannotation. Interestingly, complementation of a *pilC* mutant with constructs using either start site restored wild-type twitching motility, suggesting the first 32 amino acids are dispensable for function in PilC.

This study also investigated the possible interactions between the N-terminal and C-terminal cytoplasmic domains of PilC with the ATPases PilB and

PilT, respectively. A potential interaction of the N-terminal domain with PilB was demonstrated by *in vitro* co-affinity purification. While intensive efforts to generate a soluble fragment of the C-terminal domain for *in vitro* interaction studies were unsuccessful, a potential interaction with PilT was uncovered using site directed mutagenesis. Residues in the C-terminal domain that were highly conserved in T4P but not T2S systems were mutagenized and the mutant proteins tested for function in the *P. aeruginosa pilC* mutant. The resulting mutant strains had wild-type levels of pilus assembly, yet exhibited reduced twitching motility. These results are suggestive of disruption of a potential interaction with PilT. In conclusion, our data supports an essential role for PilC in T4P polymerization and depolymerization. Furthermore, the highly conserved nature of PilC and its orthologues in both T4P and T2S systems makes it a promising target for therapeutic efforts.

CHAPTER 2- MATERIALS AND METHODS

2.1 Bacterial Strains, genetic manipulations and growth conditions

Bacterial strains and vectors used in this study are listed in **Table 1**. Growth media included Lauria-Bertani (LB), with or without agar (Invitrogen) or, where indicated, Pseudomonas Isolation Agar (PIA) (Difco). *P. aeruginosa* strains were grown on LB supplemented where indicated with antibiotics at the following concentrations: gentamicin, 30 mg/L or 100 mg/L, carbenicillin, 200 mg/L. *E. coli* strains were grown on LB supplemented where indicated with antibiotics at the following concentrations: gentamicin 15 mg/L, kanamycin 50 mg/L, ampicillin 100mg/L. All strains were grown at 37°C for the time indicated.

Null mutant construction

PAK *pilC*, *pilT/pilC, pilT/pilN, pilT/pilO, pilT/pilP, pilC/pilM, pilC/N, pilC/O, pilC/pilP,* mutant strains were generated using the strategy as previously described (Hoang *et al.*, 1998, Burrows *et al.*, 2000). *E. coli* SM10 cells were electroporated with the plasmid pEX18Ap containing the desired gene to be mutated (example *pilC::GmFRT)* and grown on LB agar plates supplemented with gentamicin (30 mg/L). Bi-parental mating was used to transfer the plasmid was into PAK wild type cells for single mutant creation, or the desired mutant background strain (example PAK *pilT*::FRT) for double mutant creation. Briefly, The strains were grown in 5mL LB broth supplemented with appropriate

antibiotics, at 37°C for 8 h. Subsequently, the liquid culture of the two strains were mixed in three (v/v) ratios, 1:1, 1:9, 9:1 in a total volume of 1mL. The mixture was centrifuged for 3 min at 1677x g and the cell pellet re-suspended in 100µL of the supernatant. Subsequently the re-suspended mixture of each ratio was divided into three aliquots (33.3 µl each) on LB agar plates that were incubated upright at 37°C for 16 h. One spot from each ratio was re-suspended in 1ml of LB broth and 100µl plated on *Pseudomonas* isolation agar (PIA) plates, supplemented with gentamicin (100 mg/L), and incubated for 16 h at 37°C to counter-select the *E. coli* donor. Individual colonies from the PIA plates were double-patched onto LB plates with gentamicin (30 mg/L) or carbenicillin (200 mg/L) and incubated at 37°C for 16h. Cells containing the gentamicin resistance cassette inserted into the gene grew only on gentamicin-supplemented plates. The gentamicin cassette was subsequently excised by the Flp recombinase encoded by pFLP2. The colonies that grew only on gentamicin were electroporated with pFLP2 and plated on carbenicillin plates. Subsequently, the colonies were replica-plated onto gentamicin and carbenicillin plates. Cells that underwent successful excision of gentamicin cassette grew only on carbenicillin. The pFLP2 plasmid was subsequently removed using sucrose counter selection. Cells retaining the FRT scar were verified by PCR amplification using oligonucleiotides whose sequences are listed in **Table 2**.

2.2 Generation of complementation constructs/plasmid transformations

Complementation Constructs

The gene of interest was amplified by PCR using PAK chromosomal DNA as a template. Subsequently, the amplicon and the vector were digested with compatible restriction enzymes (Fermentas). The digested DNA was PCR purified, followed by ligation using T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5α, and the cells were grown on LB agar plates supplemented with appropriate antibiotics at 37°C.

Plasmid Transformations

P. aeruginosa strains were transformed with plasmids of interest by electroporation (Dower et al. 1988). A small amount of freshly grown cells was washed and re-suspended in 1 ml of nuclease free water. In an electroporation cuvette, 100 μl of the re-suspended cells and 100 ng of plasmid DNA were mixed. The cells were electroporated at 2.5 kV while time constants remained above 4 ms. Cells were re-suspended in 1 ml LB for 3-5 hs at 37°C shaking at 220 rpm then plated on a 1.5% LB agar plate with appropriate antibiotics. Resulting colonies were re-streaked on the same medium for single colonies.

Chemically-competent *E. coli* strains were transformed by heat shock (Chung et al, 1989). Commercially prepared competent cells were thawed on ice and incubated with 100 ng of plasmid DNA for 30 min with gentle mixing. Cells were then immersed in a 42°C water bath for 30 s then cooled on ice for 15 min.

Cells were re-suspended in 1 ml of LB with shaking for 3-5 h to recover. Cells were then plated on a 1.5% LB agar plate with appropriate antibiotics and incubated overnight at 37°C. Colonies arising were re-streaked on the same medium for single colonies.

2.3 Twitching motility assays

Twitching motility assays were performed as previously described (Ayers et al., 2009). Briefly, single colonies were stab inoculated with a sterile 200µl pipette tip to the bottom of 1% agar plates, in triplicate. The plates were wrapped with Parafilm and incubated at 37°C for 36 h. Post incubation, the agar was carefully removed and adherent bacteria were stained with 1% crystal violet for 10 min, followed by a wash with tap water to remove unbound dye.

2.4 Analysis of sheared surface proteins

Sheared-surface protein analyses were performed as described previously (Voisin et al., 2007). Briefly, strains of interest were streaked in a grid-like pattern on LB agar plates and incubated at 37°C for approximately 14 h. Using glass cover slips, the cells were gently scraped from the plates and re-suspended in 4.5 ml of 1X PBS (Phosphate buffered saline, pH 7.4). Surface appendages were sheared from the cells by vortexing the cell suspensions for 30 s. Subsequently, the suspensions were transferred to 1.5ml eppendorf tubes and the cells were pelleted by centrifugation at room temperature for 5 min at 11,688 x g. The supernatant was transferred to fresh micro-centrifuge tubes and re-centrifuged at

room temperature for 20 min at 11,688 x g to remove remaining cells. The supernatant was transferred to a new micro-centrifuge tube and 1/10 volume of each of 5 M NaCl and 30% polyethylene glycol (molecular weight range, ~8000) was added. Soluble proteins were precipitated on ice for 90 min. The precipitated proteins were collected by centrifugation at 11, 688 x g at 4^{o}C for 30 min. The supernatant was discarded and the tubes were re-centrifuged for 3 min. The remaining supernatant was discarded and the precipitated-protein pellet was re-suspended in 150µl of 1X SDS sample buffer [80 mM Tris (pH 6.8), 5.3% (vol/vol) 2- mercaptoethanol, 10% (vol/vol) glycerol, 0.02% (wt/vol) bromophenol blue, and 2% (wt/vol) SDS]. The samples were boiled for 10 min and separated on 15% SDS-PAGE gels. Proteins were visualized with Coomassie brilliant blue (Sigma).

2.5 Preparation of Whole Cell Lysates

Whole cell lysates were made by diluting overnight cultures grown in LB with appropriate antibiotics where indicated to an OD_{600} of 0.6, then harvesting the cells from a 1 ml aliquot by centrifugation for 2 min at 11,688 x g in a micro-centrifuge. The pellet was re-suspended in 100µl of 1x SDS sample buffer [80 mM Tris (pH 6.8), 5.3% (vol/vol) 2- mercaptoethanol, 10% (vol/vol) glycerol, 0.02% (wt/vol) bromophenol blue, and 2% (wt/vol) SDS] and boiled for 10 min. The whole cell lysate samples were subsequently subjected to western blot analysis.

2.6 Western Blot Analysis

Whole cell lysate samples were separated on 15% acrylamide gels at 80-120V. Samples were transferred to nitrocellulose membranes at 225 mA for 1 hr. The membranes were blocked in 5% (w/v) skimmed milk in 1x PBS (pH 7.0) for 1 h followed by incubation in the appropriate antisera **(Table 3)** for 18 h at 4°C. The membranes were washed 3 times in 1x PBS for 5 min, and incubated in goat-anti-rabbit IgG AP secondary antibody (Bio-Rad) as per the manufacturer's protocol. The membranes were washed 3 times in 1x PBS for 5 min, visualized with alkaline phosphatase developing reagent (Bio-Rad), as per manufacturer protocol.

2.7 Expression & Purification of the N and C-terminal domains of PilC

Expression and Purification of the N-terminal Domain of PilC:

The *pET28b::pilC1-173aa* (N-terminal 6x His-tag) expression construct was transformed into *E. coli* BL21-DE3. One ml expression trials were performed to identify optimal conditions. It was determined that induction with 1 mM of isopropyl β-D-1 thiogalactopyranoside (IPTG) for 3h at an O.D of 0.6 led to a high level expression. Expression experiments were subsequently scaled up to one liter. Western-blot analysis using anti-His and anti-PilC antibodies confirmed the expression of the N-terminal domain of PilC and the in-frame incorporation of the purification tag**.** The cell pellet was re-suspended in lysis buffer containing 1% lauryldimethylamine-oxide (LDAO), 10ng/ml of DNase and RNase and 100mg/L

benzamidine prior to lysis via French press and centrifuged at 48,258 x g to separate the clarified lysate from the unlysed cells. The lysate was collected and applied to 10 ml Ni-NTA columns containing 1ml of Ni-NTA resin. The column was subsequently washed with 20 ml Ni+_buffer (20mM Tris pH 8.0, 250 mM KCl) , and 10ml Ni+ buffer containing 15 mM, 30 mM, 150 mM and 300 mM imidazole to elute the N-terminal domain of PilC + 6x-His.

Expression and Solubility Trials of the C-terminal Domain of PilC:

The *pET151::pilC243-374* (N-terminal 6x His-tag) expression construct was transformed into *E. coli* BL21-DE3. Expression trials were performed at 16°C overnight, 25°C for 5 h, and 37°C for 3 h at 0.1mM, 0.5mM, and 1mM IPTG to determine the optimal conditions for protein expression. Induction with 1 mM of IPTG for 3 h at 37°C was optimal for the expression of the C-terminal domain of PilC.

The cell pellets were re-suspended in 20mM Tris pH 8.0, 1.4mM β-mercaptoethanol, 1mg/ml of lysozyme, 0.5M NaCl, and 0.05% LDAO. The mixture was incubated on ice for one hour and centrifuged at 13,000x g for 20 min. The supernatant was separated from the pellet, and the fractions were mixed with equal volumes of 2x SDS sample buffer, separated on a 12.5% SDS-PAGE gel at 150V for 1 h, and stained with Coomassie brilliant blue.

The lysis method of the C-terminal domain was scrutinized to determine if this parameter contributed to the observed insolubility. Instead of the detergent

based lysis method described above, sonication was used to disrupt the cell membranes. Briefly, the cell pellet from a 1L expression trial in *E. coli* BL21-DE3 was re-suspended in lysis buffer containing 1% lauryldimethylamine-oxide (LDAO), 10ng/ml of DNase and RNase and 100mg/L benzamidine prior to lysis. The cells were sonicated on ice with alternating 10 s on and 10 s off periods for a total on time of 2 min and cell debris separated at 20,000 x g for 45 min. The supernatant was then collected and the fractions were mixed with equal volumes of 2x SDS sample buffer, separated on a 12.5% SDS-PAGE gel at 150V for 1 h, and stained with Coomassie brilliant blue.

2.7 In vitro co-affinity purification

The N-terminal domain of PilC was expressed and purified as described above. The 150mM and 300mM imidazole elution fractions were collected and dialyzed in 1x PBS overnight at 4°C. The dialyzed protein was concentrated to 2μg/ml and incubated with 1 ml of Ni-NTA resin overnight at 4°C. The PAK strain of *P. aeruginosa* was streaked out on a 1.5% LB agar plate in a grid-like fashion and grown over night at 37°C. The bacterial cells were harvested by scraping with a sterile cover slip and re-suspended in 1x PBS containing 100mg/L benzamidine, 10ng/ml of DNase and RNase. The cells were sonicated on ice with alternating 10 s on and 10 s off periods for a total on time of 2 min. The cell lysates were centrifuged for 15 min at 3000x g to separate unlysed cells. The supernatant was mixed with purified bait protein + Ni-NTA resin for 2 hours at

4°C. The mixture was then applied to a pre-equilibrated gravity flow 10 ml nickel column and the flow-through was collected. Washes and elution fractions of 10 ml were collected: 1x PBS, 5 mM imidazole elution, and 300mM imidazole elution. All fractions were used for SDS – PAGE and western blot analysis as described above.

2.8 Circular dichroism spectroscopy

Circular dichroism spectroscopy was used to examine the secondary structure composition of the purified N-terminal domain of PilC 1. Briefly, the N-terminal domain was expressed and purified as described above. The 150mM and 300mM imidazole elution fractions were collected and dialyzed in 1x PBS overnight at 4°C. The protein sample was adjusted to a final concentration of 0.45 μg/ml and 0.23μg/ml prior to the assay. Absorbance was measured at intervals of 1 nm from 190 nm to 260 nm, with a data collection time of 3 s. The temperature of the sample chamber was set to 25°C. Absorbance values were transferred to Microsoft Excel where the data was normalized, averaged and converted to molar elipticity [θ] using the formula below. Control spectra containing buffer alone were collected and subtracted from the sample spectra to account for background absorbance.

[θ]=(Absorbance/1000)/[(Concentration (mg/mL)/Molecular Mass (Da)/1000)x #Amino acids]

2.9 Site-directed mutagenesis

Site-directed mutagenesis (SDM) of PilC was performed using the QuikChange Site-Directed Mutagenesis Kit (Strategene) according to the manufacturers protocol using the oligonucleotides listed in **Table 2**. Mutagenesis was performed on a pUCP20Gm::*pilC* plasmid template DNA with an annealing temperature of 55°C with an extension time of 16 min for 18 cycles. SDM reactions were digested with 1µl of 10U/µl DpnI (Fermentas) restriction endonuclease and incubated for 2 h in a 37°C water-bath. Digestion reactions were then transformed into chemically-competent *E. coli* DH5α cells that were subsequently grown overnight at 37°C on 1.5% LB agar plates supplemented with appropriate antibiotics. Mutations were verified by DNA sequencing (MOBIX; Hamilton, ON, Canada). Mutant constructs were electroporated into PAK *pilC* and *pilT/pilC* mutant strains, and transformants were selected by plating onto 1.5% LB agar supplemented with appropriate antibiotics. Phenotypes were analyzed using a twitching motility assay and sheared surface pilin preparations.

CHAPTER 3- RESULTS

3.1 The inner membrane sub-complex *pilN/O/P* genes are not essential for T4P assembly in *P. aeruginosa.*

The components of the PilM/N/O/P inner membrane sub-complex were previously shown to be required for pilus biogenesis in a number of bacteria including *Pseudomonas*, *Myxococcus*, *Thermus,* and *Neisseria* (Martin et al., 1995, Ayers et al., 2009, Nudleman et al., 2006, Rumszauer et al., 2006, Carbonnelle et al., 2006). To investigate the essentiality of the inner membrane sub-complex for T4P biogenesis in *P. aeruginosa*, we disrupted *pilN*, *pilO* and *pilP* in combination with the retraction ATPase gene, *pilT*. Sheared surface protein preparations were used to assess single, double, and complemented mutant strains for the ability to express surface pili **(Figure 3 A, B, C).** Results from single mutants confirmed previous reports that the absence of any of the *pilN/O/P* genes prevents surface pilus expression (Ayers et al., 2009). However, the simultaneous disruption of any of the *pilN/O/P* genes in combination with *pilT* resulted in the production of recoverable surface pili, comparable to levels observed for the wild type, but less than that observed for the *pilT* control. Complementation of double mutants with either *pilT* or the relevant *pilN*, *pilO* or *pilP* gene restored the expected single mutant phenotypes, and immunoblot analyses of whole cell lysates samples confirmed expression of PilN/O/P and PilT *in trans.* These data suggest that PilN, PilO and PilP are not essential for T4P

biogenesis in *P. aeruginosa,* although in their absence, pilus assembly is impaired.

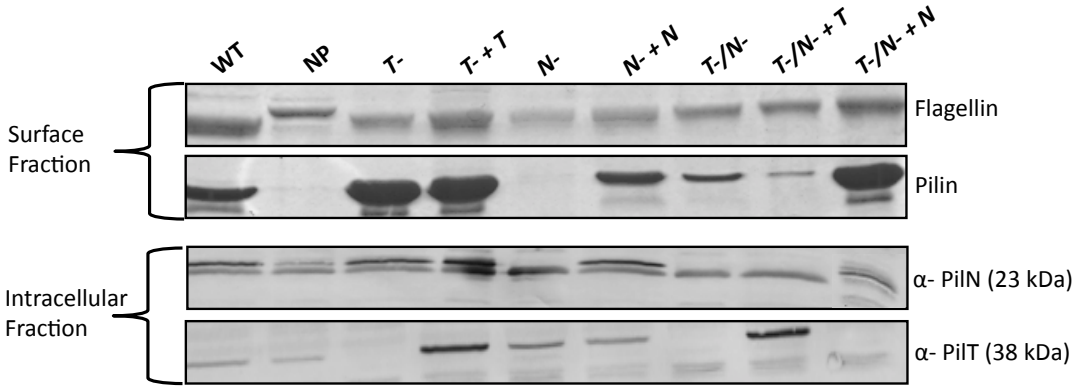

Figure 3 A: PilN is not essential for surface pilus expression in *P. aeruginosa*. i) Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*T-* (*pilT*::FRT+pUCP20Gm), *T- +T* (*pilT*::FRT+pUCP20Gm::*pilT*), *N-* (*pilN*::FRT+pUCP20Gm), *N- +N* (*pilN*::FRT+pUCP20Gm::*pilN*), *T-/N-* (*pilT*::FRT/*pilN*::FRT+pUCP20Gm), *T-/N-* +T (*pilT*::FRT/*pilN*::FRT+pUCP20Gm::*pilT*), *T-/N-+N* (*pilT*::FRT/*pilN*::FRT+pUCP20Gm::*pilN*). Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control. **ii)** Western blot analysis was conducted with intracellular fractions of the above strains with anti-PilN and anti-PilT antibodies.

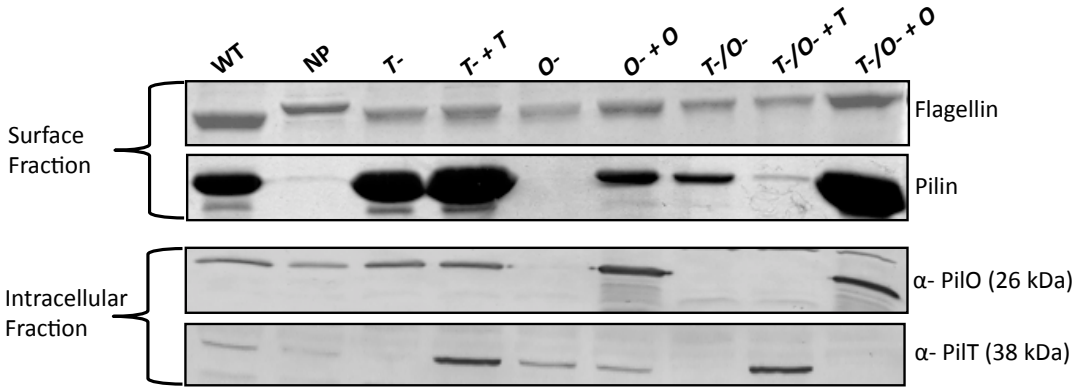

Figure 3 B: PilO is not essential for surface pilus expression in *P. aeruginosa*. i) Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*T-* (*pilT*::FRT+pUCP20Gm), *T- +T* (*pilT*::FRT+pUCP20Gm::*pilT*), *O-* (*pilO*::FRT+pUCP20Gm), *O- +O* (*pilO*::FRT+pUCP20Gm::*pilO*), *T-/O-* (*pilT*::FRT/*pilO*::FRT+pUCP20Gm), *T-/O-* +T (*pilT*::FRT/*pilO*::FRT+pUCP20Gm::*pilT*), *T-/O-+O* (*pilT*::FRT/*pilO*::FRT+pUCP20Gm::*pilO*). Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control. **ii)** Western blot analysis was conducted with intracellular fractions of the above strains with anti-PilO and anti-PilT antibodies.

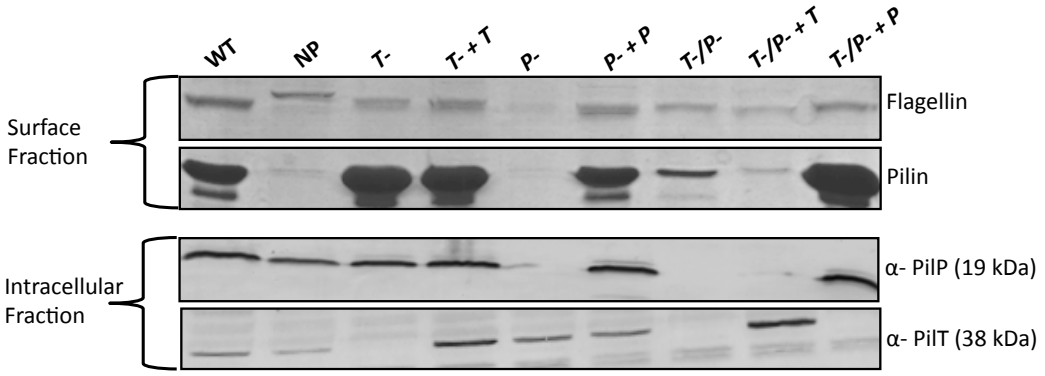

Figure 3 C: PilP is not essential for surface pilus expression in *P. aeruginosa*. i) Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm), *T-* (*pilT*::FRT+pUCP20Gm), *T- +T* (*pilT*::FRT+pUCP20Gm::*pilT*), *P-* (*pilP*::FRT+pUCP20Gm), *P- +P* (*pilP*::FRT+pUCP20Gm::*pilP*), *T-/P-* (*pilT*::FRT/*pilP*::FRT+pUCP20Gm), *T-/P-* +T (*pilT*::FRT/*pilP*::FRT+pUCP20Gm::*pilT*), *T-/P-+P* (*pilT*::FRT/*pilP*::FRT+pUCP20Gm::*pilP*). Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control. **ii)** Western blot analysis was conducted with intracellular fractions of the above strains with anti-PilP and anti-PilT antibodies.

3.2 The putative platform protein PilC is essential for surface piliation in *P. aeruginosa.*

Based on its level of conservation in T4P and T2S systems, PilC has been suggested to be essential for assembly of the pilus and pseudopilus (Nunn *et al.*, 1990; Hobbs *et al.*, 1993; Tonjum *et al.*, 1995). Conversely, studies in *N. meningitidis* suggest that its PilC orthologue is dispensable for pilus assembly (Carbonelle et al., 2006). Given this discrepancy, we determined the essentiality of PilC in the T4P system of *P. aeruginosa* by generating a *pilT/pilC* double knockout and examining its surface piliation **(Figure 4).** In contrast to *Neisseria,* the *pilC* single and double mutant strains were devoid of surface pili. Complementation of the *pilC* mutant and *pilT/pilC* mutant strains with PilC *in trans* restored surface pilus expression. Based on these results, it is clear that PilC is essential for pilus assembly in *P. aeruginosa.*

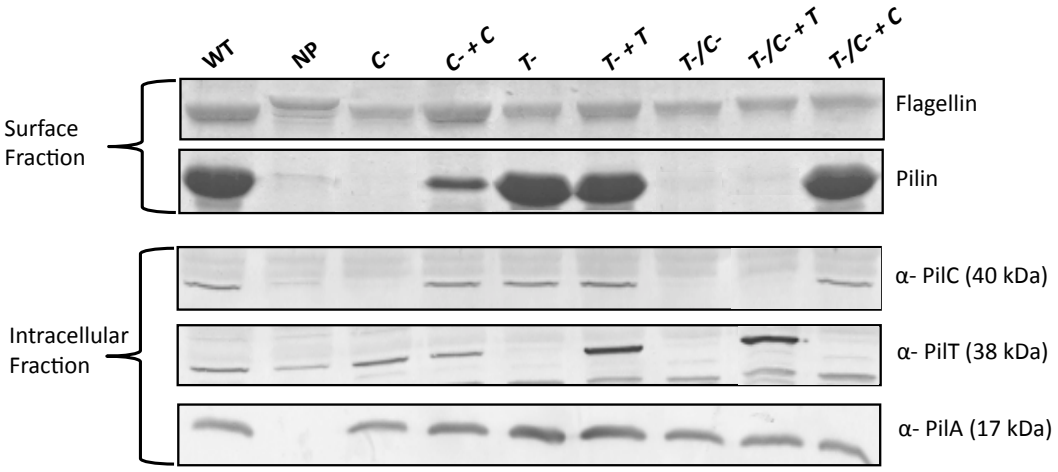

Figure 4. PilC is essential for surface pilus expression in *P. aeruginosa*. **i)** Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*C*- (*pilC*::FRT+pUCP20Gm), *C*- +*C* (*pilC*::FRT+pUCP20Gm::*pilC*), *T*- (*pilT*::FRT+pUCP20Gm), *T*- +*T* (*pilT*::FRT+pUCP20Gm::*pilT*), *T-/C*- (*pilT*::FRT/*pilC*::FRT+pUCP20Gm), *T-/C*- +T (*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilT*), *T-/C*-+*C* (*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilT*). Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control. **ii)** Western blot analysis was conducted with intracellular fractions of the above strains with anti-PilT, anti-PilT, and anti-PilA antibodies.

3.3 Both start sites of PilC produce functional proteins but the first is used *in vivo*

Analysis of the predicted protein sequences of PilC from two closely related *P. aeruginosa* strains, PAK and PAO1, showed a discrepancy in their lengths. The start site in PAK PilC was observed to begin 32 amino acids (96base pairs) before PAO1 PilC. Therefore, two potential start sites have been proposed for this protein **(Figure 5)**. Sequence alignments of the PAK and PAO1 PilC sequences with homologous platform proteins from T4P and T2S systems suggested that the first start site, annotated in the PAK sequence, was potentially correct as other proteins were similar in length to the longer version of PilC (data not shown).

To determine which start created a functional product, we created two complementation constructs, each using one of the two potential start sites named PilC (full length) or PilC (truncated) corresponding to the PAK and PAO1 **(Figure 5)**. Immunoblot analysis of the wild type, *pilC* mutant and complemented mutant strains, in PAK and PAO1, showed that both wild type strains produced a single product with a mass that corresponded to use of the first start site (43 kDa), compared to the smaller product (38 kDa) expressed from the construct using proposed second (PAO1) start site **(Figure 6A)**. Interestingly, twitching motility assays revealed that PilC expressed from either construct was capable of restoring pilus function to wild type levels **(Figure 6B).** Therefore, we suggest

that the first start site is used *in vivo,* and that the first 32 amino acids located in the N-terminal domain of PilC are not required for function.

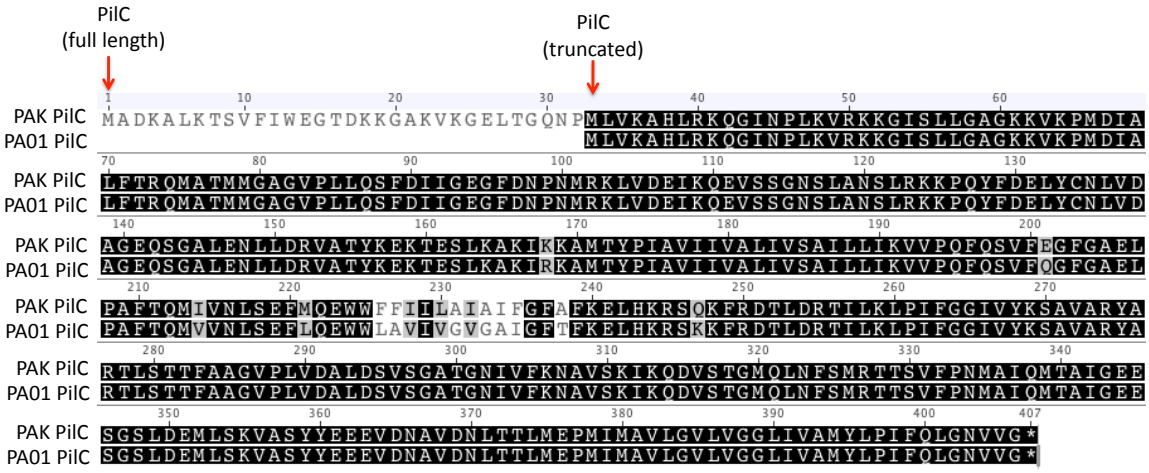

Figure 5. PilC protein sequence from PAK and PAO1 strains of *P. aeruginosa*. Protein alignment of the submitted PilC sequences in PAK (Nunn *et al.*, 1990) and PAO1 (www.pseudomonas.com). Identical amino acids are shaded in black, similar amino acids are shaded in grey. The start site of PAK PilC is annotated at position 1 and start site for PAO1 is annotated 32 amino acids later. The start sites of the constructs are highlighted with red arrows at amino acid position 1 (PilC-full length), and position 32 (PilC truncated). Alignment was performed using Geneious.

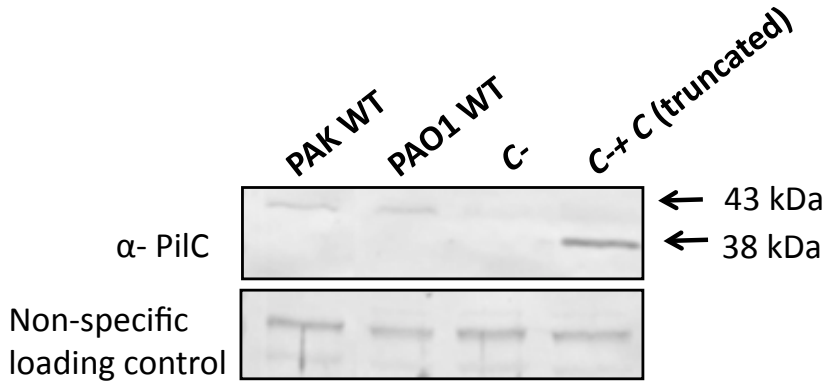

Figure 6A. The first start site of PilC is preferred in both PAK and PAO1 strains of *P. aeruginosa*.
Immunoblot analyses of whole cell lysates from the wild type PAK, wild type PAO1, *C-* (*pilC::FRT*) and C-+C [pilC::FRT+pUCP20Gm::PilC (truncated)] complemented mutant strains of *P. aeruginosa.* Samples were probed with PilC antisera. A non-specific band was used as a loading control.

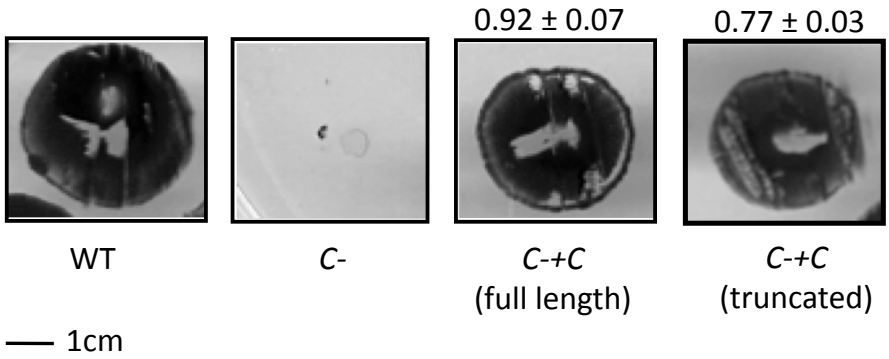

Figure 6 B. Both start sites of PilC are able to restore twitching motility. Twitching motility assay of the wild type (WT), *C-* (*pilC::FRT*) and *C-+C* complemented using constructs with the start site corresponding to the PAK (PilC full length) or PAO1 (PilC truncated) start sites. Fold changes in twitching zone area, compared to wild type, are shown above each complemented twitching zone. This assay was performed in triplicate.

3.4 The N-terminal domain of PilC interacts with the ATPase PilB

3.4.1 The absence of *pilC* has a negative impact on the stability of PilB

We tested whether the members of the PilM/N/O/P sub-complex and the platform protein, PilC affected one another's stability. Immunoblot analyses using whole cell lysates and specific antisera for the proteins of interest showed no changes in stability of PilM/N/O/P in a *pilC* mutant background, and vice versa (data not shown).

We also examined the stability of the T4P ATPases in the absence of PilC. Results of immunoblot analyses suggest that the absence of PilC destabilizes the assembly ATPase PilB. However, the levels the retraction ATPase PilT was observed to be wild type **(Figure 7)**. Also, the simultaneous disruption of *pilC* and any of either *pilM/N/O/P* resulted in ATPase levels that were comparable to those observed in the *pilC* single mutant (data not shown), suggesting that of the components tested, PilC is the only one required for PilB stability.

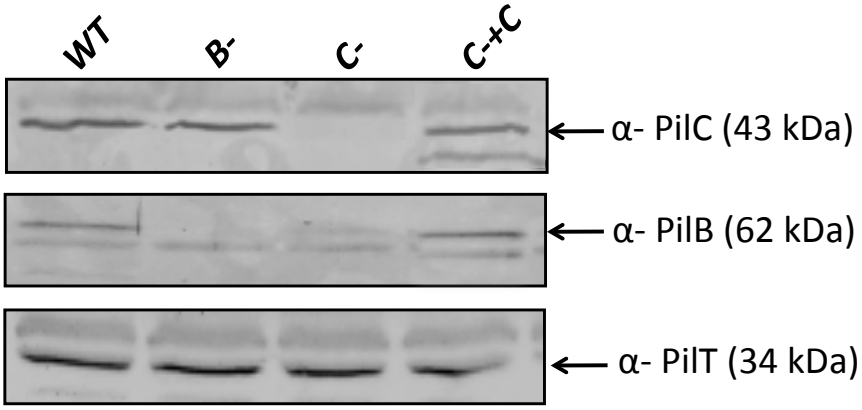

Figure 7. In the absence of PilC intracellular levels of PilB are reduced. Immunoblot analysis of whole-cell lysates of the WT (PAK wild type), *B- (pilBΩ), C- (pilC::FRT)* mutants and complemented *C-+C* (*pilC::FRT+pUCP20Gm::*PilC*)* strains of *P. aeruginosa* probed with anti-PilC, PilB, and PilT antiserum.

3.4.2 The N-terminal domain of PilC and polymerization ATPase PilB co-purify

For co-purification experiments, the N-terminal domain of PilC was expressed and purified as described in the Methods section, and circular dichroism (CD) was conducted to confirm its predicted alpha helical secondary structure. An expression construct encoding the N-terminal cytoplasmic domain was created using boundaries based on predictions from the hydropathy prediction program TMHMM (http://www.cbs.dtuc.dk/servies/TMHMM) and boundaries previously used for orthologous platform proteins. Following expression, the fragment was purified and visualized by SDS-PAGE analysis **(Figure 8).**

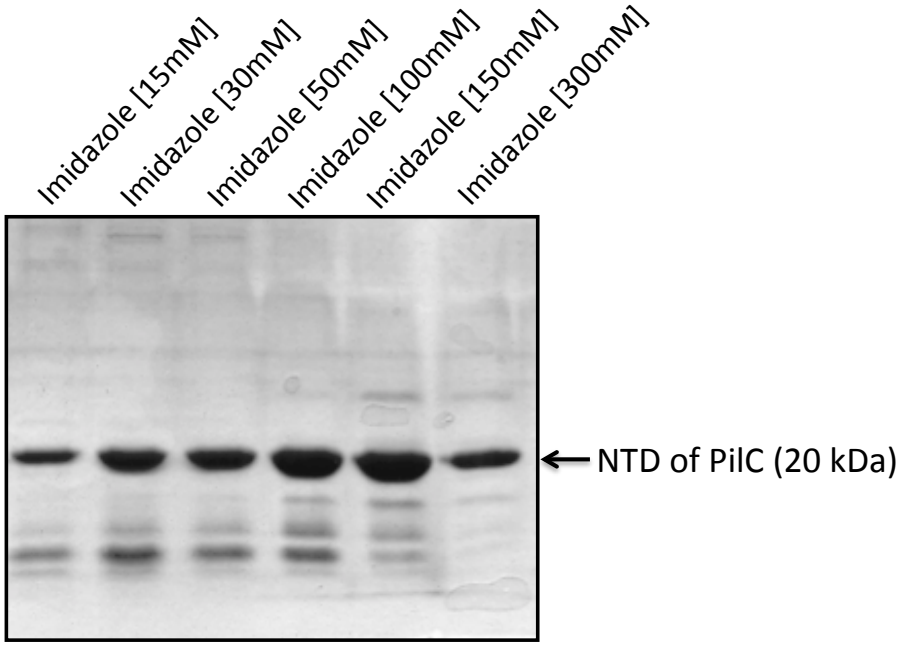

Figure 8. Purification of the N-terminal domain of PilC using nickel chromatography. Purification of the N-terminal cytoplasmic domain of PilC + N-terminal 6x-Histadine tag. Bound proteins were eluted with 15, 30, 50, 100, 150, 300mM imidazole. Elution fractions were separated on a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue.

The secondary structure content of the purified fragment of the cytoplasmic N-terminal domain was analyzed using circular dichroism spectroscopy. As predicted by bioinformatics analyses, the N-terminal domain of PilC gave a CD spectra characteristic of alpha helical proteins **(Figure 9)**, with minima of at -6000 θ at 208 and -5800 at 222 nm.

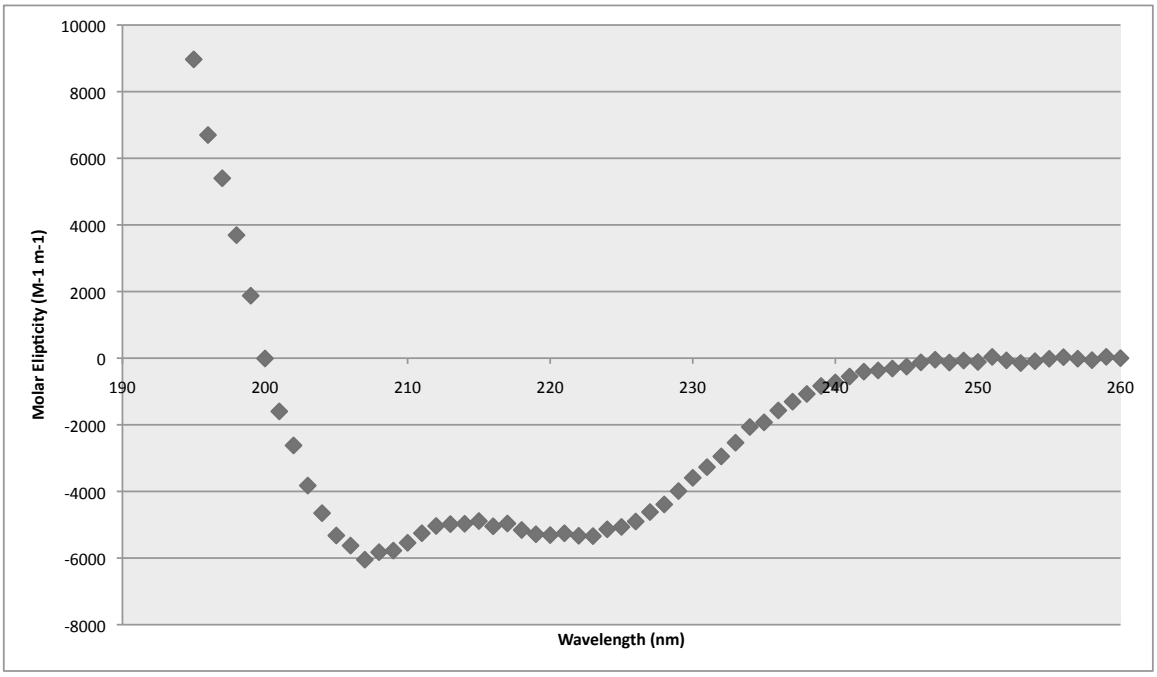

Figure 9. Circular dichroism spectroscopy analysis of the N-terminal domain PilC shows alpha helical content. Averaged curve (protein concentrations of 0.45mg/ml and 0.23mg/ml) illustrating alpha-helical fold of purified N-terminal domain of PilC+ 6xHis. Local minima were found at 222 and 208nm characteristics of alpha helix.

In vitro co-affinity purification was performed to determine potential interaction partners of the N-terminal cytoplasmic domain of PilC. An N-terminal 6x-His tag was used to immobilize this domain on charged nickel resin after incubation with whole cell lysates of strain PAK. Results show that PilB was located in the bound fraction at the highest concentration of imidazole **(Figure 10),** while the other ATPases PilT and PilU were not present in the final elution fraction (data not shown). To eliminate false positives due to non-specific binding, a control experiment in which the bait protein (N-terminal domain + 6x-His) was omitted was conducted in tandem**.** PilB was not detected in the 300mM imidazole elution fraction in the absence of the N-terminal domain of PilC **(Figure 10)**.

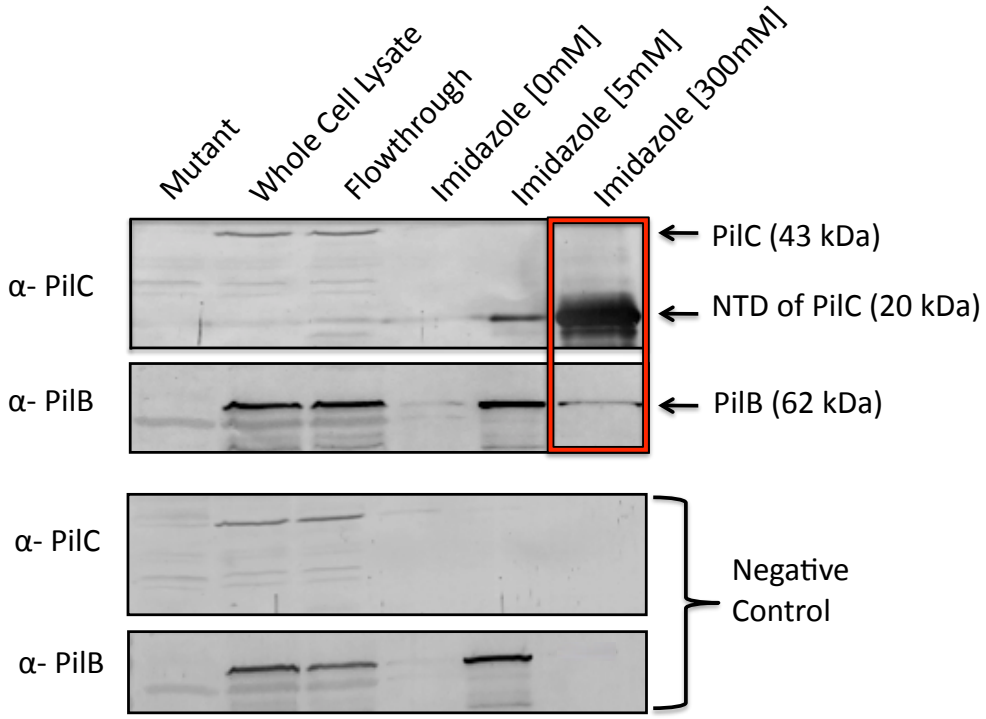

Figure 10. *In vitro* co-affinity purification of the N-terminal domain of PilC and PilB. Western blot analysis conducted on mutant (*pilB::*FRT or *pilC*::FRT*),* *P. aeruginosa* whole cell lysates*,* column flow-through, and imidazole wash fractions (0mM, 5mM, 300mM) were probed with anti-PilC and PilB antiserum. The negative-control experiment was conducted in the absence of the N-terminal domain of PilC.

3.5 The C-terminal domain of PilC potentially interacts with the ATPase PilT

Studies performed on the second cytoplasmic domain of BfpE in *E. coli* suggested this region is functionally important for retraction of the pilus (Crowther *et al.,* 2004, Milgotina et al., 2011). Apart from this evidence, no other studies, structural models, or interaction partners have been proposed for the C-terminal cytoplasmic domain of PilC or its orthologues. Extensive attempts at generating a soluble fragment of this domain for *in vitro* co-affinity purification experiments, including generation of constructs with different boundaries, use of various expression and lysis conditions, and refolding of protein from inclusion bodies, were ultimately unsuccessful (data not shown). Therefore, *in vivo* site directed mutagenesis (SDM) of the C-terminal domain in full length PilC was performed, and the consequences of the mutations tested by complementation of a *pilC* mutant.

Residues of interest (**Table 4)** were selected from sequence alignments of the C-terminal cytoplasmic domains of putative T4P and T2S system platform proteins in various species **(Figure 11)**. Residues that were conserved in both the T4P and T2S systems were omitted for two main reasons. First, the T2S system has no known retraction ATPase, indicating the conserved residues would be less likely to be involved in pilus retraction. Second, conserved residues in both the T4P and T2S system may be structurally, rather than functionally, important.

Table 4. Residues of interest in the C-terminal domain of PilC for paired site directed mutagenesis

Residues of interest for paired mutations	Mutant obtained? (Y/N)*
R245D R250D	Y
D254K K259D	Y
D254A R255D	N
R274D R277D	Y
D291K D294K	Y
F324A R327D	Y
E351K K355D	N
*Y represents mutant was obtained, N represents mutant was not obtained.	

1.T4P *Thermus thermophilus* (PilC)
2.T4P *Pseudomonas aeruginosa* (PilC)
3.T4P *Neisseria meningitidis* (PilG)
4.T4P *Escherichia coli* (BfpE)
5.T2S *Xanthomonas campestris* (XpsF)
6.T2S *Pseudomonas aeruginosa* (GspF)
7.T2S *Aeromonas hydrophila* (ExeF)
8.T2S *Erwinia chrysanthemi* (OutF)
9.T2S *Klebsiella pneumoniae* (PulF)
10.T2S *Vibrio cholerae* (EpsF)

Figure 11. Sequence alignment of the second cytoplasmic domains of putative platform proteins in the T4P and T2S systems of multiple bacteria species. Platform proteins from the T4P and T2S systems were aligned based off sequence identity. Residues are colored based on level of conservation. Red boxes highlight reputed functionally conserved residues shared between *P. aeruginosa* PilC and *E. coli* BfpE. Green boxes highlight residues uniquely conserved between T4P platform proteins also chosen for mutagenesis.

A structural model of the C-terminal cytoplasmic domain was required to determine whether the residues of interest were likely to be surface exposed and thus available for potential interactions. Although no structural studies of this domain have yet been reported (which is not surprising given its intractability), the sequence similarity between the N-terminal and C-terminal domains allows homology modeling using the N-terminal fragment structure of PilC from *T. thermophilus* as a template (Karrupiah *et al.,* 2010). Using the structural modeling algorithm I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), a model of the C-terminal domain of *P. aeruginosa* was generated and location of the residues selected for mutagenesis **(Table 4)** were mapped **(Figure 12).** All residues were substituted to the opposite charge or in the case of hydrophobic amino acids to alanine, and mutagenesis of codons was completed in pairs for rapid screening.

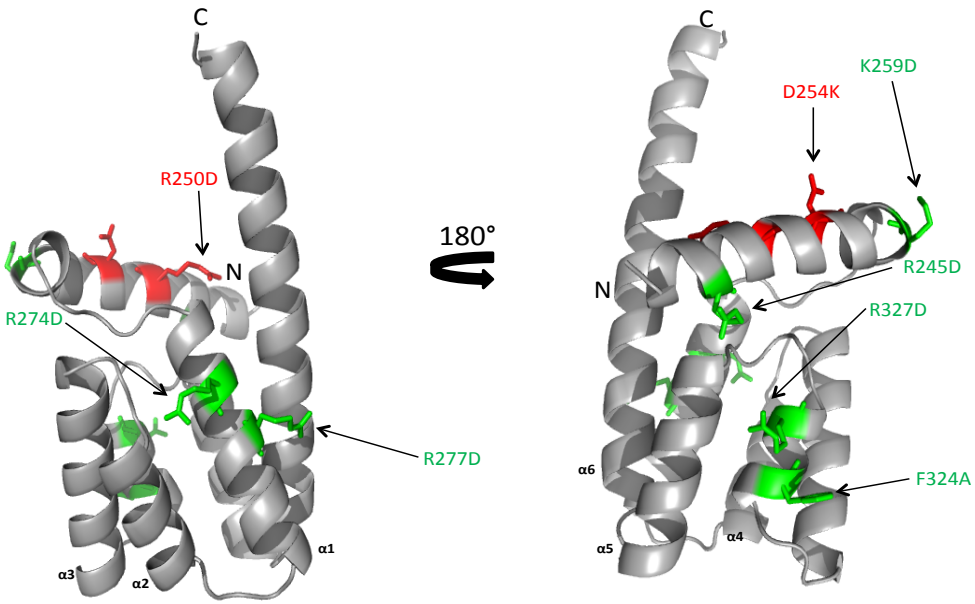

Figure 12. Structural Model of the C-terminal domain of PilC with amino acids targeted for site-directed mutagenesis. The C-terminal domain of PilC in *P. aeruginosa* was modeled using I-TASSER structural prediction algorithm (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Surface exposed residues of interest were mapped on the model and targeted for site-directed mutagenesis. Residues conserved with *E. coli* BfpE are highlighted in red. Additional resides identified by sequence analysis between platform proteins in the T4P and T2S systems are highlighted in green. Residues are to be mutagenized to the opposite charge or alanine.

The ability of each mutant protein to restore both T4P production (a measure of assembly) and twitching (a measure of retraction) was tested in a *pilC* deficient strain **(Figure 13 A, B).** Immunoblot analysis was used to determine the intracellular levels of PilC, PilT, PilA in the *pilC* + PilC SDM strains **(Figure 13 C).**

Mutations in the C-terminal domain of PilC, the domain potentially involved in pilus retraction, caused a reduction in twitching motility compared to wild type in all mutants except for F324A-R327D. The F324A-R327D mutant had wild type levels of twitching motility. Of the remaining mutants, R274D-R277D exhibited the most detrimental effect on twitching motility **(Figure 13 A)**.

All site direct mutations caused some degree of PilC instability based on the reduced amount of protein detected on immunoblots; however, the amount of surface-exposed pili in the SDM mutants was comparable to the *P. aeruginosa pilC* mutant complemented with wild type PilC, suggesting that pilus assembly was not affected by the mutations **(Figure 13 B)**. Intracellular levels of PilA were comparable to wild type. To ensure the twitching results were not due to decreased PilT levels in the mutants, we confirmed via immunoblot analsyis that intracellular levels of PilT were comparable to wild type in the PilC mutants **(Figure 13 C)**. This result confirms that stability of PilT is not dependent on levels of PilC.

A

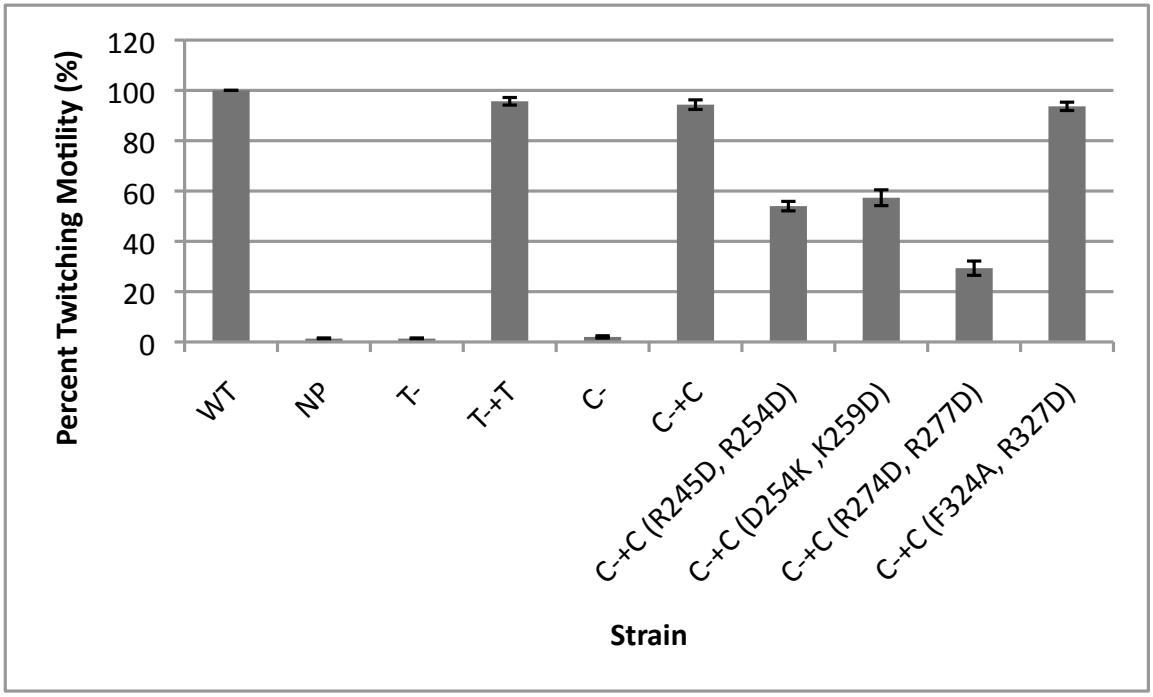

Figure 13 A. Specific mutations in the C-terminal domain of PilC reduce twitching motility in *P. aeruginosa*.
Mutations of interest were introduced into the C-terminal domain of PilC using site directed mutagenesis. The following strains:
WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*T-* (*pilT*::FRT+pUCP20Gm), *T- +T* (*pilT*::FRT+pUCP20Gm::*pilT*), *C-* (*pilC*::FRT+pUCP20Gm), *C- +C* (*pilC*::FRT+pUCP20Gm::*pilC*), *T-/C-* (*pilT*::FRT/*pilC*::FRT+pUCP20Gm), *C*-+C (R245D, R250D) [*pilC*::FRT+pUCP20Gm::*pilC*(R245D, R250D)],*C*-+C (D254K, K259D) [*pilC*::FRT+pUCP20Gm::*pilC*(D254K, K259D)], *C*-+C (R274D, R277D) [*pilC*::FRT+pUCP20Gm::*pilC*(R274D, R277D)], *C*-+C (F324A, R327D) [*pilC*::FRT+pUCP20Gm::*pilC*(F324A, R327D)],were stab inoculated, in triplicate, onto 1% agar twitching plates and incubated for 36h at 25 degrees centigrade. Twitching zones were visualized by crystal violet stain. Twitching motility of the mutants was compared to various control strains including WT normalized to 100% twitching and a *pilC* mutant complemented with PilC.

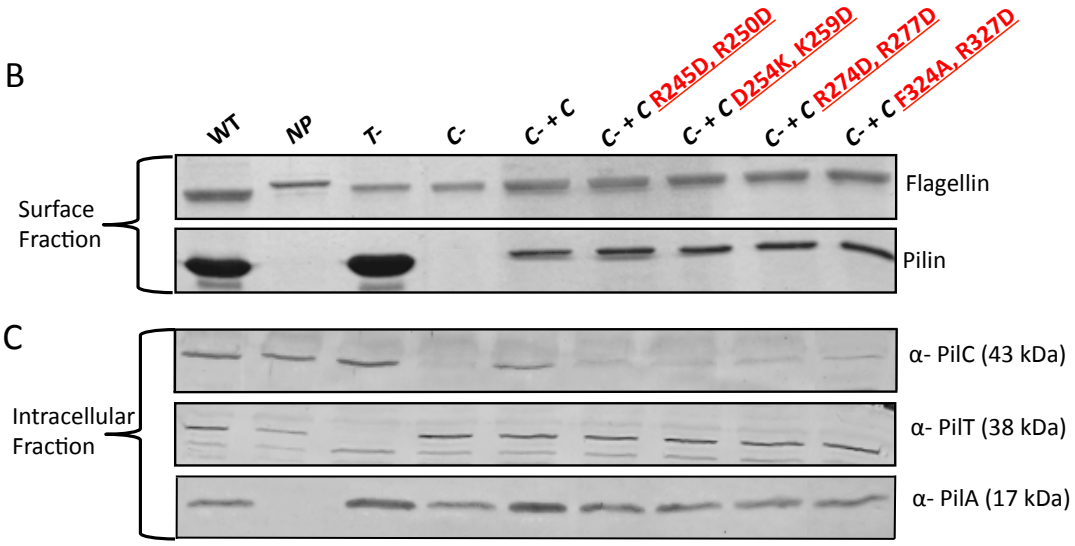

Figure 13 B. Impact of C-terminal domain mutations on surface piliation. Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*T*- (*pilT*::FRT+pUCP20Gm), *C*- (*pilC*::FRT+pUCP20Gm),and complemented strains: *C*-+C(R245D,R250D)[*pilC*::FRT+pUCP20Gm::*pilC*(R245D,R250D)],*C*-+C (D254K, K259D) [*pilC*::FRT+pUCP20Gm::*pilC*(D254K, K259D)],*C*-+C (R274D,R277D) [*pilC*::FRT+pUCP20Gm::*pilC*(R274D,R277D)],*C*-+C(F324A, R327D)[*pilC*::FRT+pUCP20Gm::*pilC*(F324A, R327D)]
Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control.

Figure 13 C. The stability of the T4P biogenesis machinery in C-terminal domain mutants. Immunoblot analysis of whole-cell lysates of the strains listed in Figure 13 B probed with anti-PilC, PilB, PilT and PilA antiserum. Site directed mutants were compared to control strains such as WT, and pilC mutant complemented with PilC.

To ensure the effects on twitching motility were not due to impaired pilus assembly, the mutant constructs were introduced into the *pilT/pilC* double mutant **(Figure 14)**. The results suggest pilus assembly is unaffected, as the PilC mutants conferred hyperpiliated phenotypes identical to the double mutant complemented with the wild type protein. These data confirm that the reduced intracellular levels of mutant PilC proteins (**Figure 13 C)** are sufficient for PilC mediated assembly. Therefore, the observed decreases in twitching motility are more likely to be due to specific effects of the point mutations on pilus retraction.

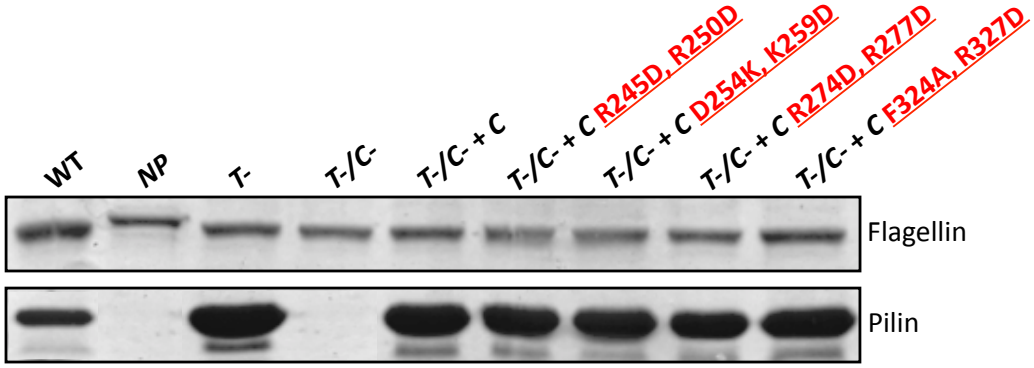

Figure 14: The C-terminal domain mutants of PilC can assemble surface exposed pili. Sheared surface preparation of WT (PAK WT+pUCP20Gm), NP (*pilA*::FRT+pUCP20Gm),*T*-(*pilT*::FRT+pUCP20Gm),*T-/C* (*pilT*::FRT/*pilC*::FRT+pUCP20Gm),and complemented mutant strains: *T-/C*-+C (*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilC*) *T-/C*-+C(R245D,R250D)[*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilC*(R245D, R250D)],*T-/C*-+C (D254K, K259D)[*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilC*(D254K, K259D)],*T-/C*-+C (R274D,R277D)[*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilC*(R274D, R277D)],*T-/C*-+C (F324A, R327D)[*pilT*::FRT/*pilC*::FRT+pUCP20Gm::*pilC*(F324A, R327D)], Samples were separated on a 15% SDS-PAGE gel, followed by Coomassie staining. The flagellin band was used as a loading control.

CHAPTER 4-DISCUSSION

4.1 The T4P system of *P. aeruginosa* potentially functions through the coordinated activities of two inner membrane sub-complexes.

The data presented in this study suggests that *P. aeruginosa* contains two inner membrane sub-complexes that work together in the T4P system **(Figure 1)**. The putative platform protein, PilC, and the assembly and disassembly ATPases— PilB and PilT, respectively—make up the "motor" component. The motor sub-complex works with a second sub-complex, consisting of the PilM/N/O/P proteins, that is suggested to align the motor with the outer membrane secretin, and to control secretin dynamics (Ayer et al., 2009).

The data presented suggests that PilC is necessary to coordinate the activity of the assembly ATPase (Py et al., 2001, Arts et al., 2007). Furthermore, in *E. coli,* the PilT (BfpF) orthologue was hypothesized to interact with the platform protein PilC (BfpE) (Crowther et al., 2004, Milgotina et al., 2011). Here we show that in *P. aeruginosa,* the N-terminal cytoplasmic domain of PilC interacts with assembly ATPase PilB, while the C-terminal cytoplasmic domain may interact with the retraction ATPase PilT.

4.2 The platform protein PilC is required for T4P assembly and disassembly in *P. aeruginosa*.

4.2.1 Proposed mechanisms of energy transduction

Although the mechanism of T4P assembly and disassembly remains elusive, models can be used to predict the complex workings of the machinery. The way in which the major pilin subunits might be transferred onto, or interact with, PilC is yet to be determined. However, we hypothesize it is likely through protein-protein interactions occurring in the inner membrane. The N-terminal helical domain of PilA is embedded in the inner membrane, as are the three predicted transmembrane domains of PilC (Craig et al., 2008). A recent study analyzed the interactions of the PilA subunit in *P. aeruginosa* with a model palmitoyl-oleoyl-phosphatidylethanolamine (POPE) membrane, and calculated the energy necessary for its extraction from this environment (Lemkul et al., 2011). Lemkul and colleagues determined the most energetically favorable orientation of PilA in the membrane, and determined the force needed to extract the monomer from the membrane to be at maximum, the use of 3 ATP molecules or 113 kJ of energy (Lemkul et al, 2011). Extraction of the PilA subunit could therefore be accomplished by the large domain movements and concerted effort of the 6 subunits that make up the ATPase (Satyshur et al., 2007). A major criticism of their study is that the membrane-mimetic environment did not account

for the contributions of other inner membrane proteins that could potentially reduce the energy barrier.

The inner membrane platform protein PilC could potentially be playing this role in *P. aeruginosa*. The hydrolysis of ATP in the cytoplasm by the ATPases (PilB or PilT) has been proposed to be coupled with a conformational change in PilC, which transfers the mechanical energy they generate across the inner membrane to the pilin subunits, directing the polymerization or de-polymerization of the pilus filament (Nunn et al., 1990, Craig et al., 2004, Ayers et al., 2010). The precise mechanism of conformational change occurring in PilC is not yet known, however, it may arise from a reorientation of the alpha helices in the cytoplasmic domains with respect to one another. More specifically, a sliding of the bundles across one another in a piston-like fashion could occur, such as in the cytoplasmic HAMP domains of *E. coli* methyl-accepting chemotaxis proteins upon binding of an appropriate ligand to the periplasmic receiver domain (Zhou *et al*., 2009). The shift from the sliding action from the helices in the HAMP system has been estimated to be approximately 2 Angstroms. The shift required to extract a pilus subunit from the membrane for pilus biogenesis in *P. aeruginosa* has been measured to be ~10 Angstroms. The crystal structure of the dimerized N-terminal domain of PilC has six times the number of helices than the HAMP domain, suggesting the sliding helix scenario could potentially explain the mechanism PilC employs for rapid pilus polymerization and depolymerization.

4.2.2 The first start site of PilC is preferred in vivo

During the course of this study, a discrepancy in the reported PilC sequences for the PAK and PAO1 *P. aeruginosa* strains was noted. This difference was due to the presence of two potential start sites that were noted when this gene was first described, suggesting a potential misannotation (Nunn et al., 1990). The PAK sequence has 32 additional amino acids (96 base pairs) at the N-terminus compared to PAO1 PilC. Immunoblot analyses determined that the first start site, corresponding to the PAK sequence, was used under native conditions in both the PAK and PAO1 strains **(Figure 6 A)**. To our surprise, complementation of motility in PAK and PAO1 strain was similar regardless of which start site was used **(Figure 6 B),** implying that the first 32 amino acids of PilC are not essential for protein function.

Yeast two-hybrid analysis studies in *E. coli* suggested that the N-terminal domain of the PilC equivalent interacted with the assembly ATPase, while the second cytoplasmic region of this protein interacted with the retraction ATPase (Crowther et al., 2004, Milgotina et al., 2011). The crystal structure of the N-terminal cytoplasmic region of the PilC homologue in the T2S system, GspF, revealed an all-helical structure that is potentially involved in dimer formation and protein-protein interactions with other T2S components (Abendroth et al., 2009). It is interesting to note that successful crystallization of this region required an N-terminal truncation that removed the region shown here to be dispensable for protein function. In *T. thermophilus*, partial proteolysis was required to remove

the first 60 amino acids of PilC before the first cytoplasmic domain was successfully crystallized (Karrupiah et al., 2010), but our data suggest that at least part of the missing information is not relevant to function. The precise role, if any, of this region the T2S or T4P system remains unknown.

4.3 The N-terminal domain of PilC plays a role in T4P biogenesis and is required for interactions with PilB.

In the T2S system, the single ATPase that powers protein secretion has been demonstrated to associate with the inner membrane sub-complexes, interacting specifically with the homologues of PilC (GspF) and PilM/N (GspL) (Py et al., 2001, Robert et al., 2005). Although none of the data presented in this study supports interactions between the PilM/N/O/P sub-complex and any of the T4P ATPases, the high conservation and genetic association of *pilB* and *pilC* homologues across T4P systems supports a potential interaction between these two gene products. We showed previously that fluorescent PilB fusions are mis-localized in a *pilC* mutant, and here that stability of the polymerization ATPase, PilB, is negatively affected when PilC is absent **(Figure 7)** (Chiang et al., 2005). Also, co-purification experiments suggest that the N-terminal cytoplasmic domain of PilC in *P. aeruginosa* interacts directly with the assembly ATPase PilB **(Figure 10)**.

Mutagenesis and interaction studies in *Xanthomonas campestris* determined PilB interacts with a soluble protein, PilZ, and in *P. aeruginosa*,

potentially with the CheY-like protein, PilG (Guzzo et al., 2009, Bertrand et al., 2010). These data suggest a signal cascade in which PilZ and PilG may positively regulate PilB function in response to environmental signals. Once activated, the mechanical force generated by a conformational change in PilB upon hydrolysis of ATP may be transferred through the platform protein, PilC, for pilus assembly to occur.

4.4 The C-terminal cytoplasmic domain of PilC plays a role in T4P function

Mutation of specific residues in the second cytoplasmic domain of the putative platform protein BfpE in *E. coli* produced an auto-aggregated strain that was unable to retract pili, suggesting an interaction with the retraction ATPase (Crowther *et al.,* 2004, Milgotina et al., 2011). The relevant residues (R198, D202, R203) are also found in PilC (R250, D254, R255). To identify additional residues of interest sequence analysis of the second cytoplasmic domain of T4P and T2S system platform proteins in various bacterial species was conducted **(Figure 11)**.

All but one pair of PilC mutant proteins generated in this study caused a decrease in twitching motility compared with the wild type PilC, suggesting that the mutations affect its function. The F324A-R327D pair of mutations did not reduce twitching motility **(Figure 13 A)**. The phenylalanine reside was predicted to be an unusually surface exposed hydrophobic residue, located distal to the membrane, and available for a potential interaction with the ATPase. This result

suggests that these specific residues may not be required retraction of the pilus. Interestingly, the mutant pair with the greatest decrease in twitching motility in *P. aeruginosa* was R274D-R277D **(Figure 13 A)**. Although the precise reason for the marked decrease in twitching motility was not investigated in this study, we postulate, that the decreased twitching phenotypes may be the result of weakened interaction between PilC and PilT caused by mutagenesis of the C-terminal domain. Single mutant generation is currently in progress to identify which amino acid changes in the mutated PilC pairs are responsible for the decreased twitching phenotypes.

4.5 PilM/N/O/P may align the motor and outer membrane sub-complexes

The conservation of PilM/N/O/P in species expressing T4P pili suggests that they serve a key role. This hypothesis was supported by studies in *N. meningitidis* demonstrating that pilus assembly relies on those proteins, and that no surface pili were found in retraction-deficient double mutants (Carbonnelle et al., 2006). However, in *P. aeruginosa,* surface pili were recovered when the retraction ATPase was simultaneously disrupted with any of the *pilN/O/P* genes.

Ayers and coworkers showed that secretin stability was reduced in the absence of *pilN*, *pilO*, and *pilP*, however, the impact was most pronounced in a *pilM* background (Ayers et al., 2009). We were unable to generate a *pilM* mutant in a retraction deficient background. Therefore, the combination of *pilT* and *pilM* mutations may be toxic due to an insufficient level of secretins to accommodate

pilus exit from the cell. A similar phenotype was observed previously in a *pilT/pilQ* mutant of *Neisseria*, where blocking retraction in the absence of the secretin led to accumulation of pili in the periplasm, impairing cell viability (Wolfgang et al., 2000).

The reasons for the observed differences between the phenotypes of *Neisseria* and *Pseudomonas* double mutants are not clear, especially considering the conserved structural homology between the inner membrane proteins. However, we postulate that the differences observed may relate to the subcellular localization of the T4P system. *Neisseria* are diplococci and express peritrichous T4P, while *P. aeruginosa* are rod shaped and express polar T4P. Interactions between PilM and the cytoskeleton have been proposed to be required for polar targeting of the T4P in *P. aeruginosa* (Luke et al., 2004). The absence of PilM in *Francisella,* and the absence of the cytoskeletal protein, MreB, in *Neisseria* and *Moraxella,* may explain peritrichous T4P expression in these organisms. Since polar localization is not required in *Neisseria*, PilM/N/O/P may have evolved a slightly different role in its machinery.

In *P. aeruginosa,* loss of PilN/O/P in a *pilT* background does not prevent pilus assembly, although surface pilus levels are substantially reduced **(Figure 3 A, B, C)**. We propose that in single mutants, pilus assembly is inefficient relative to disassembly. Assembly is impaired when any component of the sub-complex is missing, but is particularly affected by loss of PilM, which we previously showed causes decreases in all four proteins. Furthermore, interaction studies

have shown that the PilN/O heterodimer interacts with the putative secretin dynamic-associated (SDA) protein, PilP (Tammam et al., 2011). SDA proteins are proposed to aid in secretin gating (Ayers et al., 2010). Inefficient alignment, decreased secretin levels and/or inefficient secretin function may explain the reduced levels of surface piliation observed in the double mutants.

CHAPTER 5-CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions and Significance

Although the inner membrane architectures of select T2S systems have been previously characterized (Sandkvist et al., 2000, Py et al., 2001, Filloux et al., 2004, Robert et al., 2005, Johnson et al., 2006), studies identifying the organization of the homologous components required for the assembly of T4P have only begun. Through a systematic molecular approach, we have further refined our understanding of the functions of inner membrane assembly components essential for the T4P system in *Pseudomonas aeruginosa.*

We showed that the inner membrane sub-complex proteins PilN/O/P are not required for T4P biogenesis in *P. aeruginosa*. Instead, we propose that they are required for efficient alignment or gating of the assembly system. The inner membrane platform protein, PilC, is essential for surface pilus expression and twitching motility in *P. aeruginosa.* Other important findings include the potential interaction between the two N- and C-terminal cytoplasmic domains of PilC, and the ATPases PilB and PilT, respectively. We suggest that these proteins form the motor that powers the T4P system in *P. aeruginosa*. Although additional studies are required to characterize the mechanism of these interactions, these findings represent an important step forward in clarifying the proteins required for T4P function in *P. aeruginosa.*

5.2 Future Directions for PilC investigation

In-vivo cross-linking studies can be performed to identify direct protein-protein interactions. The chemical cross-linking reagents formaldehyde and DSP (dithiobis succinimidylproprionate) are commonly used, with the latter allowing for reversible cross-linking. The use of formaldehyde and DSP as cross-linkers has several advantages, including the stabilization of transient interactions often missed by *in-vitro* co-affinity purification. Furthermore, formaldehyde and DSP can be used for cross-linking in intact cells. Disadvantages to using formaldehyde and DSP are that only closely associated proteins can be cross-linked, and epitopes recognized by antibodies could be destroyed by formaldehyde modification (Klockenbusch et al., 2010).

The chemical cross-linking method has been previously used in the T2S system of *P. aeruginosa,* and interaction between the soluble portions of the pseudopilin subunits was confirmed (Durand et al., 2005). A similar cross-linking strategy will be employed with the ATPases PilB and PilT and the cytoplasmic domains of PilC.

P. aeruginosa wild type lysates, and lysates of *pilB, pilT*, *pilC, pilC/B, and pilT/C* mutants with and without addition of cross-linkers formaldehyde and DSP would then be subject to SDS-PAGE, and staining with Coomassie Blue. If an interaction was occurring between the N and C-terminal domains of PilC and the ATPases PilB and PilT there would be relative increase to the size of the band visualized, compared to the non-cross linked proteins. An interaction could also

be confirmed using Western Blot analysis using specific antisera raised against PilB, PilT and PilC. Other potential interaction partners appearing as bands on the Coomassie stained SDS-PAGE gel will be excised and sent for analysis by mass spectrometry.

Alternatively, the bacterial two-hybrid system could be used to find physical interactions between two proteins. This approach utilizes the fusion of two proteins to complementary fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase (Karimova *et al.,* 1998). If an interaction occurs between PilB and the N-terminal cytoplasmic domain of PilC, the fragments would combine to produce cAMP, triggering the transcriptional activation of an adjacent reporter gene, such as *lacZ* allowing for the selectable screening of phenotypes, and quantification of β-galactosidase activity (Py *et al.*, 2001). The two-hybrid approach has been successfully used by to determine interaction partners of putative platform proteins (Arts et al., 2007, Ball et al., 1999, Py et al., 2001, Crowther et al., 2004).

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CHAPTER 7- APPENDIX

Table 1. Bacterial strains and vectors

Strain	Relevant Characteristics	Source or Reference
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
E. coli SM10	thi-1 thr leu tonA lacY supE recA RP4-2-Tcr::Mu, Km^r; mobilizes plasmids into *P. aeruginosa* via conjugation	{Simon, R. 1983)
PAK WT	Wild type	(J. Boyd)
mPAO1 WT	Wild type	(Jacobs, M.A. 2003)
PAK NP	Tetracycline resistance cassette insertion mutation within *pilA*	(Watson, A.A. 1996)
PAK BΩ	Omega cassette insertion mutation in *pilB*	(Nunn, D. 1990)
PAK *pilC::FRT*	FRT scar at position 95 within *pilC*	This study
PAO1 *pilC::FRT*	FRT scar at position 365 within *pilC*	This study
PAK *pilM::FRT*	FRT scar at position 529 within *pilM*	(Ayers, M. 2009)
PAK *pilN::FRT*	FRT scar at position 124 within *pilN*	(Ayers, M. 2009)
PAK *pilO::FRT*	FRT scar at position 328 within *pilO*	(Ayers, M. 2009)
PAK *pilP::FRT*	FRT scar at position 86 within *pilP*	(Ayers, M. 2009)
PAK *pilQ::FRT*	FRT scar at position 570 within *pilQ*	This study
PAK *pilT*::FRT	FRT scar at position 540 *pilT*	(Whitchurch, C.B. 1991;}}
PAK *pilC::FRT/pilM::GmFRT*	FRT scar at position 95 within *pilC* and FRT-	

	flanked gentamicin insertion at position 529 within *pilM*	This study
PAK *pilC::FRT/pilN::FRT*	FRT scars at position 95 within *pilC* and 124 within *pilN*	This study
PAK *pilC::FRT/pilO::FRT*	FRT scars at position 95 within *pilC* and 328 within *pilO*	This study
PAO1 *pilC::FRT/pilT::FRT*	FRT scars at position 365 within *pilC* and 540 within *pilT*	This study
PAK *pilT::FRT/pilC::FRT*	FRT scars at position 94 within *pilC* and 540 within *pilT*	This study
PAK *pilT::FRT/pilC::FRT*+ pUCP20Gm::*pilT*	FRT scars at position 94 within *pilC* and 540 within *pilT,* complemented with *pilT*	This study
PAK *pilT::FRT/pilC::FRT*+ pUCP20Gm::*pilC*	FRT scars at position 94 within *pilC* and 540 within *pilT,* complemented with *pilC*	This study
PAK *pilN::FRT/pilT::FRT*	FRT scars at position 124 within *pilN* and 540 within *pilT*	This study
PAK *pilN::FRT/pilT::FRT*+ pUCP20Gm::*pilT*	FRT scars at position 124 within *pilN* and 540 within *pilT*, complemented with *pilT*	This study
PAK *pilN::FRT/pilT::FRT*+ + pUCP20Gm::*pilN*	FRT scars at position 124 within *pilN* and 540 within *pilT*, complemented with *pilN*	This study
PAK *pilO::FRT/pilT::FRT*	FRT scars at position 328 within *pilO* and 540 within *pilT*	This study
PAK *pilO::FRT/pilT::FRT* +pUCOPGm::*pilT*	FRT scars at position 328 within *pilO* and 540 within *pilT,* complemented with *pilT*	This study

PAK *pilO::FRT/pilT::FRT* +pUCOPGm::*pilT*	FRT scars at position 328 within *pilO* and 540 within *pilT,* complemented with *pilO*	This study
PAK *pilP::FRT/pilT::FRT*	FRT scars at position 86 within *pilP* and 540 within *pilT*	This study
PAK *pilP::FRT/pilT::FRT* +pUCP20Gm::*pilT*	FRT scars at position 86 within *pilP* and 540 within *pilT,* complemented with *pilT*	This study
PAK *pilP::FRT/pilT::FRT* +pUCP20Gm::*pilP*	FRT scars at position 86 within *pilP* and 540 within *pilT,* complemented with *pilP*	This study
Vectors		
pUCP20Gm	Shuttle vector with SmaI-flanked Gm cassette inserted into a ScaI site in *bla*	(Chiang, P. 2003)
pEX18Ap	Suicide vector used for gene replacement	(Hoang, T.T. 1998)
pCR2.1	Subcloning vector used as part of the TOPO TA Cloning Kit	Invitrogen
pilC.pEX18Ap	Suicide vector containing PAK *pilC*	This study
pilCnru.pEX18Ap	Suicide vector containing PAK *pilC* mutated to contain a *nruI* site at position 95	This study
pilC::GmFRT.pEX18Ap	Suicide vector containing PAK *pilC* disrupted with FRT-flanked gentamicin cassette at position 95	This study
$pilC_{PAO1}$.pEX18Ap	Suicide vector containing mPAO1 *pilC*	This study
$pilC_{PAO1}$::GmFRT.pEX18Ap	Suicide vector containing mPAO1 *pilC* with FRT-flanked gentamicin cassette at position 365	This study
pilM::GmFRT.pEX18Ap	Suicide vector containing	(Ayers, M. 2009)

	pilM disrupted with FRT-flanked gentamicin cassette at position 529	
pilN::GmFRT.pEX18Ap	Suicide vector containing *pilN* disrupted with FRT-flanked gentamicin cassette at position 124	(Ayers, M. 2009)
pilO::GmFRT.pEX18Ap	Suicide vector containing *pilO* disrupted with FRT-flanked gentamicin cassette at position 328	(Ayers, M. 2009)
pilT::GmFRT.pEX18Ap	Suicide vector containing *pilT* disrupted with FRT-flanked gentamicin cassette at position 540	(Asikyan, M.L. 2008)
pPS856	Source of FRT-flanked gentamicin cassette	(Hoang, T.T. 1998)
pFLP2	Suicide vector encoding flp recombinase	(Hoang, T.T. 1998)
pUCP20Gm:: *pilC*.(full length)	*pilC* complementation construct	This study
*pilC*trunc.pCR2.1	Subclone *pilC* construct with N-terminal 95bp removed	This study
pUCP20Gm:: *pilC*(truncated)	*pilC* complementation construct with N-terminal 95bp removed	This study
pUCP20Gm::*pilT*	*pilT* complementation construct	(Ayers, M. 2009)
pUCP20Gm::*pilN*	*pilN* complementation construct	(Ayers, M. 2009)
pUCP20Gm::*pilO*	*pilO* complementation construct of PilC	(Ayers, M. 2009)
pUCP20Gm::*pilP*	*pilP* complementation construct of PilC	(Ayers, M. 2009)

Table 2: Oligonucleotide sequences

Name	Oligonucleotide sequence
PilC Nru-1	5' AGGTCAAGGGCGAACTGACCGGGCAGAATCGCG ATGCTGGTGAAAGCCCATCTGCGCAAGCA 3'
PilC Nru-2	5' TGCTTGCGCAGATGGGCTTTCACCAGCATCGCGATT CTGCCCGGTCAGTTCGCCCTTGACCT 3'
PilC-1	5' AAGTCGAATTCGTCGTATCTCTGCTCGTCTC 3'
PilC-2	5' GGTTGGTACCTTATCCGACGACGTT 3'
PilC trunc-1	5' AAAAGAATTCAGGAGGATTTTATGCTGGTGAAGGCC CATCTGCG 3'
PilC trunc-2	5' GATCGGATCCTTATCCGACGACGTTGCCGAGT 3'
PilC comp fwd	5' TACTGGAATTCGCCTGGAGGAAGTCAACCGCGTG 3'
PilC comp rev	5' TACTGGGATCCTTATCCGACGACGTTGCCGAGTTGG 3'
B2 R245D R250D-1	5' GAATTGCATAAAGACTCACAAAAATTCGATGACACAC TCGACAG 3'
Rev R245D R250D-2	5'CTGTCGAGTGTGTCATCGAATTTTTGTGAGTCTTTATG CAATTC 3'
B2 D25K K259D-1	5'CGTGACACACTCAAAAGAACGATCCTCGATCTTCCCA TTTTCG 3'
Rev D25K K259D-2	5'CGAAAATGGGAAGATCGAGGATCGTTCTTTTGAGTGT GTCACG 3'
B2 D255A-1	5'CCGTGACACACTCGCCAGAACGATCCTCAAACTTCCC 3'
Rev D25A-2	5'GGGAAGTTTGAGGATCGTTCTGGCGAGTGTGTCACG G 3'
B2 R255D-1	5'CGCTCACAAAAATTCCGTGACACACTCGACGATACGA TCCTCAAACTTCCCATTTTCGG 3'
Rev R255D-2	5'CCGAAAATGGGAAGTTTGAGGATCGTATCGTCGAGTG TGTCACGGAATTTTTGTGAGCG 3'

B2 R274D R277D-1	5'TCTGCGGTCGCCGATTATGCAGATACCTTGTCCACGACC 3'
RevR274D R277D-2	5'GGTCGTGGACCAGGTATCTGCATAATCGGCGACCGCAGA 3'
B2 D291K D294K-1	5'GTTCCCCTGGTCAAAGCGCTCAAATCCGTCTCCGG 3'
Rev D291K D294K-2	5'CCGGAGACGGATTTGAGCGCTTTGACCAGGGGAAC 3'
B2 F324A R327D-1	5'GGCATGCAGCTCAACGCCTCCATGGACACCACCAGCGTCTTTC 3'
Rev F324A R327D-2	5'GAAAGACGCTGGTGGTGTCCATGGAGGCGTTGAGCTGCATGCC 3'
PilM-1	5' GTGCTAGGGCTCATAAAGAAGAAAGC 3'
PilM-2	5' TCAGTCGAAACTCCTCAACGCCAGGCCGC 3'
PilN-1	5' ATGGCACGGATCAACCTTCTACCCTGGCG 3'
PilN-2	5' TCATTTCTTGGCTCCTTGCGCAACCCCATGCT 3'
PilO-1	5' ATGAGTCTGGCCAGTTCCCTGGAAAGTCTGCGCA 3'
PilO-2	5' TCATTTCTTCAGCCCCTTGTCGTTGTAGCGAT 3'
PilP-1	5' ATGAGAGCCCGCCTGATTCTGAGCAGCCTGCT 3'
PilP-2	5' TCAGGAGCGTTCCTTGAGAGTCAGGCTGCGCGGAC3'
PilT-1	5′ GGATCCGGTGTTTTCCTTGTCCGA 3′
PilT-2	5' AAGCTTGAATCCTAGACGCAGTTC 3'

Table 3: Anti-sera and dilution factors

Anti-sera	Dilution
α-PilA	1:5000
α-PilB	1:500
α-PilC	1:1000
α-PilD	1:1000
α-PilM	1:1000
α-PilN	1:1000
α-PilO	1:5000
α-PilP	1:1000
α-PilQ	1:1000
α-PilT	1:500
α-PilU	1:5000
α-6x His (Santa Cruz)	1:3000
α-Rabbit	1:3000
α-Mouse	1:3000