CHARACTERIZATION OF MINOR PILINS

IN *PSEUDOMONAS AERUGINOSA*

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By

CARMEN L. GILTNER BSc., MSc.

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AUTHOR: Carmen Lynn Giltner, BSc. (University of Alberta), MSc. (University of Alberta)

SUPERVISOR: Dr. Lori L. Burrows

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ABSTRACT

Type II Secretion (T2S) and type IV pilus (T4P) systems in Gram-negative bacteria share many features that suggest a common ancestral origin. This study examined the role of the minor pilins FimU, PilV, PilW, PilX and PilE, as well as the putative adhesin PilY1 in both the T4P and T2S systems, and elucidated the role of these proteins in pilus assembly. Genetic analysis of the major pilin cluster and the minor pilin operon revealed that the major pilin alleles are associated with a specific set of minor pilins, and that unrelated strains of the same major pilin type have identical minor pilin genes, suggesting that the two gene clusters were horizontally acquired as a 'pilin island'. We observed that the minor pilins required a specific stoichiometric ratio for proper assembly, as overexpression either completely abolished, or significantly reduced twitching motility in mutant backgrounds. We demonstrated that the minor pilins were incorporated into the pilus fibre, and that they were dependent on PilA for surface localization. The T4P minor pilins were also shown to play a role in the secretion of effectors through the T2S system, as elastase and haemolytic phospholipase C secretion was reduced in minor pilin mutants, while overexpression of FimU or PilX significantly increased secretion of T2S exoproteins. Therefore, the minor pilins may participate in T2S substrate recognition. We found that PilY1 was not essential for assembly in the absence of retraction, but that its absence caused changes in the levels of other T4P biogenesis proteins, namely FimU, PilW, PilF and PilQ secretin multimers. Finally we show that

the minor pilin, PilX functions as a strain-specific factor, potentially through specific interactions with non-conserved residues of PilQ that are necessary to induce opening of the secretin.

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ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
Cys	Cysteine
Da	Daltons
DSL	Disulfide bonded loop
Glu	Glutamic acid
Gly	Glycine
GSP	General secretion pathway
HGT	Horizontal gene transfer
HQA	4-hydroxy-2-alkylquinolones
hr	Hour
ICU	Intensive care unit
Ile	Isoleucine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LDAO	Lauryldimethylamine-oxide
LB	Luria-Bertani
Met	Methionine
min	Minute
NBT	Nitro-Blue Tetrazolium Chloride
NMR	Nuclear magnetic resonance
ORF	Open reading frame
Phe	Phenylalanine
PQS	*Pseudomonas* quinolone signal
s	Second
T4P	Type IV pili
T2S	Type II secretion
Tyr	Tyrosine
Val	Valine

CHAPTER 1: INTRODUCTION

I. AN INTRODUCTION TO PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa is a Gram-negative motile aerobic rod, found in numerous environments including soil, water, fungi, plants, and the human body (Bodey *et al.*, 1983; Costerton,2001; Schwartz *et al.*, 2006). *P. aeruginosa* is a member of the Gammaproteobacteria, which contains medically relevant pathogens including *Escherichia coli, Yersinia pestis, Vibrio cholerae, Francisella* spp. and *Salmonella* spp. Gammaproteobacteria are classified based on their small subunit ribosomal RNA and are made up of a diverse bacterial population composed of autotrophic and chemoheterotrophic species. The Gammaproteobacteria contain the highest number of completed bacterial genomes including seven *P. aeruginosa* genomes (Winsor *et al.*, 2009; *P. aeruginosa 2192* Sequencing Project; *P. aeruginosa c3719* Sequencing Project), data from which were used in this work.

Clinical significance

P. aeruginosa is considered an opportunistic pathogen which can cause serious infections in immunocompromised individuals, including HIV and cystic fibrosis patients, and is a major cause of nosocomial infections and hospital-acquired pneumonia (Bodey *et al.*, 1983; Brennan *et al.*, 2004). Its resistance to mild antiseptics and its ability to adhere to a wide range of surfaces allow for rapid patient-to-patient transfer in hospital settings. *P. aeruginosa* has been found in sink drains, creams, hands of medical personnel (Green *et al.*, 1974), and on raw

vegetables from hospital and cafeteria kitchens (Shooter *et al.*, 1969; Shooter *et al.*, 1971). Its ability to bind to stainless steel is particularly problematic as highly recalcitrant, organized communities known as biofilms can serve as a significant hospital reservoirs (Tredget *et al.*, 1992; Hota *et al.*, 2009).

Cystic fibrosis

Cystic fibrosis patients have an autosomal recessive genetic defect that prevents them from producing the cystic fibrosis transmembrane conductance regulator (CFTR) chloride ion channel. The loss of this ion channel decreases salt transport across mucosal membranes and results in dehydration of the mucus layer. This more viscous mucus prevents clearance of bacteria, which are normally removed from the lungs through ciliary movement. Reduced clearance combined with defects in innate mucosal immunity leads to chronic bacterial lung infections that currently account for the majority of morbidity and mortality in cystic fibrosis patients. From 80-95% of cystic fibrosis patients ultimately succumb to bacterial infection-associated respiratory failure (Lyczak *et al.*, 2002).

Biofilms

Biofilms are highly organized surface associated communities of bacteria contained within an extracellular matrix (Costerton *et al.*, 1999; Mah *et al.*, 2001). Bacteria within a biofilm express a different complement of genes than their

planktonic counterparts to create this differentiated community (Sauer *et al.*, 2002). It is this altered genetic expression that is problematic in clinical settings where biofilms exhibit heightened tolerance to antiseptics, antibiotics and are able to evade the immune system (Lyczak *et al.*, 2000; Drenkard *et al.*, 2002; Leid *et al.*, 2002; Stoodley *et al.*, 2002; Drenkard,2003). In addition to the extracellular matrix, which decreases permeability to antibiotics and prevents phagocytosis by immune cells, bacteria within the biofilm enter a stationary growth state which precludes treatment with antibiotics that target actively dividing cells (Leid *et al.*, 2002; Drenkard,2003).

The biofilm lifecycle begins with initial attachment of a planktonic cell to a surface (O'Toole *et al.*, 1998; O'Toole *et al.*, 2000). Following attachment, bacterial growth creates an organized cluster of cells that are highly adherent to both the surface and to each other; these clusters are termed microcolonies (O'Toole *et al.*, 2000). Subsequent structural differentiation creates the mature biofilm, identified in *P. aeruginosa* as a mushroom, or pillar shaped structure (O'Toole *et al.*, 1998; Xi *et al.*, 2006). Type IV pili (discussed below) were shown to be essential for biofilm maturation, as they were shown to cause bacterial aggregation and were responsible for stabilizing surface contacts following initial attachment (O'Toole *et al.*, 1998). The biofilm lifecycle is complete with bacterial detachment from the biofilm and reversion back to planktonic cells where the cells can then colonize new surfaces.

II. TYPE IV PILI

Type IV pili (T4P) are multifunctional membrane anchored fibres produced on the surface of a number of Gram-negative bacteria including *Neisseria gonorrhoeae* (Meyer *et al.*, 1984), *Vibrio cholerae* (Faast *et al.*, 1989; Shaw *et al.*, 1990; Johnson *et al.*, 1994), enteropathogenic *E. coli* (Donnenberg *et al.*, 1992), and *P. aeruginosa* (Johnson *et al.*, 1986) (Figure 1.1). It has been suggested that T4P are the most widespread bacterial attachment organelle, as T4P have recently been found also in Gram-positive bacteria, including *Clostridium perfringens*, *Ruminococcus albus*, and *Streptococcus sanguis* (Henrichsen 1975; Pelicic 2008). T4P are functionally diverse and mediate adherence to epithelial cells (Saiman *et al.*, 1990), motility (Saiman *et al.*, 1990; Semmler *et al.*, 1999; Mattick 2002), DNA uptake (Mattick 2002), bacteriophage adsorption (Bradley,1974), and biofilm development (O'Toole *et al.*, 1998; Chiang *et al.*, 2003).

Twitching motility

P. aeruginosa T4P are critical for adherence to both biotic and abiotic surfaces (Saiman *et al.*, 1990; Giltner *et al.*, 2006), as well as for a unique kind of motility known as "twitching". Twitching motility involves the extension, or polymerization, and the retraction, or the depolymerization, of the major pilin subunit (PilA) and consequent movement along a surface (Bradley,1980). This flagellum-independent movement has been measured at between 0.6 and 2 mm/hr

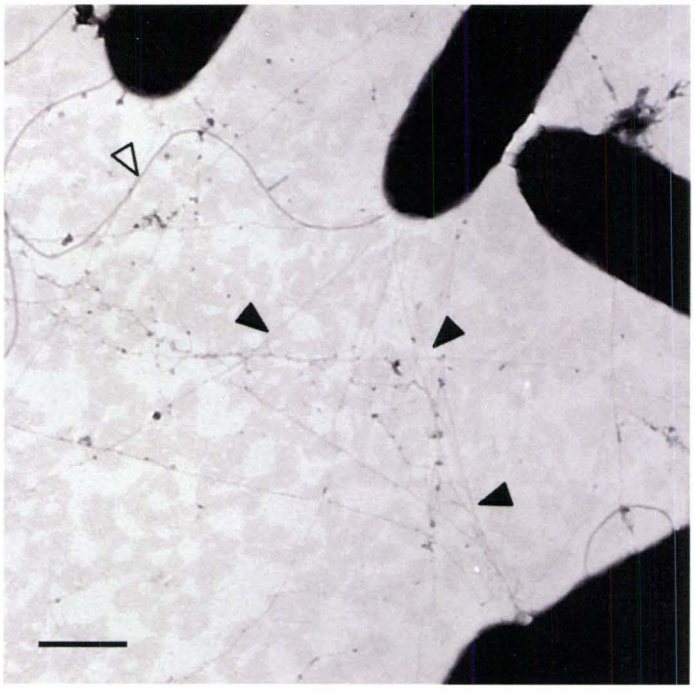

Figure 1.1. P. aeruginosa strain PAO1 showing polar flagellum and T4P.

Open arrow indicates flagella, while the closed arrow represents the long filamentous type IV pili. Bar represents 500 nm.

and is thought to be the primary role of the type IV pilus (Alm *et al.*, 1997; Semmler *et al.*, 1999). The energy required for polymerization and depolymerization of the pilus is provided by the hydrolysis of ATP by two ATPases: PilB and PilT respectively (Whitchurch *et al.*, 1991; Wolfgang *et al.*, 1998). The retraction of the pilus has been shown to be the strongest known molecular motor which can pull with forces exceeding 100 pN (Maier *et al.*, 2002; Maier *et al.*, 2004).

Classification

Historically, pili were first documented through electron microscopy as thin thread-like appendages present on surface associated cells of *Pseudomonas pyocyanca* (now *P. aeruginosa*), *E. coli*, and *Proteus mirabilis* (Houwink *et al.*, 1950). Houwink and van Iterson also observed the mutual exclusivity of flagella and pili, which were found predominantly when cultures were grown on moist or dry agar respectively (Houwink *et al.*, 1950). Following this initial report, a number of groups began to study these fibres, which were termed pili (Latin for hair or fur) or fimbriae (Latin for threads or fibres) (Duguid *et al.*, 1955; Brinton,1959); the type expressed by *P. aeruginosa* were eventually placed in a single group (type IV) defined as flexible fibres capable of mediating twitching motility (Ottow,1975).

T4P can be further divided into two classes based on their structure: the type IVa and the type IVb pili (Craig *et al.*, 2004). The type IVb pili are almost exclusively found in intestinal-colonizing bacteria such as *V. cholerae*, and enteropathogenic *E.*

coli, while the type IVa pili are found in more diverse species belonging to the Beta, Delta, and Gammaproteobacteria (Craig *et al.*, 2004). To date, a number of type IVa pilin structures have been solved through X-ray crystallography and nuclear magnetic resonance (NMR), including PilE in *N. gonorrhoeae* strain MS11, and PilA in *P. aeruginosa* strains PAK, K122-4, and 0594 (Parge *et al.*, 1995; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Craig *et al.*, 2003; Audette *et al.*, 2004a; Dunlop *et al.*, 2005; Nguyen *et al.*, 2009). Comparison of these structures reveals a generally conserved architecture of a four-stranded anti-parallel β-sheet packed on a hydrophobic N-terminal α-helix, which is predicted to form the central core of the pilus fibre (Craig *et al.*, 2006) (Figure 1.2). Structural analysis of the type IVb pilins in *E. coli* and *V. cholerae* shows that they are architecturally similar to the type IVa pilins in that they share an N-terminal hydrophobic α-helical domain and a globular C-terminal domain with an anti-parallel β-sheet, however beyond these basic features there are distinct differences between the two groups. The type IVa and IVb pilin vary in their size (the type IVb are larger than the type IVa pilins), leader peptide length (15-30 vs. 5-6 amino acids for type IVa pilins), and N-terminal residue (Met, Val, or Leu, vs. Phe for type IVa pilins) (Craig *et al.*, 2004; Xu *et al.*, 2004; Ramboarina *et al.*, 2005; Li *et al.*, 2008). Comparison of the type IVa pilin from *P. aeruginosa* strain K (PAK) and the type IVb pilin from *V. cholerae* (TcpA) reveals a significantly altered αβ-loop region, where the PAK pilin shows a minor β-sheet while the TcpA pilin shows an α-helical domain, and a modified disulfide bonded loop (DSL) region where PAK pilin

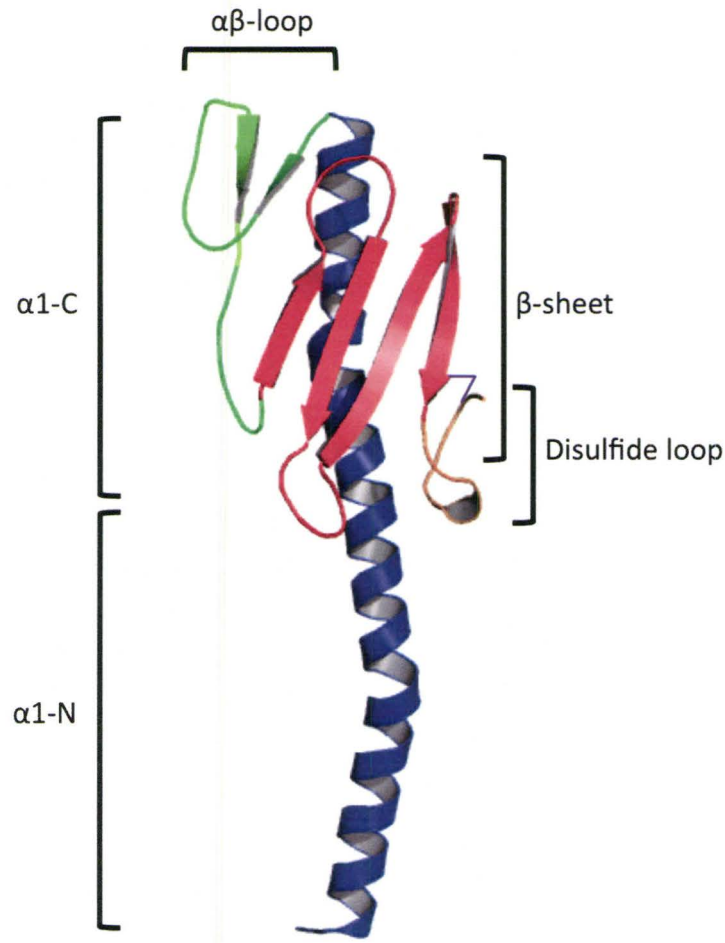

Figure 1.2. Crystal structure of P. aeruginosa pilin, PilA

Solved crystal structure of *P. aeruginosa* PAK PilA (Craig *et al.*, 2003; Hazes *et al.*, 2000). Here the α-helix is shown in blue, the αβ-loop in green, the β-sheet in red, the disulfide bonded loop in orange, and the disulfide bind in purple.

has a small loop region and the TcpA pilin has a larger (65 residues compared to 12 for PAK) more ordered DSL region which contains two α-helices and two β-strands (Hazes *et al.*, 2000; Craig *et al.*, 2003; Craig *et al.*, 2006). Ultimately, the differences observed in the surface exposed αβ-loop and DSL regions amongst type IVa and IVb pilins contribute greatly to surface variability and function of the pilus fibres, as PAK pilins display the DSL at the tip of the pilus, while the TcpA DSL is partially exposed along the length of the pilus and participates in pilin-pilin interactions (Lee *et al.*, 1994; Hazes *et al.*, 2000; Craig *et al.*, 2003).

Pilus fibre

T4P are long fibres that are typically 6 nm wide and up to several micrometers long, and composed primarily of a single 15-20 kDa protein termed pilin (Folkhard *et al.*, 1981; Soto *et al.*, 1999). Pilin fibres are assembled in the periplasm through polymerization of pilin monomers, of which an estimated 500-1000 copies form the quaternary structure known as the pilus fibre (Collins *et al.*, 2005). Extrusion of the pilus fibre through the outer membrane requires the outer membrane secretin, PilQ. The secretin was shown to have a dodecameric quaternary structure, and to be funnel shaped with the tapered end oriented towards the periplasm (Collins *et al.*, 2001). Collins and colleagues speculated that the closed conformation of the secretin would undergo a conformational change upon contact of the growing pilus to open the channel and allow passage of the pilus

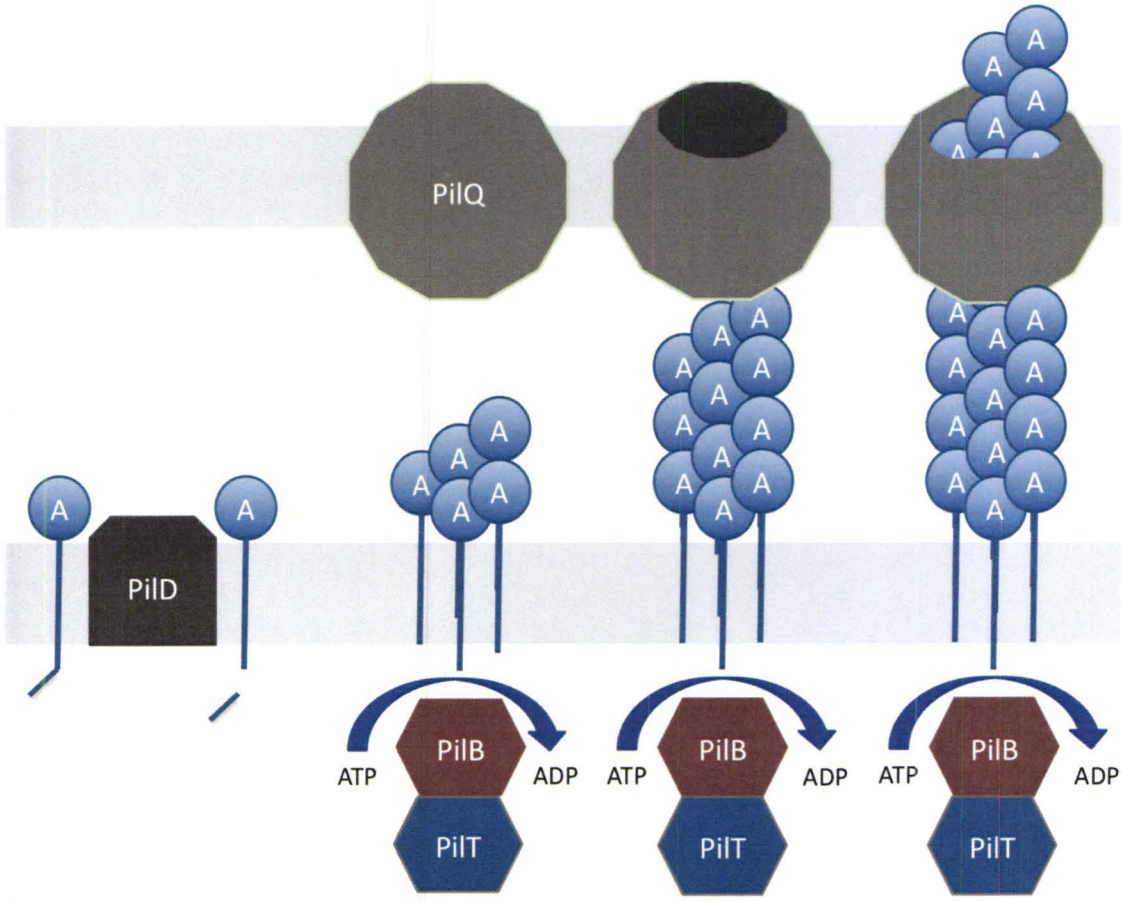

Figure 1.3. Simplified Model of the T4P system.

PilA is processed by the bifunctional enzyme PilD to create the mature pilin subunit. Processed PilA is then polymerized into the growing pilus fibre through the hydrolysis of ATP by the ATPase PilB. As the pilus is extended, it contacts the outer membrane secretin PilQ, which is predicted to undergo a conformational change to allow extrusion of the pilus through the outer membrane and into the extracellular space. Retraction of the pilus is accomplished through depolymerization of the pilus through the hydrolysis of ATP by the ATPase PilT.

fibre through the pore (Collins *et al.*, 2001; Collins *et al.*, 2003; Collins *et al.*, 2004) (Figure 1.3).

Crystallographic and cryo-electron microscopy evidence has shown the pilin monomers to be arranged in a 3-start left-handed helix with a predicted 3.6 subunits per turn (Craig *et al.*, 2003; Craig *et al.*, 2006). The helical architecture provides both flexibility and strength to the pilus, both of which are required for functional twitching motility (Maier *et al.*, 2002). Early studies identified dimers of pilin monomers remaining upon dissociation of the pilus fibre with detergent; from those data, it was hypothesized that pilin dimers were the building blocks of the pilus (Watts *et al.*, 1982). Furthermore, a pair of tyrosine residues located in the N-terminus at positions 24 and 27 were predicted to function as a dimer-dimer interface between pilin monomers (Watts *et al.*, 1982; Watts *et al.*, 1983). More recent models have suggested that single pilin monomers are added to the growing pilus fibre (Craig *et al.*, 2006), however the Tyr residues may still play key roles at the interface between monomers.

The fibre is held together through hydrophobic subunit-subunit interactions along the N-terminal α-helices which form the central shaft of the pilus (Craig *et al.*, 2006). The N-terminal α-helix can be divided into two subdomains: α1-N (spanning amino acids 1-28) and α1-C (amino acids 29-52). The α1-N region is highly hydrophobic, protrudes from the globular C-terminal domain, and is thought to form the central core of the assembled pilus fibre (Castric,1995; Craig *et al.*, 2004) (Figure 1.2). The hydrophobicity of α1-N retains individual pilin subunits in the inner

membrane for essential post-translational processing steps, and as a reservoir prior to assembly. The α1-C fragment embedded in the C-terminal globular domain is amphipathic and interacts with the anti-parallel β-sheets of the same subunit (Figure 1.2) (Craig *et al.*, 2008). In the pilus fibre, the α-helix forms helical bundles with adjacent subunits that spiral along the length of the pilus (Craig *et al.*, 2003). Along the length of a single α-helix there are three distinct helical bundles which contact adjacent subunits at residues 1-13, 4-19, and 24-39 (Craig *et al.*, 2003). Tight packing of the α-helices is facilitated by two residues, Pro22 and Gly42 in the *N. gonorrhoeae* PilE sequence, which cause kinks in the α-helix to create an S-shaped structure (Craig *et al.*, 2004). Combined, these features allow for a flexible structure that can withstand forces required for twitching motility (Maier *et al.*, 2002).

The αβ-loop links the C-terminal end of the α-helix to the first β-strand in the anti-parallel β-sheet, and was demonstrated to be structurally variable among the type IVa pilins examined to date (Parge *et al.*, 1995; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Audette *et al.*, 2004a; Craig *et al.*, 2006; Craig *et al.*, 2008; Li *et al.*, 2008; Nguyen *et al.*, 2009). In the pilin of *P. aeruginosa* strain PAK, the αβ-loop is comprised of an unmodified minor β-sheet made up of three β-strands, while that of strain K122-4 contains a short α-helix (Parge *et al.*, 1995; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Audette *et al.*, 2004a; Craig *et al.*, 2006). The αβ-loop in the pilin from *N. gonorrhoeae* has a single helical turn that is posttranslationally O-glycosylated at Ser63 (Forest *et al.*, 1999; Craig *et al.*, 2003). The distinct folds in the surface exposed αβ-loop region provide variability to the pilin structure without affecting

its ability to assemble into a fibre (Craig *et al.*, 2008). Recently, the structure of an additional type IVa pilin from *P. aeruginosa* strain Pa8110594 was found to have features similar to pilins of both type IVa and b groups, although it is predicted to preferentially fit the type IVa pilus fibre model (Nguyen *et al.*, 2009).

An anti-parallel β-sheet motif is found in both type IVa (4 strands) and type IVb pilins (5+ strands) (Craig *et al.*, 2003; Craig *et al.*, 2004), which makes up the majority of the pilin C-terminal domain. The β-sheets, which comprise the outer surface of the assembled pilus, are highly variable in sequence even within groups (Craig *et al.*, 2003; Craig *et al.*, 2004; Craig *et al.*, 2006; Craig *et al.*, 2008), however the overall structure is maintained. It is predicted that the conserved structural scaffold of the α-helix and the β-sheets preserves the integrity of the pilin molecule and participates in subunit-subunit interactions (Craig *et al.*, 2004; Craig *et al.*, 2006; Craig *et al.*, 2008).

The C-terminal domains have fewer subunit-subunit interactions than the α-helices, with positively charged grooves running between subunits in *P. aeruginosa* strains K122-4, KB7 and PAK, which provides both flexibility and potential DNA binding sites along the pilus fibre (van Schaik *et al.*, 2005; Craig *et al.*, 2006) (Figure 1.4). The natural competence of *N. gonorrhoeae* is predicted to be a direct result of non-specific binding of DNA along positively charged grooves, which may then be internalized upon retraction of the pilus (Craig *et al.*, 2006; Craig *et al.*, 2008). *P. aeruginosa*, not generally considered to be naturally competent, also exhibits

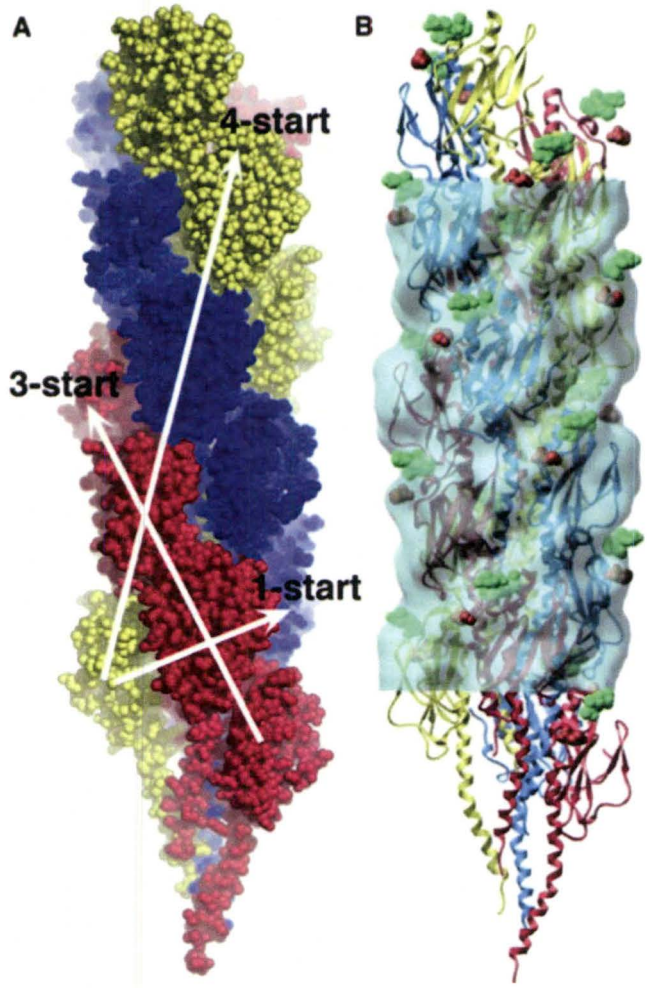

Figure 1.4. Model of the type IV pilus fibre

Cryo-EM model of *N. gonorrhoeae* T4P here a 3-start left handed helical model is proposed. (A) space-filling and (B) ribbon diagram model showing surface exposed regions of the pilus. Reproduced from Craig *et al.*, 2006 with permission from Molecular Cell, Elsevier BV.

positive grooves along the pilus surface and purified pili of strains K122-4, KB7, and PAK were able to specifically bind to DNA (van Schaik *et al.*, 2005).

The C-terminal end of the pilin's globular domain terminates with a DSL region that is implicated in function of the pilus, as mutants in either of the Cys residues or deletions within the loop region impact twitching motility (Harvey *et al.*, 2009). The DSL region protrudes from the pilin opposite the αβ-loop (Figure 1.2) however in current pilus fibre models, the DSL is buried along the length of the pilus and only exposed at the fibre's tip (Lee *et al.*, 1994; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Craig *et al.*, 2003). In *P. aeruginosa* the DSL was shown to function as an adhesin, as competitive inhibitors were specifically able to prevent DSL binding to a variety of surfaces (Giltner *et al.*, 2006). Adhesion of the DSL to surfaces was attributed to main-chain rather than side chain-based interactions, based on the hypervariability of this region (Hazes *et al.*, 2000; Giltner *et al.*, 2006). As antibodies specific to this region were able to prevent binding to and colonization of surfaces, the DSL represents an ideal target for vaccine development (Giltner *et al.*, 2006).

Pilin Diversity (Groups I-V)

Five different pilin alleles have been identified in *P. aeruginosa*, which are classified in part based on the identity of the pilin accessory protein encoded downstream of the pilin; the first of these groups to be identified were the group I and II alleles (Castric *et al.*, 1994; Kus *et al.*, 2004) (Figure 1.5). Laboratory strains

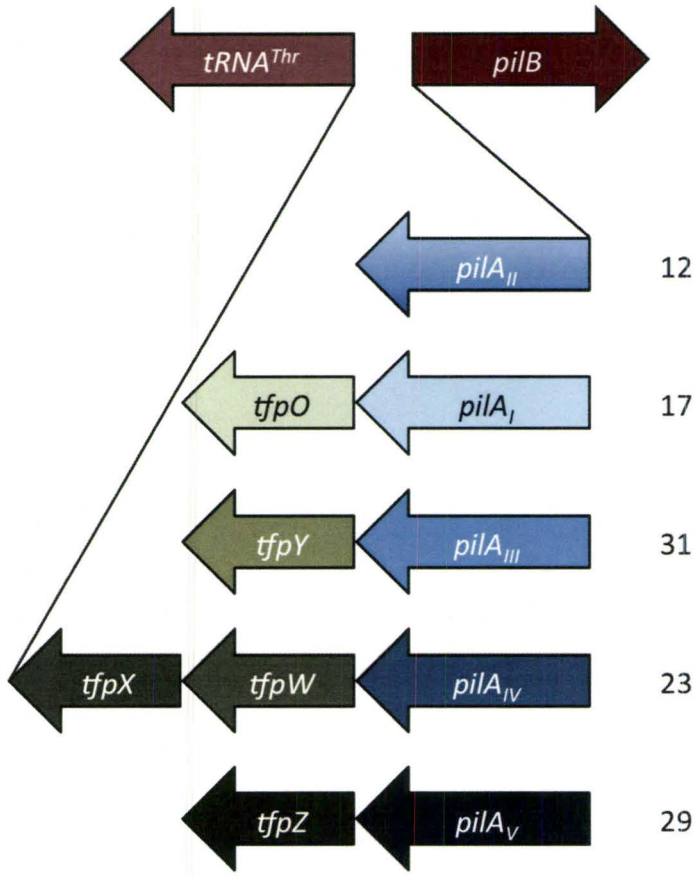

Figure 1.5. Pilin diversity in P. aeruginosa

Figure is adapted from Kus *et al.*, (2004) showing the five different pilin alleles and their respective accessory genes in *P. aeruginosa* environmental and clinical isolates. Above region depicts the genetic information located between the tRNA^{Thr} and *pilB* genes, where accessory genes are shown in green and pilin alleles are shown in blue. The group number is depicted by the subscript roman numeral on the *pilA* gene. The length of the disulfide bonded loop for each PilA allele is shown on the right.

PAO1, PAK, and PA103 express group II pilins (Pasloske *et al.*, 1985; Johnson *et al.*, 1986) which lack accessory genes and have a *tRNA^{Thr}* gene directly downstream of *pilA*. The pilins of group II have a 12-residue DSL, a PilA protein length of 143-144 amino acids, and a G+C content of 48% for this region compared to an overall 67% for the PAO1 genome (Sastry *et al.*, 1985; Spangenberg *et al.*, 1995). In contrast, the group I alleles found in the Liverpool epidemic strain (LES) and PA2192 isolates encode 148 amino acid pilins with a 17 residue DSL and have 52 %G+C content in the pilin-encoding region (Pasloske *et al.*, 1988). The group I pilins were further differentiated from those of group II by the identification of an additional open reading frame (ORF) between the *pilA* and *tRNA^{Thr}* genes, which was named *pilO* (Castric *et al.*, 1989; Castric *et al.*, 1994; Spangenberg *et al.*, 1995). To avoid confusion with the T4P structural gene *pilO,* part of the *pilMNOPQ* operon, this gene has been renamed *tfpO* (for type four pilin protein O) (Kus *et al.*, 2004). TfpO is involved in glycosylation of group I pilins at Ser148 (Castric *et al.*, 2001; Comer *et al.*, 2002).

In 1995, an additional pilin allele was identified in a single *P. aeruginosa* isolate based on its unusual sequence, and was termed group III (Spangenberg *et al.*, 1995; Kus *et al.*, 2004). Spangenberg and colleagues described the group III pilin as 173 amino acids (longer than any of the other pilin alleles), with a DSL of 31 amino acids, and a G+C content of 55% (Spangenberg *et al.*, 1995). In addition, a putative ORF encoding a protein of unknown function was found between the *tRNA* and *pilA* genes (Spangenberg *et al.*, 1995).

To determine the diversity of *P. aeruginosa* pilin alleles in environmental and clinical isolates, a comprehensive analysis of 292 isolates was performed. The results showed that 47% of the isolates tested had group I alleles, 31% had group II and 18% had group III alleles (Kus *et al.*, 2004). The remaining 4% of the population had one of two different pilin alleles, named groups IV and V. Group IV contained two isolates, Pa5196 and PA7, which coded for a PilA protein of 155 amino acids with a 23-residue DSL (Kus *et al.*, 2004; Roy *et al.*, 2010). Two ORFs were identified between the *tRNA^{Thr}* and *pilA* genes, which coded for the novel TfpW and TfpX proteins. TfpW is a predicted transmembrane protein, thought to function as a glycosyltransferase that specifically adds one or more D-arabinofuranose sugars linked in an α-1,5 configuration to Thr and Ser residues in the αβ loop region of the pilin (Voisin *et al.*, 2007; Kus *et al.*, 2008). TfpX is a predicted pilus accessory protein similar in sequence to TfpZ (group V), but its specific function is currently unknown. Group V strains encoded pilins of 154 residues with a 23 amino acid DSL that were most closely related to group III pilins (Kus *et al.*, 2004). Like groups I, II and IV, an additional ORF, named *tfpZ*, was identified between the *tRNA^{Thr}* and *pilA* genes (Kus *et al.*, 2004).

Analysis of clinical and environmental isolates revealed that group II, and group III pilin alleles were less likely to be found in cystic fibrosis isolates than in clinical and rectal samples, while group I pilins were more likely to be found in isolates from cystic fibrosis patients (Kus *et al.*, 2004). Interestingly, group IV and V were found strictly in clinical samples, however a larger environmental sample size

may reveal a more diverse habitat (Kus *et al.*, 2004). Analysis of this relatively small sample size revealed two novel pilin groups; likely examination of a greater number of isolates would yield even more pilin alleles and accessory proteins.

PilD

PilA subunits undergo two additional posttranslational processes before incorporation into the growing pilus fibre. The bi-functional enzyme PilD specifically cleaves the PilA leader peptide between Gly (-1) and Phe (+1) and subsequently mono-methylates the new N-terminal Phe (Bally *et al.*, 1991; Strom *et al.*, 1991). PilD shows high specificity for particular residues within the leader peptide, as mutational studies show complete loss of peptidase activity when Gly (-1) is mutated, and a reduced rate of proteolysis in Phe (+1) mutants (Strom *et al.*, 1991; Strom *et al.*, 1992). Single amino acid substitutions in the PilD protein demonstrated that cleavage of the leader peptide from the mature pilin is essential for pilus formation, while loss of N-methyltransferase activity does not affect surface piliation (Pepe *et al.*, 1998). *In vitro* studies have shown that peptidase and methylase activity occur independently; methylation can be blocked without affecting peptidase activity, indicating that these processes have two distinct active sites (Strom *et al.*, 1993a; Strom *et al.*, 1993b; Strom and Lory 1993; Pepe *et al.*, 1998). Proteolysis and methylation were shown to be dependent on four Cys residues predicted to reside in the N-terminal cytoplasmic loop, as addition of

sulfhydryl-reactive agents inhibited these processes (Strom *et al.*, 1993a). These two pairs of Cys residues are absent in some PilD orthologues that are capable of maintaining proteolytic activity even in the presence of a chemical inhibitor. The XcpO homologue in *Xanthomonas campestris*, which lacks all four Cys residues in the cytoplasmic loop, was able to functionally complement a *P. aeruginosa pilD* mutant (Hu *et al.*, 1995), suggesting that the Cys residues do not constitute the PilD active site. More recent studies have shown that two conserved Asp residues in the C-terminal cytoplasmic loop are essential for PilD activity, therefore PilD was re-classified as an aspartyl protease (LaPointe *et al.*, 2000).

Other proteins in the type IV pilus system (FimU, PilV, PilW, PilX, and PilE) as well as the type II secretion systems (XcpT, XcpU, XcpV, XcpW, XcpX) have a type IV pilin-like leader sequence and high sequence similarity to the major pilin subunit PilA in the N-terminal alpha helical region (Bally *et al.*, 1992; Nunn *et al.*, 1992). The major (XcpT) and minor (XcpU, XcpV, XcpW, XcpX) pseudopilins are processed by PilD (Strom *et al.*, 1994), suggesting that the pilin-like minor pilins may also be processed by PilD.

III. MINOR PILINS

P. aeruginosa has a complex assembly system with over 50 gene products that contribute to the construction of functional pili (Jacobs *et al.*, 2003). While the molecular structures of several type IV pilins have been solved (Parge *et al.*, 1995;

Hazes *et al.*, 2000; Keizer *et al.*, 2001; Audette *et al.*, 2004a; Craig *et al.*, 2006; Nguyen *et al.*, 2009), much less is known about other components of the assembly machinery. Six *P. aeruginosa* group II pilin-like proteins termed 'minor' pilins, based on their low abundance relative to PilA, were shown to be essential for twitching motility (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996), and orthologous proteins in *Neisseria gonorrhoeae* have been suggested to act by opposing retraction of the pilus (Winther-Larsen *et al.*, 2005).

The minor pilins share structural similarities to the major pilin subunit and have been identified in a number of other type IVa and IVb pili-producing species (Ramer *et al.*, 2002; Winther-Larsen *et al.*, 2005; Pelicic 2008). In *P. aeruginosa,* the minor pilins of groups I, II, and IV share the highest similarity (near 100%) while those of group III strains share approximately 60% similarity (Table 1.1). Structurally, these proteins are predicted to contain, like PilA, a single highly conserved, N-terminal α-helical transmembrane domain, and a less conserved periplasmic C-terminal domain. The conserved N-terminal leader peptide (a sequence of 6 to 12 amino acids) is required for Sec translocation (Dupuy *et al.*, 1991) and is hypothesized to be processed by the pre-pilin peptidase PilD. In fact, pilin-like proteins found in the type II secretion system (homologous to the type IV minor pilins) termed minor pseudopilins were shown to be cleaved and methylated by PilD (Strom *et al.*, 1994).

The overall similarity of the minor pilins to PilA suggests that these proteins could be assembled into the pilus along with the major pilin subunit, although this

	Percent similarity relative to PAO1						
	Group I		Group II		Group III		Group IV
	2192	LES	PAO1	CS2	PA14	C3719	PA7
PilA	53	60	100	75	45	45	47
FimU	100	100	100	100	65	65	94
PilV	100	100	100	100	75	75	93
PilW	98	100	100	99	68	68	93
PilX	100	100	100	100	75	75	94
PilY1	95	95	100	95	69	69	94
PilY2	100	99	100	100	51	51	88
PilE	100	100	100	100	61	61	97

Table 1.1. Minor pilin conservation across groups

The protein percent similarity of the major and the minor pilins in groups I-IV against the group II strain PAO1. The minor pilins are highly conserved between groups with the exception of group III species. Sequencing data for group V strains are not yet available.

idea has never been experimentally confirmed. Recent structural analyses of the pilus fibre suggest that the N-terminal α-helix of the major pilin acts as the major site for inter-subunit contact in the formation of the pilus fibre (Craig *et al.*, 2003; Craig *et al.*, 2006). Those data suggest that this conserved region of the minor pilins could function in a similar fashion. However, radio-labeling studies showed that the minor pilins were located in the inner membrane, and not found in the extracellular milieu (Alm *et al.*, 1996), suggesting that these proteins were not incorporated into the pilus fibre.

Identification of the minor pilins FimU, PilV, PilW, PilX and PilE

The *P. aeruginosa* minor pilin *pilE* was first identified in 1994 by Russell and Darzins via mutant library screens for strains resistant to pilus-specific phage (Russell *et al.*, 1994) and of the minor pilins, is predicted to be the most structurally similar to PilA (Figure 1.6). The remaining FimT, FimU, PilV, PilW, and PilX minor pilins were identified through amplification of the region upstream of *pilE* and subsequent mutagenesis of predicted ORFs (Strom *et al.*, 1991; Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996). Each minor pilin mutant was defective in twitching motility and surface piliation, with the exception of *fimT* mutants which were phenotypically wild-type (Strom *et al.*, 1991). Interestingly, *in trans* expression of *fimT* was found to complement the *fimU* mutant (Alm *et al.*, 1996), suggesting that these proteins may have similar roles

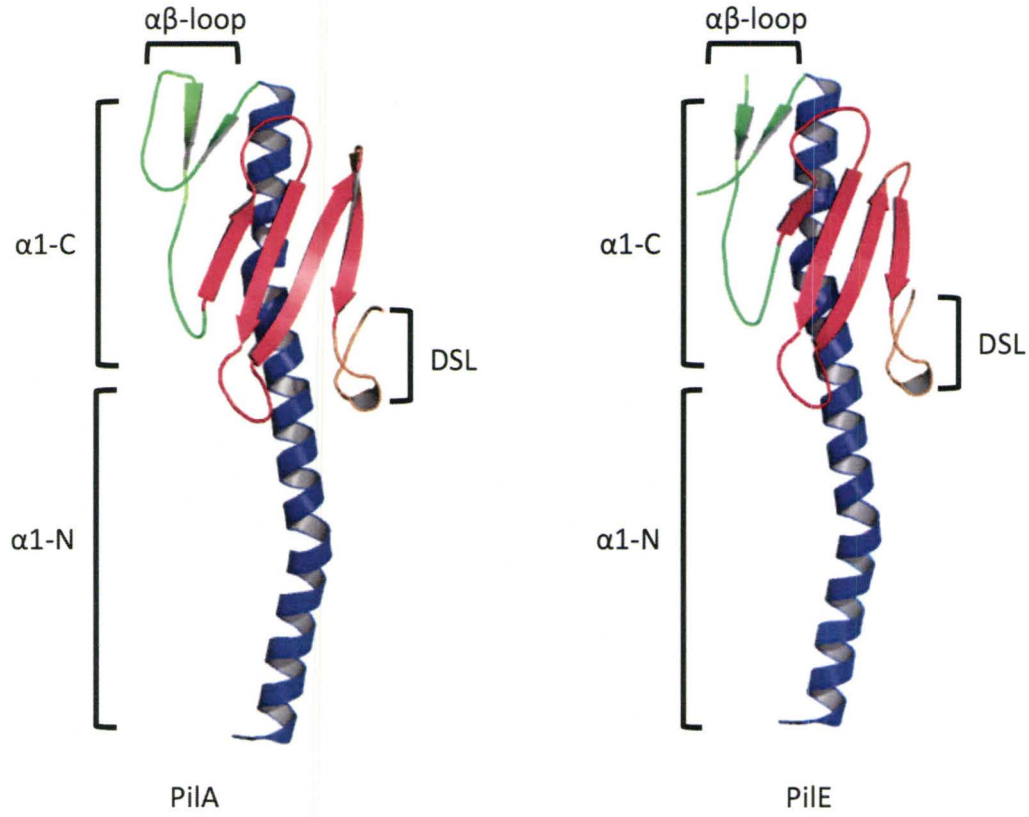

Figure 1.6. Predicted structure of PilE

The PilE sequence was threaded onto the *P. aeruginosa* PAK PilA crystal structure using the Phyre algorithm (Kelly and Sternberg, 2009). PilE has a similar N-terminal α-helical region, anti parallel β-sheet, and a C-terminal disulfide bonded loop region to that of the PilA structure. Due to sequence dissimilarity, the PilE αβ-loop region was unable to be resolved with this prediction program (Kelley and Sternberg, 2009).

	PilA	FimT	FimU	PilV	PilW	PilX	PilE
% Similarity to PilA (whole protein)	100	28	39	34	22	22	38
% Similarity to PilA (first 40 aa)	100	55	56	40	41	43	73
Predicted DSL length	12	33	32	14-42	n/a	n/a	27
Tyr residues	2	1	1	1	2	0	2

Table 1.2. Minor pilin phenotypes

The protein percent similarity of the major and the minor pilins in groups I-IV against the group II strain PAO1. The minor pilins are highly conserved between groups with the exception of group III species. Sequencing data for group V strains are not yet available.

in pilus biogenesis. Further investigation of the minor pilin genes products revealed that they were organized in an operon and had sequence similarity with the major pilin subunit, PilA (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Belete *et al.*, 2008). Like that of PilA, the N-terminal region of FimT, FimU, PilV, PilW, PilX and PilE is very hydrophobic, contains the residues essential for PilD processing (Gly -1, and Phe +1), and one or two of the conserved Tyr residues (Tyr24 and Tyr27) thought to be used in subunit-subunit interactions (Russell *et al.*, 1994) (Table 1.2). FimT has an Ala substitution in the (-1) position (Alm *et al.*, 1996). However in PilA, a Gly to Ala conversion continued to allow cleavage of the pre-pilin leader sequence (Strom *et al.*, 1991) suggesting that FimT may still undergo PilD processing.

A highly conserved Glu (+5) residue is found in the major and the minor pilins with the exception of PilX, which has an Ile (+5) substitution (Figure 1.7). Previous site-directed mutagenesis of the Glu (+5) residue in *P. aeruginosa* PilA to either a Lys or a Val did not affect PilD processing of the PilA protein, but did affect the ability of PilD to methylate the new N-terminal Phe (Pasloske *et al.*, 1988; Pasloske *et al.*, 1988; Strom *et al.*, 1992). The Glu (+5) residue has been suggested to act as a recognition site for PilA subunit incorporation into the pilus fibre (Parge *et al.*, 1995). Therefore, the absence of this residue in PilX was proposed to lead to termination of pilus elongation (Alm *et al.*, 1996), as addition of PilX to the growing fibre may preclude further PilA addition. These data suggest that the minor pilins might be capable of interacting with PilA (Russell *et al.*, 1994), however, other than

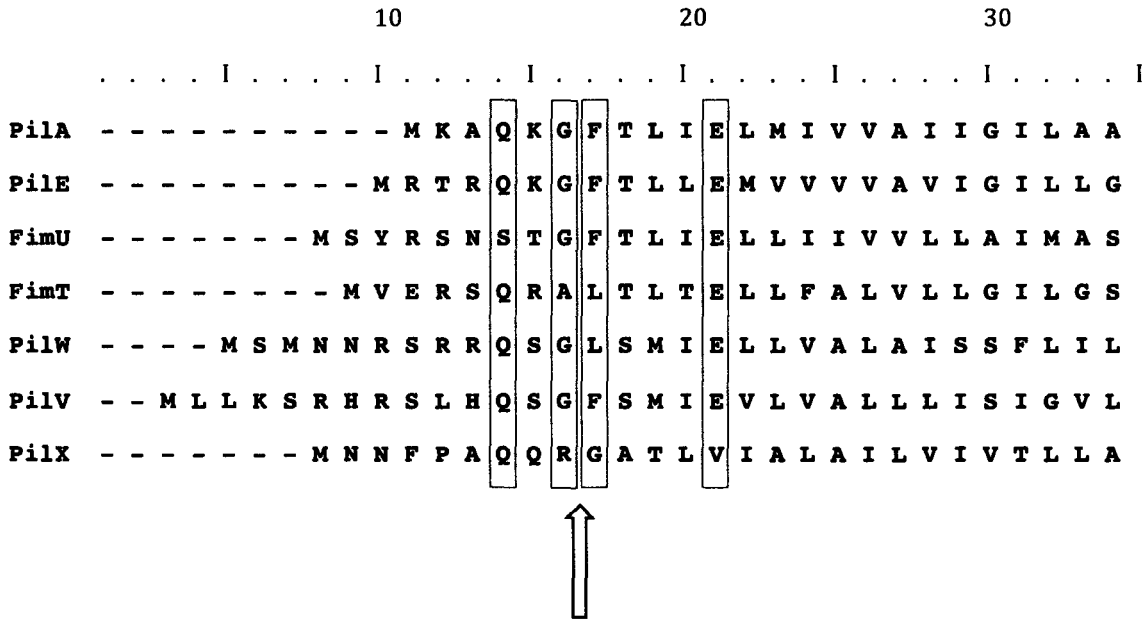

Figure 1.7. Alignment of major and minor pilin N-terminal domains

Protein alignment of PilA, PilE, FimU, FimT, PilW, PilV, and PilX. Conserved residues required for PilD processing are outlined in grey while the cleavage site is marked by the open arrow.

PilA itself, no other pilin-like protein has been found in the pilus of *P. aeruginosa* (Pasloske *et al.*, 1989; Alm *et al.*, 1996).

Similar to PilA, the C-terminal region of FimT, FimU, and PilE contains a potential DSL, however at 33, 32 or 27 amino acids in length respectively, the DSL region is twice the size of the DSL seen in group II PilA variants, and is more similar to PilA from groups III, IV and V (31, 23, and 29 amino acids respectively). PilV contains a putative DSL, however with six C-terminal Cys residues it is difficult to estimate its length (Table 1-2). The exact role of the extended DSL is unknown, however in PilA, the larger DSL seen in groups III IV and V was speculated to interact with the pilin accessory protein (Asikyan *et al.*, 2008). Both PilX and PilW lack the C-terminal Cys residues that could form a disulfide bond.

BLAST searches revealed similarity of minor pilins $FimU_H$, $PilV_I$, $PilW_J$, and $PilX_K$ to the General Secretion Pathway (GSP) family of proteins, with homologues in *Klebsiella oxytoca*, *Erwinia* spp., and *Aeromonas* spp. among others. The GspK family of proteins, like PilX, all lack the Glu at the (+5) position (Alm *et al.*, 1996; Bleves *et al.*, 1998). Interestingly, protein secretion, DNA uptake, and pilus biogenesis systems each contain one protein with a hydrophobic residue (Val, Met, Ile, Ala) at the otherwise highly conserved (+5) position.

PilY1 and PilY2

The non-prepilin protein, PilY1 is encoded between the *pilX* and *pilE* genes in the minor pilin operon. It is the largest gene in the operon spanning 3.5 kb, and its gene product was demonstrated to control the expression of the *P. aeruginosa* lipase, LipC, at the transcriptional level (Martinez *et al.*, 1999). Transposon insertion mutants in the *pilY1* gene lack twitching motility, however little is known about its role in type IV pilus assembly (Alm *et al.*, 1996; Bohn *et al.*, 2009). PilY1 shares 43% C-terminal similarity over residues 657-913 to the *N. gonorrhoeae* tip-associated adhesion protein PilC2 over residues 506-765 (Alm *et al.*, 1996). Pilus assembly in *N. gonorrhoeae* is dependent on the expression of one of two variant copies of *pilC* (*pilC1* or *pilC2*) (Rudel *et al.*, 1995). Neisserial pilus attachment to epithelial cells was shown to be a function of the PilC2 protein, as a piliated *pilC2* mutant was unable to bind epithelial cells, and exogenous PilC2 was able to competitively inhibit both *N. meningitidis* and *N. gonorrhoeae* binding to epithelial cells (Scheuerpflug *et al.*, 1999). The PilC2 protein has also been demonstrated to regulate type IV pilus retraction in *Neisseria* spp., as expression of PilC2 *in trans* in *pilC2* mutant strains prevented pilus retraction (Morand *et al.*, 2004). These data suggest that the *pilY1* gene product may play a similar role in *P. aeruginosa* type IV pili biosynthesis.

Localization studies revealed PilY1 to be in the membrane and extracellular compartments, similar to PilC2 which was localized to the outer membrane and the pilus tip (Rudel *et al.*, 1995; Alm *et al.*, 1996; Rahman *et al.*, 1997). Based on these

results, PilY1 was proposed to function as a tip-associated adhesion protein in the *P. aeruginosa* T4P system (Alm *et al.*, 1996); however, recent studies reveal a more complex and pilus-independent role for PilY1 in *P. aeruginosa*, involving protein transport, altered secretion patterns and decreased susceptibility to neutrophil attack (Bohn *et al.*, 2009). *pilY1* mutants were impaired in their ability to secrete pyocyanin, 4-hydroxy-2-alkylquinolones (HAQs) and intercellular communication molecules such as the *Pseudomonas* quinolone signal (PQS) (Bohn *et al.*, 2009). Electron microscopy revealed the presence of a truncated form (88 kDa) of PilY1 in extracellular vesicles surrounding the cells, however, the biological role of these secreted vesicles remains to be determined (Bohn *et al.*, 2009). Bohn and colleagues speculated that PilY1 is cleaved to the truncated form while it resides in the inner membrane, where it may then be carried by the extending pilus through the outer membrane to be expelled in to the extracellular milieu (Bohn *et al.*, 2009).

A recent structural study of the *P. aeruginosa* PilY1 C-terminal domain (residues 615-1163) showed that it comprises a seven-bladed β-propeller with a single calcium binding site located between β-strands 13 and 14 (Orans *et al.*, 2009). PilY1 appears to operate as a switch between pilus extension and retraction, as PilY1 in the calcium-bound state prevents PilT-mediated retraction, while the calcium-free form of PilY1 allows pilus retraction to occur (Orans *et al.*, 2009).

pilY2 is a putative 0.35 kb ORF located directly downstream of *pilY1*. Transposon mutagenesis demonstrated *pilY2* mutants to be defective in twitching motility and surface piliation (Alm *et al.*, 1996). However, little is known of the

function of *pilY2* in pilus biogenesis. Insertional mutants are predicted to have a polar affect on the downstream *pilE* gene whose start site is located in the last 17 bp of *pilY2*, therefore this putative gene was not further investigated in my work.

Regulation of the minor pilins

Recently the minor pilins were shown to be arranged in a polycistronic operon, under the positive control of the response regulator AlgR (Belete *et al.*, 2008). The response regulator was shown to be phosphorylated at Asp54, as an *algR* mutant with a single amino acid substitution (D54N) was unable to undergo phosphorylation (Whitchurch *et al.*, 2002). AlgR was initially shown to act coordinately with AlgZ as part of a two component regulatory system to control alginate biosynthesis through activation of *algD*, the first gene in the alginate biosynthesis operon (Mohr *et al.*, 1990; Mohr *et al.*, 1992; Wozniak *et al.*, 1994). Phenotypically, an *algD* mutant lacked alginate biosynthesis, but maintained wild-type twitching motility, this suggested that AlgR might regulate an additional promoter involved in type IV pilus expression (Whitchurch *et al.*, 2002). Through reverse transcriptase PCR analysis and electrophoretic mobility shift assays, Belete and colleagues showed that the *fimUpilVWXY1Y2E* genes, but not the upstream gene *fimT*, were co-transcribed, and that an unphosphorylated AlgR-D54N mutant was defective in twitching motility (Belete *et al.*, 2008). There appears to be an additional level of control of the operon, as disruption of the *pilY1* gene increased

expression of the upstream *fimUpilVWX* genes, indicative of positive feedback regulation of the whole minor pilin operon (Bohn *et al.*, 2009).

IV. TYPE II SECRETION SYSTEM

Homologous proteins have been shown to be components of the type II secretion (T2S) and T4P systems, as well as flagella and sugar binding systems in the archaea (Bleves *et al.*, 1996; Bleves *et al.*, 1998; Sauvonnet *et al.*, 2000) suggesting a common ancestral origin. The *P. aeruginosa* T2S system includes 12 different Xcp (extracellular protein deficient) proteins, XcpA and XcpP-Z. The minor pilin proteins of the T4P system share similarity with the minor pseudopilins of the Xcp system including XcpU/FimU (36 % similarity over whole protein, and 64% similarity over the first 40 amino acids of the mature pilin), XcpV/PilV (33 and 60 %), XcpW/PilW (33, and 49 %), and XcpX/PilX (27 and 43 %), however the T2S system appears to lack a PilE equivalent. The major pilin subunit (PilA) shares similarity with the major T2S subunit (XcpT) (31 and 63%), both of which are processed by the same enzyme PilD (also called XcpA) (Nunn *et al.*, 1993).

The T2S system is part of the general secretory pathway (GSP), responsible for the selective secretion of exoproteins including cellulases, pectinases, proteases, and toxins (Sauvonnet *et al.*, 2000). The GSP employs a two-step secretion process; first, proteins cross the cytoplasmic membrane to the periplasm using the Sec or Tat systems (discussed below), adopt their final tertiary structure and finally are

transferred across the outer membrane via the T2S system (Sauvonnet *et al.*, 2000). The T2S system is widely distributed among Gram negative bacteria (Cianciotto 2005). The *P. aeruginosa* T2S system is an important contributor to bacterial virulence, as it is required for release of exotoxin A, shown to directly damage endothelial cells and inhibit mammalian protein synthesis (Vasil *et al.*, 1977). Furthermore, T2S is essential for virulence in mouse model systems of *Legionella pneumophila*, which contains a set of genes similar to the *P. aeruginosa* T2S *xcp* genes (Rossier *et al.*, 2004; Soderberg *et al.*, 2004; Rossier *et al.*, 2005).

The structure of three GSP proteins, GspI, GspJ, and GspK (homologues of PilV, PilW, and PilX respectively) was solved as a heterotrimer, where GspI and J and GspI and K interact directly (Yanez *et al.*, 2007; Korotkov *et al.*, 2008; Yanez *et al.*, 2008). Recently, protein-protein interaction data revealed that the pseudopilins XcpU, V, W and X (equivalent to FimU, PilV, PilW and PilX) interact to form a heterotetramer, in which XcpV was postulated to act as a nucleator for pseudopilus formation (Douzi *et al.*, 2009). This heterotetramer is predicted to form the tip of the pseudopilus with the major pseudopilin subunit XcpT (equivalent to PilA) polymerizing at the base of this complex (Douzi *et al.*, 2009) (Figure 1.8). Because of the sequence similarity between minor pilins and minor pseudopilins, this model suggests that the minor pilins in the T4P system may similarly be incorporated into the growing pilus fibre, possibly at its tip.

Minor pseudopilins are conserved across T2S systems, and contain a conserved N-terminal α-helical region, however their sequence varies substantially

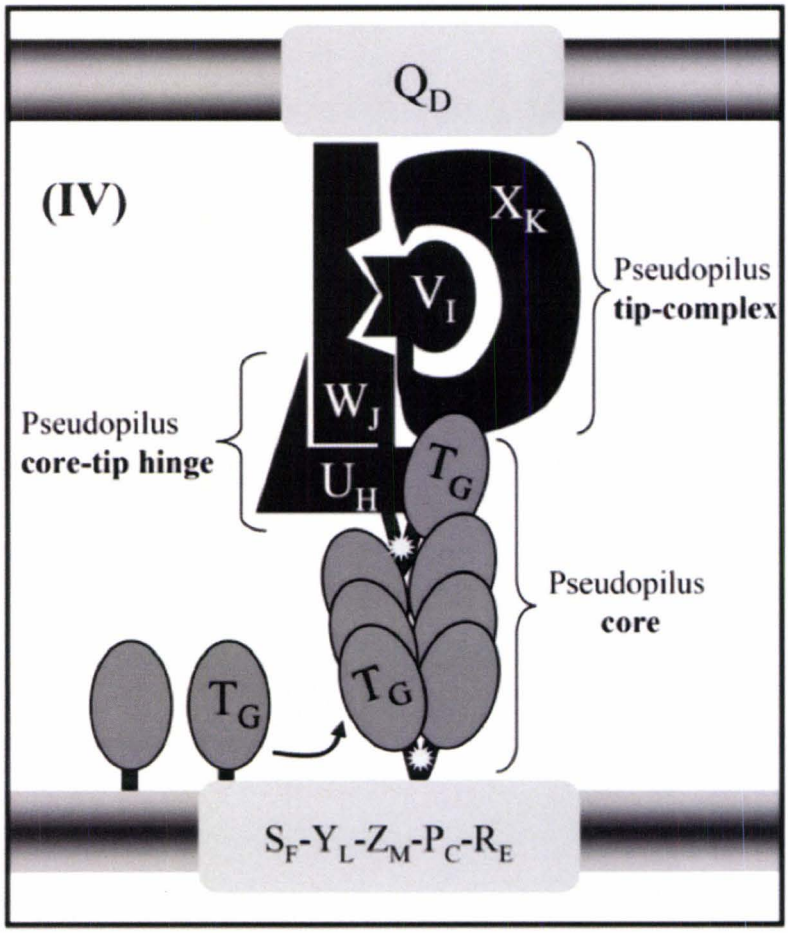

Figure 1.8. Proposed pseudopilus tip complex

XcpU is proposed to act as a linker between the major pseudopilin XcpT and the minor pseudopilin complex of XcpW-XcpV-XcpX. Model reproduced with permission from Douzi *et al.*, 2009 courtesy of the American Society for Biochemistry and Molecular Biology, Inc.

in the C-terminal domains (Lindeberg *et al.*, 1998). The minor pseudopilins were shown to be essential for secretion of exoproteins, as *gspU*, *gspV*, *gspW*, or *gspX* mutants were unable to secrete T2S dependant products (He *et al.*, 1991; Possot *et al.*, 2000; Sauvonnet *et al.*, 2000). Based on the similarities between the T4P and T2S systems, and the incorporation of both the minor pilins and pseudopilins into the fibre, we hypothesize that the T4P minor pilins may likewise be able to affect secretion of T2S exoproteins. This thesis explores the role of the minor pilins in secretion of both Sec- and Tat-dependant substrates through the T2S system.

SecYEG Translocon

The bi-functional Sec (for secretion) system is involved in the translocation of unfolded proteins across the inner membrane and insertion of membrane proteins into the cytoplasmic membrane (Natale *et al.*, 2008). Specifically, newly synthesized proteins that have 5-6 positively charged amino acids on the N-terminus of the protein (n-region), a central hydrophobic core of 10-12 amino acids (h-region) followed by a short polar region (c-region) are targeted for translocation (Figure 1.9) (Wickner *et al.*, 1991; de Keyzer *et al.*, 2003; Osborne *et al.*, 2005). The insertion and translocation of proteins is accomplished by the core SecYEG proteins, and the motor protein SecA (Luirink *et al.*, 2005). The structure of the SecYEG complex has been solved and shows SecY to be a polytopic membrane protein with 10 transmembrane segments that represents the main channel across the

cytoplasmic membrane (Beckmann *et al.*, 1997; Beckmann *et al.*, 2001; Mitra *et al.*, 2005; Rusch *et al.*, 2007). The specific targeting of proteins to the Sec system is provided by SecA and SecB, which bind to unfolded proteins as they are translated from the ribosome and chaperone them to the SecYEG core (Rusch *et al.*, 2007). While SecB is not essential for protein translocation, the SecA ATPase is, as it provides the energy required for protein transport (Wickner *et al.*, 1991; de Keyzer *et al.*, 2003; Osborne *et al.*, 2005). Proteins central to this thesis that are known to depend on the Sec translocon and carry a Sec translocation signal peptide include the major and minor pilins from T4P, the major and minor pseudopilins from T2SS, and the T2SS-secreted effector elastase and other proteases.

Tat Translocon

The twin arginine translocation (Tat) system transports fully folded proteins that carry a conserved N-terminal double Arg motif across the inner membrane (Ser/Thr-Arg-Arg-X-Phe-Leu-Lys) (Berks,1996; Sargent *et al.*, 2001). The general signal peptide motif is similar to the Sec signal with n-, h-, and c-regions however, the Tat signal n-regions are generally longer than the Sec signals (13-20 amino acids), the h-regions are less hydrophobic, and the c-regions contain a positively charged amino acid which acts as a Sec avoidance signal (Bogsch *et al.*, 1997). The integral membrane proteins TatA, TatB, and TatC have been shown to be components of the Tat secretion system (Bogsch *et al.*, 1998; Sargent *et al.*, 1998;

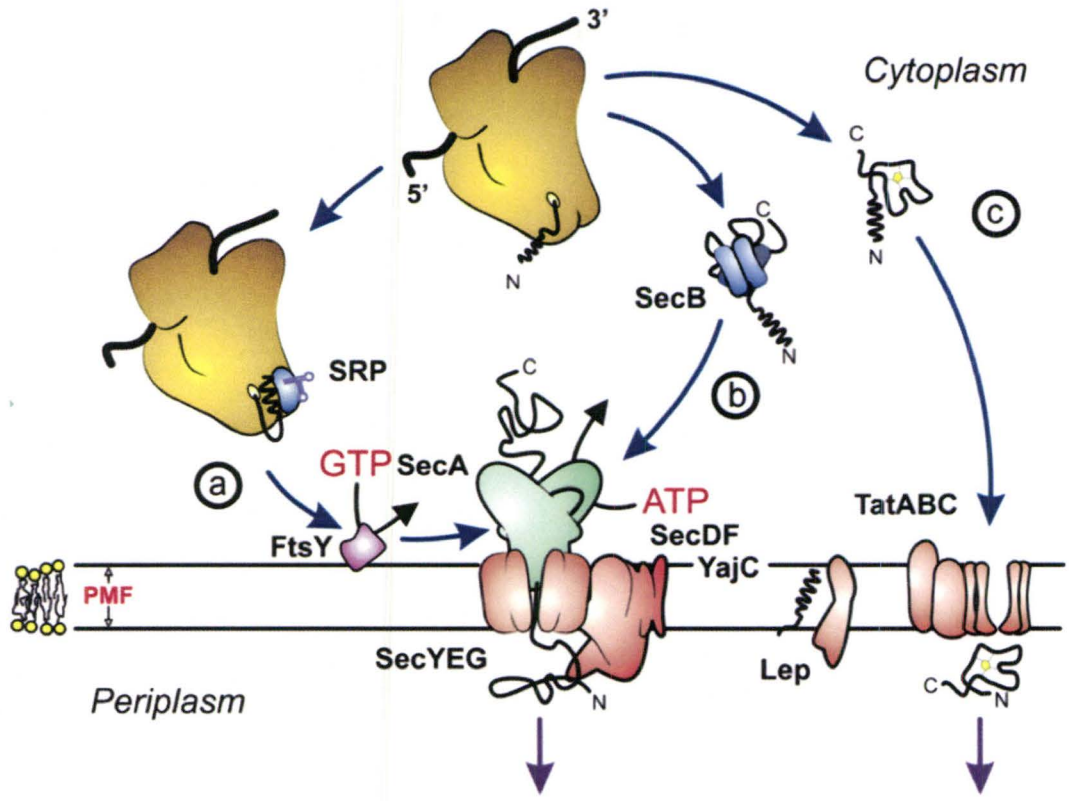

Figure 1.9. Tat and Sec-dependant secretion

Translocation of proteins across the inner membrane is accomplished in part by the Sec and the Tat systems. Sec substrates are translocated through hydrolysis of ATP in an unfolded state where they subsequently fold in the periplasm. Tat substrates do not require ATP for translocation, instead they are dependent on a protein gradient for transport. Tat systems transport fully folded proteins that contain a conserved twin arginine motif. Sec-dependent substrates are targeted to the Sec machinery via a 6-12 amino acid positively charged leader peptide, which is subsequently cleaved following translocation. Figure reproduced from Natale *et al.*, 2008 with permission from Elsevier B.V.

Weiner *et al.*, 1998; Sargent *et al.*, 1999; Orriss *et al.*, 2007). TatA and TatC comprise the minimal essential components of the Tat-translocase (Pop *et al.*, 2002; Jongbloed *et al.*, 2004). TatC is a six-pass integral membrane protein, which binds to the twin Arg motif and is predicted to be the motor of the Tat system. TatA is thought to form the pore for translocation by either weakening the membrane to allow protein transport, or by forming a ring-like structure which functions as a gated pore (Sargent *et al.*, 2001; Alami *et al.*, 2003; Gerard *et al.*, 2006; Kreutzenbeck *et al.*, 2007). Unlike the Sec system, energy from ATP is not required for protein transport; instead, proton motive force is used. Only a small ΔpH is required for protein transport, however it has been estimated that roughly 80,000 protons pass across the membrane for each Tat-substrate translocation, suggesting that Tat transport is more energetically costly than Sec transport (Cline *et al.*, 1992; Klosgen *et al.*, 1992; Robinson *et al.*, 1993; Alder *et al.*, 2003). Proteins described in this thesis that are known to undergo Tat-dependent translocation are the *P. aeruginosa* lipase LipC and the haemolytic phospholipase C (PlcH).

V. GENOMIC ISLANDS

T2S and the T4P systems are present in highly diverse species backgrounds, and components of both systems have been found in Gram-positive and Gram-negative bacteria, as well as flagella and sugar binding systems in the archeae (Bleves *et al.*, 1996; Bleves *et al.*, 1998; Sauvonnet *et al.*, 2000), which suggests a common ancestral origin. However, while the T2S and T4P systems may have a

common ancestor, the distribution of these systems amongst a wide range of species is likely to have resulted from horizontal gene transfer (HGT). That process has been studied in primarily in pathogenic species, however, recent analysis of prokaryotic genomes has shown that exchange of genetic material via HGT has been underestimated, and that HGT may play a greater role in bacterial diversity than clonal divergence and periodic selection combined (Ochman *et al.*, 2000; Dobrindt *et al.*, 2004; Langille *et al.*, 2008). In fact, the *E. coli* K12 genome is predicted to have acquired approximately 17% of its content through HGT (Lawrence *et al.*, 1998). Lawrence and Roth hypothesized that operons are selfish, and genes that are involved in a single function (i.e. minor pilins) are clustered together to optimize their horizontal transfer (Lawrence *et al.*, 1996). The selfish operon theory dictates that grouping genes of related function promotes the spread of the operon, even though it has no fitness advantage to the individual bacterium (Lawrence *et al.*, 1996).

Essential bacterial genes, termed core genes, maintain a characteristic codon usage and homogeneous G+C content throughout the genome. These genes are primarily housekeeping and essential metabolic genes, which have low mutational capacity, as mutation in these genes would decrease bacterial fitness (Hacker *et al.*, 2000). Genomic islands were initially identified by their atypical G+C content and unusual codon usage (Hacker *et al.*, 2000). Dobrint and colleagues outlined the methods for identifying genomic islands as: a) genome comparisons to identify areas containing insertions or deletions (indels); b) screening of regions adjacent to

tRNA genes, as genomic islands are frequently inserted into regions flanked by tRNA sequences; c) detection of repeat structures, transposon and insertion sequences (IS elements); and d) identification of atypical G+C content and codon usage (Dobrindt *et al.*, 2004).

Genomic vs. pathogenicity islands

Genomic islands are present in environmental isolates and generally range in function from metabolism to iron uptake (Hacker *et al.*, 2001), while pathogenicity islands are mobile elements found only in pathogenic species that carry one or more genes encoding for virulence factors. Many examples of PIs can be observed in human and plant pathogens, but are absent in non-pathogenic species (Hacker *et al.*, 2000; Hacker *et al.*, 2001). Virulence determinants such as antibiotic resistance cassettes have been shown to be horizontally transferred amongst bacterial strains and species, which facilitates multidrug resistance (Walsh,2006).

The T4P system is found in a number of pathogenic bacterial species, and mutants unable to form functional pili have been shown to have reduced virulence in mouse models. Based on the clustering of the T4P genes it is possible that the major and minor pilin operon could be horizontally co-transferred to other bacterial species, however more studies need to be performed to test this hypothesis. This thesis will look at the similarities of the minor pilin operon with known genomic

islands and present evidence that the major and minor pilin operons are likely to have been horizontally co-transferred.

VI. HYPOTHESIS

Through incorporation into the growing pilus fibre the minor pilins FimU, PilV, PilW, PilX, and PilE affect both the T4P and the T2S systems, and are organized in conjunction with the major pilin island into a pathogenicity island.

Main objectives of this thesis:

1. Assess the function of the minor pilins FimU, PilV, PilW, PilX, and PilE in the T4P system, and address whether these pilin-like proteins can be incorporated into the pilus fibre.
2. Describe the evolutionary relationship of the minor pilin operon with respect to the major pilin operon, and discuss whether cross-complementation between *P. aeruginosa* pilin allele groups can restore function.
3. Identify the role of PilY1 and the minor pilins FimU, PilV, PilW, PilX, and PilE in secretion of exoproteins through the T2S system. Address the specificity of these proteins for Sec versus Tat-dependant substrates.
4. Describe novel roles for PilY1 including altered antibiotic resistance profiles and its affect on 11 known T4P biogenesis proteins.

CHAPTER 2:

PSEUDOMONAS AERUGINOSA MINOR PILINS ARE INCORPORATED INTO TYPE IV PILI

Carmen L. Giltner, Marc Habash, and Lori L. Burrows

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I. ABSTRACT

Type IV pili (T4P) are long filamentous appendages required for both adhesion and a unique form of motility known as twitching. Twitching motility involves the extension and retraction of the pilus and requires a number of gene products, including five conserved pilin-like proteins of unknown function (FimU, PilV, PilW, PilX and PilE in *Pseudomonas aeruginosa*), termed 'minor' pilins. Here we show that maintenance of a specific stoichiometric ratio among the minor pilins was important for function as loss or overexpression of any component impaired motility. Disruption of individual minor pilin genes, or of the AlgR positive regulator of minor pilin operon expression in a strain where pilus retraction was blocked by inactivation of the PilT retraction ATPase, revealed that pili were produced, although levels of piliation were reduced relative to the *pilT* positive control. Differences in the levels of piliation of complemented strains pointed to specific roles for each protein in the assembly process, with FimU and PilX implicated as key promoters of pilus assembly on the cell surface. Using specific antibodies to each protein, we showed that the minor pilins FimU, PilV, PilW, PilX and PilE were processed by prepilin peptidase PilD and incorporated throughout the growing pilus filament. This is the first demonstration that the minor pilins, conserved among type IVa pilus-expressing bacteria, are incorporated into the fibre, and support a role for them in initiation, but not termination, of pilus assembly.

II. INTRODUCTION

Type IV pili (T4P) are produced by a number of Gram-negative bacteria including *Neisseria gonorrhoeae* (Meyer *et al.*, 1984), *Vibrio cholerae* (Faast *et al.*, 1989; Shaw *et al.*, 1990), enteropathogenic *Escherichia coli* (Donnenberg *et al.*, 1992), and *Pseudomonas aeruginosa* (Costerton *et al.*, 1999). They are long filamentous appendages characterized by their morphology and mode of assembly (Strom and Lory 1993; Alm *et al.*, 1997). *P. aeruginosa* T4P are critical for adherence to both biotic and abiotic surfaces (Hahn,1997; Giltner *et al.*, 2006), biofilm formation (O'Toole *et al.*, 1998), as well as for a unique form of motility known as "twitching" (Semmler *et al.*, 1999; Mattick 2002). Twitching motility involves the extension and retraction of the pilus through assembly and disassembly of the pilin subunits at the inner membrane, and consequent movement of the bacterium along a surface (Bradley,1980). Production of functional pili by *P. aeruginosa* requires over 50 genes involved in biogenesis of the pilin and components involved in assembly, pilus-associated chemotaxis and regulation of the system (Alm *et al.*, 1995; Martin *et al.*, 1995; Alm *et al.*, 1996; Mattick 2002; Jacobs *et al.*, 2003; Chiang *et al.*, 2008). The pilin structural subunit (PilA) is produced as a pre-pilin, with an N-terminal leader sequence of six amino acids (Strom *et al.*, 1993b). While the leader sequence is highly conserved among *P. aeruginosa* strains, there is only 47-75% overall sequence similarity between pilins of the 5 different alleles expressed by *P. aeruginosa* (Asikyan *et al.*, 2008).

Previous studies identified five pilin-like proteins, FimU, PilV, PilW, PilX, and PilE (termed 'minor' pilins due to their limited abundance relative to PilA), are essential for formation of functional pili (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996). Minor pilins are involved in both twitching motility and expression of pili on the cell surface, and are thought to have a role in pilus assembly (Russell *et al.*, 1994; Alm *et al.*, 1996; Alm *et al.*, 1996; Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006). Recently, the *fimU-pilVWXY1Y2E* gene cluster, under the control of the response regulator AlgR, was shown to be expressed as a polycistronic operon, independently of the *fimT* gene located upstream of *fimU* (Belete *et al.*, 2008). Also, the *fimT* mutant was found to have wild-type levels of surface piliation and twitching motility (Alm *et al.*, 1996; Alm *et al.*, 1996), suggesting that unlike the other minor pilins, FimT does not have a significant role in pilus assembly. A *pilY2* mutant had reduced twitching motility and surface piliation (Alm *et al.*, 1996), however disruption of the short (348 bp) *pilY2* ORF may have had polar effects on PilE expression, as the ribosome binding site and translational start site of PilE overlap with the last 17 bp of the putative *pilY2* gene. Also, *pilY2* is not universally present in the minor pilin gene clusters of other bacteria that express T4P, suggesting that it may be a pseudogene. Based on these data, we elected not to further investigate *fimT* or *pilY2.* The *pilY1* gene product has limited sequence similarity to the *pilC* gene product of *Neisseria spp.*, which functions in adherence to epithelial cells, potentially by acting as a pilus tip adhesin (Rudel *et al.*, 1995). In *P. aeruginosa*, PilY1 was localized to the outer membrane but was not specifically

associated with type IV pili (Rahman *et al.*, 1997; Bohn *et al.*, 2009). Recent structural data showed that PilY1 binds Ca^{++} and opposes pilus retraction in a calcium-dependent manner (Orans *et al.*, 2009). PilY1 also has a regulatory role, as *pilY1* mutants have increased mRNA expression profiles of *fimU, pilV, pilW,* and *pilX*; *pilE* transcript levels were similar to wild type (Bohn *et al.*, 2009). PilY1 has no sequence similarity with other minor pilins with the exception of a possible PilD cleavage site in the N-terminus of the protein (Lewenza *et al.*, 2005).

The *P. aeruginosa* minor pilins are predicted to contain a hydrophobic N-terminal α-helical domain with putative PilD cleavage and methylation sites, similar to that of the major subunit PilA (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996), and to the pseudopilins XcpTUVWX of the evolutionarily-related type II secretion (T2S) system (Nunn *et al.*, 1993; Bleves *et al.*, 1998). Processing and methylation of PilA by PilD requires three highly conserved residues: Gly (-1), Phe (+1), and Glu (+5) (Strom *et al.*, 1991a; Strom and Lory 1993) that can tolerate conservative substitutions without affecting protein methylation or cleavage (Strom *et al.*, 1991b; Pepe *et al.*, 1998).

Early radio-labeling experiments localized the minor pilins to the inner membrane, but unlike the major pilin subunit, they were not detected outside of the cell (Russell *et al.*, 1994; Alm *et al.*, 1996). More recently the *N. gonorrhoeae* orthologues of FimU-PilVWX (PilHIJK) were identified in sheared protein preparations (Winther-Larsen *et al.*, 2005), suggesting that these minor pilins were found exterior to the cell. However, it was not clear from those data whether the

proteins were incorporated into the pilus fibre or were extracted as part of the inner membrane assembly complex of the pilus during shearing of the pili. The *N. gonorrhoeae* minor pilins (PilHIJKL, with PilL being unique to *Neisseria*) and major pilin PilE were postulated to form a complex at the base of the pilus, which may interact with the PilT retraction ATPase to prevent pilus retraction from occurring during assembly, as the minor pilins were dispensable for assembly in a retraction-deficient background (Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006). Alternatively, the minor pilins were suggested to potentially alter the conformation of PilA or other-PilT interacting proteins to prevent PilT activation (Winther-Larsen *et al.*, 2005). In the T2S system, the interaction of XcpX (othologous to PilX) and the major subunit XcpT (orthologous to PilA) was postulated to cause a conformational change that elicited disassembly and degradation of XcpT (Durand *et al.*, 2005). It was clear that further work was needed to elucidate the role of the minor pilins in pilus biogenesis, and to provide experimental support for these diverse hypotheses.

Here we investigated the specific roles of the *P. aeruginosa* minor pilins in T4P biogenesis. The minor pilins were shown to be required for surface piliation without affecting the stability of the major pilin subunit PilA, but to be dispensable for assembly when retraction was blocked through mutation of PilT. However, in contrast to the situation in *Neisseria,* the levels of surface pili recovered in the absence of one or more minor pilins were substantially reduced relative to the positive control. Characterization of individual mutants showed that those lacking FimU and PilX have related phenotypes that differ from those of strains lacking PilV,

PilW or PilE. Furthermore, the stoichiometry and expression levels of the minor pilins appear to be tightly controlled in wild type cells, as over-expression of individual minor pilins *in trans* impaired surface piliation and twitching motility. Finally, we present immunogold-labeling electron microscopy evidence that the minor pilins are incorporated throughout the length of the pilus and propose a model of how the minor pilins may be acting to facilitate pilus assembly.

III. MATERIAL AND METHODS

Bacterial strains and plasmids

Bacterial strains and DNA used in this study are listed in Table 2.1. Bacteria were maintained as glycerol stocks at -80°C. *P. aeruginosa* strains were grown routinely on Luria Bertani (LB) agar plates supplemented with gentamicin (30 mg/L) and L-arabinose (0 - 0.2% [w/v]) where indicated. *Escherichia coli* was used in this study for cloning and propagation of plasmid constructs, and grown on LB supplemented with gentamicin (15 mg/L) and ampicillin (100 mg/L) respectively.

Table 2.1 List of strains and plasmids

Strain or plasmid	Relevant characteristics(s)	Source or reference
pBADGr	pMLBAD backbone with dhfr (trimethoprim resistance) replaced with aacC1 (gentamicin resistance)	(Asikyan *et al.*, 2008)
pBADGr*pilA*	PAO1 *pilA* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Harvey *et al.*, 2009)
pBADGr*fimU*	PAO1 *fimU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*pilV*	PAO1 *pilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*pilW*	PAO1 *pilW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	This study
pBADGr*pilX*	PAO1 *pilX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*pilE*	PAO1 *pilE* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pEX18AP + *pilT*::GmFRT	Gentamicin FRT insertion in the NruI site within *pilT*	(Asikyan *et al.*, 2008)
pEX18AP + *algR*::GmFRT	Gentamicin FRT insertion in the NruI site within *algR*	This study
E. coli		
DH5α	GENOTYPE: F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda-thi-1	Invitrogen
TOP10	GENOTYPE: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 ΔlacX74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
SM10	Carries plasmid RP4 with tra genes encoding conjugative pilus integrated into its chromosome, along with a KmR	(Simon *et al.*, 1983)

	marker.	
BL21-DE3	GENOTYPE: F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3)	Invitrogen
P. aeruginosa		
mPAO1	Laboratory strain	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilA*	IS*phoA*/hah transposon insertion (position 165)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*fimU*	IS*lacZ*/hah transposon insertion (position 237)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilV*	IS*phoA*/hah transposon insertion (position 122)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilW*	IS*lacZ*/hah transposon insertion (position 381)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilX*	IS*phoA*/hah transposon insertion (position 182)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilE*	IS*phoA*/hah transposon insertion (position 183)	(Jacobs *et al.*, 2003)
mPAO1 *algR*::FRT	FRT insertion in the NruI site of *algR* gene	This study
mPAO1 *pilT*::FRT	FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 Tn::*fimU*-*pilT*::GmFRT	IS*lacZ*/hah transposon insertion (position 237), and an FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 Tn::*pilV*-*pilT*::GmFRT	IS*phoA*/hah transposon insertion (position 122), and an FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 Tn::*pilW*-*pilT*::GmFRT	IS*lacZ*/hah transposon insertion (position 381), and an FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 Tn::*pilX*-*pilT*::GmFRT	IS*phoA*/hah transposon insertion (position 182), and an FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 Tn::*pilE*-*pilT*::GmFRT	IS*phoA*/hah transposon insertion (position 183), and an FRT insertion in the NruI sit of the *pilT* gene	This study
mPAO1 *algR*::FRT-*pilT*::GmFRT	FRT insertion in the NruI site of *algR* gene, and an FRT insertion in the NruI sit of the *pilT* gene	This study

mPAO1 + pBADGr *pilA*	Wild type strain with *pilA* under arabinose control in pBADGr	This study
mPAO1 + pBADGr *fimU*	wild type strain with *fimU* under arabinose control in pBADGr	This study
mPAO1 + pBADGr *pilV*	wild type strain with *pilV* under arabinose control in pBADGr	This study
mPAO1 + pBADGr *pilW*	wild type strain with *pilW* under arabinose control in pBADGr	This study
mPAO1 + pBADGr *pilX*	wild type strain with *pilX* under arabinose control in pBADGr	This study
mPAO1 + pBADGr *pilE*	wild type strain with *pilE* under arabinose control in pBADGr	This study
mPAO1 Tn::*pilA* + pBADGr*pilA*	IS*phoA*/hah transposon insertion (position 165) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*fimU* + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*pilV* + pBADGr *pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*pilW* + pBADGr *pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*pilX* + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*pilE* + pBADGr *pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*fimU-pilT*::GmFRT + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237), and an FRT insertion in the NruI sit of the *pilT* gene, complemented with *fimU* gene in pBADGr	This study
mPAO1 Tn::*pilV-pilT*::GmFRT + pBADGr *pilV*	IS*phoA*/hah transposon insertion (position 122), and an FRT insertion in the NruI sit of the *pilT* gene, complemented with *pilV* gene in pBADGr	This study
mPAO1 Tn::*pilW-pilT*::GmFRT +	IS*lacZ*/hah transposon insertion (position 381), and an FRT insertion in the NruI sit of the *pilT* gene, complemented with *pilW*	This study

pBADGr *pilW*	gene in pBADGr	
mPAO1 Tn::*pilX*-*pilT*::GmFRT + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182), and an FRT insertion in the NruI sit of the *pilT* gene, complemented with *pilX* gene in pBADGr	This study
mPAO1 Tn::*pilE*-*pilT*::GmFRT + pBADGr *pilE*	IS*phoA*/hah transposon insertion (position 183), and an FRT insertion in the NruI sit of the *pilT* gene, complemented with *pilE* gene in pBADGr	This study

Genetic manipulations

For complementation constructs, minor pilin genes were amplified via PCR from mPAO1 chromosomal DNA. Primers included EcoRI, HindIII, or PstI restriction sites for insertion into the pBADGr vector (Asikyan *et al.*, 2008). Primer sequences are listed in Table 2.2. Combinations of these primers were used to amplify *fimU*, *pilV*, *pilW*, *pilX*, and *pilE* genes. *fimU* was amplified with primers 1 and 2; *pilV* with primers 3 and 4; *pilW* with primers 5 and 6; *pilX* with primers 7 and 8; and *pilE* with primers 9 and 10 (Table 2.2). Minor pilin genes were amplified using Taq polymerase (Qiagen) and the PCR products purified (Qiagen) before digestion with relevant restriction enzymes (Table 2.2). The pBADGr vector was linearized using identical restriction enzymes and the digested PCR products and vector were ligated with Fast Ligase (Fermentas). Ligated constructs were electroporated into *E. coli* DH5α cells and their integrity verified via DNA sequence analysis (ACGT Co., Toronto, ON). Verified constructs were electroporated into relevant mPAO1 transposon mutants (for complementation) and the mPAO1 wild-type (for determination of dominant negative effects), and transformants were plated on LB containing gentamicin (30 mg/L).

RT-PCR

Bacterial strains were grown to an optical density at 600 nm of 0.7. RNA isolation was performed using the Qiagen RNAeasy kit with RNase-free DNase

Table 2.2 List of primers used in this study

Primer Number	Primer Name	Sequence (5' to 3')
1	FimU Sense	AAAAGAATTCATGTCATATCGTTCCAACTCG
2	FimU Antisense	AAAA**AAGCTT**TCAATAGCATGACTGGGGC
3	PilV Sense	AAAAGAATTCATGCTATTGAAATCGCGACACAGG
4	PilV Antisense	AAAA**AAGCTT**TCATGGCTCGACCCTGAGGGTGT
5	PilW Sense	AAAAGAATTCATGAGCATGAACAACCGCTCCCGACG
6	PilW Antisense	AAAA*CTGCAG*TCATGGCACGAGATTCCTGAGTGTCTGGC
7	PilX Sense	AAAAGAATTCATGAACAACTTCCCTGCACAAC
8	PilX Antisense	AAAA**AAGCTT**TCAGTTGGTATAGAGACGGGCGA
9	PilE Sense	AAAAGAATTCATGAGGACAAGACAGAAGGGC
10	PilE Antisense	AAAA**AAGCTT**TCAGCGCCAGCAGTCGTTG
11	PilT Sense	GGATCCGGTGTTTTCCTTGTCCGA
12	PilT Antisense	AAGCTTGAATCCTAGACGCAGTTC
13	AlgR Sense	ACGAATTCGAGCTCGGTACCCGGGCCTGAGCTTATGAAT GTCCTG
14	AlgR Antisense	AACGACGGCCAGTGCCAAGCTTCCGCCGACCGCCGTCAG A

addition. RT-PCR was performed using the Qiagen RNAeasy kit protocol as described by the manufacturer (Qiagen). No RT controls were used for each PCR reaction to verify lack of chromosomal DNA contamination in RNA preparations.

Cloning, expression and purification of the FimU protein

The *fimU* gene was amplified from mPAO1 chromosomal DNA and directionally cloned into the pET151 TOPO vector as per the manufacturer's specifications (Invitrogen). The completed construct was verified by DNA sequencing (ACGT Co., Toronto, ON) and transformed into *E. coli* BL21-DE3 cells. Transformed cells were grown in LB-ampicillin (100 mg/L) to an optical density at 600 nm of 0.4 and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) for 16 hours at 16 °C. Cells were harvested, lysed by French press and the soluble fraction was bound to a Ni affinity column (GE Healthcare). The nickel column was washed with 6-10 column volumes of wash buffer (20mM Tris pH 8.0, 10mM imidazole, 500mM KCl, 10% glycerol [v/v], 0.1% lauryldimethylamine-oxide; LDAO), and the FimU protein was eluted with 3-4 column volumes of elution buffer (20mM Tris pH 8.0, 300mM imidazole, 500mM KCl, 10% glycerol [v/v] and 0.1% LDAO). Samples from fractions of 1 ml were run on an SDS-PAGE gel to determine protein purity (data not shown).

Polyclonal antisera were generated by immunizing rabbits with 4 mg of purified FimU protein at Cedarlane Laboratories (Burlington, Ontario). Rabbits

were boosted twice before exsanguination. Sera was separated from whole blood by centrifugation, stored at -80°C and used in subsequent Western blot assays.

Electron Microscopy

Bacterial strains were grown on LB containing gentamicin (30 mg/L) or gentamicin and L-arabinose (0.2 % [w/v]). Formvar-coated carbon grids (200 mesh) supplied by the McMaster Electron microscopy Facility were wetted with 2.5 µl ddH_2O. A small amount of bacteria from the edge of a colony was picked using a sterile toothpick and dispersed in the ddH_2O by gently touching the toothpick to the liquid for 5 s. Bacteria were incubated for 5 min on the grids before negatively staining with 2.5 µl of 2 % (w/v) uranyl acetate for 45 s. Unbound bacteria and stain were removed by wicking of excess liquid from the grids using Whatman filter paper. EM pictures were taken with a JEOL JEM 1200 TEMSCAN (Peabody, MA, USA) microscope operating at an accelerating voltage of 80 kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images.

Immunogold labeling assays

Nitrocellulose-coated carbon grids (Ortega laboratory) were glow discharged for 30 s prior to incubation with purified mPAO1 pili fixed with a 0.5% gluteraldehyde suspension for 5 min followed by blocking solution (0.3% skim milk in 1 x PBS) for

30 min. Grids were incubated in a 1:10 dilution of anti-FimU, or polyclonal rabbit anti sera raised against linear epitopes of PilV (residues 137-154, RSSKPGDCDGKGSMLEIR), PilW (residues 255- 274 DKDRLYQIAKGSQTLRNLVP), PilX (residues 141-158 LPIPSGGQVNEAESPEYG), or PilE (residues 109-126 RDKTCGKLTLNQLGERGA) for 1 hr at RT. Grids were washed three times with blocking solution and incubated in goat anti-rabbit 10 nm colloidal gold (Amersham). Grids were washed three times with 1 x PBS followed by three washes with ddH2O and stained with 1% uranyl-acetate (Electron Microscopy Sciences) for 60 s. TEM pictures were taken with a JEOL JEM 1200 TEMSCAN (Peabody, MA, USA) microscope operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images.

Twitching motility assays

Bacterial twitching was performed as previously described (Gallant *et al.*, 2005). Briefly, single bacterial colonies were stab-inoculated (to the underlying plastic surface) with a sterile toothpick on 1% agar LB plates containing gentamicin (30 mg/L) or gentamicin and L-arabinose (0.2%). After incubation, the agar was carefully removed and twitching zones were visualized by staining with 1% (w/v) crystal violet for 30 minutes. Unbound dye was removed with water and plates were allowed to air dry. To determine twitching motility zones relative to wild type, the areas of 12 twitching zones from three independent experiments were

measured using ImageJ (Abramoff *et al.*, 2004). The mean of these zones are reported.

Surface Piliation Assay

Sheared surface proteins were isolated as described previously (Castric,1995; Kus *et al.*, 2004; Asikyan *et al.*, 2008) with the following modifications. Bacterial strains were streaked in a cross-hatched fashion on three 1.5% agar LB plates containing gentamicin (30 mg/L) or gentamicin and L-arabinose (concentrations of 0.01, 0.02, 0.1 and 0.2 % w/v) and grown overnight at 37 °C. Bacteria were gently scraped from the agar surface with a sterile coverslip, and resuspended in 4.5 ml of 1x phosphate-buffered saline (PBS pH 7.4). Suspensions were vortexed for 30 s to shear surface organelles and transferred to three 1.5 ml microcentrifuge tubes where the cells were harvested by centrifugation at room temperature (RT) for 5 min at 11688 x g. The supernatant was transferred to new microcentrifuge tubes and re-centrifuged at RT for 20 min at 11688 x g to remove remaining cellular debris. The supernatant was transferred to new microcentrifuge tubes and a 1/10 volume of each of 5 M NaCl and 30 % polyethylene glycol (MW 8000) was added. The mixture was incubated on ice for 1 hr to allow for precipitation of proteins. Tubes were centrifuged a final time at RT for 30 min at 11688 x g to harvest the precipitated proteins (pilin and flagellin). The three recovered pellets for each sample were pooled and resuspended in 100 µl of 1 x sodium-dodecyl-sulphate (SDS) loading buffer (80 mM Tris pH 6.8; 5.3 %

[v/v] 2-mercaptoethanol; 10 % [v/v] glycerol; 0.02 % [w/v] bromophenol blue; 2 % [w/v] SDS). Samples were boiled for 10 min and run on a 15 % SDS-PAGE gel. Precipitated proteins were visualized with Coomassie brilliant blue (Sigma).

Intracellular PilA levels

Bacterial cells recovered from the sheared surface protein procedure were resuspended to an OD_{600} = 0.6 in 1 x PBS (pH 7.4). Cells from 1 ml of this suspension were collected by centrifugation at 11688 x g for 2 min. The pellet was resuspended in 100 µl 1 x SDS loading buffer and boiled for 10 min. The whole cell lysates (10 µl) were separated on a 15 % SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane for analysis with rabbit polyclonal anti-PilA antibodies. The nitrocellulose membrane was blocked O/N with 5 % skim milk (w/v) at 4 °C. Blots were incubated with primary anti-PilA antibody at a 1:5000 dilution for 2 hr at RT, washed three times with 1 x PBS for 10 min, and incubated with a 1:3000 dilution of secondary goat-anti-rabbit IgG alkaline phosphatase antibody for 2 hr at RT. Blots were washed with PBS as above, and developed using Nitro-Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP, BioRad).

PilD Processing

The anti-PilV antibody does not bind its substrate with high affinity, therefore to observe PilD processing of PilV proteins, the mPAO1 and *pilD* mutant strains were transformed with pBADGr*pilV* and induced with 0.2% arabinose. Whole cell lysates were prepared as above.

Generation of knockouts in mPAO1

Double knockouts were generated using the biparental mating assay as previously described (Burrows *et al.*, 2000). The pEX18AP + *pilT*::GmFRT or the pEX18AP + *algR*::GmFRT plasmid was electroporated into *E. coli* SM10 cells (Simon *et al.*, 1983), and through biparental mating was transferred to mPAO1Tn::*fimU*, mPAO1Tn::*pilV*, mPAO1Tn::*pilW*, mPAO1Tn::*pilX*, or mPAO1Tn::*pilE* cells (*pilT* construct), or mPAO1 (*algR* construct). The gentamicin resistance cassette was subsequently excised by the Flp recombinase encoded by pFLP2, and the plasmid removed using sucrose counterselection (Hoang *et al.*, 1998). Double and single mutants were verified by assessing FRT disruption of the *pilT* gene (primers 11 and 12, Table 2.2), or *algR* gene (primers 13 and 14, Table 2.2) using PCR amplification.

IV. RESULTS

Complementation of twitching motility in minor pilin (fimU-pilVWXE) mutants

Major (*pilA*) and minor (*fimU*, *pilV*, *pilW*, *pilX* and *pilE*) pilin mutants of *P. aeruginosa* mPAO1 carrying an empty vector with an arabinose-inducible promoter (pBADGr) lacked twitching motility as reported previously (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996; Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006) (Figure 2.1). Each mutant was complemented with the cognate gene in pBADGr and evaluated for restoration of twitching motility. In each case, twitching motility was restored, demonstrating that the mutant phenotypes were not due to polar effects on downstream genes (Figure 2.1).

The *pilA* complementation construct was unable to restore twitching in the *pilA* mutant in the absence of L-arabinose (uninduced condition), while addition of 0.2% arabinose (induced condition) restored twitching motility to wild type levels (Figure 2.1). In contrast, the *fimU*, *pilV*, *pilW*, *pilX*, and *pilE* genes restored twitching motility in their cognate mutants to wild-type levels under uninduced conditions (where there is a small amount of residual promoter activity). However, upon induction with 0.2% arabinose, twitching motility of the *pilX* and *fimU* complemented mutants was reduced relative to the uninduced condition (72 and 22 %, of wild type respectively), while the *pilV*-, *pilW*-, and *pilE*-complemented strains became nonmotile (Figure 2.1). The loss of twitching motility in strains overexpressing the minor pilins was not due to growth defects, as growth curves for

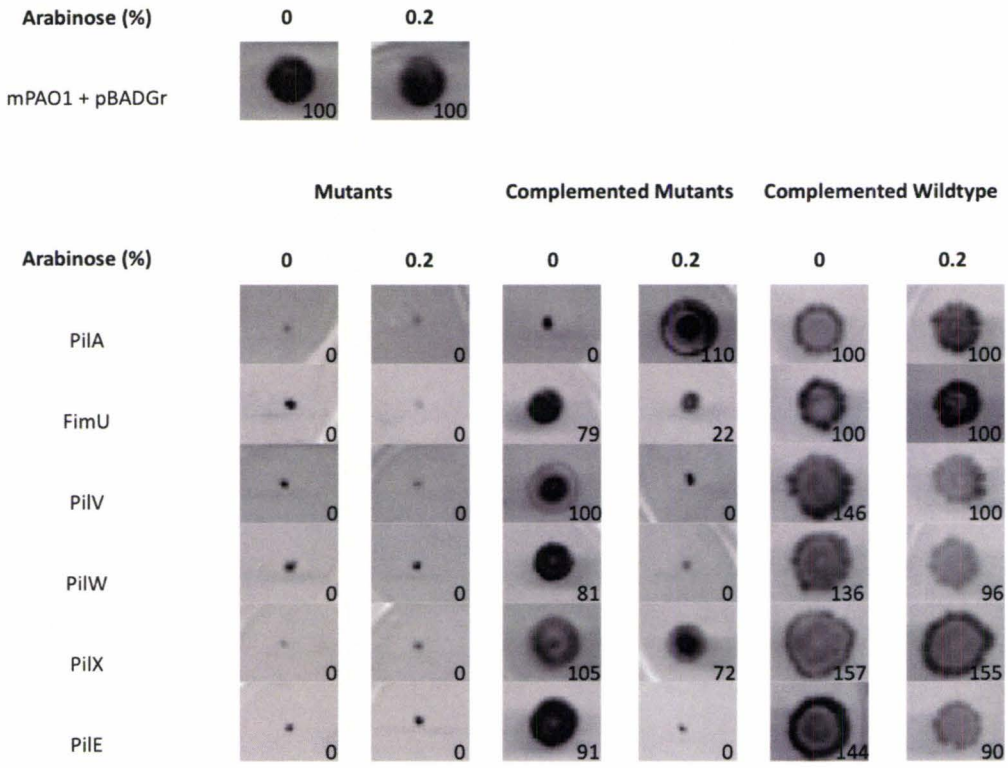

Figure 2.1. Twitching phenotypes of minor pilin mutants and complemented mutants.

Results of twitching motility assays on 1% LB agar plates, with or without 0.2% arabinose to induce expression of the cloned gene, were performed for 48 hrs at 37 °C. None of the minor pilin mutants are able to twitch, but twitching is restored to wild-type levels by complementation *in trans* with the cognate gene. Overexpression of the minor pilins at 0.2% arabinose abolishes twitching in the mutant but not wild-type backgrounds, except in the case of PilX. Numbers represent mean twitching zone area from three independent experiments where n=12. Twitching areas were measured using ImageJ (Abramoff *et al.*, 2004).

all strains at 0.2% arabinose were equivalent to wild type (data not shown). Transformation of the wild type mPAO1 strain with the same complementation constructs resulted in most cases in larger twitching zones relative to wild-type in the absence of arabinose (146, 136, 157, and 144% of wild type for *pilV, pilW, pilX* and *pilE* respectively; *fimU* transformants remained equivalent to wild-type), while over-expression of these genes restored twitching to wild-type levels for the *pilV, pilW,* and *pilE* genes. The *pilX* gene was the exception; its over-expression in the wild-type continued to increase motility to 155% of wild-type levels. The observed changes in twitching motility were not due to different levels of minor pilins, as protein levels in induced conditions were identical in the wild type and mutant backgrounds (Figure 2.S1).

Loss of minor pilins affects surface piliation but not PilA stability

Loss of twitching motility and surface piliation in the minor pilin mutants implied a role for them in stability of the major pilin subunit, pilus assembly, or function of the pilus. To differentiate between these possibilities, the levels of whole cell and sheared surface pilins recovered from mutant and complemented strains were compared (Figure 2.2). Intracellular PilA levels in the minor pilin mutants were found to be equivalent to wild-type using an anti-PilA antibody (Figure 2.2c); therefore observed changes in surface piliation were not due to changes in PilA levels. As reported for *Neisseria* spp. (Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*,

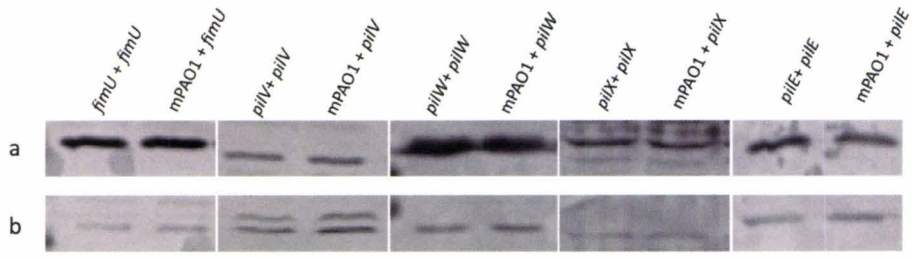

Figure 2.S1. Minor pilin levels are equivalent in wild type and mutant backgrounds.

Standardized whole cell lysates of mPAO1 *fimU*::Tn + pBADGr *fimU*, mPAO1 + pBADGr *fimU,* mPAO1 *pilV*::Tn + pBADGr *pilV*, mPAO1 + pBADGr *pilV,* mPAO1 *pilW*::Tn + pBADGr *pilW*, mPAO1 + pBADGr *pilW,* mPAO1 *pilX*::Tn + pBADGr *pilX,* and mPAO1 + pBADGr *pilX* mutants grown in the presence of arabinose (0.2%), were separated on 15% SDS-PAGE gels, and probed with anti-FimU, -PilV, -PilW, -PilX, or -PilE antibodies respectively (a). Representative loading control bands are shown in (b).

2006), no surface pili could be recovered for any of the *P. aeruginosa* minor pilin mutants (Figure 2.2), consistent with the observed loss of motility. The complemented strains yielded one of two phenotypes. Complemented *pilV*, *pilW* and *pilE* mutants had levels of surface pili equivalent to the complemented wild-type control at all arabinose concentrations tested; however, surface piliation did not correlate with twitching motility, as the recombinant strains were able to twitch only at low, but not high, levels of arabinose. Surprisingly, the complemented *fimU* and *pilX* mutants had no recoverable surface pili at any arabinose concentration (Figure 2.2) even though they twitched to some degree at all concentrations tested (not shown). Therefore, we hypothesized that the complemented *fimU* and *pilX* mutants may express very short pili, which would not be sheared from the cell by our standard isolation methods but could still mediate twitching motility.

Electron microscopy of minor pilin (fimU-pilVWXE) mutants and complemented mutants

To establish whether the minor pilin mutants could assemble any detectable surface pili, including short or aberrant forms that may not be amenable to shearing using our standard methods, we examined each of the mutants and complemented mutants by transmission electron microscopy (TEM). None of the five minor pilin mutants had detectable pili (Figures 2.3 and 2.S2). The *pilV-*, *pilW-*, and *pilE-*

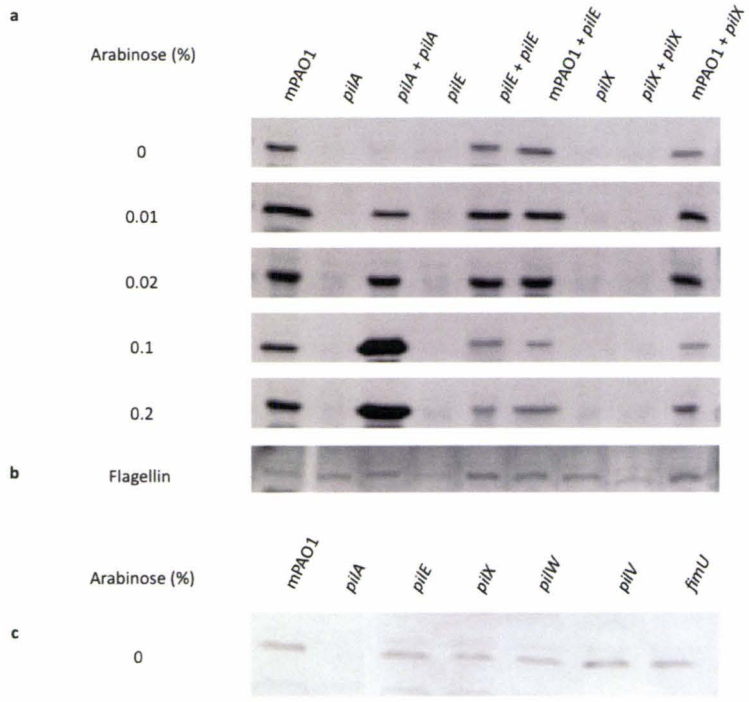

Figure 2.2. Comparison of recoverable surface pili and intracellular PilA levels among minor pilin mutants.

(a) Sheared surface proteins from the mPAO1 wild type and *pilA*, *pilE* and *pilX* mutant strains and complemented strains (grown on arabinose concentrations ranging from 0 - 0.2%) were separated on 15% SDS-PAGE gels and stained with Coomassie blue. Only the pilin band from each gel is shown. The *pilV* and *pilW* mutants have the same phenotype as the *pilE* mutant, while the *fimU* mutant has the same phenotype as the *pilX* mutant. (b) Representative flagellin bands from the 0.2% arabinose sample used as a loading control. (c) Standardized whole cell lysates of mPAO1, *pilA*, *pilE*, *pilX*, *pilW*, *pilV*, and *fimU* mutants grown in the absence of arabinose, were separated on 15% SDS-PAGE gels, and probed using an anti-PilA antibody.

complemented mutants had wild-type levels of surface piliation under uninduced conditions (Figures 2.3 and 2.S2). In contrast, pili on those fimU- and pilX-complemented mutants for which fibres could be visualized were an average of 450 and 150 nm in length, respectively compared to several micrometers reported for wild type cells (Figures 2.3 and 2.S2) (Folkhard *et al.*, 1981). These data supported the idea that complemented fimU and pilX mutants expressed functional pili too short to be sheared by vortexing.

Surface piliation is restored in a retraction-deficient background

If retraction and assembly occur independently, a decrease in assembly rates would yield a net increase in retraction, resulting in loss of external pili. In *Neisseria spp.*, surface piliation of minor pilin mutants was restored in a retraction ATPase (PilT)-deficient background (Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006). To determine if the *P. aeruginosa* minor pilins were essential for pilus assembly, *pilT* double knockouts were generated in each of the minor pilin mutant backgrounds, as well as in an *algR* mutant lacking expression of the entire minor pilin operon (Lizewski *et al.*, 2004; Belete *et al.*, 2008). In each case, surface piliation was restored, demonstrating that none of the minor pilins, individually or together, is essential for pilus assembly. However, in contrast to the situation in *Neisseria* spp., where surface piliation of minor pilin double mutants was restored to levels

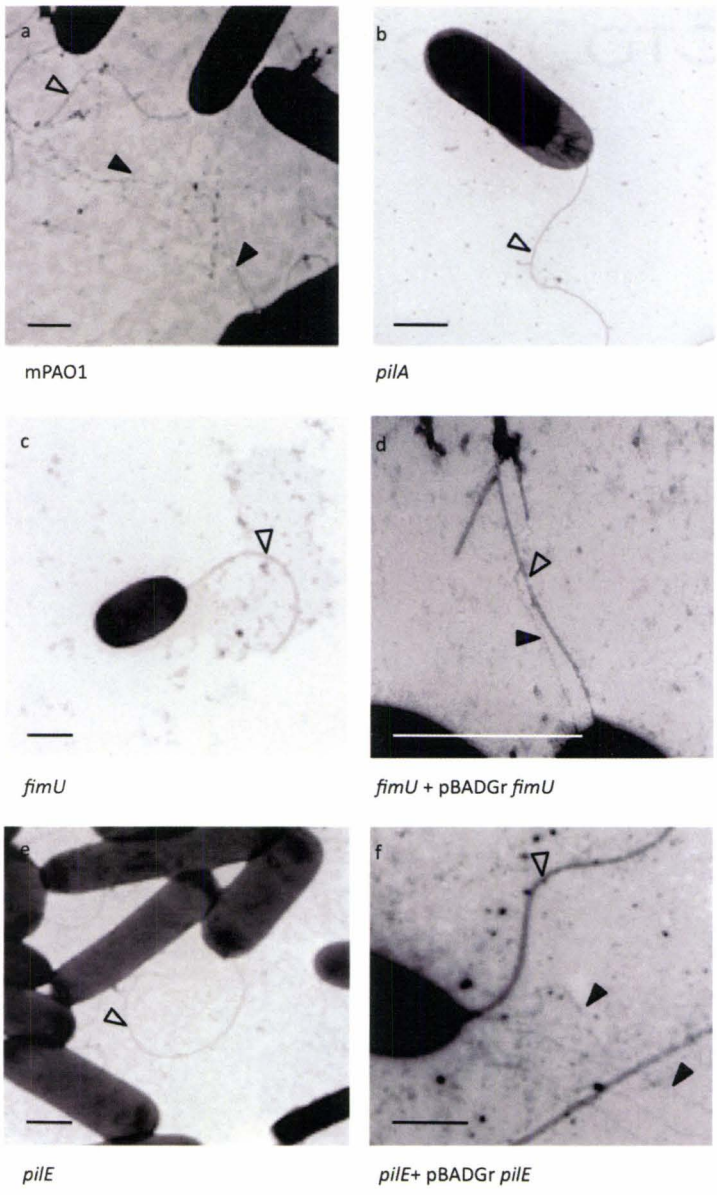

Figure 2.3. Transmission electron microscopy of minor pilin mutants and complemented mutants.

Figure 2.3. Transmission electron microscopy of minor pilin mutants and complemented mutants.

Electron micrographs of negatively-stained mPAO1 wild type (a), mPAO1 *pilA*::Tn (b), mPAO1 *fimU*::Tn (c), mPAO1 *fimU*::Tn + pBADGr *fimU* (d), mPAO1 *pilE*::Tn (e), and mPAO1 *pilE*::Tn + pBADGr *pilE* taken with a JEOL JEM 1200 TEMSCAN microscope (Peabody, MA, USA) operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images. Filled arrows indicate pili, while open arrows indicate flagella. Bar represents 500 nm.

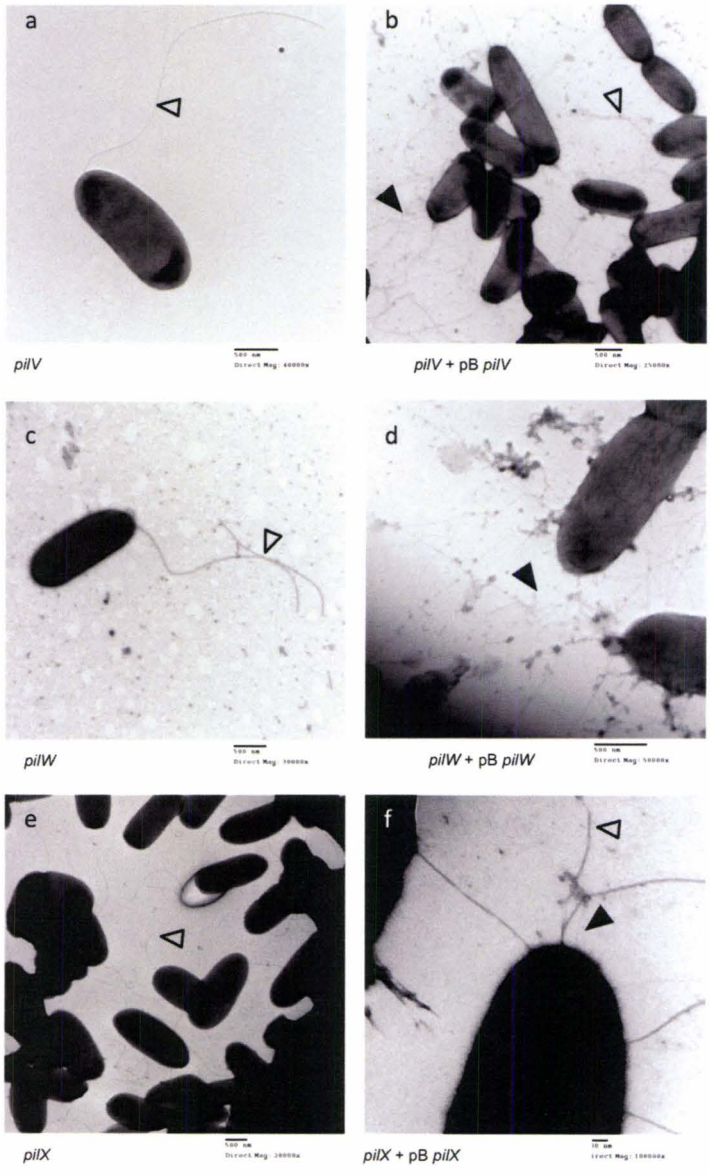

Figure 2.S2. Transmission electron microscopy of remaining minor pilin mutants and complemented mutants.

Figure 2.S2. Transmission electron microscopy of remaining minor pilin mutants and complemented mutants.

Electron micrographs of negatively-stained mPAO1 *pilV*::Tn (a), mPAO1 *pilV*::Tn + pBADGr *pilV* (b), mPAO1 *pilW*::Tn (c), mPAO1 *pilW*::Tn + pBADGr *pilW* (d), mPAO1 *pilX*::Tn (e), and mPAO1 *pilX*::Tn + pBADGr *pilX* taken with a JEOL JEM 1200 TEMSCAN microscope (Peabody, MA, USA) operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images. Filled arrows indicate pili, while open arrows indicate flagella. Bar represents 500nm.

commensurate with the pilT control (Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006), surface piliation was ~65% of the pilT control for the fimU-pilT double mutant, and more substantial decreases in piliation were observed for the other double mutants (~30, 25, 25, 45 and 20% of the pilT control in double mutants lacking pilT and pilV, pilW, pilX, pilE or algR respectively; Figure 2.4). These data show that in P. aeruginosa, minor pilins are dispensable for pilus assembly but in their absence, assembly is suboptimal.

Surface piliation in the double mutants was restored to levels consistent with the *pilT* control upon complementation of each double mutant with its cognate minor pilin gene in the absence of arabinose induction (Figure 2.4). However, upon over-expression of the minor pilins *in trans,* an interesting result was observed: while the phenotypes of the *pilT-fimU*, *pilT-pilX*, and to a lesser extent *pilT-pilE* mutants remained relatively unchanged, surface piliation in the *pilT-pilV* and *pilT-pilW* recombinant strains was reduced to that of the original minor pilin-*pilT* double mutant (Figure 2.4). These data show that over-expression of the *pilV* or *pilW* genes *in trans* is detrimental to pilus assembly.

Minor pilin processing and incorporation into the pilus

Because the minor pilin proteins have a highly conserved N-terminus resembling that of the major pilin subunit PilA, and each has a putative PilD cleavage site, it is conceivable that they are incorporated into the fibre. Alignment of the minor pilins

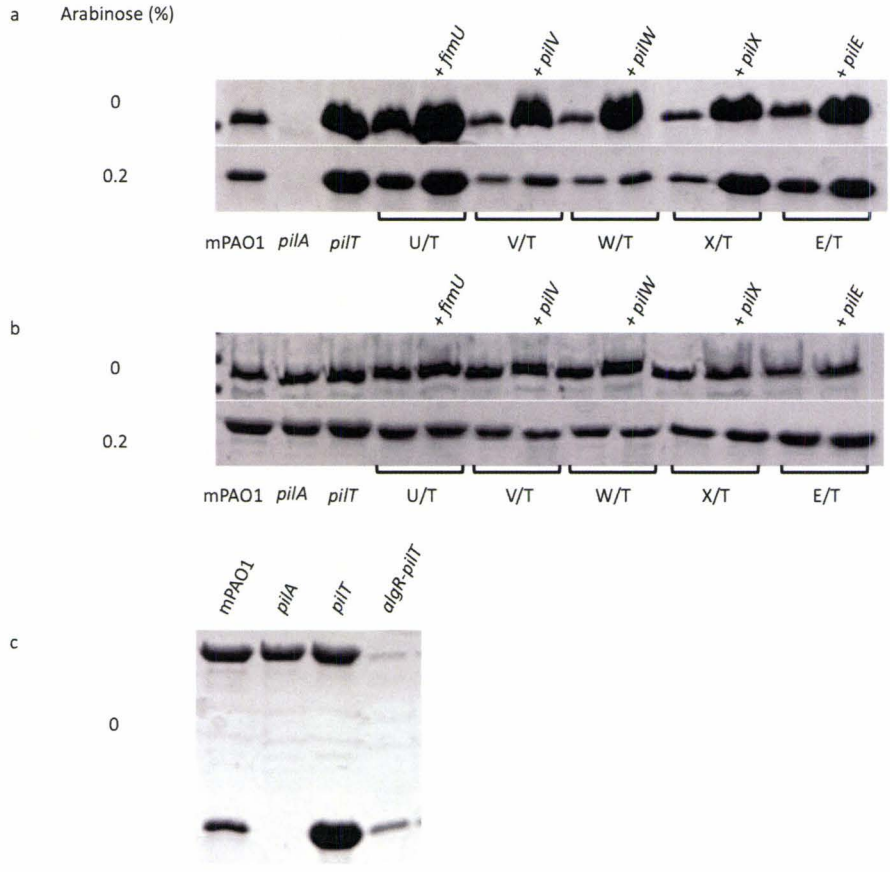

Figure 2.4. Pilus assembly is impaired in mutants lacking single minor pilins.

(a) Sheared surface proteins from the mPAO1 wt, *pilA*, *pilT*, *fimU-pilT*, *pilV-pilT*, *pilW-pilT*, *pilX-pilT*, *pilE-pilT* mutant strains as well as the respective complemented strains were separated on 15% SDS-PAGE gels and stained with Coomassie blue. The upper panel shows samples prepared from plates without arabinose, while the lower panel shows samples prepared from plates containing 0.2% arabinose. (b) Flagellin band loading controls from the pilin samples in (a). (c) Sheared surface proteins (flagellin above and pilin below) from the mPAO1 wt, *pilA*, *pilT*, and *algR-pilT* strains in the absence of arabinose.

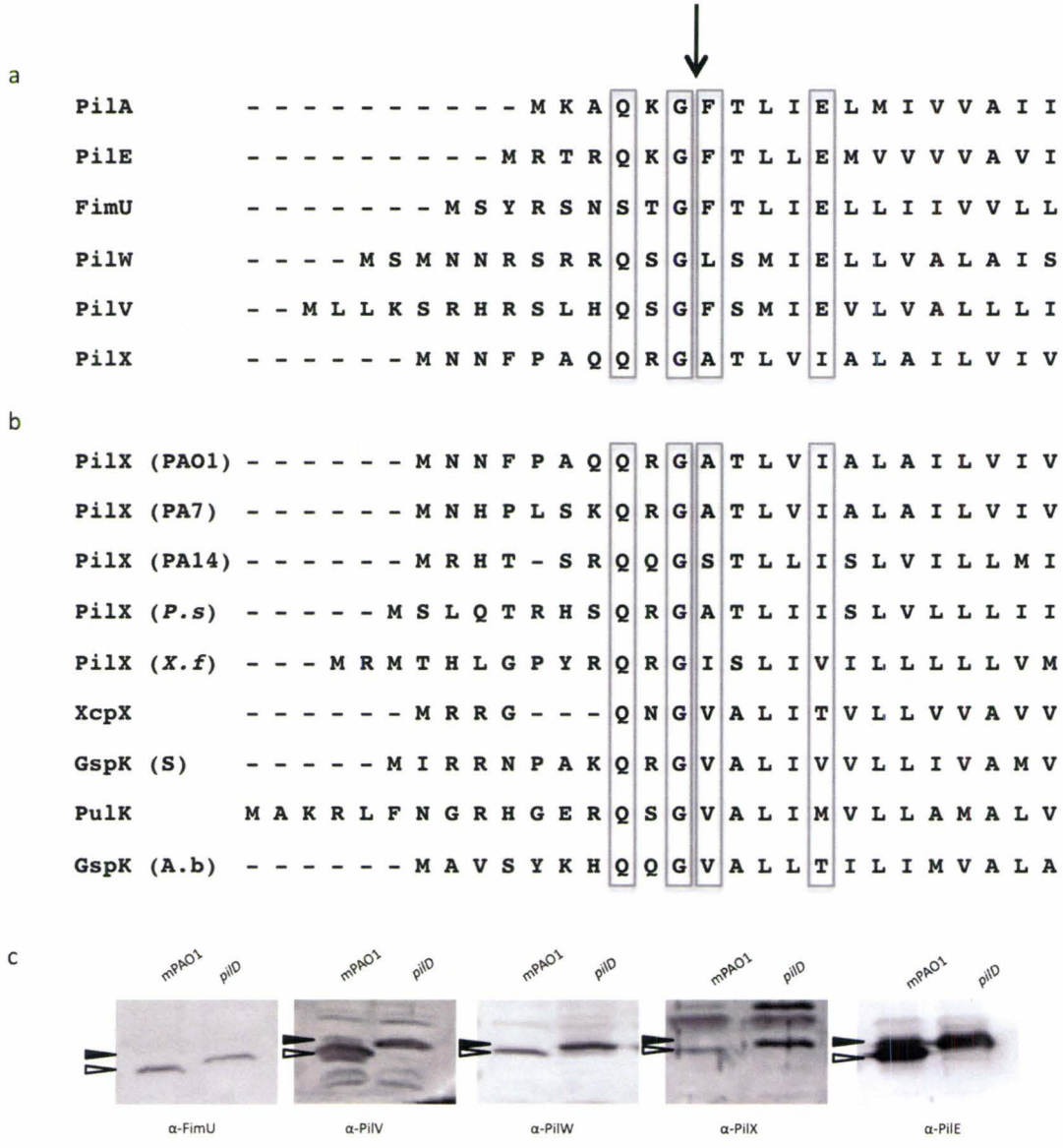

Figure 2.S3. Processing of the minor pilins by PilD.

Figure 2.S3. Processing of the minor pilins by PilD.

(a) Protein sequence alignment of *P. aeruginosa* strain mPA01 based on protein sequences from the *Pseudomonas* genome database (www.pseudomonas.com). PilD cleavage site is predicted to lie between the glycine (G) residue and the phenylalanine (F) residue. The glutamic acid residue located at the +5 position is highly conserved in the major pilin and all the minor pilins with the exception of PilX. The N-terminal region is the most highly conserved in the first 40 residues. Arrow represents PilD cleavage site of the prepilin. (b) Protein sequence alignment of PilX orthologues from type IV pilus and type II secretion systems. (P.s.) *P. stutzeri*, (X.f.) *Xyella fastidiosa*, (S) *Shewanella* spp., (M.a.) *Marinobacter algicola*, (A.b) *Acinetobacter baumannii*. (c) Standardized whole cell lysates of mPAO1 + pBADGr *fimU*, and *pilD* + pBADGr *fimU* (i), mPAO1 + pBADGr *pilV*, and *pilD* + pBADGr *pilV* (ii), mPAO1 + pBADGr *pilW*, and *pilD* + pBADGr *pilW* (iii), mPAO1 + pBADGr *pilX*, and *pilD* + pBADGr *pilX* (iv), and mPAO1 + pBADGr *pilE*, and *pilD* + pBADGr *pilE* (V) mutants grown in the presence of arabinose (0.2%), were separated on 15% SDS-PAGE gels, and probed with anti-FimU, -PilV, -PilW, -PilX, or -PilE antibodies. Filled arrow indicates the prepilin, while a decrease in the mass of the minor pilin in the wild type relative to the *pilD* mutant indicates processing by PilD, as indicated by the open arrow.

FimU-PilVWXE with PilA reveals that the characteristic Gly (-1), Phe (+1), and Glu (+5) are conserved (Figure 2.S3a), with three exceptions: PilW and PilX have a Leu (+1) and Ala (+1) substitution respectively; however, previous studies (Strom *et al.*, 1991a) showed that these residues are permissive for PilD cleavage. PilX has an additional Ile (+5) substitution, and analysis of PilX orthologues in the T4P and T2S systems reveals conservation of a hydrophobic residue at the +5 position (Figure 2.S3b). To determine if the minor pilins FimU, PilV, PilW, PilX and PilE were processed by PilD, whole cell lysates of wild-type and *pilD* mutant strains were probed with anti-FimU, -PilV, -PilW, -PilX, or -PilE sera. There was a clear increase in mass for each of the minor pilins when *pilD* was inactivated (Figure 2.S3c), confirming that they are normally processed by PilD.

Processing of the minor pilins implies that they are incorporated into the growing pilus fibre. To establish whether the minor pilins were incorporated into external pili, sheared surface proteins from the wild type, a *pilT* mutant and the *fimU-pilT* double mutant were probed with an anti-FimU antibody (Figure 2.5a). FimU was present in extracellular pili from the wild type and *pilT* mutants, but not those of the *fimU-pilT* double mutant (Figure 2.5a). Of note, FimU was not observed in sheared surface preparations in the absence of PilA while the intracellular FimU levels remained equivalent to wild type, suggesting that PilA is required for extracellular localization of FimU (Figure 2.5a). As a control to ensure that the shear forces used to remove extracellular pili from the bacterial surface were not sufficient to lyse the cells or to extract inner membrane assembly complex proteins

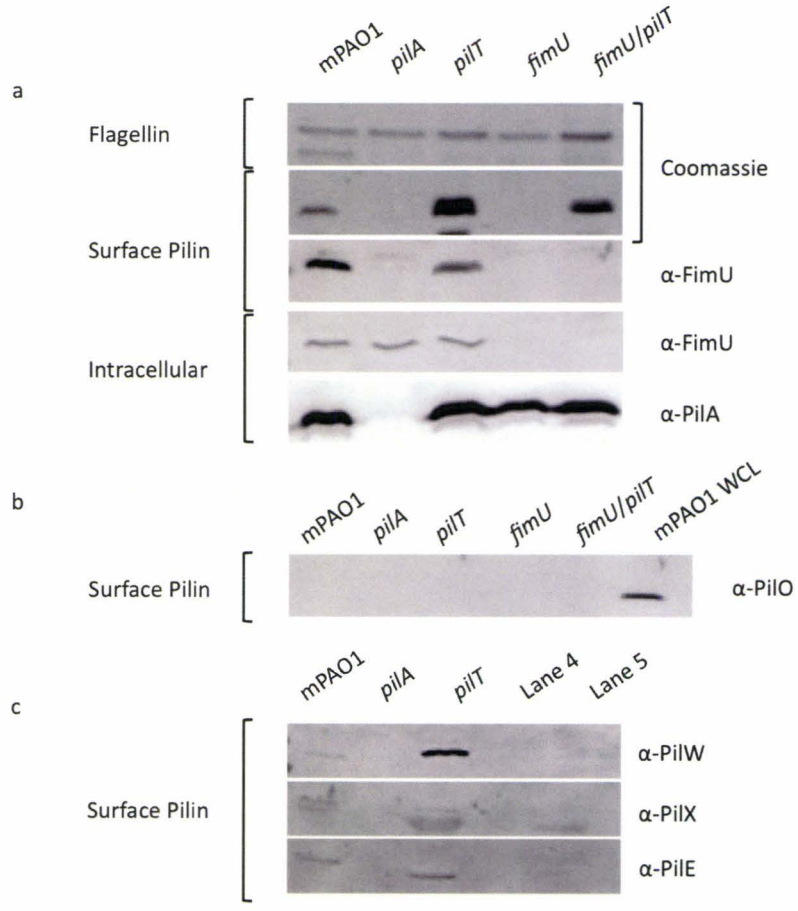

Figure 2.5. The minor pilins are present in external pili fractions.

Figure 2.5. The minor pilins are present in external pili fractions.

(a) Flagellin: sheared flagellin samples were used as a loading control, Surface Pili: Sheared surface proteins from PAO1 wt, *pilA*, *pilT*, *fimU*, and *fimU/pilT* mutant strains grown in the absence of arabinose were separated on 15% SDS-PAGE gels and stained with Coomassie blue, or visualized in Western blots using an anti-FimU antibody. Intracellular: Whole cell lysates of PAO1 wt, *pilA*, *pilT*, *fimU*, and *fimU/pilT* mutant strains were separated on a 15% SDS-PAGE gel and probed with an anti-FimU or anti-PilA antibody. (b) Sheared surface proteins from mPAO1, *pilA*, *pilT*, *fimU*, and *fimU/pilT* mutant strains, and standardized whole cell lysate of mPAO1 were separated on 15% SDS-PAGE gels and probed using an anti-PilO antibody. (c) Surface Pili: Sheared surface proteins from PAO1 wt, *pilA*, *pilT*, *pilW* (lane 4 in the anti-PilW blot), *pilW/pilT* (lane 5 in the anti-PilW blot), *pilX* (lane 4 in the anti-PilX blot), *pilX/pilT* (lane 5 in the anti-PilX blot), and *pilE* (lane 4 in the anti-PilE blot), *pilE/pilT* (lane 5 in the anti-PilE blot) mutant strains grown in the absence of arabinose, were separated on 15% SDS-PAGE gels and probed with anti-PilW, -PilX, or -PilE antibodies.

associated with the pilus, the same sheared surface preparations were blotted with antisera to PilO (an inner membrane protein involved in T4P assembly) (Ayers *et al.*, 2009). PilO could not be detected in any of the samples (Figure 2.5b), suggesting that the extracellular FimU observed is not an artifact, but is a result of its incorporation into the pilus fibre. Similar experiments revealed the presence of PilW, PilX and PilE in pilin preparations from wild type and *pilT* mutant cells, but not in their cognate mutants, suggesting that under native conditions, these minor pilins are also incorporated into the pilus (Figure 2.5c). Our current PilV antibody binds denatured PilV only with very low affinity, therefore we were unable to demonstrate the presence of PilV in sheared fractions by Western blot. However, this antisera does recognize native PilV (below). To show conclusively that the minor pilins localize to the pilus fibre, immunogold labeling of sheared surface proteins from wild type cells with anti-minor pilin antisera was performed as described in the Experimental Procedures. In each case, labeling along the pilus fibre could be detected, showing definitively that each of the minor pilins is incorporated into the pilus (Figure 2.6b and c anti-FimU, 6e and f anti-PilV, 6h and i anti-PilW, 6k and l anti-PilX, and 6n and o anti-PilE, scale bars represent 100 nm). To control for non-specific binding, immunogold labeling of *pilV-pilT, pilW-pilT, pilX-pilT* and *pilE-pilT* surface pilin preparations with anti-PilV, -PilW, -PilX, and -PilE antisera respectively was performed. Immunogold labeling was not observed on any of the *pilV-pilT* (6d), *pilW-pilT* (6g), *pilX-pilT* (6j), or *pilE-pilT* (6m) pilus fibres.

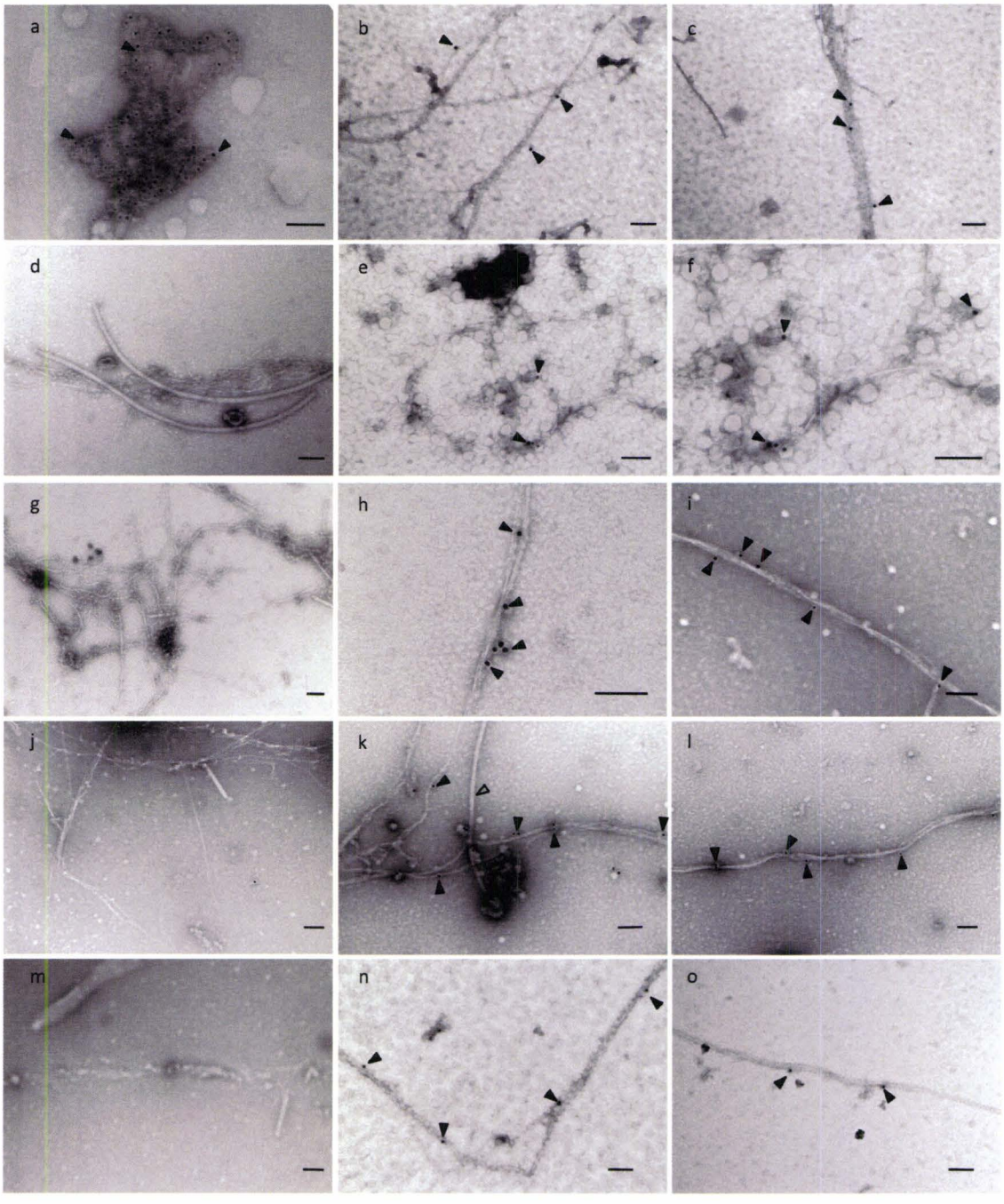

Figure 2.6. The minor pilins are incorporated throughout the pilus fibre.

Figure 2.6. The minor pilins are incorporated throughout the pilus fibre. Immunogold TEM images using anti-PilA, -FimU, -PilV, -PilW, -PilX, or -PilE antibodies and a secondary gold-conjugated antibody. Grids were negatively stained with uranyl acetate and examined with a JEOL JEM 1200 TEMSCAN microscope (Peabody, MA, USA) operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images. mPAO1 pili were probed with (a) anti-PilA, (b and c) anti-FimU, (e and f) anti-PilV, (h and i) anti-PilW, (k and l) anti-PilX, and (n and o) anti-PilE antibodies, while *pilV-pilT* pili were probed with anti-PilV antibody (d), *pilW-pilT* pili with anti-PilW (g), *pilX-pilT* pili with anti-PilX (j) and *pilE-pilT* pili with anti-PilE antibody (m). Filled arrows indicate labeling of the major pilin subunit PilA and the minor pilins FimU, PilV, PilW, PilX and PilE, while open arrows indicate flagella. Bar represents 100 nm.

Although each of the minor pilins appears to be incorporated into the pilus, it is not clear whether the proteins are incorporated singly or as a part of a complex. To investigate potential interacting partners, whole cell lysates of wild type or *fimU*, *pilV*, *pilW*, *pilX* and *pilE* mutants were analyzed for levels of the remaining minor pilins (Figure 2.7). In the *pilE* mutant, FimU levels are reduced to 20% relative to wild-type, while the loss of any of PilV, PilW, or PilX leads to increased FimU levels (370, 346, 361% of wild type respectively). Levels of PilE are reduced compared to wild-type in each of the minor pilin mutants, most notably in the *fimU* mutant (31% of wild type levels)(Figure 2.7). These data suggest a possible interaction between FimU and PilE, based on the reciprocal decrease in relative protein levels associated with loss of either of these components (Figure 2.7). However the decreased levels of PilE in each of the minor pilin mutants suggests additional interdependence between other minor pilins and PilE. The observed increases in FimU protein levels in the *pilV*, *pilW* and *pilX* mutants are not due to introduction of the transposon-specific promoters, since *fimU* is upstream of the other ORFs and therefore expressed from the native promoter. Similarly, the decreased PilW levels in the *fimU*, *pilV* and *pilX* mutants (57, 51, and 34 % of wild type respectively) are likely to be post-translational due to the presence of transposon-encoded promoters driving downstream gene expression in each of the mutants, and instead suggest potential interactions of PilW with the other minor pilins.

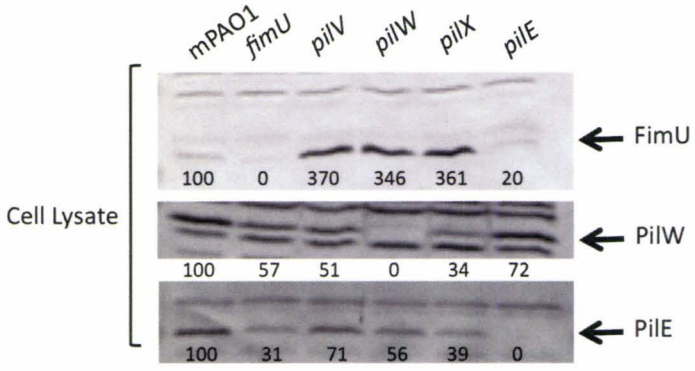

Figure 2.7. FimU and PilE expression levels in minor pilin mutants.

Whole cell lysates of mPA01, *fimU, pilV, pilW, pilX,* or *pilE* mutants were separated on a 15% SDS-PAGE gel and visualized with anti-FimU, anti-PilW or anti-PilE antibodies. Densitometric analyses are shown below each band.

V. DISCUSSION

Analysis of the minor pilin sequences with the functional interaction database STRING 8.1 (Jensen *et al.*, 2009) shows a high probability of protein-protein interactions among them, supporting our contention that the minor pilins may form one or more subcomplexes. Previous bioinformatic studies of proteins involved in pilus biogenesis in sequenced genomes of *P. aeruginosa* showed that most components are highly conserved, with the exception of the major and minor pilins (Asikyan *et al.*, 2008). However, the entire set of minor pilins covaried between genomes, suggesting that the minor pilins evolved as one functional unit (Asikyan *et al.*, 2008). In native conditions, expression of the minor pilin operon under control of AlgR would allow for co-translational formation of a stable complex required for pilus assembly. Although loss of any one of the minor pilins may preclude stable complex formation, a stable complex is not essential for assembly, as single mutants, as well as the *algR* mutant in which none of the minor pilin genes are expressed, are able to produce at least some surface pili in the absence of the retraction ATPase PilT (Figure 2.4). Interestingly, while the overexpression of individual minor pilin proteins did not markedly decrease motility when a stable complex was already present (i.e. in the wild-type background, Figure 2.1), overexpression was detrimental in the mutant backgrounds (Figure 2.1). We propose that the overexpression of individual minor pilins in the context of an unstable complex

leads to titration of other chromosomally expressed components, resulting in the net loss of function (Figure 2.8a).

Although the exact interactions among members of this hypothesized complex(es) remain to be defined, our data suggest a number of potential protein-protein interactions. A FimU-PilE interaction is likely, as loss of one component leads to a decrease in levels of the other (Figure 2.7), while the concordance of the *pilV*, *pilW*, and *pilE* mutant and complemented mutant phenotypes (Figures 2.1, 2.2, and 2.4) may suggest a potential interaction among their gene products. This observation is particularly interesting in light of recent reports in other systems demonstrating interactions between PilV and PilW orthologues. In *Neisseria,* PilI and PilJ (equivalents of *P. aeruginosa* PilV and PilW) depend upon one another for stability (Winther-Larsen *et al.*, 2005), while orthologues of PilV and PilW in the type II secretion (T2S) system of *V. vulnificus* form stable heterodimers (Yanez *et al.*, 2007). A recent *in vitro* study of interactions among minor pseudopilins from the *P. aeruginosa* T2S system showed that the PilV orthologue plays a central role in organization of a quaternary complex containing PilV, PilW, PilX and FimU orthologues (Douzi *et al.*, 2009). The PilV and PilW proteins may therefore be central players, and loss or overexpression of either of these proteins may prevent complex formation. Furthermore, the observed decrease in PilW and PilE levels in expression of PilV, PilW and PilE *in trans* at low levels does not lead to formation of short pili, suggesting that they contribute to pilus assembly in a manner different from FimU and PilX. The observed increase in FimU levels in *pilV*, *pilW* and *pilX*

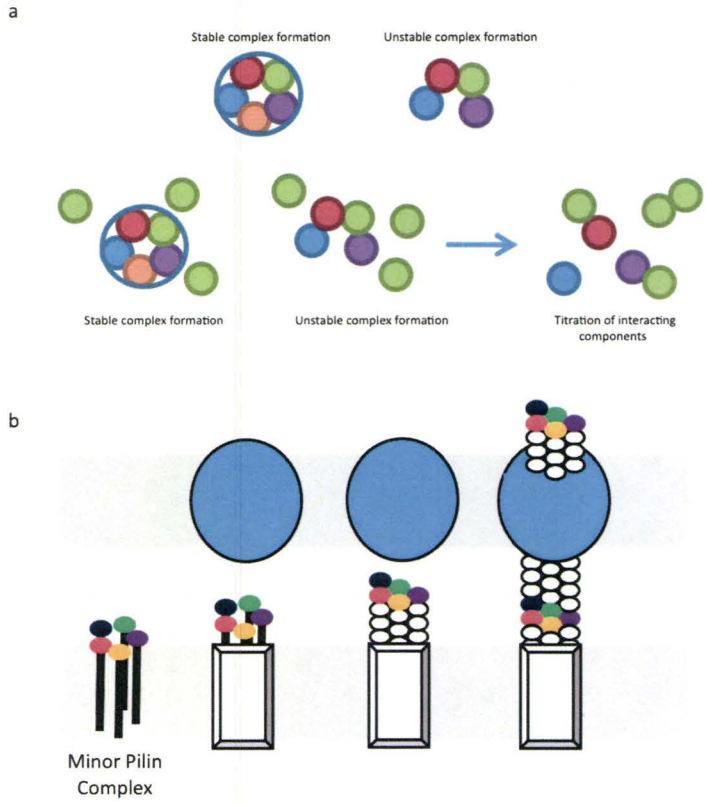

Figure 2.8. Model of the minor pilin complex and its role in pilus assembly

(a) In wild-type cells, the full complement of minor pilins are co-translationally expressed in the correct stoichiometric ratios, which leads to stable complex formation. In single minor pilin mutants, a stable complex is unable to form; therefore when a single minor pilin is expressed in trans, titration of interacting components leads to disruption of function. (b) Simplified model of the T4P system. Green circle represents the outer membrane secretin, while the rectangle represents other inner membrane components essential for piliation. The minor pilin complex, located in the inner membrane is hypothesized to aid in initiation of the pilus fibre, and be incorporated into the growing fibre.

mutants of *P. aeruginosa* compared with those in the wild type is similar to the reported upregulation of minor pilin operon expression in *pilY1* mutants (Bohn *et al.*, 2009), suggesting that there is positive feedback regulation on the *fimU* operon when one or more of its products are missing. The existence of such a positive feedback loop also supports the hypothesized requirement for stoichiometric expression of components in order to correctly form functional minor pilin subcomplexes along the pilus. *fimU* and *pilX* mutants (Figure 2.8) suggests an interdependence of these proteins, and potentially formation of a larger complex including all of the minor pilins.

In *N. gonorrhoeae*, Western blot analyses of pili from minor pilin-*pilT* double mutants showed that with the exception of the PilW orthologue, PilJ, the detection of substantial levels of individual minor pilins in sheared pilus fractions depended on expression of the others, suggesting they might be incorporated in the form of a subcomplex (Winther-Larsen *et al.*, 2005). However, the levels of individual minor pilins detected in surface-exposed pili did not correlate with the levels detected in whole cell lysates, suggesting that the stoichiometry of incorporation may be dictated by factors other than intracellular expression levels. In comparing the native levels of *P. aeruginosa* minor pilins in pili sheared from wild type versus *pilT* mutants, we observed that PilW levels were increased in *pilT* preparations (where there are more pilins) relative to wild type preparations (Figure 2.5), but FimU, PilX and PilE levels were not. Together with the evidence from *Neisseria*, these data suggest that only PilW orthologues are independently and stoichiometrically

incorporated into pili. However, it is difficult to reconcile this idea with the observation that PilV and PilW equivalents are dependent upon one another for stability and form stable heterodimers *in vitro*.

Overexpression of PilV, PilW or PilE (but not FimU or PilX) results in cessation of motility (Figure 2.1) even though recoverable surface pili continue to be produced (Figure 2.2, compare the levels of pili made by the mutant and wild type strain expressing PilE *in trans* at 0.2% arabinose). Misincorporation of single minor pilins, or partial minor pilin complexes, into the pilus may prevent their efficient depolymerization by PilT, resulting in a piliated but non-motile phenotype resembling that of a *pilT* mutant. Shorter-than-wild type pili (*fimU* and *pilX* complemented mutants) appear to mediate relatively normal rates of motility in terms of surface area covered per unit time, which has implications for understanding the mechanism underlying twitching. It is possible that either an increased speed or increased number of cycles of pilus extension and retraction allow for cells with shorter pili to travel a distance equivalent to the wild type. The ability of bacteria with short pili to demonstrate wild type levels of motility supports our previous observation that the amount of recoverable surface pili cannot readily be correlated with the size of motility zones (Harvey *et al.*, 2009).

Various models for the function of the minor pilins in pilus biogenesis have been proposed, including potential roles as primers or terminators of assembly. Alignment of minor pilins of the T4P system and minor pseudopilins from the T2S system shows a high level of conservation, particularly in the N-terminal region

(Figure 2.S3). It is of particular interest to note the unusual features of PilX (GspK in T2S nomenclature) orthologues, including lack of a Glu (+5) residue and the insertion of an unusual 'alpha' domain between β2 and β3 of the antiparallel beta sheet domain of the standard type IV pilin fold (Korotkov *et al.*, 2008). The (+5) residue has been postulated to act as a registration site for PilA as it is assembled into the pilus, by neutralizing the positive charge on the N-terminal amine of the adjacent subunit (Bleves *et al.*, 1998; Craig *et al.*, 2004). Therefore, the substitution of Ile for Glu at the (+5) position was hypothesized to allow XcpX, the PilX homologue in the *P. aeruginosa* T2S system, to act as a terminator of pseudopilus elongation (Bleves *et al.*, 1998; Filloux *et al.*, 1998), as loss of XcpX led to increased filament length, whereas overexpression of XcpX led to shorter filaments. If PilX similarly acted as a terminator of pilus extension, surface pili in a *pilX* mutant might be expected to be longer on average than those of the wild type because assembly would continue unchecked. However, our data does not favour this model, as the *pilX* mutant lacks recoverable surface pili (Figure 2.2) and our electron microscopy data clearly show further incorporation of pilin subunits subsequent to PilX incorporation into growing fibres (Figure 2.6k and 2.6l).

An alternative model proposes that the minor pilins act co-coordinately to prevent retraction of the pilus by forming a cap structure that occludes the PilT ATPase (Winther-Larsen *et al.*, 2005). That model has conceptual issues, in that PilT is cytoplasmic but the minor pilins are presumably oriented with their C-termini in the periplasm, similar to the major subunit PilA. Therefore, it is difficult to visualize

a direct interaction between these components. A second alternative model suggests that the minor pilins could alter the conformation of components that might otherwise interact with PilT in order to block its activity, but there is currently no experimental data supporting such a scenario. Unlike the results observed in the *Neisseria* system where deletion of the minor pilins in a retraction-deficient background did not affect the levels of recoverable surface pili, all of the *P. aeruginosa* retraction-deficient double mutants had reduced surface piliation relative to the *pilT* control. These data support a role for the *P. aeruginosa* minor pilins in promoting assembly or surface presentation of the pilus, rather than opposing its retraction. One or more of the minor pilins could prime assembly, or stimulate opening of the normally-closed PilQ secretin to allow exit of the pili to the exterior of the cell, similar to the scenario proposed for the T2S system (Korotkov *et al.*, 2008; Douzi *et al.*, 2009). In general, the differences in assembly levels observed among individual mutants suggests that although they may form one or more subcomplexes, each minor pilin has a specific role in pilus biogenesis.

Based on our observation that increased FimU and PilX levels result in expression of functional but very short fibres, similar to the situation reported for XcpX (Durand *et al.*, 2005), we propose that the minor pilins FimU and PilX specifically promote assembly, while the remaining minor pilins support this function. If they act as primers, overexpression of either FimU or PilX could create additional start sites for pilus assembly. Assuming a finite number of PilA monomers are present in any one cell, they would be shared among an increased

number of start sites, thus leading to the observed phenotype of short pili (Figure 2.8a). In the absence of FimU, PilX alone appears to be a capable initiator of assembly, as piliation levels of the *fimU-pilT* mutant are only partly reduced relative to the *pilT* control (65%). However, FimU appears to be a less efficient initiator, as piliation levels in the *pilX-pilT* mutant are only 25% of the *pilT* control (Figure 2.4). Alternatively, FimU may promote assembly while PilX stimulates opening of the secretin pore via its unusual alpha domain insertion. In the absence of PilX, pili could still be assembled but their passage through the secretin could be suboptimal, resulting in fewer surface-exposed fibres. Loss of all minor pilins in the *algR-pilT* mutant did not preclude pilus assembly, therefore it can still proceed in their absence; however, the levels of surface pilin were reduced, showing that under conditions were minor pilins are absent, assembly and surface exposure is inefficient.

Although previous studies suggested that the *P. aeruginosa* minor pilins remain in the inner membrane (Alm *et al.*, 1995; Alm *et al.*, 1996), more recent data in the *N. gonorrhoeae* system showed that the minor pilins could be detected in Western blots of sheared wild type pili, although they could not be demonstrated to be integral to the fibre (Winther-Larsen *et al.*, 2005). The *N. meningitidis* minor pilin PilX (which despite its name has no equivalent in the *P. aeruginosa* system), could be detected in intact pili by immunoelectron microscopy, and based on mutant phenotypes, was suggested to stabilize lateral pilus aggregation by physically opposing retraction of bundled fibres (Helaine *et al.*, 2007). In our case, Western

blots of sheared surface preparations and immunogold-labeling studies revealed the presence of FimU, PilV, PilW, PilX and PilE at multiple points along pilus fibres external to the bacterium (Figures 2.5 and 2.6). Based on the data presented here, we suggest that the minor pilins promote pilus assembly, being incorporated into the pilus as a result (Figure 2.8b). The appearance of the minor pilins at locations other than the distal end of the fibre suggest that the minor pilins can be incorporated not simply at the tips of new pili but also throughout growing pili. The corollary of this observation is that incorporation of the minor pilins, including PilX with its atypical features, is not likely to be responsible for subsequent termination of assembly or initiation of retraction events. It should be noted that we cannot rule out the possibility that the minor pilins are indeed localized only to pilus tips, and that the immunogold TEM images show staggered tips of bundled pili, rather than sites of incorporation along the fibre. However, most labeled fibres appear to have a diameter (i.e. less than 8 nm) consistent with a single pilus. Unlike the *Neisseria* PilX protein, the *P. aeruginosa* FimU and PilX proteins clearly do not oppose retraction, as expression of either FimU or PilX *in trans* in their respective mutants results in short, non-recoverable surface pili (Figure 2.2).

In summary, our evidence suggests that the minor pilins PilV/W/E may be required for formation of one or more subcomplexes that allow FimU and PilX to promote pilus assembly and possible secretin egress. Our data suggest that all of the minor pilins are incorporated into the growing pilus fibre instead of simply nucleating assembly from within the inner membrane, and none appear to be

responsible for termination of pilus elongation. These data suggest a new model for T4P biogenesis, where the minor pilin proteins and PilA monomers together form the pilus fibre. Whether the minor pilins have additional roles in adherence or other pilus-related functions will be the focus of future studies.

VI. ACKNOWLEDGEMENTS

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CHAPTER 3:

EVIDENCE FOR A PILIN ISLAND IN *PSEUDOMONAS AERUGINOSA* AND OTHER TYPE IV PILUS-EXPRESSING PSEUDOMONADS

Carmen L. Giltner, Navpreet Rana, Michael N. Lunardo, Anne Hussein,

and Lori L. Burrows

For Submission to Environmental Microbiology

Attribution: C.L.G. designed the experiments with input from L.L.B. C.L.G. performed all of the experiments but for the following exceptions, and directly supervised the participating trainees. M.L.N. generated the group III minor pilin constructs, and assisted with pilin preparations. A.H. generated group II minor pilin combination constructs. Sequencing data for the group IV and V minor pilin gene clusters was generated by N.R. and M.N.L. N.R. assisted with pilin preparations and twitching motility assays in the group III strains, and constructed the group III *algR* mutant. C.L.G. and L.L.B wrote the manuscript.

I. ABSTRACT

Bioinformatic analyses of available *Pseudomonas aeruginosa* genomes revealed that most proteins involved in type IVa pilus biogenesis are highly conserved (>90% similarity), except for the major pilin subunit PilA, and a set of minor pilin-like proteins involved in pilus assembly. Here we show that major pilin alleles are associated with a specific set of minor pilins, and that unrelated strains of the same major pilin type have identical minor pilin genes, suggesting that the two gene clusters were horizontally acquired as a 'pilin island'. Unrelated strains with group III and group V major pilins had identical minor pilin gene sets, suggesting subsequent evolution of the major pilin genes. A similar arrangement of major and minor pilin genes was identified in other Pseudomonads, supporting evolutionary linkage between the gene clusters. To address the biological significance of the observed correlation between major and minor pilin genes, cross-complementation studies between group II and group III strains were performed. With the exception of *pilX*, heterologous genes were able to complement twitching motility, although not to the same extent as the native gene in every case. Motility of a group III mutant unable to express any of its minor pilins could be restored by introduction of the group II minor pilin operon only if the group III *pilX* gene was co-introduced, providing a functional rationale for the linkage between the major and minor pilin operons.

II. INTRODUCTION

Type IV pili (T4P) are widely distributed across eubacterial genera. They are long and thin (up to 4 μm long and 6-8 nm in diameter) but very strong fibres involved in a number of processes ranging from surface attachment to electron transfer (Folkhard *et al.*, 1981; Hahn,1997; Reguera *et al.*, 2005; Giltner *et al.*, 2006). T4P are unique appendages that can be rapidly extended and retracted via polymerization and depolymerization of pilin subunits at the inner membrane in an ATP-dependent manner (Bradley,1980; Semmler *et al.*, 1999; Mattick 2002). Cycles of pilin assembly, binding of the pilus to a surface and pilin disassembly result in "twitching" motility. Although many of the components involved in pilus extension and retraction have been identified through mutagenesis studies, the exact mechanisms of pilus assembly and disassembly are still unclear.

The T4P system of *Pseudomonas aeruginosa*, an opportunistic Gram-negative pathogen, has been extensively characterized over the last two decades, and a large number of proteins involved in pilus regulation, assembly and function have been identified (Mattick 2002; Jacobs *et al.*, 2003). The pilus itself is composed of thousands of PilA subunits, which are processed prior to assembly by the PilD prepilin peptidase (Nunn *et al.*, 1990; Nunn *et al.*, 1991; Strom et al., 1993)(Figure 3.1). There are three ATPases involved in pilus assembly (PilB) and disassembly (PilT and PilU), and several proteins involved in assembly of the pili across the cell envelope (PilC, PilM/N/O/P/Q, PilF). The PilM/N/O/P/Q and F proteins form a

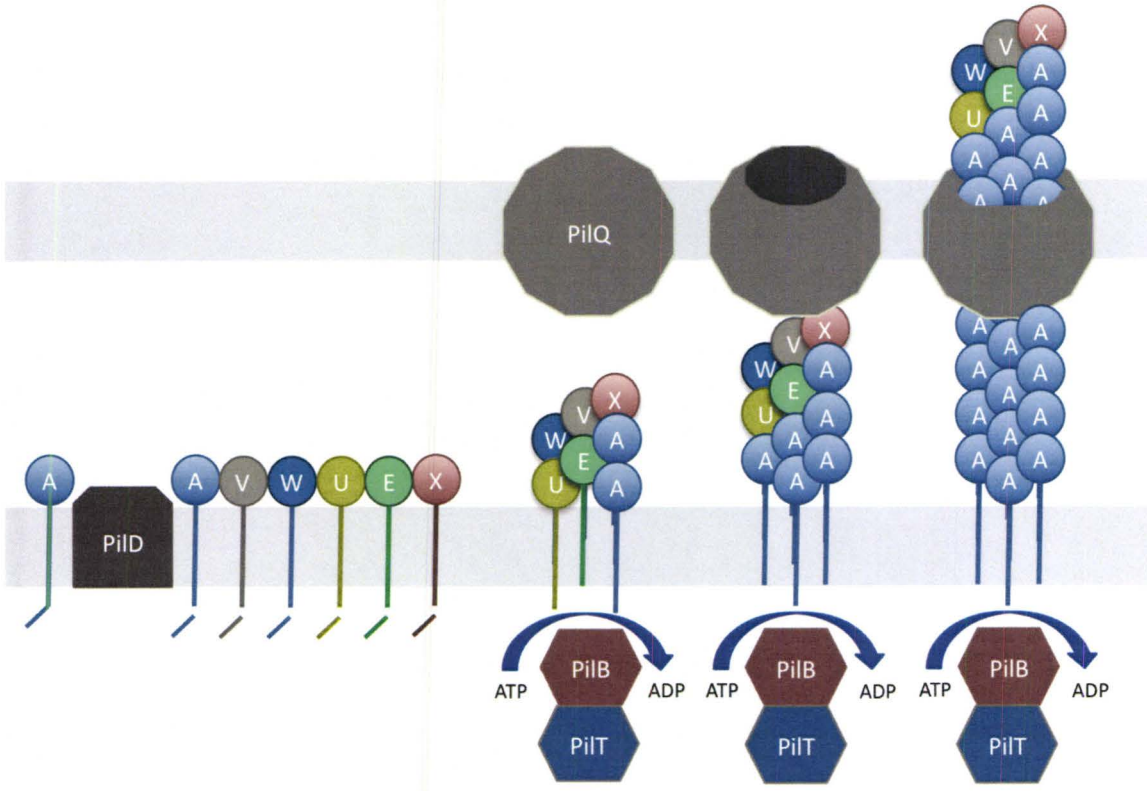

Figure 3.1. Model of Type IV Pilus System

Assembly of the type IV pilus requires over 50 gene products. Here the major biogenesis components are shown. First the major and the minor pilin subunits are processed by PilD. The minor pilins initiate polymerization of the pilus. FimU acts as the intermediate between the minor pilin complex and the major pilin.

complex that spans the entire cell envelope from cytoplasm (PilM) to outer membrane (PilF and PilQ) to allow extrusion of the pili to the exterior (Koo *et al.*, 2008; Ayers *et al.*, 2009; Sampaleanu *et al.*, 2009). The "minor" pilins are a set of low abundance pilin-like proteins encoded by the *fimU/pilV/W/X/Y1/Y2/E* gene cluster; the exact functions of these gene products are not yet clear, but they are involved in control of pilus assembly and incorporated into the pilus fibre (Giltner *et al.*, 2010). Expression of the minor pilin genes is positively controlled by the AlgR response regulator, and loss of twitching motility in *algR* mutants can be restored by provision of the minor pilin operon *in trans* (Belete *et al.*, 2008).

We showed previously that *P. aeruginosa* strains express one of five phylogenetically distinct alleles of PilA (Kus *et al.*, 2004). The pilins differ in several respects including their sequences and overall length, the size of the C-terminal disulfide-bonded loop thought to be the adhesinotope, and the association of the pilin gene with specific downstream accessory genes in distinct genetic cassettes. The pilin accessory genes were shown to be involved in pilin post-translational modification (Castric, 1995; Voisin *et al.*, 2007; Kus *et al.*, 2008) or in modulation of pilus assembly (Asikyan *et al.*, 2008). Using complementation assays, we discovered that introduction of group III, IV or V alleles of *pilA* into a group II strain lacking its cognate *pilA* gene led to poor motility (Asikyan *et al.*, 2008). Further investigation of this phenomenon suggested that the overall rate of pilus assembly was reduced in recombinant strains, leading to fewer surface pili and impaired motility. From that study we hypothesized that a mismatch between horizontally-acquired pilins and

recipient-specific pilus assembly components could potentially be responsible for the motility defect.

Bioinformatic analyses of available *P. aeruginosa* genomes showed that of 7 non-clonal genomes, there were 2 carrying group I pilin alleles, 2 carrying group II, 2 carrying group III and 1 carrying a group IV pilin; no genome with a group V pilin is yet available. Unlike the pilin protein, the majority of T4P assembly proteins were essentially identical among all genomes examined, suggesting that the assembly system in general is not involved in recognition of the pilin protein (Asikyan *et al.*, 2008). In addition to PilA, the only T4P assembly proteins that were not highly conserved among genomes were the minor pilins. However, unrelated genomes containing the same PilA allele had identical minor pilin genes (Asikyan *et al.*, 2008). Furthermore, the minor pilin genes in group I pilin-expressing genomes had high overall similarity to those of the group II genomes, and we showed that only the group I PilA was able to complement the motility of a group II mutant to levels commensurate with the group II PilA protein (Asikyan *et al.*, 2008). Together, these data led us to hypothesize that compatibility between major and minor pilins may be important for normal pilus assembly, and that in order to maintain this functional relationship, the major and minor pilin gene clusters would need to be co-transferred during horizontal DNA acquisition events as a single genomic island. Based on this hypothesis, we proposed that: a) it should be possible to predict the *pilA* allele of any strain based on its minor pilin gene complement, and vice versa; b) the minor pilin genes of group III and group V strains, which have the highest inter-

group similarity between their PilA proteins, would be more similar to one another than to the minor pilins of groups I, II or IV; c) the minor pilin genes of group IV strain Pa5196 should be identical to those of group IV strain PA7, the only other group IV strain identified to date (Kus *et al.*, 2008); and d) minor pilins from the group II strain PAO1 may not restore wild type motility in a group III (PA14) mutant and vice versa, due to incompatibility with the resident major pilin. A corollary of our observation that unrelated genomes have similar major and minor pilin gene sets is that the two gene clusters should be close enough to one another in the genome to allow for their co-transfer during horizontal acquisition of DNA. Our analysis shows that in *P. aeruginosa*, as well as other *Pseudomonas* species, the two clusters form a 'pilin island' that is consistent in size with other pathogenicity islands involved in virulence factor expression, and provide a functional rationale for a the observed linkage between the two gene clusters.

III. METHODS

Bacterial strains, genetic manipulations and growth conditions

The bacterial strains and genetic constructs used in this work are listed in Table 3.1. Cloning was performed using standard molecular methods in *E. coli*. *P. aeruginosa* strains were originally isolated as described in Kus *et al.* (Kus *et*

Table 3.1 List of strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
pBADGr	pMLBAD backbone with dhfr (trimethoprim resistance) replaced with aacC1 (gentamicin resistance)	(Asikyan *et al.*, 2008)
pBADGr*pilA*	PAO1 *pilA* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Harvey *et al.*, 2009)
pBADGr*fimU*	PAO1 *fimU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilV*	PAO1 *pilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilW*	PAO1 *pilW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilX*	PAO1 *pilX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilE*	PAO1 *pilE* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*fimU*$_{III}$	PA14 *fimU* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr*pilV*$_{III}$	PA14 *pilV* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr*pilW*$_{III}$	PA14 *pilW* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr*pilX*$_{III}$	PA14 *pilX* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr*pilE*$_{III}$	PA14 *pilE* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr*fimUpilV*	PAO1 *fimUpilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*fimUpilVW*	PAO1 *fimUpilVW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	This study
pBADGr*fimUpilVWX*	PAO1 *fimUpilVWX* sequence inserted with EcoRI and PstI restriction enzymes in the MCS	This study

	of pBADGr	
pBADGr*algR*	PAO1 *algR* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pEX18AP + *pilT*::GmFRT	Gentamicin FRT insertion in the NruI site within mPAO1 *pilT*	(Harvey *et al.*, 2009)
pEX18AP + *algR*::GmFRT	Gentamicin FRT insertion in the NruI site within PA14 *algR*	This study
pVDtac39	IncQ/P4 *mob*$^{+}$ *tac lacI*^{q}	(Deretic *et al.*, 1987)
pVDtacPIL	pVDtac39 backbone with *fimT fimU pilV pilW pilX pilY1 pilY2* and *pilE* inserted in the MCS	(Lizewski *et al.*, 2004)
E. coli		
DH5α	GENOTYPE: F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1	Invitrogen
TOP10	GENOTYPE: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 ΔlacX74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
SM10	Carries plasmid RP4 with tra genes encoding conjugative pilus integrated into its chromosome, along with a KmR marker	(Simon *et al.*, 1983)
BL21-DE3	GENOTYPE: F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3)	Invitrogen
P. aeruginosa		
mPAO1	Laboratory strain	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilA*	IS*phoA*/hah transposon instertion (position 165)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*fimU*	IS*lacZ*/hah transposon insertion (position 237)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilV*	IS*phoA*/hah transposon insertion (position 122)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilW*	IS*lacZ*/hah transposon insertion (position 381)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilX*	IS*phoA*/hah transposon insertion (position 182)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilE*	IS*phoA*/hah transposon insertion (position 183)	(Jacobs *et al.*, 2003)
mPAO1 *algR*::FRT	Wild type strain with FRT scar insertion in the NruI site within *algR*	This study
PA14 *algR*::FRT	PA14 strain with FRT scar insertion in the NruI site within *algR*	This study

mPAO1 *pilT*::FRT	Wild type strain with FRT insertion in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *pilA*	Wild type strain with mPAO1 *pilA* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *fimU*	Wild type strain with mPAO1 *fimU* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *pilV*	Wild type strain with mPAO1 *pilV* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *pilW*	Wild type strain with mPAO1 *pilW* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *pilX*	Wild type strain with mPAO1 *pilX* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 + pBADGr *pilE*	Wild type strain with mPAO1 *pilE* under arabinose control in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilA* + pBADGr*pilA*	IS*phoA*/hah transposon instertion (position 165) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*fimU* + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilV* + pBADGr *pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilW* + pBADGr *pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilX* + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilE* + pBADGr *pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*fimU* + pBADGr $fimU_{III}$	IS*lacZ*/hah transposon insertion (position 237) complemented with PA14 *fimU* gene in pBADGr	This study
mPAO1 Tn::*pilV* + pBADGr $pilV_{III}$	IS*phoA*/hah transposon insertion (position 122) complemented with PA14 *pilV* gene in pBADGr	This study
mPAO1 Tn::*pilW* + pBADGr $pilW_{III}$	IS*lacZ*/hah transposon insertion (position 381) complemented with PA14 *pilW* gene in pBADGr	This study

mPAO1 Tn::*pilX* + pBADGr $pilX_{III}$	IS*phoA*/hah transposon insertion (position 182) complemented with PA14 *pilX* gene in pBADGr	This study
mPAO1 Tn::*pilE* + pBADGr $pilE_{III}$	IS*phoA*/hah transposon insertion (position 183) complemented with PA14 *pilE* gene in pBADGr	This study
mPAO1 Tn::*fimU-pilT*::FRT	IS*lacZ*/hah transposon insertion (position 237), with an FRT scar in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilV-pilT*::FRT	IS*phoA*/hah transposon insertion (position 122), with an FRT scar in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilW-pilT*::FRT	IS*lacZ*/hah transposon insertion (position 381), with an FRT scar in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilX-pilT*::FRT	IS*phoA*/hah transposon insertion (position 182), with an FRT scar in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilE-pilT*::FRT	IS*phoA*/hah transposon insertion (position 183), with an FRT scar in the NruI site within *pilT*	(Giltner *et al.*, 2010)
mPAO1 *algR*::FRT + *fimUpilV*	Wild type strain with FRT scar insertion in the NruI site within *algR* complemented with group II *fimU* and *pilV*	This study
mPAO1 *algR*::FRT + *fimUpilVW*	Wild type strain with FRT scar insertion in the NruI site within *algR* complemented with group II *fimU*, *pilV* and *pilW*	This study
mPAO1 *algR*::FRT + *fimUpilVWX*	Wild type strain with FRT scar insertion in the NruI site within *algR* complemented with group II *fimU*, *pilV*, *pilW* and *pilX*	This study
mPAO1 *algR*::FRT + pVDtac39	Wild type strain with FRT scar insertion in the NruI site within *algR*, with the vector control pVDtac39	This study
mPAO1 *algR*::FRT + pVDtacPIL	Wild type strain with FRT scar insertion in the NruI site within *algR*, complemented with the minor pilin operon	This study
PA14 *algR*::FRT + pVDtac39	PA14 strain with FRT scar insertion in the NruI site within *algR*, with the vector control pVDtac39	This study
PA14 *algR*::FRT + pVDtacPIL	PA14 strain with FRT scar insertion in the NruI site within *algR*, complemented with the minor pilin operon	This study

al., 2004) and were maintained as glycerol stocks at -80°C. *E. coli* and *P. aeruginosa* were routinely grown on Luria-Bertani (LB) agar containing relevant antibiotics as indicated; for *E. coli*, ampicillin at 100 mg/L, gentamicin at 15 mg/L; for *P. aeruginosa*, carbenicillin at 300 mg/L and gentamicin at 30 and 100 mg/L. For PCR, chromosomal DNA templates were prepared using Instagene (Bio-Rad) as described by the manufacturer. PCR primers were designed using Geneious (Drummond *et al.*, 2009) and synthesized by ACGT Corp. (Toronto, ON).

Generation of algR mutants and pilT double mutants in mPAO1 and PA14

Both single knockouts and double knockouts were generated using the biparental mating assay as previously described (Burrows *et al.*, 2000). The pEX18AP + PA14 *algR*::GmFRT plasmid and the pEX18AP + *pilT*::GmFRT plasmid were electroporated into *E. coli* SM10 cells (Simon *et al.*, 1983). Through biparental mating pEX18AP + PA14 *algR*::GmFRT plasmid was transferred to mPAO1 and PA14 wild type strains and the pEX18AP + *pilT*::GmFRT plasmid was transferred to mPAO1FRT::*algR*, and PA14FRT::*algR* cells respectively. The gentamicin resistance cassette was subsequently excised by the Flp recombinase encoded by pFLP2, and the plasmid removed using sucrose counterselection (Hoang *et al.*, 1998). Single *algR* mutants and double *algR-pilT* mutants were verified by assessing FRT disruption of the *algR* or *pilT* genes respectively using PCR amplification (primers 1 and 2 or primers 3 and 4 respectively, Table 3.2).

Sequencing of novel minor pilin genes from group IV and V pilin-expressing strains of P. aeruginosa

No sequence information was available for the group V pilin-expressing strains, therefore we designed PCR primers based on consensus sequences derived from the alignment of minor pilin genes from groups I-IV pilin-expressing strains (group I, LES; group II, PAO1; group III, PA14; and group IV, PA7). The primers were designed to target the highly conserved 5' regions of the minor pilin genes, encoding the hydrophobic N-terminal alpha helix region of the proteins. Additional primers corresponding to the conserved regions up- and downstream of the minor pilin gene cluster were used to amplify its 5' and 3' ends (Primers 5-19, Table 3.2). Chromosomal DNA from group V strains Pa87110594 and Pa281457 as used as the templates. The PCR products were sequenced directly and additional primers were designed from the resulting sequence to complete the contigs and to sequence across regions originally amplified using the consensus primers.

To date only two isolates containing group IV pilin genes have been identified, strains PA7 and Pa5196. The genome of PA7 was sequenced previously (Dodson *et al.*, 2007). To compare the minor pilin genes of the Pa5196 group IV strain with those from other *P. aeruginosa* strains, they were amplified from Pa5196 chromosomal DNA using overlapping primers designed based on PA7 sequences (Primers 20-35, Table 3.2).

Table 3.2 List of primers used in this study

Primer Number	Primer Name	Sequence (5' to 3')
1	PA14 *algR* Sense	ACGAATTCGAGCTCGGTACCCGGGCCTGAGCTTATGAATGT CCTG
2	PA14 *algR* Antisense	AACGACGGCCAGTGCCAAGCTTCCGCCGACCGCCGTCAGA
3	mPAO1 *pilT* Sense	GGATCCGGTGTTTTCCTTGTCCGA
4	mPAO1 *pilT* Antisense	AAGCTTGAATCCTAGACGCAGTTC
5	0594 Sense 1	TGG CAA CTG GTC CTC AAT CGG CA
6	0594 Antisense 1	TTG CCG GTG ATG CGG CTT TCC AG
7	0594 Sense 2	TAC CGT TCA GCC CAG TGG ACG C
8	0594 Antisense 2	TAG CGC AAG CAG ACA CCG TAC TCA CC
9	0594 Sense A1	AAG CCT GCG CAC AGG AGA TG
10	0594 Antisense A1	GCC CAG GCC ATA CTG CCT GA
11	0594 Sense A2	AGG AAA AAG CCT GCT GGC TG
12	0594 Antisense A2	CGA GAT TGC CGG GGG TGA
13	0594 Sense A3	GGC TCT TGA CCG AGC CGG A
13	0594 Antisense A3	GCC AAG TGG CTA ACT GAC TTC CCT
14	0594 Sense B4	GGT TCG ACC AAA ACC CAG GA
15	0594 Antisense B4	ATA GGC GTA GTC AGC AAT GCC A
16	0594 Sense B5	GCG ATA ATG ACA AGG CAG CTT T
17	0594 Antisense B5	CCT AGT CCA TCC TGT TGG AAG GC
18	0594 Sense B6	CCC TTG TGA CAG CGG CTC TAC
19	0594 Antisense B6	AGA ACA TTA CCT TCT CGA TGC CC
20	5196 *fimT* Sense	GGGCGGTCAACAGGCCGATGACGCCA
21	5196 *fimU* Antisense	TGGTCGCCCTGCTGCTGGTCAGCATCGGC
22	5196 *fimT* (2) Sense	CCTTCGAGCGATGGCCGGGGTTG
23	5196 *fimU* (2) Antisense	CATCGCGTTGAGTTCCTCGCTGGC
24	5196 *pilV*	GGCCGGACGGATCCAACTCGAACC

	Sense 5196 *pilW*	
25	Antisense 5196 *pilX*	GCTCGAGACGGCCAGCAGGGTGAC
26	Sense 5196 *pilX*	CTATCCCGCCTCCAGGACCAGCC
27	Antisense 5196 *pilY1*	CCAACATCAGCGGCTGTTGGCTGACC
28	Sense 5196 *pilY1*	CGGAAAGCCCGGAATACGGCAAC
29	Antisense 5196 *pilY1* (2)	GCCAGATCGGCGAGGGTATTGGAG
30	Sense 5196 *pilY1* (3)	CCAGAACTGCTATACCCGTCGCTCC
31	Sense 5196 pilY1 (4)	GCTTACCGCCCCGGCGAACAGAC
32	Sense 5196 *pilY1* (2)	GATCGAGCCCAGCGGCAACTACG
33	Antisense 5196 *pilY1* (3)	CTGCCGATCAGCACGGTATGCCAGG
34	Antisense 5196 *pilY1* (4)	CCTCACGTCCGGCCATCAGCTCGTC
35	Antisense PA14 *fimU*	CGGTGCCGGAGACCACCACGTTC
36	Sense PA14 *fimU*	TTGGGCTAGCAGGAGGAATTCATGCGCTCTATTTGT
37	Antisense PA14 *pilV*	CCCGGGTACCATGGTGAATTCTCAGTTACAGCTGTC
38	Sense PA14 *pilV*	TTGGGCTAGCAGGAGGAATTCACCCATCTTCAAAG
39	Antisense PA14 *pilW*	CCCGGGTACCATGGTGAATTCATTTTGCTGAAGAG
40	Sense PA14 *pilW*	TTGGGCTAGCAGGAGGAATTCGTGAGAACAAGCAT
41	Antisense PA14 *pilX*	CCCGGGTACCATGGTGAATTCTCATGGCATGAGAT
42	Sense PA14 *pilX*	TTGGGCTAGCAGGAGGAATTCCTGCGCCATAC
43	Antisense PA14 *pilE*	CCCGGGTACCATGGTGAATTCTCAGTTGGTATACAG
44	Sense PA14 *pilE*	TTGGGCTAGCAGGAGGAATTCATGAAGTCGAACAGA
45	Antisense	CCCGGGTACCATGGTGAATTCTTAGCGCCAGCATTCT

Complementation of group II mutants with group III minor pilins

Complementation constructs were created as previously described (Giltner *et al.*, 2010). Briefly, group III minor pilin genes were amplified via PCR from strain PA14 chromosomal DNA. Primers included EcoRI restriction sites for insertion into the pBADGr vector (Guimond *et al.*, 2008). Primer sequences are listed in Table 3.2.

Combinations of these primers were used to amplify *fimU*, *pilV*, *pilW*, *pilX*, and *pilE* genes. *fimU* was amplified with primers 36 and 37; *pilV* with primers 38 and 39; *pilW* with primers 40 and 41; *pilX* with primers 42 and 43; and *pilE* with primers 44 and 45 (Table 3.2). Minor pilin genes were amplified using Taq polymerase (Qiagen) and the PCR products purified (Qiagen) before digestion with EcoRI restriction enzyme (Table 3.2). The pBADGr vector was linearized using identical restriction enzymes and the digested PCR products and vector were ligated with Fast Ligase (Fermentas). Ligated constructs were electroporated into *E. coli* DH5α cells and their integrity verified via DNA sequence analysis (ACGT Co., Toronto, ON). Verified group III constructs and group II constructs (Giltner *et al.*, 2010) were electroporated into relevant mPAO1 mutants and transformants were selected on LB containing gentamicin (30 mg/L).

Twitching motility assays

Bacterial twitching was performed as previously described (Gallant *et al.*, 2005). Briefly, single bacterial colonies were stab-inoculated with a sterile toothpick to the underlying plastic surface of 1% agar LB plates containing

gentamicin (30 mg/L) or gentamicin and L-arabinose (0.2%), or carbenicillin (200 mg/L) and isopropyl β-D thiogalactopyranoside (1 mM, IPTG). After incubation, the agar was carefully removed and twitching zones were visualized by staining with 1% (w/v) crystal violet for 30 minutes. Unbound dye was removed with water and plates were allowed to air dry.

Surface Piliation Assay

Sheared surface proteins were isolated as described previously (Martin *et al.*, 1995; Crowther *et al.*, 2005; Guimond *et al.*, 2008) with the following modifications. Bacterial strains were streaked in a cross-hatched fashion on three 1.5% agar LB plates containing gentamicin (30 mg/L) or gentamicin and L-arabinose (concentrations of 0.01, 0.02, 0.1 and 0.2 % w/v) and grown overnight at 37 °C. Bacteria were gently scraped from the agar surface with a sterile coverslip, and resuspended in 4.5 ml of 1x phosphate-buffered saline (PBS pH 7.4). Suspensions were vortexed for 30 s to shear surface organelles and transferred to three 1.5 ml microcentrifuge tubes where the cells were harvested by centrifugation at room temperature (RT) for 5 min at 11688 x g. The supernatant was transferred to new microcentrifuge tubes and re-centrifuged at RT for 20 min at 11688 x g to remove remaining cellular debris. The supernatant was transferred to new microcentrifuge tubes and a 1/10 volume of each of 5 M NaCl and 30 % polyethylene glycol (MW 8000) was added. The mixture was incubated on ice for 1 hr to allow for precipitation of proteins. Tubes were centrifuged a final time at RT for 30 min at

11688 x g to harvest the precipitated proteins (pilin and flagellin). The three recovered pellets for each sample were pooled and resuspended in 100 µl of 1 x sodium-dodecyl-sulphate (SDS) loading buffer (80 mM Tris pH 6.8; 5.3 % [v/v] 2-mercaptoethanol; 10 % [v/v] glycerol; 0.02 % [w/v] bromophenol blue; 2 % [w/v] SDS). Samples were boiled for 10 min and run on a 15 % SDS-PAGE gel. Precipitated proteins were visualized with Coomassie brilliant blue (Sigma).

Western blot assays

Protein samples were run on 15% SDS-PAGE gel as described above and subsequently transferred to a nitrocellulose membrane for analysis with rabbit polyclonal anti-pilin antibodies. The nitrocellulose membrane was blocked O/N with 5 % skim milk (w/v) at 4 °C. Blots were incubated with PilA, FimU, PilV, PilW, PilX, or PilE antisera (Giltner *et al.*, 2010) at a 1:5000 (PilA) or 1:1000 (FimU, PilVWXE) dilution for 2 hr at RT, washed three times with 1 x PBS for 10 min, and incubated with a 1:3000 dilution of secondary goat-anti-rabbit IgG alkaline phosphatase antibody (Sigma) for 2 hr at RT. Blots were washed with 1 x PBS as above, and developed using NBT/BCIP (BioRad).

Accession numbers

Sequences of group III and group V minor pilin operons were deposited in GenBank under the following accession numbers: Pa87110594 bankit 1335437; Pa5196 bankit 1335458.

IV. RESULTS

Minor pilin genes are identical in strains of the same major pilin type

Of the sequenced genomes of *P. aeruginosa* currently available, there is one carrying the group IV *pilA, tfpW* and *tfpX* genes (strain PA7) and none carrying the group V *pilA* and *tfpZ* genes. To test our hypothesis that strains with the same major pilin allele have the same minor pilin genes, we sequenced the minor pilin genes in the only other group IV pilin-expressing strain identified to date, Pa5196 (Guimond *et al.*, 2008), and in the group V strains Pa87110594 and Pa281457, which we showed previously by pulsed field gel electrophoresis fingerprinting to be unrelated other than by their pilin type (Kus *et al.*, 2004). The minor pilin genes of group IV strains Pa5196 and PA7 were 98% identical over 6719 nucleotides, and the minor pilin genes from the two group V strains were 99% identical over 6747 nucleotides. Therefore, strains of the same major pilin type have identical minor pilin genes. The group III and group V PilA proteins were previously shown to be more similar to one another (45.9 % amino acid identity over 179 residues) than to other *P. aeruginosa* pilins (Kus *et al.*, 2004), and therefore we predicted that their minor

pilin genes would also be more similar to one another than to those of other pilin types. Surprisingly, we found that minor pilin genes from group III and V strains are essentially identical (97.6 % over 6789 nucleotides) (Figure 3.2).

PCR verification of major and minor pilin gene relationships

To verify the observed correlation between major and minor pilin genes, we developed diagnostic PCR primers designed to amplify minor pilin gene sequences from strains of each major pilin group(s) (Table 3.2). From our collection of >350 strains of pilin-typed *P. aeruginosa* (Kus *et al.*, 2004), we randomly selected 5 strains belonging to each of pilin groups I, II, III and V (group IV comprises only the two representatives mentioned above, PA7 and Pa5196), performed PCR and sequenced the resulting PCR product. In each case, an amplicon of the expected size and sequence was obtained, confirming the observed correlation between major and minor pilin type.

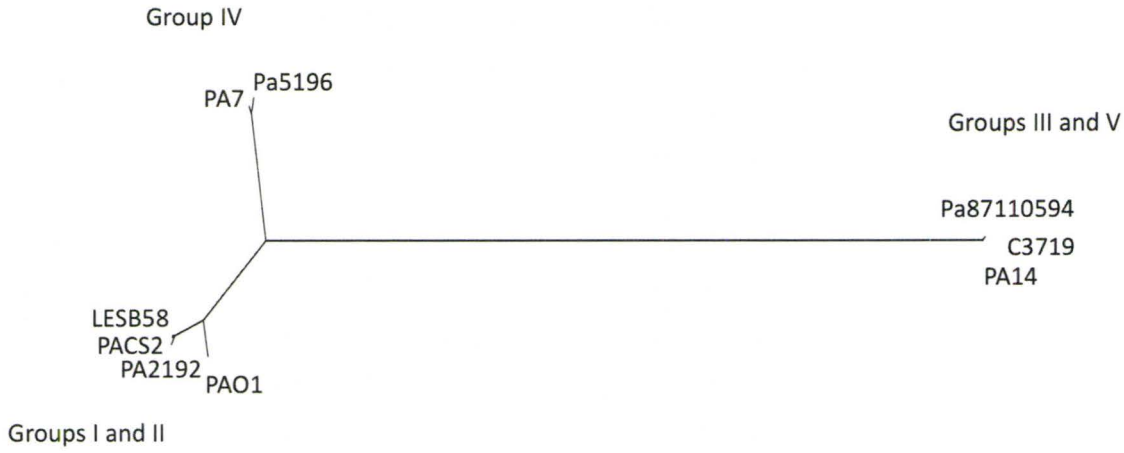

Figure 3.2. Phylogenetic relationship between pilin groups

Phylogenetic tree of the minor pilin operon was constructed using a bootstrap value of 1000. Identical trees were observed using neighbour-joining, parsomony and maximum likelihood methods. Minor pilin operons are grouped by their respective pilin allele suggesting that the major and minor pilin operons are linked. Confidence values thresholds were set to 0.9.

Major and minor pilin gene clusters in P. aeruginosa and other Pseudomonads

The reproducible association between the two gene clusters suggested that they were co-acquired through horizontal gene transfer; therefore, we examined their genetic arrangement on the *P. aeruginosa* genome. Figure 3.3 shows that the two gene clusters are separated by approximately 24.6 kb; the entire region encompassing the two clusters and the intervening genes is approximately 36 kb, which is similar in magnitude to other characterized genomic islands, which range in size from ≥10-500 kb (Hacker *et al.*, 2000; Langille *et al.*, 2008). There are three conserved, tandemly-arranged tRNA genes located between the *pilA* and minor pilin gene clusters, encoding a $tRNA^{Lys}$, $tRNA^{Pro}$ and $tRNA^{Asn}$ (Figure 3.3). The same arrangement is found in all of the *P. aeruginosa* genomes examined, regardless of the pilin type, although an additional insertion of 108 kb occurs in the PA14 genome upstream of $tRNA^{Lys}$ (Figure 3.3). Interestingly, the 108 kb insertion in PA14 contains genes for two other types of pili: the type IVb pilus components *pilL2/N2/O2/P2/Q2/R2/S2/T2/V2/M2*, as well as the *cup* gene cluster *cupD1/D2/D3/D4/D5* encoding Cup fimbriae (Figure 3.3). We also examined the genetic arrangement of orthologous genes in other *Pseudomonas* species that express T4P, including *P. fluorescens, P. syringae, P. entomophila* and *P. stutzeri.* Although the annotations of other species' genomes are incomplete with respect to T4P components, we could readily identify the two gene clusters through sequence similarity, and found that they are arranged in a similar manner (Figure 3.3). The

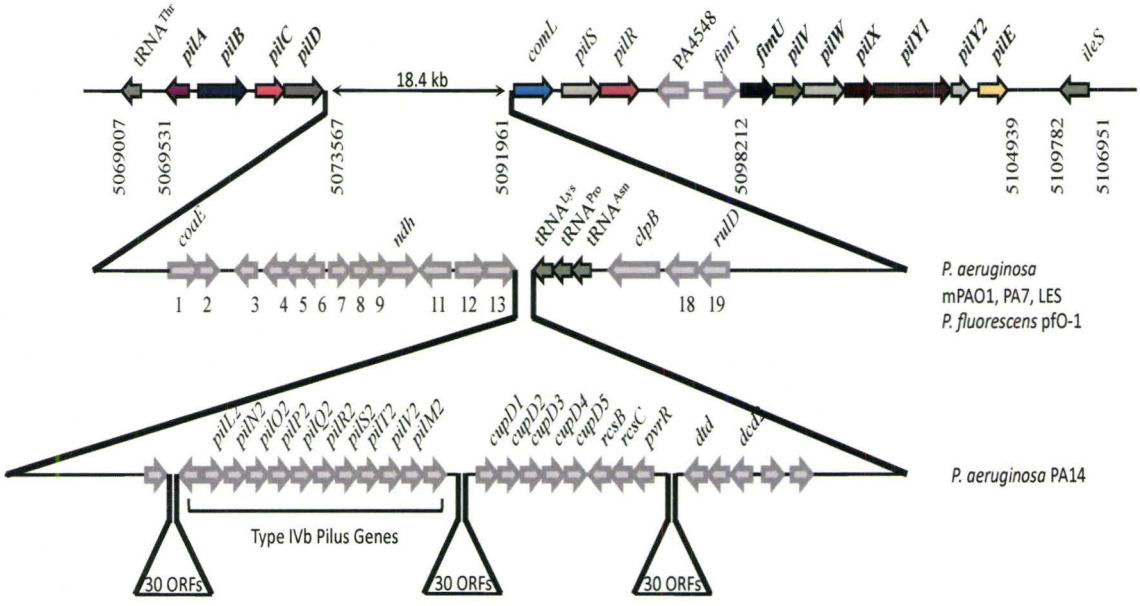

Figure 3.3. Major and Minor pilin operons are syntenic amongst Pseudomonads

P. aeruginosa strains mPAO1, PA7, and LES show identical gene distribution between the major and the minor pilin operons, while *P. fluorescence* pfO-1 shows an additional $tRNA^{Pro}$ directly upstream of the $tRNA^{Lys}$ gene. The *P. aeruginosa* strain PA14 shows an additional 108 kb insertion directly upstream of tandem tRNA sequences (above), while *P. syringae* DC3000 has a 99.2 kb insertion upstream of the *$tRNA^{Lys}$* and *P. stutzeri* an additional 90.6 kb insertion downstream of the *coaE* gene. ORF denotes open reading frame

combination of the highly conserved major pilin operon and the presence of three sequential *tRNA* genes appears to act as a hot spot for genetic insertion, as the gene order in other *Pseudomonas* spp. examined is conserved with that of mPAO1, with species-specific insertions upstream of the $tRNA^{Lys}$ gene (similar to PA14). *P. fluorescens* PfO-1 has an additional $tRNA^{Pro}$ insertion upstream of the $tRNA^{Lys}$ gene, *P. syringae* DC3000 has a 99.2 kb insertion upstream of the $tRNA^{Lys}$ and *P. stutzeri* has an additional 90.6 kb insertion downstream of the *coaE* gene. The *P. syringae* insertion has an additional $tRNA^{Pro}$, similar to *P. fluorescens*, and cluster of chemotaxis genes (among others), while the *P. stutzeri* insertion contains cell shape and type II secretion elements (data not shown).

In *P. aeruginosa*, the mol%GC content of the *pilA* gene ranges from 49% (strain PAO1) to 55% (strain PA14), well below the genome average of 67% (Stover *et al.*, 2000), while that of the *pilB/C/D* genes is 59-61%, 49-55% and 65%, respectively. The minor pilin genes have a higher mol%GC ranging from 61% to 65%, closer to the species average. In total, there are 24 ORFs between *pilD* and *fimU* in *P. aeruginosa* (Figure 3.3), most of which are annotated as hypothetical genes. However, notable genes include *pilR/S*, encoding a two-component regulatory system that was previously shown to be required for transcription of the *pilA* gene, and *comL*, which in species other than *P. aeruginosa* has been shown to be involved in T4P-mediated competence for DNA uptake. Comparison of the intervening ORFs among *P. aeruginosa* genomes showed that they are identical in all strains, with the exception of the PA14 insertion. The boundaries of the 'pilin island'

are defined by a highly conserved $tRNA^{Thr}$ gene located upstream of the *pilA* gene cluster and highly conserved biosynthetic genes including *ileS* (isoleucine tRNA synthetase) downstream of the minor pilin gene cluster (Figure 3.3). Based on these analyses, it appears that the major and minor pilins were co-acquired through horizontal gene transfer, and that insertions between these clusters (*P. aeruginosa* PA14, *P. syringae*, *P. fluorescens* and *P. stutzeri*) may have been secondary events mediated by insertions at conserved tRNA genes.

Functional conservation among minor pilins in P. aeruginosa

The minor pilin genes are essential for twitching motility in *P. aeruginosa* (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996). Our previous complementation data showed that group III major pilins did not restore wild type motility when introduced into group II *pilA* mutants (Asikyan *et al.*, 2008), suggesting that compatibility between major and minor pilins is important for optimal function, although this concept was not tested directly. Therefore, we took a two-pronged approach to investigating whether minor pilins of divergent sequence (i.e. those of groups II and III) were able to substitute for one another *in vivo*. First, we took advantage of the observation of Belete and colleagues (Belete *et al.*, 2008) that the response regulator AlgR was required for transcription of the minor pilin operon, and that expression of the minor pilin genes *in trans* in an *algR* mutant could restore motility. An *algR* mutant of group II strain mPAO1 ($algR_{II}$) was

generated as described in the Methods, and we verified that it was no longer capable of twitching motility (Figure 3.4). To determine the minimum number of minor pilins required for wild type surface piliation, complementation constructs expressing one or more group II minor pilin genes were introduced into the $algR_{II}$ mutant. Complemented strains were assessed for twitching motility and surface piliation (Figure 3.5). The *algR* mutant was unable to twitch or express surface pili until the full set of minor pilin genes was provided (Figure 3.5). To determine whether these results were consistent among strains, we generated an *algR* mutant of group III strain PA14 as described in the Methods. Similar to the $algR_{II}$ mutant, the $algR_{III}$ mutant was no longer capable of twitching motility (Figure 3.4). We also assessed the ability of the $algR_{III}$ mutant to assemble pili in a retraction-deficient background lacking the PilT retraction ATPase. As we showed previously for an $algR_{II}$-*pilT* mutant (Giltner *et al.*, 2010), the $algR_{III}$-*pilT* strains were capable of assembling surface pili (Figure 3.4). The PA14 $algR_{III}$ mutant was then transformed with either pVDtacPIL encoding the entire mPAO1 minor pilin operon or the pVDtac39 vector control (Deretic *et al.*, 1987; Lizewski *et al.*, 2004). Although we and others have shown that pVDtacPIL complements twitching motility and surface piliation in $algR_{II}$ mutants of PAO1 (Lizewski *et al.*, 2004; Belete *et al.*, 2008; Giltner *et al.*, 2010), the construct did not complement either phenotype in the group III

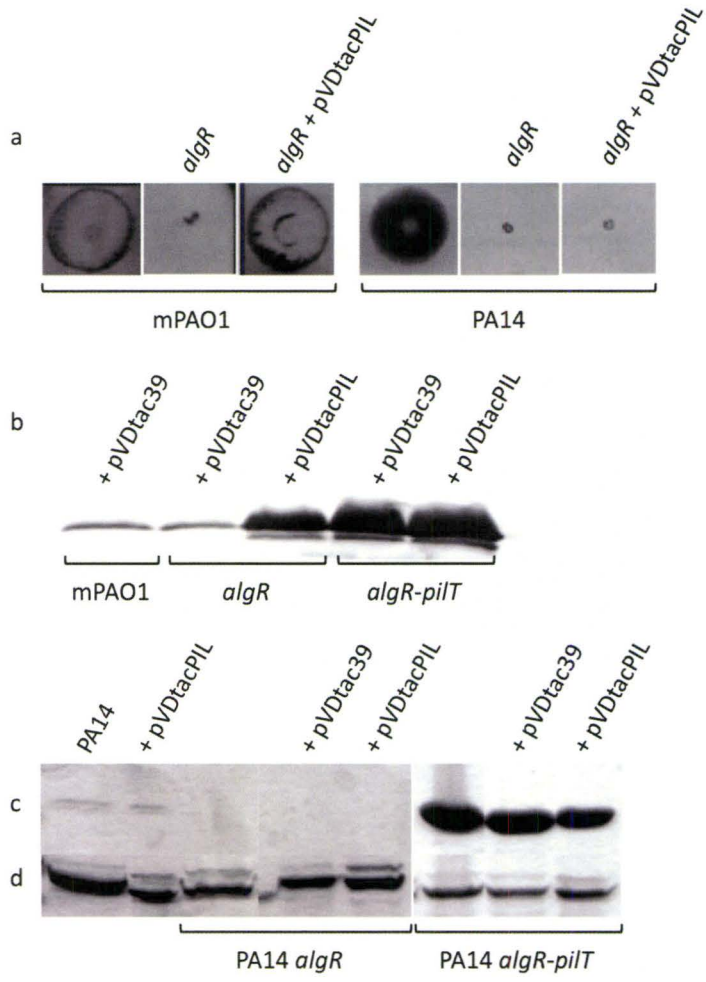

Figure 3.4. Group II minor pilins do not complement group III algR mutants

Figure 3.4. Group II minor pilins do not complement group III algR mutants

(a) Bacterial strains of mPAO1, mPAO1-*algR*, PA14, PA14-*algR*, and *algR* mutants complemented with either the empty vector (pVDtac39) or the entire minor pilin operon (pVDtacPIL) were stab-inoculated through 1% LB-agar to the underlying plastic surface and were grown for 24 hours. Twitching zones were visualized with 1% crystal violet staining. Twitching motility assay shows loss of motility in an *algR* mutant, which is restored to wild type levels upon complementation with the whole minor pilin operon (pVDtacPIL) in the mPAO1 background. However the group II minor pilins can not complement the group III PA14-*algR* mutant. (b) Sheared pilin preparations of strains mPAO1, *algR*, *algR-pilT*, and complemented mutants were run on a 12.5 % SDS-PAGE gel, transferred to nitrocellulose and probed with an anti-PilA (group II) antibody. (c) Sheared pilin preparations of strains PA14, *algR*, *algR-pilT*, and complemented mutants were run on a 12.5 % SDS-PAGE gel and stained with Commassie-brilliant blue. (d) Flagellin loading control.

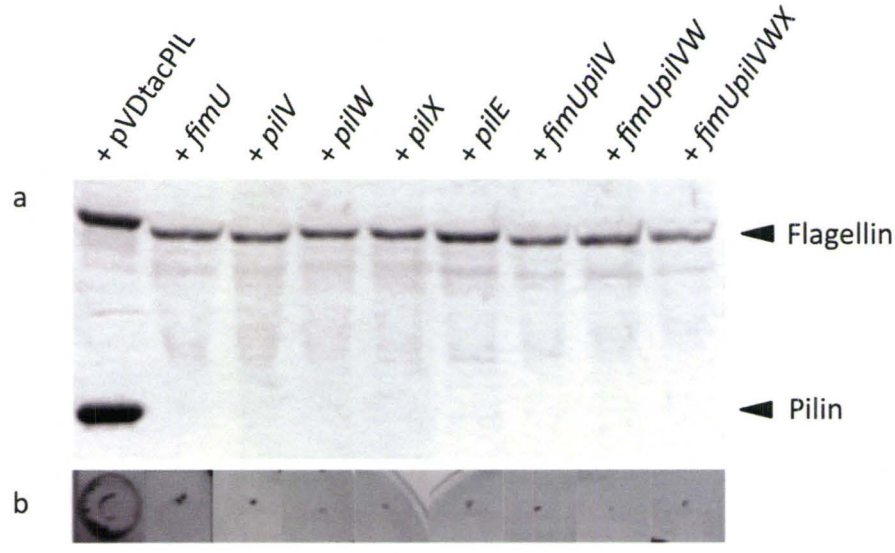

Figure 3.5. The entire minor pilin complement is required for functional surface piliation

(a) Purified pilin preparations of mPAO1-a*algR*$_{II}$, mutants complemented with *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *fimUpilV*, *fimUpilVpilW*, *fimUpilVpilWpilX*, or the full minor pilin operon (pVDtacPIL) were run on a 15% SDS-PAGE gel and stained with Commassie-brilliant blue. (b) Bacterial strains of mPAO1-a*algR*$_{II}$, mutants complemented with *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *fimUpilV*, *fimUpilVpilW*, *fimUpilVpilWpilX*, or the full minor pilin operon (pVDtacPIL) were stabbed inoculated on a 1.0 % LB agar plate and grown for 24 hours. Agar was removed and plastic surface was stained with crystal violet (1.0 %).

background (Figure 3.4), suggesting that one or more of the group II minor pilins do not function in the group III background.

To ensure that the minor pilins were being expressed at similar levels in different backgrounds, whole cell lysates of mPAO1, PA14, $algR_{II}$, $algR_{III}$, $algR_{II}$ + $algR_{II}$ and $algR_{III}$ + $algR_{III}$ were probed with antibodies raised against group II FimU, PilV, PilW, PilX, or PilE respectively (Giltner *et al.*, 2010) (Figure 3.6). The group II FimU, PilV, PilW, PilX and PilE proteins were detected, however the group II antibodies were not cross-reactive with group III minor pilins (Figure 3.6 and data not shown).

As an alternate strategy to assess the functional relatedness of the minor pilins from different groups, we complemented group II mPAO1 mutants lacking individual minor pilin genes (Giltner *et al.*, 2010) with their counterparts from group III strain PA14. The overall amino acid similarities between the mPAO1 and PA14 gene products range from 61% for PilE to 75% for PilV and PilX, although the similarities are higher in the highly conserved N-terminal regions and lower at the C-termini. We compared twitching motility of the recombinant strains to that of strains expressing their native gene *in trans* from the same promoter/vector combination. The levels of expression of each group II protein were determined by Western blot with specific antibodies (Figure 3.7). Complementation of the group II mutants with the equivalent group III genes *in trans* decreased motility by ~6, 3, 33, and 8 % relative to complementation with the group II minor pilin *fimU*, *pilV*, *pilW*,

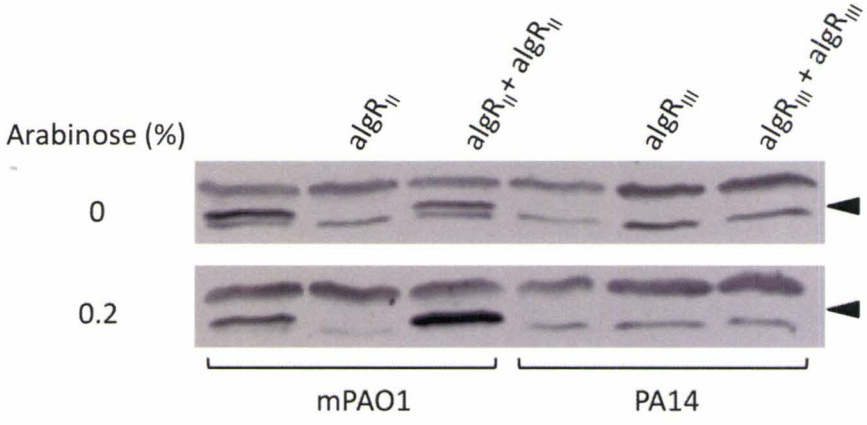

Figure 3.6. Group II minor pilin antibodies are not cross-reactive with group III minor pilins

Whole cell lysates of mPAO1, PA14, *algR*$_{II}$, *algR*$_{III}$, *algR*$_{II}$ + *algR*$_{II}$ and *algR*$_{III}$ + *algR*$_{III}$ were run on a 15% SDS-PAGE gel and transferred to nitrocellulose. Protein expression levels of the minor pilins were visualized using a 1:1000 dilution of anti-FimU antibody. Similar to the major pilin antibody, the group II minor pilin antibodies were not cross reactive with the group III minor pilins.

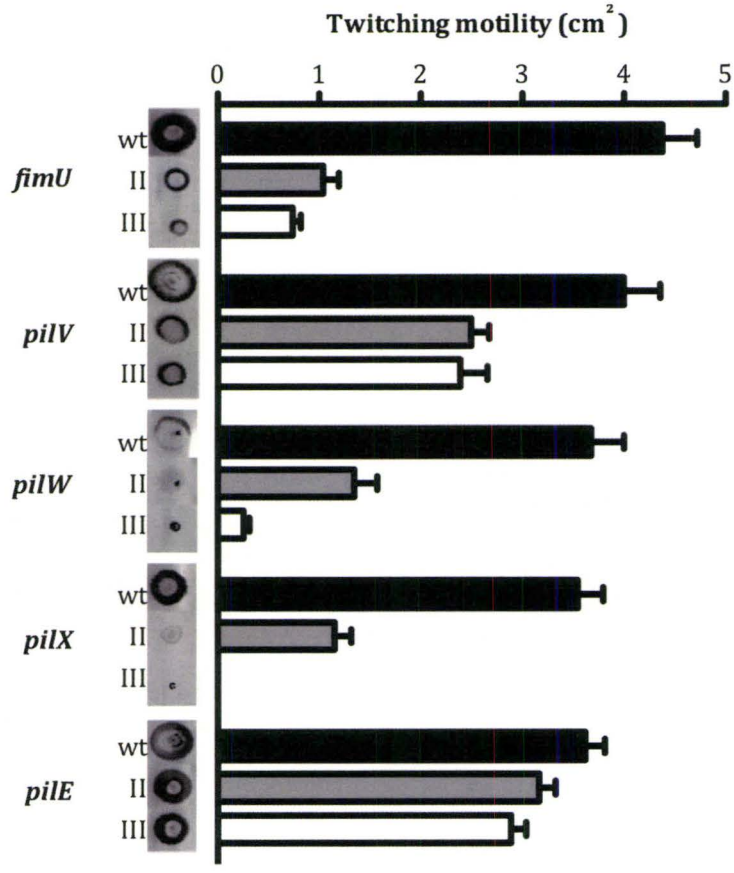

Figure 3.7. Group III minor pilins cannot complement Group II mutants

Bacterial strains of mPAO1, *fimU*, *pilV*, *pilW*, *pilX*, *pilE* mutants and their respective complemented strains were stab-inoculated through 1% LB-agar to the underlying plastic surface and were grown for 24 hours. Twitching zones were visualized with 1% crystal violet staining. Twitching motility zones of wild type and group II minor pilin mutants complemented with either the groups II or group III cognate gene were measured using ImageJ (Ambramoff *et al.*, 2004) for replicates of 3 where n=12. Representative twitching zones are seen on the left, while average twitching zones are seen on the left.

or *pilE* strains respectively; however no motility was observed with the $pilX_{III}$ complementation construct (Figure 3.7). Previously, we have shown that group II complementation constructs were able to complement twitching to wild type levels, but that twitching motility did not correlate directly with surface piliation (Giltner *et al.*, 2010). Consistent with our previous results, no surface piliation was observed in a sheared surface protein assay for the $PilX_{II}$- or the $PilX_{III}$-complemented strains (Figure 3.8). However, when these strains were probed with an anti-PilA antibody, a small amount of surface pilin was detected for the $PilX_{II}$ but not the $PilX_{III}$ complemented strain (Figure 3.8). As we have previously shown that the $PilX_{II}$ complemented strain was able to twitch to ~72 % relative to wild type twitching (Giltner *et al.*, 2010) the complete absence of surface pilin in the $PilX_{III}$ complement was consistent with the lack of twitching observed for this strain (Figure 3.8). While levels of surface piliation were equivalent in the group II or III complemented *pilV* and *pilE* mutants, complementation of the $fimU_{II}$ mutant with $fimU_{III}$ increased surface piliation by ~55% relative to the mutant complemented with the group II gene, while complementation of the $pilW_{II}$ mutant with $pilW_{III}$ decreased surface piliation by ~50% compared to complementation with the cognate gene (Figure 3.8).

We then assessed the extent of surface piliation in retraction-deficient group II minor pilin mutants complemented with the relevant group III minor pilin gene.

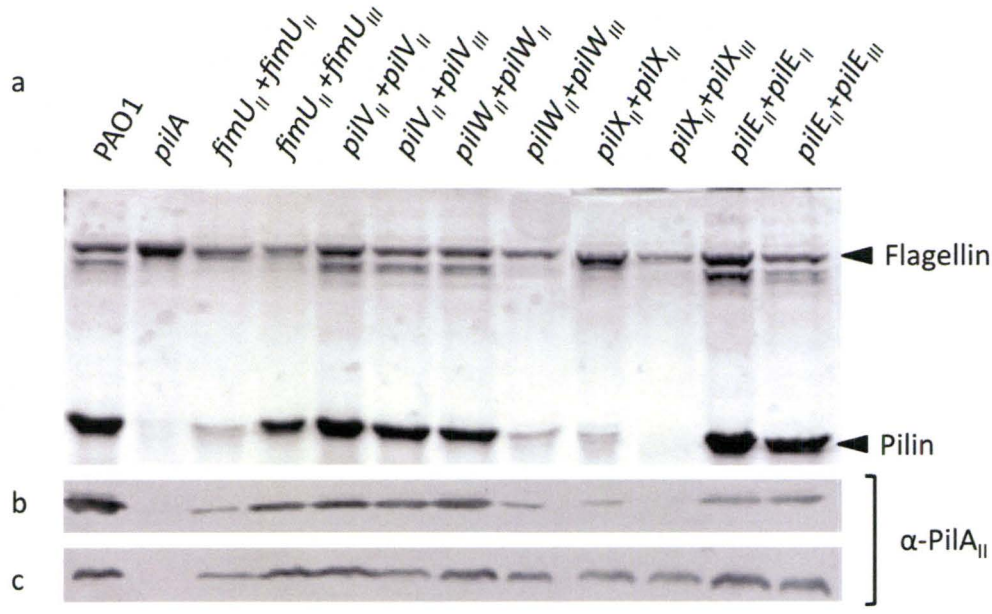

Figure 3.8. Group III minor pilins can complement group II mutants with the exception of pilX

(a) Purified pilin preparations of mPAO1, the *pilA* mutant and the minor pilin complemented strains *fimU* +*fimU*$_{II}$, *fimU* +*fimU*$_{III}$, *pilV* + *pilV*$_{II}$, *pilV* + *pilV*$_{III}$, *pilW* + *pilW*$_{II}$, *pilW* + *pilW*$_{III}$, *pilX* + *pilX*$_{II}$, *pilX* + *pilX*$_{III}$, *pilE* + *pilE*$_{II}$, and *pilE* + *pilE*$_{III}$ were run on a 15% SDS-PAGE gel and stained with Commassie-brilliant blue. Surface pilin preparations (b) or whole cell lysates (c) of mPAO1, the *pilA* mutant and the minor pilin complemented strains *fimU* +*fimU*$_{II}$, *fimU* +*fimU*$_{III}$, *pilV* + *pilV*$_{II}$, *pilV* + *pilV*$_{III}$, *pilW* + *pilW*$_{II}$, *pilW* + *pilW*$_{III}$, *pilX* + *pilX*$_{II}$, *pilX* + *pilX*$_{III}$, *pilE* + *pilE*$_{II}$, and *pilE* + *pilE*$_{III}$ were run on a 15% SDS-PAGE gel, transferred to nitrocellulose, and probed with an anti-group II PilA antibody.

Complementation of the *pilW-pilT* mutant with either group II or III *pilW* restored surface piliation to *pilT* background levels (~111 and 95 % respectively). Complementation with the group II minor pilin lead to an increase in surface piliation relative to the group III complement in *fimU*, *pilV*, and *pilX* strains (~227 vs. 186 for *fimU*, ~287 vs. 120 for *pilV*, and ~142 vs. 48 % for *pilX* group II and III complements respectively), while conversely, *pilE* mutant strains had more recoverable surface pili when complemented with the group III cognate gene (~79 vs. 114 % for group II and III respectively; Figure 3.9). Interestingly, while an increase of 55% in piliation was observed in the $fimU_{III}$-complemented relative to the $fimU_{II}$-complemented $fimU_{II}$ mutant, the $fimU_{II}$ complemented $fimU_{II}$-*pilT* mutant had a 41% increase in surface piliation relative to complementation with $fimU_{III}$ (Figures 3.8 and 3.9).

PilX is a group-specific factor

As surface piliation and twitching motility were not restored in $algR_{III}$ mutants complemented by the group II minor pilin operon, or in group II *pilX* mutants complemented by the group III pilX gene, we hypothesized that PilX was acting as a group- specific factor. To validate this hypothesis, $algR_{III}$ mutant was transformed with both the group II minor pilin operon (expressed from pVDtacPIL) and the arabinose-inducible construct expressing $pilX_{III}$ (pBADGr $pilX_{III}$), and tested for twitching motility. Under uninduced conditions, twitching motility was restored

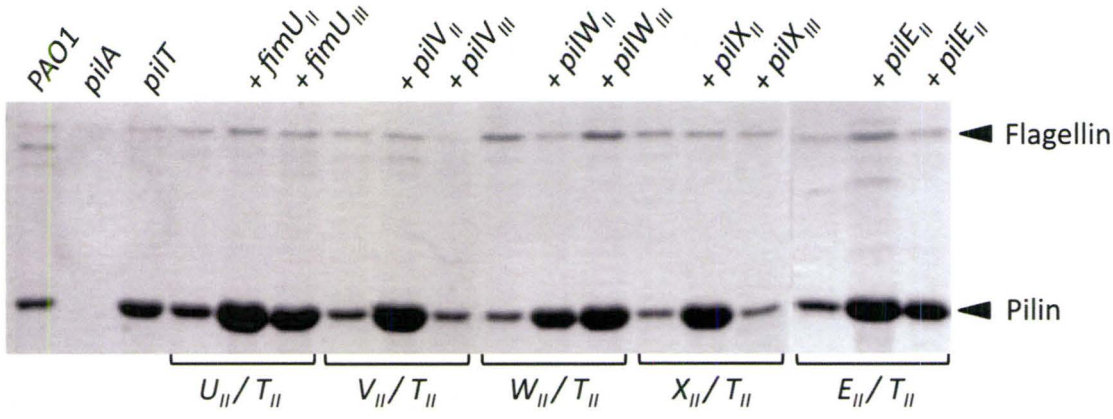

Figure 3.9. Surface piliation is restored in a pilT mutant background regardless of pilin source

Surface pilin preparations of the minor pilin complemented strains *fimU-pilT*, *fimU-pilT* +*fimU*$_{II}$, *fimU-pilT* +*fimU*$_{III}$, *pilV-pilT*, *pilV-pilT* + *pilV*$_{II}$, *pilV-pilT* + *pilV*$_{III}$, *pilW-pilT*, *pilW-pilT* + *pilW*$_{II}$, *pilW-pilT* + *pilW*$_{III}$, *pilX-pilT*, *pilX-pilT* + *pilX*$_{II}$, *pilX-pilT* + *pilX*$_{III}$, *pilE-pilT*, *pilE-pilT* + *pilE*$_{II}$, and *pilE-pilT* + *pilE*$_{III}$ were run on a 15% SDS-PAGE gel and stained with Commassie-brilliant blue.

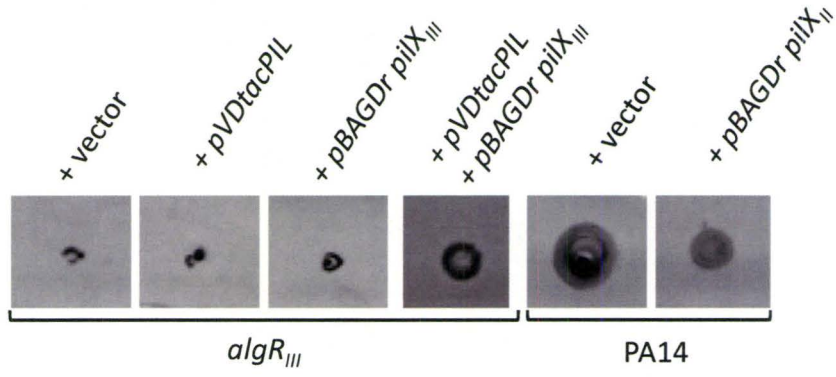

Figure 3.10. PilX is the group specific factor

Bacterial strains of *algR$_{III}$* + pVDtac39, *algR$_{III}$ + pVDtacPIL*, *algR$_{III}$* + pBADGr *pilX$_{III}$*, *algR$_{III}$ + pVDtacPIL + pBADGr pilX$_{III}$*, PA14 wildtype and PA14 + pBADGr *pilX$_{II}$* were stab-inoculated through 1% LB-agar to the underlying plastic surface and were grown for 24 hours. Twitching zones were visualized with 1% crystal violet staining.

demonstrating that expression of the cognate PilX protein is essential for twitching motility (Figure 3.10).

V. DISCUSSION

Horizontal gene transfer plays a significant role in the evolution of bacterial species; in fact, transfer of genomic islands is one of the main sources of adaptability for prokaryotes and viruses (Ochman *et al.*, 2000; Langille *et al.*, 2008). Genomic islands are thought to be more prevalent in bacteria that live in close proximity with a large and diverse range of bacterial species, especially in the case of environmental biofilms; this situation permits access to a wide range of horizontal gene transfer options (Dobrindt *et al.*, 2004). The 'pilin island' identified here is found in an array of bacterial species, including *P. aeruginosa*, a ubiquitous environmental bacterium and prolific biofilm former.

Historically, genomic islands have been identified through %GC contents that deviate significantly from background levels (Dobrindt *et al.*, 2004). The %GC content of the *pilABCD* cluster meets this criterion but that of the minor pilin operon does not, suggesting that simply scanning for regions of skewed GC content may result in an under-representation of the total number of genomic islands in a species (Pundhir *et al.*, 2008). The *pilABCD* cluster of strains PA7 and PA14, but not that of PAO1 or LES, is recognized by algorithms such as PredictBias, which looks for skewed GC content and potential signatures of horizontal gene transfer such as bacteriophage or tRNA genes (Pundhir *et al.*, 2008). However, PredictBias does not

pick up the minor pilin gene cluster that clearly varies between *Pseudomonas* genomes in synchronicity with the *pilABCD* genes. These data suggest that the boundaries of genomic islands may less obvious than previously appreciated.

Genomic islands often contain tRNA genes, which serve as site-specific recombination sites within the chromosome (Langille *et al.*, 2008). This feature is also present in many lysogenic bacteriophage species and conjugative plasmids (Cheetham *et al.*, 1995; Dimopoulou *et al.*, 2002). The pilin island identified here contains 4 tRNA genes, one downstream of the *pilA* gene and three more in tandem between the *pilA/B/C/D* and the minor pilin gene clusters (Figure 3.3). The *tRNA* genes located between *pilA/B/C/D* and *fimU/pilV/W/X/Y1/Y2/E* may act as recombinational hot spots, as additional sequences are present upstream of the tandem *tRNA* genes in PA14, *P. fluorescens*, and *P. syringae*. The order of the *pilA/B/C/D* and the *fimU/pilV/W/X/Y1/Y2/E* clusters, as well as the shared open reading frames in the region between these clusters, is identical, suggesting that the 108 kb, 99.2 kb, 90.6 kb and $tRNA^{Lys}$ insertions (in PA14, *P. syringae, P. stutzeri,* and *P. fluorescens* respectively) are likely to have been secondary gene acquisition events.

Our complementation studies show that individual group III minor pilin genes cannot restore motility in their respective group II minor pilin mutants to the same extent as the native gene (Figure 3.7), suggesting that recombination between the two gene clusters at the tRNA hotspots would result in some loss of function. Previously we showed that optimal pilus functionality is dependent on the presence

of the pilin accessory protein encoded downstream of PilA, present in *P. aeruginosa* groups I, III, IV, and V (Asikyan *et al.*, 2008). There appears to be evolutionary pressure on PilA to co-evolve with its accessory protein, as a chimeric construct expressing $PilA_V$ with the group III accessory protein TfpY did not increase twitching motility to the levels observed when $PilA_V$ was expressed with the cognate TfpZ protein (Asikyan *et al.*, 2008). Furthermore, of the five pilin alleles in *P. aeruginosa,* those of group II strains which lack an accessory protein show the largest within-group variation in pilin sequence (Kus *et al.*, 2004), suggesting an increased exploration of sequence space is possible when the requirement for co-evolution is not present. Similar results are seen with sequence phylogenies where the minor pilins are correlated with specific accessory proteins (Figure 3.2). The results suggest that PilA, the accessory protein (where present) and the minor pilins experience evolutionary pressure to co-evolve.

Additional drift of PilA sequence after acquisition of the island is likely, since group III and V pilins and accessory proteins have only partial sequence identity (Asikyan *et al.*, 2008), but both groups have identical minor pilin genes. Similarly, the TfpW and TfpX accessory proteins of group IV strains PA7 and Pa5196 are 57 and 47% identical, respectively, while their minor pilins are identical (Kus *et al.*, 2008). In *P. aeruginosa*, most components of the type IV pilus assembly system appear insensitive to changes in pilin sequence, since the inner membrane complex proteins (PilM,N,O,P,Q), and ATPases (PilB, T, U) are identical even though PilA sequences vary from 45 – 75% identity. The ability of the assembly system to

accommodate diverse pilins allows for changes in *pilA* sequence in response to antigenic drift, horizontal gene acquisition and niche exploitation without loss of function; however, it appears that this adaptation is only possible if the pilin island is initially transferred as a unit, as minor pilins do not function optimally with heterologous pilins (Figure 3.7).

From our bioinformatic analyses, it appears that the mPAO1 pilin island is inserted between the $tRNA^{Thr}$ upstream of *pilA* and the highly conserved gene coding for an isoleucyl-tRNA synthetase (*ileS*) found at the distal end of the pilin island (Figure 3.3). Co-acquisition of the *pilA/B/C/D* and *fimU/pilV/W/X/Y1/Y2/E* gene clusters is also supported by the presence within the island of the *pilS* and *pilR* genes encoding a key two-component regulatory system essential for *pilA* expression. Acquisition of the entire island would ensure that expression of the pilin would continue should a similar regulatory mechanism be absent in the new background. In contrast, the gene required for positive regulation of minor pilin gene expression, *algR*, is located outside the pilin island, suggesting that either there must be sufficient conservation of the promoter region to ensure continued minor pilin transcription, or that integration of the minor pilin operon of *P. aeruginosa* into the AlgR regulon was a post-acquisition event. Similar integration of genes outside a genetic island into its regulatory circuitry has been reported for the SPI-2 pathogenicity island of *Salmonella typhimurium* (Tomljenovic-Berube *et al.*, 2010).

The genetic organization of the pilin island is conserved among *P. aeruginosa* strains and other Pseudomonad species. Individual genes within the minor pilin

cluster appear to have co-evolved, as cross-complementation with individual heterologous minor pilins did not restore pilus function to the same extent as the homologous protein; in the case of PilX, no piliation or motility was recovered. The levels of surface piliation observed for a *pilX*$_{II}$-*pilT* mutant complemented with *pilX*$_{III}$ were consistent with those of the *pilX*$_{II}$-*pilT* mutant alone, indicating that PilX cannot function in a heterologous context. The structure of a PilX homologue from the type II secretion system of *V. cholerae* showed an unusual alpha helical domain (termed an arrowhead) inserted between β-strands 2 and 3 that is predicted to interact with the secretin and facilitate its opening for passage of the pilus/secretion pseudopilus (Forest 2008; Korotkov *et al.*, 2008). Therefore, we hypothesize that PilX may be a strain-specific factor responsible for efficient secretin opening. Previously we showed that overexpression of *pilX* resulted in expression of short pili, which is consistent with our hypothesis that PilX aids in secretin opening, as increased levels of PilX would increase the number of assembly initiation or secretin opening events (Giltner *et al.*, 2010).

The sequence similarity between group II and III minor pilins is relatively high (i.e. 65 and 75% similarity for the FimU and PilX components, respectively), however this difference is sufficient to result in altered phenotypes in the heterologous background. Group II *fimU* mutants had increased surface piliation when complemented with *fimU*$_{III}$ compared with *fimU*$_{II}$ (Figure 3.8). Based on this observation, we hypothesize that FimU$_{III}$ is able to assemble into the group II pilus, however the rate of pilus depolymerization may be slower than normal, resulting in

more recoverable pilin on the cell surface. This hypothesis is supported by our double knockout mutant data, which shows that surface piliation in the same mutants is equivalent when retraction is blocked, suggesting that there are no inherent differences in the levels of pilus assembly whether *fimU*$_{II}$ or *fimU*$_{III}$ is provided (Figure 3.9).

Previously, we proposed that FimU may promote assembly while PilX stimulates opening of the secretin pore via its unusual alpha domain insertion (Giltner *et al.*, 2010). Our current results support this hypothesis, as complementation with the group III *pilX* gene into a *pilX*$_{II}$ mutant does not result in surface piliation; while cross complementation with *fimU*$_{III}$ in the *fimU* mutant does (Figure 3.8). Therefore if the native PilX is present (i.e. in the *fimU* mutant), either *fimU*$_{II}$ or *fimU*$_{III}$ can restore surface piliation. The opposite situation may be true in a *pilX* mutant, where although the native FimU protein can initiate pilus assembly, the secretin cannot be efficiently opened in the absence of PilX (Figure 3.9).

In summary, our evidence suggests that the minor pilin operon was horizontally transferred as part of a pilin island, which includes the major pilin gene cluster and intervening sequences. It appears that the tandem tRNAs present between the major and minor pilin operons are hot-spots for genetic insertion, as PA14 and four other Pseudomonad species analyzed had additional insertions in this region. Our data also suggest that subsequent to horizontal gene transfer, the major and minor pilins have co-evolved such that cross complementation between strains may no longer result in full function. We have determined that the strain-

specific factor responsible for this lack of cross complementation is PilX, and we hypothesize that its role is to open the PilQ secretin. Direct evidence of the interaction between PilX and the secretin will be the focus of future studies.

VI. ACKNOWLEDGEMENTS

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CHAPTER 4:

PSEUDOMONAS AERUGINOSA TYPE IV MINOR PILINS ARE INVOLVED IN EXOPROTEIN SECRETION

Carmen L. Giltner and Lori L. Burrows,

For submission to PLoS Pathogens

I. ABSTRACT

Type II Secretion (T2S) and type IV pilus (T4P) systems in Gram-negative bacteria share many features that suggest a common ancestral origin. In *Pseudomonas aeruginosa*, which expresses both systems, the minor pseudopilins XcpU, XcpV, XcpW, and XcpX are homologous to the minor pilins FimU, PilV, PilW, and PilX. The major T4P subunit PilA and the major T2S subunit XcpT were previously shown to interact, implying potential intersection between the two systems. Here, we asked whether the T4P minor pilins were involved in T2S secretion of exoproteins. Knockouts of any of the minor pilin genes substantially reduced secretion of specific effectors, while overexpression of either FimU or PilX significantly increased effector secretion. The minor pilins appear to be in part responsible for T2S substrate selectivity, as exotoxin A, elastase and phospholipase C levels were specifically reduced in minor pilin mutants while those of other effectors were not. Double mutants of the minor pilins FimU or PilX and the outer-membrane secretin PilQ had levels of secretion equivalent to *fimU* and *pilX* mutants, confirming secretion occurred through the T2S and not the T4P system. Finally, overexpression of *pilV, pilW* or *pilE in trans* in complemented mutants reduced effector secretion below levels of the negative control, suggesting that stoichiometric incorporation of minor pilins is important for normal T2S. This is the first demonstration that the T4P minor pilins play a role in the secretion of effectors

through the Xcp system, and suggests that intersection between the T2S and T4P systems is more extensive than previously appreciated.

II. INTRODUCTION

The type II secretion (T2S) system is a widely distributed mechanism for the secretion of enzymes and toxins from Gram negative bacteria. In the opportunistic pathogen *Pseudomonas aeruginosa*, the T2S system is important for pathogenesis, as it is responsible for secretion of a number of virulence factors including exotoxin A, elastases, proteases, lipases, phospholipases, and alkaline phosphatases (Ball *et al.*, 2002; Filloux 2004). T2S is the second of two independent secretion events: first, exoproteins carrying either a Sec or Tat-dependent signal sequence are translocated across the inner membrane by the relevant system; and second, fully folded proteins are secreted from the periplasm to the cell's exterior through the strain-specific T2S system. At least two separate T2S systems have been characterized in *P. aeruginosa* (Ball *et al.*, 2002; Filloux 2004; Michel *et al.*, 2007), and orthologous gene products that may be components of additional systems can be readily identified by homology searches of available *P. aeruginosa* genomes. In *P. aeruginosa*, proteases and lipases are translocated via the Xcp (extracellular protein deficient) system, and alkaline phosphatase is secreted via the Hxc (Homologuous to Xcp) system (Py *et al.*, 2001; Ball *et al.*, 2002; Filloux 2004; Robert *et al.*, 2005a; Robert *et al.*, 2005b). Both the Xcp and the Hxc systems are composed of 11 proteins, XcpP-Z and Hxc P-Z

respectively, and share the prepilin processing enzyme PilD (XcpA) with the type IV pilus (T4P) system (Filloux 2004). The Xcp system is regulated by quorum sensing, and therefore active under conditions of high cell density such as during stationary phase, while the Hxc system is only active under phosphate-limited conditions (Chapon-Herve *et al.*, 1997; Ball *et al.*, 2002; Wagner *et al.*, 2003).

Many of the components that make up the T2S machinery are structurally similar to members of the T4P system. Examples include the major pilin and pseudopilins PilA, XcpT, and HxcT, the secretins PilQ, XcpQ and HxcQ, the inner membrane lipoprotein PilP, with integral membrane proteins XcpP, and HxcP, the ATPases PilB, XcpR, and HxcR, and the platform proteins PilC, XcpS, HxcS (Figure 4.1) (Bitter *et al.*, 1998; Planet *et al.*, 2001). Interestingly, the inner membrane protein XcpY has domains with structural similarity to both the cytoplasmic PilM protein and the inner membrane PilN protein (Figure 4.1), suggesting they have a similar function in the two systems (Ayers *et al.*, 2009; Sampaleanu *et al.*, 2009). The minor (low abundance) pilins also share sequence similarity with the minor pseudopilins, particularly in the α-helical N-terminal domain (FimU with XcpU, PilV with XcpV, PilW with XcpW, and PilX with XcpX), and both the T4P and T2S minor components are processed by PilD (Nunn *et al.*, 1993; Giltner *et al.*, 2010).

The similarity between the T2S and T4P systems was further solidified when the major pseudopilin XcpT was shown to form a pilus-like fibre on the surface of the cell when overexpressed (Sauvonnet *et al.*, 2000; Durand *et al.*, 2003; Vignon *et al.*, 2003). It was proposed that this fibre represented an uncontrolled elongation of

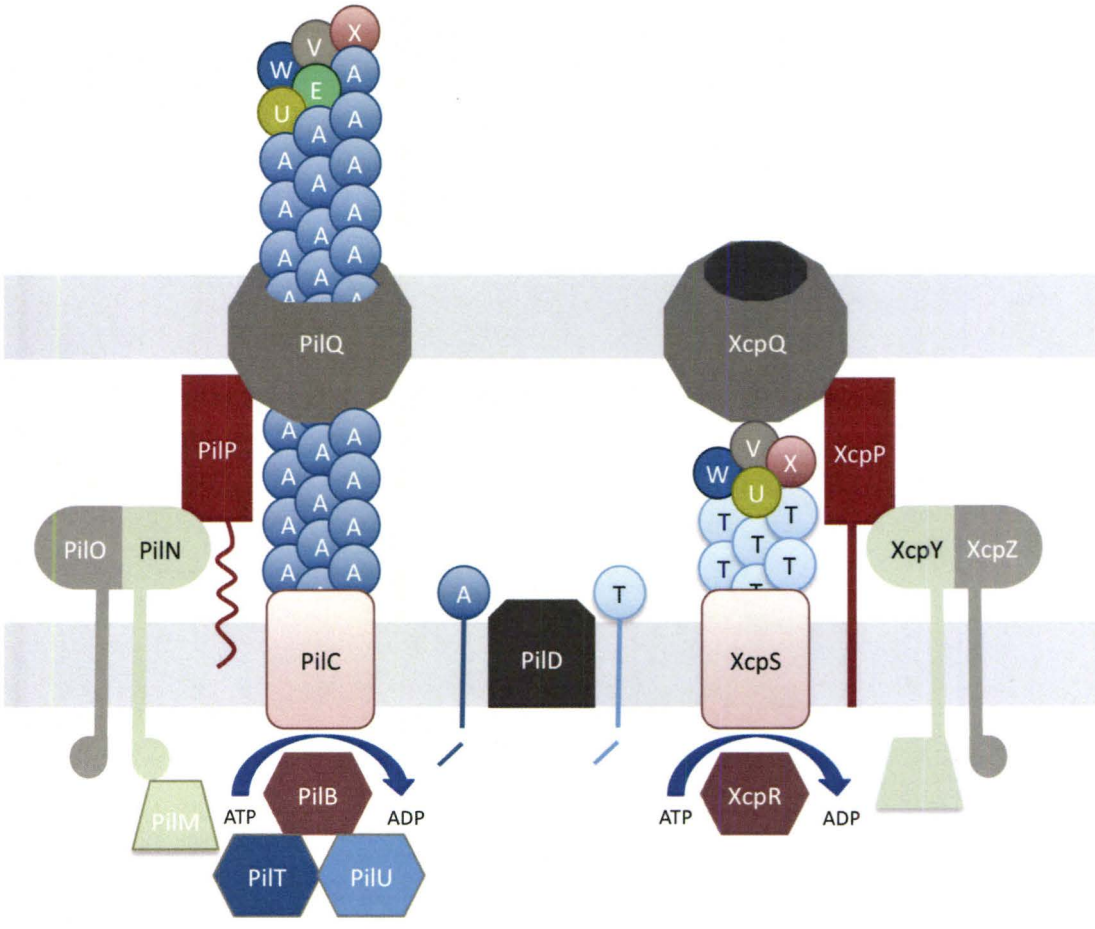

Figure 4.1. Similarities of the Type IV Pilus and the Type II Secretion Systems

Simplistic model predictions of the type IV pilus and the type II secretion systems above illustrate the similarities between the two systems. Proteins predicted to play similar roles in these related systems are coloured in a similar fashion. The PilD/XcpA protein, responsible for pre-pilin and pseudopre-pilin processing, is the only protein known to be shared between the two systems. Note that the type IV pilus system has multiple ATPase proteins (PilB, PilT, and PilU) where only one ATPase (XcpR) has been identified in the type II secretion system. A second T2S system was identified in *P. aeruginosa* (Hxc) which has similar nomenclature as the Xcp system.

the normally short pseudopilus, whose length is predicted to be determined by the distance between the inner and the outer membranes (Sauvonnet *et al.*, 2000; Vignon *et al.*, 2003). Similar studies in the T2S system of *Klebsiella oxytoca* showed the T2S fibre to be a left-handed helical structure, where the α-helices of the major subunit comprised the central core of the fibre, similar to T4P fibre models (Craig *et al.*, 2003; Kohler *et al.*, 2004; Craig *et al.*, 2006). Interestingly, extrusion of a pseudopilus fibre and secretion of exoproteins appeared to be mutually exclusive, as activity of the T2S effector elastase was significantly decreased upon overexpression of XcpT (Durand *et al.*, 2003).

The minor pseudopilins are evolutionarily well conserved and have been shown in a number of bacterial species to play a role in pseudopilus assembly, however their exact function remains unknown. Recent structural evidence suggests that the minor pseudopilins have a conserved N-terminal α-helix, and an αβ-loop similar to PilA (Yanez *et al.*, 2007; Korotkov *et al.*, 2008; Yanez *et al.*, 2008; Lam *et al.*, 2009). These structural data combined with our recent finding that the T4P minor pilins are incorporated into the pilus fibre (Giltner *et al.*, 2010) suggest that the minor pseudopilins may likewise be incorporated into the pseudopilus. Co-affinity purification and surface plasma resonance studies have shown that the major and minor pseudopilins interact in a specific and ordered fashion to form an XcpU-XcpW-XcpV-XcpX complex that is predicted to form the tip of the pseudopilus (Hu *et al.*, 2002; Durand *et al.*, 2005; Douzi *et al.*, 2009). Cross-linking studies also showed interactions between XcpT and each of XcpU, XcpW and XcpV (Lu *et al.*,

1997), suggesting a close proximity of the major pseudopilin to the minor pseudopilin components. XcpX was also shown to interact with the major pseudopilus component XcpT, and to play a role in length determination of the fibre, as an *xcpX* mutant displayed abnormally long pseudopili, while XcpX overexpression decreased pseudopilus length (Durand *et al.*, 2005). Durand and colleagues proposed that the interaction between XcpX and XcpT causes destabilization of XcpT and that wild type expression levels are required to maintain the native length of the pseudopilus (Durand *et al.*, 2005).

The high degree of similarity between the T2S and T4P systems (Bitter *et al.*, 1998; Planet *et al.*, 2001; Ayers *et al.*, 2009; Sampaleanu *et al.*, 2009) suggests that each may retain some functions of the other. Not only can the T2S systems of *P. aeruginosa* and *K. oxytoca* can form pilus-like fibres upon overexpression of the major pseudopilin, XcpT or PulG respectively (Sauvonnet *et al.*, 2000; Durand *et al.*, 2003; Vignon *et al.*, 2003), but the T4P systems of *V. cholerae* and *Francisella tularensis* were shown to secrete virulence factors (Kirn *et al.*, 2003; Hager *et al.*, 2006; Forsberg *et al.*, 2007). These data suggest that a functional overlap exists between the T4P and T2S systems.

Although the structural components of the T2S system have been identified, little is known about how the T2S system discriminates exoprotein effectors from resident periplasmic proteins (Russel,1998). No conserved linear motif that could represent a secretion signal has been identified in T2S substrates, suggesting that substrate selection may require a three-dimensional recognition site. Secreted

substrates are highly diverse, and even exoproteins from the same bacterial species lack sequence similarity and conserved strucutrual motifs, as seen in the exotoxin A and elastase enzymes from *P. aeruginosa* (Allured *et al.*, 1986; Thayer *et al.*, 1991). Furthermore, secretion of substrates was found to be species specific, as the *K. pneumoniae* pullulanase enzyme could not be secreted by *P. aeruginosa,* and that cellulase and elastase secretion were not interchangeable amongst *Erwinia* and *Pseudomonas* species respectively (de Groot *et al.*, 1991; Py *et al.*, 1991; Cooper *et al.*, 1993). Biochemical analysis of individual exoproteins led to identification of key amino acid residues that are essential for translocation across the outer membrane (Wong *et al.*, 1991; Connell *et al.*, 1995; Chapon *et al.*, 2000). The *P. aeruginosa* lipase LipA was poorly secreted when either D20N or T180I substitutions were made (Hausmann *et al.*, 2008). A Q3K substitution in the heat-labile enterotoxin (LT) of enterotoxogenic *E. coli* caused periplasmic accumulation of LT, but the same mutation in the homologous cholera toxin (CT) from *V. cholerae* had no effect on CT secretion (Mudrak *et al.*, 2010). These data suggest that there is no conserved consensus sequence that dictates substrate recognition. The specificity may therefore be dictated by the T2S system, however little is known about which components of the T2S apparatus come into contact with the secreted substrates (Russel,1998; Johnson *et al.*, 2006).

Here we show that, in addition to their previously demonstrated role in pilus assembly and twitching motility via the T4P system, the T4P minor pilins FimU, PilV, PilW, PilX and PilE, as well as the non-pilin like PilY1 protein expressed by the minor

pilin operon of *P. aeruginosa* have additional roles in secretion of exoproteins by the T2S system. We show that the range of T2S effectors that are secreted is altered in T4P minor pilin mutants and hypothesize that minor pilins may participate in substrate identification. The dual roles of the minor pilins in T4P formation and secretion of T2S products makes these components interesting targets for anti-virulence strategies.

III. MATERIAL AND METHODS

Bacterial strains, genetic manipulations and growth conditions

The bacterial strains and genetic constructs used in this work are listed in Table 4.1. Cloning was performed using standard molecular methods in *E. coli*. *E. coli* and *P. aeruginosa* were routinely grown on Luria-Bertani (LB) agar containing relevant antibiotics where indicated; for *E. coli*, ampicillin at 100 mg/L, gentamicin at 15 mg/L; for *P. aeruginosa*, carbenicillin at 300 mg/L and gentamicin at 30 and 100 mg/L. For PCR, chromosomal DNA templates were prepared using Instagene (Bio-Rad) as described by the manufacturer. PCR primers were designed using Geneious (Drummond *et al.*, 2009) and synthesized by ACGT Corp. (Toronto, ON).

Table 4.1 List of strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
pBADGr	pMLBAD backbone with dhfr (trimethoprim resistance) replaced with aacC1 (gentamicin resistance)	(Asikyan *et al.*, 2008)
pBADGr*fimU*	mPAO1 *fimU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	Giltner *et al.*, 2010
pBADGr*pilV*	mPAO1 *pilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	Giltner *et al.*, 2010
pBADGr*pilW*	mPAO1 *pilW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	Giltner *et al.*, 2010
pBADGr*pilX*	mPAO1 *pilX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	Giltner *et al.*, 2010
pBADGr*pilE*	mPAO1 *pilE* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	Giltner *et al.*, 2010
pBADGr *pilY1*	mPAO1 *pilY1* sequence inserted with EcoRI restriction enzyme in the MCS of pBADGr	This study
pBADGr *algR*	mPAO1 *algR* sequence inserted with XbaI and HindIII restriction enzymes in the MCS of pBADGr	This study
pEX18AP + algR::GmFRT	Gentamicin FRT insertion in the EcoRV site	Giltner *et al.*, 2010
pEX18AP + *pilQ::GmFRT*	Gentamicin FRT insertion in the EcoRV site	This study
pVDtac39	IncQ/P4 *mob*$^{+}$ *tac lacI*^{q}	(Deretic *et al.*, 1987)
pVDtacPIL	pVDtac39 backbone with *fimT fimU pilV pilW pilX pilY1 pilY2* and *pilE* inserted in the MCS	(Lizewski *et al.*, 2004)
E. coli		
DH5α	GENOTYPE: F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1	Invitrogen
TOP10	GENOTYPE: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 ΔlacX74 *rec*A1 *ara*D139	Invitrogen

SM10	Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G Carries plasmid RP4 with tra genes encoding conjugative pilus integrated into its chromosome, along with a KmR marker.	(Simon *et al.*, 1983)
P. aeruginosa		
mPAO1	Laboratory strain	(Jacobs *et al.*, 2003)
mPAO1 Tn::*fimU*	IS*lacZ*/hah transposon insertion (position 237)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilV*	IS*phoA*/hah transposon insertion (position 122)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilW*	IS*lacZ*/hah transposon insertion (position 381)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilX*	IS*phoA*/hah transposon insertion (position 182)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilE*	IS*phoA*/hah transposon insertion (position 183)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilY1*	IS*phoA*/hah transposon insertion (position 3486)	(Jacobs *et al.*, 2003)
mPAO1 *algR*::FRT	Wild type strain with FRT scar insertion in the NruI site within *algR*	This study
ΔPQP$_A$Q$_A$	Quadruple deletion mutant: *xcpP-xcpQ-xphA-xqhA*	(Michel *et al.*, 2007)
mPAO1 *xcpR*::Tn	IS*lacZ*/hah transposon insertion (position 1509)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*fimU* + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	Giltner *et al.*, 2010
mPAO1 Tn::*pilV* + pBADGr *pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	Giltner *et al.*, 2010
mPAO1 Tn::*pilW* + pBADGr *pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	Giltner *et al.*, 2010
mPAO1 Tn::*pilX* + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	Giltner *et al.*, 2010
mPAO1 Tn::*pilE* + pBADGr *pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	Giltner *et al.*, 2010
mPAO1 Tn::*pilY1* + pBADGr *pilY1*	IS*phoA*/hah transposon insertion (position 3486) complemented with cognate gene in	This study

	pBADGr	
mPAO1 *algR*::FRT + pBADGr *algR*	Wild type strain with FRT scar insertion in the NruI site within *algR* complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*pilX-pilQ*::FRT	IS*phoA*/hah transposon insertion (position 182) and a FRT insertion in the *pilQ* gene	This study
mPAO1 Tn::*fimU-pilQ*::FRT	IS*lacZ*/hah transposon insertion (position 237) and a FRT insertion in the *pilQ* gene	This study
mPAO1 Tn::*pilX-pilQ*::FRT + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182) and a FRT insertion in the *pilQ* gene, complemented with cognate gene in pBADGr	This study
mPAO1 Tn::*fimU-pilQ*::FRT + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237) and a FRT insertion in the *pilQ* gene complemented with cognate gene in pBADGr	This study

Generation of algR and pilY1 complementation constructs

The *algR* and pilY1 complementation constructs were constructed through amplification of mPAO1 chromosomal DNA with primers 3 and 4 (*algR*) and 5 and 6 (*pilY1*). Purified PCR product was digested with XbaI and HindIII (*algR*) or EcoRI (*pilY1*) and subsequently ligated into the linearized pBADGr vector cut with identical restriction enzymes (algR) or into PCR 2.1 Advantage Kit (Clonetech) for use in TA cloning (Invitrogen). The resulting PCR 2.1 *pilY1* construct was digested with EcoRI, and the *pilY1* insert was gel purified (Quiagen), and ligated using Quick ligase (Fermentas) into the linearized pBADGr vector cut using identical restriction enzymes. Constructs were verified by sequencing (MOBIX) and electroporated into their respective mutants.

Generation of fimU-pilQ and pilX-pilQ double knockouts in mPAO1

Double knockouts were generated using the biparental mating assay as previously described (Burrows *et al.*, 2000). The pEX18AP + *pilQ*::GmFRT plasmid was electroporated into *E. coli* SM10 cells (Simon *et al.*, 1983), and through biparental mating was transferred to mPAO1 *fimU*::Tn and mPAO1 *pilX*::Tn cells. The gentamicin resistance cassette was subsequently excised by the Flp recombinase encoded by pFLP2, and the plasmid removed using sucrose counterselection (Hoang *et al.*, 1998). Double mutants were verified by assessing FRT disruption of the *pilQ* gene using PCR amplification (primers 1 and 2, Table 4.2).

Table 4.2 List of primers used in this study

Primer Number	Primer Name	Sequence (5' to 3')
1	mPAO1 *pilQ* Sense	TCGCGCCTCGGGATCGCTTTGCT
2	mPAO1 *pilQ* Antisense	AAAAGCTTGGCGTAGTTCACCTGGA
3	*algR* sense	GGTACCCGGGGATCCTCTAGAATGAATGTCCTGATTGTCGATG
4	*algR* Antisense	TCCGCCAAAACAGCCAAGCTTTCAGAGCTGATGCATCAGAC
5	*pilY1* sense	GCCCGTCTCTATACCAACTGATCGAGCCTCGCATGAAA
6	*pilY1* Antisense	GCAGCACTTTCATATCAGTTCTTTCCTTCGATGGGGC

Type II secretion plate assays

Plate-based secretion assays were performed to determine protein secretion of general proteases, lipases, and phospolipase C. Bacterial strains (mPAO1, *algR, pilA, fimU, pilV, pilW, pilX, pilE, pilY1, fimU-pilQ, pilX-pilQ, xcpP-xcpQ-xphA-xqhA* and respective complemented mutants) were grown for 36 hours on either tryptic soy broth (TSB)-skim milk plates (1.5% skim milk) to test general protease secretion, blood-agar plates (TEKnova) to test for secretion of hemolysins, or lipid-agar plates (50 mM MOPS, 40 mM K2HPO4, 25 mM NaH2PO4, 7.5mM NH4SO4, 0.4mM MgSO4, 0.5% (v/v) olive oil, 40ug/mL Neutral Red) to test for secretion of lipases. The protocol for lipid agar plates was adapted from Kagami *et al.* (1998) (Kagami *et al.*, 1998). Exoprotein secretion is identified by a zone of clearing around bacterial colonies on the skim milk and blood agar plate assays, or bacterial growth on lipid plates. Areas of the bacterial colony size and zone of clearing were measured using ImageJ (Abramoff *et al.*, 2004) and reported as a ratio relative to wild type.

Elastase and exotoxin A antibody generation

Purified elastase (LasB) and exotoxin A (ToxA) proteins (Sigma) were used to boost rabbits twice before exsanguination (Cedarlane). Terminal bleeds were found to specifically recognize their target proteins, LasB or ToxA respectively, in both purified protein and whole cell lysate samples. These antibodies were used in subsequent immunoblot experiments.

Elastase assays

Qualitative elastase assays were performed using an elastin-congo-red conjugate (Sigma). Bacterial strains (mPAO1, *algR*, *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *pilY1*, *fimU-pilQ*, *pilX-pilQ*, *xcpP-xcpQ-xphA-xqhA* and respective complemented mutants) were grown on tryptic soy broth (TSB) containing 0.05% elastin-congo-red, or TSB containing 0.05% elastin-congo-red with 0.2 % [w/v] L-arabinose for 65 hours. Areas of the bacterial colony size and zone of clearing were measured using ImageJ (Abramoff *et al.*, 2004).

The EnzCheck elastase assay kit (Mol Probes) was employed to determine presence of extracellular elastase as previously described (Mol Probes). Briefly, liquid bacterial cultures were grown for 22 hours (OD_{600}=1.6-1.8) whereupon 1 ml culture was centrifuged at 11688 x g for 5 minutes, the cellular fraction was washed twice in 1.0 ml ddH_2O to remove any contaminating extracellular elastase, standardized to OD_{600}=0.6 and saved for immnoblot assays (below), while the supernatant was retained for kinetic assays. BODIPY Fl-elastin (10 μl) was added to 100 μl sample supernatant in 1 x Molecular probes reaction buffer and the amount of fluorescence produced due to the activity of elastase was monitored over 1 hour in a fluorescence multi-well plate reader (Synergy 4 Microplate reader, BioTek) with excitation at 485 nm and emission detection at 530 nm.

Localization of LasB and ToxA in minor pilin mutants

Cellular samples (above) were resuspended in 100 µl of 1 x sodium-dodecyl-sulphate (SDS) loading buffer (80 mM Tris pH 6.8; 5.3 % [v/v] 2-mercaptoethanol; 10 % [v/v] glycerol; 0.02 % [w/v] bromophenol blue; 2 % [w/v] SDS), while 50 µl of 3 x SDS loading buffer was added to 100 µl of the supernatant samples. Samples were boiled for 10 min, run on a 15 % SDS-PAGE gel, and subsequently transferred to a nitrocellulose membrane for analysis with rabbit polyclonal anti-LasB or anti-ToxA antibodies. The nitrocellulose membrane was blocked overnight with 5 % skim milk (w/v) at 4 °C. Blots were incubated with primary anti-LasB antibody at a 1:5000 dilution, or anti-ToxA antibody at a 1:1000 dilution for 2 hr at RT, washed three times with 1 x phosphate-buffered saline (PBS pH 7.4) for 10 min, and incubated with a 1:3000 dilution of secondary goat-anti-rabbit IgG alkaline phosphatase antibody (Sigma) for 2 hr at RT. Blots were washed with PBS as above, and developed using NBT/BCIP (BioRad). Presence of unprocessed elastase was seen at 53.3 and 51 kDa and the mature elastase was observed as a band at 33 kDa, while exotoxin A was observed as a band corresponding to 66 kDa.

IV. RESULTS

Minor pilin mutants are defective in Sec-dependent exoprotein secretion

Previously characterized non-polar major (*pilA*) and minor (*fimU*, *pilV*, *pilW*, *pilX* and *pilE*) pilin mutants of *P. aeruginosa* were used for this study (Giltner *et al.*,

2010). Additional mutants in *pilY1* and *algR* were generated as described in the Methods. As reported previously, both mutants lacked twitching and surface piliation, and both phenotypes were restored upon complementation with the relevant gene *in trans* (Chapter 3 Figure 4 and Chapter 5 Figures 1 and 2).

Function of the T2S system was first investigated by assessing the proteolytic activity of strains grown on 1.5% skim milk plates (Michel *et al.*, 2007). The negative controls, an *xcpR* single mutant and a quadruple deletion mutant *xcpP-xcpQ-xphA-xqhA* (ΔPQP_AQ_A) had small zones of clearing around the colony on this medium, confirming previous reports of residual non-specific protease activity in T2S mutants (Michel *et al.*, 2007) (Figure 4.2). Disruption of any of the minor pilin genes substantially reduced protease secretion compared to wild type, although a residual zone of clearing similar to that of the negative controls was observed for the mutant strains, suggesting that the minor pilin mutants were also defective in T2S (Figure 4.2).

Complementation of *fimU* and *pilX* mutants resulted in 188 ± 13.6 and 274 ± 3.9% increases respectively in the zone of clearing relative to wild type under uninduced conditions, and 238 ± 8.4 and 254 ± 25.5% increases respectively when inducing conditions were used (Figure 4.2). The zones of protease secretion of the major pilin, minor pilin, and quadruple secretion mutants were unaffected by the addition of arabinose (Figure 4.2). No membrane blebbing or other anomalies were observed when cells were viewed under the electron microscope (Chapter 2,

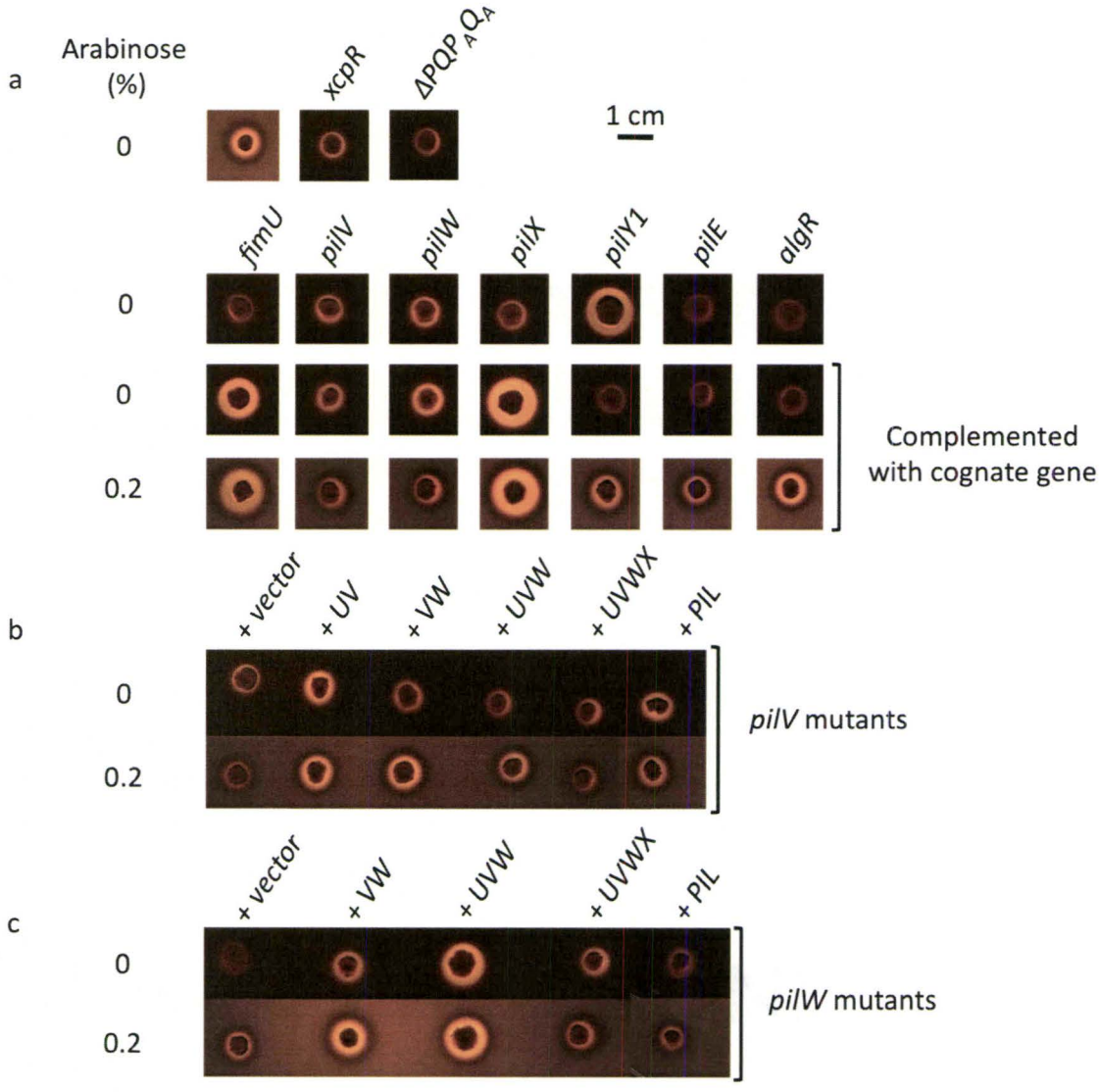

Figure 4.2. Type II secretion of proteases on milk agar plates

Figure 4.2. Type II secretion of proteases on milk agar plates

(a) Single colonies of mPAO1, *xcpR*, ΔPQP$_A$Q$_A$, *fimU*, *pilX*, *pilV*, *pilW*, *pilY1*, *pilE*, *algR* and respective complemented mutants were inoculated on skim milk plates. Plates were incubated for 36 hours at 37°C. Colonies were then assessed for protease secretion, visualized as a zone of clearing around the colonies. Single colonies of pilV (b) or pilW (c) mutant strains complemented with pBADGR (empty vector), *fimUpilV* (pilV mutants only), *pilVW*, *fimUpilVW*, *fimUpilVWX* or pVDtacPIL (PIL; *fimTU-pilVWXY1Y2E*) were inoculated on skim milk plates. Plates were incubated for 36 hours at 37°C. Colonies were then assessed for protease secretion, visualized as a zone of clearing around the colonies. Bar represents 1 cm.

Figures 3 and S1), indicating that increased protein release in the *fimU*- and *pilX*-complemented strains was not due to protein leakage through an impaired outer membrane. The size of protease secretion zones of the *pilE*-complemented strain was unaffected by changes in arabinose concentration; however, wild type secretion could not be restored in the *pilV*- and *pilW*-complemented mutants at any arabinose concentration tested (Figure 4.2). Interestingly, the *pilY1* mutant had increased secretion zones (224 ± 12.9%) relative to wild type suggesting a potentially negative role for PilY1 in T2S.

Similar trends in secretion of T2S substrates as seen on skim milk plates were observed in a Congo red-elastin plate assay. Like the ΔPQP_AQ_A mutant, which had no clearing either around or beneath the colony, no detectable degradation of elastin by minor pilin (*fimU*, *pilV*, *pilW*, *pilX*, and *pilE*) mutants was detected (Figure 4.3), suggesting that they are impaired in T2S of elastase. Complementation of the *pilE* mutant restored the zone of clearing to wild type levels under induced conditions, while complementation of *fimU* and *pilX* pilin mutants increased observed zones of elastin degradation by 117 ± 4.7 and 114 ± 5.1% (uninduced), and by 129 ± 19.02 and 201 ± 10.45 % (induced) respectively (Figure 4.3). Loss of PilY1 led to a 164 ± 15.5% increase in elastase secretion relative to wild type, which was restored to wild type levels upon complementation by *pilY1* in trans. In contrast, complementation of the *pilV* and *pilW* mutants did not restore elastase secretion, as exhibited by a lack of clearing around the colonies (Figure 4.3).

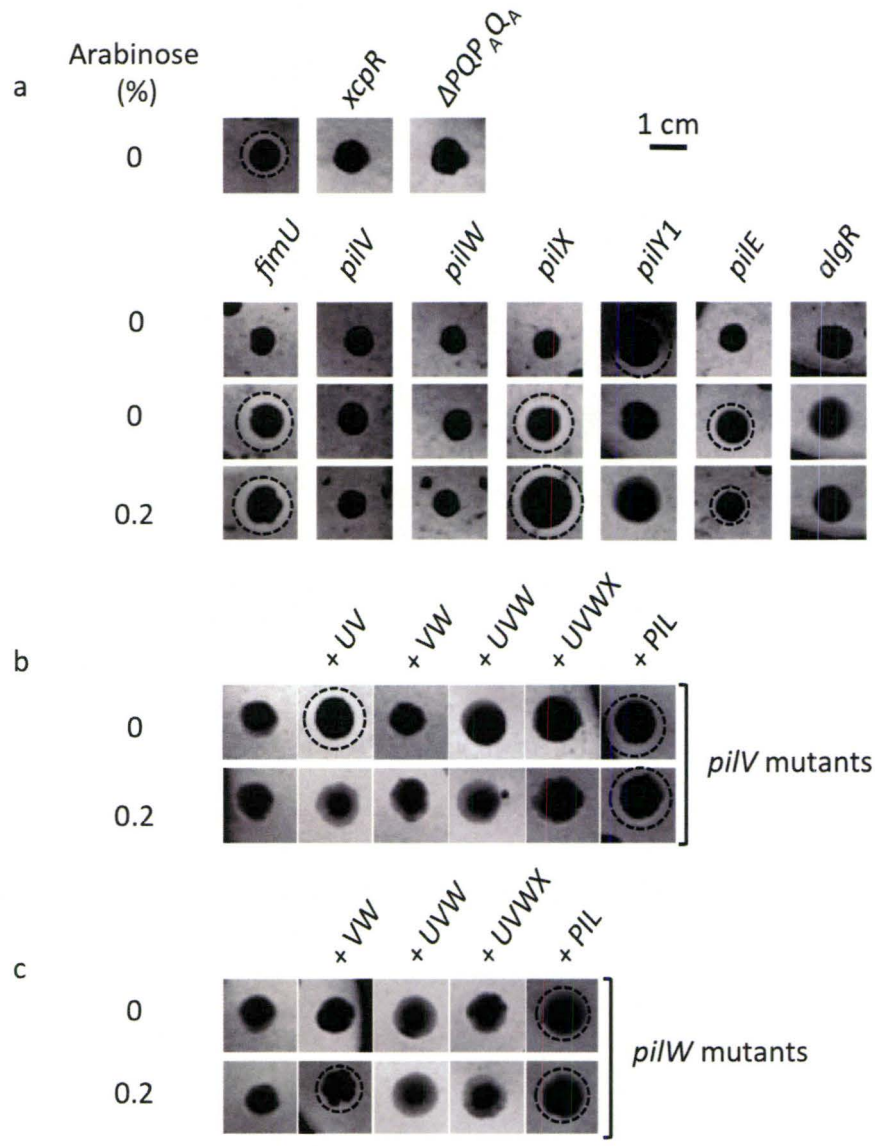

Figure 4.3. Type II secretion of elastase on elastin-congo-red plates

Figure 4.3. Type II secretion of elastase on elastin-congo-red plates

(a) Single colonies of mPAO1, *xcpR*, ΔPQP$_A$Q$_A$, *fimU*, *pilX*, *pilV*, *pilW*, *pilY1*, *pilE*, *algR* and respective complemented mutants were inoculated on elastin-congo red plates. Plates were incubated for 65 hours at 37°C. Colonies were then assessed for protease secretion, visualized as a zone of clearing around the colonies. Single colonies of pilV (b) or pilW (c) mutant strains complemented with pBADGR (empty vector), *fimUpilV* (pilV mutants only), *pilVW*, *fimUpilVW*, *fimUpilVWX* or pVDtacPIL (PIL; *fimTU-pilVWXY1Y2E*) were inoculated on elastin-congo red plates. Plates were incubated for 65 hours at 37°C. Colonies were then assessed for protease secretion, visualized as a zone of clearing around the colonies. Zones of clearing are indicated by a dashed line.

The *pilV* and *pilW* mutants have previously been shown to be non-polar (Giltner *et al.*, 2010), therefore our inability to complement their secretion defect was surprising. T2S orthologues of PilV and PilW have been shown to form heterodimers (Yanez *et al.*, 2007; Forest 2008; Korotkov *et al.*, 2008); therefore, we hypothesized that correct stoichiometry of the PilV and PilW proteins may be important for function. When the *pilV* and *pilW* mutants were complemented using pBADGr *pilVW* under induced conditions, secretion zones on skim milk and elastin plates were restored to wild type levels (Figures 4.2b, 4.2c, 4.3b, and 4.3c), suggesting that a 1:1 ratio of PilV to PilW is required. To further elucidate the role of each of the minor pilins in secretion, *pilV* and *pilW* mutants were complemented with constructs containing different combinations of minor pilins. Secretion by the *pilV* + *fimUpilV* mutant was increased to levels equivalent to the complemented *fimU* strain. However, complementation of *pilV* and *pilW* mutants with *fimUpilVW* showed opposing effects on secretion; *pilV* mutants complemented with the three genes did not secrete to wild type levels, while the *pilW* mutant complemented with the same construct had increased zones of secretion (229 ± 15.6 %) relative to wild type. The increase in secretion seen in *pilW* + *fimUpilVW* is reminiscent of the phenotype of *fimU* + *fimU* and *pilV* + *fimUpilV* complemented mutants, suggesting that overexpression of genes upstream of the mutation (in this case *fimU* and *pilV* in the *pilW* mutant) increases secretion levels (Figures 4.2 and 4.3). However, upon complementation of *pilV* and *pilW* mutants with either *fimUpilVWX* or the entire minor pilin operon (expressed from pVDtacPIL; (Lizewski *et al.*, 2004)), secretion

levels reverted to those seen in the ΔPQP_AQ_A mutant (Figures 4.2 and 4.3), suggesting that overexpression of the entire minor pilin operon is detrimental to secretion.

To ensure that any increase in secretion observed in the complemented minor pilin mutants was through the T2S rather than the T4P system, double mutants lacking the T4P secretin PilQ were generated in the *fimU* or *pilX* backgrounds as described in the Methods. Double mutants were complemented either with the empty vector or the cognate minor pilin gene and were assessed for exoprotein secretion on skim milk plates (Figure 4.4). In each case, the complemented *fimU-pilQ* or *pilX-pilQ* double mutants had a larger zone of clearing than the wild type, although not to *fimU* + *fimU* or *pilX* + *pilX* levels, suggesting that the changes in secretion caused by alterations in minor pilin levels is likely T2S-specific.

Quantification of elastase secretion

The LasB (elastase) protein is expressed as a 53.6 kDa pre-pro-protein, which undergoes two subsequent post-translational modifications. Following cleavage of the leader peptide, the 51 kDa pro-protein undergoes autocatalytic cleavage into 18 and 33 kDa fragments that remain non-covalently associated prior to secretion to inhibit protease activity of the active 33 kDa enzyme (Bever *et al.*,

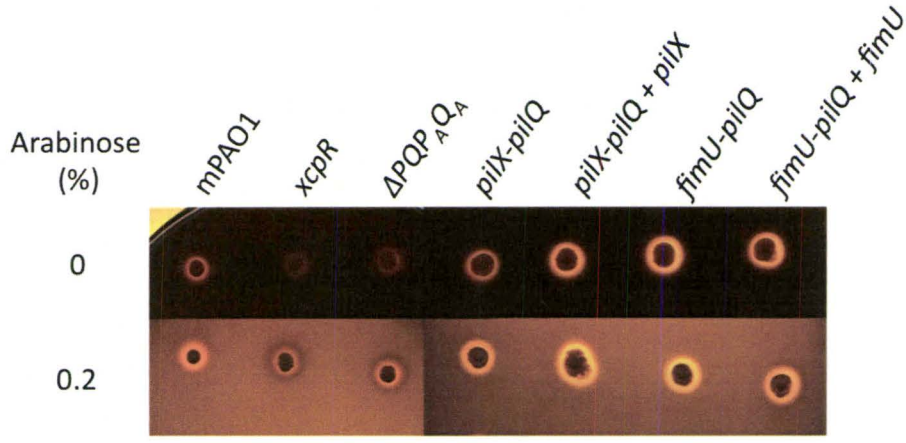

Figure 4.4. Protease secretion in complemented mutants is T2SS specific

(a) Single colonies of mPAO1, *xcpR*, ΔPQP$_A$Q$_A$, *fimU-pilQ*, *fimU-pilQ* + *fimU*, *pilX-pilQ*, and *pilX-pilQ* + *pilX* were inoculated on skim milk plates. Plates were incubated for 36 hours at 37°C. Colonies were then assessed for protease secretion, visualized as a zone of clearing around the colonies. Similar to single minor pilin mutants, increased secretion is observed in *pilX-pilQ* and *fimU-pilQ* double mutants showing that secretion does not occur through the T4P secretin, PilQ. Protease secretion in the absence of arabinose indicates a leaky promoter.

1988; Bever *et al.*, 1988; Kessler *et al.*, 1988; Kessler *et al.*, 1992; McIver *et al.*, 1993; Kessler *et al.*, 1994; Kessler *et al.*, 1998). Following co-secretion of the 18 and 33 kDa products, the smaller fragment is degraded and the active elastase enzyme released (Kessler *et al.*, 1988; Tommassen *et al.*, 1992). To quantify the levels of extracellular elastase, bacterial strains (mPAO1, *algR*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE*, ΔPQP_AQ_A, and their respective complemented strains) were grown for 22 hours and cellular and supernatant fractions were separated as described in the Methods. Whole cell lysates and supernatants were probed with an anti-LasB antibody, and supernatants were tested for elastase activity using an elastin-BODIPY-FL conjugate as a substrate. Levels of secreted elastase relative to the wild type (set at 100%) are shown in Figure 4.5a. The *fimU*, *pilV*, *pilW*, *pilX* and *pilE* mutants had levels of elastase secretion between those of the wild type and the quadruple mutant negative control, suggesting that loss of these proteins is detrimental to elastase secretion (Figure 4.5a). In contrast, the *algR* and *pilY1* mutants had 144 ± 3.5% and 219 ± 6.6% of wild type secretion levels (Figure 4.5a). The *algR* phenotype appeared to be intermediate, potentially due to the lack of expression of both the minor pilins (which decreases secretion) and *pilY1* (which increases secretion). Complementation of *fimU*, *pilV*, or *pilX* mutants with their cognate genes increased elastase activity relative to wild type (195 ± 2.5, 188 ± 4.5, and 226 ± 7.1% respectively), while complementation of *pilW*, *pilE*, *pilY1*, and *algR* decreased

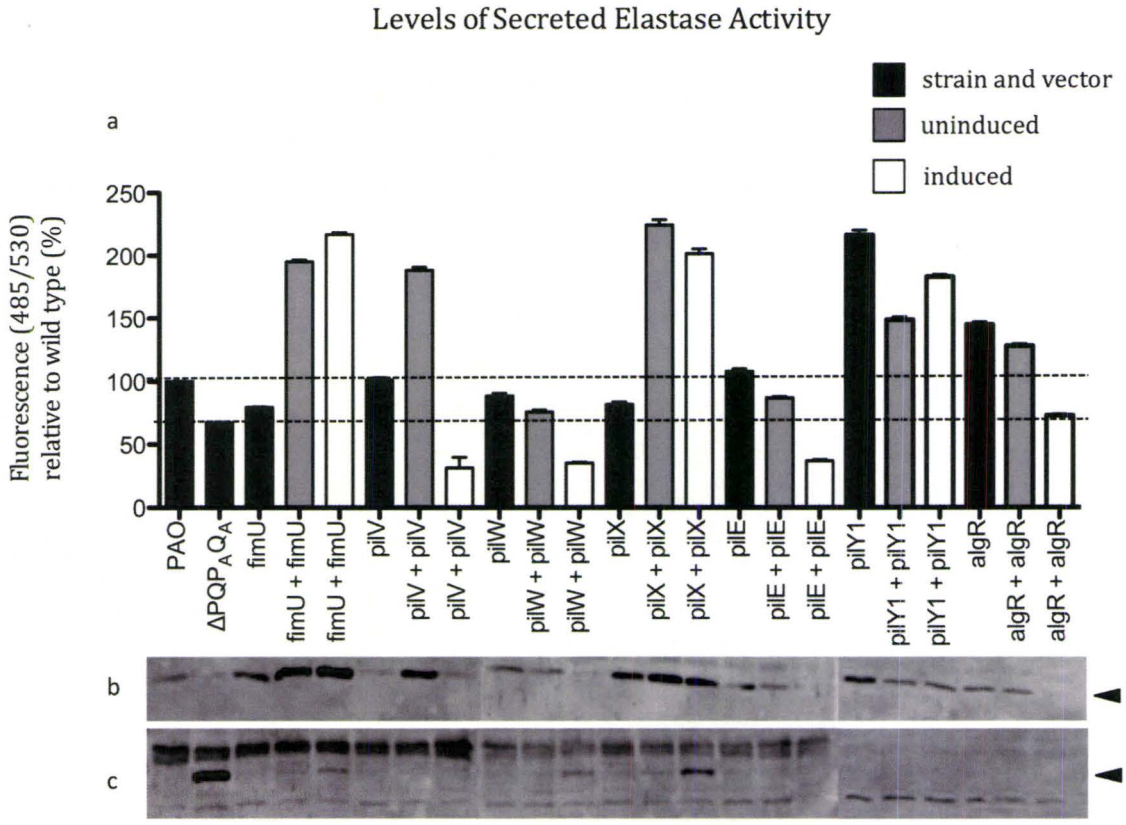

Figure 4.5. Minor pilin mutants are defective in elastase secretion

(a) Bacterial cultures of mPAO1, ΔPQP$_A$Q$_A$, *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *pilY1*, *algR* (black), respective complemented strains induced with 0.2 % arabinose (white) or uninduced (grey) were grown for 22 hours before harvesting. Supernatant samples were incubated with elastin conjugated BODIPY-FL, and measured for relative fluorescence A485/530. Relative fluorescence data at one hour are reported. Immunoblot assays of supernatant (b) or whole cell lysate (c) were probed with anti-LasB antibody. Arrow indicates LasB band at 33kDa (b) and 51 kDa (c).

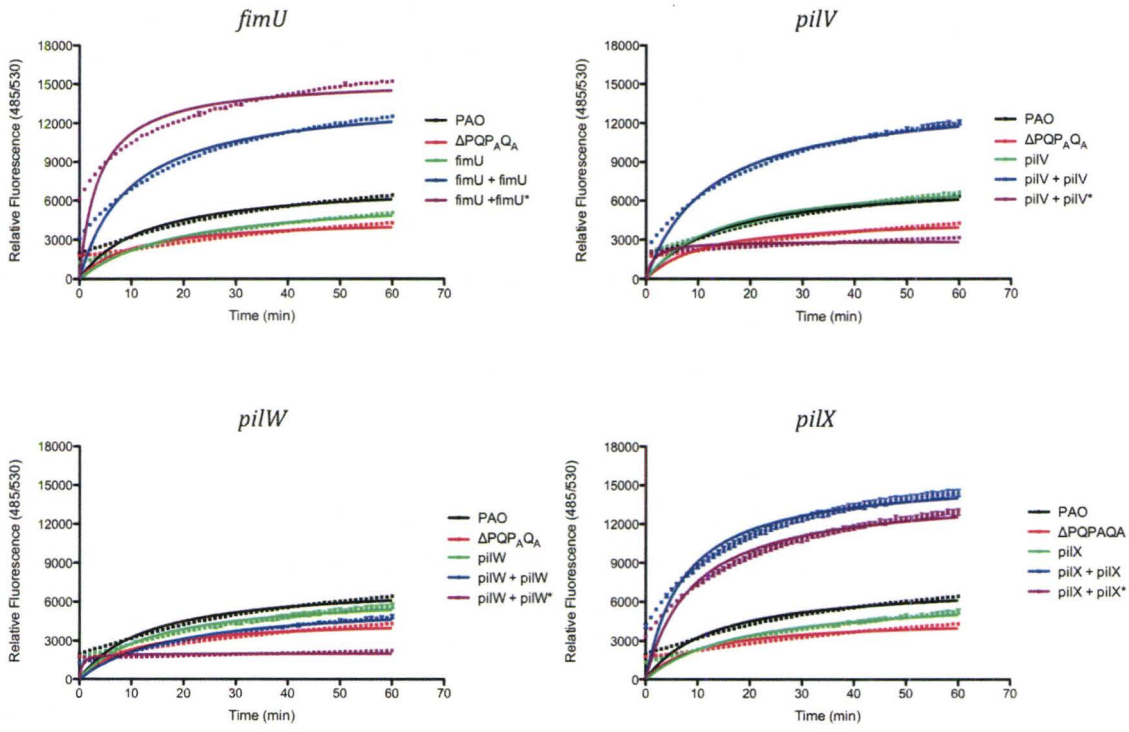

Figure 4.S1. Elastase activity over time

Bacterial cultures of mPAO1 (black), ΔPQP$_A$Q$_A$ (red), *fimU*, *pilV*, *pilW*, or *pilX* (green), and respective complemented strains induced with 0.2 % arabinose (purple) or uninduced (blue) were grown for 22 hours before harvesting. Supernatant samples were incubated with elastin conjugated BODIPY-FL, and measured for relative fluorescence A485/530 over a course of one hour. Graphs were plotted with Prism GraphPad 5.

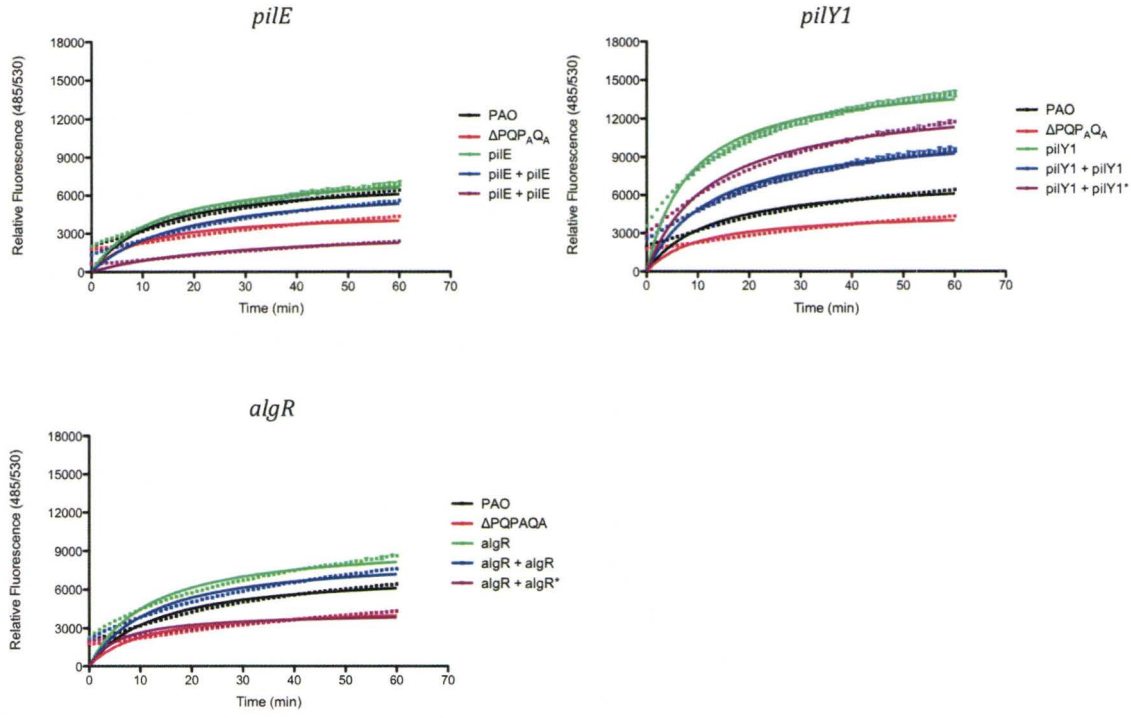

Figure 4.S2. Elastase activity over time

Bacterial cultures of mPAO1 (black), ΔPQP$_A$Q$_A$ (red), *pilE*, *pilY1*, or *algR* (green), and respective complemented strains induced with 0.2 % arabinose (purple) or uninduced (blue) were grown for 22 hours before harvesting. Supernatant samples were incubated with elastin conjugated BODIPY-FL, and measured for relative fluorescence A485/530 over a course of one hour. Graphs were plotted with Prism GraphPad 5.

secretion relative to wild type (76 ± 2.7, 87 ± 1.3, 148 ± 4.1, and 128 ± 2.4% respectively). Interestingly, induction of *fimU* and *pilY1* expression in the complemented mutants increased elastase activity relative to levels observed in the uninduced state, while induction of expression of other minor pilins decreased secretion relative to the uninduced state (Figure 4.5a). Overexpression of *pilV*, *pilW* and *pilE* were of particular interest as it suppressed elastase activity to levels below that seen in the negative ΔPQP_AQ_A mutant control (Figure 4.5a). For relative fluorescence over time, see the supplementary data (Figures 4.S1 and 4.S2). Of note, the results of these liquid-based assays differ from the above plate-based assays where no secretion was observed for the *pilV*- and *pilW*-complemented mutants (Figure 4.3), suggesting that growth conditions can affect the observed phenotypes.

Western blot analysis using anti-LasB antisera showed intracellular accumulation of a 51 kDa product (representing the associated 18 and 33 kDa fragments) in the ΔPQP_AQ_A mutant, and no LasB was detected in the supernatant fraction (Figure 4.5b). Therefore, the levels of elastase activity measured for the negative ΔPQP_AQ_A mutant control equated to background. The *fimU*-, *pilW*- and *pilX*-complemented mutants showed intracellular accumulation of the 51 kDa product, although to a lesser extent than the negative control. LasB was detected in the supernatant fraction of the *fimU* and *pilX* complements but not the *pilW*-complemented mutant (Figure 4.5b).

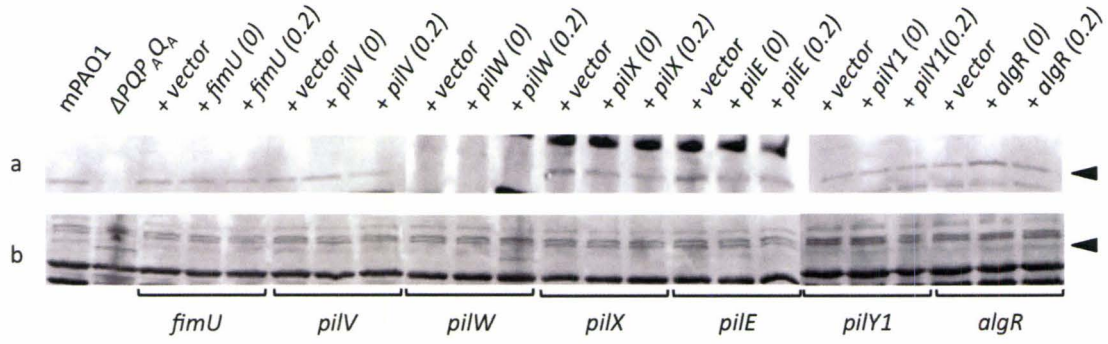

Figure 4.6. Minor pilin mutants are unable to secrete Exotoxin A

Bacterial cultures of mPAO1, ΔPQP$_A$Q$_A$, *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *pilY1*, *algR* and respective complemented strains were grown for 22 hours before harvesting (0) represents uninduced conditions while (0.2) represents induction with 0.2 % arabinose. Supernatant (a) and cellular (b) fractions were separated and run on a 12.5 % SDS-PAGE gel, transferred to nitrocellulose and probed with an anti-ToxA antibody. Arrow indicates ToxA band at 66 kDa.

Exotoxin A assays

To determine if secretion of other T2S effectors was affected by changes in T4P minor pilin levels, levels of intracellular and extracellular exotoxin A were quantified using an anti-ToxA antibody. An intracellular accumulation of exotoxin A was observed in the ΔPQP_AQ_A mutant, while no exotoxin A was detected in the supernatant fraction (Figure 4.6), confirming that the secretion of exotoxin A is specific to the T2S system (Voulhoux *et al.*, 2000). All of the minor pilin mutants with the exception of *pilW* were able to secrete exotoxin A (Figure 4.6). Complementation with *pilW* alone did not restore the secretion phenotype, similar to the phenotype observed in plate-based protease and elastase assays (Figures 4.2 and 4.3). Overexpression of *pilW* led to an intracellular accumulation of exotoxin A demonstrating that a higher-than-native level of *pilW* inhibits secretion (Figure 4.6). Exotoxin A secretion was restored to wild type levels in the *pilW* mutant upon complementation with *pilVW* or *fimUpilVW* under uninduced conditions, however overexpression of *fimUpilVW* or *fimUpilVWX* decreased levels of secreted exotoxin A (Figure 4.7). Although the *pilV* mutant was able to secrete exotoxin A, arabinose induction of any of the complementation constructs lead to the loss of extracellular exotoxin A (Figure 4.7). Interestingly, the *algR* mutant, which lacks *pilW* expression, was able to secrete exotoxin A, suggesting that the loss of secretion observed in the *pilW* mutant is not the dominant phenotype. Furthermore, the uninduced *algR*-complemented mutant showed increased exotoxin A secretion relative to the

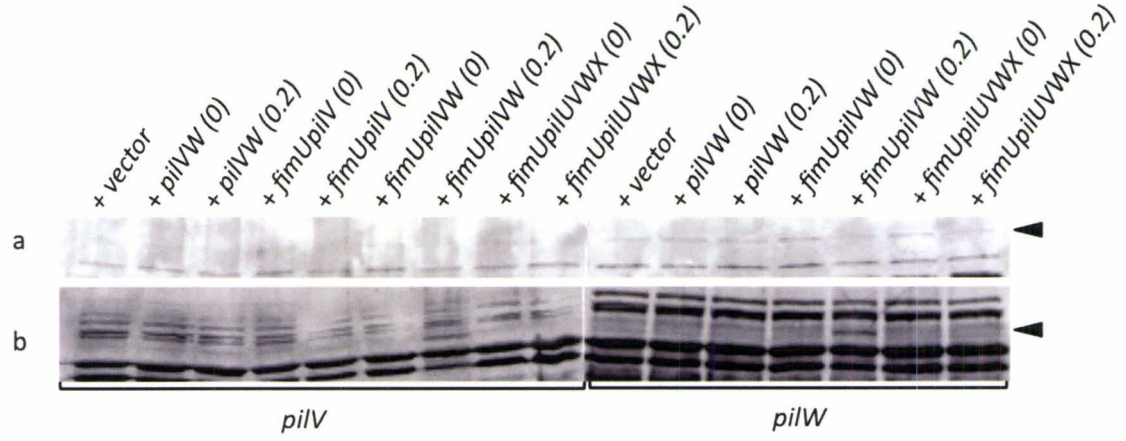

Figure 4.7. Minor pilin overexpression decreases secretion of Exotoxin A

Bacterial cultures of mPAO1, ΔPQP$_A$Q$_A$, *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *pilY1*, *algR* and respective complemented strains were grown for 22 hours before harvesting (0) represents uninduced samples while (0.2) represents induction with 0.2 % arabinose. Supernatant (a) and cellular (b) fractions were separated and run on a 12.5 % SDS-PAGE gel, transferred to nitrocellulose and probed with an anti-ToxA antibody. Arrow indicates ToxA band at 66 kDa.

induced *algR*-complemented construct (Figure 4.7), suggesting that overexpression of the minor pilin operon has detrimental effects on secretion.

Minor pilin mutants secrete Tat-dependent exoproteins

To determine whether alterations in T4P minor pilin levels affect secretion of both Sec-dependent and Tat-dependent T2S substrates, secretion of the Tat-dependent substrates lipase (LipC) and the hemolytic phopholipase C (PlcH) was evaluated. The ability of bacterial strains to secrete lipases was measured by testing for growth on lipid-minimal media plates (Kagami *et al.*, 1998). The minor pilin (*fimU, pilV, pilW, pilX, pilY1* and *pilE*) mutants, the regulatory mutant *algR*, and the double mutants *fimU-pilQ* and *pilX-pilQ* grew on lipid plates to the same extent as the wild type (Figure 4.8). The ΔPQP_AQ_A mutant was not able to grow on lipid plates (Figure 4.8), showing that secretion of lipases by other mutants tested occurred through the T2S system. Secretion of PlcH was tested on sheep blood-agar plates, where a positive result was defined as a zone of clearing around the colony (Tay *et al.*, 1995). The quadruple ΔPQP_AQ_A mutants were unable to secrete haemolysin (Figure 4.8), verifying that the Xcp system is responsible for PlcH secretion. All minor pilin mutants were able to secrete haemolysin (Figure 4.8), however the morphology of the colonies was atypical, with a smaller zone of clearing compared to wild type (57, 54, 48, 53, and 40% compared to wild type in *fimU, pilV, pilW, pilX* and *pilE* strains respectively). Loss of *pilY1* or *algR* also

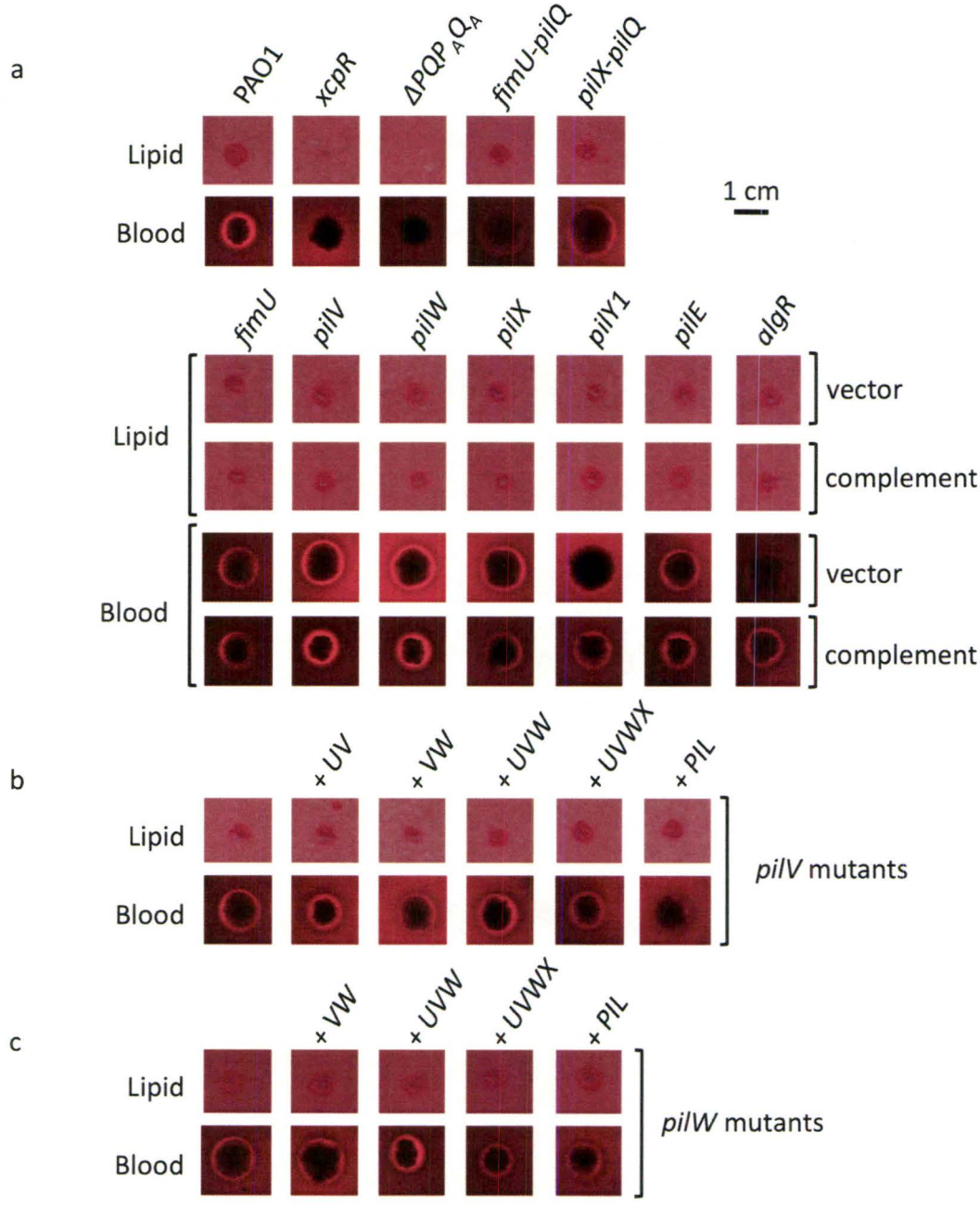

Figure 4.8. Minor pilin mutants have reduced PlcH secretion

Figure 4.8. ***Minor pilin mutants have reduced PlcH secretion***

(a) Single colonies of mPAO1, *xcpR*, ΔPQP$_A$Q$_A$, *fimU-pilQ*, *pilX-pilQ*, *fimU*, *pilX*, *pilV*, *pilW*, *pilY1*, *pilE*, *algR* and respective complemented mutants (*) were inoculated on lipid or blood agar plates. Plates were incubated for 36 hours at 37°C. Colonies were then assessed for lipid secretion visualized as growth, or hemolysin secretion visualized as a zone of clearing around the colonies. Single colonies of pilV (b) or pilW (c) mutant strains complemented with pBADGR (empty vector), *fimUpilV* (*pilV* mutants only), *pilVW*, *fimUpilVW*, *fimUpilVWX* or pVDtacPIL (the entire minor pilin operon) were inoculated on lipid and blood agar plates. Plates were incubated for 36 hours at 37°C. Colonies were then assessed for assessed for lipid secretion visualized as growth, or hemolysin secretion visualized as a zone of clearing around the colonies.

decreased haemolytic activity as 40 and 41% of wild type zones were observed for those strains (Figure 4.8). Complementation of each of the minor pilin, pilY1 or algR mutants with their cognate genes in trans restores haemolysin secretion to wild type levels.

V. DISCUSSION

As the components that make up the T2S machinery are structurally similar to members of the T4P system, and there are several examples of functional overlap between systems, we sought to determine whether the T4P minor pilins could also function in the T2S system. Loss of the minor pilins was observed to affect secretion of specific exoproteins, therefore we hypothesized that the minor pilins may be involved in substrate selection. Analysis of known T2S proteins revealed that the N-terminus of the *Erwinia chrysanthemi* OutD secretin, and the PDZ protein interaction domain of the pilotin OutC were shown to be integral for species-specific substrate recognition. Chimera formation involving the exchange of the N-terminus of OutD in *E. chrysanthemi* for that of *E. carotovora* allowed for species specific secretion of *E. carotovora* exoproteins, while deletion of the PDZ domain rendered OutC non-functional (Bouley *et al.*, 2001). Bouley and colleagues suggested that exoproteins contain variable secretion signals that are recognized by different regions of OutC and OutD (Bouley *et al.*, 2001). Selectivity therefore, may involve other members of the T2S apparatus to form a multi-point three dimensional recognition domain. The

proximity of the minor pseudopilin complex to the XcpP pilotin (homologue of OutC) and the XcpQ secretin (homologue of OutD), makes the minor pseudopilins ideal candidates for determination of substrate recognition. Minor pseudopilins are conserved across T2S systems, and contain a conserved N-terminal α-helical region, however their sequence varies substantially in the C-terminal domains (Lindeberg *et al.*, 1998). The diversity in the minor pilin and minor pseudopilin sequences between bacterial species and strains may therefore contribute to the species-specific recognition of exoproteins. Loss of any of the minor pseudopilin genes *outH, outI, outJ* or *outK* in *E. chrysanthemi* or *pulH, pulI, pulJ* or *pulK* in *K. pneumoniae* was shown to prevent secretion of T2S-dependent products (He *et al.*, 1991; Possot *et al.*, 2000; Sauvonnet *et al.*, 2000). We therefore hypothesize that the minor pilins and minor pseudopilins, in combination with other T2S factors, are involved in substrate recognition through a three-dimensional multi-point contact (Figure 4.9).

Based on our results, the minor pilins appear to fall into three categories: (a) elastase secretion is unaffected in liquid cultures of *pilV, pilW* or *pilE* mutants, while complementation with the cognate gene under induced conditions decreases secretion of elastase to levels below the ΔPQP_AQ_A control; (b) loss of *fimU* or *pilX* decreases secretion of elastase slightly (80% of wild type), however upon complementation, secretion increases to approximately double that of wild type; and (c) loss of *pilY1* increases secretion of elastase, which returns to wild type levels upon complementation *in trans* (Figure 4.4). Marked differences in elastase secretion between the liquid and plate-based assays were observed, particularly in

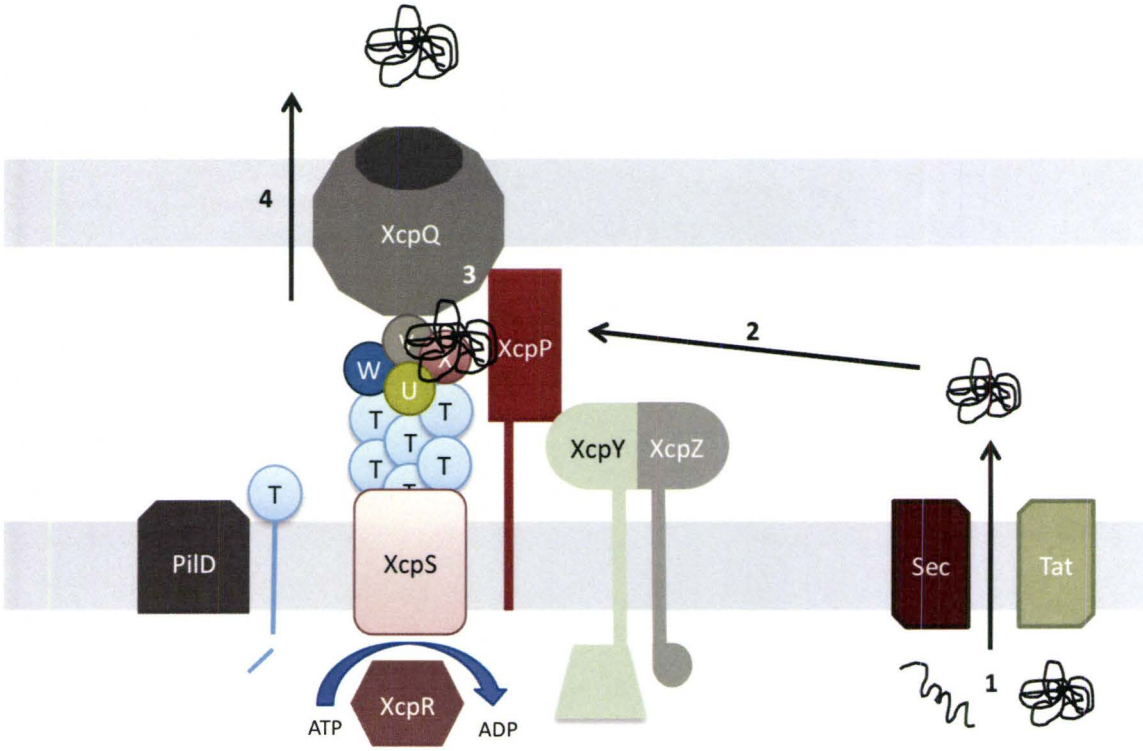

Figure 4.9. Model: Minor pilins may participate in selection of T2SS substrates

Proposed model of how minor pilins may participate in substrate specificity. (1) Proteins are translocated across the inner membrane in folded state by the Tat system, or an unfolded state by the Sec system, where upon Sec-dependant products are folded and exoproteins are shuttled to the T2S system (2). Substrates are recognized by four main proteins: XcpP, XcpQ, PilX, and FimU (3). The diversity of the minor pilins between bacterial species allows for species specific substrate the *pilV*, *pilW*, and *pilE* mutants where no elastase activity was observed on plates

(Figure 4.3). Bertrand and colleagues observed a decrease in surface piliation when strains were grown in liquid cultures (Bertrand *et al.*, 2010), suggesting that T4P are produced preferentially on solid surfaces. As T4P are predominantly expressed when cells are grown on a surface (Bertrand *et al.*, 2010), the secretion recognition. (4) The binding of the substrate to the Xcp system triggers translocation across the outer membrane to the extracellular meiux.

phenotype may also be sensitive to changes in viscosity, as secretion trends for the minor pilin mutants and complemented mutants were identical on skim milk and elastin-Congo red plates (Figures 4.2 and 4.3).

Our results indicate that loss of the minor pilins FimU, PilV, PilW, PilX, and PilE significantly reduce the secretion of proteases, specifically elastase, and exotoxin A, but not lipases. It is enticing to speculate that the minor pilins may function in recognition of Sec- but not Tat-dependent substrates, as elastase and exotoxin A are secreted in a Sec-dependent manner, while most lipases contain a twin arginine motif for Tat-dependent secretion. However other *P. aeruginosa* lipases have been shown to be Sec-dependent substrates (Rosenau *et al.*, 2000), as selective inhibition of the SecA ATPase in *E. coli* resulted in a cytoplasmic accumulation of lipase (Rosenau *et al.*, 2000). Furthermore, when grown on blood plates only partial clearing was observed around the minor pilin mutants, suggesting that secretion of the Tat-dependent hemolytic phospolipase C (PlcH) is decreased in these mutants. Translocation of PlcH requires two chaperone proteins, PlcR1 and PlcR2 (Cota-Gomez *et al.*, 1997; Stonehouse *et al.*, 2002). While PlcH is

Tat-dependant, its chaperone PlcR2, which does not carry a signal sequence, is proposed to piggyback on PlcH through the Tat-translocon, while PlcR1 has been shown to be Sec-dependent. In the periplasm, PlcH is predicted to be transferred from PlcR2 to PlcR1, which then accompanies PlcH through the Xcp system and out of the cell (Cota-Gomez *et al.*, 1997; Stonehouse *et al.*, 2002) (M. L. Vasil, personal communication). Therefore, the minor pilins may preferentially affect Sec-dependant exoproteins, but in the case of PlcH, it may be the chaperone PlcR1 rather than PlcH whose secretion is impacted, thereby affecting PlcH secretion.

Although loss of any of the minor pilins inhibits secretion of exoproteins, a significant increase in secretion was observed specifically upon overexpression of FimU or PilX (Figures 4.2 and 4.3). However this appears to be conditional upon their expression context, as complementation with the entire minor pilin operon does not appear to significantly increase secretion (Figures 4.2 and 4.3). Previously we have suggested that both FimU and PilX may be involved in initiation of surface pilus assembly, as overexpression of either protein results in short, but still functional pili (Giltner *et al.*, 2010). These proteins may also stimulate pseudopilus assembly in the T2S system, leading to an increase in exoprotein secretion. The T2S pseudopilus has been predicted to function as a piston to either push exoproteins though the secretin or upon retraction, to permit the egress of specific exoproteins (Hobbs *et al.*, 1993; Pugsley,1993; Nouwen *et al.*, 1999; Nunn,1999; Nouwen *et al.*, 2000; Filloux 2004). If the T2S system indeed functions in this manner, then an

increased number of pseudopilus assembly events would directly increase the amount of secreted product.

Recently, the PilV T2S homologue XcpV was shown to play a central role in minor pseudopilus complex formation, as XcpV was proposed to act as a nucleator of pseudopilius assembly that first binds XcpX and then XcpW (Douzi *et al.*, 2009). Our results support a similar role for the XcpV orthologue PilV, as its overexpression (and therefore potential titration of other components) completely abolishes secretion of exotoxin A and elastase (Figures 4.2 and 4.3). Likewise, overexpression data suggests that PilV function is particularly sensitive to fluctuations in stoichiometry, as overexpression of complementation constructs containing *pilV* (*fimUpilV, fimUpilVW, fimUpilVWX*) eliminated exotoxin A secretion (Figure 4.7). We propose that overexpression of PilV titrates the PilX and PilW components (required for formation of a minor pilin subcomplex) away from their site of action to prevent secretion. Although a PilE orthologue is not present in the T2S system, PilE may play a role similar to PilV or PilW, as overexpression of PilE reduces protease and elastase secretion (Figures 4.2, 4.3 and 4.5).

Our data show that overexpression of FimU and PilX enhances secretion of elastase (Figures 4.2, 4.3 and 4.5). A 1:1 stoichiometry between PilV and PilW appears to be important for function, as overexpression of only one of these proteins blocks secretion of exoproteins to a greater extent than a quadruple T2S mutant, as seen under induced conditions. We hypothesize that the minor pilins are

involved in substrate selectivity, and that a loss of a single minor pilin decreases the efficiency of exoprotein secretion by the T2S system.

VI. ACKNOWLEDGMENTS

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CHAPTER 5:

MULTIPLE ROLES FOR PILY1 IN *PSEUDOMONAS AERUGINOSA* PHYSIOLOGY

Carmen L. Giltner, Rami El Sabai, Colin Rous, Anne Hussain,

and Lori L. Burrows

Attribution: C.L.G. and L.L.B designed the experiments, and C.L.G directly supervised participating trainees. C.L.G. did the experiments with the following exceptions: R.E.S. made the double *pilY1-pilT* mutant, and C.R. for generated the *pilY1* complementation construct. A.H. assisted with Western blots.

I. ABSTRACT

Pseudomonas aeruginosa is an important opportunistic pathogen that requires functional type IV pili (T4P) to establish and maintain infection. Several proteins are required for a unique form of motility mediated by T4P termed 'twitching', including the putative adhesin protein PilY1. In addition to twitching motility, PilY1 has been implicated in pilus formation and a number of additional virulence-related functions, including contributions to killing of neutrophils, and control of type II secretion of exoproteins. To better understand PilY1-specific functions, we investigated which T4P biogenesis proteins were affected by the loss of *pilY1*. PilY1 was shown to be required for surface piliation without affecting the stability of the major pilin subunit PilA, but to be dispensable for assembly when retraction was blocked through mutation of PilT. PilY1 may also decrease multimerization of the PilQ secretin mutants, due to decreased intracellular levels of the outer membrane pilotin, PilF, indicating a possible role for PilY1 in PilF stability. Furthermore, *pilY1* mutants were found to have altered antibiotic resistance profiles and a truncated cellular shape compared to wild type cells. Together these data show that while PilY1 is not essential for pilus assembly, it directly influences a number of T4P biogenesis products.

II. INTRODUCTION

Type IV pili are thin (0.5-0.6 nm), long (several μm) filamentous appendages that are responsible for a unique kind of motility known as twitching (Folkhard *et al.*, 1981; Semmler *et al.*, 1999; Mattick 2002). Twitching motility involves repeated cycles of polymerization and depolymerization, where the pilus is able to extend through the secretin (PilQ), attach to a surface, and retract to pull the bacterium forward. In *Pseudomonas aeruginosa*, the secretin is a dodecameric complex of PilQ subunits whose multimerization is dependent on PilF, as Western blot analysis showed a loss of SDS-resistant multimers in *pilF* mutants (Koo *et al.*, 2008). The pilus, extruded through the secretin pore, is composed primarily of PilA monomers, although the incorporation of five other pilin-like proteins has recently been shown (Giltner *et al.*, 2010). Of the large number of gene products that have been implicated in pilus biogenesis (Mattick 2002; Jacobs *et al.*, 2003) the specific roles of only a subset have been defined. The gene products FimU, PilV, PilW, PilX, PilY1, PilY2 and PilE are encoded by a polycistronic operon positively controlled by the response regulator AlgR. They are required for pilus biogenesis and thus essential for twitching motility, as knockouts in any of these genes gives a non-motile phenotype (Russell *et al.*, 1994; Alm *et al.*, 1995; Alm *et al.*, 1996; Alm *et al.*, 1996). The minor pilins, FimU, PilV, PilW, PilX and PilE have been shown to be cleaved by the bifunctional enzyme PilD before incorporation into the growing pilus fibre, and may be responsible for efficient opening of the secretin (Giltner *et al.*, 2010). These gene products are predicted to be structurally similar to PilA, while the PilY1

protein, encoded by the same operon, does not share this predicted structure. Although a potential PilD cleavage site was identified at the N terminus (Lewenza *et al.*, 2005) PilY1 is not considered to be a minor pilin.

PilY1 is a 128 kDa multifunctional protein that has been shown to function in pilus biogenesis, secretion of the lipase enzyme LipC, phenazines and quinolones, and to play roles in adherence to human epithelia and resistance to killing by polymorphonuclear cells (Bohn *et al.*, 2009; Heiniger *et al.*, 2010). PilY1 shares C-terminal similarity to the *Neisseria gonorrhoeae* pilus tip-associated adhesion protein PilC2 (43% over residues 657-914 versus residues 506 -765, respectively) (Alm *et al.*, 1996). The *pilC2* gene was shown to function as the pilus adhesin, as exogenous PilC2 was able to prevent binding of *N. gonorrhoeae* and *N. meningitidis* to epithelial cells, and piliated *pilC2* mutants were similarly unable to bind epithelial cells (Scheuerpflug *et al.*, 1999). However, localization studies of PilY1 have shown that it resides in the membrane, and in extracellular vesicles, however conflicting evidence has been reported regarding its localization to/with the pilus (Bohn *et al.*, 2009; Heiniger *et al.*, 2010). Although Bohn and colleagues looked specifically at the pilus fibre, no PilY1-labeling was observed (Bohn *et al.*, 2009) however, a recent study has shown that PilY1 is likely to be pilus associated (Heiniger *et al.*, 2010). The discrepancy may have arisen due to differences in antibody affinity, as the Tummler antibody was generated from an N-terminal peptide (positions 338-352), while the Wolfgang antibody was generated from a purified C-terminal fragment of PilY1 (Bohn *et al.*, 2009; Heiniger *et al.*, 2010).

Recently, the structure of the PilY1 C-terminal domain (amino acids 615 to 1163), which is the region similar to the PilC2 protein in *Neisseria* spp., was determined. The structure shows a modified β-propeller fold and a conserved calcium-binding domain (Orans *et al.*, 2009). Inhibition of calcium binding via a single amino acid substitution (D859A), or by addition of exogenous Ca^{++} chelators was shown to prevent surface piliation unless PilT-mediated pilus retraction was blocked. Orans and colleagues concluded that calcium binding was important as a switch for inducing a PilY1 configuration capable of antagonizing PilT retraction activity (Orans *et al.*, 2009).

Conflicting reports on the localization of PilY1, and the absence of any in-depth studies regarding the role of PilY1 within the T4P system, led us to examine the affect of PilY1 members of the T4P system. Here we show that PilY1 affects the expression levels of a number of known pilus biogenesis proteins, including PilF and PilQ. Loss of PilY1 decreased intracellular PilF levels, which appeared to affect the stability of the PilQ multimer, and supplementation of *pilF* in trans to *pilY1* mutants restored wild-type levels of PilQ multimerization. Furthermore, we show a PilY1-dependent alteration in cell morphology that is also observed in minor pilin, *algR*, and *pilD* mutants, where cells are shorter and wider than wild type cells. Finally, we show that the loss of *pilY1*, but not of other members of its operon, alters the antibiotic sensitivity profile of the cells. These data, as well as those from other groups, point to a complex contribution of PilY1 to *P. aeruginosa* physiology.

III. MATERIAL AND METHODS

Bacterial strains and plasmids

Bacterial strains and DNA used in this study are listed in Table 5.1. Bacteria were maintained as glycerol stocks at -80°C. *P. aeruginosa* strains were grown routinely on Luria Bertani (LB) agar plates supplemented with gentamicin (30 mg/L) or carbenicillin (200 mg/L) and L-arabinose (0 - 0.2% [w/v]) where indicated. *Escherichia coli* was used in this study for cloning and propagation of plasmid constructs, and grown on LB supplemented with gentamicin (15 mg/L) and ampicillin (100 mg/L) respectively.

Genetic manipulations

For complementation constructs, the *pilY1* gene was amplified via PCR from mPAO1 chromosomal DNA. Primer sequences are listed in Table 5.2. Combinations of these primers were used to amplify the full-length *pilY1* gene. *pilY1* was amplified from mPAO1 chromosomal DNA with primers 1 and 2, using Advantage 2 PCR Kit (Clonetech) for use in TA cloning with the PCR2.1 vector (Invitrogen). The *pilY1* product was digested out of the PCR2.1 vector with EcoRI and gel purified (Qiagen). The pBADGr vector was linearized using EcoRI and the digested *pilY1* product and vector were ligated with Quick Ligase (Fermentas). Ligated constructs were electroporated into *E. coli* DH5α cells and verified via DNA sequence analysis (ACGT Co., Toronto, ON). Verified constructs were electroporated into relevant mPAO1

Table 5.1 List of strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
pBADGr	pMLBAD backbone with dhfr (trimethoprim resistance) replaced with aacC1 (gentamicin resistance)	(Asikyan *et al.*, 2008)
pBADGr*fimU*	PAO1 *fimU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilV*	PAO1 *pilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilW*	PAO1 *pilW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilX*	PAO1 *pilX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr*pilE*	PAO1 *pilE* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner *et al.*, 2010)
pBADGr *pilY1*	PAO1 *pilY1* sequence inserted with the EcoRI restriction enzyme in the MCS of pBADGr	Chapter 4
pVDtac39	IncQ/P4 *mob*$^{+}$ *tac lacI*^{q}	(Deretic *et al.*, 1987)
pVDtacPIL	pVDtac39 backbone with *fimT fimU pilV pilW pilX pilY1 pilY2* and *pilE* inserted in the MCS	(Lizewski *et al.*, 2004)
E. coli		
DH5α	GENOTYPE: F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1	Invitrogen
TOP10	GENOTYPE: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 ΔlacX74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
P. aeruginosa		
mPAO1	Laboratory strain	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilA*	IS*phoA*/hah transposon instertion (position 165)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*fimU*	IS*lacZ*/hah transposon insertion (position 237)	(Jacobs *et al.*, 2003)

mPAO1 Tn::*pilV*	IS*phoA*/hah transposon insertion (position 122)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilW*	IS*lacZ*/hah transposon insertion (position 381)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilX*	IS*phoA*/hah transposon insertion (position 182)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilY1*	IS*phoA*/hah transposon insertion (position 1407)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*pilE*	IS*phoA*/hah transposon insertion (position 183)	(Jacobs *et al.*, 2003)
mPAO1 Tn::*sltB1*	IS*phoA*/hah transposon insertion (position 535)	(Jacobs *et al.*, 2003)
mPAO1 *algR*::FRT	Wild type strain with FRT scar insertion in the NruI site within *algR*	Chapter 3
mPAO1 Tn::*fimU* + pBADGr *fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilV* + pBADGr *pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilW* + pBADGr *pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilX* + pBADGr *pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 Tn::*pilE* + pBADGr *pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 FRT::*algR-pilT*::FRT	Wild type strain with FRT scar insertion in the NruI site within *algR* and an FRT insertion in the NruI site within *pilT*	Chapter 3

Table 5.2 List of primers used in this study

Primer Number	Primer Name	Sequence (5'-3')
	pilY1	
1	Sense	GCCCGTCTCTATACCAACTGATCGAGCCTCGCATGAAA
	pilY1	
2	Antisense	GCAGCACTTTCATATCAGTTCTTTCCTTCGATGGGGC
	pilT	
3	Sense	GGATCCGGTGTTTTCCTTGTCCGA
	pilT	
4	Antisense	AAGCTTGAATCCTAGACGCAGTTC

transposon mutants (for complementation), and transformants were plated on LB containing gentamicin (30 mg/L).

Twitching motility assays

Bacterial twitching was performed as previously described (Gallant *et al.*, 2005). Briefly, single bacterial colonies were stab-inoculated (to the underlying plastic surface) with a sterile toothpick on 1% agar LB plates containing gentamicin (30 mg/L) or carbenicillin (200 mg/L) and L-arabinose (0.2%). After incubation, the agar was carefully removed and twitching zones were visualized by staining with 1% (w/v) crystal violet for 30 minutes. Unbound dye was removed with water and plates were allowed to air dry. To determine twitching motility zones relative to wild type, the areas of 6 twitching zones from three independent experiments were measured using ImageJ (Abramoff *et al.*, 2004). The mean of these zones are reported.

Surface piliation assay

Sheared surface proteins were isolated as described previously (Castric,1995; Kus *et al.*, 2004; Asikyan *et al.*, 2008) with the following modifications. Bacterial strains were streaked in a cross-hatched fashion on three 1.5% agar LB plates containing gentamicin (30 mg/L) or gentamicin and L-arabinose (concentrations of 0.01, 0.02, 0.1 and 0.2 % w/v) and grown overnight at

37 °C. Bacteria were gently scraped from the agar surface with a sterile coverslip, and resuspended in 4.5 ml of 1x phosphate-buffered saline (PBS pH 7.4). Suspensions were vortexed for 30 s to shear surface organelles and transferred to three 1.5 ml microcentrifuge tubes where the cells were harvested by centrifugation at room temperature (RT) for 5 min at 11688 x g. The supernatant was transferred to new microcentrifuge tubes and any remaining cellular debris removed by centrifugation at RT for 20 min at 11688 x g. The supernatant was transferred to new microcentrifuge tubes and a 1/10 volume of each of 5 M NaCl and 30 % polyethylene glycol (MW 8000) was added. The mixture was incubated on ice for 1 hr to allow for precipitation of proteins. Precipitated proteins (pilin and flagellin) were collected by centrifugation at RT for 30 min at 11688 x. The three recovered pellets for each sample were pooled and resuspended in 100 µl of 1 x sodium-dodecyl-sulphate (SDS) loading buffer (80 mM Tris pH 6.8; 5.3 % [v/v] 2-mercaptoethanol; 10 % [v/v] glycerol; 0.02 % [w/v] bromophenol blue; 2 % [w/v] SDS). Samples were boiled for 10 min and run on a 15 % SDS-PAGE gel. Precipitated proteins were visualized with Coomassie brilliant blue (Sigma).

Electron Microscopy

Bacterial strains were grown on LB containing gentamicin (30 mg/L) or gentamicin and L-arabinose (0.2 % [w/v]). A small amount of bacteria from the edge of a colony were picked using a sterile toothpick and dispersed in 200 µl

ddH_2O. Samples were applied to previously glow-discharged carbon-coated grids by floating grids on a 10 µl drop of bacterial suspension. Bacteria were incubated for 5 min on the grids before negatively staining with 2.5 µl of 2 % (w/v) uranyl acetate for 45 s. Unbound bacteria and stain were removed by wicking of excess liquid from the grids using Whatman filter paper. EM pictures were taken with a JEOL JEM 1200 TEMSCAN (Peabody, MA, USA) microscope operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images.

Generation of pilY1-pilT double knockouts

Double knockouts were generated using the biparental mating assay as previously described (Burrows *et al.*, 2000). The pEX18AP + *pilT*::GmFRT plasmid was electroporated into *E. coli* SM10 cells (Simon *et al.*, 1983), and through biparental mating was transferred to mPAO1Tn::*pilY1* cells. The gentamicin resistance cassette was subsequently excised by the Flp recombinase encoded by pFLP2, and the plasmid removed using sucrose counterselection (Hoang *et al.*, 1998). Double mutants were verified by assessing FRT disruption of the *pilT* gene using PCR amplification (primers 3 and 4, Table 5.2).

Antibiotic resistance profiles

Bacterial strains mPAO1, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE* and *sltB1* were grown to OD_{600} = 0.6. Standardized cultures were diluted to OD_{600} = 0.25 and 100 µl of diluted culture was plated on Mueller Hinton agar plates (Difco). Paper disks infused with imipenem (10 µg), carbenicillin (100 µg), piperacillin (75 µg), cefotaxime (5 µg), ciprofloxacin (1 µg) or tobramycin (10 µg) were placed on the agar surface and incubated over night as per manufacture's protocol (Oxoid). Antibiotic sensitivity is seen as a zone of clearing around the antibiotic disk.

IV. RESULTS

Twitching motility of a pilY1 *mutant is restored upon complementation*

As previously reported, the *pilY1* mutant used for this work was unable to twitch (Alm *et al.*, 1996), however complementation with the full-length *pilY1* construct restored twitching motility to ~70% of wild type under induced conditions (Figure 5.1). Contrary to the minor pilins, twitching motility in *pilY1* complemented mutants was directly related to the arabinose concentration, suggesting that the function of PilY1 is not subject to the same stringent stoichiometric constraints as the minor pilins expressed from the same operon (Giltner *et al.*, 2010). No surface piliation was observed in *pilY1* mutants (Figure 5.1), consistent with their lack of motility. Furthermore, complementation of the *pilY1* mutant under uninduced conditions, which results in a twitching zone of

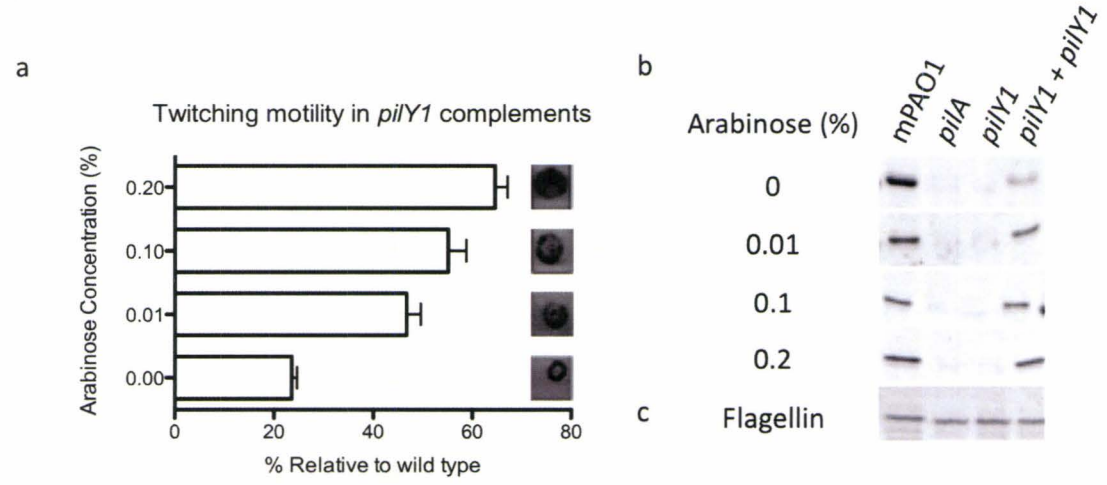

Figure 5.1. Twitching motility and surface piliation are complementable in pilY1 mutants

(a) Results of twitching motility assays on 1% LB agar plates, with arabinose concentrations ranging from 0-0.2 % [w/v] to induce expression of the cloned gene, were performed for 48 hrs at 37 °C. *pilY1* mutants are unable to twitch, however twitching is restored to ~70 % of wild-type by complementation *in trans* with the cognate gene. Mean twitching zone area from three independent experiments where n=12 are represented with representative zones shown on the right. Twitching areas were measured using ImageJ (Abramoff *et al.*, 2004). (b) Sheared surface proteins from the mPAO1 wild type and *pilA*, and *pilY1* mutant strains and complemented strains (grown on arabinose concentrations ranging from 0 - 0.2%) were separated on 15% SDS-PAGE gels and stained with Coomassie blue. Only the pilin band from each gel is shown. (c) Representative flagellin bands from the 0.2% arabinose sample used as a loading control.

~23% relative to wild type, caused a decreased level of surface piliation, while induction with 0.2% arabinose resulted in wild type levels of surface piliation (Figure 5.1). This result is concordant with our previous data, which shows that the amount of surface piliation can not necessarily be directly correlated to the amount of twitching observed (Giltner *et al.*, 2010).

To determine if PilY1 was essential for pilus assembly, a double knockout of *pilY1* and *pilT* was generated (see Methods). Surface piliation was restored, demonstrating that PilY1 is dispensable for pilus assembly in a retraction-deficient background. These data are consistent with our previous observations where surface piliation was restored in *algR-pilT* double knockouts, demonstrating that abrogating expression of the minor pilin operon containing the *pilY1* gene does not abolish surface piliation (Giltner *et al.*, 2010). However, levels of surface pili in the *pilY1* double mutant were not commensurate with the *pilT* control (Figure 5.2). Similarly, *pilY1-pilT* double knockouts in *P. aeruginosa* strain PAK were shown to have surface piliation that was reduced compared with the *pilT* control, but complementation with *pilY1* resulted in levels equivalent to the *pilT* mutant (Heiniger *et al.*, 2010). Together these data show that while assembly is suboptimal, PilY1 is dispensable for assembly when retraction was blocked through mutation of *pilT*.

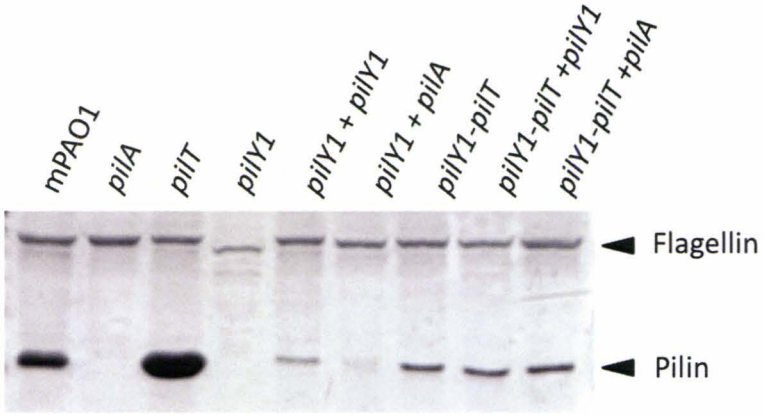

Figure 5.2. Surface piliation is restored in a retraction deficient background

(a) Surface pilin preparations of bacterial strains mPAO1, *pilA*, *pilT*, *pilY1*, *pilY1-pilT* and respective complemented strains were run on a 15% SDS-PAGE gel and stained with Commassie brilliant-blue.

PilY1 affects PilQ multimerization

To determine what role PilY1 may be playing in T4P biogenesis, expression levels of 11 different T4P biogenesis proteins (PilA, FimU, PilW, PilM, PilN, PilO, PilP, PilT, PilF, PilQ and PilB) were compared using specific antibodies in wild type and *pilY1* mutants. Loss of PilY1 increased the intracellular level of FimU (~1075 ± 53.6% of wild type, Table 5.3), consistent with a previous study where an 21.7-fold increase in *fimU* mRNA levels were detected in *pilY1* mutants (Bohn *et al.*, 2009). Bohn and colleagues (Bohn *et al.*, 2009) also showed that *pilW* mRNA levels were increased in *pilY1* mutants (19.5-fold increase relative to wild type), however our data show that the intracellular levels of PilW protein are decreased in both *pilY1* and *pilY1-pilT* mutants (~10 ± 2.0 and 19 ± 1.9% of wild type respectively). Intracellular PilW levels are restored to approximately 50 ± 16.4% of wild type upon complementation with *pilY1* (Table 5.3).

Although *pilA* mRNA levels were found to be unchanged in a *pilY1* mutant (Bohn *et al.*, 2009), loss of *pilY1* also lead to a decrease in intracellular PilA expression levels (~62 ± 23.3% of wild type levels). We therefore complemented the *pilY1* mutant with *pilA* to ensure that the absence of surface piliation in the *pilY1* mutant was not due to decreased intracellular PilA levels. Upon complementation, the *pilY1* + *pilA* mutant was unable to assemble surface pili, despite increased levels of intracellular PilA (Figure 5.3), therefore its piliation defect does not arise from reduced levels of pilin.

	% relative to wild type						
Arabinose (%) / Antibody (anti-)	*pilT*	*pilY1*	*pilY1-pilT*	*pilY1+pilY1*		*pilY1-pilT +pilY1*	
	0	0	0	0	0.2	0	0.2
PilA	106	62	89	85	107	94	124
PilT	0	118	0	93	103	0	0
FimU	91	1075	1435	1181	509	1291	465
PilW	97	10	19	61	50	20	56
PilB	125	156	135	140	113	186	121
PilM	160	135	142	136	93	140	101
PilN	100	89	100	107	105	86	95
PilO	88	87	101	81	120	102	122
PilP	92	104	102	106	162	98	141
PilF	73	89	75	65	118	75	132
PilQ Monomers	123	142	155	115	133	147	90
PilQ Multimers	59	62	60	85	54	62	108

Table 5.3. Levels of pilus biogenesis proteins in mutants of interest

Standardized whole cell lysates of mPAO1, *pilT*, *pilY1*, *pilY1-pilT*, and complemented strains were grown in the absence or presence of arabinose (0.2%), were separated on 15% SDS-PAGE gels, and probed with anti-PilA, PilT, FimU, PilW, PilB, PilM, PilN, PilO, PilP, PilF or PilQ antibodies. Resulting Western blots were analyzed with Image J (Abramoff *et al.*, 2004), mean values of 3 independent experiments are shown.

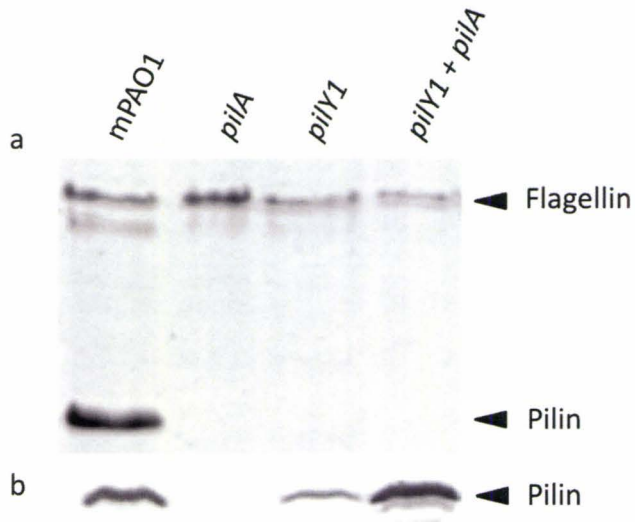

Figure 5.3. Overexpression of PilA does not restore surface piliation in a pilY1 mutant

(a) Surface pilin preparations of bacterial strains mPAO1, *pilY1*, *pilA*, *pilY1* and *pilY1* complemented with *pilA*, were run on a 15% SDS-PAGE gel and stained with Commassie brilliant-blue. (b) Standardized whole cell lysates of mPAO1, *pilY1*, *pilA*, *pilY1* and *pilY1* complemented with *pilA*, were run on a 15% SDS-PAGE gel, and transferred to nitrocellulose. Western blots were probed with an anti-PilA antibody.

To determine if the loss in surface piliation observed in the *pilY1* mutant was due to changes in ATPase levels such as decreased PilB (leading to loss of assembly) or increased PilT (leading to increased retraction), we analyzed PilB and PilT levels. Interestingly, an increased level of PilB was observed in the *pilY1* mutant (~156 ± 11.7% of wild type levels), which was restored to wild type levels upon complementation with *pilY1*, while PilT intracellular levels were unaffected (Table 5.3). Furthermore, the increase in PilB levels may be an affect of PilY1 on post-translational PilB stabilization, as no increase of gene expression was observed (Bohn *et al.*, 2009)

As PilY1 was previously shown to localize to membranes, likely outer membranes, during fractionation studies (Alm *et al.*, 1996), we analyzed levels of the outer membrane secretin PilQ and the PilF lipoprotein responsible for PilQ multimerization. Immunoblot analysis showed a decreased level of PilF in *pilY1* mutants (~89 ± 3.3% relative to wild type), which was restored to wild type levels upon complementation with *pilY1* (Table 5.3). Likewise, levels of PilQ multimers were decreased in the *pilY1* mutant (~62 ± 25% relative to wild type), and multimer levels were restored to ~85 ± 24% of wild type in the *pilY1* complemented mutant (Table 5.3). PilQ monomer levels were increased in a *pilY1* mutant, consistent with loss of multimerization, however, these results need to be replicated to ensure that the loss of multimerization of PilQ is significant. To determine whether the decreased level of PilQ multimers was due to the observed decrease in PilF, *pilY1* mutants were complemented with either PilF, or a non-lipidated PilF variant which

carries a single amino acid substitution from cysteine to alanine at residue 18 (Koo *et al.*, 2008), and personal communication, J. Koo). Complementation of the *pilY1* mutant with either *pilY1* or *pilF* restored multimer levels close to that of wild type (~85 ± 44 and 74 ± 36% respectively) while complementation with the *pilFC18A* resulted in only 40 ± 11% of wild type multimer levels. These data suggest that the loss of PilQ multimers observed in the *pilY1* mutant strain may be due to decreased PilF levels (Figure 5.4), however replication of these data is required to determine whether they are statistically significant.

The PilM/N/O/P proteins were shown to form an inner membrane complex that affects the stability of the outer membrane secretin (Ayers *et al.*, 2009), therefore we tested the abundance of these proteins in the absence of PilY1. PilM levels were increased in a *pilY1* mutant background (~135 ± 10% of wild type), while PilN and PilO levels were slightly decreased (~89 ± 2 and 87 ± 4.8% of wild type respectively, Table 5.3). Upon complementation with *pilY1*, PilM and PilN levels returned to wild type, however PilO levels increased to ~120 ± 12% that of wild type (Table 5.3). Loss of *pilY1* did not affect cellular levels of PilP, however, like PilO, PilP levels increased to ~162 ± 21 and 141 ± 6.7% respectively of wild type in the *pilY1* and *pilY1-pilT* mutants when they were complemented with *pilY1* in trans (Table 5.3).

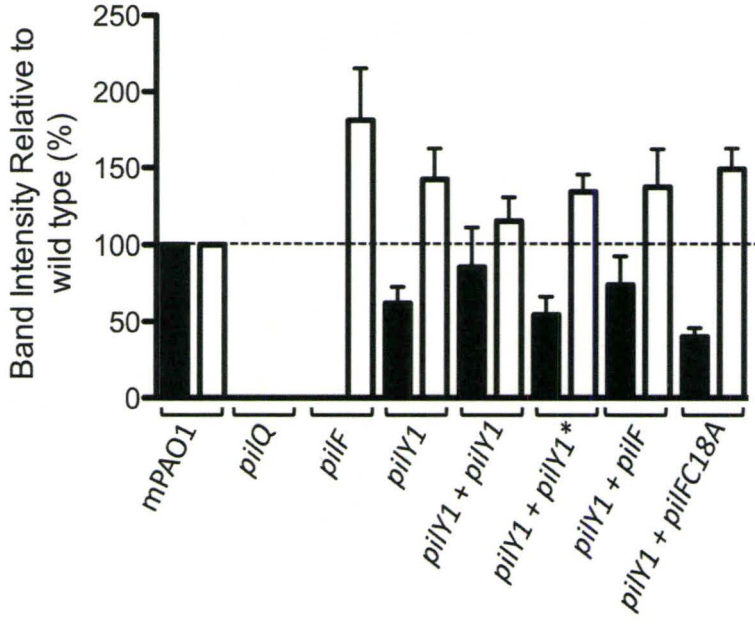

Figure 5.4. Complementation of PilF in a pilY1 mutant restores PilQ multimerization

Standardized whole cell lysates of mPAO1, *pilQ*, *pilF*, *pilY1* and *pilY1* complemented with either *pilY1*, *pilF* or a non-functional PilFC18A variant, were run on a 15% SDS-PAGE gel, and transferred to nitrocellulose. Western blots were probed with an anti-PilQ antibodies. Densitometries of western blots from four independent experiments were analyzed with Image J (Abramoff *et al.*, 2004). PilQ multimers are represented by black bars while monomers are represented by white bars.

Mutation of pilY1 alters cell morphology

During electron microscopy of *pilY1* mutants to assess their piliation status, we noted that relative to wild type cells, the *pilY1* mutants were shortened in their lengthwise dimension with wider central section, resembling an egg. However, under arabinose induction, the complemented *pilY1* mutant regained wild type cell shape, suggesting that the loss of *pilY1* contributes to this aberrant morphology (Figure 5.5 and Table 5.4). Interestingly, a mutant of the minor pilin operon response regulator, *algR*, showed a similar morphology, suggesting that the observed phenotype may be due to lack of *pilY1* expression. To see if any other members of the minor pilin operon affected cell shape, length and width of $n \geq 100$ randomly selected cells of *fimU*, *pilV*, *pilW*, *pilX*, and *pilE* mutants were measured using ImageJ (Abramoff *et al.*, 2004) and the length to width ratio determined (Table 5.4). A shortened length and the increased width similar to that observed for the *pilY1* mutants was observed for the *fimU*, *pilV*, *pilW*, and *pilX* mutants, however the *pilE* mutant retained wild type morphology (Table 5.4). Each of the minor pilins has been shown to be processed by the pre-pilin peptidase, PilD (Giltner *et al.*, 2010) and PilY1 has a putative PilD cleavage site (Lewenza *et al.*, 2005); therefore to determine whether PilD processing was required to maintain wild type cell shape, a *pilD* mutant was analyzed. The *pilD* mutant was truncated in length and increased in width compared to the wild type, suggesting that processing of the minor pilins is required for maintenance of wild type cell morphology.

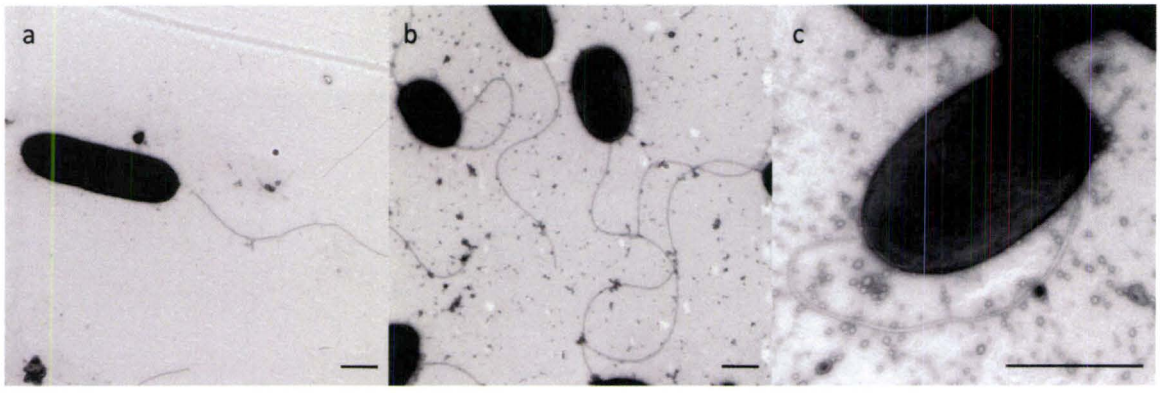

Figure 5.5. Loss of PilY1 alters cellular morphology

Electron micrographs of negatively-stained mPAO1 wild type (a) and mPAO1 *pilY1*::Tn (b and c) taken with a JEOL JEM 1200 TEMSCAN microscope (Peabody, MA, USA) operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA). AmtV600 software was used to capture digital images. Bars represent 100 nm.

Strain	Length	Diameter	Ratio L:D
PAO1	1.77 ± 0.40	0.52 ± 0.06	3.43
pilY1	1.48 ± 0.29	0.73 ± 0.08	2.06
pilY1 + pilY1	2.03 0.40	0.66 ± 0.08	3.11
algR	1.37 ± 0.24	0.54 ± 0.07	2.59
pilD	1.61 ± 0.28	0.69 ± 0.05	2.36
fimU	1.45 ± 0.26	0.58 ± 0.07	2.51
pilV	1.54 ± 0.30	0.62 ± 0.06	2.49
pilW	1.51 ± 0.28	0.56 ± 0.06	2.70
pilX	1.78 ± 0.30	0.66 ± 0.07	2.70
pilE	1.72 ± 0.33	0.59 ± 0.08	2.94

Table 5.4. Loss of PilY1 or any of the minor pilins alters cell shape morphology

Electron micrographs of negatively-stained mPAO1, *fimU*, *pilV*, *pilW*, *pilX*, *pilE*, *pilD*, *algR*, *pilY1* and *pilY1* complemented strains were taken with a JEOL JEM 1200 TEMSCAN microscope (Peabody, MA, USA) operating at an accelerating voltage of 80kV with a 4 Megapixel digital camera (AMT (Advanced Microscopy Techniques Corp.) Danvers, MA, USA) and measured for their length and width using Image J (Abramoff *et al.*, 2004). Mean cell length and diameter measurements of mPAO1, *pilY1*, *pilY1* + *pilY1*, *algR*, *fimU*, *pilV*, *pilW*, *pilX*, *pilE* strains where n ≥ 100 are represented in the table.

Antibiotic susceptibility profiles are altered in pilY1 mutants

As cell shape was altered in various minor pilin and *pilY1* mutants, we hypothesized that the properties of the cell envelope may be perturbed. A consequence of such a change could be altered antibiotic susceptibility profiles, as has been observed for other cell envelope mutants in our lab (Scheurwater, Matos and Burrows, unpublished data). Therefore, standardized cultures of mPAO1 and *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE* and *sltB1* (encoding a lytic transglycosylase whose absence alters *P. aeruginosa* resistance profiles; Scheurwater, Matos and Burrows, unpublished data) mutants were grown on Mueller-Hinton media in the presence of antibiotic disks of imipenem, carbenicillin, piperacillin, cefotaxime, ciprofloxacin or tobramycin (see Methods for concentrations). The antibiotic sensitivity profiles for the *fimU*, *pilV*, *pilW*, *pilX* and *pilE* mutants were similar to wild type. However, the *pilY1* mutant was completely resistant to cefotaxime at 5 μg but more susceptible to carbenicillin and ciprofloxacin compared with the wild type (Figures 5.6 and 5.7). Interestingly, the antibiotic profile of the *pilY1* mutant was identical to that of the *sltB1* lytic transglycosylase mutant (Figure 5.6; Scheurwater, Matos and Burrows, unpublished data).

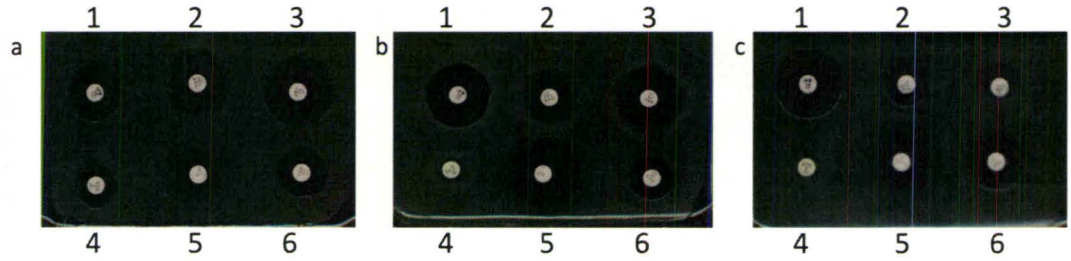

Figure 5.6. Loss of PilY1 affects antibiotic resistance profiles

Antibiotic profiles of mPAO1 (a) and *pilY1* mutant (b) against the β-lactams imipenem (1), carbenicillin (2), and piperacillin (3), the cephalosporin, cefotaxime (4), the fluoroquinolone, ciprofloxacin (5) and the aminoglycoside, tobramycin (6). The *pilY1* mutant appears to be more resistant to cefotaxime (4), but more sensitive to ciprofloxacin (5) compared to wild type, similar to what is seen in a peptidoglycan *sltB1* mutant (c).

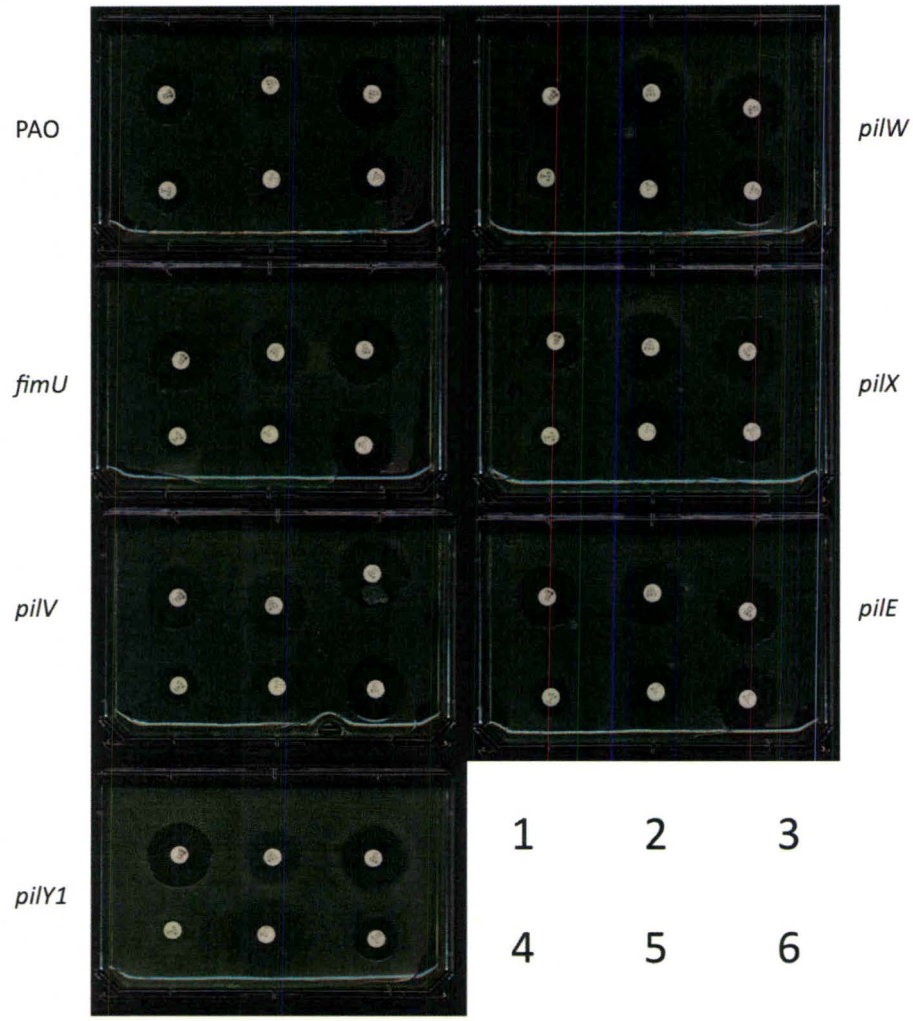

Figure 5.7. Loss of minor pilin retains wild type antibiotic resistance profile

Antibiotic profiles of mPAO1, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1* and *pilE* strains against the β-lactams imipenem (1), carbenicillin (2), and piperacillin (3), the cephalosporin, cefotaxime (4), the fluoroquinolone, ciprofloxacin (5) and the aminoglycoside, tobramycin (6).

V. DISCUSSION

PSORTb software predicts that PilY1 harbors a Sec-dependent signal sequence and that it localizes to the outer membrane and extracellular fractions (Gardy *et al.*, 2005). Concordant with these predictions, previous studies have shown that PilY1 from strain PAK is located in the insoluble membrane and soluble fractions (Alm *et al.*, 1996). More recent studies found PilY1 to be localized to extracellular vesicles in the *P. aeruginosa* strain PAO1, and to the cytosolic, inner membrane and supernatant fractions in the *P. aeruginosa* strain PA14, however PilY1 was not detected in outer membrane fractions in PA14 under native conditions (Bohn *et al.*, 2009; Kuchma *et al.*, 2010). Kuchma and colleagues also observed that PilY1 was not present in the supernatant fractions in the absence of PilA, suggesting that the loss of surface piliation impacts PilY1 localization (Kuchma *et al.*, 2010). In support of this observation, PilY1 was recently shown to localize to/with the pilus in strain PAK (Heiniger *et al.*, 2010). We observed that intracellular levels of PilA were reduced in an mPAO1 *pilY1* mutant, but that complementation of a *pilY1* mutant with *pilA* did not restore surface piliation. Heiniger and colleagues (Heiniger *et al.*, 2010) showed that intracellular pilin levels were not significantly different from wild type in PAK *pilY1* mutants although no surface pili were produced. Together these data show that while there may be strain-specific differences in the levels of intracellular pilin in *pilY1* mutants, the surface localization of PilY1 and PilA is mutually dependent.

Complementation of the *pilY1* mutant with its cognate gene resulted in a partial restoration of twitching motility (~70% of wild type). The mutant used for this work has a transposon insertion at position 1407 in the *pilY1* gene, and may therefore continue to express a competing but non-functional N-terminal fragment that prevents full restoration of twitching motility. Therefore, a full deletion mutant of *pilY1* may be required to allow complementation of wild-type twitching motility.

mRNA expression profiling of *pilY1* mutants showed previously that *fimU, pilV, pilW* and *pilX* were upregulated (Bohn *et al.*, 2009), suggesting that in addition to AlgR regulation, a positive feedback loop controls expression of the minor pilin operon. We showed previously that similar to the *pilY1* mutant, intracellular levels of FimU were increased in *pilV*, *pilW*, and *pilX* mutants, while intracellular levels of PilW were decreased in *fimU*, *pilV*, and *pilX* mutants (Giltner *et al.*, 2010). Here we saw by Western blot analysis a concordant increase in intracellular FimU levels in the *pilY1* mutant, however, PilW levels were decreased to ~10% relative to wild type (Table 5.3). The difference in *pilW* mRNA expression levels reported for *pilY1* mutants (Bohn *et al.*, 2009) and the observed intracellular PilW levels suggests potential post-translational regulation of PilW stability that may depend on the presence of PilY1.

Previously, we showed that overexpression of FimU or PilX increased protease secretion via the T2S system, and that loss of *pilY1* similarly increased secretion (Chapter 4). We hypothesized that the increase in secretion observed in the *pilY1* mutant was due to the resulting upregulation of the minor pilin operon

leading to increased FimU and PilX levels. This increased secretion phenotype may also explain the observed resistance to polymorphonuclear neutrophil killing by *pilY1* mutants (Bohn *et al.*, 2009). Furthermore, Bohn and colleagues observed that *pilY1* and *pilW* mutants had heightened persistence in murine airways (Bohn *et al.*, 2009). They believed that the phenotype of their *pilW* mutant was the result of its polar effect on expression of the downstream *pilY1* gene. Based on our data, it is formally possible that the enhanced fitness of the *pilY1* strain may be due specifically to the loss of *pilW* rather than *pilY1*, as intracellular levels of PilW are decreased in the *pilY1* mutant (Table 5.3).

Loss of *pilY1* led to increases in levels of proteins that would promote pilus assembly, such as PilB, while PilT levels remained constant (Table 5.3). This result implies the existence of a sophisticated regulation system, which may sense defects in twitching or pilus assembly, and subsequently upregulate components that could restore those processes. Recently, PilY1 has been shown to affect surface associated behaviors; in particular, overexpression of *pilY1* was shown to inhibit swarming motility in a wild type PA14 background (Kuchma *et al.*, 2010). However, PilY1 overexpression in the wild type background did not alter twitching motility (Kuchma *et al.*, 2010), which suggests that overexpression of PilY1 may preferentially select for twitching motility over swarming motility.

Western blot analyses showed that *pilY1* mutants appear to have fewer PilQ multimers and reduced levels of PilF (Table 1 and Figure 4). However, *pilY1* mutants are still able to make ~60% of wild type multimer levels, indicating that

decreased multimer formation is not the sole reason for loss of twitching motility. It was shown previously that untethering of PilF from the outer membrane through site directed mutagenesis of its lipidation site resulted in PilQ multimerization in both the inner and the outer membranes (Koo *et al.*, 2008). Therefore, we hypothesize that loss of PilY1 may prevent PilF localization to the outer membrane, and that the multimers observed in *pilY1* mutants may be localized to the inner membrane. This idea is particularly relevant in light of the altered cell shape and antibiotic resistance profiles observed in the *pilY1* mutant. Addition of a lytic transglycosylase inhibitor to *E. coli* cells was found to produce shortened cells (Reid *et al.*, 2004a; Reid *et al.*, 2004b). It was predicted that the 'stubby' phenotype was due to inhibition of peptidoglycan biosynthesis in the cylindrical portion of the cells, which requires the activity of lytic transglycosylases to generate breaks in the sacculus for insertion of new material (Reid *et al.*, 2004a; Reid *et al.*, 2004b). These data were particularly intriguing as we found *pilY1* mutants to have the same antibiotic sensitivity profile as the *P. aeruginosa* lytic transglycosylase *sltB1* mutant (Figure 5.6 and Scheurwater, Matos and Burrows, unpublished data). We therefore hypothesize that the altered cellular morphology in *pilY1* mutants may be due to a similar pertubation of lytic transglycosylase activity, or more generally, peptidoglycan metabolism; however, more studies need to be conducted to understand the link between the phenotypes of *pilY1* and lytic transglycosylase mutants.

PilY1 has been implicated in many pilus-independent aspects of *P. aeruginosa* physiology, including protein transport, altered secretion patterns (Chapter 4) and decreased susceptibility to neutrophil attack (Bohn *et al.*, 2009). We have shown that loss of PilY1 alters levels of a number of T4P gene products, particularly specific minor pilins, PilF and PilQ (Table 5.3 and Figure 5.4), and that PilY1 mutants have altered resistance and cellular morphology compared to wild type. We hypothesize that the translocation of PilY1 from the inner to the outer membrane may be dependent on the minor pilin proteins, as they have previously been shown to affect secretion of T2S-dependant exoproteins (Chapter 4), however more work needs to be done to elucidate the PilY1 mechanism of action.

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CHAPTER 6:

SUMMARY AND CONCLUSIONS

I. SUMMARY OF DISSERTATION AND DISCUSSION

This dissertation examines the function of the *P. aeruginosa* minor pilins and describes several new roles for these proteins including their incorporation into the pilus fibre and participation in secretion of exoproteins through the T2S system. The non-pilin like protein PilY1 was found to affect expression levels of several T4P biogenesis proteins, cell shape and resistance to specific antibiotics. Significantly, the major pilin cluster and the minor pilin operon were demonstrated to form a pathogenicity island, which is conserved amongst Pseudomonads. The newly elucidated roles of the minor pilins described in this work suggests a new model for type IV pilus and type II secretion pseudopilus biogenesis pathways.

The role of PilX

In a comprehensive study of over 300 *P. aeruginosa* strains from different sources, five phylogenetically distinct *pilA* alleles were identified (groups I-V) (Kus *et al.*, 2004). The major pilin component of the T4P (PilA) was found to vary in its amino acid sequence, where *P. aeruginosa* strains belonging to the same pilus group have more similar pilins (i.e. 75% similarity between PACS2 and PAO strains), while pilins from different groups have less similarity (i.e. 45% similarity between PAO1 and PA14 or C3719 strains) (Asikyan *et al.*, 2008). Although other pilus biogenesis machinery proteins (such as PilB and PilT) were shown to be highly conserved across *P. aeruginosa* groups implying that the assembly machinery can work with a

wide variety of pilins, cross-complementation between groups II and III did not restore full motility (Asikyan *et al.*, 2008).

Five pilin-like proteins were hypothesized to participate in group-specific recognition of the pilin allele as the sequence of these proteins varied between groups (Asikyan *et al.*, 2008). We found that with the exception of PilX, the group III minor pilins were able to complement twitching motility in group II minor pilin mutants, although not to wild type levels. Surprisingly, PilX, along with PilV, are the most highly conserved minor pilins across strains, with 75 % protein similarity between groups II and III (Asikyan *et al.*, 2008); therefore the lack of complementation by PilX was unexpected. Structural studies of the T2S PilX homologue, GspK, have shown an unusual alpha helical domain located between strands β2 and β3, which has been termed an arrowhead (Forest 2008; Korotkov *et al.*, 2008). Contact of this unique region with the secretin has been predicted to facilitate secretin opening and subsequent release of exoproteins in the T2SS (Forest 2008; Korotkov *et al.*, 2008); however how PilX achieves this remains unknown. Analysis of the group II and III PilX amino acid sequences show that while they are 58% identical, there are significant differences in the sequence. The sequential differences are particularly apparent between regions 97 to 142, where a number of non-polar residues in the mPAO1 sequence are replaced with polar charged residues in the PA14 sequence. For example consensus residues 136 to 139 glycine, threonine, leucine, tryptophan in mPAO1, are replaced with aspartate, lysine, aspartate, arginine in PA14. Additionally, the PA14 PilX sequence contains

an insertion of six amino acids, three of which are charged. Overall, the PA14 PilX sequence (between residues 97 to 139) appears to have a pattern of alternating charge, with a total of 5 positively charged and 4 negatively charged residues, while only 2 positively charged residues are present in the same region for mPAO1. These data suggest that the charged residues in the group III PilX may cause the observed strain-specific complementation data.

Further analysis of the highly conserved PilQ protein of strains mPAO1 and PA14 (94.8% identity), does show a small region (consensus residues 477-515) of disparity between the sequences. Concordant with the group III PilX sequence, alternating charges are also observed in the PA14 PilQ sequence between residues 494 and 514, where only two negative charges (glutamate and aspartate) are observed in the same region of mPAO1. These results indicate that the alternating charged group III PilX may interact with the alternating charges in the group III PilQ, while the two positively charged lysines in the group II PilX may interact with the two negative charges in the group II PilQ, to efficiently to open the pore.

The predicted structural similarities between PilX and its T2S homologues suggested that PilX might also play a role in the T2S system. PilX, along with the other minor pilin mutants, were found to be deficient in elastase and PlcH secretion (Chapter 4). Additionally, overexpression of PilX was found to substantially increase exoprotein secretion through the T2SS, suggesting that additional PilX may stimulate more pseudopili to open the secretin, thus resulting in an amplified release of exoproteins. Furthermore, PilX may be more readily able to substitute or

augment XcpX in secretion based on their conserved isoleucine substitution at the +5 position (Chapter 4). Although recent site directed mutagenesis studies of T2S exoproteins identified specific residues to be responsible for secretion, a consensus amino acid sequence for the selectivity of exoproteins in the T2SS is largely unknown (Mudrak *et al.*, 2010). If PilX is in fact responsible for secretin opening, the interaction appears to be quite specific, as cross complementation of the secretin between species, or even strains, prevents T2S secretion (de Groot *et al.*, 1991; Py *et al.*, 1991; Cooper *et al.*, 1993). This role for PilX as a strain-specific factor is supported by our cross complementation data, where an *algR*$_{III}$ mutant was able to twitch only upon complementation with the group II minor pilin operon and the group III PilX (Chapter 3). However, it would be interesting to determine whether the PilX T2S homologue, XcpX, was likewise strain specific. Together these data led us to propose a new model for both T4P and T2S systems.

The role of FimU

Like PilX, overexpression of FimU allowed for twitching motility, and substantially increased secretion of T2S exoproteins (Chapters 2, 3 and 4). However unlike PilX, cross complementation of group II or III *fimU* in a *fimU*$_{II}$ background restored twitching motility and surface piliation. We hypothesize that PilX makes contact with the secretin to open the pore, while FimU facilitates polymerization initiation events to build the pilus or pseudopilus. This hypothesis is supported by

our double knockout studies of minor pilin mutants in a *pilT* mutant background, where in the absence of *fimU*, *pilX* is able to open the pore and allow surface piliation, while in the absence of *pilX*, the pore does not open efficiently, and only basal levels of surface piliation are observed (Chapter 2 Figure 4).

Like PilX, FimU also appears to have a strain-specific role, as the affinity of $fimU_{III}$ for its group II interaction partners may be increased. This theory is supported by the observed reduction in twitching motility and the increase in surface piliation in the cross-complemented mutant, suggesting that it is able to assemble pili, but the difference in affinity of $FimU_{III}$ for its group II versus group III interaction partners may reduce the rate of pilus depolymerization, leaving more recoverable pili on the cell surface and reducing twitching motility (Chapter 3). The proposed model of the minor pseudopilin complex suggests that the FimU homologue (XcpU) is the last pseudopilin to be assembled into the complex, and that it directly contacts the major pseudopilin (XcpT) (Douzi *et al.*, 2009). If the T4P minor pilins form a similar complex, loss of $FimU_{II}$ may decrease the efficiency of subsequent $PilA_{II}$ polymerization, as $PilW_{II}$ (the proposed penultimate minor pilin in the complex) and $PilA_{II}$ may not interact efficiently. Therefore, in our cross-complementation experiments, $FimU_{III}$ may have altered affinity for either $PilA_{II}$ or $PilW_{II}$, although this concept has not yet been tested directly. We propose that FimU acts as an intermediate between the minor pilin complex and the major pilin, and therefore that changes in FimU levels or sequence affect the polymerization of the pilus (Chapters 2 and 3). The proposed interaction site between XcpU and XcpT is

within the highly conserved N-terminal domains of each protein (Douzi *et al.*, 2009). Alignment of the N-terminal domains of group II and group III FimU reveals four significant changes in amino acid composition in the first 41 amino acid prepillin residues; particularly, these residues are clustered between residues 25 and 41. A non-polar residue in the group III FimU was replaced with a polar residue in the group II FimU at positions 35 and 37, while a polar residue (group III) was replaced with a charged residue (group II) at position 34. Finally, a positively charged arginine (group III), was replaced by a negatively charged glutamic acid (group II) at position 41. The local clustering of these amino acid substitutions suggests that the general change from non-polar to polar charged groups between the FimU proteins of groups III and II could account for the hypothesized change in affinity between FimU and PilA (Chapter 3).

The role of PilV, PilW and PilE

For most motility and secretion phenotypes tested, *pilV* and *pilW* mutants behaved in an identical fashion, with the exception of exotoxin A secretion (Chapter 4), where *pilV* but not *pilW* mutants were able to secrete exotoxin A. PilV and PilW appear to form a functional complex at a 1:1 ratio, since in general their co-expression *in trans* is able to restore secretion phenotypes. Both the levels and stoichiometry of these proteins appear to play an important role in the T4P and the T2S systems, as twitching motility and secretion can only be restored by low protein

expression levels, while overexpression of either of these proteins abolishes twitching motility and secretion of exproteins (Chapters 2 and 4). Homologues of PilV (EspI) and PilW (EspJ) were co-crystallized as a heterodimer (Yanez *et al.*, 2007), therefore the notion of a 1:1 ratio of PilV to PilW is not unprecedented. In context of the proposed minor pseudopilin complex, PilW and PilV homologues are predicted to fit between PilX (top of the complex) and FimU (bottom of the complex) (Chapter 1 Figure 8), suggesting that overexpression of either PilV or PilW may titrate away interacting components to prevent FimU and PilX function. Interestingly, the PilW orthologue PilJ was shown to be incorporated independently of the other minor pilins, as PilJ was detected in the purified pilin preparation of each of the minor pilin mutants (Winther-Larsen *et al.*, 2005). Douzi and collegues (Douzi *et al.*, 2009) have shown that integration of the pseudopilins into the complex requires a highly ordered incorporation starting with the tertiary complex of XcpW-V-X, followed by XcpU via interaction with XcpW. Based on these results, we hypothesize that PilW is the first to enter the pseudopilus. We have previously shown that surface piliation is seen in the absence of FimU (Chapter 2 Figure 4), therefore if FimU is the terminal minor pilin, these data suggest that PilW is sufficient for sub-optimal pilus assembly. If PilW is the first minor pilin to be incorporated into the pilus it would also explain the observation that PilW appears in the pilus in a ratio consistent with the amount of isolated pili, while levels of the other minor pilins are not changed with increased piliation (Chapter 2 Figure 5).

Phenotypes of PilE were similar to those of PilV and PilW, however lack of a PilE Xcp homologue makes it difficult to speculate about the role of this protein in the context of the minor pilin complex. Like PilV and PilW, overexpression of PilE is detrimental to both twitching motility and secretion of T2 exoproteins (Chapters 2 and 4), suggesting that PilE may support the role of FimU and PilX in a similar fashion. Although we know that it is found in the pilus, how PilE fits into the minor pilin complex remains unknown, therefore interaction studies to determine what proteins contact PilE will be the focus of future studies.

The role of PilY1

PilY1 is a 128 kDa multifunctional protein that has been shown to function in pilus biogenesis, secretion of the lipase enzyme LipC, phenazines and quinolones, and play roles in adherence to human epithelia, resistance to killing by polymorphonuclear cells, and indirectly affects cyclic-di-GMP levels (Bohn *et al.*, 2009; Heiniger *et al.*, 2010; Kuchma *et al.*, 2010). Due to ~43% C-terminal similarity to the *Neisseria gonorrhoeae* pilus tip-associated adhesion protein PilC2 (Alm *et al.*, 1996; Scheuerpflug *et al.*, 1999), PilY1 was initially predicted to function as the pilus adhesin. However until recently, PilY1 was not observed to be localized to the pilus (Alm *et al.*, 1996; Bohn *et al.*, 2009; Heiniger *et al.*, 2010). Heiniger and colleagues have now shown PilY1 to be present in sheared surface protein preparations, and to be required for attachment to epithelial cells (Heiniger *et al.*, 2010); furthermore,

the role of PilY1 in pilus biogenesis has been proposed to counteract pilus retraction when it is in the calcium bound state (Orans *et al.*, 2009). We therefore analyzed intracellular levels of 11 T4P biogenesis proteins and observed a marked decrease in both PilQ multimerization and intracellular PilF expression in a *pilY1* mutant, suggesting that PilY1 may directly or indirectly facilitate PilQ multimerization.

Electron microscopy and membrane fractionation data have led to the idea that PilY1 is secreted into the extracellular milieu (Alm *et al.*, 1996; Bohn *et al.*, 2009; Kuchma *et al.*, 2010). These data are particularly interesting based on our findings that secretion was increased in a *pilY1* mutant, while its overexpression inhibited secretion (Chapter 4). The upregulation of FimU and PilX in the *pilY1* mutant may be responsible for increased secretion, as overexpression of either protein has the same phenotype (Chapter 4). This idea is consistent with the observation *pilY1* mutants had increased polymorphonuclear neutrophil killing (Bohn *et al.*, 2009). It would therefore be interesting to determine if the complemented *fimU* and *pilX* mutants were likewise increased in polymorphonuclear neutrophil killing. Previously we have seen that expression levels of FimU, PilW and PilE are altered in minor pilin mutants (Giltner *et al.*, 2010) consistent with the phenotype of the *pilY1* mutant. Based on some of the similarities in phenotype between minor pilin and *pilY1* mutants, we hypothesized that the minor pilins may be involved in secretion of PilY1; however, more work needs to be done to fully elucidate the role of PilY1.

New model for T4P and T2S systems

The current model for pilus assembly involves the co-translation of prepilin across the inner membrane to the periplasmic face along the signal recognition particle (SRP) targeting route of the Sec-dependent pathway (Arts *et al.*, 2007). As the PilA prepilin subunits are predisposed to self-association due to their highly hydrophobic termini (Pugsley,1996; Audette *et al.*, 2004b), co-translation of the pilin-SRP complex likely prevents premature pilin aggregation (Arts *et al.*, 2007). Following Sec translocation, prepilins are both cleaved and methylated by PilD (Bally *et al.*, 1991; Strom *et al.*, 1991a) before polymerization into the pilus fibre through the PilQ secretin. Prior to our work, the pilus fibre was thought to be largely, if not entirely, composed of PilA.

The predicted structural similarity of the major and the minor pilins suggested that the minor pilins would be processed by PilD in a similar manner. Indeed, all the minor pilins were shown to contain a putative Sec-dependent signal sequence and be processed by PilD (Lewenza *et al.*, 2005; Giltner *et al.*, 2010). Immunogold labeling showed that the minor pilins are localized to the pilus, requiring revision of the model of pilus assembly to one in which the minor pilins are integral components of the fibre and incorporated along its length. This suggests that the minor pilins may facilitate diversity of the pilus, similar to pilus modifications (Kus *et al.*, 2008). Furthermore, we have shown that PilX likely contacts the secretin to facilitate opening and extrusion of the pilus fibre, while

FimU likely aids in polymerization of the major pilin subunit by acting as an intermediate between the minor pilin complex and PilA.

II. FUTURE DIRECTIONS

Generation of chromosomally FLAG-tagged Phosholipase C

The loss of minor pilins FimU, PilV, PilW, PilX, and PilE and PilY1 decreased the zone of clearing on blood plates relative to wild type levels (Chapter 4, Figure 8). To ensure the observed zone of clearing was due to the loss of PlcH we need to generate a method to detect native levels of PlcH. Therefore, to determine the relative amount of PlcH in the intracellular versus extracellular fractions, a chromosomally FLAG-tagged PlcH will be engineered in the mPAO1, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE*, *fimU-pilQ*, and *pilX-pilQ* strains. Western blot analysis of 22 hr cultures will be probed with an anti-FLAG antibody to determine whether both the wild type and the minor pilin mutants are able to secrete PlcH.

Secretion of PilY1

To determine whether the minor pilins are required for PilY1 export, we sought to determine the location of PilY1 in the minor pilin mutants. Anti-PilY1 sera has been requested from the Wolfgang laboratory for the purpose of PilY1 identification. They showed that deletion of the *fimU-pilE* genes prevented surface

exposure of PilY1 expressed *in trans*, but did not report the mechanistic basis for this observation (Heiniger *et al.*, 2010). Whole cells of mPAO1 and *fimU*, *pilV*, *pilW*, *pilX*, *pilY1* and *pilE* mutants will be washed in buffer to remove any PilY1 located in the supernatant, and bacterial suspensions will be directly spotted onto nitrocellulose membranes. The dot blot will be probed with anti-PilY1 antibody to assess the presence of PilY1 in the outer membrane. Supernatant from these strains will be concentrated and probed for PilY1, as previous reports suggest PilY1 can be detected with a 300 fold concentration of the supernatant (Kuchma *et al.*, 2010). If the minor pilins are involved in PilY1 secretion than PilY1 will not be detected in the supernatant or cell associated fractions, however if the minor pilins are not involved in PilY1 secretion, PilY1 will be detected in both fractions.

Localization of PilF in a PilY1 mutant

The lipoprotein PilF was previously shown to be required for the localization and multimerization of PilQ (Koo *et al.*, 2008). Mutagenesis of the putative lipidation site was found to delocalize PilF and lead to PilQ multimer formation in both the inner and outer membranes (Koo *et al.*, 2008). Loss of PilY1 abrogated twitching motility, however the observed reduction in PilQ multimer formation was not large enough to account for the lack of motility (Chapter 5). As intracellular levels of both PilF and PilQ multimers are decreased in a *pilY1* mutant, we will determine whether the outer membrane localization of PilF is aberrant in a *pilY1*

mutant background by using sucrose gradients fractionate inner and outer membranes of mPAO1, *pilF*, *pilQ*, and *pilY1* strains as described previously (Koo *et al.*, 2008). Fractions will be run a 15 % SDS-PAGE gel and probed with anti-PilF and anti-PilQ antisera. If PilY1 is required for PilF localization, than PilQ multimers will be detected only in the inner membrane fraction in a *pilY1* mutant background, however if PilY1 is not required for localization of PilF, multimers will be detected only in the outer membrane.

Interaction partners of the minor pilins

For a more detailed understanding of how the minor pilins are assembled into the T4P and their role in the T2SS, their interacting components need to be elucidated. N-terminally truncated minor pilin constructs with C-terminal cleavable histidine tags are already in hand, therefore these constructs will be used to purify each of the minor pilins FimU, PilV, PilW, PilX, and PilE. Interacting partners and their hierarchal assembly into the minor pilin complex can be elucidated through dynamic light scattering methods. A systematic analysis of the minor pilins will be accomplished by initially running predicted interacting partners together over a nickel column. For example, as PilW (EspJ) and PilV (EspI) homologues were shown to form a stable heterodimer (Yanez *et al.*, 2007), PilW-His and PilV (cleaved of the histidine tag) will be run over a Ni-affinity column. A shift in the elution time of the PilW-His + PilV combination compared to the elution time of PilW-His alone would

indicate an interaction between these proteins. In turn, each of the purified truncated minor pilin proteins would likewise be run over a Ni-affinity column to determine their interacting partners, or groups. Based on these data, the truncated minor pilins may then be used in crystallization, and co-crystallization studies, to further elucidate the configuration of the complex.

III. SIGNIFICANCE AND CONCLUSIONS

T4P are essential virulence factors for the opportunistic pathogen *P. aeruginosa*, as non-piliated strains were shown to have reduced virulence in mice models, and *pilT* and *pilU* mutants were not capable of dissemination (Farinha *et al.*, 1994; Zolfaghar *et al.*, 2003; Alarcon *et al.*, 2009a; Alarcon *et al.*, 2009b; Heiniger *et al.*, 2010). These highly conserved structures are therefore essential virulence factors, which play a role in attachment to surfaces, and development of biofilms (Saiman *et al.*, 1990; O'Toole *et al.*, 1998; Chiang *et al.*, 2003; Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; Giltner *et al.*, 2006). The surface exposure of the pilus makes it an ideal candidate for vaccine development, however it is essential to understand all the components that make up the pilus fibre. This work has characterized 5 pilin-like proteins of unknown function, showing that are incorporated into the pilus fibre, and are essential for both surface piliation and secretion of T2S products (Chapters 2 and 4). Therefore targeting of these proteins with small molecule inhibitors would simultaneously target two virulence systems at once.

Furthermore, the minor pilins' sequences are more conserved across species compared to PilA, making these ideal candidates for vaccine development.

In addition to furthering our understanding of the assembly and composition of the pilus fibre, we have contributed to an enhanced understanding of the evolutionary history of the acquisition of these essential virulence factors. We have shown that the major pilin cluster and the minor pilin operon were likely part of a single pathogenicity island acquired through horizontal gene transfer, and that this genetic arrangement is conserved across *P. aeruginosa* strains and other Pseudomonads (Chapter 3). Understanding the composition and potential for acquisition of genetic elements encoding virulence factors are essential tools for the goal of treatment of disease caused this ubiquitous opportunistic pathogen.

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