Role of XcpT in Type IV pili of *Pseudomonas aeruginosa*

THE *PSEUDOMONAS AERUGINOSA* MAJOR PSEUDOPILIN XCPT IS INCORPORATED INTO THE TYPE IV PILUS UNDER NATIVE CONDITIONS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2011) Hamilton, Ontario

TITLE: *Pseudomonas aeruginosa* major pseudopilin XcpT is incorporated into the Type IV pilus under native conditions

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NUMBER OF PAGES: x, 92

Abstract

Retractable surface appendages Type IV pili (T4P) are one of the major virulence determinants in the opportunistic pathogen *Pseudomonas aeruginosa* (Pa), that is the leading cause of mortality in CF patients. T4P are heteropolymers composed of the major-pilin subunit PilA and the less-abundant minor pilins (MPs), FimU/PilV/W/X/E. Pilins share high sequence and structural similarity with pseudopilins (XcpT/U/V/W/X), that are proposed to form a periplasmic-structure in the evolutionarily related Type II secretion system (T2SS). Similar to T4P system, the T2SS is a multi-subunit complex that spans the inner (IM) and the outer (OM) membranes. It involves a two-step process facilitating the secretion of toxins into the extracellular milieu from the periplasm.

Using immunogold TEM analysis and Western blot we identified, under native conditions, the major pseudopilin of T2SS XcpT, is incorporated into the T4P appendage, thus appearing on the surface. This is in contrast to previous studies reporting, the otherwise periplasmic structure, the pseudopilus appears on the surface only upon over-expression of XcpT. Further, we identified this incorporation is strictly dependent on PilA expression, such that levels of surface-XcpT co-varied with the levels of surface-PilA. However, XcpT incorporation into the T4P fiber did not affect T4P-mediated twitching motility or T2SS-mediated elastase secretion. Based on these observations we proposed two explanations. Firstly, given the similarity between XcpT and type IV pilins, it is possible the pseudopilin is recognized by the T4P machinery and therefore is incorporated into the pilus. Secondly, since XcpT incorporation does not affect T4P-mediated motility, it may affect other properties of T4P, such adherence during biofilm formation,

previously associated with surface-exposed pseudopilus. In addition, we also identified enhanced expression of *fimU* and *pilX* MPs drastically increased elastase secretion, through a yet to be discovered mechanism. Regardless, our results present an alternative role of both minor pilins and XcpT in their non-native systems suggesting there is more overlap between the T4P and T2S systems than previously appreciated. Further exploration of this overlap will aid in the study of the two systems in Pa, as well as in other pathogens.

Acknowledgements

I would like to express my appreciation to my supervisor Dr. Lori Burrows for giving me the opportunity to be part of her team, for her expertise, time and most importantly for her patience. It was truly an honor and a privilege to work with her on this memorable journey that started with tentative steps but ended with an experience of lifetime. I would also like to thank my committee members Dr. Murray Junop and Dr. Eric Brown for their constructive comments, continuous support and encouragement.

I would like to acknowledge Dr. Carmen Giltner for being a great mentor/tutor, for providing assistance with lab techniques, for critiquing my work and most importantly for establishing the grounds for my project. A very special thanks to Herlinder for being a great friend, a helpful peer, for her continuous support, her valuable comments and for those insightful discussions. I would like to extend my sincerest thanks to Sarra, Ylan, Edie, Uyen and Anthony for making this a fun and an enjoyable learning experience.

The most special thanks goes to my family for their unconditional support, love and advice throughout my studies.

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List of Abbreviations and symbols

Abbreviations	Meaning
Ara	L-arabinose
ATP	Adenosine-5'-triphosphate
°C	degree Celsius
DSL	Disulfide bonded-loop
E/C	Extracellular fraction
Fig	Figure
Grp(s)	Group(s)
h(s)	Hour(s)
I/C	Intracellular fraction
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
L	Liter(s)
LB	Luria-Bertani media
µg	Microgram(s)
µL	Microliter(s)
mL	Milliliter(s)
MPs	Minor pilins
min	Minute(s)
NP	*pilA* mutant
PBS	Phosphate buffered saline
OM	Outer membrane
Pa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
s	Second(s)
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T2S	Type II secretion
T2SS	Type II secretion system
T4P	Type IV pilus
TEM	Transmission electron microscopy
WT	Wild-type

CHAPTER 1 - INTRODUCTION

Introduction

Pathogenic bacteria have complex organelles that confer the ability to establish infection in the host (Gooderham & Hancock, 2009). The Type IV pilus (T4P) and Type II secretion (T2S) systems are two key virulence determinants of *Pseudomonas aeruginosa* (Pa) that allow the bacteria to colonize and establish infection in the host. Pa is an opportunistic pathogen that infects individuals with compromised immune systems, such as patients undergoing chemotherapy, HIV-positive individuals or pregnant women (Stover et al, 2000). It is one of the most common pathogens found in the lungs of CF patients, causing chronic infections that are largely responsible for morbidity and mortality (Fridkin SK, 1999). Its ability to thrive in range of environments and to cause cross infections in hospitals and clinics make it the third most common nocosomal pathogen in North America (Ikeno T, 2007; Stover et al, 2000).

Pa infections are difficult to treat due to its large array of defense mechanisms, such as efflux pumps, partial impermeability of the outer membrane (OM), and production of β-lactamases and aminoglycoside-modifying enzymes, which protect the organism from antimicrobial agents (Strateva & Yordanov, 2009). Most Pa infections proceed in three stages, colonization, invasion and disease dissemination. Colonization begins with attachment to the host epithelial cells, which is mediated by flagella, adhesion proteins and primarily by retractable surface appendages called Type IV pili (T4P) (Mattick, 2002). During colonization, the bacteria employ several virulence factors to

evade the host immune system, altering signaling pathways to develop a niche for growth. Pa has several secretion systems that release virulence factors in the extracellular milieu or directly into the host (Bleves et al, 2010). T2SS, also known as the general secretory pathway (GSP) is highly conserved among gram-negative pathogens and involved in the transport of toxins and hydrolases from the periplasm to the cell exterior (Filloux et al, 1998). Structural and functional similarity between components of T4P and T2S system suggest a common evolutionary origin (Ayers et al, 2010).

1.1 The Type IV pilus system

T4P are surface appendages expressed in gram-negative and some gram-positive bacteria. They serve as a major virulence determinant in various pathogenic bacteria, such as *Neisseria gonorrhoeae, Moraxella bovis, Dichelobacter nodosus* and Pa (Mattick, 2002). In addition to surface adhesion, other functions of the T4P include DNA uptake, cell signaling, phage binding, biofilm formation, signal transduction and motility (Craig et al, 2004; Merz et al, 2000). Studies done by Chi et al (1991) and Farinha et al (1994) showed that bacterial colonization and virulence are greatly reduced in the absence of T4P (E Chi, 1991; Farinha et al, 1994). To develop therapeutic agents targeting the initial stage of Pa infection, it is crucial to understand the structural and functional organization of the T4P system. Pa T4P are long fibers composed of the major subunit PilA and low abundance minor pilins (MPs) FimU/PilV/W/X/E. A recent study by Giltner *et al.,* (2010) proposed that FimU and PilX act as the key initiators of pilus assembly, such that when overexpressed they may create multiple initiation sites for pilus assembly that share

the limited number of PilA monomers in the cell, resulting in shorter pili. Overexpression of PilV/W/E possibly affects formation of a stable initiation complex, preventing proper function of FimU or PilX. Based on these observations, it was suggested these five MPs form an initiation complex that facilitates assembly, but which is dispensable if retraction is blocked, trapping abnormal pili on the cell surface.

The regulation, assembly and function of the T4P in Pa requires ~50 gene products (Whitchurch et al, 1991). The pilins, transported to the inner membrane through the Sec pathway are cleaved and methylated by a prepilin peptidase PilD, that is central to both the T4P and T2S systems (Nunn et al, 1990; Nunn & Lory, 1991) (Fig 1). Subsequently, they are assembled into a heteropolymer with the aid of the inner-membrane complex (PilC/M/N/O/P/FimV) and cytoplasmic ATPases, PilB/PilT/PilU (Ayers et al, 2009; Coil & Anne, 2010; Nunn et al, 1990; Wehbi et al, 2010; Whitchurch et al, 1991). PilB energizes the assembly process while retraction is facilitated by PilT and potentially PilU, a PilT paralogue (Whitchurch & Mattick, 1994). The assembled heteropolymer exits the cell through the outer membrane (OM) secretin, PilQ that is potentially stabilized by PilF (Koo et al, 2008; Martin et al, 1993).

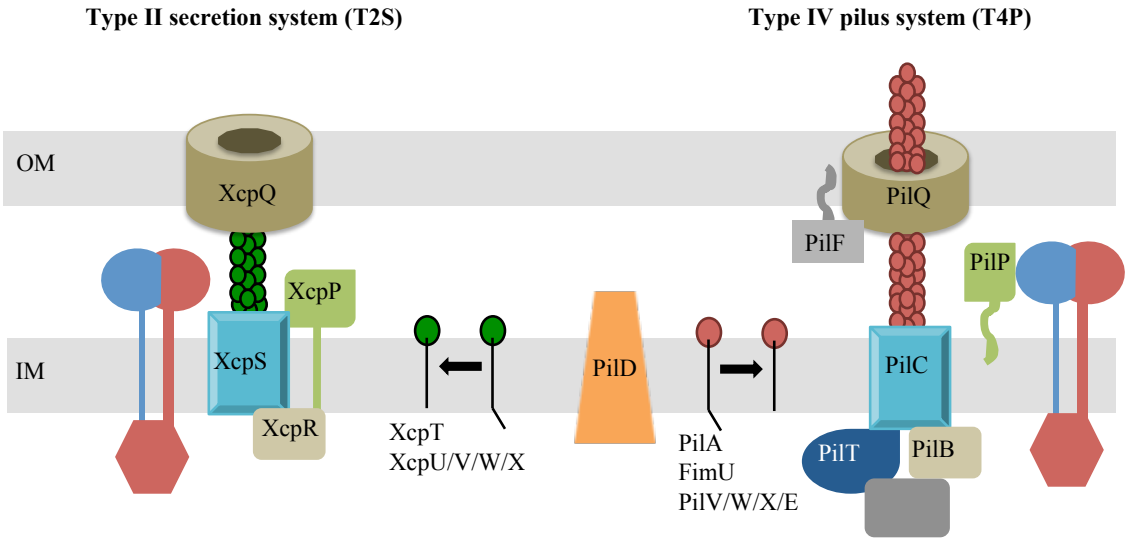

Fig 1: T4P and T2S systems share homologous components. T4P and T2S systems are multi-subunit complexes, which span the IM and the OM. Major subunit PilA and minor pilins of T4P share N-terminal sequence homology with the T2S pseudopilins (XcpT-X). Processing of pilins and pseudopilins by shared enzyme PilD is indispensible for their function. Both systems consist of an inner membrane complex (red, blue and green) that potentially stabilizes other components of the system, and a platform protein PilC (XcpS). The OM secretin PilQ (XcpQ) provides a channel for exoprotein or pilus translocation across the OM in T2SS or T4P respectively. PilB and PilT mediate pilus extension and retraction respectively. On the contrary, T2S lacks a retraction ATPase and XcpR facilitates assembly.

1.2 Type II secretion system (T2SS)

The T2SS is a multi-protein complex that spans the inner and the outer membrane of gram-negative bacteria (Fig 1). Secretion through the T2SS is a two- step process. In the first step, the proteins are transported to the periplasm through the Sec or Tat pathways, followed by T2SS-mediated translocation across the OM into the cell exterior (Filloux, 2004; Voulhoux et al, 2001).

The T2SS was first identified in the pathogen *Klebsiella oxytoca*, responsible for the secretion of pullulanase (d'Enfert et al, 1987). It is also found in various plant and animal pathogens, including *Vibrio cholerae*, *Erwinia chrysanthemi, Xantomonas campestris* and *L. pneumophila* (de Groot et al, 1991; He et al, 1991; Hu et al, 1992; Liles et al, 1998; M Sandkvist, 1997). In Pa, two functional T2SSs have been defined: the Xcp system that secretes exotoxin A, lipases, phospholipase C, alkaline phosphatase or elastase (LasB), and the Hxc (**H**omologous to **X**cp **C**omplex) that allows the secretion of low-molecular-weight alkaline phosphatase LapA (Ball et al, 2002; Tommassen, 1992).

T2SS is composed of at least 12 gene products and several of these are homologous to components of the T4P system (Hobbs & Mattick, 1993) (Fig 1). XcpQ, homologue of PilQ, is an outer membrane secretin that allows translocation of exoproteins across the OM (Bitter et al, 1998). Unlike the T4P, the T2SS only has a building ATPase (XcpR, a PilB homologue) and lacks a retraction ATPase (PilT in the T4P system). As a result, the mechanism underlying the T2SS retraction is not yet clear.

One of the major components of the T2SS is a group of five proteins (XcpT-X) that are termed pseudopilins based on their structural and functional similarity to the Type

IV pilins (Nunn & Lory, 1992) (Fig 1). Pilins and pseudopilins are inner membrane proteins that are transported to the IM via the Sec pathway (Arts et al, 2007). The most abundant of these proteins is XcpT, a homologue of PilA (22% identity), while the less abundant pseudopilins XcpU, XcpV, XcpW and XcpX are homologous to the Type IV MPs FimU, PilV, PilW and PilX respectively. Like Type IV pilins, the pseudopilins have an amphipathic N-terminal α-helix that anchors the periplasmic C-terminal globular domain to the inner membrane. The C-terminal domain consists of an αβ-loop and β-sheet domain but lacks the disulfide bond loop (DSL), characteristic of the Type IV pilins (Alphonse et al, 2010). The DSL region has been implicated in mediating Pa adhesion to host cells and abiotic surfaces (Giltner et al, 2006; Lee et al, 1994; Sheth et al, 1995). Instead, the C-terminus of the pseudopilins is stabilized by a calcium-binding site (Korotkov et al, 2009)

In addition, like PilA and MPs, the pseudopilins are the substrates of an inner membrane prepilin peptidase PilD/XcpA, that cleaves a short, basic N-terminal leader peptide (Fig 2) (Nunn & Lory, 1991). Based on these characteristics, the pseudopilins were proposed to form a short, pilus-like structure, the 'pseudopilus', which is only long enough to span the periplasm. Studies in Pa, *K. oxytoca* and *X. campestris* showed that overproduction of the major pseudopilin (XcpT, PulG or XpsG, respectively) results in the assembly of a pseudopilus on the cell surface, providing support for the pilin-like properties of pseudopilins (Durand et al, 2003; Nien-Tai HU, 2002; Sauvonnet et al, 2000). In the two current models for T2SS, the pseudopilus is proposed to remain in the periplasm under native conditions, and either act as a plug, regulating the toxin passage

through XcpQ, or as a piston undergoing rapid retraction/assembly to pushing substrates through XcpQ (Filloux et al, 1998).

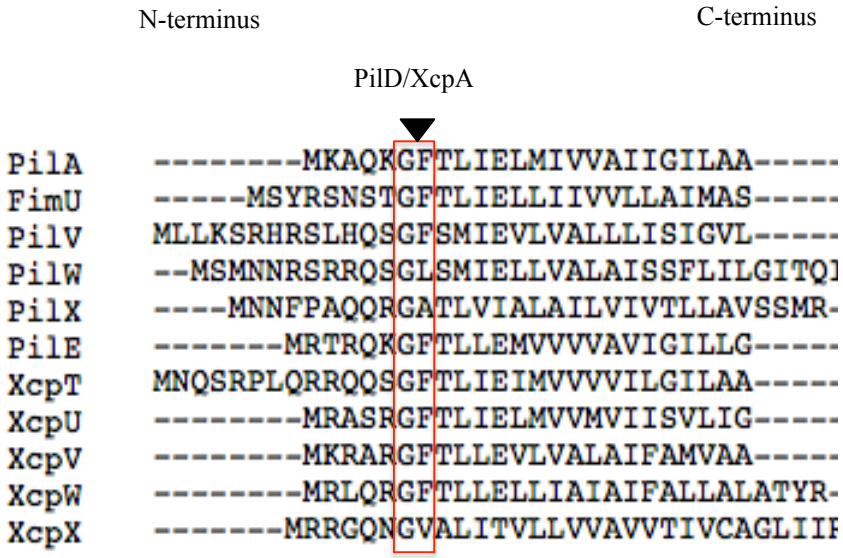

Fig 2: N-terminus sequence alignment of major, minor pilins and pseudopilins: Type IVa pili of Pa is composed of major subunit PilA and minor pilins (FimU/PilV/W/X/E). Homologues of these proteins, called pseudopilins (XcpT-X) are found in the evolutionary conserved T2SS, where they assemble into a putative-periplasmic structure, named the pseudopilus. Sequence homology between the pilins and pseudopilins is limited to the hydrophobic N-terminal α-helical leader sequence that is processed by prepilin peptidase (PilD/XcpA), central to both the T2S and T4P systems.

1.3 Significant findings

Structural and functional similarities between the T4P and T2S systems are suggestive of potential interactions between their components. The ability of major pseudopilins XcpT and PulG of Pa and *K. oxytoca* respectively to polymerize into a pilus-

like fiber supports the idea that they are functionally similar to the Type IV pilins (Jelsbak & Kaiser, 2005; Sauvonnet et al, 2000; Vignon et al, 2003). Further, cross-linking studies in Pa showed that PilA forms heterodimers with XcpT and the minor pseudopilins XcpU/V/W/X, indicating the pilins and pseudopilins have compatible structural features that allow them to interact (Lu et al, 1997). Using immunogold electron microscopy, Sauvonnet et al., (2000) showed that the *E.coli* K-12 pilin protein PpdD is able to assemble into a pilus-like structure using the Pul secretion apparatus when both were expressed in *E. coli*, indicating that interactions between the pilins and T2S system are structurally feasible (Sauvonnet et al, 2000).

In our study, we investigated the possible association between the T4P and the pseudopilin XcpT. In contrast to the results of earlier studies, XcpT was shown to be present on the cell surface under native conditions. The T4P major pilin, PilA was shown to be required for XcpT surface expression, such that changes in T4P surface piliation were reflected in the levels of XcpT on the surface. However, levels of secretion in a *pilA* mutant were comparable to the positive control, suggesting XcpT surface expression under native conditions is independent of the T2SS.

Subsequent TEM and immunogold studies showed that XcpT is incorporated into the pilus fiber, however this affected neither T4P assembly nor function. In addition, the T2SS minor pseudopilin XcpV reduced XcpT and PilA levels on the surface, while the minor pseudopilin XcpX increased them. However, changes in surface piliation did not affect twitching motility, suggesting that the absolute amount of surface pili is poorly correlated with levels of motility, as shown previously (Giltner et al, 2010a; Harvey et al,

2009). Lastly, XcpR, the T2S polymerizing ATPase was shown to be required for native levels of XcpT surface assembly, but to be dispensable for surface expression in a retraction-deficient strain (*pilT::*FRT). These data suggest that both T4P and T2S components govern the assembly of XcpT into the T4P fiber. Since this association affects the function of neither system, the biological purpose of XcpT incorporation is not yet clear. In brief, our data suggests there is more cross-talk between the two systems than previously thought and that studying these shared mechanisms will further our understanding of the two systems. Further, given that the surface appendages are good targets for vaccine development, the identification of a novel but highly conserved subunit XcpT of T4P fiber will aid in the development of broad-spectrum drugs.

Chapter 2: Material and Methods

2.1 Bacterial strains, genetic manipulations and growth conditions

Bacterial strains used for this study are listed in Table 1. *Pseudomonas aeruginosa* strains were grown on Luria-Bertani (LB) agar plates supplemented with 30 mg/L gentamicin or 200 mg/L kanamycin or 200 mg/L carbencillin where indicated. *E. coli* strains were grown on LB supplemented with 15mg/L gentamicin or 100 mg/L kanamycin or 100 mg/L ampicillin. All strains were grown at 37°C for the time indicated.

Generation of *xcpR*::Tn/*pilT*::FRT knockout :

Biparental mating assay was employed to generate double knockouts as described previously (Burrows et al, 2000). *E.coli* SM10 cells were electroporated with the plasmid pEX18Ap + *pilT::GmFRT* and grown on LB agar plates supplemented with gentamicin (30 mg/L) (Simon et al, 1983). Subsequently, the plasmid was transferred into mPAO1 *xcpR::Tn* cells though biparental mating. Briefly, the two strains were grown in 5 ml LB broth supplemented with appropriate antibiotics, at 37°C for 8 h. Subsequently, the liquid culture of the two strains were mixed in thee (v/v) ratios, 1:1, 1:9, 9:1 in a total volume of 1ml. The prepared mixture was centrifuged for 3 min at 1677xg and the cell pellet was resuspended in 100µl of the supernatant. Subsequently the resuspended mixture of each ratio was divided into three spots (33 µl) on LB agar plates that were incubated upright at 37°C for 16 h. One spot from each ratio was resuspended in 1ml of LB broth and plated (100 µl) on Pseudomonas isolation (PIA) agar plates, supplemented with gentamicin (100 mg/L), and incubated for 16 h at 37°C. Individual colonies from the PIA plates were

double-patched onto gentamicin (30 mg/L) and carbenicillin (200 mg/L) and incubated at 37°C for 16h. Cells containing the gentamicin resistance cassette inserted into the gene grew only on gentamicin-supplemented plates. The gentamicin cassette was subsequently excised by the Flp recombinase encoded by pFLP2 (Hoang et al, 1998). The colonies that grew only on gentamicin were electroporated with pFLP2 and plated on carbenicillin plates. Subsequently the colonies were replica-plated onto gentamicin and carbenicillin plates. Cells that underwent successful excision of gentamicin cassette grew only on carbenicillin. The pFLP2 plasmid was subsequently removed using sucrose counterselection. Cells with an insertion of the FRT scar in the *pilT* gene were verified by PCR amplification (primers listed in Table 2).

2.2 Generation of complementation constructs

For complementation constructs, gene of interest was amplified by PCR using mPAO1 chromosomal DNA as a template. Subsequently, the amplicon and the vector were digested with compatible restriction enzymes (Fermentas). The digested DNA was PCR purified, followed by ligation using T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5α, and the cells were grown on LB agar plates supplemented with appropriate antibiotics at 37°C.

2.3 Twitching motility assays

Twitching motility assays were performed as previously described (Gallant et al, 2005). Using a sterile pipette tip (P10), bacterial colonies were stab-inoculated into LB

agar plate (1% agar), to the interface between the agar and the plastic. The plates were incubated at 37°C for 36 h. Subsequently, the LB agar was removed and twitching zones were visualized by incubating the plate with 1% (wt/vol) crystal violet for 30 min. Residual dye was removed by rinsing the plate with lukewarm water (Gallant et al, 2005)

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2.4 Analysis of sheared surface proteins

Sheared-surface protein analyses were performed as described previously (Voisin et al, 2007). Strains of interest were streaked in a grid-like pattern on LB agar plates and incubated at 37°C for approximately 14 h. Using glass cover slips, the cells were gently scraped from the plates and resuspended in 4 ml of 1x PBS (Phosphate buffered saline, pH 7.4). Surface appendages were sheared off the cells by vortexing the cell suspensions for 30 s. Subsequently, the suspensions were transferred to 1.5ml eppendorf tubes and the cells were pelleted by centrifugation at room temperature for 5 min at 11,688 x g. The cell pellet was saved for analysis of intracellular levels of protein. The supernatant was transferred to new microcentrifuge tubes and recentrifuged at room temperature for 20 min at 11,688 x g to remove the remaining cells. The supernatant was transferred to a new microcentrifuge tube and 1/10 volume of each of 5 M NaCl and 30% polyethylene glycol (molecular weight, 8000) was added. Incubating the suspensions on ice for 80 min precipitated the soluble proteins. The precipitated proteins were collected by centrifugation at 11, 688 x g for 30 min. The supernatant was discarded and the tubes were re-centrifuged for 3 min. The remaining supernatant was discarded and the precipitated-protein pellet was resuspended in 100µl of 1x SDS loading buffer [80 mM

Tris (pH 6.8), 5.3% (vol/vol) 2- mercaptoethanol, 10% (vol/vol) glycerol, 0.02% (wt/vol) bromophenol blue, and 2% (wt/vol) SDS]. The samples were boiled for 10 min and separated on 15% SDS-PAGE gels. Proteins were visualized with Coomassie brilliant blue (Sigma) or subjected to western blot analysis.

2.5 Preparation and analysis of whole cell lysates

The cell pellets collected after the first centrifugation step during the isolation of sheared surface proteins were resuspended in 1x PBS and standardized to $O.D_{600}$ = 0.6. 1 ml of the standardized cell suspension was centrifuged at 11,688 x g for 3 min. The supernatant was discarded and the cell pellet was resuspended in 1X SDS loading buffer. The intracellular fractions were subjected to western blot analysis.

2.6 Immunoblot analysis

Prepared intracellular and sheared surface samples 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. The samples were boiled for 10min and separated on 15% SDS-Page gel. Subsequently they were transferred onto nitrocellulose membrane. The membrane was blocked overnight with 5% skim milk (w/v) at 4°C. The blots were incubated with primary anti-PilA antibody at a 1:5000, 1:3000 anti-XcpT, 1:5000, anti-PilA, 1:1000, anti-FimU or 1:1000 anti-PilW antibody for 1h at 37°C shaking. The blots were then washed three

times with 1 x PBS for 10minutes and subsequently incubated with secondary goat-anti-rabbit IgG alkaline phosphotase antibody at 1:3000 dilution for 1 hours at room temperature. The blots were washed three times with 1 x PBS for 10 minutes, and developed using NBT/BCIP (BioRad). Densitometric analysis were performed using Image J (Abramoff et al, 2004).

2.7 Type II secretion plate-based assays

Elastase secretion on solid surfaces was investigated using tryptic soy broth (TSB)-skim milk agar plates (1.5% skim milk) (Lu et al, 1997). Plates were incubated for approximately 15 h at 37°C. Secretion was assessed by comparing the zone of clearing around the colony to the positive (WT) and negative (*xcpQ/xcpP/xqhA/xphA* mutant, denoted ΔPQPAQA) controls (Michel et al, 2007).

2.8 Type II secretion liquid-based assays

Strains of interest were grown in 5ml LB supplemented with appropriate antibiotics at 37°C for approximately 16 h. Subsequently, 1ml of the overnight cultures were sub-cultured in a total of 10ml LB and grown for 22 h at 37°C.

The strains were standardized to $O.D_{600}$ = 0.6 and 1 ml of the standardized culture were centrifuged at 11,688 x g for 3 min. The supernatant was transferred to a new microcentrifuge tube and the pellet was washed once with deionized water (100µl) and re-pelleted. Subsequently it was resuspended in 100 µl of 1x SDS loading buffer. For western blot analysis, 100 µl of the supernatant (extracellular fraction, E/C) was

resuspended in 50 µl of 3x SDS-loading buffer. The samples were boiled for 10 min and 8 µl of the sample was loaded.

2.9 Transmission electron microscopy (TEM) and immunogold labeling

TEM and immunogold labeling was performed as described previously (Giltner et al, 2010a). Bacterial strains were grown on LB plates supplemented with gentamicin (30mg/L) for 16 h. A small amount of cells from the edge of a colony were removed with a toothpick and gently resuspended in 50 µl of filtered Nuclease Free Water (Qiagen). Formvar-coated (200-mesh) nickel grids (supplied by the McMaster Electron Microscopy Facility) were incubated with 50µL of the cell suspension for 2 min and subsequently blocked in 0.3% milk solution [0.3% skim milk in 1x phosphate-buffered saline (PBS)] for 30 min. The grids were incubated with 1:10 dilution of anti-FimU, anti-PilA or anti-XcpT antiserum for 1h at room temperature. Grids were washed three times with 1x PBS, for 2 min per wash and then incubated with goat anti-rabbit 10-nm colloidal gold conjugated secondary antibodies (Amersham) for 1 h at room temperature. Grids were again washed three times with 1x PBS, for 2 min per wash followed by staining with 1% uranyl-acetate (Electron Microscopy Sciences) for 60 s. TEM pictures were taken with a JEOL JEM 1200 TEMSCAN (JEOL) microscope operating at an accelerating voltage of 80 kV with a 4- megapixel digital camera (Advanced Microscopy Techniques Corp.). AmtV600 software was used to capture digital images.

2.10 Bioluminescence reporter assay

Promoter region (331bp upstream of start site plus 29 bps following start site) of the gene encoding for exoprotein elastase, *lasB* was cloned in BamHI-XhoI site of pMS402 plasmid, containing the lux operon (*luxCDABE*). The construct and the empty vector were introduced into strains of interest. Strains containing either plasmid were grown in LB liquid media supplemented with gentamicin (30mg/L) and/or kanamycin (200 µl/ml), overnight for 16 h at 37°C, shaking at 200 rpm. The next day, the strains were sub-cultured by adding 1*ml* of the overnight culture to fresh LB media containing appropriate antibiotics. All strains were grown and standardized to OD_{600} = 0.2. Prior to the assay, the white, clear bottom 96 well plate was UV sterilized for 15 min. LB liquid media was used as a blank. 100 µl of each standardized culture was added to the 96 well plate and repeated three times for each culture. The plated were sealed with a plastic film. Luminescence and OD_{600} were measured simultaneously for 20-24 h, with 15 min time intervals using Synergy HT Multi-Mode Microplate Reader. The values were reported as relative light units/OD_{600}.

CHAPTER 3 – RESULTS

3.1 XcpT is expressed on the cell-surface under native conditions

Previous studies have reported XcpT is expressed on the surface as a pseudopilus only when it is overexpressed (Durand, Bernadac et al. 2003). To study its surface expression under native conditions, levels of intracellular and sheared surface proteins in a WT background were compared to the mutant (*xcpT::Tn*). Wild type (WT), NP (non-piliated, pilA mutant) and *xcpT* (*xcpT::Tn*) strains were transformed with either the vector control (pBADGr, an L-arabinose inducible vector that permits a small amount of expression under uninduced conditions in Pa), the pilA gene in trans (+ *pilA*) under uninduced (0% ara) or induced conditions (0.1% ara) or *xcpT* gene in trans (+ pB*xcpT*) (Fig 3).

Sheared surface fractions were separated on 15% SDS-PAGE gel and protein bands were visualized with Coomassie brilliant blue (Fig 3a). The flagellin band, which is not affected by changes in piliation or secretion, was used as a loading control. Alternatively, the surface fractions were probed with anti-XcpT or anti-PilA (Fig 3a). XcpT was present on the surface of the WT strain under native conditions and was absent in an *xcpT* mutant. To illustrate that surface levels of XcpT are dependent on the amount of XcpT present in the cell, pB*xcpT* was introduced in the WT strain. As expected, there was an increase in both intracellular and extracellular levels of XcpT relative to the WT (Fig 3ab). Likewise, complementation of the *xcpT* mutant with pB*xcpT* resulted in an increase in both intracellular and surface levels of XcpT relative to the WT and the mutant.

Relative to WT, levels of surface PilA were lower in an *xcpT* mutant complemented with pBADGr or its cognate gene, possibly due to differences in the WT strain and the background strain of *xcpT* mutant.

To determine whether changes in levels of XcpT affected twitching motility in these strains, the relative sizes of their twitching zones were compared (Fig 4). Twitching motility was inhibited only in strains lacking *pilA* expression. Complementation studies showed that introduction of pB*xcpT* restores secretion in the *xcpT* mutant under uninduced conditions (Fig 3c). However, further increase in *xcpT* expression inhibits secretion, consistent with previous studies suggesting, overexpression leads to the formation of pseudopilus on the surface, which blocks the secretin (Durand, Bernadac et al. 2003).

3.2 Surface expression of XcpT is dependent upon PilA

Several studies have reported that the Type IV pilins, particularly PilA, interact with the pseudopilins and possibly play a role in secretion (Lu, Motley et al. 1997; Sauvonnet, Vignon et al. 2000). To determine whether XcpT was present on the cell surface in the absence of PilA, the levels of surface XcpT in mutants lacking PilA were assessed (Fig 3). In the absence of PilA (NP), surface expression of XcpT was completely inhibited (Fig 3a). To establish whether this phenotype was due to the lack of PilA, the *pilA* mutant was complemented with its cognate gene in trans (pB*pilA*), under uniniduced (0% ara) and induced conditions (0.1% ara). As reported previously, in a *pilA* mutant, restoration of WT levels of surface PilA requires induction of pB*pilA* with 0.1% ara

(Giltner, Habash et al. 2010). Similarly, we found that PilA levels were only partially restored at 0% ara, and a substantial increase was observed upon induction with 0.1% ara. Likewise, complementation of *pilA* mutant with its cognate gene, under induced conditions (0.1% ara) restored XcpT surface expression to 210% of WT.

Previous studies have reported over-expression of *xcpT* results in the formation of a pseudopilus on the cell-surface (Durand, Bernadac et al. 2003). To investigate whether surface XcpT can be recovered in a NP strain, XcpT was over-expressed in the NP background. A significant increase in surface XcpT levels was observed relative to the vector control. Over-expression of *pilA* in a WT strain (WT + *pilA*) under induced conditions did not result in a corresponding increase in XcpT levels. Intracellular XcpT and PilA levels were compared to ensure changes in their surface levels were not due to effects on protein expression (Fig 3b). An increase in either intracellular PilA or XcpT was observed only when their respective genes are over-expressed.

To examine the correlation between surface expression of XcpT and T2S, strains of interest were inoculated on skim milk agar plates. Secretion was assessed qualitatively by measuring the area of the zone of casein hydrolysis around the colony, due to the activity of several T2S-dependent proteases including LasA, LasB, PmpA and PrpL (Braun P 1998; Fox A 2008; Bleves, Viarre et al. 2010) (Fig 3c). The negative control is quadruple deletion mutant (QM) *xcpP-xcpQ-xqhA-xphA*, that lacks the outer membrane secretin and the associated accessory protein of the two characterized T2S systems in Pa (Michel, Durand et al. 2007). A small zone of clearing was observed around the colony due to the residual activity of non-specific proteases as reported previously (Michel, Durand et al.

2007). Complementation of the *xcpT* mutant with its cognate gene (at 0% ara) restored secretion to WT levels however, over-production (0.1% ara) reduced secretion to QM levels, consistent with previous reports (Durand, Bernadac et al. 2003). Secretion in the NP strain lacking surface XcpT was comparable to that of WT, suggesting lack of surface exposed XcpT does not affect secretion (Fig 3c).

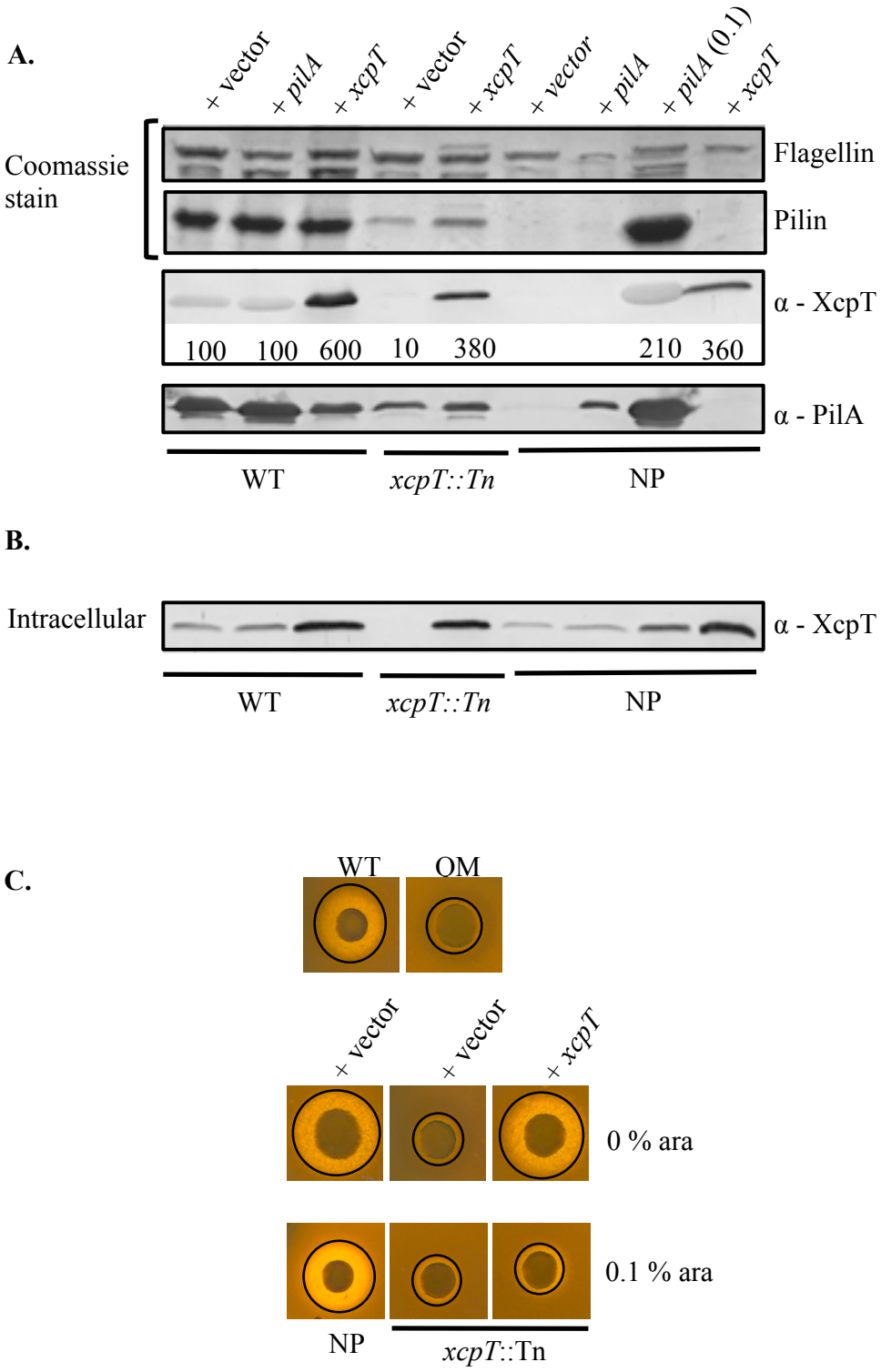

Fig 3: PilA is required for XcpT-surface expression but not T2SS

Fig 3: PilA is required for XcpT surface expression but not T2SS. a) Sheared-surface preparations of strains of interest were separated on SDS-PAGE gel, stained with Coomassie brilliant blue stain or probed with anti-XcpT and anti-PilA antiserum. WT cells with pB*xcpT* have increased amounts of XcpT on the surface comparing to WT with the vector control. No XcpT is detected on the surface in a *pilA* mutant (NP), however it is restored upon complementation with *pilA* in trans, under induced conditions (0.1% ara). Overexpression of *xcpT* in a NP strain restores surface XcpT to greater than WT levels. Flagellin band is used as a loading control. **b)** Intracellular PilA and XcpT levels are equivalent in all strains with vector control and increased in strains over-producing either *pilA* or *xcpT* genes. **c)** Strains were then assessed for protease secretion using skim-milk agar plates. Zone of clearing around the colony was observed for WT and NP strains. QM strain was used as a negative control. Secretion in *xcpT* mutant was restored upon complementation with pB*xcpT* in trans under uninduced conditions, but abolished upon over-expression (0.1% ara). Densitometric analyses for each band are shown.

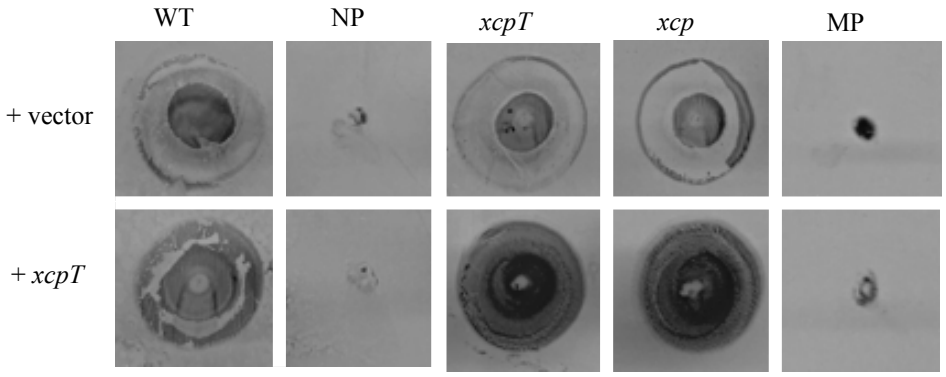

Fig 4: Overexpression of *xcpT* does not affect T4P-mediated twitching motility. WT, NP (*pilA* mutant), negative control QM expressing with vector control (pBADGr) or pB*xcpT* were stab inoculated in 1% agar plate (0% ara). Following the removal of the agar, the plates were stained with crystal violet. Overexpression of *xcpT* in NP strain does not restore twitching motility. Twitching motility is unaffected in *xcpT* and *xcp* mutants (representative of *xcpR, xcpU,V,W,X* mutants). Twitching was abolished in all minor pilin mutants, and was not restored upon *xcpT* overexpression

3.3 PilA is required for XcpT surface expression under native conditions

Based on our results, we hypothesized that XcpT was either incorporated into the T4P or that its assembly was dependent on the major pilin subunit PilA. Regardless, if surface expression of XcpT were dependent on PilA, it would be affected by mutations in the T4P biogenesis machinery. To investigate whether PilA is required for XcpT assembly on the surface, T4P retraction was blocked in a NP strain (NP/*pilT::*FRT) (Fig 5). As shown previously, to study genes required for T4P assembly, the retraction process is blocked, consequently all pili assembled is expressed on the surface.

In a NP/*pilT::*FRT, no XcpT was present on the surface (Fig 5). However, upon complementation with *pilA* in trans, under uninduced (0% ara) and induced (0.1% ara), XcpT was restored to *pilT::*FRT levels. Upon complementation of NP/*pilT::*FRT with *pilT* in trans, surface expression of XcpT and PilA was abolished. Therefore, levels of XcpT on the surface correlate with those of PilA.

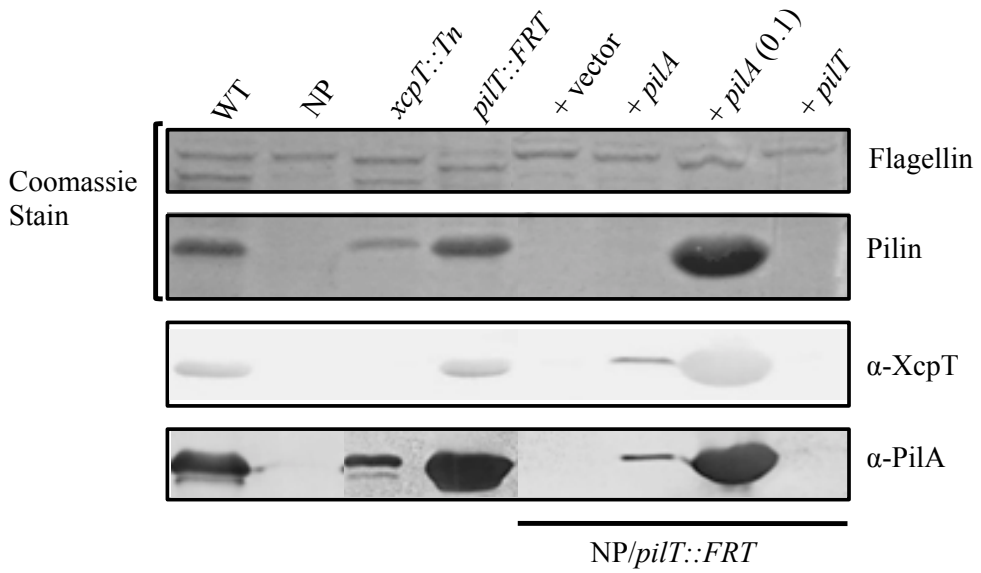

Fig 5: Levels of surface-XcpT co-varies with surface-PilA levels. Sheared surface preparation of NP/*pilT* double knockout strain to determine whether PilA is required for XcpT-surface expression. **a)** Surface-fractions were separated on a SDS-PAGE gel, stained with Coomassie brilliant blue or probed with anti-PilA and anti-XcpT. Surface-expression of XcpT in an NP/pilT mutant complemented with vector control is comparable to the negative control (*xcpT* mutant). Expression is partially restored upon complementation with PilA (NP/pilT + pilA) under uninduced conditions. Complementation under induced conditions (0.1% ara) restores expression to more than *pilT::FRT* levels. Surface expression in a *pilT* mutant is relatively higher than WT. The *xcpT* mutant is shown as a negative control. Expression is restored upon complementation with its cognate gene in trans.

3.4 XcpT surface assembly is dependent on PilA in other *P.aeruginosa* strains

To establish that XcpT surface expression is dependent on the major subunit PilA regardless of the genetic background, two additional strains of Pa were studied. As reported previously, Pa strains can be divided into five groups based on the sequence similarity of PilA and the presence of accessory proteins involved in the post-translational modification of the pilin subunit (Asikyan et al, 2008; Kus et al, 2008; Kus et al, 2004). The three strains analyzed for this study were PAO1 (Group II), PAK (Group II) and Pa5196 (Group IV). Sheared-surface proteins from strains of interest were separated on SDS-PAGE gel and either stained with Coomassie brilliant blue (Fig 6a) or probed with anti-PilA (5196), anti-PilA (PA01) or anti-XcpT (Fig 6a). Complementation of Pa5196 NP strain with its cognate gene in trans restored intracellular and surface PilA expression to WT levels. However, in PAO1 and PAK, expression was restored to WT only under induced conditions (0.1%).

In the absence of PilA, surface assembly of XcpT was perturbed in Pa5196 and PAO1, yet it was unaffected in the PAK NP strain. Upon complementation of the NP strain with its cognate gene in trans without induction, levels of XcpT in Pa5196 and PAK were comparable to the vector control. However, when PilA expression is induced with 0.1% arabinose, XcpT levels in Pa5196 are restored to WT levels, whereas in PAK, a significant increase (178% of WT) is observed. Likewise, in PAO1, *xcpT* expression in a NP strain is restored to WT levels under induced conditions. Intracellular XcpT levels were equivalent in all strains, suggesting variation in surface expression was not due to changes in intracellular protein-expression (Fig 6b).

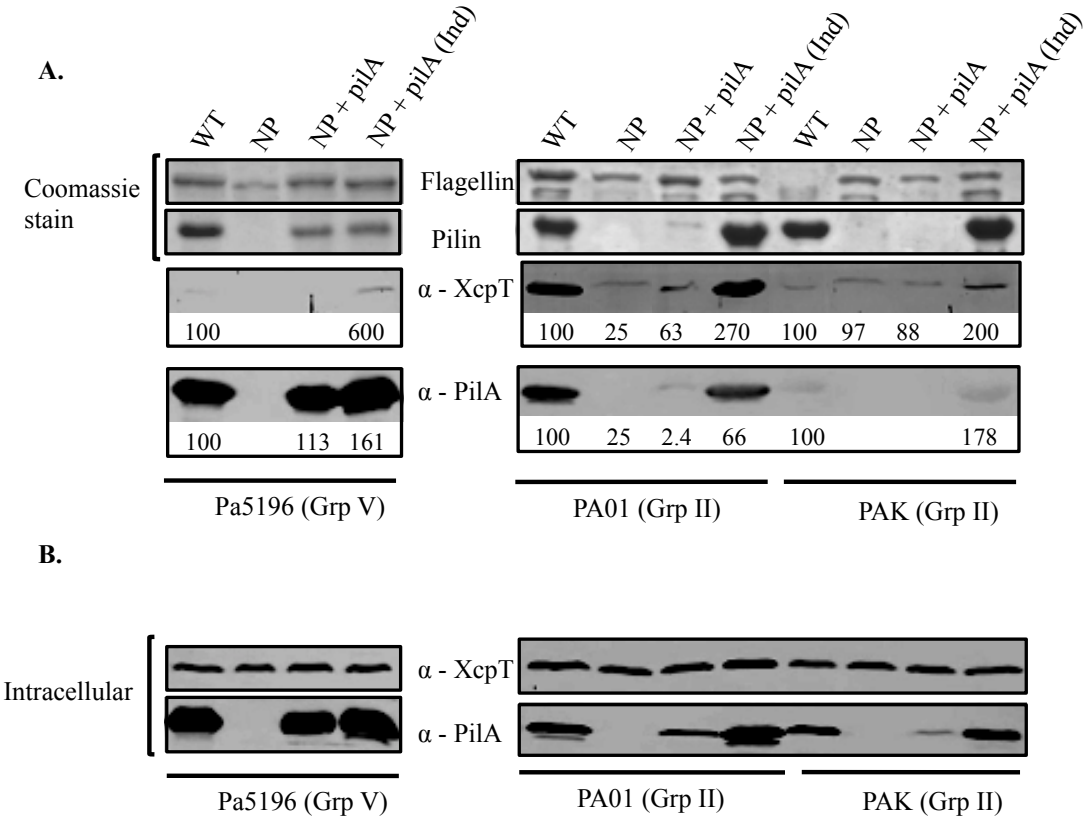

Fig 6: PilA dependent surface-expression of XcpT is also observed in other *P.aeruginosa* strains. Sheared surface preparation of wild type PAO1, PAK, Pa5196, their respective *pilA* mutants and *pilA* mutants complemented under uninduced (0% L-arab) and induced (0.1% L-arab) conditions. **a)** Proteins were separated on SDS-PAGE gel and stained with Coomassie brilliant blue or probed with anti-PilA and anti-XcpT. Overexpression of *pilA* increases surface-piliation and XcpT surface-expression in all three strains. **b)** Standardized intracellular fractions were probed with anti-XcpT and anti-PilA antibodies and levels of both proteins are equivalent in all strains unless over expressed. Densitometric analyses for each band are shown.

3.5 XcpT is incorporated into the Type IV pilus

Western blot analyses showed that under native conditions, levels of XcpT on the surface were correlated to those of PilA. Using TEM and immunogold labeling, we asked whether XcpT was incorporated into the T4P fiber (Fig 7). However, very few pili were labeled and some cells showed no labeling at all. Further, the absence of a pseudopilus in the WT confirmed that the presence of XcpT on the surface is related to its incorporation into the T4P. Consistent with western blot analysis, in the absence of the pilus (NP + vector), no labeling was observed on the surface. Overexpression of XcpT in a NP strain led to the formation of a pseudopilus that was evenly labeled.

As expected, no labeling was detected in the negative control (*xcpT* mutant) complemented with pBADGr. Complementation of the *xcpT* mutant with pB*xcpT* led to the assembly of a pseudopilus that appeared as a bundled fiber on the surface, evenly labeled with anti-XcpT antibodies (Fig 7).

To further examine the potential incorporation of XcpT into T4P, a hyper-piliated strain (*pilT::*FRT) was analyzed. As observed with the WT strain, very few pilus fibers per cell were sparsely labeled. Complementation of the mutant with its cognate gene restored WT levels of surface piliation, however few cells had XcpT labeled pili (Fig 7). Together with the Western blot results, the data suggests that XcpT is present in surface pili but at very low levels.

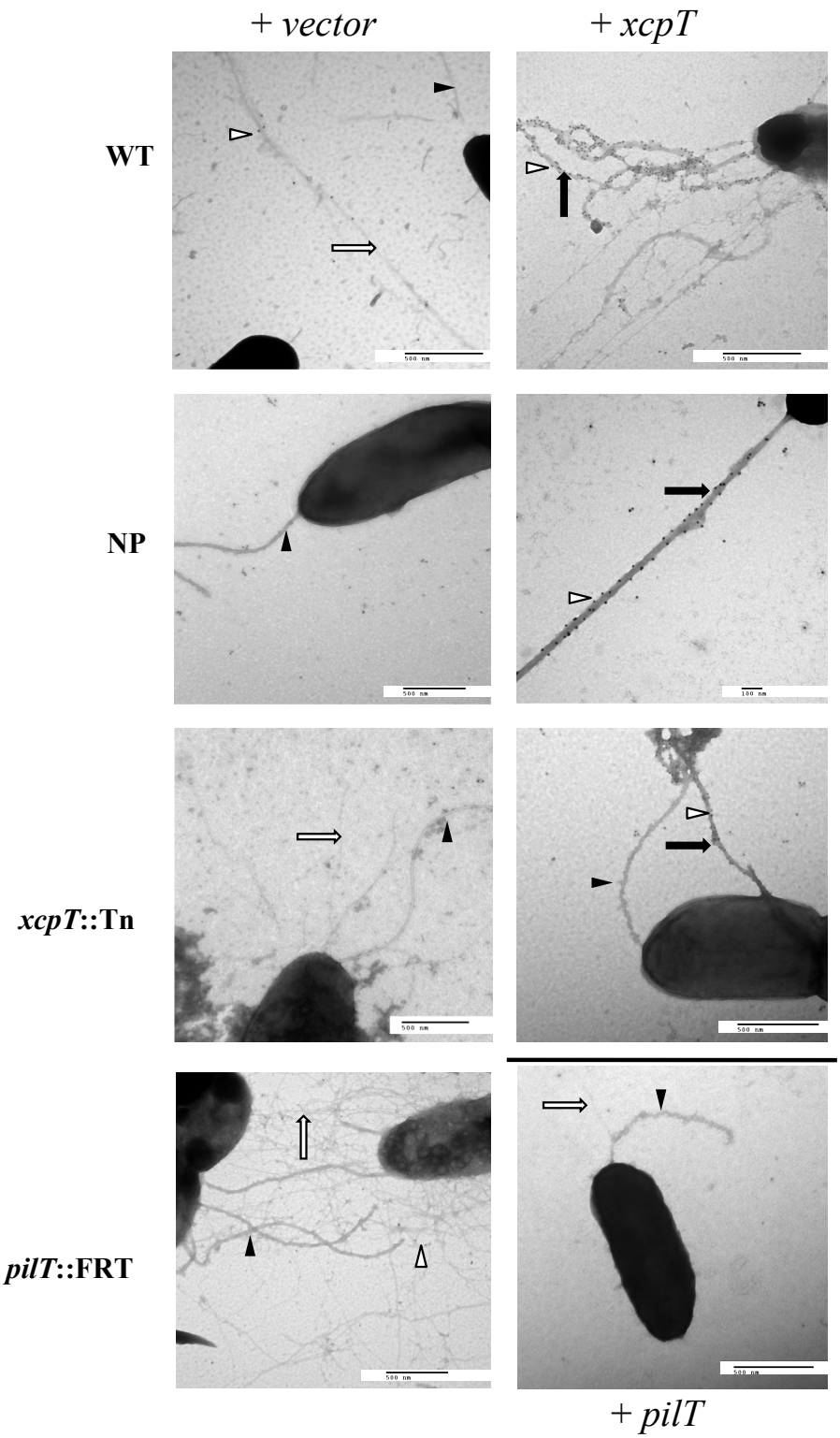

Fig 7: XcpT is incorporated into T4P fiber

Fig 7: XcpT is incorporated into T4P fiber. Immunogold TEM images of PA01 wild type (WT) cells and NP (*pilA::*Tn), *xcpT::*Tn and *pilT::*Tn mutants with the indicated vectors were probed with anti-XcpT antibodies and a secondary gold-conjugated anti-body. Grids were negatively stained with uranyl acetate and examined with a JEOL JEM 1200 TEMSCAN microscope. AmtV600 software was used to capture digital images. XcpT labeling was detected in T4P fiber in WT cells and was absent in the mutant and NP strain. Introduction of pB*xcpT* in trans, resulted in the formation of bundled pseudopili, labeled with XcpT.
Pointers: Flagella ▲, Pili ⇧, Labelled protein △, Pseudopilus ⬆

3.6 FimU and PilX affect XcpT surface expression

Previous studies in *Neisseria* and *Pseudomonas* reported that MPs are incorporated into the T4P and potentially facilitate the assembly and retraction processes (Giltner et al, 2010a; Winther-Larsen et al, 2005). To determine whether these proteins also affect XcpT expression, sheared surface fractions of minor pilin mutants and their respective complemented strains were analyzed under uninduced conditions (Fig 8).

Sheared-surface fractions were separated using SDS-PAGE and stained with Coomassie brilliant blue stain or probed with anti-PilA or anti-XcpT. Relative to WT, levels of both surface PilA and XcpT were significantly reduced in the minor pilin mutants (Fig 8). Complementation of *pilV, pilW* and *pilE* mutants with their cognate gene restored XcpT or PilA levels to WT. However, complementation of *fimU* and *pilX* mutants with their cognate gene restored neither XcpT nor PilA levels to WT. This is consistent with previous studies, showing that over-production of FimU or PilX in trans does not restore surface piliation or twitching motility to WT levels (Giltner et al, 2010a). To confirm that this wasn't due insufficient levels of FimU and PilX, the expression of two proteins was induced with 0.1% arabinose, and yet it did not restore either PilA or XcpT (Fig S1). Intracellular PilA and XcpT levels are equivalent in all strains suggesting

that the observed changes in surface expression were not due to changes in protein expression.

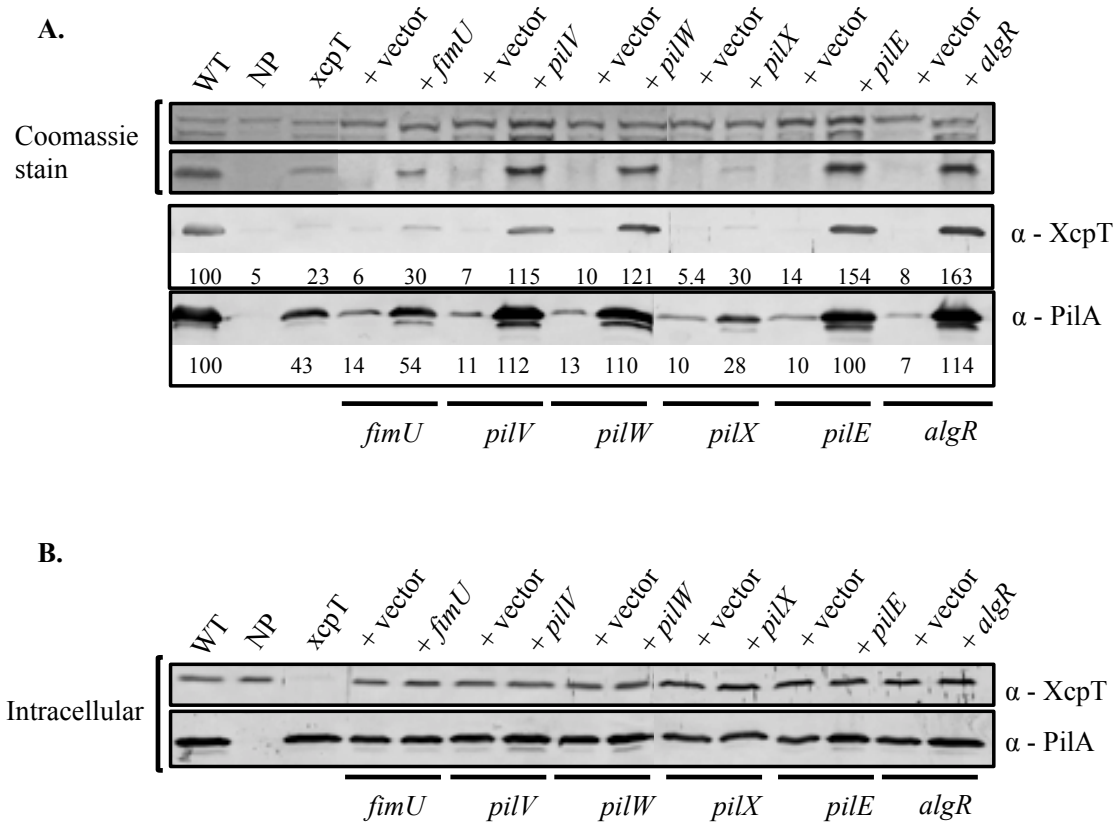

Fig 8: Surface expression of XcpT is not restored in *fimU* and *pilX* mutants complemented with their cognate genes in trans. a) Sheared-surface proteins separated on SDS-PAGE gel. Pilin subunits or XcpT appears at approximately 15kDa. Flagellin is used as a loading control. Surface proteins probed with anti-XcpT and anti-PilA antisera. WT is used as a postive-control and *xcpT* mutant is used as a negative control. NP mutant lacks surface pili and surface-xcpT. XcpT surface expression is drastically reduced in minor pilin (MP) single mutants. Surface-PilA and XcpT levels are not restored to WT levels in *fimU* and *pilX* complemented strains. **c)** Intracellular PilA and XcpT levels are equivalent in all strains. Densitometric analyses for each band are shown.

Earlier studies showed levels of MPs are interdependent on each other such that in the absence of *pilV, pilW* or *pilX*, FimU levels increase. Hence, to determine whether the observed decrease in XcpT levels in *fimU* and *pilX* complemented strains is due to changes in the levels of other MPs, individual MPs and combinations of MPs were introduced in an *algR* mutant (Fig 9).

In the absence of AlgR, levels of surface PilA are reduced and no XcpT is expressed on the surface. Amount of PilA and XcpT are restored, upon complementation with pB*algR* in trans. Introduction of individual MPs in an *algR* mutant background does not restore WT levels of surface XcpT or PilA, suggesting all MPs are required. To determine, whether the *algR* phenotype is due to the lack of MPs, since AlgR also controls other systems, the entire minor pilin operon (pVDTacpil) was introduced in trans. Both surface XcpT and PilA levels were restored to WT levels (Fig 9).

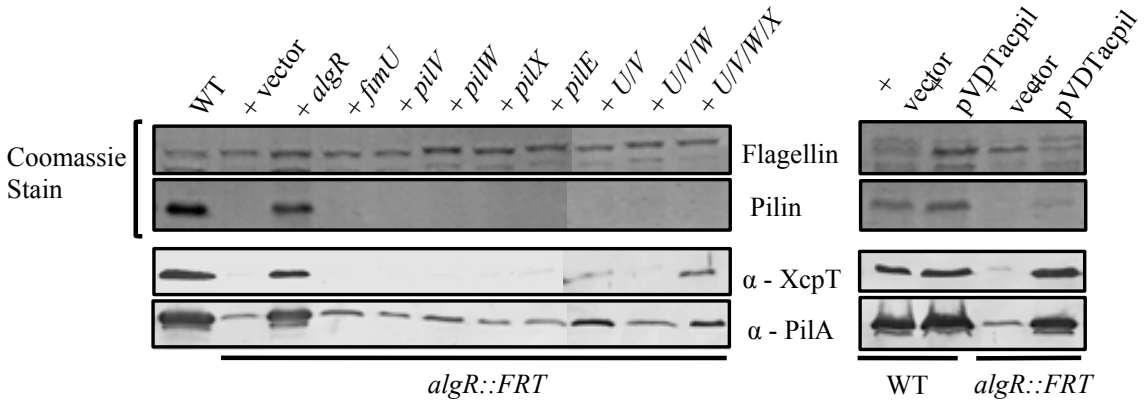

Fig 9: Expression of all minor pilins is required for WT expression of surface-XcpT and PilA. Sheared-surface fractions of strains were separated on SDS-PAGE gel, followed by Coomassie staining or western blot analysis with anti-PilA or anti-XcpT antibodies. The *algR* mutant was complemented with individual (*algR* + *fimU,* + *pilV,* + *pilW*, + *pilX,* + *pilE*) or different combinations of minor pilins (*algR* + *UV,* + *UVW,* + *UVWX*). WT and *algR* mutant were also introduced the entire minor pilin operon (pVDTacpil) or with vector control (pVDTac39). Restoration to WT levels of surface-XcpT and PilA is observed only in *algR* + pVDTacpil strain. **b)** Intracellular levels of XcpT are equivalent in all strains.

3.7 XcpT and PilA surface expression are reduced in a *xcpV* mutant and increased in a xcpX mutant

Durand et al. (2005) showed that when over-expressed in an *xcpV* mutant, XcpT was unable to assemble into a pseudopilus, suggesting that *xcpV* is required for the formation of a pseudopilus (Durand, Bernadac et al. 2003). In contrast, increased levels of pseudopili were observed in an *xcpX* mutant, suggesting it acts as a putative terminator of pseudopilus assembly (Bleves, Voulhoux et al. 1998; Durand, Michel et al. 2005). To determine whether these pseudopilins have the same effect on XcpT surface expression under native conditions, we analyzed the sheared-surface preparations of single minor pseudopilin mutants complemented with vector control (pBADGr) or their cognate gene in trans, under uninduced and induced (0.1% ara) conditions (Fig 10).

Relative to WT, levels of surface XcpT and PilA were reduced in an *xcpU* and *xcpW* mutants complemented with vector control (pBADGr). However, they were more reduced in an *xcpV* mutant, complemented with pBADGr. Although the levels of surface PilA were also reduced in these mutants, no effect on twitching motility was observed (Fig 4). In contrast, the *xcpX* mutant had increased levels of XcpT on the surface, yet it also had increased levels of PilA on the surface (Fig 10). Intracellular XcpT and PilA levels were equivalent in all strains.

Relative to WT, levels of surface XcpT in *xcpU* and *xcpW* mutants complemented with their relevant genes remained unchanged, whereas a slight increase was observed in an *xcpV*-complemented strain. On the contrary, complementation of *xcpX* mutant with its cognate gene resulted in reduced surface XcpT levels to WT (Fig 10c). To determine

whether further increase in the levels of the four pseudopilins would restore WT levels of XcpT, the mutants were complemented with their cognate genes under induced conditions. Levels of surface *xcpT* in *xcpU/V/W/X* single mutants complemented with their cognate gene (0.1% ara) were comparable to the mutants with vector control. However, *xcpX* mutant complemented with its relevant gene (0.1% ara) resulted in a significant decrease in XcpT levels, consistent with the earlier studies, suggesting XcpX controls the length of the pseudopilus (Durand, Michel et al. 2005).

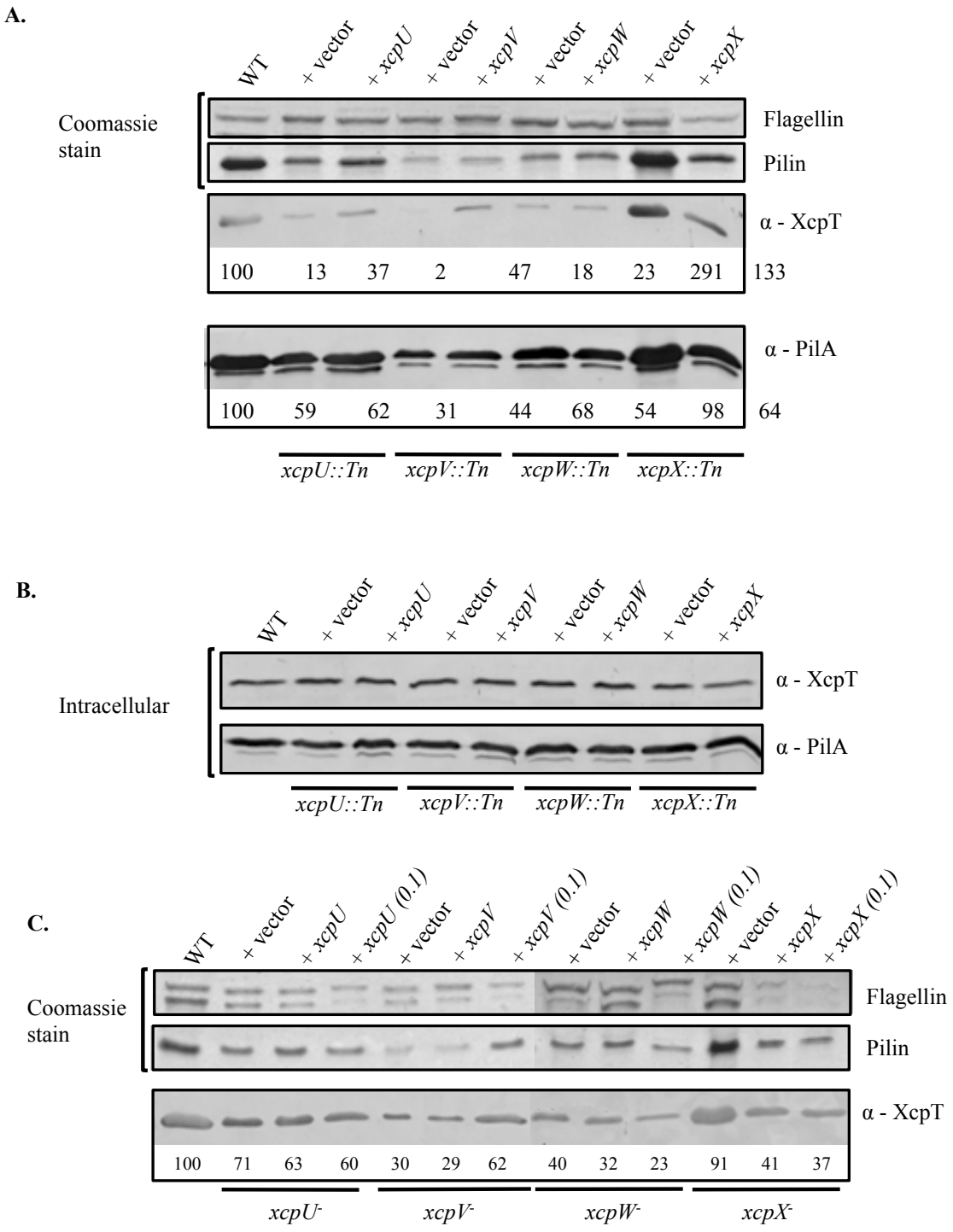

Fig 10: Pseudopilins modulate XcpT surface-expression under native conditions

Fig 10: Pseudopilins modulate XcpT surface expression under native conditions. Sheared-surface fractions of single pseudopilin mutants (*xcpU/V/W/X)* complemented with vector control (pBADGr) or their cognate gene were separated on a SDS-PAGE gel, followed by Western blot analysis or staining with Coomassie brilliant blue. Both surface (a) and intracellular fractions (b) were probed with anti-XcpT and anti-PilA. **a)** Levels of surface XcpT and PilA were significantly reduced in the *xcpV* mutant and increased in the *xcpX* mutant relative to WT. Complementation with cognate gene under uninduced conditions (0% ara) did not restore WT levels of surface XcpT or PilA in any of the four mutants. **b)** Intracellular PilA and XcpT levels are equivalent in all strains. **c)** Surface fractions of mutants complemented with cognate genes under induced conditions (0.1% ara). Densitometric analyses for each band are shown.

3.8 XcpR is not essential for XcpT surface expression

XcpR is the only T2SS ATPase identified, and similar to PilB, it is postulated to promote pseudopilus assembly (Ball et al, 1999; Filloux et al, 1998). To determine whether it is required for pseudopilus assembly under native conditions, we compared the levels of surface XcpT in an *xcpR* single and *xcpR/pilT* double mutants. In the absence of *xcpR*, surface and intracellular levels of XcpT were drastically reduced relative to WT. Introduction of *xcpT* in trans, restored intracellular levels of XcpT, however surface levels increased only slightly, suggesting XcpR facilitates assembly/incorporation of XcpT into the growing T4P fiber, but is not essential (Fig 11). Alternatively, reduced levels of intracellular XcpT may result due to polar effect on *xcpT* expression in an *xcpR* mutant. Therefore, additional studies need to be conducted to determine whether the *xcpR* mutant has polar effects on downstream genes.

Interestingly, there was a dramatic increase in surface levels of XcpT in an *xcpR/pilT* mutant complemented with vector control whereas intracellular levels remained the same as in the *xcpR* mutant (Fig 11). Complementation with *pilT* in trans restored both intracellular and surface XcpT to levels similar to the *xcpR* mutant. Overproduction

of XcpT in the double mutant restored intracellular levels to levels consistent with the *xcpR* mutant and further increased surface XcpT levels relative to WT.

As expected in a T4P retraction-deficient background, levels of surface PilA were higher in all three mutants lacking PilT (*pilT::*FRT, *xcpR::*Tn/*pilT*::FRT and *xcpR::*Tn/*pilT*::FRT + *xcpT*). Intracellular levels of PilA were equivalent in all strains. However, *xcpT* and *xcpR* mutants had relatively low levels of PilA on the surface, which can be attributed to slight differences in the background strain compared with the WT strain used in this study, as mentioned above. As previously shown, elastase secretion is completely abolished in an *xcpR* mutant and is not restored upon complementation with *xcpT* in trans (Fig 11c).

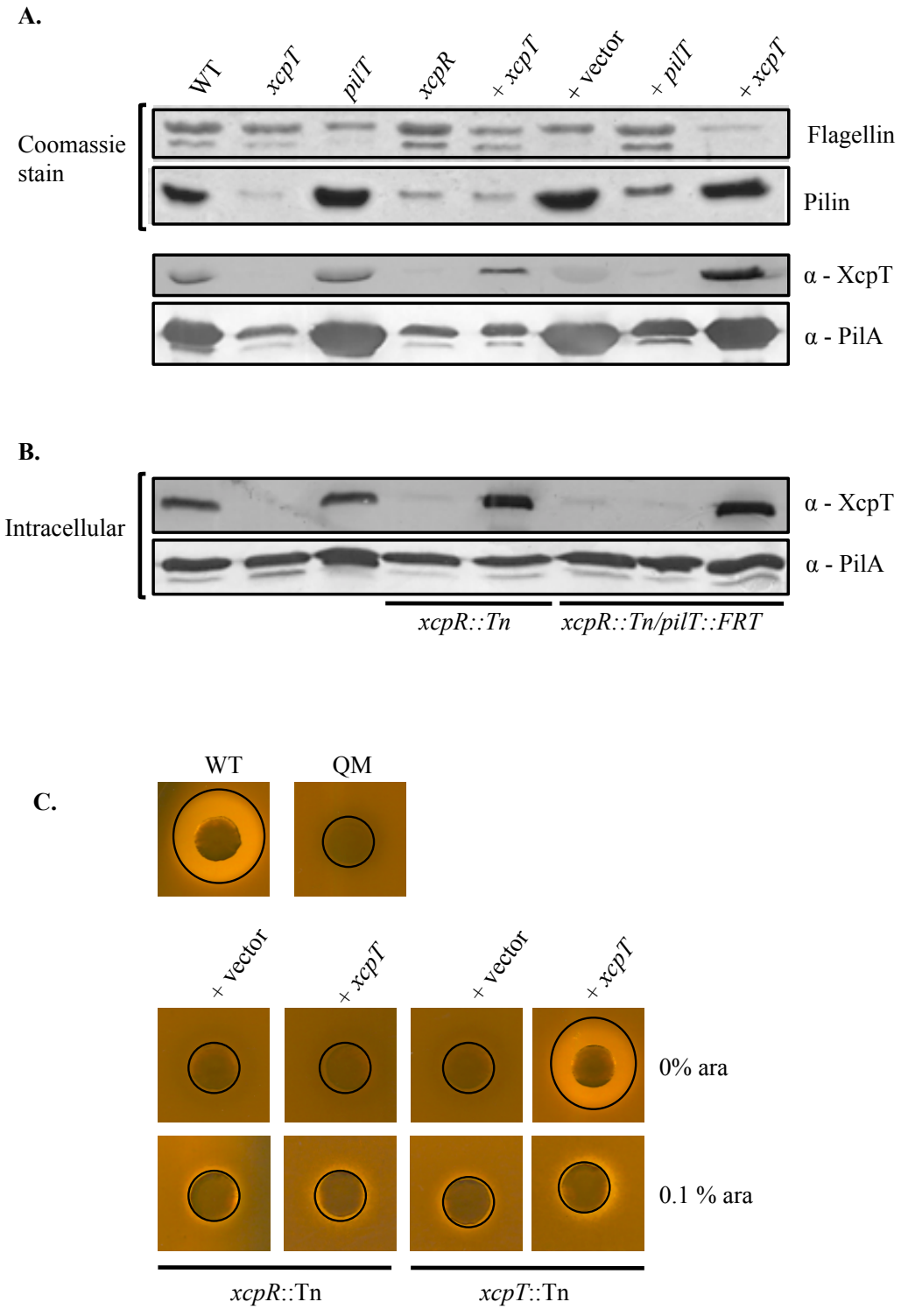

Fig 11: XcpR, a T2SS ATPase is not required for XcpT assembly on the surface

Fig 11: XcpR, a T2SS ATPase is not required for XcpT assembly on the surface. a) Sheared-surface samples of WT, *xcpT::*Tn*, xcpR::*Tn*, xcpR::*Tn*/pilT::*FRT strains and their respective complemented strains were separated on SDS-PAGE gel, stained with Coomassie brilliant blue stain or probed with anti-XcpT or anti-PilA. Levels of XcpT in the *xcpR* mutants are reduced, however in an *xcpR/pilT* mutant, they are greatly increased. **b)** Intracellular levels of XcpT are reduced in an *xcpR* mutant; however, they are equivalent in all other strains. **c)** Protease secretion was assessed by inoculating strains on 1.5% skim-milk agar plates and comparing the zone of clearing around the colony. WT is used as a positive-control and QM is used as a negative-control. Secretion is completely abolished in an *xcpR* and *xcpT* mutant. Complementation with cognate gene in trans under uninduced conditions restores secretion in an *xcpT* mutant.

CHAPTER 4 – DISCUSSION

Type II secretion and Type IV pilus systems are two of the major virulence factors in *P. aeruginosa,* contributing to motility, adhesion and host-cell toxicity and promoting its survival. Bioinformatics and structural studies suggest the two systems are highly similar, sharing common components, architecture and function (Ayers et al, 2010). Among the major components of the T2SS is a set of five pseudopilins that are proposed to assemble into a pilus-like complex, similar to their counterparts in T4P system.

4.1 XcpT is expressed on the surface under native conditions and T4P major pilin PilA is required for XcpT

Our study focused on investigating the role of T4P appendage in the surface expression of XcpT, the major pseudopilin in the T2SS, equivalent to PilA. Previous studies suggested that the minor pseudopilins form a putative complex that remains in the periplasm under native conditions and potentially appears on the surface, as a pseudopilus, upon overexpression of XcpT (Durand et al, 2003; Hu et al, 2002). Here we showed that XcpT is present on the surface under native conditions and does not require overexpression of the pseudopilin (Fig 1).

Although XcpT was present in the sheared-surface fraction of the WT strain, it does not form a pseudopilus on the surface as shown using TEM immunogold labeling (Fig 7). However, overexpression of *xcpT* in the WT background resulted in the assembly of a distinct appendage on the surface, identified as a pseudopilus using immunogold labeling

(Fig 7). Hence, these results indicate under native conditions XcpT appears on the surface but does not assemble into a pseudopilus.

4.2 The T4P major pilin PilA is required for XcpT expression on the surface

Previous studies have reported that PilA potentially interacts with the T2SS pseudopilins and participates in elastase secretion (Lu et al, 1997). Interestingly, we observed that in the absence of PilA, surface XcpT levels are significantly reduced. This defect was restored upon complementation with *pilA* in trans suggesting the effect was solely due to PilA (Fig 1). PilA-driven surface expression of XcpT, suggested that either XcpT is incorporated into the T4P, or PilA regulates its expression on the surface. The former scenario is more likely, as using TEM and immunogold labeling we showed that XcpT is incorporated into the pilus under native conditions (Fig 7). Given the sequence similarity (22% identical, 35% similar) between the two proteins and their shared structural fold, it is possible that XcpT is being recognized as a potential T4P subunit by the assembly apparatus. Further, Sauvonnet et al., (2000) showed, *E.coli* K-12 pilin protein PpdD is able to assemble into a pilus like structure using the Pul secreton when both are expressed in *E.coli*, indicating interactions between the pilins and the T2SS system are structurally feasible (Sauvonnet et al, 2000). Hence, it is possible that the XcpT subunit is being recognized by the T4P machinery and is getting assembled into the pilus.

However, XcpT was neither detected in all pilus fibers nor incorporated evenly throughout the pilus, as shown previously with labeling of T4P with PilA or MPs (Giltner

et al, 2010a). The purpose of XcpT in T4P is yet to be determined, but based on our data and the current model of T2SS, suggesting the pseudopilins are degraded as the pseudopili are retracted; incorporation into the T4P may serve to control the amount of XcpT available for pseudopilus formation (Filloux et al, 1998). Alternatively, XcpT might be involved in T4P-mediated processes other than twitching motility, such as biofilm formation and adherence. Our data suggests XcpT is dispensable for twitching motility and surface piliation, since neither is affected in an *xcpT* mutant (Fig 3, 4).

To confirm PilA-associated XcpT surface expression, we also studied other strains of Pa (Fig 6). Increasing *pilA* expression (0.1% ara) resulted in a corresponding increase in surface XcpT in all three strains investigated (PAO1, PAK, Pa5196), suggesting this phenotype is not specific to PAO1. Pa is one of the few pathogens that has evolved to express both systems, therefore the interaction between the components could be a mere coincidence, instead of a functional requirement (Ayers et al, 2010). Therefore, additional studies are to be conducted to identify the purpose of XcpT in T4P.

Furthermore, we observed that the PilA-XcpT association is group specific. Complementation of PAO1 (group II) *pilA* mutant with group III, IV, V *pilA* with or without their associated accessory proteins, were unable to restore WT levels of XcpT in PAO1 *pilA* mutant (data not shown). Whereas, PilA and the MPs exhibit low sequence similarity across the groups, XcpT is highly conserved among the Pa strains (Asikyan et al, 2008; Giltner et al, 2010b). Inability of PilA from different strains to complement native levels of twitching motility or surface piliation in PAO1 was proposed to be due to group-specific interactions between the MPs and PilA (Asikyan et al, 2008; Giltner et al,

2010b). Therefore, lack of the group-specific interactions prevents the assembly of heterologous pilin in a PAO1 background, which in turn reduces the amount of XcpT incorporated. Hence, MPs could exert their effect on the pseudopili indirectly, by controlling the amount of PilA expressed on the surface (Giltner et al, 2006).

Previous studies have linked T4P biogenesis to secretion, however, this study is the first showing that the T2S component, major subunit XcpT is part of the Type IV pilus. Studies in *Francisella tularensis* reported although a gene cluster encoding T2S components is missing, secretion is mediated by gene products homologous to T4P components (Forsberg & Guina, 2007). These studies and the extensive structural similarity between the two systems are suggestive of a cross talk between the two systems.

4.3 Role of minor pilins in the expression of surface XcpT and toxin secretion

In addition to PilA, we observed the XcpT surface expression was significantly reduced in both the *algR* and individual MP mutants. With the exception of *fimU* and *pilX*, complementation of each mutant with its cognate gene in trans, restored XcpT and PilA to WT surface levels (Fig 8). A previous study characterizing the role of MPs in the T4P system showed that the complementation of *fimU* and *pilX* mutants in trans does not restore surface piliation (Giltner et al, 2010a). This is consistent with the proposed role of FimU and PilX as the initiators of pilus assembly, such that overexpression either leads to the formation of many start sites. It was proposed that given the finite pool of PilA subunits, they might be divided among these start sites, resulting in more numerous but

shorter pili. On the other hand, PilV/W/E are proposed to form a sub-complex required for FimU and PilX to initiate assembly (Giltner et al, 2010a; Winther-Larsen et al, 2005). In the absence of one or more of these components, the sub-complex is disturbed affecting surface piliation and indirectly affecting levels of XcpT on the surface. Hence, reduced levels of XcpT in the *fimU* and *pilX* complemented strains (0% ara) are likely due to decreased surface piliation. Giltner *et al*., (2010) reported that the intracellular levels of MPs are inter-dependent. For example, FimU levels increase drastically in the absence of *pilV/W/X* and likewise PilE levels are reduced in all minor pilin mutants (Giltner et al, 2010a). Consequently, it is not possible to study the phenotype of individual MPs using single minor pilin mutants. Using an *algR* mutant, the expression of the entire operon was reduced and subsequently single minor pilin genes or successive combinations of these genes were introduced to the *algR* mutant (Fig 9). However, WT levels of XcpT were not restored in any of these strains, unless the mutant was complemented with the entire minor pilin operon (pVDtacpil or pvpil). This is consistent with previous findings that the MPs form a stable complex required for pilus assembly (Giltner et al, 2010a).

To determine whether MPs are required for XcpT assembly, we analyzed MP mutants in a retraction deficient background. Surface piliation and PilA levels were significantly reduced in *pilV/pilT, pilW/pilT, pilE/pilT* and *algR/pilT* mutants relative to *pilT::*FRT as shown previously, explaining the reduced levels of surface XcpT in these mutants. These results suggest that XcpT assembly into the T4P is solely dependent on the levels of surface PilA. These data are consistent with previous cross-linking studies,

showing XcpT (~ 17 kDa) is able to form a homodimer (~34 kDa) and a heterodimer with PilA (Lu et al, 1997).

4.4 Pseudopilus assembly and secretion are independent processes

To assess whether lack of XcpT on the surface in a *pilA* mutant affects secretion, we examined the secretion of the major T2S protease elastase using skim-milk plate-based assay (Fig 3c). Secretion in NP strain is comparable to WT, suggesting expression of XcpT on the surface is independent of its role in secretion. Regardless, surface expression of XcpT at native levels is indispensible for secretion, suggesting XcpT is potentially a part of two complexes, a periplasmic pseudopilus and T4P (Fig 1).

Previous studies investigating the assembly of XcpT pseudopilus reported that overexpression of XcpT inhibits elastase secretion, because the extension of the resulting pseudopili through the secretin blocks the passage for substrate secretion (Durand et al, 2003). However, using TEM and immunogold analysis, we observed that an *xcpT* mutant complemented with pB*xcpT* (0% ara) expressed pseudopili (Fig 7), yet this strain has normal levels of secretion (Fig 3c). The vector pBADGr has a leaky, arabinose inducible promoter that produces sufficient levels of XcpT at 0% arabinose to complement secretion and induce formation of a pseudopilus in an *xcpT* mutant. This indicates that the pseudopilus assembly and toxin secretion are two independent processes. Vignon *et al.,* (2003) showed previously that a single amino acid mutation (E5V and E5A) in PulG (XcpT homologue in *Klebsiella*) abolishes pullulanase secretion but does not affect pseudopilus assembly (Vignon et al, 2003). Likewise, studies have suggested the T4P

secretin (PilQ) and the Hxc secretin can permit Xcp pseudopilus egress, permitting effector secretion through the XcpQ secretin to continue.

However, as previously reported, at high levels of XcpT (0.1% ara), elastase secretion is inhibited, suggesting at sufficiently high levels, pseudopili block all available XcpQ secretins (Fig 1) (Durand et al, 2003). Alternatively, a significant increase (0.1% ara) in XcpT levels may potentially disturb the normal assembly of the pseudopilus thereby inhibiting toxin secretion. Similar effects are observed in the T4P system, where maintenance of specific stoichiometric ratios of MPs and major pilins is required for the proper function of the T4P system (Giltner et al, 2010a).

4.5 The role of pseudopilins in the expression of XcpT on the surface

Previous studies have reported that of the four minor pseudopilins, only XcpV and XcpX have an observable effect on the formation of the XcpT pseudopilus (Douzi et al, 2009; Durand et al, 2005). To determine whether these pseudopilins affect the surface expression of XcpT under native conditions, we analyzed XcpT surface expression in mutants lacking each of the four minor pseudopilins and in their respective complemented strains (Fig 10). XcpT surface expression was slightly reduced in *xcpU, xcpV* and *xcpW* mutants, but was partially restored upon complementation with the relevant genes at 0.1% arabinose (Fig 10c). Lack of full restoration suggests that the pseudopilins assemble in a specific stoichiometric ratio, that like the MPs if not maintained affects the assembly of the final structure (Giltner et al, 2010a).

Levels of XcpT were slightly reduced in an *xcpV* mutant and significantly higher in an *xcpX* mutant relative to *xcpU* and *xcpW* mutants. These results are consistent with previous studies in Pa that suggested XcpV initiates pseudopilus assembly, because when over-produced in an *xcpV* mutant, XcpT failed to assemble into a pseudopilus (Douzi et al, 2009). In contrast, XcpX in Pa and *Klebsiella oxytoca* (PilK) is postulated to control the length of the pseudopilus (Durand et al, 2005; Sauvonnet et al, 2000; Vignon et al, 2003). Unlike other pseudopilins, XcpX has a large α-domain inserted between β2 and β3 of the C-terminal β-sheet that is proposed to act as an "arrow-head", facilitating the opening of the secretin for substrate secretion. However, the large domain is postulated to block the pseudopilus extension beyond the periplasm, which can be overcome upon overexpression (Durand et al, 2005; Forest, 2008; Korotkov & Hol, 2008b). Therefore, in the absence of XcpX, there is an increase in pseudopilus formation by XcpT or PulG. Our data show that XcpX and XcpV also affect XcpT surface expression under native conditions, indicating these pseudopilins also regulate XcpT incorporation into the pilus (Fig 10). Although this observation suggests the minor pseudopilins are potentially incorporated into the pilus, it is likely that they are incorporated, as a complex, because structural studies suggest specific interactions between the minor pseudopilins is required for the formation of a stable sub-complex. For instance, crystal structures of three minor pseudopilins of *E.coli*, shows the GspI/J/K (XcpV/W/X) complex likely forms at the tip of the pseudopilus, and XcpU and XcpT subunits are incorporated proceeding this complex (Korotkov & Hol, 2008a). Likewise, a structure of EpsI and EpsJ (PilV and PilW homologues in *Vibrio vulnificus*) heterodimers shows complex formation is

facilitated by specific structural features (Yanez et al, 2008) Hence, based on these observations, it is possible that the minor pseudopilins are incorporated into the pilus, but if so, they are likely to be found at the tip of the pilus.

In addition to changes in XcpT levels, we also observed similar changes in the levels of surface PilA. These results suggest that levels of XcpT and PilA on the surface are interdependent. However, the levels of recoverable surface pili did not correlate with twitching motility, since pseudopilin mutants and their complemented strains had equivalent motility zones (Fig 4). These data are consistent with our previous observations that the size of the twitching zones does not reflect the amount of surface pili (Giltner et al, 2010a; Harvey et al, 2009).

Based on these data, we hypothesize that changes in the levels of pseudopilins and PilA affects the interaction between these components in the IM, which consequently affects the assembly of the pilus and the pseudopilus. For example, the absence of XcpV could increase XcpW's affinity for XcpT, such that the interaction between PilA and XcpT is affected, reducing the amount of PilA-XcpT complex in the pilus. This hypothesis is consistent with the previous study showing that over-expression of minor pseudopilins disrupts the PilA-XcpT interaction in the inner membrane (Lu et al, 1997). Since the subunits could be cross-linked prior to PilD cleavage, it is possible the pilins and pseudopilins interact prior to processing and as they are ready to be assembled the processing facilitates their release from the IM.

Based on the structural similarity and potential evolutionary relationship between the two systems, it is likely that the pseudopilins and pilins are recognized by both

systems. Consequently, the minor pseudopilins may assemble into the T4P, thereby facilitating its dynamics.

4.6 XcpR is not required for XcpT surface expression under native conditions

In the absence of the T2SS assembly ATPase XcpR, both intracellular and surface levels of XcpT are substantially reduced relative to WT (Fig 11). Low surface levels suggest that XcpT continues to be incorporated into the pilus, however due to reduced intracellular levels, fewer subunits are being assembled. Overexpression of *xcpT* in an *xcpR* mutant restores both intracellular and surface levels of XcpT, suggesting that XcpR is dispensable for XcpT surface expression. In addition, it also indicates when expressed at chromosomal levels, intracellular XcpT is likely regulated by XcpR, such that *xcpT* expression is reduced in the absence of XcpR. Expressing XcpT in trans, increased both intracellular and extracellular XcpT, possibly due to removal of regulatory control. However, to conform these effects are not due to polar effects on *xcpT* expression, *xcpR* mutant will be complemented with its cognate gene. Regardless, such interplay between the T2SS components was reported in earlier studies. Overexpression of XcpT was shown to destabilize XcpP, whereas overexpression of PulG (XcpT in *K.oxytoca*) destabilized XcpR among other proteins (Durand et al, 2003; Possot et al, 2000). This is suggestive of a potential interplay between the T2S components, explaining reduced levels of XcpT in an *xcpR* mutant.

To examine, whether XcpR is specifically affecting the incorporation of XcpT into the pilus, we studied the XcpT assembly in an *xcpR/pilT* double mutant. The mutant

had increased levels of XcpT on the surface, but intracellular levels were comparable to those of the *xcpR* mutant. This result suggests that loss of retraction leads to increased incorporation of XcpT in the T4P, due to increased levels of PilA. Likewise, this also suggests, XcpR is not required to energize the incorporation of XcpT into the pilus.

4.7 Conclusions/Significance

Structural and functional similarities between the evolutionarily related T4P and T2S systems are suggestive of possible interactions between their components. The major pseudopilin of T2SS, XcpT, is incorporated into the T4P and therefore their levels co-vary, such that changes in levels of surface PilA result in a corresponding change in levels of surface XcpT. Another important finding was that the T2S components also play a role in the assembly of T4P, since changes in minor pseudopilins also affect levels of surface PilA. However, none of these changes had an effect on the functioning of either system (i.e. secretion or twitching) indicating the association simply regulates the amount of intracellular pilins and pseudopilins, indirectly affecting the pilus and pseudopilus assembly. However, to identify the purpose of this interaction, further studies testing the pathogen's virulence, adherence and biofilm formation need to be conducted.

4.8 Future directions

What role do the minor pilins/pseudopilins play in formation of XcpT/PilA fiber?

Based on our observations and previous studies, it is evident that XcpX controls the length of the pseudopilus and XcpV potentially acts as the initiator (Douzi et al, 2009;

Durand et al, 2005). However, we showed they also had an effect on PilA surface expression, suggesting they possibly play a role in the biogenesis of Type IV pilus. This introduces a novel characteristic of T2S minor pseudopilins that should be explored to understanding the functioning of the both systems.

Lack of XcpX increased both surface PilA and XcpT levels suggestive of a role in pilus retraction. However, it is unclear whether XcpX exerts this effect by controlling incorporation of XcpT or both XcpT and PilA into the pilus. Double mutant of *xcpX/xcpT* will be created to identify whether XcpX can exert this effect in the absence of XcpT. Reduced levels of surface PilA in an *xcpX/xcpT* double-mutant would indicate, XcpX specifically interacts with XcpT to control the length/amount of the surface piliation.

In addition, TEM studies should be conducted to confirm whether surface piliation in an *xcpX* mutant is comparable to the hyperpiliated *pilT::*FRT strain (Fig 7). Increased surface piliation has been linked to the formation of dense biofilms and increased adherence (Chiang & Burrows, 2003). Further, Durand *et al*., (2003) reported cells expressing surface pseudopilus (in pilus/flagella deficient strain) are able to form biofilms relative to cells with no pseudopilus (Durand et al, 2003). Based on these results, we hypothesize, by controlling the levels of XcpX, the cell controls the levels of XcpT incorporated into the pilus, and hence the extent of bacterial adherence.

In addition to XcpT, there is a possibility that the minor pseudopilins also get incorporated into the T4P and thereby regulate levels of surface piliation. Similar to XcpT studies, sheared-surface preparations and TEM immunogold labeling will be performed for the four minor pseudopilins.

Structural analysis of XcpT and PilA interaction

To further understand the assembly process and how different components allow the incorporation of XcpT into the pilus, it is important to study the specific interactions between XcpT and PilA and how the pseudopilin fits into the T4P model. Previously, cross-linking studies have reported a possible interaction between the two proteins, in addition to PilA interaction with other pseudopilins (Lu et al, 1997). To confirm and further characterize this interaction, methods to study protein-protein interactions will be employed, such as site-directed mutagenesis, protein-affinity chromatography, co-crystallization or pull-down assays will be used (Phizicky & Fields, 1995).

How does PilA affect T2S?

Preliminary data from our lab reported Pa strains with single-amino acid substitutions in the C-terminal disulfide bonded-loop (DSL) of PilA have altered levels of secretion on skim-milk agar plates (unpublished data, Harvey, H.). This suggests PilA specifically interacts with a component involved in protease secretion. Given our data, there is a possibility that PilA-XcpT interaction regulates secretion under certain conditions. Mutations in the DSL region potentially alter the interaction between PilA and XcpT, such that either the pseudopilus assembly is perturbed or the assembly/retraction cycles are unregulated. To test this hypothesis, surface expression of XcpT and secretion of other substrates of T2SS, such as exotoxin A, phospholipase C and lipase will be analyzed in *pilA* mutant complemented with mutated *pilA* gene in trans.

Does XcpT require processing by PilD for incorporation into the T4P?

Processing of pseudopilins and pilins is essential for the assembly of the putative pseudopilus and T4P (Nunn & Lory, 1991; Nunn & Lory, 1992). To determine whether expression of XcpT on the surface requires processing by PilD, site-directed mutagenesis will be conducted in the N-terminal cleavage site of XcpT. Subsequently, the assembly and expression of XcpT on the surface will be analyzed. This would establish that the incorporation of XcpT into the T4P is similar to PilA or MPs.

What other components influence the formation and expression of XcpT and PilA on the surface?

Ability of both T2S components (pseudopilins and XcpR) and T4P components (MPs and PilT) to facilitate assembly of XcpT PilA fiber suggests other components may also play a role. Incorporation of XcpT in T4P implies that the pilus machinery recognizes the XcpT subunit and incorporates it into the growing pilus and T2SS ATPase is not required. The next step would be to identify additional T2S or T4P system components essential for XcpT surface expression. Sheared-surface protein analysis and TEM/immunogold labeling of T2S and T4P components will be employed to compare the levels of surface XcpT and PilA in mutants of T2SS and T4P system.

The idea that the pseudopilus can use the T4P secretin, PilQ, to exit the cell suggests that pilus fibers containing XcpT may also use the T2S secretin, in addition to PilQ. To study whether the T4P secretin is essential for XcpT surface expression, a single PilQ-deletion mutant and QM/*pilQ* mutants will be created (Durand et al, 2003). As

mentioned previously, the QM lacks two of three possible secretins, and by mutating PilQ, XcpT surface assembly should be completely abolished (Michel et al, 2007). On the contrary, deleting PilQ alone should have the same effect if the XcpT/PilA fiber is using the T4P secretin.

Detection of XcpT in T4P suggests by analogy that the Type IV pilins may be incorporated into the pseudopilus. However, lack of *pilA* does not affect secretion. A plausible explanation could be the MPs are able to substitute for PilA in T2S. Therefore, a strain carrying deletion in major and MP genes will be analyzed for XcpT surface expression and secretion of T2S substrates.

In brief, identifying components involved in the formation of PilA-XcpT containing fiber, will help understand the overlap the between the T4P and T2S systems and their function.

CHAPTER 5 – ROLE OF MINOR PILINS IN SECRETION

5.1 INTRODUCTION

5.1.1 Minor pilins

Minor pilins (MPs) are a set of low-abundance (relative to PilA) proteins that are essential for the assembly of functional pili (Alm et al, 1996; Alm & Mattick, 1995; Alm, 1996; Giltner et al, 2010a; Russell & Darzins, 1994). The MP gene cluster *fimU/pilV/W/X/Y1/Y2/E* is encoded as a polycistronic operon that is positively regulated by AlgR, part of a two-component system AmrZ/AlgR (Belete et al, 2008). In addition to the MP operon, AlgR also regulates synthesis of alginate, a polymer that gives Pa a mucoid phenotype (Belete et al, 2008; Lizewski et al, 2004). A plasmid carrying the MP operon is able to restore twitching motility in an *algR* mutant, suggesting there are no additional AlgR-regulated genes required for T4P assembly (Belete et al, 2008).

Recent studies conducted in *N.gonorrhoeae* showed that the five MPs PilH-L (FimU/PilV/W/X/E homologues, respectively), like the major pilin subunit PilE, are processed by PilD and incorporated into the pilus (Winther-Larsen et al, 2005). Similarly, Pa MPs were shown to be required for twitching motility and reported to be incorporated throughout the pilus (Giltner et al, 2010a). Subsequent studies showed that FimU and PilX potentially act as the key initiators of pilus assembly, such that when overexpressed, shorter pili resulted. On the other hand, overexpression of PilV/W/E inhibits pilus biogenesis. Based on these observations, Giltner *et al.,* (2010) suggested the five MPs form an initiation complex that facilitates assembly, but is dispensable in retraction-deficient backgrounds (Giltner et al, 2010a).

Extensive progress in our understanding of the structural and the functional roles of the MPs has been made. Recently, bioinformatics and functional analyses of the MPs from different strains of Pa revealed that the MP operon and the major pilin gene cluster (*pilABCD)* constitute a pilin island (Giltner et al, 2010b). Proper function of the major pilin requires expression of the corresponding MPs, or MPs from a related group.

Although we have progressed in our understanding of the genetic organization, functional and structural characteristics of these low-abundance proteins, a few studies have suggested MPs (FimU/PilV/W/X/E) are not exclusive to the T4P system and potentially play a role in T2SS (discussed in chapter 1).

5.1.2 Common regulatory pathways controlling T2S and T4P systems

In addition to the structural and functional similarities between pilins and pseudopilins discussed in Chapter 1, there are regulatory pathways connecting the two systems (Fig 3). Pa has two quorum-sensing systems, LasR-LasI and RhlR-RhlI. LasI is an autoinducer synthase gene that synthesizes 3-oxo-C12-HSL (N-[3-oxo-dodecanoyl]-L-homoserine lactone). The *lasR* gene codes for a transcriptional activator protein that binds 3-oxo-C12-HSL and activates genes encoding for virulence factors, including LasB (Fig 3) (Venturi, 2006). Likewise, RhlI synthesizes C4-HSL (N-butyrylhomoserine lactone) which binds transcriptional regulator protein RhlR, and activates transcription of genes involved in both the T2S and T4P systems (Venturi, 2006). Regulation of both systems by the alternative sigma factor RpoN σ^{54} connects the T2S and T4P systems (Potvin et al, 2008). RpoN and the PilR-PilS two-component regulatory systems together are required

for PilA expression (Hobbs et al, 1993). Similar to PilR-PilS, expression of MP operon is regulated by AmrZ, a potential sensor and AlgR, a response regulator (Whitchurch et al, 1996). In addition to regulating MPs, AlgR also represses *rhlR* expression, indirectly controlling the T2SS (Morici et al, 2007).

Our preliminary data suggests that expression levels of MPs modulate the secretion of T2S proteases, such that overexpression of *fimU* and *pilX* lead to increased secretion of elastase on skim-milk agar plates (Fig 1). We propose that MPs may regulate pathways connecting the two systems.

5.2 RESULTS

5.2.1 Overexpression of *fimU* and *pilX* result in increased elastase secretion

Using skim milk plate assays, we showed that inactivation of individual MPs does not alter protease secretion, such that the size of zone of lysis is comparable to WT (Fig. 12). The QM (*xcpP-xcpQ-xphA-xqhA*) lacks the two outer membrane secretins and associated proteins required for T2S, therefore it is negative for secretion (Michel et al, 2007). Complementation of *fimU* and *pilX* mutants with their cognate genes resulted in increased protease secretion, as demonstrated by a larger zone of hydrolysis. On the contrary, *pilV*, *pilW* and *pilE* single mutants complemented with their respective genes had WT levels of secretion.

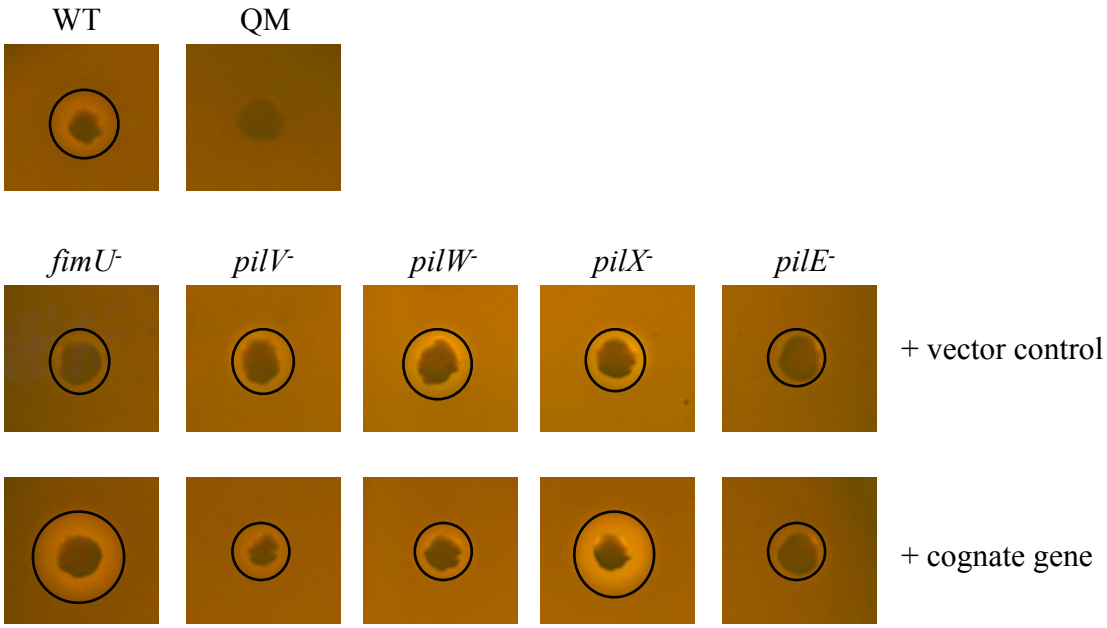

Fig 12: FimU and PilX increase elastase secretion. Minor pilin mutants (*fimU, pilV, pilW, pilX, pilE*) complemented with vector control or their cognate gene in trans were grown on 1.5% skim milk agar plates. Protease secretion was measured by observing the relative size of zone of lysis around the colony. QM was used as a negative control. Elastase secretion in minor pilin mutants is comparable to WT. However, expression of *fimU* and *pilX* from a leaky promoter in their respective mutants increases secretion.

5.2.2 Minor pilins are not incorporated into the pseudopilus

We hypothesized that incorporation of specific MPs into the putative pseudopilus may allow them to facilitate elastase secretion. To determine whether MPs are incorporated into the pseudopilus, *xcpT* was overproduced in a NP background to form a pseudopilus on the cell surface (Fig 13). Recall, *xcpT* is cloned into pBADGr that has a leaky promoter, producing more than native levels of XcpT. Subsequently, surface appendages (flagella, pili or pseudopili) were sheared and probed with anti-PilA, anti-XcpT, anti-FimU or anti-PilW antibodies (the only MP antibodies available at the time of this study). Absence of immunoreactive bands for FimU or PilW in sheared-surface fractions of NP + *xcpT* cells suggested that the MPs are not incorporated into the pseudopili (Fig 14). FimU and PilW MPs were detected only in WT sheared surface preparation, as expected since T4P pili are assembled allowing expression of MPs on the surface.

To confirm these results, NP cells over-expressing *xcpT* were subjected to TEM immunogold labeling with anti-FimU or anti-PilA antisera (Fig 14). Absence of PilA or FimU labeling along the pseudopili further conforms that there is unlikely to be physical cross talk between the pilins and the pseudopilins. As a control, both WT and NP vector controls were subjected to labeling with anti-PilA, and as expected labeling was observed only on WT cells expressing surface pili (Fig 14).

Based on these studies, we proposed that the MPs do not become physically incorporated into the pseudopilus but that they might affect regulatory pathways controlling expression of T2SS components, including the pseudopilus.

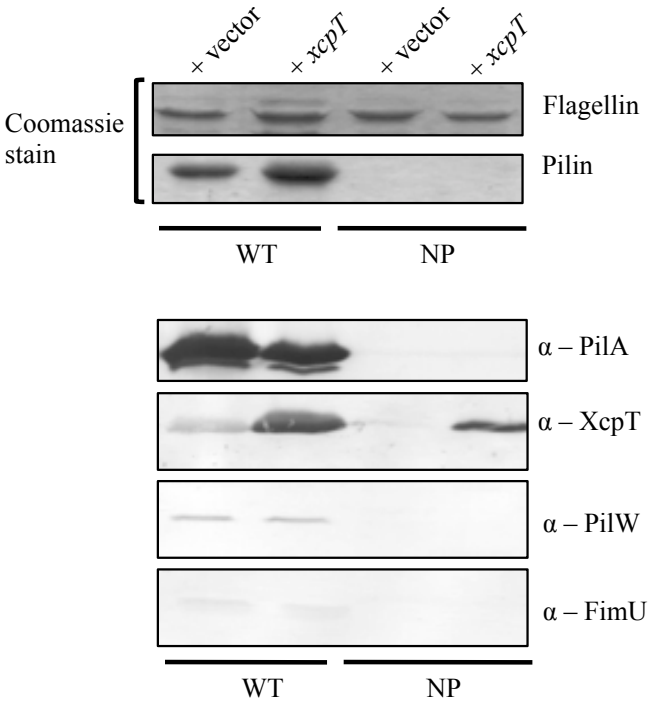

Fig 13: Minor pilins are not incorporated into the pseudopilus. WT or NP (*pilA* mutant) with either vector (pBADGr) or pB*xcpT* were grown on solid media supplemented with 0.2% Arab. Proteins sheared off of the surface were separated on the SDS-PAGE and subsequently immunoblotted. Overexpression of the *xcpT* results in surface exposed pseudopilus that would otherwise remain in the periplasm. Absence of PilW and FimU immunoreactive bands in the sheared surface preparation of NP + *xcpT* indicates the MPs are not incorporated into the pseudopilus. Decrease in surface piliation upon overexpression of *xcpT* in WT is evident in the Coomassie stained gel. Flagellin band was used as a loading control.

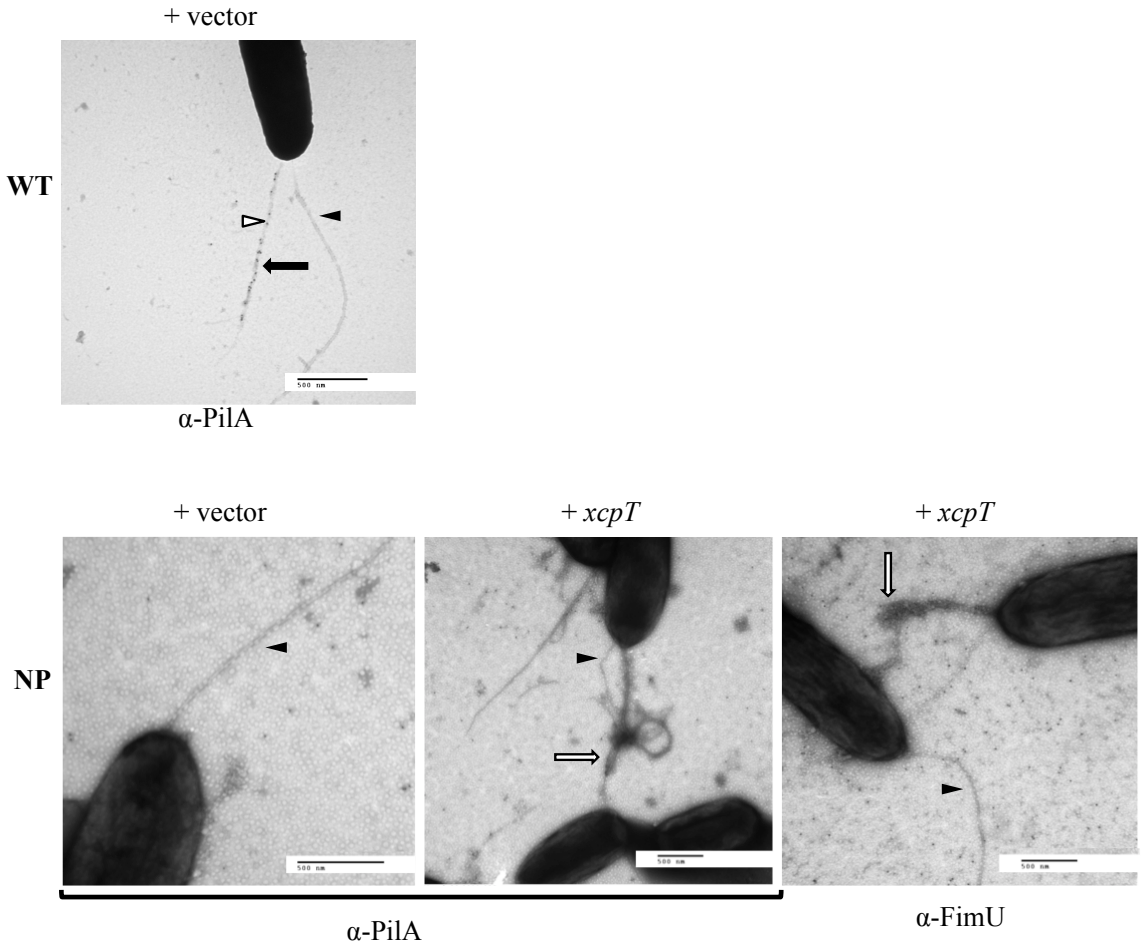

Fig 14: Type IV minor pilins are not incorporated into the T2SS pseudopilus. TEM and immunogold TEM images of PA01 wild type (WT) and NP (*pilA::Tn*), *xcpT::Tn* using anti-XcpT, anti-PilA and anti-FimU antibodies and a secondary gold-conjugated anti-body. No PilA or FimU labeling was detected in the pseudopilus formed in a NP + pB*xcpT* cells expressing surface-pseudopili. Grids were negatively stained with uranyl acetate and examined with a JEOL JEM 1200 TEMSCAN microscope. AmtV600 software was used to capture digital images. Pointers: Flagella ▲, Pili ⇧, Labelled protein △, Pseudopilus ⬆

5.2.3 Minor pilins do not regulate elastase expression

One reason that *fimU* and *pilX*-overexpressing strains have larger zones of hydrolysis on skim milk plates could be due to increased expression of the effector, elastase, potentially owing to the common regulatory pathways controlling both T2S and T4P systems (Fig 15). To determine whether MPs regulate *lasB* (elastase) expression, a lux-reporter assay was developed (Duan, 2003; Rust et al, 1996). The *lasB* promoter sequence was cloned into pMS402 carrying a promoterless *luxCDABE* cassette to create pMSL and introduced into WT, *algR,* single MP mutants and complemented MP mutants. Reporter activity was measured for ~ 14 h and reported as relative light units (RLU) divided by OD_{600}.

Promoter activation in WT cells peaked at approximately 5 h under the growth conditions used (Fig 16), corresponding to the beginning of stationary phase. This result is consistent with the findings showing quorum-system regulated genes, which includes the T2SS components and substrates are expressed at the early-stationary phase (Medina et al, 2003). Relative to WT, magnitude of activation in an *algR* mutant was roughly twice that of WT (Fig 16).

Activation in the MP mutants complemented with either the empty vector or their relevant gene is comparable to WT (Fig 16). Complementation of the *fimU* mutant with its cognate gene (U+U pMSL), drastically reduced promoter activity, which is in contrast to the hyper-secretion phenotype observed on plates (Fig 12). However this particular strain grew poorly for unknown reasons, explaining reduced promoter activity (Fig 16). Based on these results it is unlikely that the MPs are regulating elastase expression.

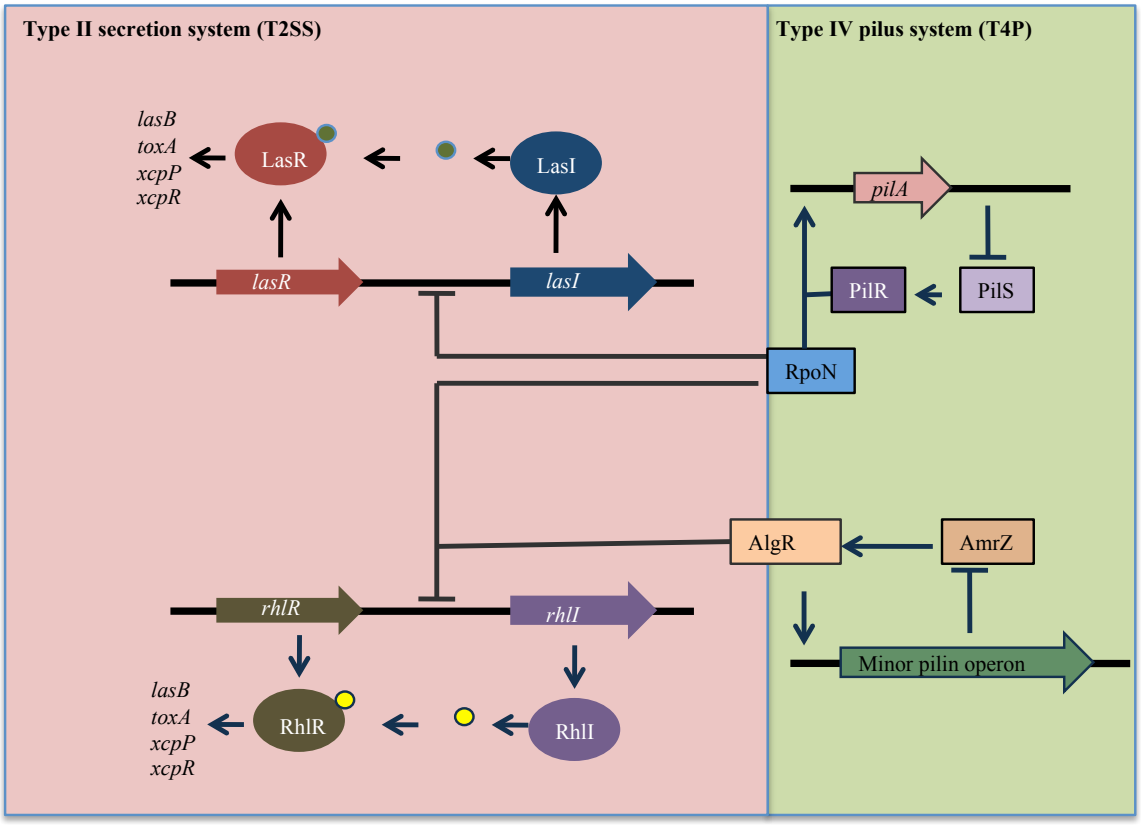

Fig 15: Common regulatory pathways connect the T4P and the T2S systems. Flow chart illustrating the interconnection between the quorum sensing systems (LasR/I and RhlR/I) controlling the T2SS and the sigma factor RpoN, PilR/S pathway regulating PilA expression. LasI encodes for 3-oxo-C12-HSL (N-[3-oxodode- canoyl]-L-homoserine lactone) that upon binding to LasR results in self-induction and expression of T2SS components and exoproteins. Similarly, RhlI synthesizes C4-HSL (N-butyrylhomoserine lactone) which binds to the transcriptional regulator RhlR and subsequently activates transcription of the T2SS components and exoproteins. RpoN σ^{54} in conjunction with PilR/S two component regulatory system positively regulates PilA transcription. RpoN also negatively regulates *lasI* and *rhlI* expression. Expression of minor pilin operon is regulated by two-component regulatory system AmrZ/AlgR. Also, AlgR negatively regulates RhlI expression. A proposed role for AmrZ/AlgR in the T2S and T4P system is illustrated.

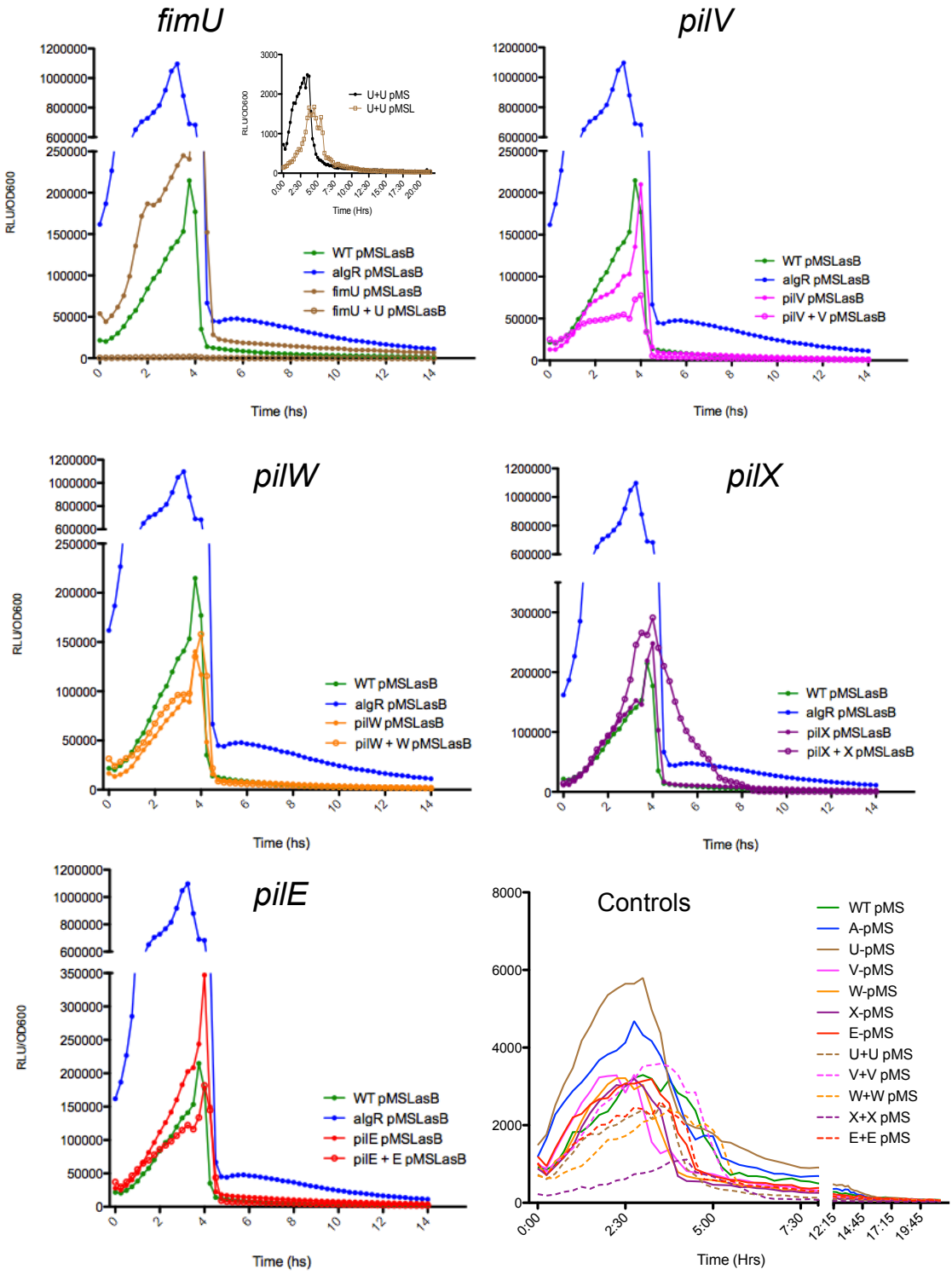

Fig 16: Minor pilins have no effect on *lasB* expression

Fig 16: Minor pilins have no effect on *lasB* expression. The *algR,* the minor pilin mutant(s) and their respective complemented strains were transformed with *lasB* promoter in a lux-reporter plasmid (pMS402). Strains were grown for 16h at 37°C and subsequently sub-cultured to OD_{600} = 0.2. The standardized cultures were used for the assay and samples were measured in triplicates. Promoter activity is reported as a ratio of relative light units to OD_{600}, and was measured for ~ 24 hrs with Synergy 4 Microplate reader, BioTek. An increase in promoter activity is observed in an *algR* mutant relative to WT. Activity in MP mutants complemented with vector control or their cognate genes is comparable to WT.

5.2.4 PA14 minor pilins cannot reproduce phenotypes observed upon complementation with cognate gene

To determine whether the secretion phenotypes observed were specific to a subset of MPs, single PAO1 MP mutants were complemented with their respective PA14 MP genes (encoded on pBADGr) in trans (Fig 17). The level of elastase in the supernatant (extracellular E/C) was measured by Western blot with an anti-LasB antibody (Fig 17). QM that is completely devoid of elastase secretion was used as a negative control (Michel et al, 2007). In the absence of any of the five MPs, elastase secretion was unaffected, as it is comparable to WT levels (Fig 17). Upon complementation with the cognate gene in trans, an increase in extracellular elastase was observed in both *fimU* and *pilX* mutants (Fig 17). This is consistent with the plate-based studies indicating overexpression of *fimU* or *pilX* results in a hyper-secretion phenotype (Fig 12).

Interestingly, in the absence of AlgR an increase in elastase secretion is observed in PAO1, which is consistent with the increased *lasB* promoter activity in this strain (Fig 16). However, complementation with the MP operon in trans did not restore extracellular elastase to WT levels (Fig 17b). On the contrary, elastase secretion in PA14 *algR* mutant was relative to WT.

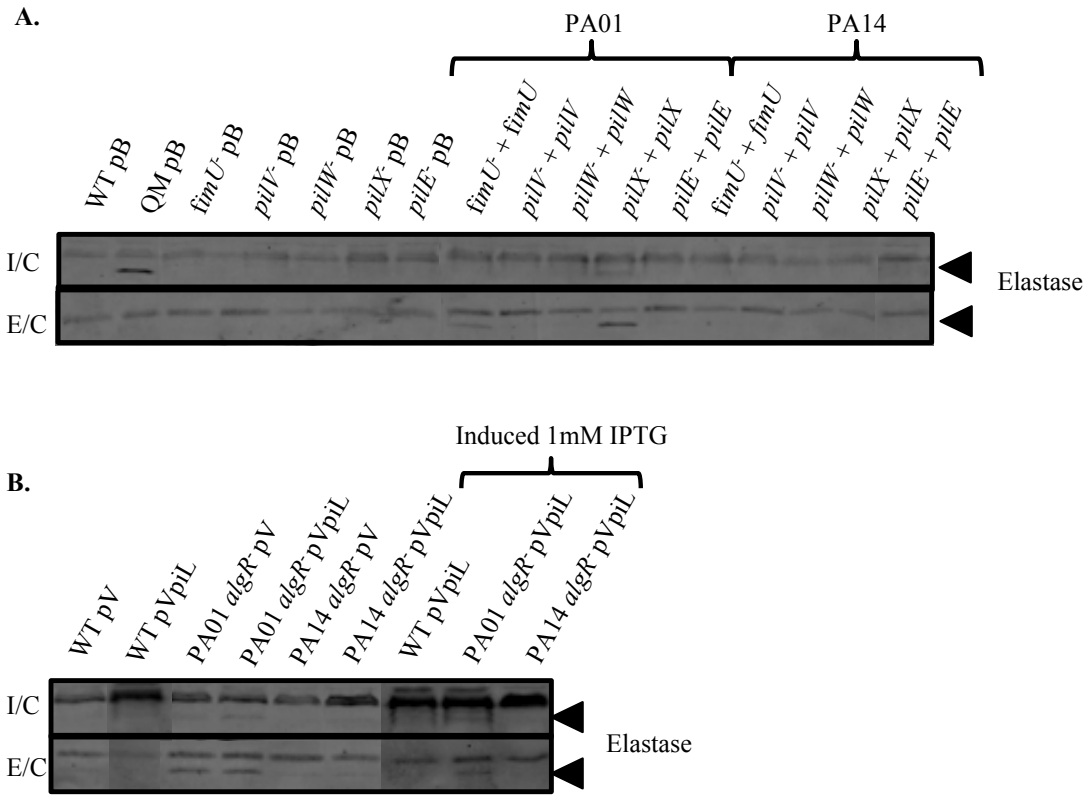

Fig 17: Group III (PA14) minor pilins do not complement Group II (PA01) minor pilin mutants. Overexpression of FimU and PilX increases elastase secretion. PA01 single minor pilin mutants were complemented with their cognate gene or their PA14 homologue in an pBADGrand evaluated for elastase secretion. Strains of interest were grown in liquid LB media for 16 hrs and subsequently sub-cultured (0% ara) and standardized to OD_{600} = 0.6. Cell pellet (intracellular I/C) and supernatant (extracellular E/C) fractions were probed with anti-LasB antisera. QM was used as a negative control, which has accumulated protein in the I/C fraction. Elastase secretion in minor pilin mutants with vector or their PA14 homologue is comparable to WT. Complementation of FimU or PilX increases secretion drastically comparing to WT. Lack of AlgR increases elastase secretion this is not restored upon complementation with the minor pilin operon in IPTG inducible vector (pVpil). PA14 *algR* mutant does not display the same phenotype. Arrows point to the elastase immunoreactive band. Minor pilin expression was further induced with 1mM IPTG.

5.3 DISCUSSION

The structural and functional similarities between the two systems are suggestive of a potential cross talk among their components. Previous studies and our preliminary data showed the specific MPs potentially modulate T2SS-mediate secretion. In chapter one, we showed the major pseudopilin XcpT is incorporated into the T4P fiber, raising the possibility that the MPs may also be incorporated into the pseudopilus, directly modulating secretion. Although only limited literature describing how the T2SS recognizes its substrates is available, it is thought that the process involves substrate interaction with the pseudopilus and the OM secretin, XcpQ (Filloux et al, 1998; Forest, 2008; Reichow et al, 2010). Therefore, we hypothesized that physical incorporation of the MPs into the pseudopilus could facilitate secretion of select substrates. However, Western blot analysis of sheared pseudopili generated by XcpT over expression showed that the MPs FimU and PilW are not detectable, suggesting three possibilities: either the pseudopilus is made solely of XcpT; MPs are not incorporated into the pseudopili, or they are incorporated but at levels below the detection limit of the technique. Consequently, TEM and immunogold labeling were performed and they showed no evidence for MPs in the pseudopilus preparations (Fig 14).

Based on these observations, an alternative hypothesis was proposed, that the MPs instead regulate the expression of the T2SS substrates, through common regulatory pathways (Fig 15). Using the lux-reporter assay, we showed that the MPs do not affect elastase expression. The magnitude and kinetics of promoter activity in MP mutants complemented with vector control or their cognate gene was comparable to levels

observed in the WT strain (Fig 16). However, as expected, *lasB* promoter activation in an *algR* mutant was relatively higher due to relief of inhibition of *lasB* expression (Fig 15). This result is consistent with the previous findings showing that AlgR represses Rhl quorum-sensing system that in turn activates the expression of the T2SS components, *xcpP*, *xcpR* and *lasB* (Morici et al, 2007).

MPs neither physically affect substrate secretion nor regulate gene expression. However, based on our previous observations that FimU and PilX affect XcpT surface expression, we propose that the MPs are possibly regulating the composition and functioning of the pseudopilus. Structural and functional studies have shown, the pseudopilus interacts with the substrate in the periplasm and upon extension, allows it to exit through the secretin (Forest, 2008). Therefore, as discussed earlier if MPs interact with the pseudopilins in the inner membrane, they may potentially affect the ratio of minor pseudopilins or XcpT incorporated into the pilus. As result, they may indirectly, through an influence on the pseudopilus composition, regulate the amount of substrate secreted.

In addition, preliminary studies performed by C.Giltner showed over-expression of *fimU* or *pilX* does not affect secretion of other T2SS-substrate, including lipase, exotoxin A nor hemolytic phospholipase C secretion. These results further support our proposed rationale that the MPs potentially affect substrate secretion by modulating the pseudopili composition and dynamics.

Cross-complementation data further suggests the MPs are playing a structural role rather than a regulatory one, in mediating T2S. Upon complementation of *fimU* and *pilX*

mutants with their cognate gene in trans, an increase in elastase secretion was observed (Fig 17). However, heterologous expression of PA14 *fimU* and *pilX* genes did not give the hyper-secretion phenotype, suggesting FimU and PilX play a structural role in mediating elastase secretion. This is consistent with the earlier plate-based studies indicating overexpression of *fimU* or *pilX* in their native background results in a hyper-secretion phenotype (Fig 12). On the other hand, *pilV, pilW* and *pilE* mutants complemented with vector control or their cognate gene had WT levels of secretion, consistent with the plate-based assays (Fig 12). Interestingly, the same sort of phenotypic division (FimU/PilX and PilV/W/E) of the five MPs was observed with regards to the T4P function (Giltner et al, 2010a). Overexpression of *fimU* and *pilX* was shown to inhibit twitching motility and surface piliation, whereas PilV/W/E were shown to be essential for pilus assembly.

Interestingly, in the absence of AlgR an increase in elastase secretion is observed in PAO1, which is not restored to WT levels upon complementation with PAO1 MP operon in trans (Fig 17b). This suggests the increase in secretion in an *algR* mutant is not due to reduction in the MPs, but instead due to an increase in *lasB* expression (Fig 15). This is consistent with our previous findings suggesting secretion is not affected in the absence of individual MPs (Fig 12,17). Further, this result is also consistent with the lux-reporter assay showing that in the absence of *algR*, *lasB* promoter activity is markedly increased (Fig 16). Interestingly, elastase secretion was comparable to WT in PA14 *algR* mutant, and it did not increase upon complementation with PAO1 MP operon under uninduced or induced (1mM) conditions. These results suggest that the MPs are playing a group-specific and a structural role in mediating secretion. An undetectable level of

elastase in WT PA14 supernatant suggests relative to PAO1 the strain secretes very low levels of elastase. However, to determine whether PA14 MPs, specifically FimU and PilX, effect elastase secretion, single MPs will be over-expressed in the WT background.

Based on our study, it is evident that the MPs are playing a role in secretion mediated by T2SS, specifically FimU and PilX. Further, preliminary data by C.Giltner and this study shows MPs affect secretion of only select substrates, mainly the secretion of elastase. Secondly, it is evident they are neither getting incorporated into the pseudopilus, nor regulating the expression of elastase. Hence, we propose two hypotheses: MPs are physically interacting with the pseudopilins in the IM, which may potentially affect their interactions with other pseudopilins hence indirectly affecting the composition of the assembled pseudopili. Recall, Lu *et al*., (1997) showed over-expression of pseudopilins disrupts the interaction between XcpT and PilA in the IM, supporting our hypothesis that interactions between pilins and pseudopilins may affect their interactions with their own system (Lu et al, 1997). Alternatively, they could be regulating the expression of T2SS components, under the control of quorum-sensing systems (Fig 15).

Our study has identified a novel relationship between the two of the major virulence systems in *P.aeruginosa*. Prior to this study, prepilin peptidase PilD was the only known shared-component between the two systems. However, identification of MPs in T2SS suggests that there is more overlap between the two systems than previously appreciated. Further characterization of the role of MPs in T2SS will help in the understanding of the T2SS-mediated secretion process as well as their role in T4P system.

Clarification of the nature of the overlap and roles of specific components will point to common points of vulnerability and inform a rational design of comprehensive pilus-based vaccines.

5.3.2 Future directions

Do minor pilins interact with pseudopilins in the inner membrane?

Our results suggest MPs are physically regulating elastase secretion and based on our previous observations (Chap 1), they are likely interacting with the pseudopilins in the inner membrane. To determine whether the MPs interact with the pseudopilins in the inner-membrane, cross-linking studies, similar to the ones conducted by Lu *et al.,* (1997) will be employed. Subsequently, site-directed mutagenesis will be utilized to disrupt these interactions and analyze the resulting effect on secretion. These studies would help understand if MPs modulate the composition of the pseudopili, then how is this process initiated.

Do minor pilins affect the expression of other T2SS components?

Model illustrating the pathways, which connect the T4P and T2S systems, suggest expression of *xcpP* and *xcpR* are also controlled by the quorum-sensing systems. To determine whether their expression is affected by changes in the levels of MPs, *lux*-reporter assay will be employed.

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CHAPTER 7 – APPENDIX

7.1 Supplementary figure

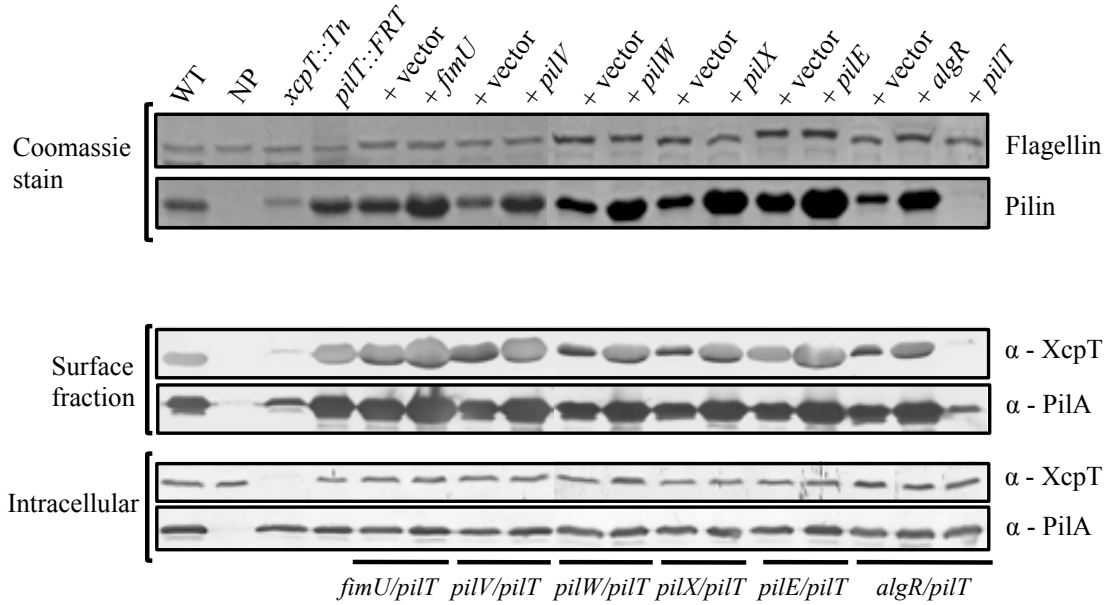

Fig S1: Minor pilins are not required for surface expression of XcpT. XcpT assembly on the surface was assessed by mutating PilT in individual minor pilin mutants. Sheared-surface proteins from WT, NP, *xcpT*::Tn, *pilT*::FRT and minor pilin/*pilT* double mutants were separated on SDSPAGE gel and stained with Coomassie brilliant blue or probed with anti-PilA and anti-XcpT antiserum. Surface PilA levels are reduced in *pilV/pilT*, *pilW/pilT* and *pilX/pilT* and relative decrease in XcpT in these strains is observed. XcpT or PilA surface expression in *fimU/pilT* and *pilE/pilT* is comparable to the *pilT* mutant. Intracellular PilA and XcpT levels are equivalent in all strains.

7.2 Figures and Tables

Table 1.0 Bacterial strains and constructs

Strain or plasmid	Relevant characteristic(s)	Source or reference
Plasmids		
pBADGr	pMLBAD backbone with dhfr (trimethoprim resistance) replaced with aacC1 (gentamicin resistance)	(Asikyan et al, 2008)
pBADGr*pilA*$_{II}$	PAO1 *pilA* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*fimU*	mPAO1 *fimU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*pilV*	mPAO1 *pilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*pilW*	mPAO1 *pilW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*pilX*	mPAO1 *pilX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*pilE*	mPAO1 *pilE* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010a)
pBADGr*fimU/pilV*	PAO1 *fimUpilV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010b)
pBADGr*fimU/pilV/W*	PAO1 *fimUpilVW* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010b)
pBADGr*fimU/pilV/W/X*	PAO1 *fimUpilVWX* sequence inserted with EcoRI and PstI restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010b)
pBADGr*algR*	mPAO1 *algR* sequence inserted with XbaI and HindIII restriction enzymes in the MCS of pBADGr	(Giltner et al, 2010b)
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)

pBADGr*xcpT*	mPAO1 *xcpT* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*xcpU*	mPAO1 *xcpU* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*xcpV*	mPAO1 *xcpV* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*xcpW*	mPAO1 *xcpW* sequence inserted with NcoI and HindIII restriction enzymes in the MCS of pBADGr	This study
pBADGr*xcpX*	mPAO1 *xcpX* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	This study
pUCP20Gm	pUCP20 with SmaI-flanked Gm cassette inserted into unique ScaI site within bla	(Chiang & Burrows, 2003)
pUCP20*pilT*	mPAO1 *pilT* sequence inserted with BamHI and HindIII restriction enzymes in the MCS of pUCP20Gm	(Chiang & Burrows, 2003)
pFLP2	Source of the Flp recombinase used to excise the FRT-flanked Gm resistance cassette following mutagenesis	(Hoang et al, 1998)
pEX18AP + *pilT*::GmFRT	Gentamicin FRT insertion in the EcoRV site	(Asikyan et al, 2008)
pBADGr$pilA_{PAK}$	PAK *pilA* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Asikyan et al, 2008)
pBADGr$pilA_{5196}$	Pa5196 *pilA* sequence inserted with EcoRI and HindIII restriction enzymes in the MCS of pBADGr	(Kus et al, 2008)
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE* gene; ori of pRO1614	(Duan & Surette, 2007)
pMSL	mPAO1 *lasB* promoter sequence (331bp upstream of start site plus 29 bps following start site) cloned in BamHI-XhoI site of pMS402	This study

***E.coli* strains**

DH5α	Genotype: F′ Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK−mK+)phoA supE44 lambda− thi-1	Invitrogen
SM10	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 strains**

mPAO1	Laboratory strain – Group II	(Jacobs et al, 2003)
mPAO1 *pilA*::Tn	ISphoA/hah transposon insertion (position 165)	(Jacobs et al, 2003)
mPAO1 *fimU*::Tn	IS*lacZ*/hah transposon insertion (position 237)	(Jacobs et al, 2003)
mPAO1 *pilV*::Tn	IS*phoA*/hah transposon insertion (position 122)	(Jacobs et al, 2003)
mPAO1 *pilW*::Tn	IS*lacZ*/hah transposon insertion (position 381)	(Jacobs et al, 2003)
mPAO1 *pilX*::Tn	IS*phoA*/hah transposon insertion (position 182)	(Jacobs et al, 2003)
mPAO1 *pilE*::Tn	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
mPAO1 *pilT*::FRT	Wild type strain with FRT insertion in the NruI site within *pilT*	(Giltner et al, 2010a)
ΔPQP_AQ_A	Quadruple deletion mutant: *xcpP-xcpQ-xphA-xqhA*	(Jacobs et al, 2003)
mPAO1 *xcpR*::Tn	IS*lacZ*/hah transposon insertion (position 1509)	(Jacobs et al, 2003)
mPAO1 *xcpT*::Tn	IS*lacZ*/hah transposon insertion (position 271)	(Jacobs et al, 2003)
mPAO1 xcpU::Tn	IS*phoA*/hah transposon insertion (position 433)	(Jacobs et al, 2003)
mPAO1 *xcpV*::Tn	IS*lacZ*/hah transposon insertion (position 73)	(Jacobs et al, 2003)
mPAO1 *xcpW*::Tn	IS*lacZ*/hah transposon insertion (position 17)	(Jacobs et al, 2003)

mPAO1 *xcpX*::Tn	IS*lacZ*/hah transposon insertion (position 270)	(Jacobs et al, 2003)
mPAO1 *xcpT*::Tn + pBADGr*xcpT*	IS*lacZ*/hah transposon insertion (position 271) complemented with cognate gene in pBADGr	This study
mPAO1 *xcpU*::Tn + pBADGr*xcpU*	IS*phoA*/hah transposon insertion (position 433) complemented with cognate gene in pBADGr	This study
mPAO1 *xcpV*::Tn + pBADGr *xcpV*	IS*lacZ*/hah transposon insertion (position 73) complemented with cognate gene in pBADGr	This study
mPAO1 *xcpW*::Tn + pBADGr*xcpW*	IS*lacZ*/hah transposon insertion (position 17) complemented with cognate gene in pBADGr	This study
mPAO1 *xcpX*::Tn + pBADGr *xcpX*	IS*lacZ*/hah transposon insertion (position 270) complemented with cognate gene in pBADGr	This study
mPAO1 *fimU*::Tn + pBADGr*fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilV*::Tn + pBADGr*pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 Tn::*pilW* + pBADGr*pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilX*::Tn + pBADGr*pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilE*::Tn + pBADGr*pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
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 + pBADGr*pilA*	Wild type strain with mPAO1 *pilA* under arabinose control in pBADGr	(Giltner et al, 2010a)
mPAO1 + pBADGr*xcpT*	Wild type strain with mPAO1 *xcpT* under arabinose control in pBADGr	This study
mPAO1 *pilA*::Tn + pBADGr*pilA*	IS*phoA*/hah transposon instertion (position 165) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)

mPAO1 *xcpT*::Tn + pBADGr*xcpT*	IS*phoA*/hah transposon instertion (position 165) complemented with cognate gene in pBADGr	This study
mPAO1 *pilA*::Tn + pBADGr*xcpT*	IS*phoA*/hah transposon instertion (position 165) with *xcpT* under arabinose control in pBADGr	This study
mPAO1 *fimU*::Tn + pBADGr*fimU*	IS*lacZ*/hah transposon insertion (position 237) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilV*::Tn + pBADGr*pilV*	IS*phoA*/hah transposon insertion (position 122) complemented with cognate gene in pBADGr	(Giltner *et al.*, 2010)
mPAO1 *pilW*::Tn + pBADGr*pilW*	IS*lacZ*/hah transposon insertion (position 381) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilX*::Tn + pBADGr*pilX*	IS*phoA*/hah transposon insertion (position 182) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilE*::Tn + pBADGr*pilE*	IS*phoA*/hah transposon insertion (position 183) complemented with cognate gene in pBADGr	(Giltner et al, 2010a)
mPAO1 *algR*::FRT + pBADGr*fimUpilV*	Wild type strain with FRT scar insertion in the NruI site within *algR,* complemented with mPAO1 *fimU/pilV*	(Giltner et al, 2010b)
mPAO1 *algR*::FRT + pBADGr*fimUpilVW*	Wild type strain with FRT scar insertion in the NruI site within *algR,* complemented with mPAO1 *fimU/pilV/W*	(Giltner et al, 2010b)
mPAO1 *algR*::FRT + pBADGr*fimUpilVWX*	Wild type strain with FRT scar insertion in the NruI site within *algR,* complemented with mPAO1 *fimU/pilV/W/X*	(Giltner et al, 2010b)
mPAO1 + pVDTac39	mPAO1 with pVDTac39	(Giltner et al, 2010b)
mPAO1 + pVDTacpil	mPAO1 with pVDTac39	(Giltner et al, 2010b)
mPAO1 *algR*::FRT + pVDTac39	Wild type strain with FRT scar insertion in the NruI site within *algR,* with pVDTac39	(Giltner et al, 2010b)
mPAO1 *algR*::FRT + pVDTacpil	Wild type strain with FRT scar insertion in the NruI site within *algR,* with pVDTacpil	(Giltner et al, 2010b)
mPAO1 *fimU*::Tn + pBADGr*fimU*$_{III}$	IS*lacZ*/hah transposon insertion (position 237) complemented with PA14 *fimU* gene in pBADGr	(Giltner et al, 2010b)

mPAO1 *pilV*::Tn + pBADGr*pilV*$_{III}$	IS*phoA*/hah transposon insertion (position 122) complemented with PA14 *pilV* gene in pBADGr	(Giltner et al, 2010b)
mPAO1 *pilW*::Tn + pBADGr*pilW*$_{III}$	IS*lacZ*/hah transposon insertion (position 381) complemented with PA14 *pilW* gene in pBADGr	(Giltner et al, 2010b)
mPAO1 *pilX*::Tn + pBADGr*pilX*$_{III}$	IS*phoA*/hah transposon insertion (position 182) complemented with PA14 *pilX* gene in pBADGr	(Giltner et al, 2010b)
mPAO1 *pilE*::Tn + pBADGr*pilE*$_{III}$	IS*phoA*/hah transposon insertion (position 183) complemented with PA14 *pilE* gene in pBADGr	(Giltner et al, 2010b)
mPAO1 *pilV*::Tn - *pilT*::FRT	IS*phoA*/hah transposon insertion (position 122), with an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010a)
mPAO1 *pilW*::Tn - *pilT*::FRT	IS*lacZ*/hah transposon insertion (position 381), with an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010a)
mPAO1 *pilX*::Tn - *pilT*::FRT	IS*phoA*/hah transposon insertion (position 182), with an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010a)
mPAO1 *pilE*::Tn - *pilT*::FRT	IS*phoA*/hah transposon insertion (position 183), with an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010a)
mPAO1 *algR*::FRT - *pilT*::FRT	FRT scar insertion in the NruI site within *algR* and an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010a)
mPAO1 *fimU*::Tn - *pilT*::FRT + pBADGr*fimU*	IS*lacZ*/hah transposon insertion (position 237), with an FRT scar in the NruI site within *pilT* complemented with *fimU* in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilV*::Tn - *pilT*::FRT + pBADGr*pilV*	IS*phoA*/hah transposon insertion (position 122), with an FRT scar in the NruI site within *pilT* complemented with *pilV* in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilW*::Tn - *pilT*::FRT + pBADGr*pilW*	IS*lacZ*/hah transposon insertion (position 381), with an FRT scar in the NruI site within *pilT* complemented with *pilW* in pBADGr	(Giltner et al, 2010a)
mPAO1 *pilX*::Tn - *pilT*::FRT + pBADGr*pilX*	IS*phoA*/hah transposon insertion (position 182), with an FRT scar in the NruI site within *pilT* complemented with *pilX* in pBADGr	(Giltner et al, 2010a)

mPAO1 *pilE*::Tn - *pilT*::FRT + pBADGr*pilE*	IS*phoA*/hah transposon insertion (position 183), with an FRT scar in the NruI site within *pilT,* complemented with *pilE* in pBADGr	(Giltner et al, 2010a)
mPAO1 *algR*::FRT - *pilT*::FRT	FRT scar insertion in the NruI site within *algR* and an FRT scar in the NruI site within *pilT*	(Giltner et al, 2010b)
mPAO1 *algR*::FRT - *pilT*::FRT + pBADGr*algR*	FRT scar insertion in the NruI site within *algR* and an FRT scar in the NruI site within *pilT,* complemented with *algR* in pBADGr	(Giltner et al, 2010b)
mPAO1 *xcpR*::Tn - *pilT*::FRT	IS*lacZ*/hah transposon insertion (position 1509) and an FRT scar in the NruI site within *pilT*	This study
mPAO1 *xcpR*::Tn - *pilT*::FRT + pUCP20*pilT*	IS*lacZ*/hah transposon insertion (position 1509) and an FRT scar in the NruI site within *pilT,* complemented with *pilT* in pUCP20Gm	This study
mPAO1 *xcpR*::Tn - *pilT*::FRT + pBADGr*xcpT*	IS*lacZ*/hah transposon insertion (position 1509) and an FRT scar in the NruI site within *pilT,* with *xcpT* gene in pBADGr	This study
mPAO1 + pMSL	mPAO1 with pMSL	This study
mPAO1 *pilA*::Tn + pMSL	ISphoA/hah transposon insertion (position 165) with pMSL	This study
mPAO1 *fimU*::Tn + pMSL	IS*lacZ*/hah transposon insertion (position 237) with pMSL	This study
mPAO1 *pilV*::Tn + pMSL	IS*phoA*/hah transposon insertion (position 122) with pMSL	This study
mPAO1 *pilW*::Tn + pMSL	IS*lacZ*/hah transposon insertion (position 381) with pMSL	This study
mPAO1 *pilX*::Tn + pMSL	IS*phoA*/hah transposon insertion (position 182) with pMSL	This study
mPAO1 *pilE*::Tn + pMSL	IS*phoA*/hah transposon insertion (position 183) with pMSL	This study
mPAO1 *algR*::FRT + pMSL	Wild type strain with FRT scar insertion in the NruI site within *algR* with pMSL	This study
mPAO1 *fimU*::Tn + pBADGr*fimU* + pMSL	ISlacZ/hah transposon insertion (position 237) complemented with cognate gene in pBADGr with pMSL	This study
mPAO1 *pilV*::Tn + pBADGr*pilV* + pMSL	ISphoA/hah transposon insertion (position 122) complemented with cognate gene in pBADGr with pMSL	This study

mPAO1 *pilW::*Tn + pBADGr*pilW* + pMSL	ISlacZ/hah transposon insertion (position 381) complemented with cognate gene in pBADGr with pMSL	This study
mPAO1 *pilX::*Tn + pBADGr*pilX* + pMSL	ISphoA/hah transposon insertion (position 182) complemented with cognate gene in pBADGr with pMSL	This study
mPAO1 *pilE::*Tn + pBADGr*pilE* + pMSL	ISphoA/hah transposon insertion (position 183) complemented with cognate gene in pBADGr with pMSL	This study

***P.aeruginosa* – Additional strains**

PA14	Clinical isolate – Group III	(Rahme et al, 1995)
Pa1244	Clinical isolate – Group I	P. Castric
PAK	Laboratory strain – Group II	J. Boyd
Pa5196	Rectal isolate – Group IV	(Kus et al, 2004)
Pa1457	Laboratory strain – Group V	(Kus et al, 2004)
PAK *pilA*::Tc^r	Tet^R cassette inserted into PAK *pilA* gene	(Watson et al, 1996)
Pa5196 *pilA::*FRT	PA14 strain with FRT scar insertion in the *pilA*	(Watson et al, 1996)
PAK *pilA*::Tc^r + pBADGr*pilA*	PAK *pilA* mutant complemented with its cognate gene	(Asikyan et al, 2008)
Pa5196 *pilA::*FRT + pBADGr*pilA*	Pa5196 *pilA* mutant complemented with its cognate gene	(Kus et al, 2008)
PA14 *algR*::FRT	PA14 strain with FRT scar insertion in the NruI site within *algR*	(Giltner et al, 2010b)
PA14 *algR*::FRT + pBADGr*algR*	PA14 *algR* mutant complemented with its cognate gene	(Giltner et al, 2010b)
PA14 *algR*::FRT + pVDTac39	PA14 strain with FRT scar insertion in the NruI site within *algR* with pVDTac39	(Giltner et al, 2010b)
PA14 *algR*::FRT + pVDTacpil	PA14 strain with FRT scar insertion in the NruI site within *algR* with pVDTacpil	(Giltner et al, 2010b)

Table 2.0: Primer sequences

Primer No.	Primer	Sequence
1	XcpT sense primer	5` GCCCGAATTCATGAATCAGAGCCG 3`
2	XcpT anti-sense primer	5` CCCAAGCTTCAGTTGTCCCAGTTG 3`
3	XcpU sense primer	5` CTAGCAGGAGGAATTCATGCGGGCTTCGCGCGGC 3`
4	XcpU anti-sense primer	5’ CAAAACAGCCAAGCTTTCATCGCCGCGCCACCTCG 3`
5	XcpV sense primer	5` CTAGCAGGAGGAATTCATGAAGCGCGCGCGTGG 3`
6	XcpV anti-sense primer	5` CAAAACAGCCAAGCTTTCATGGCTGGCTCCCGAGG 3`
7	XcpW sense primer	5` GAGGAATTCACCATGGATGAGGCTACAGCGGGGC 3`
8	XcpW anti-sense primer	5` CAAAACAGCCAAGCTTTCATTCCGGCGCCCCC 3`
9	XcpX sense primer	5` TAGCAGGAGGAATTCATGAGGCGCGGGCAGAAC 3`
10	XcpX anti-sense primer	5` CAAAACAGCCAAGCTTTCATCGCTCGTCCTTCTTC 3`
11	PilT sense primer	5′ GGATCCGGTGTTTTCCTTGTCCGA 3′
12	PilT anti-sense primer	5’ AAGCTTGAATCCTAGACGCAGTTC 3’
13	LasB sense primer	5` AAAAAAGAGCTCCCAGAAAGCGTGCAACTGATGATCG 3`
14	LasB anti-sense primer	5` AAAAAACCTAGGCAACAGGTCAAGCGTAGAAACCTTCTTCAT 3`