University of Alberta

P. aeruginosa type IV pilin receptor binding domain functions as an adhesin for abiotic surfaces

by

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Abstract

In this study, I show that *Pseudomonas aeruginosa* binds to abiotic surfaces in a concentration-dependent, saturable manner during the initial stages of biofilm formation. *P. aeruginosa* type IV pili were found to mediate binding to stainless steel in addition to polystyrene and polyvinylchloride. The role of the C-terminal receptor binding domain in mediating binding to steel surfaces was examined using synthetic peptides of this receptor binding domain. The interaction of pili with steel was specifically inhibited by synthetic peptides and effectively inhibited the binding of viable homologous and heterologous *P. aeruginosa* strains to steel.

Furthermore, *P. aeruginosa* monomer and nanotubes were used to investigate the structure of the native pilus. The pilus structure was found to be somewhat distinct but very similar to that of the nanotube, and these results strongly support a recently published model of the pilus fiber which is based on a left-handed three-start helix.

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Abbreviations

ABTS	2,2'-Azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]
AlgR	Transcriptional regulator required for alginate production and twitching motility
Asialo GM_1	Glycosphingolipid
ATP	Adenosine triphosphate
BECs	Buccal epithelial cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFU	Colony forming units
Cys	Cysteine
ΔK122-4	Truncated K122-4 pilin monomer
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENaC	Epithelial Na^+ (sodium) channel
ExeE	Protein secretion system in *Aeromonas hydrophila*
FimL	Regulator of Vfr
FliC	Flagellin protein
g	Gravity
H^+	Proton

hr	Hour
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
K122-4	*Pseudomonas aeruginosa* strain K122-4 (wild type)
KB7	*P. aeruginosa* strain KB7 (wild type)
Ki	Kinetic inhibition
kV	Kilovolts
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
MotA	Flagellar motor protein
MotB	Flagellar motor protein
mm	Millimeter
MS ring	Integral membrane protein of the flagellar basal body
nm	Nanometer
ml	Milliliter
PAK	*P. aeruginosa* strain K (wild type)
PAK2pfs	*P. aeruginosa* strain defective in pilus retraction
PAKMS591	*P. aeruginosa* strain K with non-functional FliC
PAKNP	*P. aeruginosa* strain K with non-functional PilA
PAKBΩ	*P. aeruginosa* strain K defective in pilus assembly
PAKDΩ	*P. aeruginosa* strain K defective in pre-pilin modification
PAO	*P. aeruginosa* strain O (wild type)
PilA	Structural component of the type IV pilus

PilB	ATPase responsible for retraction of the pilus
PilD	Type IV pili biogenesis protein with protease and N-methylation activity
PilR	Response regulator for type IV pili
PilS	Sensor kinase for type IV pili
PilT	ATPase responsible for extension of the pilus
PilU	ATPase responsible for extension of the pilus
PulE	Pullulanase secretion protein in *Klebsiella oxytoca*
PulC	Type II secretion system found in *Klebsiella pneumoniae* (PilD homologue)
PVC	Polyvinylchloride
OutE	Protein secretion system in *Erwinia* spp.
r.m.s.d.	Root mean square deviation
RNA	Ribonucleic acid
RPM	Rotations per minute
RpoN	Gene product involved in regulation
RT	Room temperature
sad	*s*urface *a*ttachment *d*efective phenotype
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TcpJ	PilD homologue in *Vibrio cholerae*
μg	Microgram
μl	Microliter

μM	Micromolar
Vfr	cAMP-binding protein; regulator

Chapter 1

Introduction

1.1 An Introduction to *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative motile aerobic rod, which can be found in numerous environments including soil, water, fungi, plants, and the human body (Bodey *et al.*, 1983; Costerton, 2001; Schwartz *et al.*, 2006). *P. aeruginosa* is a member of the γ-proteobacteria, and is considered to be an opportunistic pathogen which can cause serious infections in immunocompromised individuals, including HIV and CF patients, and is a major cause of nosocomial infections and hospital-acquired pneumonia (Bodey *et al.*, 1983; Brennan and Geddes, 2004; Cunha, 2001). The ubiquitous environmental distribution of this bacterium suggests that it can colonize a large number of environments and environmental surfaces.

1.2 Biofilms

Highly organized surface associated bacterial communities, or biofilms, are found in numerous environments (Mah and O'Toole, 2001). These bacterial communities are contained within an extracellular matrix (composed of polysaccharides, LPS, outer membrane fractions, and DNA) which functions to connect and structure the mature biofilm (Costerton *et al.*, 1995; Whitchurch *et al.*, 2002). The biofilm itself, consisting of cells and various exopolymers, contains void spaces or water channels which are conserved in both mixed and single species bacterial biofilms (Costerton *et*

al., 1995; Davey and O'Toole G, 2000; Wimpenny *et al.*, 2000; Xi *et al.*, 2006). *P. aeruginosa* biofilms develop from the initial attachment of bacteria to a surface (O'Toole and Kolter, 1998). Following attachment, bacterial cells aggregate to form a microcolony or a tightly adherent group of cells, which are precursors to a mature biofilm (O'Toole and Kolter, 1998). The mature biofilm forms a unique structure consisting of a mushroom shaped protrusion from the attached surface (O'Toole and Kolter, 1998; Xi *et al.*, 2006). Furthermore, bacteria within a mature biofilm can coordinate their actions and in essence function as a multicellular organism (Fuqua and Greenberg, 1998).

1.2.1 *P. aeruginosa* Biofilms

P. aeruginosa is an effective and common opportunistic pathogen of humans causing serious infections in cystic fibrosis, intensive care, burn, and immuno-compromised patients (Bodey *et al.*, 1983; Costerton, 2001; Pier, 1985). Initial binding of the bacterium to an abiotic or a cellular substratum is considered by many to be the initial stage of colonization for both biofilm formation (Watnick and Kolter, 2000) and initiation of an infection (Beachey, 1981). Recently, *P. aeruginosa* biofilms have been associated with chronic infection of cystic fibrosis patients (O'Toole *et al.*, 2000; Singh *et al.*, 2000). In addition to chronic infection, *P. aeruginosa* biofilms contribute to morbidity of patients with medical implants including catheters (Khaled *et al.*, 2001; Kumon *et al.*, 1997), prosthetics (McNeil *et al.*, 2001) and stainless steel implants (Traverso *et al.*, 2005). In the natural environment, *P. aeruginosa* is thought to exist predominantly in biofilms (Costerton, 2001); these biofilms have been found on many

surfaces from ice cream plants and water pipes to contact lenses (Gigola *et al.*, 2006; Gunduz and Tuncel, 2006; Mah and O'Toole, 2001). This is particularly problematic for treatment of infection, as bacteria existing within a biofilm are intrinsically more resistant to both antimicrobials and antiseptics (Costerton *et al.*, 1995), with a resistance 10-1000 fold higher than that of planktonic cells (Costerton *et al.*, 1999; Gristina *et al.*, 1987; Hogan and Kolter, 2002; Nickel *et al.*, 1985; Prosser *et al.*, 1987). However, resistance to antiseptics or antibiotics is not solely determined by the thickness of the biofilm, as hydrogen peroxide was able to penetrate a biofilm mutant deficient in catalase production but not a wild type biofilm (Stewart *et al.*, 2000). This suggests that the hydrogen peroxide resistance is due to a concentration of catalase in the biofilm, rather than the actual thickness. Currently, *P. aeruginosa* is thought to utilize an anaerobic growth mode within the biofilm, which would limit the amount of bacterial produced hydrogen peroxide (Yoon *et al.*, 2002).

1.2.2 Biofilm Formation

P. aeruginosa readily binds to stainless steel (Stanley, 1983; Vanhaecke *et al.*, 1990), and other abiotic substrates to form highly recalcitrant, organized communities known as biofilms (Blenkinsopp *et al.*, 1992; Johansen *et al.*, 1997; Leake *et al.*, 1982). *P. aeruginosa* biofilms on stainless steel surfaces can serve as a significant hospital reservoir for infection of susceptible patients (Tredget *et al.*, 1992). Biofilm development is divided into four main stages: the first stage involves planktonic cells binding to a surface (O'Toole and Kolter, 1998; Parsek and Greenberg, 2000). Using the extracellular organelle flagella, bacteria can move through liquid media to make

contact with the substrate surface (O'Toole and Kolter, 1998). The second step occurs once the bacterium has reached the surface and flagella and other adhesins mediate tight surface binding (O'Toole and Kolter, 1998). Third, cell proliferation or aggregation creates an attached monolayer of cells. With the proper signals, including type IV pili and the *sad* genes, (so called for mutants presenting a *s*urface *a*ttachment *d*efective phenotype), cells will aggregate to form a microcolony (O'Toole and Kolter, 1998). In fact, both categories of *sad* genes, those affecting the type IV pili, and those affecting flagella, were found to be defective in attachment to the plastic surface, suggesting a role for both type IV pili and flagella in biofilm formation (O'Toole and Kolter, 1998). Fourth, intercellular signaling is an important process in the development of a mature biofilm (Shapiro, 1998). This intercellular signaling is accomplished by small molecules, known as acyl-homoserine lactones, which are secreted from bacterial cells to enter neighbouring cells. When the concentration of homoserine lactones reaches a threshold concentration, they can affect translation of gene products. This process is known as quorum sensing (Shapiro, 1998). When quorum sensing is activated, microcolonies will differentiate into a mature biofilm consisting of mushroom or column-like structures (O'Toole and Kolter, 1998).

Type IV pili are essential for the normal development of *P. aeruginosa* biofilms as mutants lacking functional pili are not able to develop past the microcolony stage in static or flow biofilm systems (Klausen *et al.*, 2003a; O'Toole and Kolter, 1998). However, it is believed that type IV pili mediated-adhesion, but not the movement of the pili along the surface, is required for microcolony formation, as PilT and PilU mutants, defective in twitching motility, were able to form dense unstructured biofilms (Chiang

and Burrows, 2003). Although cap formation (the top of the mushroom shaped mature biofilm) in the mature structured biofilm is dependent on twitching motility, type IV pili are not necessarily required for unstructured biofilm formation, as a pilus mutant can still form a dense unstructured monolayer of cells (Klausen *et al.*, 2003b). Following microcolony formation, column or mushroom shaped organized cellular masses begin to develop (O'Toole and Kolter, 1998). In these masses, bacteria grow in an exopolysaccharide matrix composed of polysaccharides, proteins, and DNA, which serves as a bacterial interconnecting matrix component (Allesen-Holm *et al.*, 2006; Whitchurch *et al.*, 2002). Despite extensive studies on initial stages of adhesion and microcolony formation and genomic studies focusing on biofilm null mutants, the signal to develop a mature biofilm remains unknown (O'Toole *et al.*, 1999; Sauer and Camper, 2001; Vallet *et al.*, 2001; Whiteley *et al.*, 2001). O'Toole (2003) suggests that the diversity of the genes influencing biofilm development advocates a much more complex system than anticipated, where many pathways may work in concert for biofilm development (O'Toole, 2003).

1.3 Bacterial Attachment and Movement

P. aeruginosa is able to cause a variety of infections and can establish a colony on a diverse range of surfaces. The ability of bacteria to move towards nutrients, and then remain attached to these surfaces, is essential for a bacterial population to successfully compete. How *P. aeruginosa* utilizes both flagella and type IV pili to move and to attach to surfaces will be discussed below.

1.3.1 Flagella

Bacterial flagella are complex cellular machines, which require the participation of a multitude of gene products for assembly and regulation (McCarter, 2006). Flagella are found in both Gram positive and Gram negative bacteria and are required for swimming, and tumbling motility (Larsen *et al.*, 1974). Specifically, *P. aeruginosa* flagella have been implicated in tethering and adhering to epithelial cells with low affinity through the cellular receptor asialo GM_1 (Feldman *et al.*, 1998; Ramphal *et al.*, 1996) and to the ENaC channel, responsible for sodium adsorption (Kunzelmann *et al.*, 2006). Flagella were found to bind preferentially and with greater affinity to monosialo-GM_1 than to the glycosphingolipid asialo-GM_1, whereas the reverse was true for purified type IV pili (Feldman *et al.*, 1998). Furthermore, the presence of additional glycosphingolipid did not prevent the flagella from binding to the receptor, suggesting that this interaction is not specific as it is for type IV pili (Feldman *et al.*, 1998; Sheth *et al.*, 1994). The presence of the flagella is therefore an important factor in the initial colonization of surfaces and plays a significant (although not fully understood) role in biofilm formation (O'Toole and Kolter, 1998). The structure and function of this complex organelle and the process of its assembly will be discussed below.

1.3.1.1 Structure and Function

Pseudomonas flagella are filamentous proteinaceous polar appendages approximately 15 μm in length (Macnab, 1992), which through a cork-screw-like motion, can efficiently move the organism through liquid media (Berg and Anderson, 1973; Silverman and Simon, 1972). The flagellar structure spans the entire cell

envelope of Gram negative bacteria (DePamphilis, 1971), and consists of three differentiated structural regions: the basal body, the hook, and the filament (Beatson *et al.*, 2006).

The primary flagellar component is flagellin (FliC), which forms the main structural protein of the flagella filament. FliC has been shown to have conserved C-terminal and N-terminal domains in alignments of 202 different flagellar structures (Beatson *et al.*, 2006). The flagellar filament consists of up to 30,000 flagellin monomers, which are assembled in a helical array to form a hollow filament. Analysis of a recently crystallized high-resolution truncated form of FliC (Chiu *et al.*, 2006; Samatey *et al.*, 2001) showed surface exposed residues of the filament to vary substantially across bacterial flagella, while the inner region was highly conserved (Beatson *et al.*, 2006). As the FliC sequence is so highly conserved in the N- and C-termini across the bacterial flagella sequences analyzed, it is likely that the core structure is maintained in all flagellin monomers (Beatson *et al.*, 2006).

The flagellar basal body is a complex of roughly 20 proteins whose function is to transfer energy generated from the intracellular motors to the extracellular filament. To power the helical motion of the flagella, two motor proteins are required: MotA and MotB (Berg, 2003; Francis *et al.*, 1994). These transmembrane proteins surround the MS ring and form a proton channel across the cytoplasmic membrane (Zhou *et al.*, 1998a). Although the interactions between the MS ring and the Mot proteins has been investigated (Walz and Caplan, 2000; Zhou *et al.*, 1998a; Zhou *et al.*, 1998b), how flagellar movement is coupled to H^+ flow is not well understood.

1.3.1.2 Assembly

Flagella assembly is accomplished in an inside-out manner, where the first structure to be assembled is the MS-ring (Kubori *et al.*, 1992; Suzuki *et al.*, 1978; Suzuki and Komeda, 1981). This observation was first recognized by Suzuki *et al.*, (1978) when deletions in the MS-ring abolished flagella formation, whereas mutations in other regions, such as the hook, resulted only in incomplete flagellar structures (Suzuki *et al.*, 1978). This was later found to be in direct contrast to the formation of type IV pili, which are assembled from the base of the pilus (Craig *et al.*, 2006). Furthermore, while the flagella is a self-assembly of 25 different proteins (Namba and Vonderviszt, 1997), the type IV pilus is assembled through the efforts of greater than 50 gene products (Jacobs *et al.*, 2003).

1.3.2 Type IV Pili

1.3.2.1 Structure and Function

Native type IV pili, which mediate bacterial attachment to substrates (Bodey *et al.*, 1983; Woods *et al.*, 1980), are long filamentous polar appendages that extend from the bacterium. The type IV pilus is typically 6 nm wide and up to several micrometers long (Folkhard *et al.*, 1981). An estimated 500-1000 copies of the functional monomeric pilin subunit, PilA, form the quaternary structure known as the native pilus. Interestingly, dissociation of pili with the non-ionic detergent n-octyl-β-glucoside yields dimers of the PilA subunit which could form the building block of the mature pilus (Watts *et al.*, 1982, 1983). In addition, upon removal of the detergent these dimers

formed very thin filaments; a structure which is similar to that found in protein nanotubes (See section 1.4). Upon closer inspection, tyrosine residues located in the N-terminus at positions 24 and 27 were found to result in a dimer-dimer interface in the native pilus (Watts *et al.*, 1982, 1983). The PilA pilin tertiary structure exhibits a characteristic pilin fold that positions an N-terminal α-helix onto a four-stranded anti-parallel β-sheet (Figure 1.1) (Audette *et al.*, 2003, 2004). The N-terminal α-helix, highly conserved among *P. aeruginosa* strains and other Gram-negative bacteria that produce type IV pili, is highly hydrophobic and comprises the pilus oligomerization domain in related pili structures (Park *et al.*, 2001). The four-stranded anti-parallel β-sheet is anchored in the pilus structure via the N-terminal α-helix which may serve as a molecular scaffold to support and anchor the globular C-terminal domain to the filament of the pilus (Figure 1.2) (Audette *et al.*, 2004; Craig *et al.*, 2004; Hazes *et al.*, 2000). The C-terminal receptor binding domain, composed of the last 17 residues of the pilin monomer, forms a shallow depression on the solvent exposed surface of the fully formed pilus structure (Figures 1.2 and 1.3) (Audette *et al.*, 2004; Craig *et al.*, 2006; Hazes *et al.*, 2000) and has been shown to mediate binding to multiple surfaces including stainless steel and plastics (Giltner *et al.*, 2006). Furthermore, the C-terminal receptor binding domain of *P. aeruginosa* contains an internal disulfide loop between residues 129 and 142, utilizing PAK PilA sequence nomenclature, and is responsible for specific adhesion to the GalNAc-β-D-(1,4)-Gal moieties of asialo-GM_1 (Figure 1.3) (Irvin *et al.*, 1989; Sheth *et al.*, 1994).

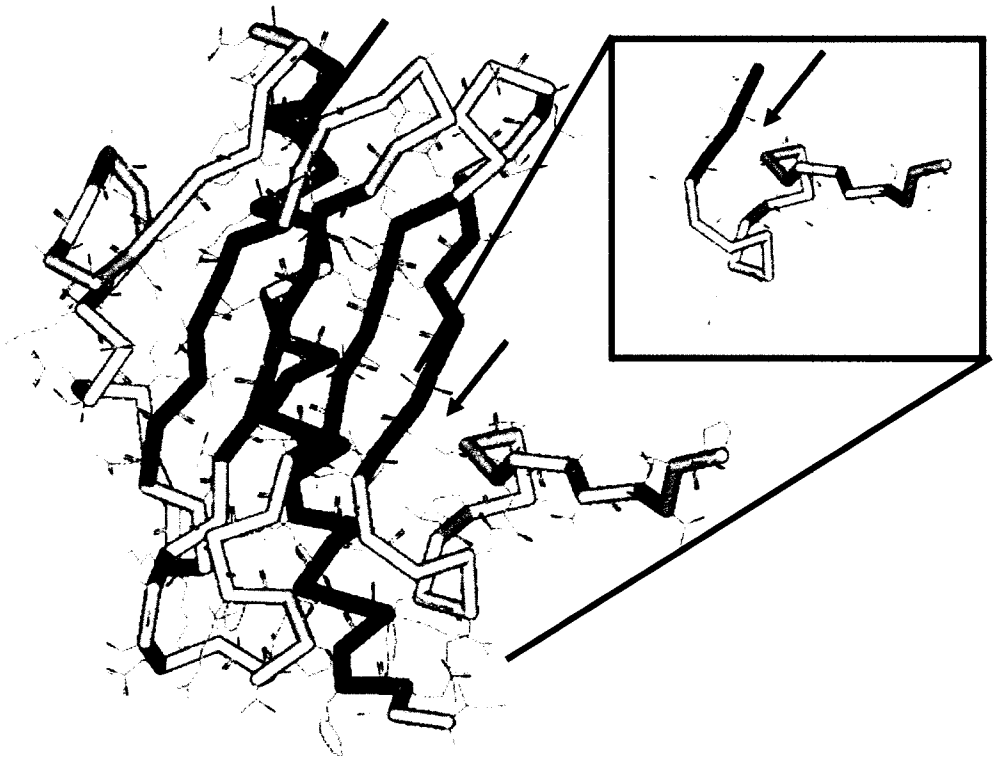

Figure 1.1 K122-4 Pilin Structure

Structure of the truncated *P. aeruginosa* strain K122-4 type IV pilin structural subunit, PilA (Audette *et al.*, 2004). The C-terminal binding domain is indicated by the inset box. The N-terminal R-helix (R1-C) is in red; the β-sheet is in cyan; and the coil regions are in grey. The two disulfide bridges observed in ΔK122-4, between Cys 57 and 93 and between Cys 129 and 142, are shown in yellow, and are highlighted with green arrows. This figure was created using the WebLab ViewerPro version 3.7 program.

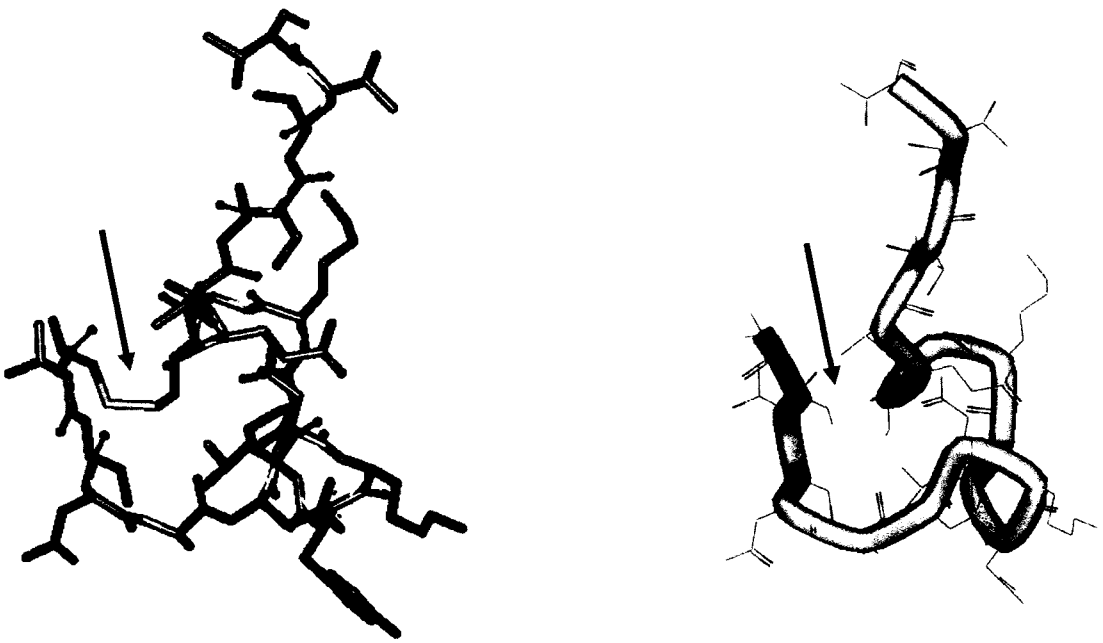

Figure 1.2 Receptor Binding Domains

Model of the *P. aeruginosa* strain K122-4 (left) and PAK (right) type IV pilin structural subunit (PilA) C-terminal receptor binding domain. The disulfide bridges observed in between Cys 129 and 142, are shown in yellow and highlighted with a green arrow. These models were generated using the WebLab ViewerPro version 3.7 program.

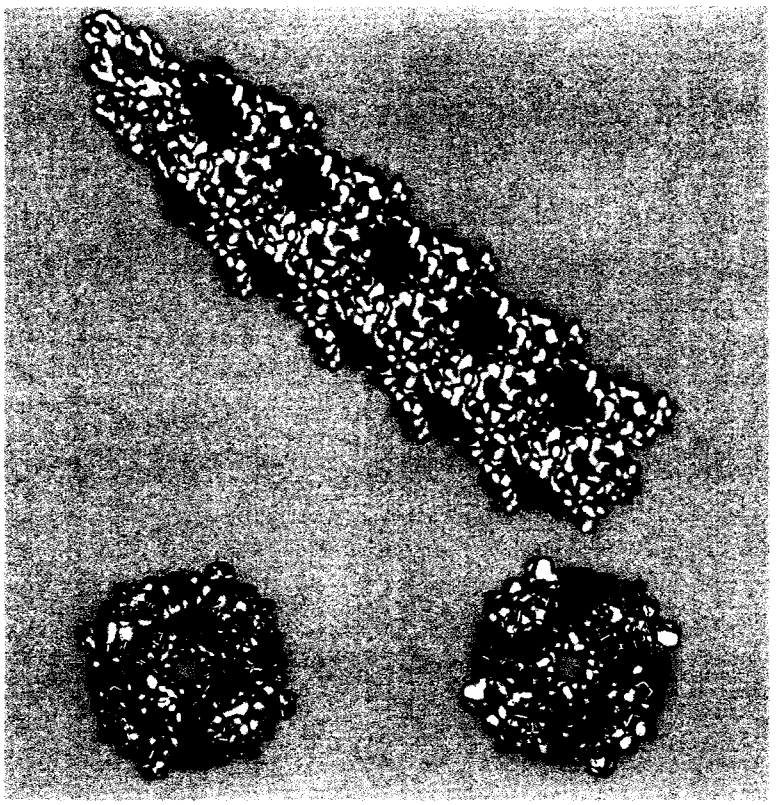

Figure 1.3 Helical Pilus Model

Proposed model of the *P. aeruginosa* strain K type IV pilus structure (Hazes *et al.*, 2000). The α-helices are shown in yellow. The surface electrostatics are shown, blue represents positive, red negative, and white neutral charge. In addition, the C-terminal receptor binding domain in green is somewhat displayed (a significant portion of the receptor binding domain is occluded due to interfacial packing) along the solvent exposed length of the pilus and multiple exposed receptor binding domain sites are present at the tip, but not the base of the pilus. This model was generated using the WebLab ViewerPro version 3.7 program.

1.3.2.2 Classification

Type IV pili are produced by a number of Gram negative bacteria including, *Neisseria gonorrhoeae* (Meyer et al., 1984), *Vibrio cholerae* (Faast *et al.*, 1989; Shaw and Taylor, 1990), enteropathogenic *Escherichia coli* (Donnenberg et al., 1992), and *P. aeruginosa* (Johnson et al., 1986). Type IV pili are divided into two subsets: type IVa and type IVb pili. The type IVa pili are those pili belonging to *P. aeruginosa* and *Neisseria gonorrhoeae*, while the type IVb pili are those belonging to intestinal-colonizing bacteria such as *Vibrio cholerae* and *Escherichia coli* (Craig *et al.*, 2003). Type IVb pili are similar to type IVa pili with respect to the conserved N-terminal α-helix, structural scaffold, and the receptor binding domain disulfide bond. However, the location of the disulfide bond in type IVa pili is located roughly between residues 129 and 142 in the C-terminal receptor binding domain, while the disulfide bond in the type IVb pili is located across a larger area, such as 155 and 211 in *V. cholerae* TcpA (Strom and Lory, 1993). The sequence of the C-terminal globular domain, the length of the leader sequence and the N-terminal methylated residue are also different (Craig *et al.*, 2003; Strom and Lory, 1993). For both the type IVa and IVb pilins the assembly mechanism is quite complex, involving a secretion system that requires over 50 gene products for the construction of functional pili (Jacobs *et al.*, 2003; Peabody *et al.*, 2003; Wolfgang *et al.*, 2000). While structural studies have elucidated the molecular structures of several type IV pilins (Audette *et al.*, 2004; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Parge *et al.*, 1995), little is known concerning the quaternary structure of the pilus. Several pilus fiber models have been proposed and include both right and left-handed single start helices (Hazes *et al.*, 2000; Keizer *et al.*, 2001; Parge *et al.*, 1995),

and multi-start helix models (Craig *et al.*, 2003; Craig *et al.*, 2004). Interestingly, most experimental and molecular modeling studies support a 3-start left-handed helix model for type IVb pilins (Craig *et al.*, 2003; Xu *et al.*, 2002), similar to the only current multi-start helix model of a type IVa pilus developed by Craig *et al.*, (2003). The three-start helical model is based on three individual strands of PilA monomers constituting the pilus structure. This may be more easily understood by visualizing a maypole with three individual ribbons wrapping around a pole (representing the central channel of the pilus structure). Each ribbon represents a string of PilA monomers which wrap around the central channel giving rise to a left-handed helix. Furthermore, at the tip of the pilus, three PilA monomers are accessible rather than one as in the single start helical models, which allows for a surface interaction with greater avidity. Recently, this *N. gonorroheae* type IVa pili model was confirmed to be arranged in a 3-start helical manner (Craig *et al.*, 2006). Although multiple models have been postulated for the type IVa pilins, the exact quaternary structure of the pilus remains undetermined.

1.3.2.3 Twitching Motility

Twitching motility is a unique bacterial movement caused by the extension and retraction of type IV pili along a surface (Bradley, 1980; Henrichsen, 1983; Whitchurch *et al.*, 2005). This flagella-independent movement has been measured at between 0.6 mm/hr and 2 mm/hr and is thought to be the primary role of the type IV pilus (Alm and Mattick, 1997; Semmler *et al.*, 1999). The depolymerization of pilin monomers causes the retraction of the pilus and works in concert with polymerization to assemble new pilin monomers to form the mature pilus structure (Strom and Lory, 1991). This process

allows the pilus tip to remain bound to the surface while the bacterium is pulled closer to the surface (Burrows, 2005). Evidence for the depolymerization of pili was found when twitching was impaired by RNA phage adsorption to the pilus (Bradley, 1972); as the bulky RNA phage bound to the pilin surface, pilin subunits could not be retracted past the region where the phage had bound, and pili decreased in length only 50 %. This indicates that the phage bound at various locations along the pilus, and prevented retraction of the pilus past the location of adsorption to prevent full retraction and twitching motility.

Twitching motility requires additional proteins for extension and retraction of the pilus. PilT, and PilB are essential for functional pili. PilT and PilB have a high degree of homology (Whitchurch *et al.*, 1991), and share two highly conserved putative nucleotide binding domains (Thompson *et al.*, 1988). Nucleotide binding proteins contain both a Walker A box (responsible for binding to the γ-phosphate group of ATP) and a Walker B box (responsible for hydrolysis of the ATP molecule) (Walker *et al.*, 1982), and are required for energy production. As neither PilT or PilB possess large regions of hydrophobic amino acids (characteristic of a transmembrane domain) nor a leader sequence common to secreted proteins, PilB and PilT are hypothesized to reside in the cytoplasm and function as nucleotide binding proteins (Strom and Lory, 1993). Unlike PilT, which forms a hyperpilliated mutant phenotype, PilB mutants are unable to express pili on the surface of the bacterium, even though the level of *pilA* transcription is unaffected (Alm and Mattick, 1997). Homologues of these nucleotide binding proteins have been implicated in the movement of other proteins across bacterial membranes in other systems including a pullulanase secretion protein (PulE) in

Klebsiella oxytoca, and protein secretion systems in *Erwinia* spp. (OutE) and *Aeromonas hydrophila* (ExeE) (d'Enfert *et al.*, 1989; Jiang and Howard, 1992; Noonan and Trust, 1995).

An additional pili biogenesis gene *pilU* was found when a transposon mutagenesis library was created to identify twitching motility mutants. Mutants lacking the ability to twitch were isolated and the mutated regions were mapped and localized to what is now called the *pilU* gene (Whitchurch and Mattick, 1994). Located directly downstream of *pilT*, *pilU* was found to share homology with nucleotide binding proteins as well as with PilT (Whitchurch and Mattick, 1994). Although both PilU and PilT give similar hyperpilliated mutant phenotypes, PilU is still sensitive to bacteriophage PO4 infection while PilT mutants are resistant (Whitchurch and Mattick, 1994). As PilU and PilT mutants are unable to retract the assembled pilus, and PilB mutants are unable to assemble the pilus, it is not surprising that these proteins are essential for catalyzing the forward and reverse movement in twitching motility (Alm and Mattick, 1997).

Twitching motility is dependent not only on the ATP-binding proteins previously discussed, but also on the presence of a functional pilus. Pilus biogenesis is highly conserved amongst type IVa pili, as expression of heterologuous type IVa pilin genes (such as those of *Moraxella bovis* or *N. gonorrhoeae*) in a *P. aeruginosa* pili mutant led to formation of functional heterologous pili (Beard *et al.*, 1990; Hoyne *et al.*, 1992). Following PilA synthesis, a short N-terminal leader sequence of six amino acids is cleaved before assembly into the mature pilus structure. PilD is a highly hydrophobic transmembrane protein located in the cytoplasmic membrane (Strom *et al.*, 1993) that has been shown to act as both a leader peptidase as well as to participate in the

methylation of the first amino acid in the mature pilin monomer (Nunn *et al.*, 1990; Nunn and Lory, 1991, 1992). Cleavage of the leader sequence is primarily dependent on two residues, first the glycine residue located in position –1, as mutational studies exchanging the glycine for an alanine residue removed the peptidase activity (Strom and Lory, 1991), and second the glutamate residue located in position +5, as mutational studies at this position decreased the degree of methylation (Macdonald *et al.*, 1993). Mutations in the *pilD* gene prevent formation of functional pili, although full length pilin, including the leader sequence, can still be found in the periplasm (Nunn and Lory, 1991). Further mutational analysis of PilD revealed that a 20-fold reduction in the N-methylation reaction can still yield functional pili, suggesting that not every pilin monomer has to be methylated to form the pilus structure (Pepe and Lory, 1998). Furthermore, although methylation of the first amino acid of the mature pilin subunit does not seem to be necessary for pilus assembly, it may be necessary for pilus function in conditions not able to be replicated in the laboratory (Pepe and Lory, 1998). PilD homologues have been found in other bacteria that express type IV pili, including *N. gonorrhoeae* (PilD) (Lauer *et al.*, 1993), *Vibrio cholerae* (TcpJ) (Kaufman *et al.*, 1991), as well as in a type II secretion system found in *Klebsiella pneumoniae* (PulC) (Pugsley and Reyss, 1990; Whitchurch *et al.*, 1991). This suggests a common mechanism in type IVa and IVb pilin and type II secretion processing. Importantly, these homologues are highly similar in the cytoplasmic domain, which is noted as containing the active site for both the leader peptidase activity and the N-methylation activity (Strom and Lory, 1991, 1993). Furthermore, purified *P. aeruginosa* PilD was able to cleave not only *P.*

aeruginosa, but also purified *N. gonorrhoeae* immature pilin monomers (Nunn and Lory, 1991) emphasizing a conserved function of the PilD enzyme.

1.3.2.4 Regulation

As regulation of pilus expression is essential for energy conservation, it is not surprising that type IV pili are highly regulated. As with aspects of pilus biogenesis, regulation of the type IVa and type IVb pili differ. Type IVa pili contain a conserved promotor for the *pilA* gene that contains an RpoN-dependent promoter motif, and is therefore transcribed by the RNA-polymerase with the alternative sigma factor, σ^{54} (Ishimoto and Lory, 1989; Johnson *et al.*, 1986; Kustu *et al.*, 1989). Expression of the σ^{54} holoenzyme requires transcriptional regulator binding upstream of the active site (Kustu *et al.*, 1989). Type IVb pili however, do not contain this conserved RpoN motif and the structural pilin gene is therefore transcribed by the major sigma factor σ^{70} (Ishimoto and Lory, 1992). In this case, an additional level of regulation is seen with the two-component regulatory system of *pilR* and *pilS*, whose products express the response regulator and the sensor kinase respectively (Boyd and Lory, 1996; Ishimoto and Lory, 1992; Strom and Lory, 1993). Another significant regulator of *P. aeruginosa* type IV pilus expression is the transcriptional regulator AlgR, which is required for alginate production and twitching motility (Whitchurch *et al.*, 1996). Decreases in both twitching motility and virulence have been documented with *algR* mutants, and although the exact mechanism of action remains unknown, two AlgR controlled operons have been identified (Lizewski *et al.*, 2004).

In addition to these transcriptional regulators, there are a number of post-transcriptional regulators that influence pilus expression. Vfr and FimL are suggested to form components of two coordinated pathways to control twitching motility (Whitchurch *et al.*, 2005). Vfr is a cAMP-binding protein, which controls many functions in *P. aeruginosa* including pilus biogenesis, twitching motility, catabolic repression, and down-regulation of flagella (Beatson *et al.*, 2002); while FimL controls pilus assembly and twitching motility through Vfr regulation (Whitchurch *et al.*, 2005). As mutations in the *fimL* gene did not effect production of the major pilin subunit, PilA, FimL is suggested to regulate pilus assembly rather than biogenesis (Whitchurch *et al.*, 2005).

1.4 Nanotubes

1.4.1 Protein Nanotubes

Deletion of the N-terminal α-helix of the PilA subunit allows for the ready expression of a highly soluble monomeric pilin protein (Figure 1.1) (Hazes *et al.*, 2000). Importantly, the pilin momomer is structurally equivalent to the full-length pilin to a 1.6 Å r.m.s.d. (or the root mean square deviation, which gives the average distance between the backbones of the superimposed proteins) (Hazes *et al.*, 2000). Monomeric PilA when exposed to a hydrophobe (e.g., C11-SH), oligomerized to form long hollow fibrous protein nanotubes (Figure 1.4) (Audette *et al.*, 2004). These nanotubes were shown to have a diameter of approximately 6 nm and be up to several hundred

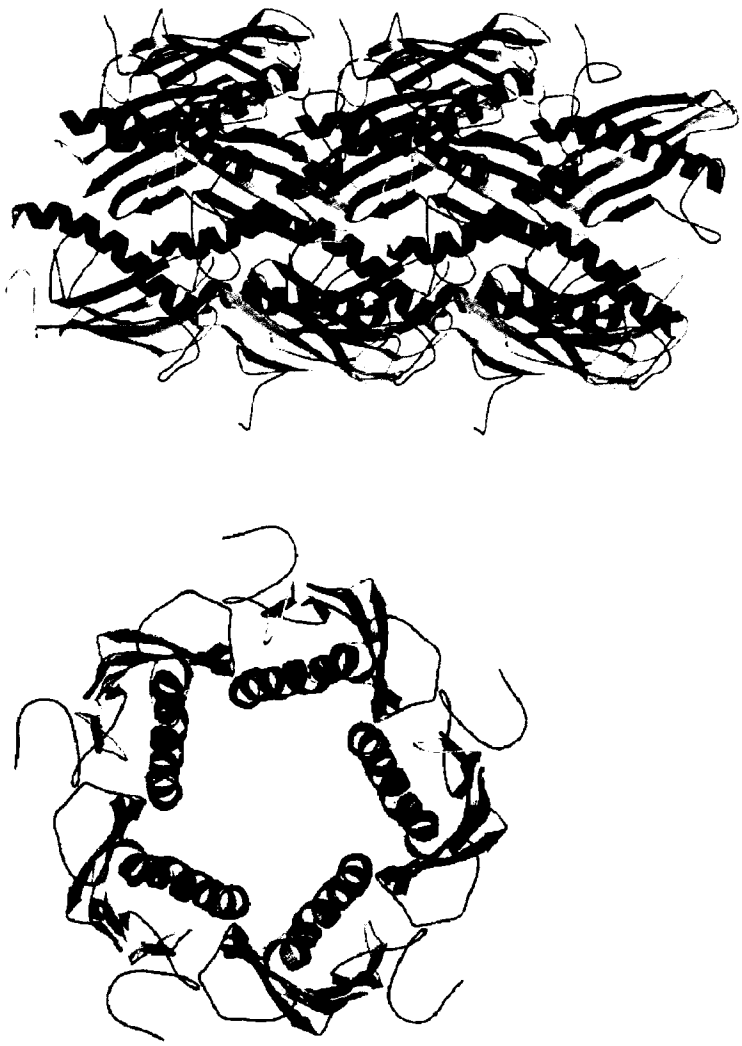

Figure 1.4 Protein Nanotube

Figure 1.4 Protein Nanotube

Proposed model of *P. aeruginosa* strain K122-4 truncated protein nanotube (Audette *et al.*, 2004). The N-terminal α-helices are shown in blue; the anti-parallel β-sheets are depicted by arrows; and the C-terminal receptor binding domain is shown by a terminal red loop. The top image looks into the protein nanotube, while the bottom image depicts the side view of the protein nanotube.

micrometers in length (Audette *et al.*, 2004), significantly longer than the native pilus structure.

1.5 Abiotic Surfaces Examined

1.5.1 Stainless Steel

Stainless steel is a ferrous alloy with a minimum of 10% chromium content, which is categorized by a grading system denoting the metal alloy composition and usage (Jones, 1996b). Grade 304 is the most common representing approximately 70% of all steel produced (Jones, 1996b). This grade is also known as 18/8 steel as it has a composition that includes 18% chromium content and 8% nickel content (Jones, 1996b). Grain boundaries are produced at the interface of the polycrystalline metal which disrupts the electron flow (Jones, 1996a). Chromium is an important addition to the ferrous alloy, as it allows for higher resistance to oxidation than iron alone (Jones, 1996b). When chromium is exposed to oxygen it forms a passivation barrier of chromium III oxide along the steel surface. The passivation layer is impervious to water and oxygen, and thus protects the underlying metal from oxidation, but is thin to allow the metal to remain shiny (Brantley and Eliades, 2001). Nickel is the third major component of stainless steel and serves to stabilize the crystalline structure and to make the metal less brittle at low temperatures (Jones, 1996b). Carbon can also be added to stainless steel, however, the greater the carbon content, the greater the rigidity of the steel. Thus, with a low carbon content to make the steel more workable, and a high

chromium content to ensure long life of the steel, it is not surprising that grade 304 stainless steel is the most common form of steel to be manufactured.

1.5.2 Polystyrene

Polystyrene was first discovered in 1839 by Eduard Simon when he performed the first recorded polymerization reaction (Scheirs and Priddy, 2003). First commercialized in 1931, polystyrene is composed of a linear polyethylene chain with laterally attached phenyl rings to alternating carbons (Scheirs and Priddy, 2003). The styrene monomer is manufactured from petroleum and then polymerized to form a transparent, light, and relatively rigid plastic (Scheirs and Priddy, 2003). The most commonly used polystyrene is a mixture of isobutene and styrene, which forms a foamed polystyrene, or Styrofoam (Scheirs and Priddy, 2003). Furthermore, the tissue culture polystyrene plates used in this study are manufactured using ionizing radiation, to be more hydrophillic and therefore more readily bind cells than the regular polystyrene plates (Cleland *et al.*, 2003).

1.5.3 Polyvinylchloride

Polyvinylchloride is a common and widely used plastic, manufactured for use in plastic piping, children's toys, catheters, intravenous tubing, and vinyl records (Scheirs and Priddy, 2003). This versatile material was first discovered independently by Henri Regnault in 1835 and by Eugen Baumann in 1872. However, it was not until 1926, when Waldo Semon developed a method to make the relatively brittle polymer more

malleable, that polyvinylchloride became commonplace (Encyclopaedia Britannica Inc., 1994). Polyvinylchloride is linear in structure with chloride ions located on alternating carbon molecules; each vinyl chloride monomer is composed of two adjoining carbons surrounded by hydrogen ions and one chloride ion, which upon polymerization form the rigid polyvinylchloride polymer (Bornehag *et al.*, 2004). With the addition of phthalate plasticizers to change the chemical consistency of the plastic (to make it more flexible), health concerns arose over the release of these phthalate additives (Katami *et al.*, 2002). Burning of polyvinylchloride is the primary cause of phthalate release into the atmosphere and has been linked to the formation of angiosarcoma of the liver (Creech and Johnson, 1974; Katami *et al.*, 2002). However, polyvinylchloride remains the most commonly used plastic because of its inexpensive cost and ease of assembly.

1.6 Research Objectives

The aim of this thesis was to examine the initial *P. aeruginosa* colonization of a number of abiotic surfaces, and investigate how this initial interaction leads to biofilm formation. To test this, the initial colonization of a diverse substrate range including stainless steel, polyvinylchloride, and polystyrene was used. *P. aeruginosa* cells and purified pili were employed to assess the initial colonization of surfaces in a number of experiments including direct binding assays, antibody inhibition, and synthetic peptide inhibition.

To further characterize the interaction of *P. aeruginosa* type IV pili with abiotic surfaces, a protein based nanotube composed of a truncated form of the pilin structural subunit was employed. The structurally distinct areas of the type IV pilus involved in

binding to substrates was then examined. This thesis contributes a further understanding as to how the initial bacterial colonization of abiotic surfaces occurs.

Chapter 2

Materials and Methods

2.1 Bacterial strains, DNA, and Antibody Sources

The *P. aeruginosa* strains used in this study were PAK, PAK 2Pfs (Bradley and Pitt, 1974), PAK-BΩ (a 2 kB Ω fragment containing a transcriptional terminator from pHP45 inserted into the pilB gene) PAK-DΩ (contains the same transcriptional terminator was inserted into the pilD gene) (Koga *et al.*, 1993) PAKMS591 (contains the gentamicin cassette from a pPC110 inserted into the FliC gene) (Starnbach and Lory, 1992) PAKNP (contains the tetracycline cassette from pB322 inserted into the pilA gene) (Saiman *et al.*, 1990) K122-4 (a clinical isolate from a cystic fibrosis patient in Toronto which possesses both pili and flagella) (Pasloske *et al.*, 1988) and KB7 (an isolate containing both pili and flagella) (See Table 2.1 for list of strains and their phenotypes) (Wong *et al.*, 1995). Several of these strains were generously provided by Dr. Jessica Boyd (NRC Institute for Marine Biosciences, Halifax, Nova Scotia). The strain PAK 2Pfs, a multi-piliated retraction deficient strain, was used for the purification of pili only and not used in experimental conditions. The phenotypes of the *P. aeruginosa* strains with respect to expression of pili were experimentally verified by Western blotting with anti-PAK pilus specific anti-sera and by direct Enzyme-Linked ImmunoSorbent Assay (ELISA) with whole cells and heat inactivated whole cells (to determine the presence of surface exposed pili) (Figure 2.1). Heat denatured and viable

P. aeruginosa strain	Phenotype	Mutation	Reference
PAKwt	Wild type	None	(Bradley and Pitt, 1974)
PAOwt	Wild type	None	(Holloway, 1955)
KB7wt	Wild type	None	(Paranchych *et al.*, 1990)
K122-4	Wild type	None	(Pasloske *et al.*, 1988)
PAK2pfs	Hyperpilliated	Pilus retraction deficient	(Bradley and Pitt, 1974; Paranchych *et al.*, 1979)
PAK- BΩ	No surface pili	Pilus assembly ATPase deficient	(Koga *et al.*, 1993)
PAK-DΩ	No surface pili	Pre-pilin leader peptidase deficient	(Koga *et al.*, 1993)
PAKNP	No surface pili	PilA deficient	(Saiman *et al.*, 1990)
PAKMS591	No surface flagella	Flagellin deficient	(Starnbach and Lory, 1992)

Table 2.1 Table of *P. aeruginosa* Strains

Phenotypes of *P. aeruginosa* strains used in this study and their respective mutations.

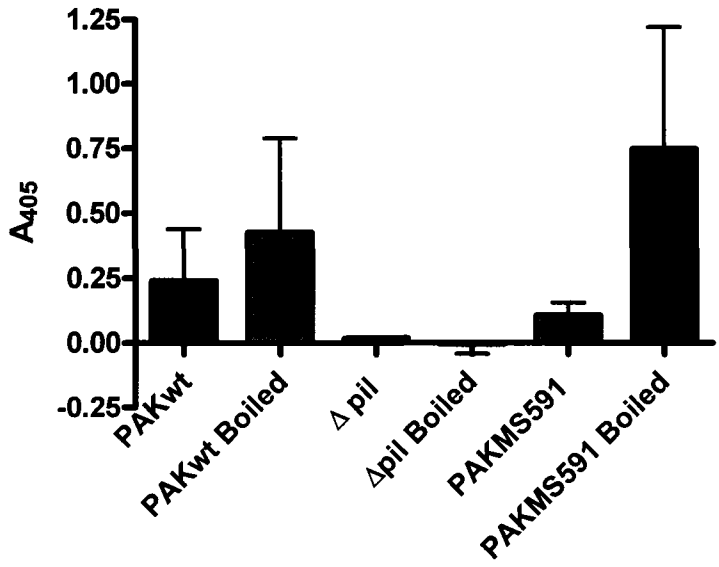

Figure 2.1 Surface Expression of Pili

P. aeruginosa strains PAKwt, PAKMS591, and Δpil whole cells and heat inactivated whole cells were assessed for surface exposed pili. Bacteria were boiled to denature membranes, or not boiled. Presence of pili was detected using PK99H (an anti-PAK pilin antibody) in a 1:3000 dilution in Buffer A. Bacteria were incubated with antibody for one hour and were subsequently pelleted via centrifugation, and the supernatant was removed. Following five washes with Buffer A, secondary antibody (1:3000 dilution) was added and incubated for an additional hour. Bacteria were again pelleted via centrifugation, washed and ABTS was used as substrate. These experiments were performed by Dr. Erin van Schaik.

cells were added to polystyrene microcentrifuge tubes and incubated with monoclonal anti-PAK pili antibody PK99H (which recognizes the 134-140 region of the PilA monomer). Bacteria were pelleted by centrifugation (for 10 minutes) and the supernatant was removed. Bacteria were then washed five times with Buffer A (PBS pH 7.4 containing 0.05 % BSA) prior to addition of secondary goat anti-mouse HRP antibody, and pili were detected using substrate buffer (0.01 M sodium citrate buffer pH 4.2 containing 1 mM 2,2'-Azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt (ABTS) (Sigma) and 0.03% (v/v) hydrogen peroxide). Surface exposed pili were detected using PAK cells, and internal PilA monomers were detected by first boiling the cells to denature the membrane. Pilus expression was also experimentally verified by testing the sensitivity to type IV pilus specific phage (Table 2.2), and by monitoring the twitching motility of the strains (Table 2.3). Phage sensitivity was measured by plating a lawn of bacteria on a Luria-Bertani broth (LB) agar plate and 1 μl of PO4 phage was inoculated in the centre of the bacterial lawn. Plates were incubated for 24 hours at 37°C and plaques were measured quantitatively (Table 2.2). Twitching motility independent of flagella motility, was determined using a low agar (1%) LB plate. Single *P. aeruginosa* bacterial colonies were punctured through the LB agar to the underlying plastic surface. Plates were incubated for 48 hours and twitching was assessed based on the movement of bacteria from the inoculation site (Table 2.3). *P. aeruginosa* was routinely grown at 37°C in LB or LB supplemented with 50 μg/ml tetracycline (Sigma) for PAKNP, 100 μg/ml of gentamicin for PAKMS591, or 50 μg/ml of streptomycin for strains PAK-BΩ and PAK-DΩ. The polyclonal antibodies generated against the PAK pili and associated

P. aeruginosa strain	Phage sensitivity
PAKwt	+++
PAOwt	+++
KB7wt	+++
K122-4	++
PAK2pfs	-
PAK- BΩ	-
PAK-DΩ	-
PAKNP	-
PAKMS591	+++

Table 2.2 Phage Sensitivity

P. aeruginosa sensitivity to type IV pilus specific phage, P04. PAK, PAO, KB7, K122-4, PAK2pfs, PAK-BΩ, PAK-DΩ, PAKNP, and PAKMS591 were grown in the presence of bacteriophage P04, and observed for plaques at 24 hours. Plaques were measured and scored based on their size (+++ represents a plaque diameter of 0.5 cm or more, ++ represents between 0.49 and 0.25 cm diameter, + represents a plaque less than 0.24 cm diameter, and – represents no plaque seen).

P. aeruginosa strain	Twitching Motility Diameter (mm)
PAKwt	**12.2**
PAOwt	**14.5**
KB7wt	**6.8**
K122-4	**4.0**
PAK2pfs	**0**
PAK- BΩ	**0**
PAK-DΩ	**0**
PAKNP	**0**
PAKMS591	**10.4**

Table 2.3 Twitching Motility

P. aeruginosa strains: PAK, PAO, KB7, K122-4, PAK2pfs, PAK-BΩ, PAK-DΩ, PAKNP, and PAKMS591 were evaluated for twitching motility. Bacteria twitching along a surface create a distinct translucent halo on the side of the agar closest to the surface, which is distinct from flagellar motility.

pre-immune antisera used in this study have been reported previously (Paranchych *et al.*, 1979).

2.2 Biotinylation

2.2.1 Biotinylation of *P. aeruginosa* Cells

Biotinylation of bacteria was performed as previously described with the following modifications (Yu *et al.*, 1996). Harvested cells were suspended in 5 ml of phosphate buffered saline (PBS) (pH 6.8) with 75 µl of 20 mg/ml biotinamidocaproate N-α-hydroxysuccinimidyl ester dissolved in dimethylsulfoxide and incubated at 22°C with agitation (200 RPM) in a water bath shaker for 1 hour (hr). Cells were harvested by centrifugation (10,000 x *g* for 10 minutes at 4°C) and washed 4 times before resuspension in PBS pH 6.8. Viable counts were performed before and after biotinylation.

2.2.2 Biotinylation of Purified Pili

PAK pili were purified from PAK 2Pfs as described previously (Paranchych *et al.*, 1979). The purity and integrity of the pili were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy (Figure 2.2). The procedure used for the biotinylation of the purified pili has been previously described (Yu *et al.*, 1996). Following biotinylation, pili were dialyzed over 48 hours in three changes of PBS pH 7.4. The ability of the biotinylated pili to bind to asialo-GM_1 and GM_1 was determined as previously described (Lee *et al.*, 1994)

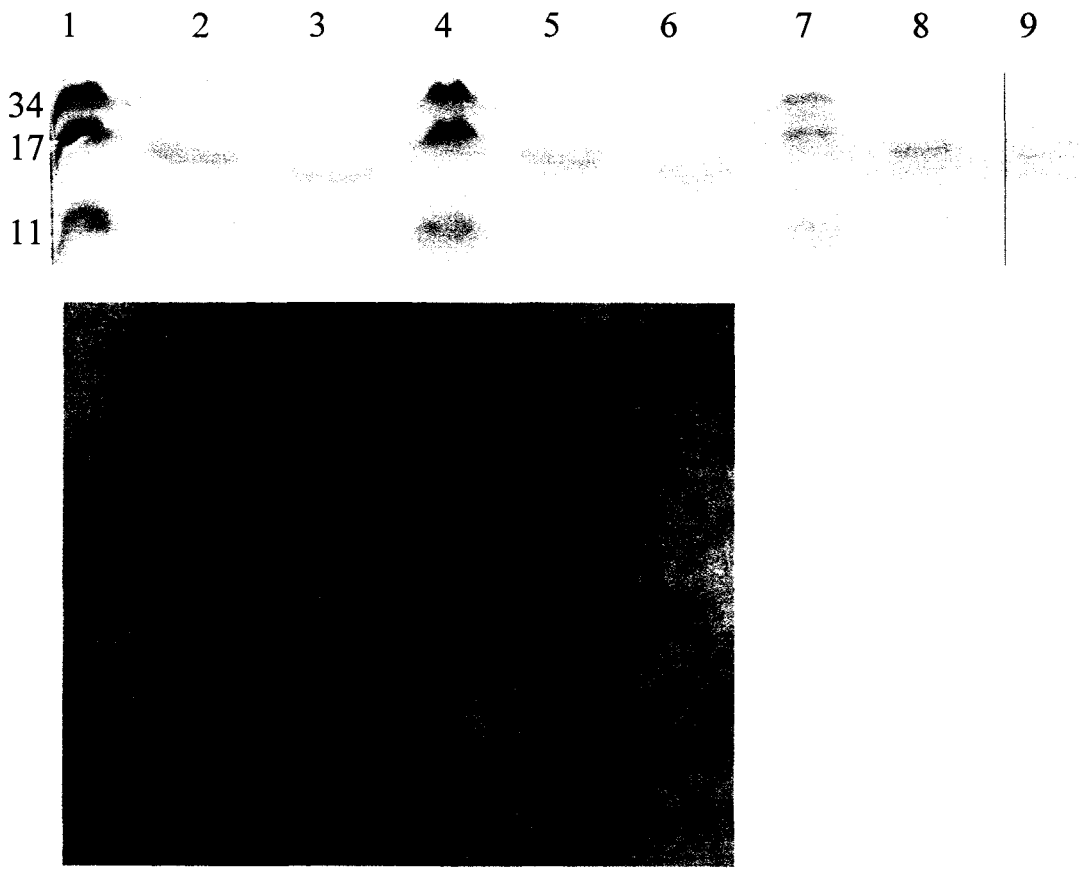

Figure 2.2 SDS-PAGE and EM of Pili

Figure 2.2 SDS-PAGE and EM of Pili

Purity and integrity of purified type IV pili was determined with SDS-PAGE (Sambrook *et al.*, 1989), and electron microscopy. Prestained protein ladder (Fermentas) seen in lanes 1, 4 and 7; PAK pili before final cesium chloride gradient purification lane 2 (here pili may have contaminants contributing to the observed higher molecular weight) and following final cesium chloride gradient lane 3, PAO pili before (lane 5) and after (lane 6) final cesium chloride gradient; and K122-4 pili before (lane 8) and after (lane 9) final cesium chloride gradient. The cesium chloride gradient is used in the final stage of pilus purification. Pili were removed following ultracentrifugation (seen as a distinct band at the cesium chloride density of approximately 1.3 mg/ml) and were dialyzed to remove any remaining cesium chloride. The second band (lanes 3, 6, and 9) represents the final purification stage after dialysis of the cesium chloride.

and the binding specificity for asialo-GM_1 was confirmed to establish the functional binding activity of the pili following biotinylation (Figure 2.3).

2.3 Peptide Synthesis

2.3.1 Synthetic Receptor Binding Domain Peptides

The peptides described in Table 2.4 were synthesized as the N-α-acetylated free carboxyl form, except for PAK(134-140) which was synthesized as the N-α-acetylated and C-terminal amide form, by solid-phase peptide synthesis and purified by reversed-phase High Performance Liquid Chromatography (HPLC) as previously reported (Wong *et al.*, 1992; Wong *et al.*, 1995). Peptides containing two cysteine residues were air oxidized to generate the disulfide bridged form of the peptide (Campbell *et al.*, 1995). Peptides were donated by Dr. R. S. Hodges, and were assessed for purity via HPLC on a C-18 column, and the molecular mass was determined by electrospray mass spectrometry as previously described (Wong *et al.*, 1995).

2.3.2 Trypsinized Bovine Serum Albumin Peptides

Bovine serum albumin (BSA) (Biotech grade Fisher) was dissolved in PBS pH 7.4, and heat denatured by boiling in a water bath for 1 hr. Trypsin (50 µl of a 1 mg/ml solution) was then added to a 10 mg/ml heat denatured BSA solution and incubated at room temperature (RT) overnight with gentle agitation. Trypsin was heat inactivated via boiling water bath for 1 hr before use in peptide inhibition assays as described in section 2.5.2.

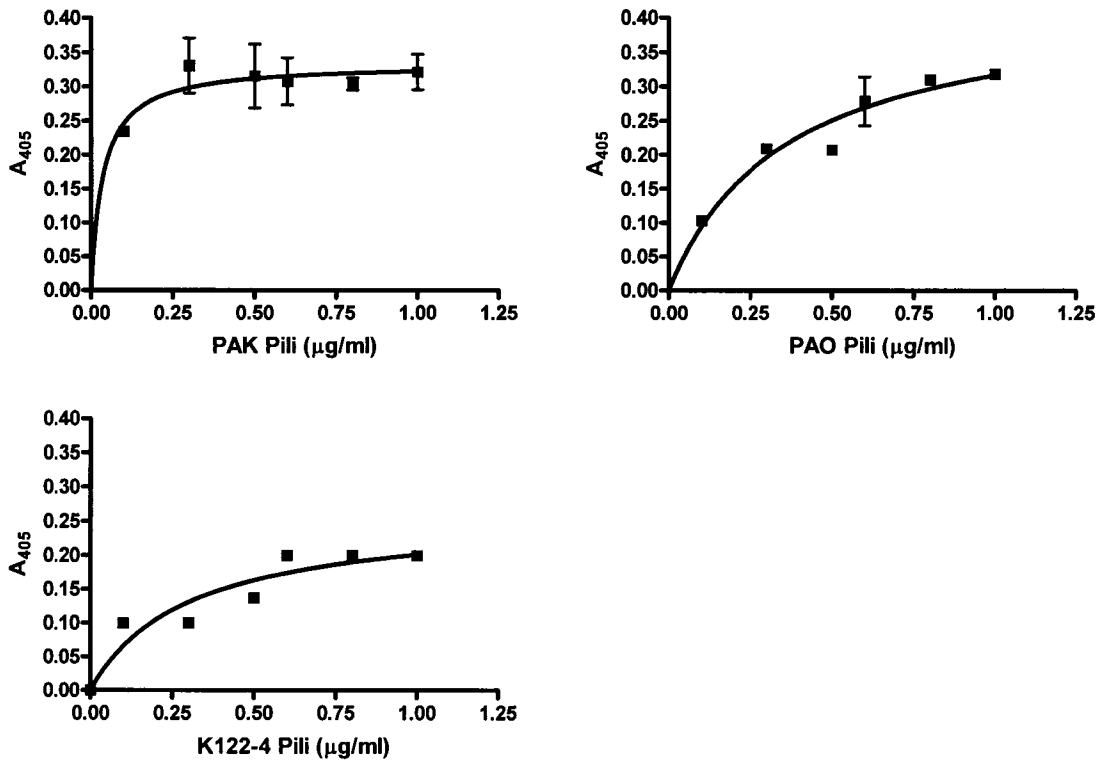

Figure 2.3 Pili Binding to Asialo-GM_1

Binding of biotinylated *P. aeruginosa* PAK, PAO, and K122-4 pili, to asialo-GM_1. As pili were able to bind to the glycosphingolipid asialo-GM_1, as previously described by Lee *et al.*, (1994), the biotinylated pili were shown to be functional.

Peptide	Sequence
PAK(128-144)ox	Ac-K-***C***-T-S-D-Q-D-E-Q-F-I-P-K-G-***C***-S-K-OH
PAK(134-140)	Ac-D-E-Q-F-I-P-K-amide
PAK(117-125)	Ac-T-L-T-R-T-A-A-D-G-OH
PAK(22-52)	Ac-P-Q-Y-Q-N-Y-V-A-R-S-E-G-A-S-A-L-A-S-V-N-P-L-K-T-T-V-E-E-A-D-P-OH
PAO(128-144)$_{OX}$_Scrambled	Ac-N-***C***-P-D-F-D-P-T-K-K-G-M-Q-A-***C***-T-S-OH
PAO(128-144)C129A/C142A_Scrambled	Ac-N-**A**-P-D-F-D-P-T-K-K-G-M-Q-A-**A**-T-S-OH
PAO(128-144)ox	Ac-A-***C***-K-S-T-Q-D-P-M-F-T-P-K-G-***C***-D-N-OH

Table 2.4 Peptide Sequences

Synthetic peptides and peptide sequences employed or referred to in this study:

Peptides were synthesized by solid phase and were N-α-acetylated with a free carboxyl except for PAK(134-140) which was synthesized as the N-α-acetylated amide peptide due to its short length. Peptides with a formed disulfide bridge are identified by "ox", and those cysteine residues involved in disulfide bond formation are shown in italics. Peptides were generously donated by Dr. R. S. Hodges.

2.4 Binding Assays

2.4.1 Stainless steel binding assay

2.4.1.1 *P. aeruginosa* Cells and Pili

Grade 304 stainless steel 2B finish plates or coupons (20 gauge, 1 mm thick and 7.6 by 11.5 cm) were washed in 95% ethanol for 10 min, and rinsed with distilled water. Immediately before the binding studies, coupons were washed with 20 ml of acetone for one min with gentle agitation and rinsed with distilled water. Steel coupons were then assembled into a Schleicher and Schuell Minifold™ System (Mandel Scientific). The Minifold system creates 96 wells each 9 mm in diameter, which was used as an ELISA plate in further experiments. Biotinylated viable PAK cells or purified PAK pili (biotinylated or unbiotinylated) were added (100 µl /well in replicates of six) to the stainless steel manifold and incubated at 37°C for one hr with gentle agitation. The manifold was subsequently washed 5 times with 250 µl /well Buffer A. Binding was assessed using either streptavidin-horseradish peroxidase (HRP) or polyclonal PAK antibodies and secondary goat anti-rabbit HRP (BioRad). Substrate buffer was added (125 µl /well) and the manifolds were incubated at RT for 10 min with shaking at 150 rpm. The absorbance was determined at 405 nm using a Multiskan Plus version 2.01 plate reader following transfer of the reaction solution to 96 well flat-bottomed micro titer plates (Corning).

Cellular concentrations in these assays ranged from 10^8-10^{12} CFU/ml. A 10^{12} CFU/ml concentration is feasible when the total number of bacteria possible in a ml is

calculated. The average *P. aeruginosa* bacterial cell dimensions (0.5 μm diameter and 1.3 μm length) give a volume of 0.25 μm^3. Therefore, the total possible number of bacterial cells in one ml is 4×10^{12}. Although the steel surface area would allow only 3×10^8 bacteria binding end-on, the cellular concentration may reflect a need for quorum sensing and a threshold concentration of autoinducers (when in high enough concentration activate quorum sensing regulated genes).

2.4.1.2 *P. aeruginosa* Monomer and Nanotubes

Grade 304 stainless steel 2B finish coupons were prepared as previously described (Giltner *et al.*, 2006). Briefly, steel coupons were immersed in ethanol for 10 minutes, scrubbed with a soft brush to remove any remaining organic material, rinsed with distilled water and immersed in acetone for one minute. The steel coupon was then rinsed in distilled water and immediately placed into the Schielder and Schell Minifold system. Purified K122-4 monomer or nanotubes or K122-4 purified pili (100 μl/well) were added in replicates of six to the steel manifold and incubated with gentle agitation for one hour at 37°C. Steel coupons were washed five times with Buffer A. Binding was assessed using a polyclonal anti-K122-4 antibody followed by secondary goat anti-rabbit HRP (BioRad). Substrate buffer was added to steel manifolds as described (Giltner *et al.*, 2006).

2.4.2 Buccal Epithelial Cell Assay

Corning 24-well tissue culture treated ELISA plates were incubated with 500 μl of a 1 μg/ml poly-L-lysine solution at 75°C overnight before washing for 15 minutes

with PBS 3 times. Glutaraldehyde (25%, 125 µl/well) was incubated for one hour at 37°C. Wells were washed as previously stated. Buccal epithelial cells, harvested from 10 healthy volunteers, were filtered through a fine (70 µl) nylon mesh (Spectrum) and added to the prepared ELISA plate for overnight fixation at 37°C. Binding studies were performed as described above.

2.4.3 Direct Binding of Peptides to Steel

To determine whether the observed binding inhibition of the synthetic pilin receptor binding domain was due to interactions of the peptide with the steel surface or due to interactions with the peptide and the cell or pilus, direct binding of the synthetic receptor binding domain to stainless steel was tested. The binding of the synthetic peptides to steel was determined by a modified immuno-assay that has been previously described (Yu *et al.*, 1996). Synthetic peptides PAK(128-144)ox and PAK(134-140) were prepared in Buffer A to a concentration of 0 to 51 µM. The peptides were then added directly to wells (100 µl/well in replicates of six) formed on the steel surface and incubated for one hour at 37°C without agitation. Following five washes with Buffer A (250 µl /well), a 1:5000 dilution of PK99H was added to each well (100 µl /well), incubated for one hour and washed as described. Secondary antibody goat anti-mouse IgG HRP (100 µl /well of a 1:3000 dilution) was added and again incubated for one hour. ABTS substrate solution was added (125 µl /well) and allowed to incubate for 25 minutes before the absorbance at 405 nm was determined.

2.4.4 Antibody Recognition

Purified K122-4 or PAK pili, monomer, or nanotubes in equivalent protein concentrations (10 μg/ml) in coating buffer (0.01M sodium carbonate, pH 9.5) were added to 96-well microtitre plates (Corning) as described previously (Engvall, 1980). Plates were then blocked with 5% BSA for one hour at 37°C. Pili, monomer, and nanotubes were detected with either the polyclonal anti-K122-4 pili or the polyclonal anti-PAK pili antibody in dilutions ranging from 10^{-2} to 10^{-7}, and incubated for one hour. Secondary antibody goat anti-rabbit IgG HRP (100 μl /well of a 1:3000 dilution) was added and again incubated for one hour. ABTS was used as substrate which was incubated for 10 minutes before the absorbance at 405 nm was determined as described above. These experiments were performed by Dr. Erin van Schaik.

2.5 Inhibition Assays

2.5.1 Antibody Inhibition Studies

Biotinylated viable PAK whole cells or biotinylated purified PAK pili were mixed with 50 μl of a 10^{-2} dilution of pilus specific antibody or, for controls, pre-immune rabbit serum in PBS buffer (pH 7.4) were added with a vortex mixer and incubated for one hour at 37°C. (Note that all antibodies test were initially set to the same titer via an ELISA employing purified pili as an antigen as follows: purified pili were attached to polystyrene plates, where serially diluted antibodies were added to the pili). Concentrations of antibody giving rise to a 0.200 A_{405} reading within five minutes were used for the remainder of the experiments. The cell or pili mixture was used for

binding assays as previously described (Yu *et al.*, 1996). Concentrations of biotinylated PAK cells or biotinylated PAK pili ranged from zero to 3 x 10^{12} CFU/ml and zero to 1.5 μg/ml respectively. The steel surface was washed five times with Buffer A, incubated with 100 μl of either a rabbit anti-IgG HRP (for the polyclonal antibody) or mouse anti-IgG HRP (for the monoclonal antibodies). After a one hour incubation at 37°C, the steel surface was washed as described above, and ABTS substrate solution (125 μl/well) was added for 15 minutes. The two pilus specific antibodies used in this study, a polyclonal anti-PAK pili antibody (Lee *et al.*, 1989), and monoclonal antibody PK99H (Doig *et al.*, 1990) have been previously described. Rabbit pre-immune serum, which had previously been determined to be free of anti-*P. aeruginosa* antibodies by ELISA (Figure 2.4), was utilized as a control.

2.5.2 Peptide Inhibition Assays

Synthetic peptides PAK(128-144)ox, PAK(117-125), PAK(134-140), PAO(128-144)ox, PAK(128-144)oxK130I, PAO(128-144)ox_Scrambled and PAO(128-144)C129A/C142A_Scrambled were dissolved in Buffer A (See Table 2.4 and Table 3.1 for sequences) and incubated with either 10^8 CFU/ml biotinylated viable PAKwt cells or 0.75 μg/ml of biotinylated purified PAK pili such that the final peptide concentration ranged from 51 nM to 51 μM. The samples were then utilized directly in a steel surface binding assay as described above. These peptides were synthesized by W. Y. Wong and K. K. Lee. The Ki values for PAK(128-144)ox peptides with both PAK pili and PAK cells were determined using Prism 4 curve fitting.

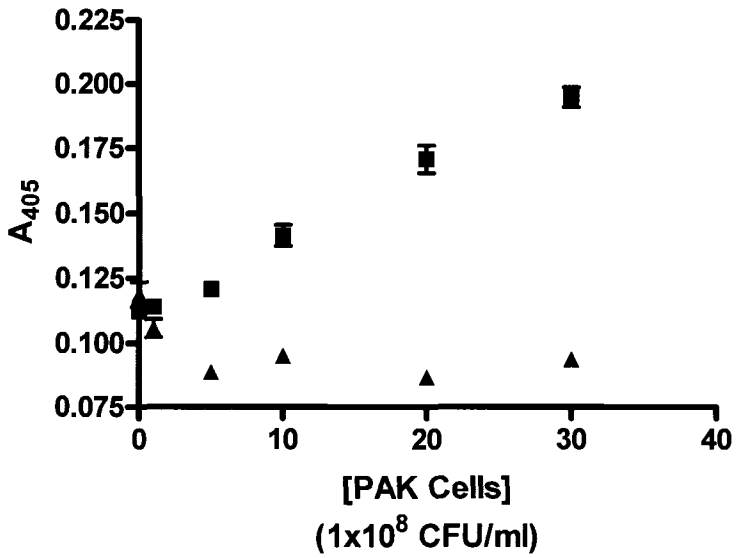

Figure 2.4 Antibody Reactivity

A direct ELISA to ensure the rabbit pre-immune serum was free of anti-*P. aeruginosa* antibodies was performed. PAK viable cells were allowed to bind to asialo-GM_1. Either rabbit pre-immune serum (▲) or polyclonal anti-PAK pili (■) antibodies were added for one hour. ELISA plate was washed with Buffer A and secondary antibody was added for an additional hour, and ABTS was used as substrate.

2.6 Acridine Orange Staining and Microscopy

Stainless steel plates, prepared and utilized in bacterial binding studies as described above, were incubated in 1 mM acridine orange stain for one min, and thoroughly rinsed with distilled water. Bacterial concentrations were 10^8 CFU/ml. Coupons were visualized using a Leitz Laborlux K microscope equipped with a MSP4 camera, and 40X Neoflour lens with epifluorescent illumination. Micrographs were recorded with Kodak Colormax 35 mm film, processed and digitally scanned immediately after film processing.

2.7 Purification and Formation

2.7.1 Monomer Formation and Purification

Monomer formation and purification were performed as previously described (Hazes *et al.*, 2000). Briefly, residues 29-144 of the pilin monomer were expressed in the *Escherichia coli* BL21 expression plasmid. Expression of the pilin monomer was induced using 1 mM isopropyl β-D-thiogalactopyranoside, and the resulting periplasmic protein was harvested by osmotic shock. The protein was purified using sequential cation-exchange chromatography with a CM-cellulose column as previously described (Keizer *et al.*, 2001).

2.7.2 Protein Nanotube Formation

K122-4 protein nanotube formation was performed as outlined in Audette *et al.* (2004). Briefly, 1.1 M of a linear hydrophobe (C11-SH) in methanol containing DTT

and EDTA was added to K122-4 pilin monomers described above, and incubated overnight at room temperature, triggering nanotube formation. Protein nanotubes were then assessed via electron microscopy (Figure 2.5). Technical assistance from Dr. Gerald Audette was greatly appreciated.

Figure 2.5 PAK Pili EM

Negatively stained electron microscopy image of formed *P. aeruginosa* K122-4 pilin nanotubes. Bar represents 100 nm.

Chapter 3

Results

3.1 Initial Colonization of Stainless Steel is Dependent Upon Type IV Pili in *P. aeruginosa*

While the involvement of type IV pili in biofilm formation on abiotic surfaces has been well documented (Klausen *et al.*, 2003a; O'Toole and Kolter, 1998), the molecular basis for this involvement has not been firmly established. *P. aeruginosa* pilins are characterized by a highly conserved N-terminal α-helix and a semi-conserved C-terminal disulfide loop region but display minimal sequence similarity through the bulk of the protein (Figure 3.1-a). However, structural studies indicate that *P. aeruginosa* pilins are strikingly similar (Figure 3.1- b) (Audette *et al.*, 2004; Craig *et al.*, 2003; Hazes *et al.*, 2000). As the first step in biofilm formation is attachment to a surface, and pili are involved in adherence, I sought to investigate the ability of *P. aeruginosa* wild-type strains PAK, PAO, K122-4, and KB7, which have considerable differences in their pilin sequences, to bind stainless steel directly (Figure 3.1-a, b). *P. aeruginosa* strains PAK, PAO, K122-4, and KB7 were observed to readily bind to stainless steel surfaces (Figure 3.2-a, d, g, and j), in agreement with previous results (Stanley, 1983; Vanhaecke *et al.*, 1990). Strains PAO and PAK bound more readily to stainless steel than either K122-4 or KB7 (Figure 3.2-a, d, g, and j). *P. aeruginosa* strain PAKMS591 (Starnbach and Lory, 1992) a *fliC*⁻ non-flagellated mutant of PAK, was bound at considerably reduced levels compared to the parental PAK strain (Figure

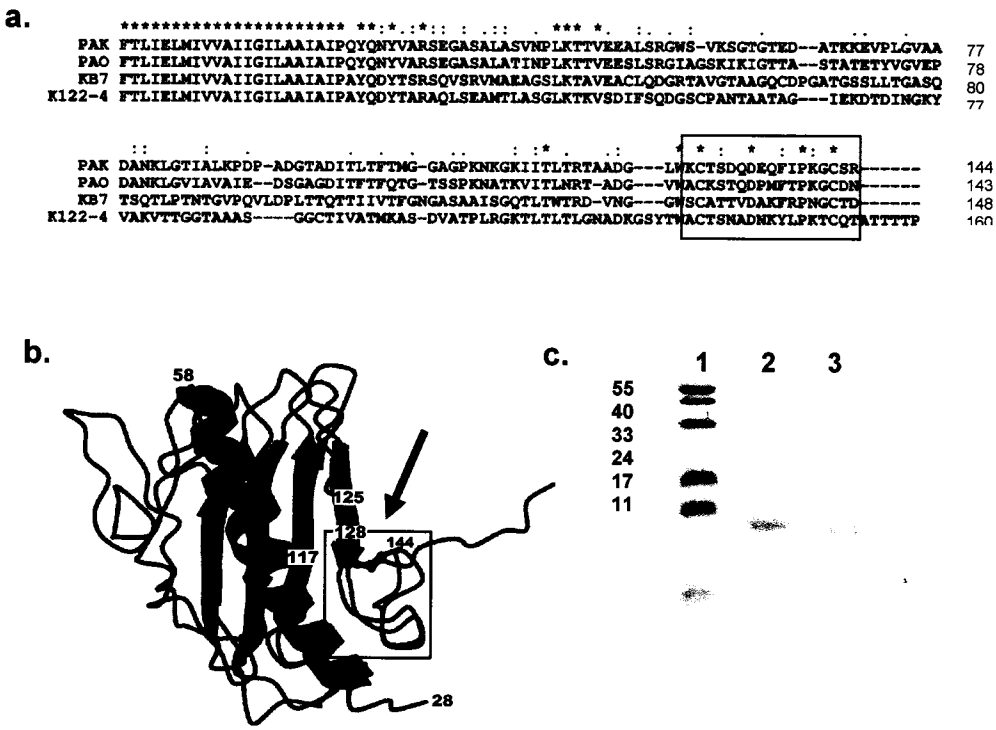

Figure 3.1 Sequence Structure and Purity of PAK Pilin

Figure 3.1 Sequence Structure and Purity of PAK Pili

a. Full-length pilin sequences of *P. aeruginosa* strains PAK, PAO, KB7 and K122-4. Boxed area represents disulfide loop region of residues 128–144 of the PAK sequence. The disulfide loop region contains an epithelial cell binding domain and displays a conserved antigenic epitope despite extensive sequence variation.

b. Structural overlay of *P. aeruginosa* strains PAK (red) and K122-4 (blue) truncated monomeric pilins. Disulfide loop region is highlighted by boxed area, cysteine residues are shown in black and the disulfide bonds are shown in green and yellow for PAK and K122-4 strains respectively and are depicted with a green arrow.

c. Fifteen % SDS-PAGE gel (Sambrook *et al.*, 1989), lane 2 shows pili preparation before final cesium chloride gradient and lane 3 shows a single non-contaminated band of pilin after density ultracentrifugation. Lane 1 represents a pre-stained protein ladder (Fermentas).

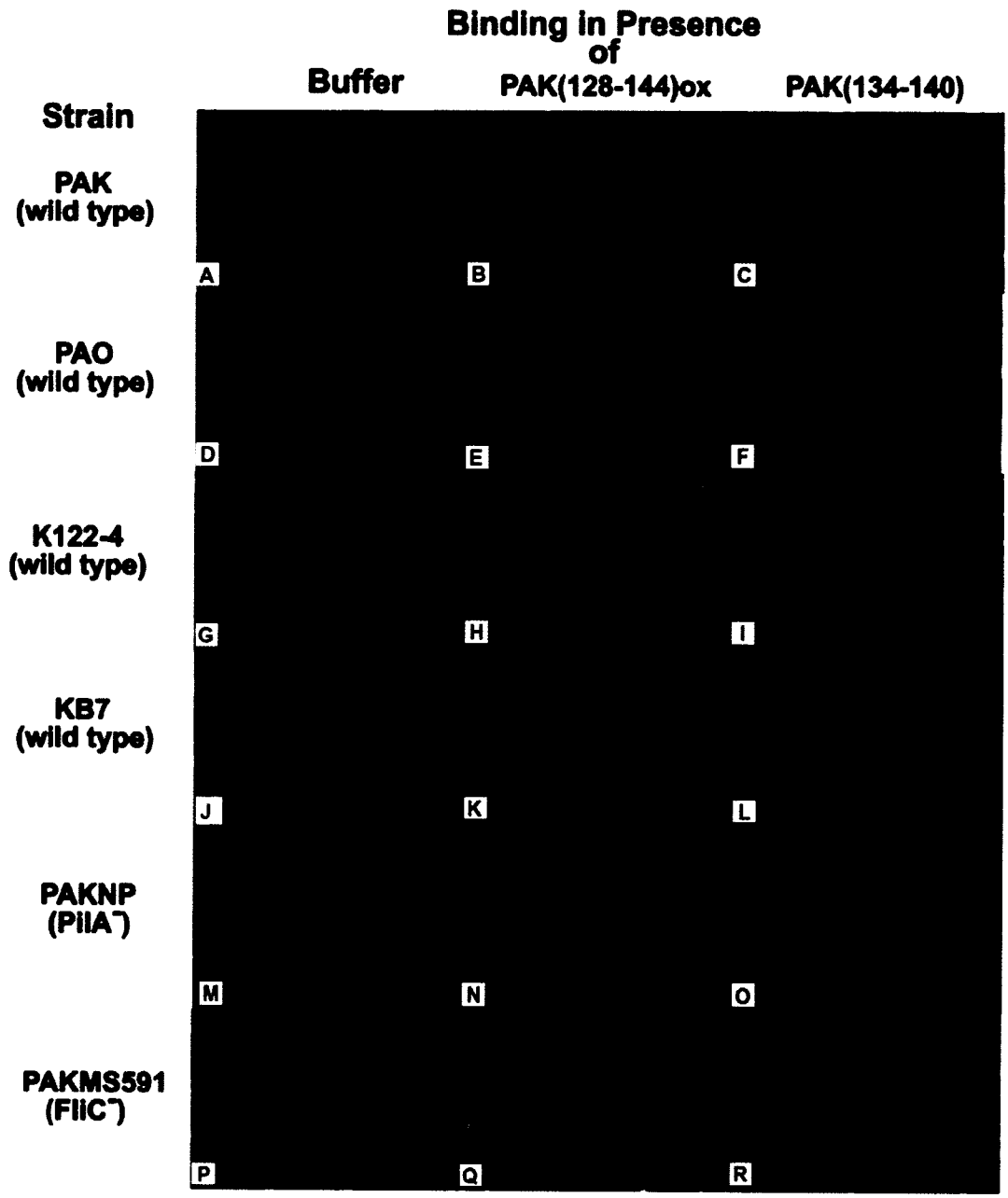

Figure 3.2 Acridine Orange Staining

Figure 3.2 Acridine Orange Staining

Epifluorescence microscopy of stainless steel after binding of *P. aeruginosa* strains PAK (panels A, B and C), PAO (panels D, E and F), K122-4 (panels G, H and I), KB7 (panels, K and L), PAKNP (panels M, N and O) and PAKMS591 (panels P, Q and R). Viable cells (10^8 CFU/ml) were allowed to incubate directly (panels A, D G, J, M and P) with a stainless steel grade 304 surface. The cells were then stained using acridine orange. Note that bound *Pseudomonas* cells are stained orange while green fluorescence indicates non-specific interaction of the fluorochrome with the surface. (See Section 2.6 for method).

P. aeruginosa strains PAK, PAO, K122-4, KB7, PAKNP and PAKMS591 were incubated with the synthetic peptide PAO(128–144)ox (51 nM) (panels B, E, H, K, N and Q respectively) or with synthetic peptide PAK(134–140) (52 mM) (panels C, F, I, L, O and R respectively).

3.2-a, and p). *P. aeruginosa* strain PAKNP, a non-piliated *pilA*$^{-}$ mutant of PAK (Saiman *et al.*, 1990), did not bind to steel surfaces (Figure 3.2-m), suggesting that *P. aeruginosa* adherence to stainless steel may be mediated by type IV pili. To further investigate the initial colonization of stainless steel, the binding kinetics of PAK cells and pili to steel were examined in a quantitative manner employing viable biotinylated cells and purified biotinylated PAK pili. PAK cells (Figure 3.3a, Figure 3.4), and purified PAK pili (Figure 3.3b) were observed to bind to steel surfaces in a saturable, concentration dependent manner while PAKNP biotinylated cells (Figure 3.3a, Figure 3.4) did not bind appreciably to the steel surface. Biotinylation had no effect on the ability of the purified pili to bind stainless steel as identical binding kinetics were observed with native pili when anti-PAK pili antibodies were utilized to quantify binding (Figure 3.5). Furthermore, addition of low concentrations of purified PAK pili inhibited the binding of biotinylated PAK cells to steel utilizing a direct inhibition method (Figure 3.3c).

I established that *P. aeruginosa* stained with acridine orange were readily visualized by epifluorescence microscopy following binding to stainless steel (Figure 3.1-c). *P. aeruginosa* cells were visualized as fluorescent orange rods bound to areas of the steel that fluoresced green either through non-specific interaction of the fluorochrome with grain boundary regions or due to non-specific interaction of the fluorochrome with organic material that interacted with the grain boundaries in the steel. Green fluorescent material was observed with both strains PAK and PAKNP (Figure 3.2-b, -n, Figure 3.6-a). Furthermore, *Pseudomonas* sp. has recently been shown to colonize preferentially with grain boundaries (Sreekumari *et al.*, 2001).

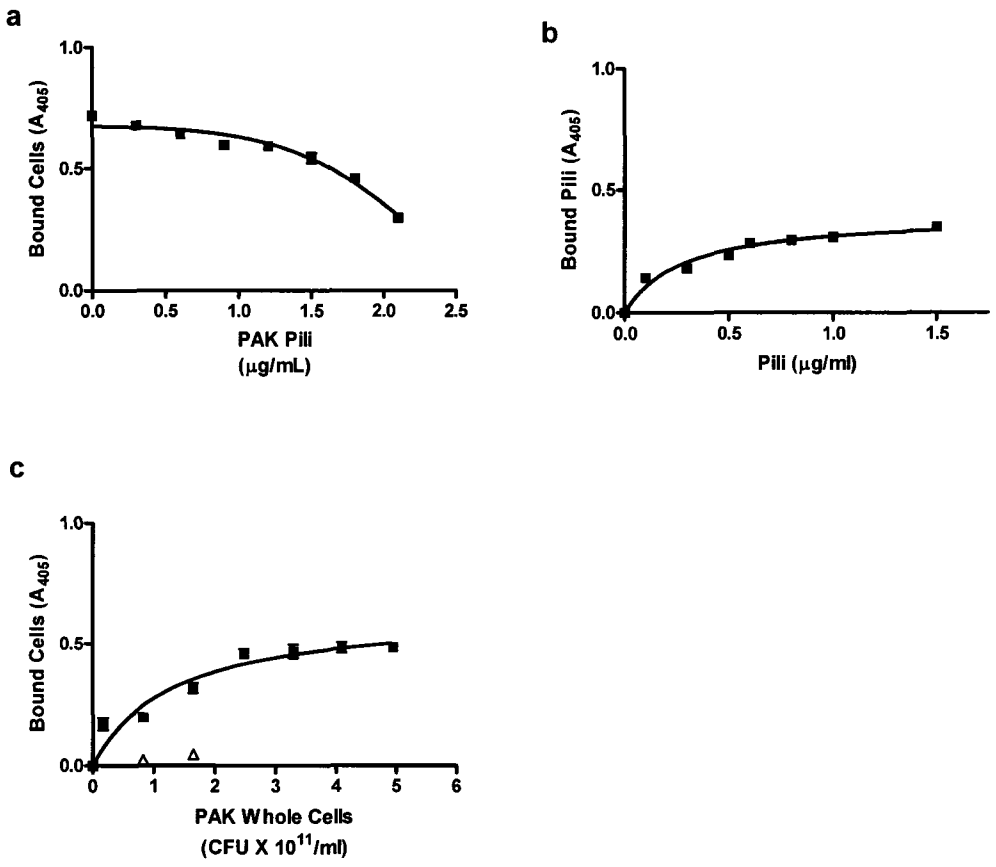

Figure 3.3 Pili and Cells Binding to Stainless Steel

Figure 3.3 Pili and Cells Binding to Stainless Steel

a. Inhibition of viable biotinylated-PAK whole cell binding to stainless steel surfaces by purified homologous unlabelled pili. Pili and bacteria (10^{11} CFU/ml) were mixed and then directly added to the stainless steel surface. The symbols and bars in this and subsequent figures report the mean ± SEM (where two individual experiments employing six replicates for each data point were used).

b. Binding of biotinylated PAK pili to stainless steel. Biotinylated pili were washed and suspended in 10 mM PBS pH 7.4, and allowed to bind to stainless steel for 60 min at 37°C. Plates were washed three times with Buffer A before addition of streptavidin HRP for an additional hour. Following a final washing, ABTS was used as substrate, and plates were read at A_{405}.

c. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) and a pilin-deficient strain PAKNP (Δ) to stainless steel at cell densities of 10^{11} CFU/ml. PAK cells bound to the stainless steel surface area were detected using ABTS as a substrate.

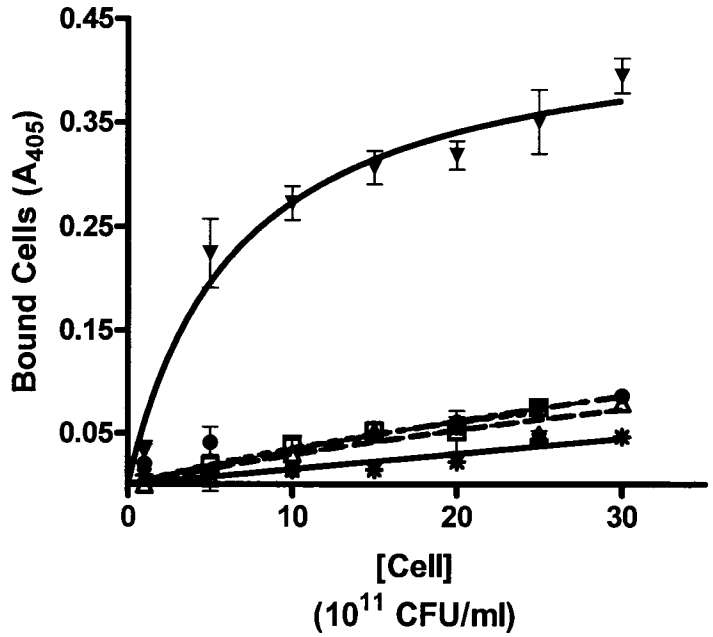

Figure 3.4 ***P. aeruginosa*** **Mutants Binding to Stainless Steel**

Binding of biotinylated viable cells of *P. aeruginosa* strains PAKwt (▼), PAKMS591 a FliC$^-$ strain (Δ), PAK-BΩ a PilB$^-$ strain (○), PAK-DΩ a PilD$^-$ strain (□), and PAKNP a PilA$^-$ strain (*) binding to stainless steel. The quantity of PAK cells bound to the stainless steel surface area was determined by measuring the amount of biotin bound to the stainless steel surface employing a modified ELISA with streptavidin-HRP and utilizing ABTS as a substrate.

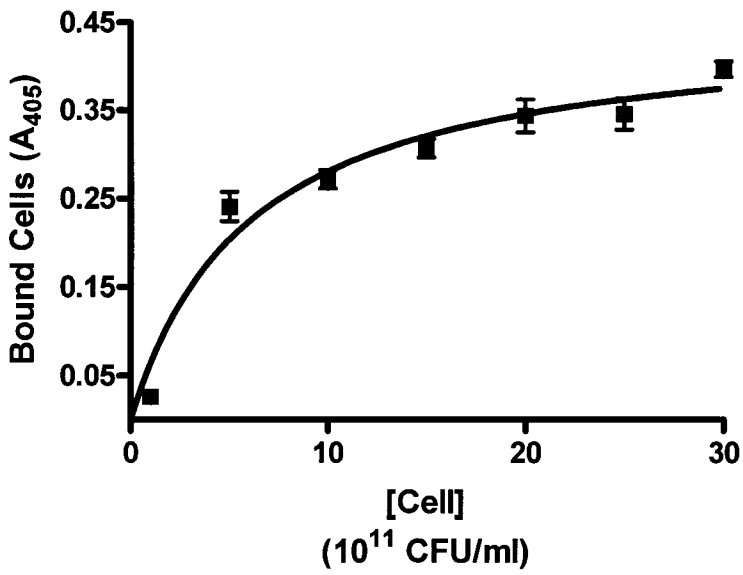

Figure 3.5 Antibody Detection of Pili Binding to Stainless Steel

PAK pili binding stainless steel visualized with polyclonal anti PAK pili antibody and secondary antibody utilizing ABTS as substrate. Note that binding is similar to that seen with biotinylated pili (see Figure 3.3b).

3.6 *P. aeruginosa* Cells Binding to Stainless Steel and Pili EM

Epifluorescence micrograph of acridine orange stained PAK wild-type cells (**a**), and PAKNP (PilA$^-$strain) (**b**) bound to stainless steel. Bacterial concentrations were 10^8 CFU/ml. Bacterial cells fluoresce orange, while grain boundaries fluoresce green due to non-specific staining. The bar represents 5 μm. **(c)**. Electron micrograph picture of PAK pili. The bar represents 100 nm.

3.2 Effect of Flagella and Type IV Pilin Mutations on Binding to Steel

Previous studies using *P. aeruginosa* FliC$^-$ and PilB$^-$ strains have established the importance of flagella and type IV pili during the initiation and development of biofilms on abiotic surfaces in static cultures (O'Toole and Kolter, 1998). To further establish that type IV pili mediate binding to stainless steel and to determine if flagella are involved in binding, a quantitative analysis of adherence to steel was performed. Two new mutants were chosen to assess the different binding phenotypes associated with mutant pili. In both cases these *P. aeruginosa* mutants cannot form a functional pilus; however the stage of pilus assembly that has been mutated differs. PAK-BΩ has an omega fragment inserted into the PilB gene (an ATPase responsible for assembly of PilA monomers into the native pilus structure), and therefore cannot form a pilus. PAK-DΩ has an omega fragment inserted into the PilD gene (a leader peptidase and N-terminal methylase, responsible for cleaving a 6 amino acid leader sequence and subsequent methylation of the first residue of the mature peptide), therefore although PilA is produced it cannot be incorporated into the pilus structure.

The binding to steel of PAK-BΩ (*pilB*$^-$ mutant), a strain that does not assemble pili but does express PilA (Koga *et al.*, 1993), was compromised relative to wild-type and equivalent to that observed for PAKNP and PAKMS591 (Figure 3.4). ELISA evidence indicated that PAKMS591 has fewer surface exposed pili than does the PAK wild type strain (Figure 3.7). Strain PAK-DΩ (*pilD*$^-$ mutant), which lacks the pre-pilin peptidase and therefore does not assemble pili on the cell surface (Koga *et al.*, 1993), bound to steel at levels equivalent to strains PAKNP, PAKMS591, and PAK-BΩ (Figure 3.4). Although the binding curves differ slightly among mutant strains, all pilus

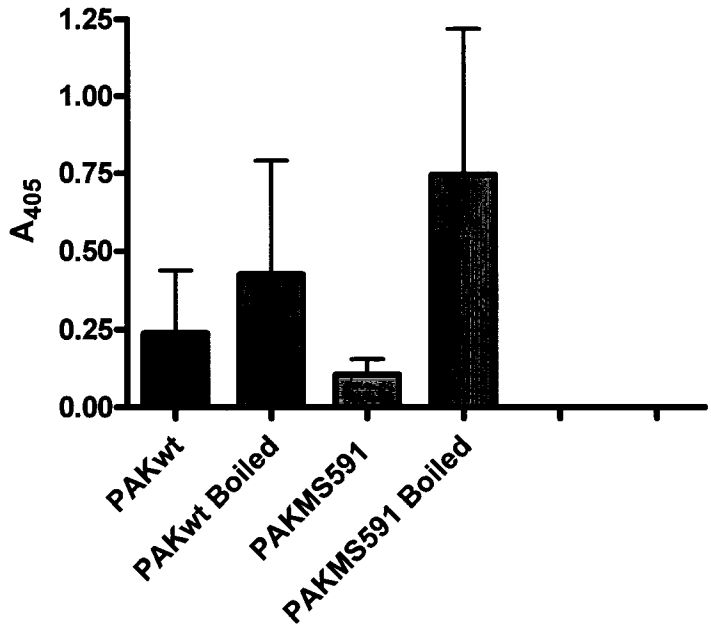

Figure 3.7 Surface Exposed Pili

ELISA evidence indicates that PAKMS591 has fewer surface exposed pili than does the PAK wild-type strain. Bacteria were boiled to denature membranes, or not boiled. Presence of pili was detected using PK99H (an anti-PAK pilin antibody). Bacteria were centrifuged and the supernatant was removed. Following five washes with Buffer A, secondary antibody was added, bacterial were washed via centrifugation and ABTS was used as substrate. These experiments were performed by Dr. Erin van Schaik.

deficient strains (PAKNP, PAK-BΩ, and PAK-DΩ), and PAKMS591, bound significantly less strongly to steel than wild-type (Figure 3.4). These results indicate that any mutation which abolishes the production of functional pili also reduces the ability to bind to stainless steel.

3.3 Binding to Stainless Steel

3.3.1 Type IV Pili

To determine whether the C-terminal receptor binding domain of the pilus was responsible for adherence to stainless steel, as for BECs (Irvin *et al.*, 1989; Schweizer *et al.*, 1998), I employed monoclonal antibodies specific for residues in the C-terminal binding domain in an inhibition assay. Monoclonal antibody PK99H recognizes residues 134-140 of PAK PilA (Wong *et al.*, 1992), inhibits pilus mediated binding to respiratory epithelial cells (Irvin *et al.*, 1989), and confers protection from challenge with strain PAK in a mouse infection model (Sheth *et al.*, 1995). Addition of rabbit polyclonal anti-PAK pili antibodies (Lee *et al.*, 1989) but not rabbit pre-immune serum strongly inhibited the binding of biotinylated PAK cells and pili (Figure 3.8) to steel in a dose-dependent manner. These data suggest that the type IV pili mediate binding to the steel surface. Murine monoclonal antibody PK99H, that recognizes PAK PilA residues 134-140 exposed at the tip of the pilus (Lee *et al.*, 1994; Wong *et al.*, 1992), significantly inhibited the binding of PAK cells and pili to steel (Figure 3.8). The inhibition of PAK binding to stainless steel by PK99H suggests that the C-terminal

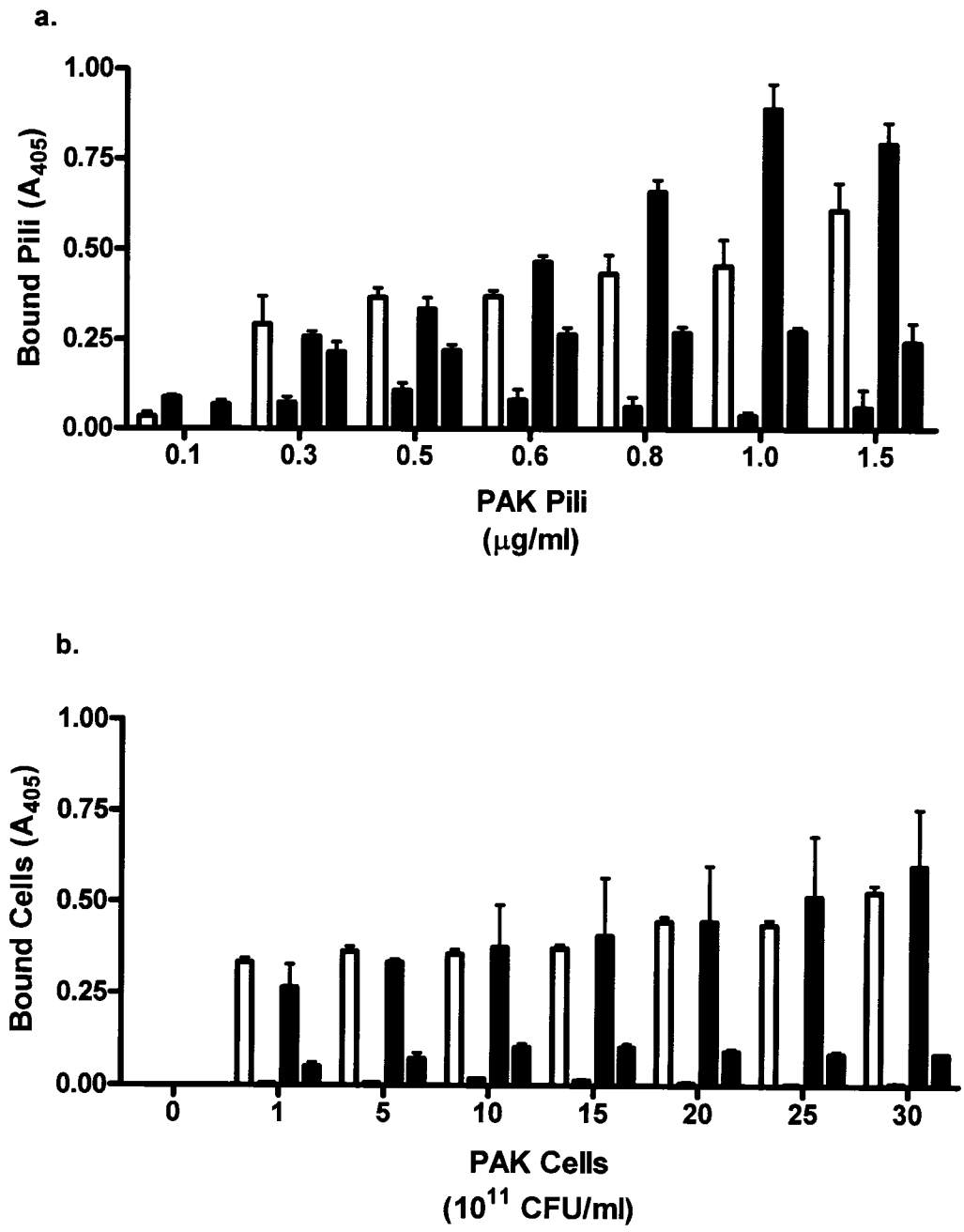

Figure 3.8 Antibody Inhibition

Figure 3.8 Antibody Inhibition

a. Antibody inhibition of the binding of viable biotinylated PAKwt cells to stainless steel relative to PAKwt cells in buffer (white bar), exposed to rabbit polyclonal anti-PAK pili antisera (specific for PAK pili) (red bar), rabbit pre-immune antisera (where no antibodies specific for the type IV pilus should be present) (green bar), or murine monoclonal antibody PK99H (specific for PAK pili) (blue bar). Antibodies specific for PAK pili were used at equivalent activity concentrations (1:3000 dilution) and the pre-immune serum was used at a 1:3000 dilution.

b. Antibody inhibition of the binding of biotinylated PAK pili to stainless steel relative to PAK pili in buffer (white bar) exposed to rabbit polyclonal anti-PAK pili antisera (specific for PAK pili) (red bar), rabbit pre-immune antisera (where no antibodies specific for the type IV pilus should be present) (green bar), or murine monoclonal antibody PK99H (specific for PAK pili) (blue bar). Antibodies specific for PAK pili were used at equivalent activity concentrations (1:3000 dilution) and the pre-immune serum was used at a 1:3000 dilution.

disulfide loop region of pilin, which contains an epithelial cell binding domain (Irvin *et al.*, 1989), may also function to mediate attachment to steel surfaces.

3.3.2 Monomer and Nanotubes

Truncated monomeric pilin subunits lack the 28 residues of the N-terminal α-helix, and therefore do not assemble into a native pilus fiber (Hazes *et al.*, 2000). However, exposure of this truncated monomer to a hydrophobe causes oligomerization of multiple pilin monomers to form long thin nanotubes, which have a similar appearance to native pili (Compare Figure 3.6-c with Figure 3.9) (Audette, 2004). These nanotubes can reach lengths up to 100 μm, which is considerably longer than native type IV pili (Folkhard *et al.*, 1981) (Figure 3.9).

As the protein nanotubes are a self-assembled structure of monomers that lack the N-terminal α-helix, I sought to determine whether the functional characteristics of the nanotubes resembled those of the native pilus, a structure that is assembled through the efforts of over 50 separate proteins in *P. aeruginosa* (Jacobs *et al.*, 2003; Mattick, 2002), and in particular whether the nanotubes retained the ability to bind to steel surfaces with high affinity.

Pili, pilin monomer, and nanotubes were tested for binding to stainless steel. In direct binding assays pili, monomer, and nanotubes, were able to bind to steel in a concentration dependent and saturable manner (Figure 3.10).

Additionally, pili, monomer, and nanotubes were immobilized on a 96-well microtitre plates, and the structure of the pilus and nanotube was investigated employing a polyclonal anti-K122-4 pili serum. Homologous pili, monomer, and nanotubes were

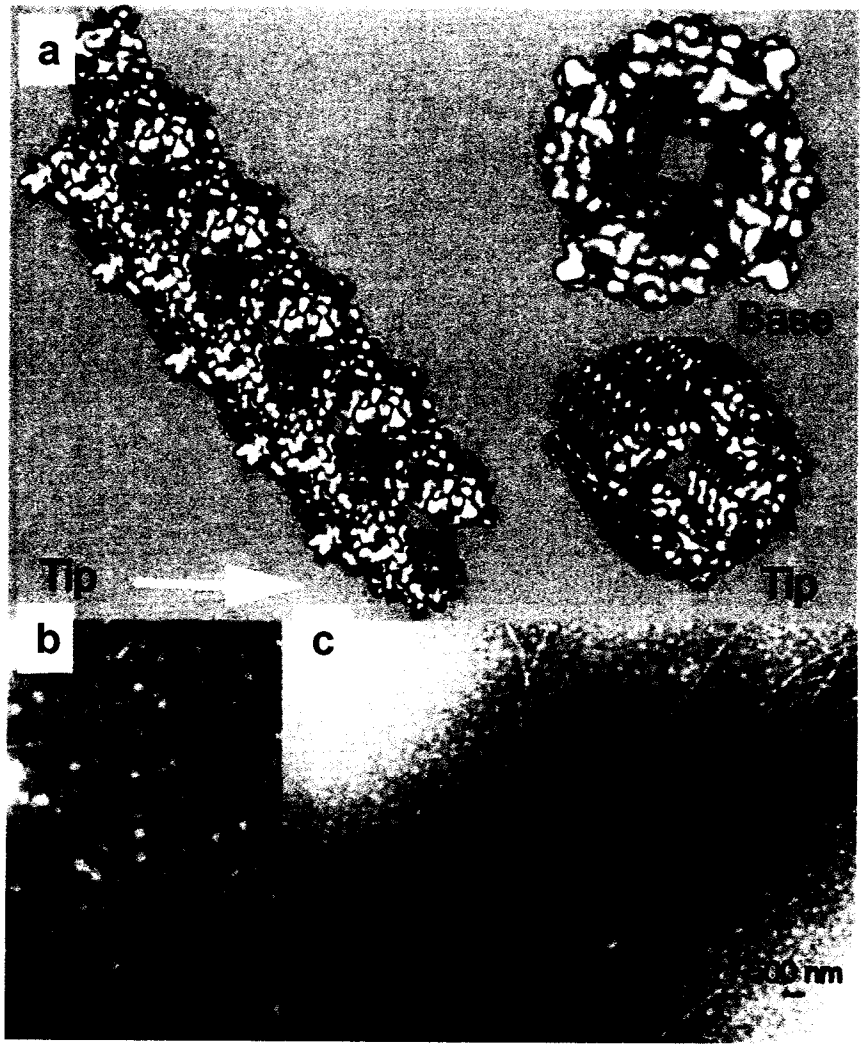

Figure 3.9 Nanotubes Model and EM

Figure 3.9 Nanotubes Model and EM

a. The 3-start left-handed helix model for PAK pilin has been previously described (Craig *et al.*, 2004) and was generously provided by Lisa Craig. The pilin protein nanotube surface representation model has been created using Web Viewer Pro with a truncated PAK monomeric pilin (as the rotation and translation function was not obtained) lacking the first 28 amino acid residues, and therefore the N-terminal α-helices found in a native pilus. The surface electrostatics are shown, blue represents positive, red negative, and white neutral charge. In addition, the C-terminal receptor binding domain in green is somewhat displayed (a significant portion of the receptor binding domain is occluded due to interfacial packing) along the solvent exposed length of the pilus and multiple exposed receptor binding domain sites are present at the tip, but not the base of the pilus.

b. Negatively stained electron micrograph of purified pili following staining with 1% aqueous ammonium molybate pH 7.0 using a Hitach H-700i EM operating at an accelerating potential of 75 kV, the bar represents 250 nm.

c. Negatively stained electron micrograph of *P. aeruginosa* strain K122-4 truncated monomeric pilin oligomerized to form a protein nanotubes, the bar represents 500 nm.

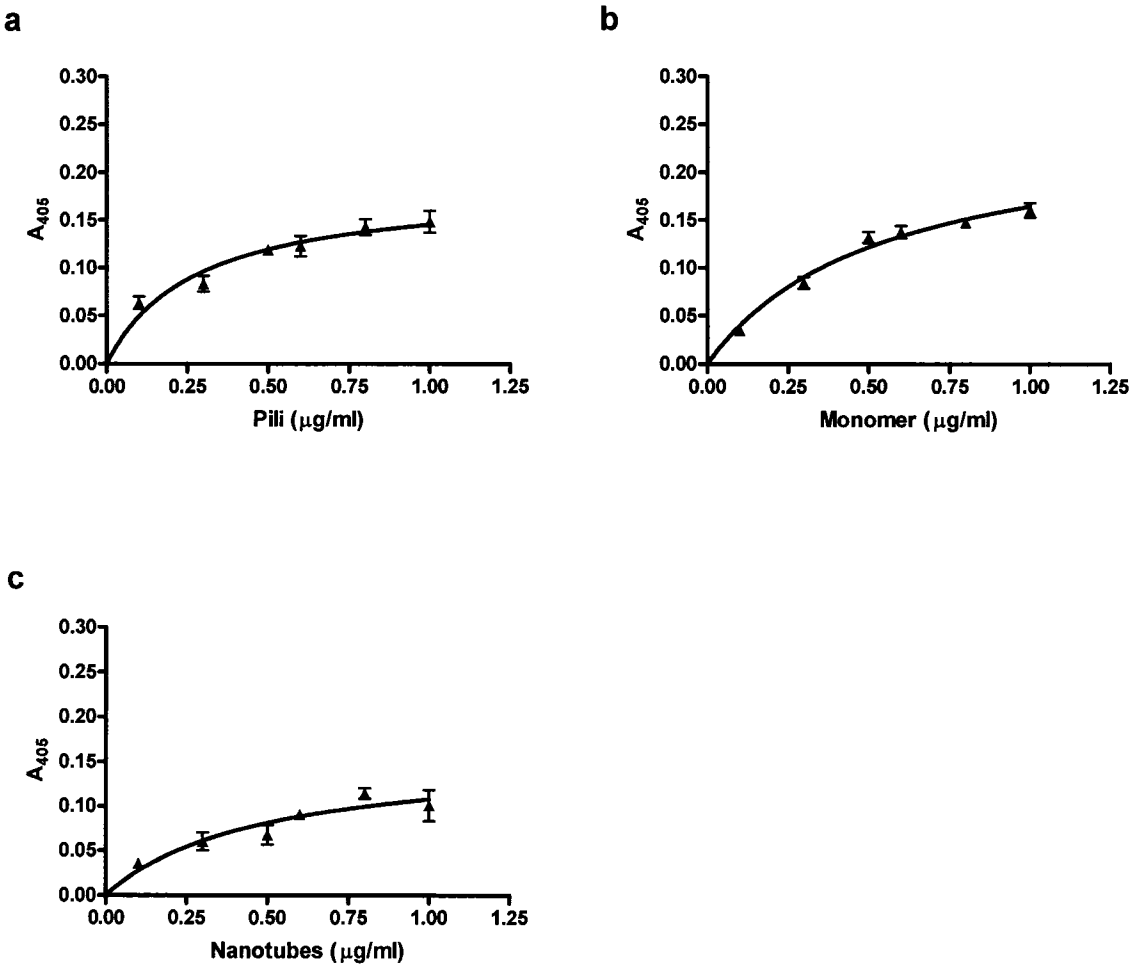

Figure 3.10 Pili Monomer and Nanotube Binding to Stainless Steel

Figure 3.10 Pili Monomer and Nanotube Binding to Stainless Steel

Between 0 and 1.0 μg/ml of K122-4 pili **(a)** K122-4 monomer (Δ 1-28) **(b)** and K122-4 nanotubes **(c)** binding to Grade 304 stainless steel surface. A modified ELISA assay was utilized to determine the amount of pili, monomer, and nanotubes bound to the stainless steel. After incubating and washing pili, monomer or nanotubes, steel coupons were incubated with polyclonal anti-K122-4 antibody in Buffer A, followed by secondary-HRP antibody in Buffer A and ABTS was used as substrate. Error bars in this and subsequent figures represent the ±SEM (experiments were performed in triplicate with individual experiments using six replicates).

all recognized at equivalent antibody titres (Figure 3.11). This shows that antibody epitopes are presented consistently through the pili, monomer and nanotubes. However, when a polyclonal anti-PAK pili serum was employed to structurally assess conserved epitopes, the monomer and the nanotubes were not recognized, even at high antibody concentrations (Figure 3.11). This suggests that the quaternary structure of the pilus is different from that of the nanotube, although the binding kinetics remain similar (Figure 3.10). The anti-K122-4 pili serum and the anti-PAK pili serum experiments were both performed by Dr. Erin van Schaik.

3.4 Synthetic Peptide Inhibition of *P. aeruginosa* Binding

3.4.1 *P. aeruginosa* Type IV Pili

As *P. aeruginosa* strains vary in their ability to bind to steel surfaces (Figure 3.2-a, d, g, and j) I sought to determine whether the PAK pilin receptor binding domain, PAK(128-144)ox, could inhibit the binding of other *P. aeruginosa* strains (See Table 2.4 for list of peptide sequences). Utilizing microscopy, I found that at very low concentrations (51 nM), PAK(128-144)ox substantially inhibits the binding of strains PAO, K122-4, and KB7 (compare Figure 3.2-d, g, and j with Figure 3.2-e, h, and k) while very high concentrations (52 μM) of PAK(134-140) have a minimal effect on binding to steel (compare Figure 3.2-a, d, g, and j with Figure 3.2- c, f, i, and l). The pilin receptor binding domain sequences of strains PAK, PAO, K122-4 and KB7 vary substantially (Figure 3.1-a, and b, Table 3.1) but all these receptor binding domains

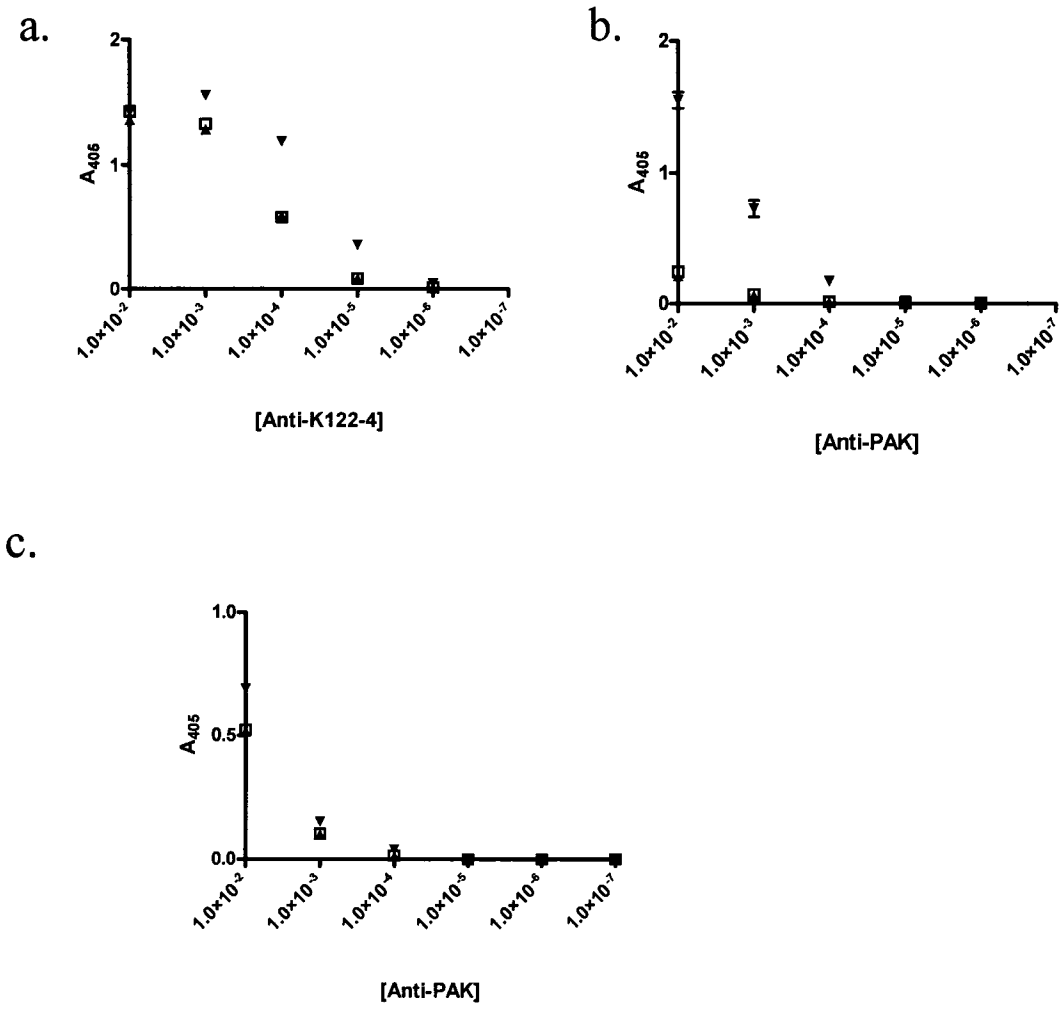

Figure 3.11 Antibody Detection of Monomer Pili and Nanotubes

Figure 3.11 Antibody Detection of Monomer Pili and Nanotubes

100 μg/ml of K122-4 pili (▼), monomer (□), or nanotubes (▲) immobilized in 96-well ELISA plates, were detected with either the polyclonal anti-K122-4 pili (a) or the polyclonal anti-PAK pili (b) antibody in dilutions ranging from 10^{-2} to 10^{-7}. 100 μg/ml of PAK pili (▼) and PAK monomer (□) immobilized in 96-well ELISA plates were incubated with polyclonal anti PAK pili antibody in dilutions ranging from 10^{-2} to 10^{-7} (c) and allowed to incubate for one hour. Secondary antibody goat anti-rabbit IgG HRP (100 μl /well of a 1:3000 dilution) was added and again incubated for one hour and ABTS was used as substrate.

P. aeruginosa Strains	Receptor Binding Domain Sequence
PAKwt	K-***C***-T-S-D-Q-D-E-Q-F-I-P-K-G-***C***-S-K
PAOwt	A-***C***-K-S-T-Q-D-P-M-F-T-P-K-G-***C***-D-N
KB7wt	S-***C***-A-T-T-V-D-A-K-F-R-P-I-V-G-***C***-T-D
K122-4wt	A-***C***-T-S-N-A-D-N-K-Y-L-P-K-T-***C***-Q-T

P. aeruginosa Strains	References
PAKwt	(Campbell *et al.*, 1995; Hazes *et al.*, 2000)
PAOwt	(Campbell *et al.*, 1995; Hazes *et al.*, 2000)
KB7wt	(Campbell *et al.*, 1995)
K122-4wt	(Campbell *et al.*, 1995; Hazes *et al.*, 2000)

Table 3.1 Pilin Receptor Binding Domain Sequences

Table of C-terminal K122-4 (residues 138-154), PAK (residues 128-144), PAO (residues 127-143) and KB7 (residues 132-148) pilin receptor binding domain sequences. Cysteine residues which create a disulfide bond are shown in italics.

display a conserved antigenic epitope and compete for epithelial cell surface receptors (Sheth *et al.*, 1995).

Antibody inhibition assays suggested that pilus mediated binding to stainless steel and specifically the C-terminal receptor binding domain of the pilin, might play a role in binding. Therefore, inhibition binding assays were utilized to test the ability of synthetic C-terminal receptor binding domains to inhibit the adherence of *P. aeruginosa* to steel. The native PAK pilin C-terminal receptor binding domain (consisting of residues 128-144) mediates binding to GalNAc-β-D-Gal containing glycoconjugates (Sheth *et al.*, 1994). A synthetic peptide of the PAK pilin receptor binding domain (PAK(128-144)ox) is able to inhibit the binding of both PAK wild-type cells and PAK pili to steel surfaces with apparent K_i's of 4 nM and 0.2 nM respectively (Figure 3.12-a, and Figure 3.13-a). PAK(128-144)ox also inhibited the binding of PAKMS591 (compare Figure 3.2-p with Figure 3.2-q) but had no effect on the binding of PAKNP (compare Figure 3.2-m and Figure 3.2-n). The peptide PAK(134-140) constitutes a portion of the receptor binding domain and has been demonstrated to bind with low affinity to respiratory epithelial cells (Yu *et al.*, 1996). PAK(134-140) did not inhibit binding of PAK wild-type cells or PAK pili to steel surfaces, even at the exceptionally high peptide concentration of 52 nM, where the PAK(128-144)ox peptide was able to inhibit binding at concentrations of 5.1 μM (Figure 3.12-b, and Figure 3.13-b). Additionally, it did not inhibit the binding of strains PAO, K122-4 or KB7, in contrast to the synthetic receptor binding domain peptide PAK(128-144)ox (Figure 3.2). In addition PAK(22-52), a peptide derived from the N-terminal α-helix (residues 1-58) which should be buried in the native pilus fiber did not inhibit binding of PAK wild-

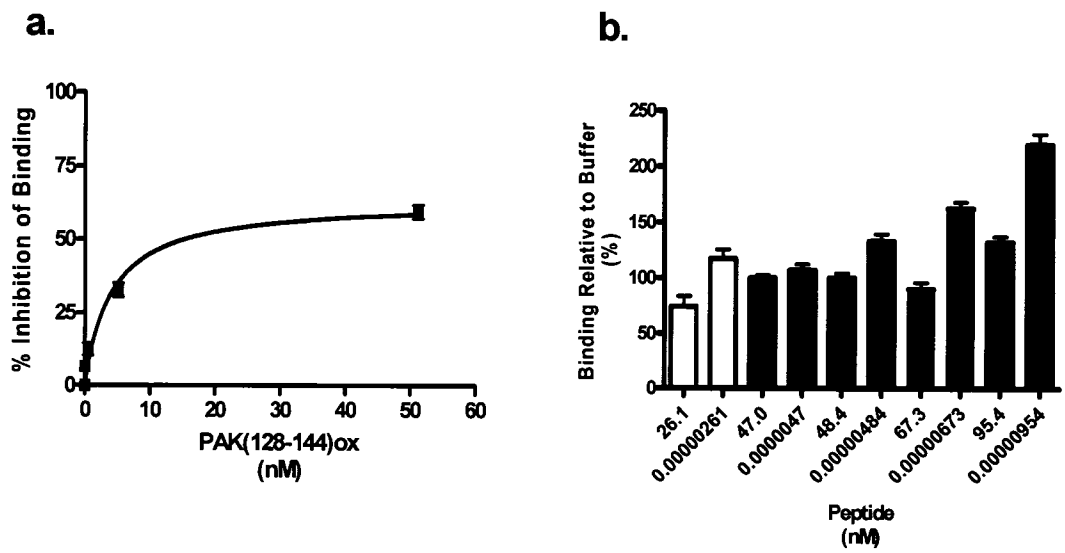

Figure 3.12 Effect of Synthetic Peptides on PAK Cells Binding to Steel

Figure 3.12 Effect of Synthetic Peptides on PAK Cells Binding to Steel

a. Inhibition of biotinylated PAKwt cell binding (10^8 CFU/ml) to stainless steel by the synthetic peptide PAK(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells. The apparent Ki of the peptide inhibition of PAKwt binding to steel is ~4 nM as determined by Prism 4 curve fitting.

b. Bar graph of the effect of various synthetic peptides on the binding of biotinylated PAKwt cells (10^8 CFU/ml) to stainless steel. Synthetic peptides consisting of the PAK PilA sequences PAK(22–52) (white bar) a portion of the N-terminal α-helix which is buried in the pilus fibre, PAK(117–125) (red bar) a solvent exposed sequence of PilA located N-terminally to the receptor binding domain, and PAK(134–140) (black bar) a sequence from the PilA receptor binding domain that has low affinity for mucosal cell surface receptors. Two scrambled peptide sequences were utilized as further controls, PAO(128–144)ox_Scrambled (blue bar), a PAO PilA receptor binding domain scrambled sequence that retains the intrachain disulfide bond and PAO(128–144)C129A/ C142A_Scrambled (green bar) a linear variant of the initial scrambled sequence where the two cysteine residues have been replaced by alanine residues. See Table 2.4 and Table 3.1 for a list of sequences. Here an increased binding is seen with PAK(117-125) rather than an inhibition.

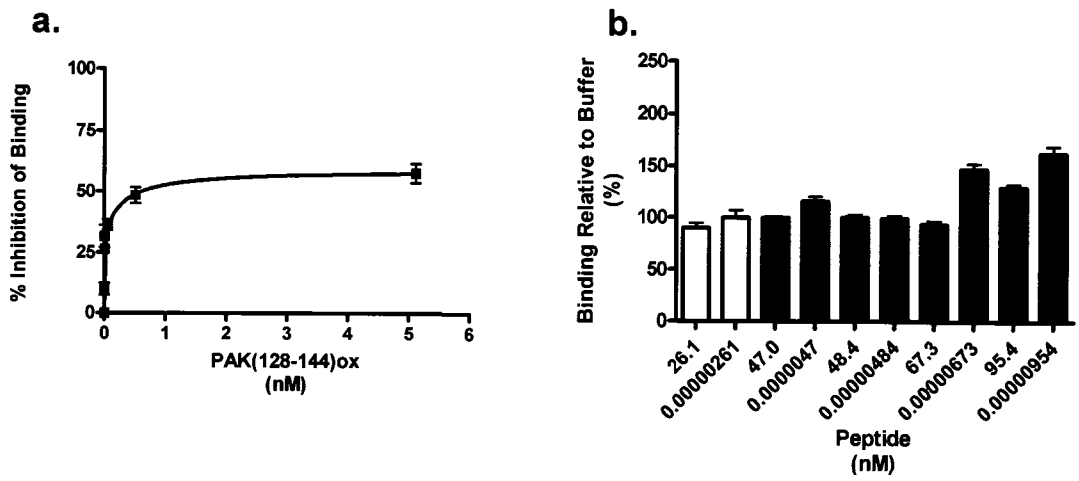

Figure 3.13 Effect of Synthetic Peptides on PAK Pili Binding to Steel

Figure 3.13 Effect of Synthetic Peptides on PAK Pili Binding to Steel

a. Inhibition of biotinylated PAK pili (0.75μg/ml) binding to stainless steel by the synthetic peptide PAK(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells. The apparent Ki of the peptide inhibition of PAK pilus binding to steel is ~0.2 nM as determined by Prism 4 curve fitting.

b. Bar graph of the effect of various synthetic peptides on the binding of biotinylated PAK pili (0.75μg/ml) to stainless steel. Synthetic peptides and symbols are as for Figure 3.12-b. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations. Indeed, PAK(117–125) appears to enhance the binding of pili to steel rather than inhibiting binding.

type cells (Figure 3.12-b) or PAK pili (Figure 3.13b) to stainless steel. Furthermore the PAK(117-125) peptide consisting of a portion of β-strands 3 and 4 (which models of the pilus fiber suggest will be displayed on the fiber surface) (Hazes *et al.*, 2000) (see Table 2.4 for peptide sequences and Figure 3.1-b) had any effect, even at high concentrations, on the binding of PAK wild-type cells (Figure 3.12-b) or PAK pili (Figure 3.13b) to steel surfaces. These data suggest that the α-helical region, residues 22-52, and the β-sheets, residues 117-125, do not participate in pilus-mediated binding to stainless steel. To determine whether the ability to interact with steel surfaces was a general attribute of the C-terminal receptor binding domain or a specific property of the PAK receptor binding domain, the inhibitory binding capacity of the PAO pilin receptor binding domain was examined. The synthetic PAO pilin receptor binding domain, PAO(128-144)ox was observed to inhibit pilus-mediated binding to stainless steel in a manner similar to the native PAK peptide (Figure 3.14-a). As a further control, peptides obtained through the trypsinization of bovine serum albumin were utilized to confirm that the inhibition of binding was sequence specific and not a common property of peptides. No inhibition of adherence was observed for PAK pili even at high peptide concentrations (Figure 3.14-b). To further confirm the specific nature of the receptor binding domain's interaction with steel surfaces two additional control peptides, a scrambled PAO pilin receptor binding domain PAO(128-144)ox_Scrambled, and a linear variant of that sequence where the two cysteine residues have been replaced by alanine residues to eliminate the disulfide bridge, PAO(128-144)C129A/C142A_Scrambled, were utilized to assess the relative importance of sequence versus amino acid composition. Neither of the scrambled sequences were able

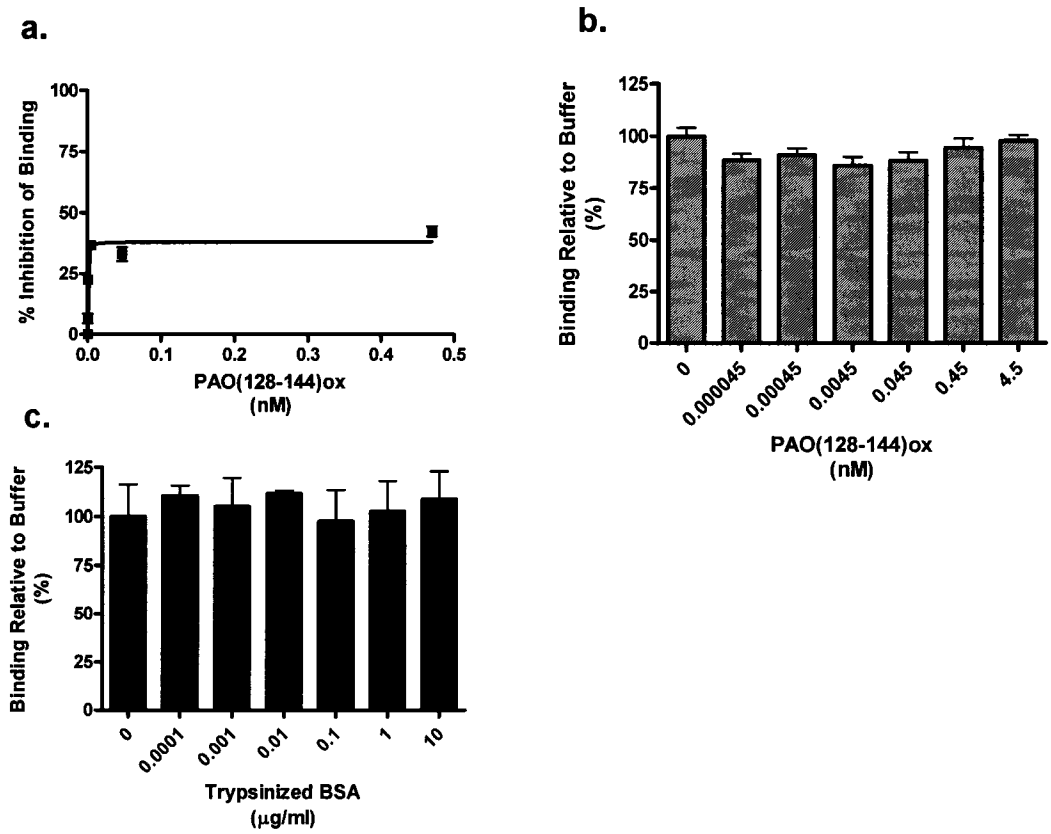

Figure 3.14 Effect of PAO Peptides and Trypsinized Peptides on Cells Binding to Steel

Figure 3.14 Effect of PAO Peptides and Trypsinized Peptides on Cells Binding to Steel

a. Competitive inhibition of biotinylated PAKwt (10^8 CFU/ml) cell binding to stainless steel by the synthetic peptide PAO(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells. PAO(128-144)ox peptide was serially diluted from 0.47 nM to 0.000047 nM.

b. Bar graph of the effect of PAO(128– 144)oxK130I (▨) on the binding of biotinylated PAK pili (0.75 μg/ml) to stainless steel. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations.

c. Bar graph of the effect of trypsinized peptide (▩) on the binding of biotinylated PAK pili to stainless steel. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations.

to inhibit binding of PAK wild-type cells or PAK pili to steel surfaces, even at very high peptide concentrations (Figure 3.12-b, and Figure 3.13-b). A PAO pilin receptor binding domain peptide with a single point mutation, PAO(128-144)oxK130I has a higher affinity for human buccal epithelial cells than stainless steel (Figure 13.4-a, and Figure 3.15-c) and was unable to inhibit *P. aeruginosa* whole cells adherence or pili binding to steel (Figures 3.12, 3.13 and 3.14). As the native PAO(128-144)ox peptide gave a 35% inhibition of bacterial cells binding to stainless steel and an 85% inhibition of bacterial cells binding to BECs, the change of the single amino acid (K130I) was able to modify binding ability of the peptide. This indicates that the amino acid sequence and the three-dimensional structure rather than the amino acid composition of the native receptor binding domain is important for binding to stainless steel. To determine whether the receptor binding domain inhibited binding to steel by a competitive mechanism or by interacting with *P. aeruginosa* cells or pili, the ability of PAK(128-144)ox to bind to stainless steel was determined using the monoclonal antibody PK99H as a probe. PK99H has been demonstrated to bind to both PAK(128-144)ox and PAK(134-140) when these peptides are bound to a cell surface receptor (Irvin *et al.*, 1989; Yu *et al.*, 1996). PAK(128-144)ox bound with high affinity to stainless steel compared to PAK(134-140), which bound only marginally to the steel surface at very high concentrations (Figure 3.15-a, b). Table 3.2 shows a comparison of peptide inhibition percentages.

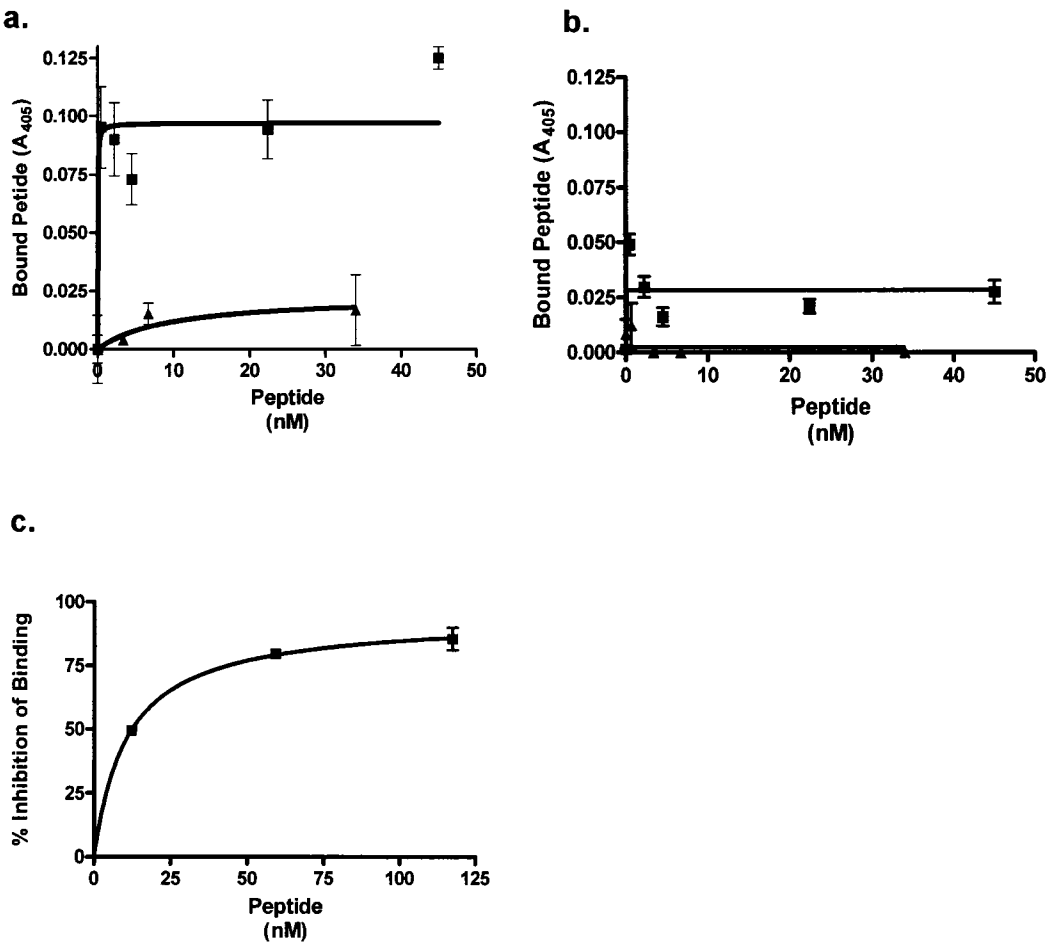

Figure 3.15 Synthetic Peptides Binding to Steel and Inhibition of PAO Bacterial Cells Binding to BECs

Figure 3.15 Synthetic Peptides Binding to Steel and Inhibition of Cells Binding BECs

a. Binding of PAK(128–144)ox (■) and PAK(134–140) (▲) to stainless steel as determined by a direct immunoassay employing murine monoclonal antibody PK99H. PK99H binds with high affinity to both of these peptides (Doig *et al.*, 1990; Wong *et al.*, 1992) even when the peptides are bound to receptors (Irvin *et al.*, 1989; Yu *et al.*, 1996). Note that PAK(128–144)ox binds with high affinity to steel while PAK(134–140) binds only slightly at very high concentrations.

b. Binding of PAK(128–144)ox (■) and PAK(134–140) (▲) to stainless steel as determined by a direct immunoassay employing biotinylated peptide. Note that PAK(128–144)ox binds with high affinity to steel while PAK(134–140) binds only slightly at very high concentrations.

c. Inhibition assay using BECs and wild-type PAO bacterial cells with PAO(128–144)oxK130I peptide (containing a single point mutation) in increasing concentrations. BECs were attached to a tissue culture-treated polystyrene plate (See Materials and Methods section 2.4.2 for details). Following attachment biotinylated PAO bacterial cells (10^{10} CFU/ml) and increasing concentrations of PAO(128-144)K130I peptide were added to the wells for one hour. Plates were washed using Buffer A and a 1:30,000 dilution of streptavidin HRP was added for an additional hour. Polystyrene plates were again washed before addition of ABTS substrate.

	Substrate	PAK(128-144)ox Inhibition (%)	PAO(128-144)ox Inhibition (%)	PAO(128-144) K130I Inhibition (%)
PAKwt cells	Stainless steel	55	ND	0
PAKwt pili		53	ND	ND
PAOwt cells	Stainless steel	ND	35	ND
PAOwt cells	BECs	ND	ND	85
K122-4 pili	Stainless steel	50	ND	ND
K122-4 monomer	Stainless steel	60	ND	ND
K122-4 nanotubes	Stainless steel	50	ND	ND

Table 3.2 Peptide Inhibition Comparisons

Comparison of peptides PAK(128-144)ox, PAO(128-144)ox, and PAO(128-144)K130I ability to inhibit cellular, pilus, monomer, and nanotube binding to stainless steel and BECs. ND = not determined.

3.4.2 Monomer and Nanotubes

As K122-4 pilin monomer and nanotubes were able to bind to stainless steel (see section 3.3.2), and as I have shown that pili bind to stainless steel via the C-terminal receptor binding domain (see section 3.1), I sought to test the ability of the synthetic peptide PAK(128-144)ox to inhibit pilin monomer and pilin nanotube binding to steel. As anticipated from the whole cell binding studies (Figure 3.2), PAK(128-144)ox was able to inhibit binding of the native K122-4 pili (Figure 3.16). The inhibition observed was not as great as that observed for the PAK cells and the homologous PAK(128-144)ox peptide inhibition (Figure 3.2, Figure 3.12-a, and Figure 3.13-a), however, as there are differences in binding among pili this is not surprising. The K122-4 monomer and nanotube were both inhibited by the PAK(128-144)ox peptide to a greater extent than that observed for the native pili (Figure 3.16). This suggests that the quaternary structure of the native pili is important for binding, and that more than one binding site is involved in binding of pili and nanotubes to steel. However, the number of binding sites is limited in both the pili and the nanotubes, as PAK(128-144)ox peptide competes for binding to steel at low concentrations. In addition, pili and nanotubes have a much higher affinity relative to the monomer, this suggests that there are multiple binding sites, and a similar number of binding sites in the purified pili and nanotube (Figure 3.16). Furthermore, the affinity of the pili and the nanotube for stainless steel is remarkably similar even though nanotubes have a far greater molecular weight (See Table 3.2), which again points to a similar number of binding sites on the pili and the nanotubes, and the similarities between the natural and synthetic structures.

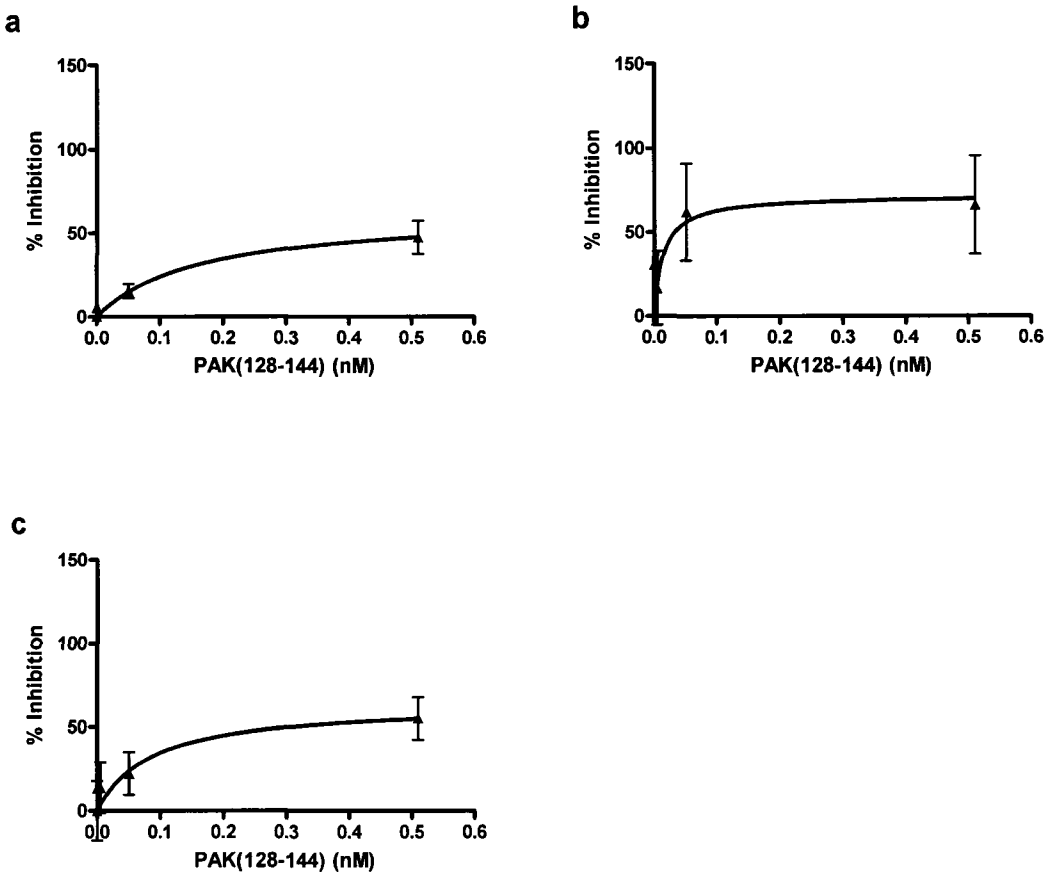

Figure 3.16 Peptide Inhibition of Pili Monomer and Nanotubes

Inhibition of 0.75 μg/ml of each K122-4 pili (**a**) K122-4 monomer (**b**) and K122-4 nanotubes (**c**) to stainless steel by PAK(128-144)ox peptide ranging in concentration from 0 to 0.51 nM. Steel coupons were incubated with polyclonal anti-K122-4 antibody followed by secondary-HRP antibody and ABTS was used as a substrate.

3.5 Binding to Other Abiotic Surfaces

As type IV pili have been implicated in biofilm formation on polystyrene and polyvinylchloride surfaces, I sought to determine whether the C-terminal receptor binding domain functions to mediate attachment to a variety of abiotic surfaces. PAK whole cells and pili were found to bind in a concentration dependent and saturable manner to both polyvinylchloride and polystyrene plates (Figure 3.17). The murine monoclonal antibody PK99H significantly inhibited binding to both polyvinyl chloride and polystyrene surfaces (Fig. 3.18). These data indicate that type IV pili mediated binding to these plastic surfaces is dependant on the C-terminal receptor binding domain.

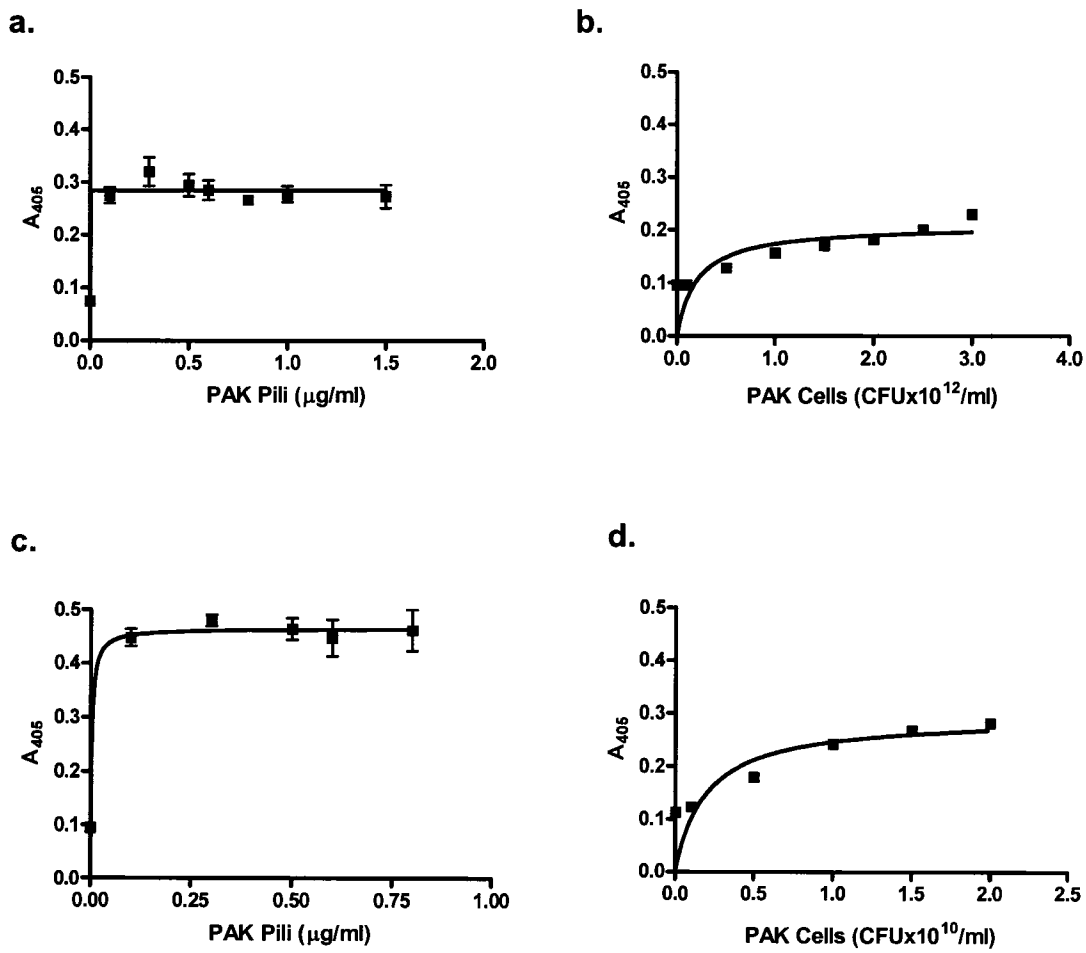

Figure 3.17 PAK Cells and Pili Binding to Plastics

Figure 3.17 PAK Cells and Pili Binding to Plastics

a. Binding of biotinylated PAK pili (■) to polystyrene plastic. Biotinylated pili were washed and suspended in 10 mM PBS pH 7.4, and allowed to bind to polystyrene for 60 min at 37°C.

b. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) to polystyrene at cell densities of 10^{12} CFU/ml.

c. Binding of biotinylated PAK pili (■) to polyvinylchloride plastic.

d. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) to polyvinylchloride at cell densities of 10^{10} CFU/ml.

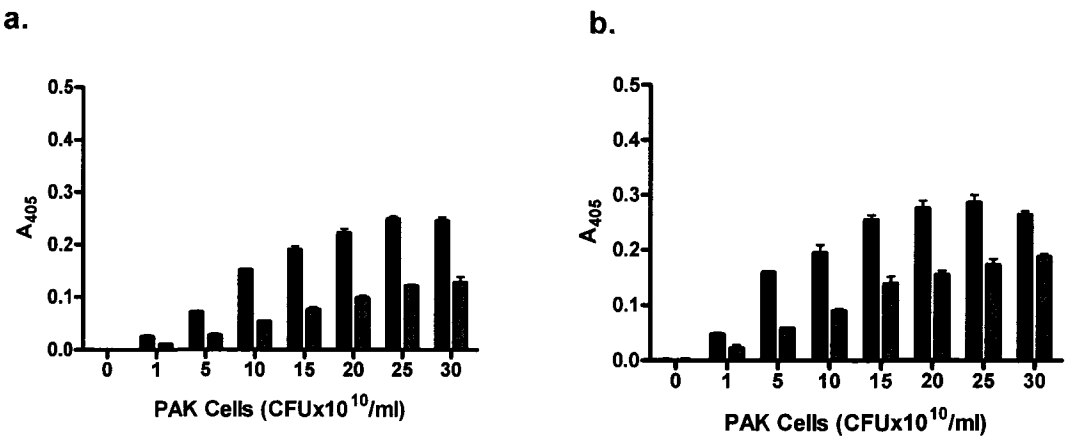

Figure 3.18 Antibody Inhibition of PAK Cells Binding to Plastic

Antibody inhibition of the binding of viable biotinylated PAKwt cells (10^{10} CFU/ml) to polyvinylchloride (**a**) or polystyrene (**b**) relative to PAKwt cells in buffer (■) or exposed to murine monoclonal antibody PK99H (▩) at a 1:1000 dilution.

Chapter 4

Discussion

4.1 *P. aeruginosa* Type IV Pilin Receptor Binding Domain Functions as an Adhesin for Abiotic Surfaces

The aggressive colonization of stainless steel surfaces is of enormous industrial and medical significance. *P. aeruginosa* infections are prevalent in burn units where large stainless steel tubs known as hydrotherapy units are often used to treat patients with severe burns (Tredget *et al.*, 1992). Tredget *et al.* (1992) have demonstrated a significant decrease in *P. aeruginosa* infection rates in burn units where stainless steel hydrotherapy units were removed. Biofilm formation on stainless steel and other substrata, has been widely investigated (Arnold *et al.*, 2004; Balazs *et al.*, 2004; Groessner-Schreiber *et al.*, 2004; Lomander *et al.*, 2004). Rough stainless steel surfaces more readily develop biofilms compared with smooth, or electropolished steel (Bagge *et al.*, 2001; Balazs *et al.*, 2004; Characklis and Marshall, 1990; Lomander *et al.*, 2004; Vanhaecke *et al.*, 1990). I sought to clarify the role of the *P. aeruginosa* type IV pilus in the initial colonization of abiotic surfaces, particularly with stainless steel given the classic genetic evidence that flagella are more likely responsible for the initial stages of *P. aeruginosa* biofilm formation (O'Toole and Kolter, 1998).

Initial binding of *P. aeruginosa* to stainless steel was found to be concentration dependent and exhibit classical saturation kinetics (Figure 3.3). *P. aeruginosa* pili clearly play a major role in mediating whole cell binding to stainless steel as (1) pili-

deficient strains are unable to adhere, (2) pili bind to steel in a concentration-dependent, saturable manner, and (3) pili inhibit whole cell binding in a direct inhibition assay (Figure 3.3).

While extracellular polysaccharide has long been proposed to play a major role in mature biofilms (Stoodley *et al.*, 2002), type IV pili protein subunits may provide the initial attachment to abiotic surfaces (Figure 3.2 and 3.3). As type IV pili are not the only adhesins employed by *P. aeruginosa* cells, other adhesins may contribute to cellular adherence. Alginate, the primary exopolysaccharide of *P. aeruginosa*, is produced in high quantities in the mucoid phenotype to create a sticky exocellular matrix (Jain and Ohman, 2004; Ramsey and Wozniak, 2005). Carbohydrates, and in particular alginate, have long been recognized as the "glue" to stick biofilms to surfaces (Costerton *et al.*, 1981), and alginate has been shown to be an epithelial cell adhesin (Doig *et al.*, 1987). However, *P. aeruginosa* alginate binding to epithelial cells is strain dependent suggesting a structural diversity (Doig *et al.*, 1987). Although alginate has been implicated in the formation of biofilms, and has been demonstrated to increase bacterial resistance to phagocytes (Pier *et al.*, 1986; Pier *et al.*, 2001, Stoodley et al., 2002), Wozniak *et al.* (2003) demonstrated that one structure of alginate did not have a significant effect on the initial binding of *Pseudomonas* to abiotic surfaces (Wozniak *et al.*, 2003). Further, I have demonstrated that type IV pili are involved in the initial adherence for several *P. aeruginosa* strains. Evidence of an initial involvement of protein in biofilm formation on copper surfaces has been reported previously (Bremer and Geesey, 1991). Interestingly, a variety of proteins have the ability to mediate tight interactions with abiotic surfaces, such as the *Mytilus edulis* (a sea mussel) foot proteins

which are capable of mediating interactions with a variety of abiotic surfaces including metals and plastics (Suci and Geesey, 2001). A direct role for protein in mediating interactions with an abiotic surface, particularly a metal surface, is thus not without precedent.

While O'Toole and Kolter (1998) found that flagella play a significant role in biofilm formation, I found that *P. aeruginosa* lacking flagella yet expressing pili were able to bind to steel (Figure 3.2 and 3.4). In addition, any strain lacking the ability to assemble functional pili were unable to bind (Figure 3.2 and 3.4). The difference between my findings and those of O'Toole and Kolter (1998) may reflect the ability of bound cells to remain surface attached and differentiate into a microcolony, and subsequently a biofilm, as my data reports on initial colonization events. Moreover, O'Toole and Kolter (1998) used polyvinylchloride as a substrate for binding whereas stainless steel was the major abotic surface employed in my studies. Pili may therefore play a more important role in the colonization of stainless steel than polyvinylchloride. Further, experimental conditions were not constant between the O'Toole and Kolter (1998) experiments and mine with stainless steel; O'Toole and Kolter (1998) allowed bacterial adhesion at 30°C for 10 hours whereas mine were performed at 37°C for one hour. It is possible there may be a temperature regulated control for pilus expression, as an environmental signal for the autophosphorylation of PilS (the histidine kinase of the two-component regulatory system for transcription of PilA) has not yet been determined.

The C-terminal receptor binding domain plays an important role in the initial binding to stainless steel (Figure 3.2), as peptides to other regions of the pilus were

unable to inhibit purified pili or whole cell binding to stainless steel (Figure 3.12 and Figure 3.13). However, it is unclear whether the quaternary structure of the native pilus, and subsequently the placement of the C-terminal binding domain in the pilus, also plays a role in binding to stainless steel surfaces. To investigate this possibility, the truncated pilin nanotube was employed. The molecular mass of nanotubes is significantly higher than that of native pili as the nanotubes are considerably longer than pili, which are made up of thousands of native PilA monomers (Nassif *et al.*, 1994; Parge *et al.*, 1995; Rudel *et al.*, 1995). It is possible that the nanotube could have a very high avidity for surfaces if several receptor binding sites were displayed on the nanotube, rather than the very limited number of receptor binding domains displayed only at the tip of the native pilus (Lee *et al.*, 1994). As nanotubes spontaneously self assemble following exposure of the monomeric pilin to a hydrophobe, the quaternary structure of the nanotubes could be significantly different from native pili that are assembled through the concerted action of a large number of dedicated proteins. Furthermore, due to packing differences of the pilin subunits in nanotubes, a large number of receptor binding domains could be displayed in the nanotubes relative to that of the native pilus. Nanotubes were able to bind to Grade 304 stainless steel in a concentration dependent and saturable manner (Figure 3.10). Therefore, the ability of the pilus to bind to stainless steel is not a sole property of the pilus structure, as the truncated pilin monomer, and formed nanotubes can bind; this is in direct agreement with the peptide inhibition data (Figure 3.10, Figure 3.12, and Figure 3.13). The C-terminal receptor binding domain is therefore important, as the monomer (lacking the N-terminal domain) was able to bind to steel. In addition, a synthetic peptide of the C-

terminal receptor binding domain was able to inhibit binding while control peptides are unable to do so (Figure 3.12 and Figure 3.13); these data support the C-terminal receptor binding domain as possessing the main adhesive property of the pilus. Moreover, the N-terminal α-helix does not appear to contribute to the interaction of pili with steel (Figure 3.10), as the pilin monomer, nanotubes (neither which contain the N-terminal α-helix) and pili, all bind to steel and saturate binding sites on steel at roughly the same protein concentration.

4.2 Inhibition Assays

Polyclonal anti-PAK pili and PK99H antibodies inhibited pilus-mediated binding to stainless steel, indicating that the pili, and specifically the C-terminal receptor binding domain, residues 128-144 of PAK PilA, may mediate the interaction with steel (Figure 3.8). Further, as the polyclonal anti-PAK pili antibody was able to inhibit binding (Figure 3.8) and a 100% inhibition is not seen with the synthetic peptide PAK(128-144)ox (Figure 3.12 and Figure 3.13), other parts of the pilus may play a minor role in pilus binding to abiotic surfaces. However, as PK99H, which binds to residues 134-140 of the pilus structure showed a significant inhibition of purified pili and whole cells binding to steel, the major interaction with stainless steel is specific to the C-terminal receptor binding domain (Figure 3.8).

To confirm that residues 128-144 were mediating the interaction between pili and steel, a variety of synthetic peptides were used in inhibition assays. Strikingly, PAK(128-144)ox effectively inhibited the adherence of heterologous *P. aeruginosa* strains to stainless steel even at low concentrations (Figure 3.2-b, e, h, and k) indicating

that the ability to bind to steel through the C-terminal binding domain is conserved among *P. aeruginosa* strains. The ability of the synthetic receptor binding domain, PAO(128-144)ox, to inhibit the binding of both PAK cells and pili to steel further supports my hypothesis that the C-terminal pilin receptor binding domain of the various pilins mediates binding to steel in addition to mediating binding to human respiratory epithelial cells.

Peptides with limited affinity for steel have been identified by phage display methodology and their affinity for steel has been correlated to their amino acid composition (Zuo *et al.*, 2005). The interaction of the receptor binding domain with steel is not simply a function of the peptide amino acid composition as two scrambled sequences of the PAO receptor binding domain (one retaining the disulfide bridge and the other a linear variant where the two cysteine residues are replaced with alanine residues) failed to inhibit binding to steel (Figure 3.12-b and 3.13-b). The PilA receptor binding domain binds to steel with high affinity in a sequence specific manner indicating that binding is likely dependent upon both the sequence and three dimensional structure of the peptide rather than the amino acid composition. The steel binding function is sequence specific as PAO(128-144)oxK130I failed to inhibit binding to steel even though the affinity of this peptide for human buccal epithelial cells was enhanced (compare Figure 3.14-b with Figure 3.15-c). This observation suggests that the ability to bind to steel and human epithelial cells can be differentiated. Indeed, the short synthetic peptide PAK(134-140) which has previously been demonstrated to have a low affinity for human buccal epithelial cells (Yu *et al.*, 1996) did not inhibit binding to steel (Figure 3.2, Figure 3.12, and Figure 3.13). These results indicate that

the two binding functions are readily differentiated by a single point mutation (K130I), suggesting that the molecular basis of the interaction with steel and epithelial cells is quite specific, although both are dependant on the C-terminal receptor binding domain. By changing the positively charged lysine residue for a neutral isoleucine residue the peptide is unable to interact with the steel surface; this suggests that the positive charge is important in binding to stainless steel. Further, as the electron flow is disrupted at grain boundaries (the area between the crystalline iron structures) the negatively charged electrons may more easily interact with the positive lysine residue.

A residue from the α-helix, PAK(22-52) synthetic peptide was unable to inhibit binding of pili or cells to the stainless steel surface (Figure 3.12-b and 3.13-b). This result is in agreement with the nanotube data which depicts that native pili, monomer and nanotube bind in a similar fashion to stainless steel (Figure 3.10). As nanotubes lack the N-terminal PilA domain, and PAK(22-52) did not inhibit binding of pili to steel, this again suggests that the C-terminal receptor binding domain is an important aspect in pilus binding.

Type IV pili have also been implicated in biofilm formation on plastic surfaces, so I examined the ability of the PilA receptor binding domain to mediate adherence to two widely used plastics, polyvinylchloride and polystyrene. Direct binding assays demonstrate that PAK whole cells and pili adhere to plastics in a concentration dependent and saturable manner (Figure 3.17 and 3.18). As well, the monoclonal antibody PK99H was able to inhibit *P. aeruginosa* binding to both polyvinylchloride and polystyrene plates (Figure 3.17 and 3.18). These data suggest that the C-terminal

receptor binding domain is involved not only in adherence to stainless steel and buccal epithelial cells, but also to other abiotic substrates including plastics.

The PilA C-terminal receptor binding domain, displayed at the tip of the type IV pilus, mediates direct binding to both biotic and abiotic surfaces, although the pilus is able to bind both substrates the affinities differ by several orders of magnitude (Figure 3.3 and Figure 3.17). However, as the single point mutation in the C-terminal receptor binding domain increased the affinity for BECs, it abolished the affinity for stainless steel. This supports my hypothesis that the receptor binding domain has retained sequence and structural elements required for adherence to a variety of surfaces. Therefore, I have determined that the C-terminal receptor binding domain is responsible for adherence to stainless steel, and although the sequence varies widely between strains, attachment via this C-terminal receptor binding domain is not strain specific. As *P. aeruginosa* is a ubiquitous organism, the ability to bind to as many surfaces as possible would facilitate growth and survival. Moreover, the conserved binding ability of the C-terminal receptor binding domain, although the sequence may differ dramatically between strains, maintains as many binding functions as possible for the organism. This also has implications for twitching motility, a form of bacterial movement based on the extension, binding, and retraction of the pilus along a solid surface (Mattick, 2002). With a conserved binding ability, twitching along a multitude of surfaces is possible. Furthermore, twitching motility has been shown to be necessary for development of biofilms, and as the majority of bacteria in the natural environment have been reported to form biofilms, the ability to bind, twitch and form microcolonies, gains even more significance.

To determine whether functional receptor binding domains in nanotubes are in fact limited, peptide inhibition studies were performed on nanotube binding to stainless steel. The synthetic peptide PAK(128-144)ox corresponds to the PilA receptor binding domain (residues 128-144) at the C-terminal end of the monomeric pilin subunit (Irvin *et al.*, 1989), and is required for adherence to both biotic and abiotic surfaces (Figure 3.2, 3.3, 3.15). This synthetic peptide, oxidized to form an internal disulfide bond, has been demonstrated here to specifically inhibit *P. aeruginosa* strain K122-4 binding to stainless steel (Figure 3.2), and was also able to prevent binding of native K122-4 pili to stainless steel (Figure 3.16). This suggests that type IV pilus binding is dependent on the C-terminal 128-144 region of pilin, in agreement with the synthetic peptide inhibition assays (Figure 3.12, 3.13). Furthermore, the PAK(128-144)ox peptide was able to inhibit heterologous bacterial strains (namely PAO, KB7 and K122-4) indicating that these interactions are not strain specific (Figure 3.2). Interestingly, the PAK(128-144)ox peptide was also able to inhibit binding of nanotubes to stainless steel in inhibition assays (Figure 3.16). Therefore, the self-assembly of protein nanotubes must allow for the C-terminal receptor binding domain to be surface exposed within the structure. Furthermore, the exposure of the C-terminal receptor binding domain on the protein nanotube must be limited, since the apparent affinity is similar to that of the native pilus (Figures 3.10 and 3.16). This suggests that while the assembly process of the nanotube differs from that of native pili, the number of exposed binding domains remains similar. Moreover, the number of C-terminal receptor binding domains must be limited in its availability for binding, as peptides were able to inhibit nanotube binding. If the nanotube was able to bind along its length (of several hundred μm) through the C-

terminal receptor binding domain, the avidity of the interaction would show the binding of the nanotube to be a high affinity interaction. In this case, the amount of peptide needed to remove the nanotube structure from the steel surface would be much higher than that needed to remove the native pilus structure (2-5 μm in length). However, if the nanotube (like the pilus) was able to bind only through the tip of the structure, the number of exposed binding sites would be similar to the pilus. This is what was observed with the nanotube, as both pili and nanotube show roughly a 50% inhibition curve (Figure 3.16).

A notably lower concentration (0.0005 nM for the monomer vs. 0.05 nM for pili and nantotubes) of PAK(128-144)ox peptide is required to inhibit the monomeric pilin subunit from binding to stainless steel (70% inhibition curve), than for either the nanotubes or pili (50% inhibition curve) (Figure 3.16). The assembly process in both the nanotube and the pilus requires multiple PilA monomers (Audette *et al.*, 2004; Folkhard *et al.*, 1981). Therefore, it is not surprising that a higher concentration of peptide is required to prevent nanotube and pili binding, which may display multiple receptor binding domains. As the C-terminal receptor binding domain is exposed only at the tip of the pilus (Lee *et al.*, 1994), and given the remarkable structural and functional similarities of nanotubes and pili (Audette *et al.*, 2004), it appears that the overall packing of truncated pilin monomers in nanotubes is surprisingly similar to that found in native pili. If residues 128-144 were exposed along the length of the nanotube, I would expect a significantly higher avidity and apparent affinity of the nanotube for stainless steel. As the affinity in direct binding and inhibition studies suggests that the apparent affinities (50% inhibition curve) for nanotubes and pili are roughly equivalent

(Figures 3.10 and 3.16), the combined length and molecular mass of the nanotube precludes the exposure of the binding domain along the length of the nanotube. Given the 40 fold higher avidity of PAK(128-144)ox (Kd 0.4879 μg/ml for nanotubes and 0.0122 μg/ml for pili) for both native pili and nanotubes, relative to that observed for monomeric pilin binding to stainless steel, I suspect that 2 or 3 receptor binding domains at the tip of the pilus (and nanotube) interact with the steel surface. The observed avidity strongly supports the previous observations of thin filaments coalescing into a multi-start helix fiber to form the protein nanotube (Audette *et al.*, 2004), and supports previous multi-start pilus fiber modeling studies (Craig *et al.*, 2004). Therefore, the functional C-terminal receptor binding domain must be displayed at the tip of the nanotube, similar to pili. Consequently, protein nanotubes bind with very high affinity (with a KD value, or the value at which 50% of the nanotubes have bound to the steel surface of 0.4879 μg/ml) (Figure 3.10) to stainless steel surfaces to create a novel metallo-biomolecular interface. Furthermore, a recent study has determined that the *N. gonorrhoeae* type IV pili is constructed in a three-start helical manner, where three separate helical pilin filaments form the pilus fiber (Craig *et al.*, 2006). This allows three separate binding domains to be exposed at the tip of the pilus structure (Craig *et al.*, 2006). In combination with my results of the pilus versus monomer affinity for stainless steel, this suggests that the three-start helical structure may be conserved in type IV pili.

4.3 Conserved Structural Pilus Epitopes

Although the nanotubes show a relative valency similar to native pili (Figure 3.11), they may not exactly resemble the intact pilus structure. As complex cellular machinery is required to generate the native pilus structure (Jacobs *et al.*, 2003; Mattick, 2002), while a spontaneous hydrophobe-induced oligomerization of truncated PilA subunits is all that is required to form nanotubes, I sought to investigate the potential for conserved antigenic epitopes displayed on the K122-4 pili, monomeric pilin, and nanotube structures. In order to examine the structural characteristics of the pili, the differential recognition of pili and nanotubes by pilus-specific antibodies was explored. Immobilized pili, monomer and nanotubes were recognized with equivalent antibody concentrations (10^{-2} to 10^{-7}) when using polycolonal anti-K122-4 sera (Figure 3.11). Considering that the truncated K122-4 monomer and nanotubes are derived from native K122-4 pilin, this result was expected, as an earlier study demonstrated that the major antigenic determinant of the pilus was residues 82-110, which compose part of the β-sheet of the pilin monomer (Sastry *et al.*, 1985). Since all three structures (monomer, pilus and nanotube) present this epitope, the differences in antibody affinities were, as expected, minimal (Figure 3.11).

To further distinguish the quaternary structures of the pilus and nanotubes, I employed a polyclonal serum generated against *P. aeruginosa* strain K (PAK) pili. This anti-PAK serum was generated using intact pili from a distinct *P. aeruginosa strain* (PAK), yet has been demonstrated to be somewhat cross-reactive to pili isolated from strain K122-4 (R.T.I., unpublished data). The anti-PAK pili serum was able to recognize both PAK pili and PAK monomer (Figure 3.11). The K122-4 and PAK pilins

exhibit very low sequence similarity (29%) even though their core tertiary structure is very similar (Audette *et al.*, 2004). Thus, the anti-PAK serum assesses structurally conserved epitopes on the intact pilus. The polyclonal anti-PAK antibody was not able to recognize either the monomer or the nanotubes even at high antibody titers (Figure 3.11), indicating that the anti-PAK sera recognizes an epitope present in native K122-4 pili that is absent or altered in the monomer and nanotube. The monomer does not assemble into a quaternary structure, and the nanotube cannot form an identical structure to that of the pilus, as it lacks the N-terminal α-helices. Therefore, the polyclonal anti-PAK pili antibody epitopes must recognize the quaternary structure of the pilus involving 2 or more PilA subunits; thus the monomer would not be recognized, and the nanotube due to differential packing of the truncated PilA subunits must also not create these anti-PAK pili epitopes. Interestingly, the left-handed three start pilus fiber yields a structure where the N-terminal α-helices (which are highly conserved in the PAK and K122-4 pilins) are displayed periodically along the pilus fiber (Craig *et al.*, 2004; Craig *et al.*, 2006), thus my results potentially offer experimental evidence that supports the three-start helix fiber model. Therefore, while the K122-4 nanotubes retain similar relative valencies, their quaternary structure is somewhat altered relative to that of the native pilus. The nanotubes therefore are a useful tool to further understand the role of the quaternary pilus structure in binding to stainless steel.

4.4 Future Experiments

As the PAK(128-144)ox peptide has been shown to inhibit binding with high affinity (with a Ki of 4.0 nM for whole cells) (Figure 3.12), and this inhibition has been

shown to inhibit heterologous *P. aeruginosa* strains (Figure 3.2), this peptide may be used in medical or industrial applications to prevent *P. aeruginosa* adherence. The peptide could be incorporated into the stainless steel surface to inhibit adherence to medical implants and surgical tools, and could prevent patient to patient *P. aeruginosa* transmission in burn wards where hydrotherapy units are used. The peptide PAK(128-144)ox peptide has been shown in this study to directly bind to stainless steel (Figure 3.15), therefore a solution of peptide could function to coat the steel surface. As the peptides bind to the stainless steel, the binding sites available for *P. aeruginosa* adherence is decreased, therefore a peptide coating would act as a preventative barrier for adherence. Furthermore, *P. aeruginosa* has been shown to bind to polyvinylchloride and tissue cultured polystyrene plastics (Figure 3.17). The PAK(128-144)ox peptide could also be used to coat the polyvinylchloride used in catheters and I.V. tubes. An additional application of this peptide could be in contact lens solution to prevent the occurrence of *P. aeruginosa* ulcerative keratitis of the cornea.

The change of a single amino acid in the PAO(128-144)ox peptide prevented inhibition of *P. aeruginosa* to stainless steel (Figure 3.14-b). Therefore the reverse, a peptide with higher affinity for the steel surface may also be found. Using twenty pools of peptides, each containing a different amino acid at the 130 position, and randomized at non-conserved residues (among *P. aeruginosa* strains), a peptide with higher affinity for the stainless steel surface may be found. Such a peptide could be even more effective at preventing *Pseudomonas aeruginosa*, and likely, other organisms with type IV pili, from binding to steel.

Additionally, a *P. aeruginosa* mutant that would constitutively express autoinducers (or the molecules that mediate quorum sensing) at high concentrations might remove the need for adding such high bacterial concentrations (e.g., 10^{13} CFU/ml) to my substrate binding assays. High cell concentrations are required for the activation of quorum sensing regulated genes (Redfield, 2002) and this might be what was occurring in my binding assays. Therefore, a bacterium expressing abnormally high concentrations of autoinducers would activate quorum sensing genes, without the presence of a large bacterial population. Binding could then be assessed on a smaller scale (e.g., $\leq 10^{9}$) and would ensure that the high affinity binding seen in these experiments is not due to selection of a sub-population.

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