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THE UNIVERSITY OF ALBERTA

STRUCTURE AND FUNCTION OF *P. AERUGINOSA* PILI

BY

© DAWN L. MACDONALD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Structure and Function of *Pseudomonas aeruginosa* Pili submitted by Dawn L. Macdonald in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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For my father, John H. Macdonald, who has supported all my endeavours in life, and for my grandfather, Robert E. Airdrie, who will always hold a special place in my heart.

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing significant morbidity and mortality in cystic fibrosis, cancer, and burn patients. *P. aeruginosa* produces polar pili which promote colonization by their ability to adhere to epithelial cell surfaces. Pili are composed of 15 kDa subunits known as pilin which have a highly conserved N-terminus, a central hypervariable region, and a semi-conserved C-terminus. The N-terminus contains a short 6-7 amino acid leader sequence which is proteolytically cleaved and the N-terminal phenylalanine is methylated by the PilD protein. Amino acids in the N-terminus of the mature protein play a role in these processing events. The invariant Gly at position -1 to the cleavage site is involved in recognition of the cleavage reaction (Strom and Lory, 1991). The fifth position Glu is necessary for recognition by the methylation actvity of the PilD protein. Alteration of the fifth position Glu to Asp resulted in markedly reduced levels of pilin mRNA.

The adherence domain of pilin is located in the C-terminal disulphide loop which is exposed at the tip of the pilus (Lee *et al.*, 1994). Alignment of pilin prototype sequences revealed the presence of four conserved, four semi-conserved, and six non-conserved residues in this region. Attachment studies of isogenic constructs expressing one of four pili types found that KB7 was bound most efficiently, followed by PAO, PAK, and K122. Point mutations in the disulphide loop of PAK indicated that three non-conserved residues (E135, Q136, I138) were important for binding to A549 human lung pneumocyte cells and to purified putative glycolipid receptors. Pili prototypes may not bind to the same epitopes on the cell surface receptor. The accessibility of the specific epitopes on the receptor may be important for binding because of the more restricted conformation of the receptor in the natural membrane. TLC overlay assays determined that putative glycolipid receptors for pili are

asialoGM1 and asialoGM2. AsialoGM1 has been found in substantial amounts in human lung tissue suggesting that asialoGM1 may be the receptor for *P. aeruginosa* pili.

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CHAPTER I INTRODUCTION

A. Introduction

P. aeruginosa is the major etiological agent responsible for chronic lung infections in cystic fibrosis (CF) patients and is known to be a pathogen in osteomyelitis, ear, eye, and urinary infections, and nosocomial pneumonias (Kohler and White, 1979). Corneal degradation as a result of *P. aeruginosa* keratitis can be a serious problem for users of extended wear contact lenses (Hazlett *et al.*, 1993). *P. aeruginosa* is classified as an opportunistic pathogen and is capable of causing significant morbidity and mortality in patients immunocompromised by severe burns and cancer (Bodey *et al.*, 1983).

Pseudomonas aeruginosa is known to produce extracellular, polar, filaments called pili with a diameter of 5.2 nm and an average length of 2.5 nm (Folkhard *et al.*, 1981). The pili of *P. aeruginosa* contribute to its virulence by promoting adherence to epithelial and mucosal cell surfaces (Paranchych *et al.*, 1986; 1990; Irvin *et al.*, 1989).

Pili are composed of thousands of 15-kDa pilin monomers helically arranged with a pitch of 4.1 nm and five subunits per turn (Watts *et al.*, 1983). Pili also act as receptors for several pilus-specific bacteriophages (Bradley and Pitt, 1974) and are responsible for a type of locomotion called twitching motility (Bradley, 1980). *P. aeruginosa* belongs to the NMePhe, or type 4 class of pili which is so named because of the unique N-terminal residue. Type 4 pili are present on many other Gram negative pathogens such as *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquifaciens, Dichelobacter nodosus* (previously *Bacteroides nodosus*)(Elleman, 1988), *Vibrio cholereae* (Shaw and Taylor, 1990), and the inducible bundle forming pilus of enteropathogenic *Escherichia coli* (Giron *et al.*, 1991). All type 4 pilins possess a conserved hydrophobic N-terminal region comprised of the first 29 amino acids (Paranchych *et al.*, 1986). Hypervariable regions in the central portion of the mature protein are responsible for antigenic variations that result

in immunologically defined pilins. DNA sequencing and extensive hybridization studies have revealed eight distinct but related pilin prototypes in *P. aeruginosa* (Paranchych *et al.*, 1990; Castric and Deal, 1994).

B. Pili Biogenesis

The promotor of *pilA*, the structural gene for pilin is recognized by RNA polymerase containing the alternative sigma factor RpoN (Ishimoto and Lory, 1989). Initiation of transcription of RpoN-dependent genes requires additional regulatory proteins, which for the pilin gene are encoded by *pilR and pilS* (Ishimoto and Lory, 1992). The amino acid sequences suggest that PilR and PilS belong to the two-component environmentally-responsive family of transcriptional activators involved in signal transduction, where PilS is autophosphorylated and transfers the phosphate to PilR (Ishimoto and Lory, 1992). PilS, like other sensor proteins of two component systems, is possibly a histidine protein kinase (Hobbs *et al.*, 1993). In the model proposed by Hobbs and coworkers (1993) activated PilR binds to the DNA upstream of the *pilA* promoter region (possibly the *sipR* sequence found to be important for transcription by Pasloske *et al.*, 1989). Looping of the DNA presumably brings the bound PilR in contact with RpoN containing RNA polymerase at the promoter (Hobbs *et al.*, 1993). Phosphorylated PilR interacts with the RpoN-RNA polymerase and facilitates open complex formation promoting transcription (Ishimoto and Lory, 1992). Translation of the pilin mRNA proceeds and the pilin precursors are localised to the inner membrane in a different manner than in general protein export.

In general export, proteins are localized to the membrane in a SecA-dependent fashion and in some cases with the SecB chaperone (Schatz and Beckwith, 1990). Interaction with an integral membrane complex containing SecY and SecE proteins and cleavage of the signal sequence on the periplasmic side of the inner membrane occurs (Schatz and Beckwith, 1990). The signal sequence in this case is characterized

by one or two positively charged residues followed by a 20-30 amino acid hydrophobic region and a consensus cleavage site (von Heijne, 1986).

Export of type 4 pilins differs in that it has no requirement for Sec proteins. The N-terminus contains a ~30 amino acid hydrophobic region necessary for localization to the inner membrane (Strom and Lory, 1987). Preceeding this region is a short positively charged leader sequence that differs from those found on most exported proteins. The leader sequence is processed by a membrane bound specific enzyme PilD, which is also responsible for processing components of the protein export apparatus of *Pseudomonas aeruginosa* (Nunn and Lory, 1992; Nunn and Lory, 1993). *PilD* and other genes necessary for expression of pili are not under the control of RpoN as is *pilA*, possibly because of the involvement of PilD in the processing of components of extracellular secretion (Koga *et al.*, 1993). The proposed consensus sequence for processing by PilD, G-][-F-T-L-I-E-X-X-X- where][is the cleavage site, and preceeds the hydrophobic region in this case. Processing of prepilin is a two step process, the first being cleavage of the leader sequence. The final step involves the methylation of the N-terminal phenylalanine. PilD is responsible for cleavage of the leader peptide and methylation of the N-terminal phenylalanine to form mature pilin (Strom *et al.*, 1993a; Nunn and Lory, 1993). Substitution of the -1 position glycine, relative to the cleavage site, inhibitied the leader peptidase cleavage and prevented assembly of the monomers into pili (Strom and Lory, 1991). Altering the -1 glycine in *N. gonorrhoeae* pilin affected the cleavage reaction and pilus assembly in the same manner (Koomey *et al.*, 1991). A mutant in which amino acids 4-7 were deleted from the mature pilin sequence resulted in 8% cleavage compared to wild-type pilin (Pasloske *et al.*, 1988) indicating that other residues participate in leader peptidase recognition. Examination of the available protein sequences shows that the -1 glycine is conserved in all type 4 pilins (Sohel *et al.*, 1993; Strom and Lory, 1992; Elleman, 1988). The recognition site for the leader peptidase activity of PilD is centred around

the -1 glycine, but extends into the N-terminus of the mature protein since deletion of amino acids 4-7 results in significantly reduced cleavage.

Recent studies suggested that the +1 phenylalanine of pilin is not essential since substitutions of this amino acid did not affect processing and assembly (Strom and Lory, 1991). Members of the NMePhe pili class that do not have Phe as the first amino acid are still processed in the same fashion. *V. cholerae* has methionine (Shaw and Taylor, 1990) and the bundle forming pili of enteropathogenic *E. coli* have a post-translationally modified leucine in this position (Sohel *et al.*, 1993). Amino acid deletion and substitution in the short leader sequence of type 4 pili have shown that it is required for the cleavage (Strom and Lory, 1991). The net positive charge within the sequence, however, is evidently not required for leader peptidase cleavage (Strom and Lory, 1992). The only other invariant residue in the leader sequence is the fifth position glutamate (Sohel *et al.*, 1993; Strom and Lory, 1992; Elleman, 1988). Mutations of the invariant glutamate residue to a lysine and to a valine have generated pilin that was cleaved but not methylated (Pasloske and Paranchych, 1988; Strom and Lory, 1991). The Glu to Lys mutant could only be assembled in the presence of wild-type N-methylated pilin monomers to form heterologous pili (Pasloske *et al.*, 1989). Post-translational N-methylation is necessary for the assembly of the monomers into pili, but not for membrane translocation, as these fifth position mutants were not assembled but were localized to the inner and outer membranes. Fusion protein studies demonstrated that the leader sequence plus the first 45 N-terminal amino acids are the minimum sequence for export (Strom and Lory, 1987).

The major donor of methyl groups in bacteria is *S*-adenosylmethionine, which is located in the cytoplasm (Kaufman *et al.*, 1991). It has been shown that *in vitro*, PilD is capable of transferring the methyl group of *S*-adenosylmethionine to the N-terminal phenylalanine of cleaved pilin and XcpW (Strom *et al.*, 1993b). PilD contains a relatively hydrophilic 80 amino acid domain with four cysteines that is located in the

cytoplasm (Strom *et al.*, 1993a). Mutations of these cysteine residues resulted in decreased levels of peptidase and methylase activity, suggesting that the active sites of PilD may be in this region (Strom *et al.*, 1993a). These same authors have also shown that the methyl donour interacts directly with one or more of the cytoplasmically located cysteines. It is likely then that proteolytic cleavage and transmethylation occur on the cytoplasmic side of the inner membrane. The purpose of methylation is not certain, but it may be required for interaction of the pilin with other proteins or membrane components involved in the assembly process, or it could allow the pilin to be transported to the cell surface for assembly (Dupuy *et al.*, 1992). The long hydrophobic segment retained by the mature pilin may anchor the subunit in the inner membrane for the next assembly step.

The transport and assembly process is carried out by PilB and PilC, since mutations in these genes result in fully processed pilin localized in the inner membrane that is not assembled (Nunn *et al.*, 1990). Analysis of the deduced amino acid sequences, suggests PilB is a cytoplasmic protein and PilC may be an integral membrane protein (Nunn *et al.*, 1990). PilB is hydrophilic and contains a short ATP-binding motif which is also present in other secretion proteins such as XcpR of *P. aeruginosa* (Bally *et al.*, 1992) and PulE of *K. oxytoca* (Pugsley, 1991). This highly conserved ATP-binding domain is referred to as Walker box A and is composed of the sequence GXXGXG (Walker *et al.*, 1982). PilB also possesses a second less strongly conserved nucleotide binding sequence present in only a few nucleotide binding proteins (Strom and Lory, 1993). Substitution of serine for glycine in the Walker box of PilB and XcpR inhibited assembly and blocked the secretion of extracellular enzymes respectively (Turner *et al.*, 1993). These results suggest that ATP hydrolysis may provide energy for pilus assembly and secretion of extracellular proteins. Similarly, mutation of residues in the Walker box of *Bacillus subtilis* led to a loss of functionality (van der Wolk *et al.*, 1993). PilB may function similarly to SecA which

couples ATP hydrolysis and translocation across the membrane. ATPase activity of SecA is activated by interaction with the precursor protein, the phospholipid bilayer, and integral membrane proteins (van der Wolk *et al.*, 1993). PilB may interact with processed pilin, PilC, and the inner membrane activating the ATPase activity to translocate and assemble a pilus strand across the inner membrane into the periplasm. Alternatively, PilB and XcpR may be part of a phosphorylation system or they may interact with nucleotides other than ATP which are involved in assembly or secretion.

The actual mechanism of pilus assembly is not known, but work on two proteins PilQ and PilT has shed some light on this subject. The concepts presented in the following discussion on pili biogenesis are presented in figure I.1. PilQ has the highest homology to the *omc* gene product of *N.gonorrhoeae* which forms a high molecular weight complex in the outer membrane. PilB and PilC would participate in the assembly of pilin monomers in a helical array, forming a pilus strand in the periplasm (as discussed previously). This strand would interact with PilQ which serves as a porthole in the outer membrane (Martin *et al.*, 1993). Remodelling of the peptidoglycan may be involved in pilus assembly and that of other exported proteins (Martin *et al.*, 1993). PilT is involved in twitching motility, colony morphology, bacteriophage susceptibility, and possibly the depolymerization/retraction of the pilus (Whitchurch *et al.*, 1991). Pili are also antigenic targets as anti-pili antibodies are produced during infection. PilT mutants retain the ability to form pili but were unable to exhibit any twitching motility, supporting the involvement of PilT in pilus retraction (Whitchurch *et al.*, 1991). The actual mechanism of twitching motility may the rapid polymerization and depolymerization of pili (Bradley, 1980). The ability to polymerize and depolymerize pili would afford the organism protection against the host defense system. PilT is hydrophilic overall, has two domains characterisic of nucleotide triphosphate-binding proteins and is likely located in the cytoplasm (Lauer *et al.*, 1993). PilT is highly homologous to PilB (Whitchurch *et al.*, 1991) which suggests

that polymerization and depolymerization may occur in a similar fashion. Depolymerization may be an adaptation to evade the host defense mechanisms as pili have been shown to mediate nonopsonic phagocytosis of *P. aeruginosa* in cystic fibrosis patients (Paranchych *et al.*, 1986). It appears that pili are important for the initiation of infection and then are no longer present when the infection is persistent and the organisms have switched to mucoidy, which may be an alternate mechanism of host defense evasion.

Four newly identified and characterized *pil* genes, *pilG, pilH, pilI,* and *pilJ*, were shown to be required for pili production (Darzins, 1994). Sequencing and expression studies revealed PilG has significant homology to CheY, the single domain response regulator (Darzins, 1993). In fact, both the structurally important hydrophobic core residues and the key active site residues of CheY were found in the predicted amino acid sequence of PilG. Two PilG mutants were unable to produce pili or perform twitching motility, but showed no inhibition of flagellar motility which curtailed the possibilty of PilG being involved in chemotaxis (Darzins, 1993). The function of PilG may be to receive an environmental signal and transduce the signal, perhaps via phosphotransfer, to the pilus assembly machinery for regulation of pili production. The gene replacement mutant of *pilH* produced pili, but had an unusual circular swirling pattern to the twitching motility (Darzins, 1994). The role of PilH may be to influence the direction of twitching motility. Insertion mutants of *pilI* and *pilJ* resulted in a drastic reduction in pili formation and twitching motility (Darzins, 1994). Predicted amino acid sequences of *pilH, pilI,* and *pilJ* revealed significant homology to chemotactic signal-transduction proteins of enteric bacteria and *Myxococcus xanthus* (gliding bacteria)(Darzins, 1994). Perhaps the function of these four Pil proteins is to serve as a signal transduction regulation system for twitching motility by controlling polymerization and depolymerization of pili. It is also possible

that twitching motility is a by-product of the response of the bacteria to environmental signals that regulate pili production via these four proteins.

The subunit-subunit interactions between pilin molecules are not yet known. Spectroscopic studies revealed that the two tyrosines (residues 24 and 27) in the N-terminal hydrophobic region were exposed to the surrounding solvent in pilin dimers (octyl glucoside dissociation), but were completely inaccessible in the intact pili (Watts *et al.*, 1983). These findings suggested that the hydrophobic region proximal to the tyrosine residues may be involved in the subunit-subunit interactions.

The processing of pilin is of interest because pili are a virulence factor and also because pilin contains an atypical signal sequence that is present on other proteins involved in export of extracellular proteins. Both the leader sequence cleavage and the methylation of the resulting N-terminal residue are mediated by the bifunctional enzyme PilD (Strom *et al.*, 1993b). Studies have shown that the recognition site for the leader peptidase activity of PilD is centred around the the -1 glycine residue (Strom and Lory, 1991). The glutamate in the fifth position of the mature pilin protein appeared to be important for the transmethylase activity of PilD (Pasloske and Paranchych, 1988).

C. Pili as Adhesins

The ability of *P. aeruginosa* to adhere to the upper respiratory tract has been correlated to the ability of this organism to persist and colonize the respiratory tract (Woods *et al.*, 1980; Johanson *et al.*, 1979). The mechanism responsible for the initial colonization of epithelial surfaces is not well understood at the molecular level. It is believed that non-mucoid *P. aeruginosa* are the initial colonizers in lungs of CF patients. There is a phase shift from non-mucoid to mucoid after the initial colonization, and it is this form that is associated with chronic infection (Pier, 1985).

Adhesin based vaccines have been useful in the field of veterinary medicine and they are employed in the prevention of enterotoxigenic *E. coli* infection (Klemm,

1985). Protection from *Dichelobacter nodosus* infection in sheep has been achieved by the use of a purified pili vaccine (Stewart *et al.*, 1982). The study of the adherence process carries potential for alternate ways of treating and preventing infections.

The attachment process for *E. coli* type 1 pili involves special proteins at the tip of the pilus rather than the major structural subunit of the pilus (Hanson and Brinton, 1988). Tip proteins have not been found associated with the *P. aeruginosa* pili, and it has been demonstrated that the receptor-binding function of pili resides in the pilin subunit itself (Lee *et al.*, 1989a; Irvin *et al.*, 1989). The presence of high affinity and low affinity binding sites on human buccal epithelial cells for *P. aeruginosa* was reported and it was proposed that the high affinity binding sites were the pili receptors and the low affinity binding sites were alginate receptors (McEachran and Irvin, 1985). Purified whole pili and anti-pilus antiserum inhibited adherence of *P. aeruginosa* bacteria to polymorphonuclear leukocytes and to human buccal epithelial cells (BEC's) (Paranchych *et al.*, 1986). There are other adhesins present on *P. aeruginosa* since pure pili cannot completely block binding to BEC's (Doig *et al.*, 1990), and pilin minus mutants can adhere although at significantly lower levels (Saiman *et al.*, 1990). Alginate (Rampha. and Pier, 1985), outer membrane protein OMPH2 (Sexton and Reen, 1992) and exoenzyme S (Lingwood *et al.*, 1991; Baker *et al.*, 1991) have also been shown to mediate adherence of *P. aeruginosa*. PAK and PAO1 pili can bind directly to mucin and have the ability to partially inhibit bacterial binding to mucin (Sajjan *et al.*, 1992). It is important to note that in these same studies, pili also bound BSA and other unrelated proteins, suggesting that the binding of pili to mucin was a non-specific type of binding and mucin is not a receptor for pili. In a model of chronic mucosal *P. aeruginosa* colonization in the mouse, RpoN (alternative sigma factor) and pilin-deficient mutants produced a lower level of colonization and 40% of colonized animals were able to eliminate the bacteria (Pier *et al.*, 1992). Similarly, other studies have found piliated bacteria bound efficiently

whereas nonpiliated bacteria hardly bound at all to mouse epidermal cells regardless of the state of flagellation (Sato and Okinaga, 1987). The adherence to the mouse epidermal cells could be inhibited by anti-pilus antiserum supporting the concept of pilus-mediated adherence to cell surfaces.

Binding of *Pseudomonas aeruginosa* to bovine trachea epithelial monolayers could be decreased by 65% with the addition of purified pilin (Saiman *et al.*, 1990) or by the presence of anti-pilin monoclonal antibody (Saiman *et al.*, 1989). The remaining 35% adherence is presumably a function of other adhesins or other accessory factors which contribute to the binding. The most adherent bacteria were piliated, motile, and accumulated exoproducts (phospholipase C and proteinases) under low phosphate conditions (Saiman *et al.*, 1990). It should be noted that both pilin and flagellin are under the control of the alternative sigma factor RpoN (Totten *et al.*, 1990) and the majority of strains that initially colonize cystic fibrosis lungs are motile (Luzar *et al.*, 1985).

The C-terminal region of pilin is semi-conserved and contains an intrachain disulphide loop. Adherence to human buccal epithelial cells was blocked only by monoclonal antibodies against the C-terminal region of pilin (Doig *et al.*, 1990) and Fab fragments directed against the disulphide loop peptide (Lee *et al.*, 1989b). Synthetic peptides of the C-terminal disulphide loop region of pilin were able to bind to BEC's and to inhibit the binding of pure pili (Irvin *et al.*, 1989). These studies emphasize the importance of the C-terminal disulphide loop in the adherence of *P. aeruginosa* to epithelial cells.

D. Cell Surface Receptors for Pili

Sphingolipids are well suited to be cell surface receptors because they can be composed of a wide variety of carbohydrates. Assuming that sphingolipids are found only in the outer leaflet of the plasma membrane, they would make up 30-60% of the lipids in that layer (Yamakawa and Nagai, 1978). Many bacteria have demonstrated

adherence to carbohydrate sequences on cell surface glycosphingolipids. *E. coli* type 1 pili bind mannose rich oligosaccharides, whereas pap pili (adherence pili) bind to glycolipids containing the disaccharide Galα1-4Gal (Stromberg *et al.*, 1990). *Crytococcus neoformans, Candida albicans,* and other yeasts bound specifically to lactosylceramide (Galβ1-4Glcβ1-1Cer) (Jimenez-Lucho *et al.*, 1990). Substitution of other sugars for the lactosyl residue (asialoGM1 and asialoGM2) blocked binding, as did substitution with other glycolipids containing internal lactose sequences. These findings are in contrast with those that used bacteria such as *Neisseria gonorrhoeae* (Stromberg *et al.*, 1988a), *Propionibacterium granulosum* (Stromberg *et al.*, 1988b), and *Bordetella pertussis* (Toumanen *et al.*, 1988) which bind to glycolipids with internal lactose sequences as well as to lactosylceramide.

Pseudomonas aeruginosa and other pulmonary pathogens have been shown to bind to fucosylasialoGM1, asialoGM1 and asialoGM2, which contain the common disaccharide GalNAcβ1-4Gal (Krivan *et al.*, 1988a,b). These same bacteria did not bind to galactosylceramide, glucosylceramide, lactosylceramide, trihexosylceramide, GM1, GM2, GM3, and several other glycolipids, which suggested that an internal or terminal GalNAcβ1-4Gal disaccharide without sialyl residues is the minimum requirement for adherence of these bacteria. Supporting the previous findings, asialoGM1 was found to be the receptor for *P. aeruginosa* on the wounded ocular surface of mice (Hazlett *et al.*, 1993). Sugar inhibition data obtained during earlier studies found that sialic acid inhibited adhesion of *P. aeruginosa* to trypsinized BEC's when preincubated, but enhanced adhesion to untrypsinized BEC's or when not preincubated (McEachran and Irvin, 1985). These findings led Baker *et al.*, 1990 to investigate binding of *P. aeruginosa* to sialylated glycolipids. The resulting study demonstrated binding of *P. aeruginosa* to asialoGM1, asialoGM2, lactosylceramide, and sialic acid containing glycosphingolipids. The lactosylceramide in this case contained a hydroxylated ceramide. The hydroxylation of ceramide appears to affect

the conformation of the sugars and is relevant for bacterial binding (Karlsson, 1989). In addition, a non-pilus adhesin was found to bind to type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) disaccharide determinants present in breast milk (Ramphal *et al.*, 1991). The process of colonization and the situation at the putative binding site are complex, the obvious complication stemming from the bacteria themselves. *P. aeruginosa* has several adhesins (alginate, pili, exoenzyme S, OMPH2) and these adhesins may have more than one binding specificity. It has been shown that pili mediate adherence to various cell types and that this adherence can be blocked by anti-pili antibodies (Pier *et al.*, 1992; Sato and Okinaga, 1987; Saiman *et al.*, 1989; Doig *et al.*, 1990), but the nature of the receptor for pilus-mediated adherence to the epithelium remains unclear. Doig and coworkers (1989) suggest that the pili receptors may be cell surface glycoproteins on BEC's because they were able to demonstrate binding of pili to immobilized BEC glycoproteins. Lee and coworkers (1994) found that pili from *P. aeruginosa* strains PAK and PAO bound to the glycolipid asialoGM1. There may be more than one receptor on epithelial cells for pili, and there could be receptor variation within the pilin prototypes.

Immunoelectron microscopy studies using MAb PK99H (epitope located in the disulphide loop) revealed that the C-terminal disulphide loop is exposed at the tip of the pilus (Lee *et al.*, 1994). MAb PK99H was able to inhibit the binding of PAK pili to asialoGM1, which supports the idea that the adherence domain lies in the disulphide loop region and also shows that asialoGM1 binding is accomplished by the pilus tip.

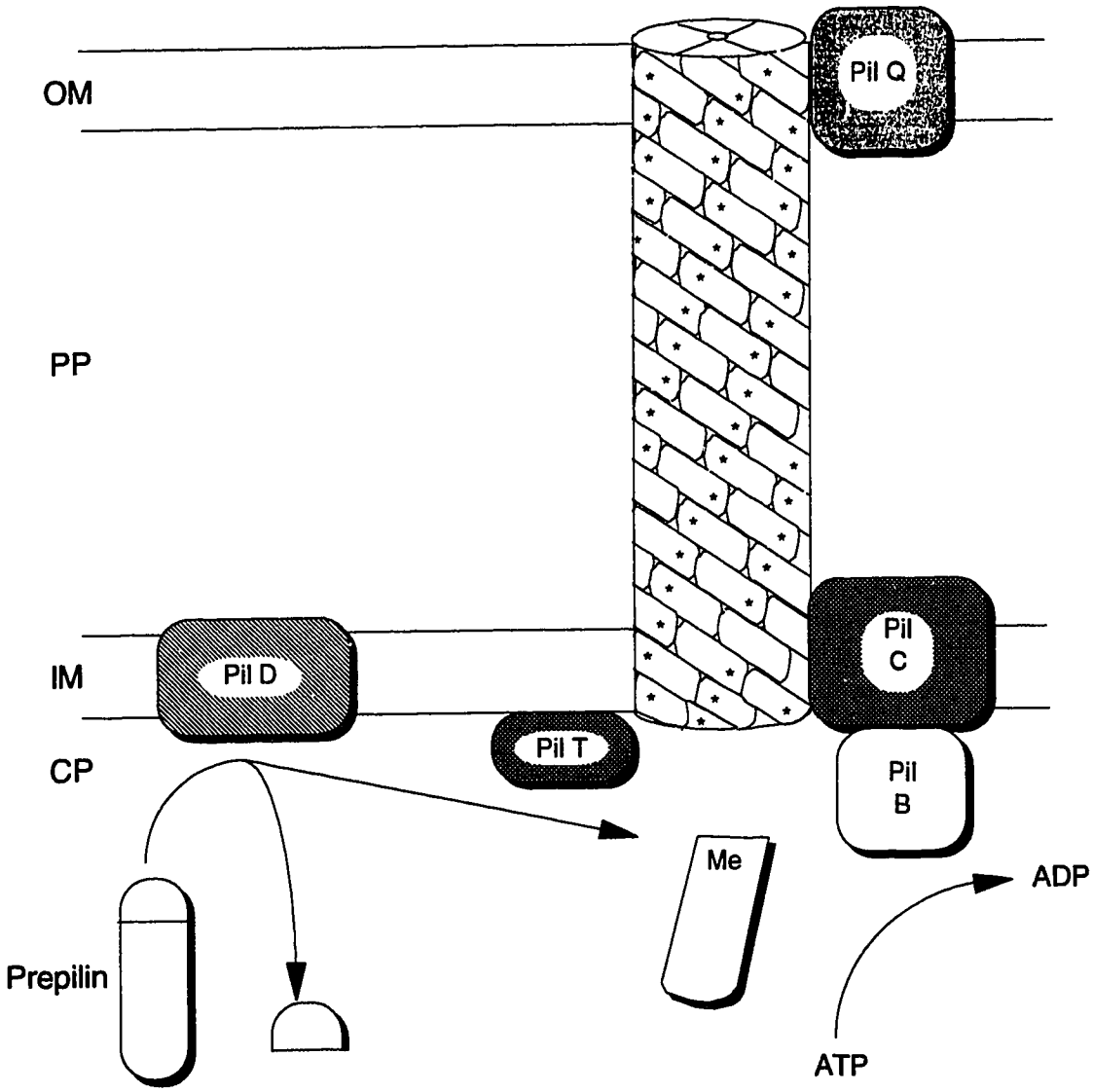
E. Aims and scope of this study

The aims of this study were two-fold: the first being to more clearly define the the recognition site for the PilD transmethylase, and the second being to identify the adhesion domain in the C-terminal region of pilin and the cell surface receptor. The scope of the study was to further investigate the role of the fifth position glutamate and neighbouring residues in the recognition of *P. aeruginosa* pilin by the PilD

transmethylase. The results of these studies are presented in this thesis and have helped to partially define the recognition site for the PilD transmethylase.

The molecular events responsible for the interaction of the C-terminal disulphide loop with the epithelial cell surface receptors are not yet understood. Thin layer chromatography (TLC) overlay assays are a quick and straightforward method to determine the binding of purified pili to an array of cell surface glycolipids and phospholipids. The scope of the study was further expanded by examining the binding of five pili prototypes to purified glycolipids and phospholipids by using TLC overlay assays. In addition, an adhesion assay was developed during the course of this study to determine the binding capabilities of different pilin prototypes to human lung pneumocyte cells. The differences in avidities with which the pili prototypes adhered to human lung pneumocyte cells prompted further investigation into the role of particular amino acids in the C-terminal disulphide loop region in cellular adhesion. Site-directed mutagenesis was performed in this study on both conserved and non-conserved residues within the disulphide loop of PAK pilin to assess the importance of individual amino acids in epithelial cell adhesion. This work also helped identify residues that function in the adherence of pili to human lung pneumocyte cells and to purified putative glycolipid receptors.

Figure I.1. A diagramatic illustration of the current concepts in the assembly of pili. The abreviations signify the following: OM - outer membrane, PP - periplasmic space, IM - inner membrane, CP - cytoplasm, Me - N-methylphenylalanine, PilB - cytoplasmic protein involved in assembly, PilC - integral membrane protein involved in assembly, PilD - leader peptidase/transmethylase, PilQ - outer membrane protein involved in assembly, PilT - cytoplasmic protein involved in depolymerization. The subunit orientation and shape in the pilius strand is taken from Watts *et al.*, (1983).


OM
PP
IM
CP
Pil Q
Pil D
Pil T
Pil C
Pil B
Me
ADP
Prepilin
ATP

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CHAPTER II MUTATIONS IN THE FIFTH POSITION GLUTAMATE IN *PSEUDOMONAS AERUGINOSA* PILIN AFFECT THE TRANSMETHYLATION OF THE N-TERMINAL PHENYLALANINE[1]

INTRODUCTION

Pseudomonas aeruginosa produces polar pili which belong to the NMePhe or type 4 pili family. Type 4 pilins all share a highly conserved N-terminal hydrophobic region of 29 amino acids. The pilin precursor contains a 6 to 7 amino acid leader sequence, which is cleaved by a special leader peptidase to leave an N-terminal phenylalanine which becomes methylated.

The unique leader peptidase is encoded by the gene *pilD*, adjacent to the pilin structural gene, and has been purified and characterised (Strom *et al,* 1991). The transmethylase activity was also found to reside on the PilD protein, making it a bifunctional enzyme (Strom *et al.*, 1993). The recognition sites for the two enzymatic activities of PilD are likely located in the conserved N-terminal region, since type 4 pilins are all processed in a similar manner. Mutational analysis by Strom and Lory (1991), and Pasloske and Paranchych (1988) have demonstrated that amino acid substitutions in the pilin N-terminus affected the processing of pilin. Substitution of the position -1 glycine, relative to the cleavage site, inhibited the cleavage of the leader peptide and prevented the assembly of the pilin monomers into pili, whereas substitution of the +1 position phenylalanine did not affect the leader peptide cleavage or the subsequent assembly of pili (Strom and Lory 1991). Mutation of the fifth position glutamate to valine (Strom and Lory 1991) or to lysine (Pasloske and Paranchych 1988) resulted in the inhibition of the methylation and subsequent

1. A version of this chapter has been published. Macdonald, D.L., Pasloske, B.L., and Paranchych, W. 1993. Can. J. Micro. **39**: 500-505.

assembly, but not the leader peptide cleavage. A deletion mutant in which amino acids 4-7 were deleted from the N-terminus (Δ4 deletion mutant) resulted in a 90% reduction in leader peptide cleavage and no methylation of the pilin monomers. The objective of the following study was to examine the role of the fifth position glutamate in the recognition of pilin by PilD by generating a glutamate to alanine mutation in the fifth position. In addition, a valine to glutamate mutation in the fifth position of the Δ4 deletion mutant was generated to determine if other N-terminal amino acids aside from the glutmate were involved in PilD recognition.

These studies suggest that the recognition site of the leader peptidase and the transmethylase activities may be comprised of key residues in the leader region plus the extreme N-terminus of the mature pilin protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strain DH5α *hsdR recA lacZYAΔ80 lacZM15,* Bethesda Research Laboratories) was used for all the cloning experiments. *E. coli* strain TG2 (*supE hsd* 5 *thi* (*lac-proAB*) (*srl-recA*)306::Tn10(tet^{r})F'[*traD*36*proAB+lacI*^{q}*lacZ* H15) was used as a host for M13mp18 phage and for template preparation. *P. aeruginosa* PAO1 (American Type Culture Collection 25247) was obtained from D.E. Bradley (Memorial University, St. John's, Newfoundland, Canada). The pilin-minus strain BLP3 was made from PAO1 as described in Pasloske *et al.* (1989). The plasmid pUCP18 was provided by Herbert Schweizer, University of Calgary, Calgary, Alberta, Canada (Schweizer 1991). The plasmids constructed and used for this study are outlined in Table II.1. All the strains used in this study were composed of BLP3 as the background strain plus the plasmid pUCP18 alone or pUCP18 containing the wild-type or a specifically mutated pilin gene.

Media and Antibiotics

Luria-Bertani (LB) medium was prepared as described previously (Maniatis *et al.* 1982). Pseudomonas Isolation Agar (PIA) (Difco, Detroit, Michigan, USA) was prepared as recommended. Antibiotic concentrations were 50 μg/ml for ampicillin and 300 μg/ml for carbenicillin (Cb).

Mutagenesis and cloning

The nucleotide changes required to generate the Δ4 Val to Glu and the Glu to Ala mutations in the pilin gene are illustrated in Figure II.1. The mutagenesis was performed according to the method of Zoller and Smith (1982) with the following changes. The oligonucleotides Δ4 Val to Glu (dTTG ATC GAG GTT GCG) and Glu to Ala (dTTG ATC GCA CTG ATG AT) were purchased from the Regional DNA Synthesis Laboratory, Calgary, Alberta. The M13 -20 primer was used as a second primer in the primer extension reaction. The closed circular DNA was not separated from the unligated and incompletely extended molecules, but was transformed directly into competent *E. coli* TG2. The resultant plaques were screened by plaque hybridization using the mutagenic primer as the probe, and verified by sequencing. The cloning procedure is outlined in Figure II.2. In this procedure, the 1.2 kilobase fragment containing the pilin gene with the desired mutation was excised from M13mp18 RF with *Hind III* and cloned into the *Hind III* site of the vector pUCP18. Wild-type PAK pilin, the Δ4 deletion mutant, and a previous Glu to Lys mutant (Pasloske and Paranchych, 1988) were also cloned into the *Hind III* site of pUCP18. All DNA manipulations were conducted according to the methods of Maniatis *et al.* (1982). Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD). and sequencing reagents were purchased from New England Biolabs (Beverly, MA).

Membrane Fractionation, Immunoblotting, and N-terminal Protein Sequencing

Membrane fractionation and protein sequencing were undertaken as outlined previously (Pasloske and Paranchych 1988). Briefly, the protein gel (15% SDS-PAGE) of the membrane fractions was transferred to polyvinylidene difluoride (PVDF) for 2 hour at 200mA. The PVDF was stained for 30 seconds with coomassie blue, and destained for no longer than 5 minutes. The pilin bands from the inner membrane fractions were cut out and applied directly to the Applied Biosystems Sequencer. Immunoblotting was performed as described previously (Pasloske *et al.* 1988). Briefly, the protein gel was transferred to nitrocellulose as stated for the PVDF transfer. The nitrocellulose was reacted with anti-PAK pili antiserum followed by incubation with ^{125}I-labeled protein A. Autoradiography of the nitrocellulose was used to detect the proteins cross reacting with the antiserum. The N-methylphenylalanine standard was synthesized as previously described (Frost *et al.*, 1978). The degree of methylation of the terminal phenylalanine was determined by calculating the area of the phenylalanine and N-methylphenylalanine peaks from the N-terminal sequencing HPLC tracings. In triplicate experiments, the amount of N-methylation differed by ±5% for all pilins except when glu was changed to ala in which case they differed by ±15%.

Bacterial transformation

The recipient *P. aeruginosa* strain was grown in LB broth to an OD_{600} of 0.5 and centrifuged for 5 minutes at 5800 x g. The cell pellet was resuspended in 0.1 volumes of TSS medium (LB broth plus 10% PEG, 5% DMSO and 40 mM $MgCl_2$). Plasmid DNA (1 µg), prepared by the method of Birnboim and Doly (1979), was added to 180 µl of the forementioned cell suspension and the mixture was put on ice for 30 minutes. The mixture was heat shocked at 42°C for 45 seconds, added to 400 µl of LB broth, then incubated at 37°C for 30 minutes. Transformants were selected on Pseudomonas Isolation Agar plates containing 300 µg/ml carbenicillin.

Bacteriophage sensitivity assay

P. aeruginosa transformants were cultured in LB broth to OD_{600} 0.5 and 50µl was spread onto Cb-LB plates. Five microlitres of each of the four pilus-specific phage (PO4, pf1, B9, f116) were placed on one quarter of the plate and it was incubated at 30°C overnight. The titres of the four phage were between $10^{10} \cdot 10^{12}$ pfu/ml. The PO4, f116, and pf1 phage were provided by Dr. D. Bradley, Memorial University, St. Johns, Newfoundland, Canada (Bradley and Pitt 1974). B9 phage was provided by Dr. R.E.W. Hancock, University of British Columbia, Vancouver, British Columbia, Canada. A zone of clearing indicated lysis by the respective phage.

Electron microscopy

Transformants were grown on Cb-LB overnight at 30°C. Phosphate buffered saline (PBS) was pipetted onto the tilted agar plates. The electron microscope grid was floated for one minute on the PBS that collected at the edge of the plate and then stained with 1% sodium phosphotungstate. The grids were examined with a Philips EM300 electron microscope.

Secondary Structure Predictions

The secondary structure of the N-terminal region of the mutated pilin proteins was determined using the method of Garnier *et al.*, (1978). The predictions were performed using PCGENE, the nucleic acid and protein sequence analysis software system, IntelliGenetics Inc. (Geneva, Switzerland).

RESULTS

Two mutations were made in the N-terminus of pilin to examine the role of the fifth position glutamate and neighbouring residues in recognition of pilin by the PilD bifunctional enzyme. DNA sequencing verified the presence of the fifth position Glu to Ala and the Δ4 Val to Glu mutations in the pilin gene. N-terminal protein sequencing

confirmed that the pilin produced by all mutants and wild-type transformants contained the correct primary protein structure (see Table II.1).

Sensitivity to pilus-specific phage was used to assess the piliation of the mutant and wild-type transformants. PAK pili exhibit sensitivity to all four phage, whereas PAO pili exhibit sensitivity to PO4, B9, and f116 only. The PAK and PAO wild-type strains gave the correct sensitivity pattern as controls (Table II.2). All of the mutant strains were resistant to the four pilus-specific phage as shown in Table II.2, suggesting that the strains either did not contain intact pili or contained pili which did not promote phage infection. The PAK wild-type transformant (BLP3 (pDM101)) was sensitive to all four phage and the control vector transformant (BLP3 (pUCP18)) was resistant to all four phage as expected. Pili were not detectable on the surface of the four mutant and control vector strains by electron microscopy (Table II.2) which revealed lack of pili as the cause of the observed phage resisitance. Pili were detected on the surface of the wild-type transformant which indicated, along with the phage sensitivity data, that PAK pili were assembled by this construct. None of the mutants were able to assemble pili.

The state of methylation of the wild-type and mutant transconjugants, as determined by N-terminal protein sequencing, are presented in Table II.3. The N-terminal pilin mutants of Strom and Lory (1991) and Pasloske and Paranchych (1988), are also noted in Table II.3. PAK wild-type pilin was > 90% methylated as was expected. The degree of methylation of the mutant pilins varied depending on the amino acid substitution. The Δ4 Val to Glu was not methylated as was the case with the Glu to Val mutant of Strom and Lory. The Glu to Ala mutant was 40-66% methylated and the Glu to Lys mutant was 12% methylated in accordance with previously recorded results (Pasloske *et al.*, 1989).

Immunoblot analysis assessed the effect of the mutations on transport and translocation of pilin to and across the inner membrane. The presence of pilin in the

inner membrane indicated transport to the inner membrane. Translocation across the inner membrane was indicated by the presence of pilin in the outer membrane. The immunoblot (Figure II.3) showed the presence of pilin in the inner and outer membranes of the three mutants and the wild-type transformant, pilin was produced by the BLP3 background strain containing only the vector. Although the N-terminal mutant pilins were unable to assemble pili, their ability to transport to, and translocation across the inner membrane was not affected. Small cytoplasmic pools of pilin were seen in the Glu to Ala and the Glu to Lys mutant strains. A smaller molecular weight band was also present in the inner membrane of these same two mutants, but this band was also present in the positive control of purified PAK pili. The molecular weight of this band was approximately 8 Kd as determined by 15% SDS-PAGE. This band probably represents a degradation product. There is a possibility that the band resulted from a specific cleavage similar to that producing S-pilin in *N. gonorrhoeae* (Jonsson *et al.* 1992; Haas *et al.* 1987). The pilin band of the outer membrane fractions of all the mutants migrated slightly more slowly than the inner membrane pilin band.

DISCUSSION

Mutations introduced into the highly conserved N-terminal region of the pilin protein had profound effects on its processing and assembly into pili. Deletion of 4 amino acids of the mature N-terminus resulted in greatly diminished leader peptide cleavage (Pasloske and Paranchych, 1988). Proteolytic processing was restored by re-establishing glutamate in the fifth position. These two observations suggest that the recognition site for the leader peptidase may include the fifth position glutamate. However, since three other mutations at position five affected the methylation reaction but not the proteolytic processing (see Table II.3), it appears that the recognition site

of the transmethylase activity, but not the leader peptidase activity, may require the glutamate residue at position five.

It was shown previously that glycine at position -1 to the cleavage site is required for complete proteolytic processing (Strom and Lory 1991). Although signal peptidase I and II cleavage sites are commonly preceeded by small amino acids, Strom and Lory suggested that glycine is not a recognition amino acid, but is the only amino acid allowing sufficient rotation of the phi (N-Cα) and psi (Cα–C=O) bond angles for propilin to attain conformational recognition by the leader peptidase. *Neisseria gonorrhoeae* also requires glycine in the -1 position of pilin precursors for proteolytic processing, but not for membrane translocation (Koomey *et al.* 1991). The mutant pilin containing the four amino acid deletion was translocated across the inner membrane but not proteolytically cleaved, indicating that processing is not a prerequisite for translocation. It has been suggested that deletion of the four amino acids may have affected the secondary structure, increasing the beta sheet and decreasing the alpha helix potential (Pasloske and Paranchych 1988). This in turn, led to a reduction in recognition of the cleavage site by the leader peptidase. Computer analysis suggests that replacing Val with Glu in position 5 of the deletion mutant partially restores the alpha helix potential that is present in the wild-type pilin in this region. The four deleted amino acids may not have a recognition function, but may be necessary to attain the correct conformation for proteolytic cleavage. This situation is not unlike that suggested by Strom and Lory (1991) for the Gly at position -1 in which it is the only amino acid allowing sufficient rotation of the phi and psi angles to attain the conformation for cleavage. The Glu substitution for Val in the Δ4 deletion mutant, increases the alpha helix potential of the N-terminal region so that the putative conformation required for proteolytic processing can be achieved. Alternatively, the four deleted amino acids may form a part of the recognition site and only the glycine residue is conformationally required. The Glu to Val mutant of Strom and Lory is

processed and has a diminished helical potential similar to the Δ4 mutant which is not processed. Replacement of the Val in the Δ4 deletion mutant with Glu allows the propilin of this mutant to be efficiently processed by the leader peptidase. The fact that this Val to Glu Δ4 propilin was completely processed could be taken to indicate that the leader peptidase recognition site extends into the amino terminal of the mature pilin. However, a Glu residue is not specifically required at position 5 relative to the cleavage site for propilin cleavage since the three mutations of the fifth position Glu were all processed relatively efficiently.

The three mutants at position five of the mature pilin sequence all affect the methylation of the terminal Phe and not the leader peptidase cleavage. The methylation reaction was inhibited to a greater extent by the valine and lysine substitutions which are less conservative than the alanine substitution. The fact that none of the mutant pilins were completely methylated and none were assembled into intact pili suggests that methylation may play an important role in pilus assembly. Strom and Lory (1991) hypothesized that methylation is required for recognition of the pilin by the assembly machinery to initiate the formation of intact pili, or to stabilize the subunit-subunit interactions during the assembly process. The methylation is not amino acid specific since replacement with a variety of other residues had no effect on processing (Strom and Lory, 1991). Therefore the recognition site for the transmethylase is located elsewhere in the pilin molecule. From the data presented in this paper and that reported by others (Strom and Lory 1991; Pasloske and Paranchych 1988; Pasloske *et al.* 1989) it appears that the fifth position glutamate is contained in the recognition site of the transmethylase activity. The recognition site for the transmethylase also appears to extend into the amino terminus of the mature pilin since the Val to Glu Δ4 mutant was not methylated. The two enzymatic activities are performed by a single bifunctional protein, hence the recognition sites of the two enzymatic processes overlap in the amino terminus of the mature pilin protein. The Gly in position -1

relative to the cleavage site is a key residue in the cleavage reaction and the Glu at position 5 is a key residue in the methylation reaction.

All of the mutant pilins were translocated across the cytoplasmic membrane, indicating that none of the changed residues are responsible for translocation competence. This supports the proposal of Strom and Lory (1991) that the leader peptides of type 4 pilins do not interact with the bacterial membranes in promoting translocation but function in pilus assembly. It is the entire N-terminal hydrophobic region itself that functions as a leader sequence for the secretion of the pilin protein (Strom and Lory 1987). Pilin was present in the cytoplasmic/periplasmic fractions of the Glu to Ala and the Glu to Lys mutants. This was not seen with the Glu to Val mutant of Strom and Lory (1991), suggesting the presence of a cytoplasmic precursor or pilin released into the periplasm following translocation.

In the immunoblot, it was also noted that the pilin in all outer membrane fractions was of slightly higher molecular weight (see figure II.3). This observation has not been reported by other investigators and may represent an assembly intermediate or an additional modification that is required for pilus assembly. We cannot dismiss the possibility that this phenomenon may be specific to the pilin-minus background strain, or to the presence of the expression vector.

In conclusion, the fifth position glutamate is required for recognition of pilin by the transmethylase activity of the PilD bifunctional enzyme. The recognition sites of the the leader peptidase and the transmethylase activities overlaps in the N-terminus of the mature pilin protein.

Table II.1. Description of plasmids

PLASMID	REFERENCE	PILIN EXPRESSED BY THE CONSTRUCT	N-terminal Sequence*
pDM101	This study	PAK wild-type pilin	FTLIELMIVVAIIG
pDM5400	Pasloske and Paranchych 1988	deletion of amino acids 4-7 of the mature pilin	FTL- - - -IVVAIIG
pDM420	This study	Val to Glu mutation in position +5 of pDM5400	FTL- - - -IEVAIIG
pDM110	This study	Glu to Ala mutation at position +5 in wild-type PAK pilin	FTLIALMIVVAII G
pDM5009	Pasloske and Paranchych 1988	Glu to Lys mutation at position +5 in wild-type PAK pilin	FTLIKLMIVVAII G

*N-terminal sequences were obtained from the inner membrane pilin band. The bands were cut out from the PVDF (polyvinylidene difluoride) membrane that the protein gel was transferred to and placed directly into the Applied Biosystems Sequencer. The dashes indicate deleted amino acids.

Table II.2. Phage Sensitivity of *P. aeruginosa* BLP3 containing plasmids expressing mutant and wild-type pilin genes.

BACTERIAL STRAIN	PO4	f116	B9	pf1	Pili by TEM
PAK wild type	S	S	S	S	+
PAO wild type	S	S	S	R	+
BLP3 (pDM101)	S	S	S	S	+
BLP3 (pUCP18)	R	R	R	R	-
BLP3 (pDM5400)	R	R	R	R	-
BLP3 (pDM420)	R	R	R	R	-
BLP3 (pDM110)	R	R	R	R	-
BLP3 (pDM5009)	R	R	R	R	-

Note : R = resistance and S = sensitivity to the four pilus-specific bacteriophage PO4, f116, B9, and pf1. P04, f116, and B9 bind to the side of the pilus and pf1 binds to the tip of PAK pili not PAO pili. TEM refers to transmission electron microscopy.

Table II.3. Characterization of the N-termini of pilins isolated from the cytoplasmic membrane o
P. aeruginosa BLP3 transconjugants expressing wild-type and mutant pilin genes.

PILIN TYPE	PREPILIN	PILIN UNMETHYLATED	PILIN METHYLATED	REFERENCE
PAK wild type	0%	0%	>90%	This study
Pilin Δ 4	90%	10%	0%	Pasloske and Paranchych, 1988
Pilin Δ 4 V_5-E_5	0%	100%	0%	This study
Pilin E_5-K_5	0%	88%	12%	This study and Pasloske and Paranchych, 1988
Pilin E_5-V_5	0%	100%	0%	Strom and Lory, 1991
Pilin E_5-A_5	0%	33-60%	40-66%	This study

Figure II.1

Nucleotide and amino acid sequence of the N-termini of the wild-type and mutant pilins. The arrow indicates the leader peptidase cleavage site, with the numbering representing the amino acids relative to the mature pilin protein. The solid line indicates the G to A nucleotide change for the Glu to Lys mutant. The dashed line indicates the A to C nucleotide change for the Glu to Ala mutant, and the dotted line indicates the T to A change for the Δ 4 Glu to Val mutant.

PAK Propilin Mutants

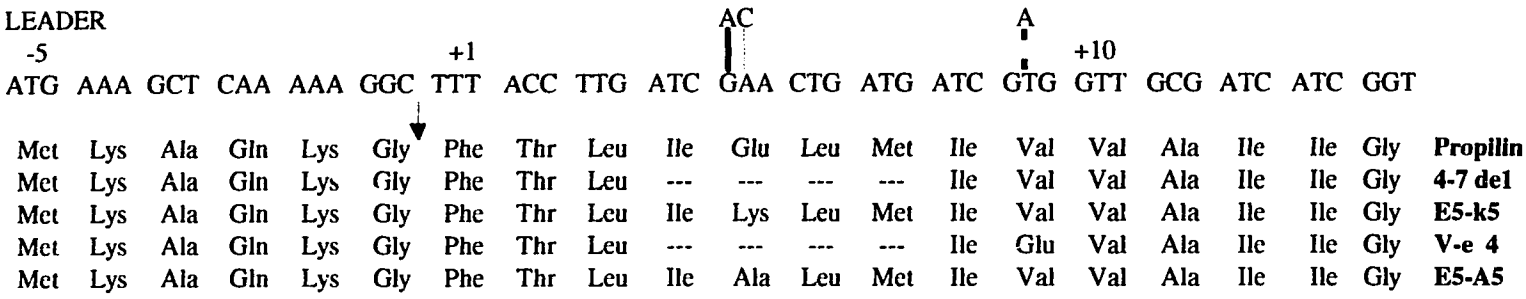


Figure II.2

Construction of the plasmids for the expression of wild-type and mutant pilin genes. The vector pUCP18 was linearized with *Hind III* and then ligated with the 1.2 Kb fragment containing the wild-type or mutant pilin gene. The arrows indicate the direction of transcription of the genes indicated. Amp encodes a β-lactamase which gives carbenicillin resistance in *P. aeruginosa*. *Pil A* is the pilin gene. SF is a stabilizing fragment that allows the plasmid to replicate in *P. aeruginosa*. Refer to Table 1 for the pilin product expressed by each plasmid.

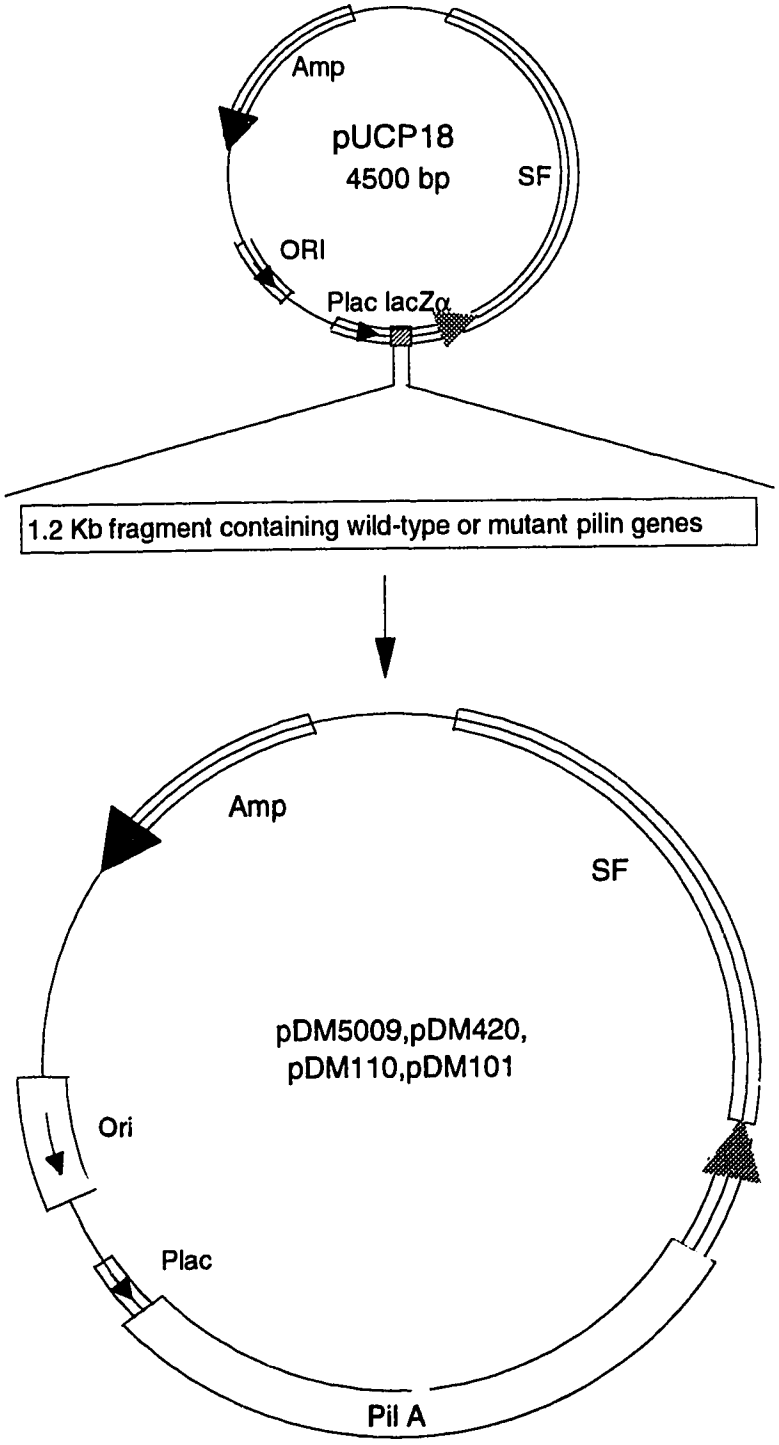
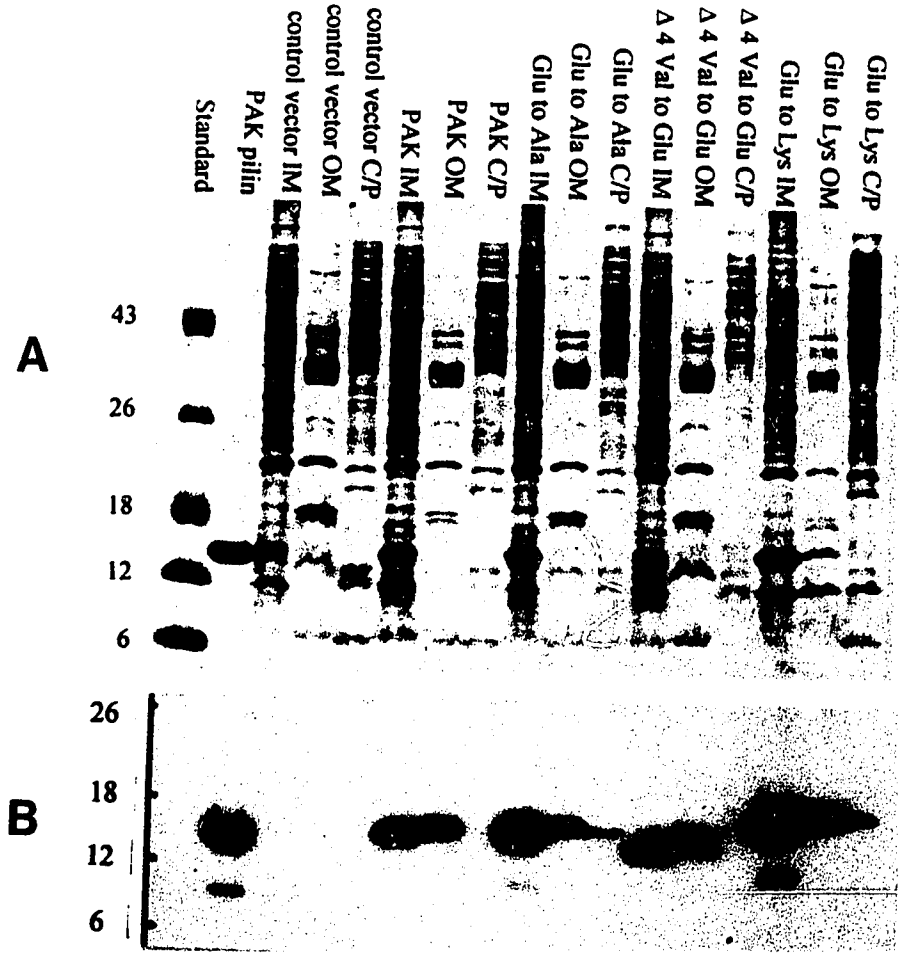

Amp
pUCP18
4500 bp
SF
ORI
Plac lacZα
1.2 Kb fragment containing wild-type or mutant pilin genes
Amp
SF
pDM5009,pDM420,
pDM110,pDM101
Ori
Plac
Pil A

Figure II.3

SDS-PAGE of membrane fractionations and immunoblot of *P. aeruginosa* BLP3 containing plasmids expressing the wild-type and mutant pilin genes. Approximately equal amounts of the cytoplasmic and outer membrane fractions of the BLP3 transconjugants were subjected to 15 % SDS-PAGE. IM = inner membrane, OM = outer membrane, C/P = cytoplasmic and periplasmic fraction.

A. Coomassie-Blue stained gel of the membrane fractions with 4 μg of purified PAK pili as a standard.

B. An immunoblot of the gel in (A) using PAK anti-pilus antiserum. The molecular weight standards (x 10^3) are marked on the left-hand of the figures.


A
B
43
26
18
12
6
Standard
PAK pilin
control vector IM
control vector OM
control vector C/P
PAK IM
PAK OM
PAK C/P
Glu to Ala IM
Glu to Ala OM
Glu to Ala C/P
Δ 4 Val to Glu IM
Δ 4 Val to Glu OM
Δ 4 Val to Glu C/P
Glu to Lys IM
Glu to Lys OM
Glu to Lys C/P

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CHAPTER III GLUTAMATE TO ASPARTATE MUTATION IN THE FIFTH POSITION OF PILIN LEADS TO A MAJOR REDUCTION IN THE EXPRESSION OF *pilA*-specific mRNA.

INTRODUCTION

Pseudomonas aeruginosa pilin belongs to the type 4 family of pilins, which share a highly conserved amino terminal region. The conserved N-terminal region is composed of a positively charged 6 or 7 amino acid leader sequence and a stretch of 29 hydrophobic amino acids. Prior to pilus assembly, the leader is removed and N-methylation of the terminal phenylalanine occurs. The post-translational N-methylation appears to be a requirement for the assembly of the subunits into the intact pilus, since mutations which allow the leader cleavage but not the methylation are not assembled (Pasloske and Paranchych, 1988; Strom and Lory, 1991; Macdonald *et al.*, 1993).

P. aeruginosa propilin is processed by the PilD protein, which is a bifunctional enzyme possessing both the leader peptidase and the transmethylase activities (Strom *et al.*, 1993). The concensus sequence for PilD enzyme recognition is G-][-F-T-L-I-E- where][is the cleavage site (Strom *et al.*, 1993a). The Gly preceeding the cleavage site is conserved in type 4 pilin and pilin-like proteins of the protein excretion apparatus (Nunn and Lory, 1992; Sohel *et al.*, 1993) and is neccesary for the cleavage of *P. aeruginosa* (Strom and Lory, 1991) and *N. gonorrhoeae* (Koomey *et al.*, 1991) pilins. Mutations of the fifth position glutamate resulted in the inhibition of the methylation and subsequent assembly, but not the leader peptide cleavage (Strom and Lory 1991; Pasloske and Paranchych 1988; Macdonald *et al.*, 1993). Examination of protein sequences reveals that glutamate is absolutely conserved in the fifth position of all type 4 pilins and related proteins (Dupuy *et al.*, 1991; Nunn and Lory, 1992; Elleman, 1988; Sohel *et al.*, 1993). The present study explores the question of whether

glutamate itself or just a positive charge is required in the fifth position for recognition by the transmethylase domain of PilD. Site-directed mutagenesis was used to change the fifth position glutamate to aspartate in *P. aeruginosa* pilin. In a Pil$^-$ background strain, the Glu to Asp mutant was assessed for pili and pilin expression using phage sensitivity, electron microscopy, and immunoblotting. Northern blot analysis was used to determine the level of *pilA*-specific mRNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strain DH5α (*hsdR recA lacZYAΔ80 lacZM15*), (Bethesda Research Laboratories) was used for all the cloning experiments. *E. coli* strain TG2 (*supE hsd* 5 *thi* (*lac-proAB*) (*srl-recA*)306::Tn10(tet^r)F'[*traD*36*proAB*+*lacI*^{q}*lacZ* H15) was used as a host for M13mp18 phage and for template preparation. The pilin-minus background strain *P. aeruginosa* PAKp was previously described (Pasloske and Paranchych, 1988). The plasmid pUCP18 was provided by Herbert Schweizer (Schweizer 1991).

Media and Antibiotics

Trypticase Soy Broth (TSB) and Pseudomonas Isolation Agar (PIA) (Difco, Detroit, Michigan, USA) were prepared as recommended. Antibiotic concentrations were 50 μg ml^{-1} for ampicillin and 300 μg ml^{-1} for carbenicillin (Cb).

Mutagenesis and Recombinant DNA Techniques

The nucleotide changes required to generate the Glu to Asp mutation in the pilin gene is illustrated in Figure III.1. The mutagenesis was performed according to the method of Kunkel *et al.*, (1987). The oligonucleotide Glu to Asp (dACC TTG ATC GAC CTG ATG ATC) was purchased from the Department of Microbiology DNA Synthesis Laboratory, Edmonton, Alberta. *E. coli* strain RZ1032 (HfrKL16 PO/45 [*lysA* (61-62)] *dut1*, *ung1*, *thi1*, *relA1* Zbd-279::Tn10, *supE44*) was the strain

used to generate the uracil containing template DNA. The *dut1 ung1* genotype signifies that this strain is unable to excise and repair uracils incorporated into the DNA. The mutagenesis procedure was completed as outlined in Figure III.1. The resultant plaques were screened by plaque hybridization (Sambrook *et al.*, 1989) using the mutagenic primer as the probe. The blots were washed in 1X SSC (0.15m NaCl, 0.015M sodium citrate) at a temperature of T_m -2°C to remove the probe from colonies not containing the mutation. The presence of the desired base change was verified by sequencing with the Sequenase kit (United States Biomedical Corporation, Cleveland, Ohio). The verified mutant pilin gene was cloned into the *Hind III* site of pUCP18 as previously described (Macdonald *et al.*, 1993). All DNA manipulations were conducted according to the methods of Sambrook *et al.* (1989). Enzymes were supplied by Boehringer Mannheim (Laval, Quebec).

Membrane Fractionation and Immunoblotting

Membrane fractionation and protein sequencing was undertaken as outlined previously (Pasloske and Paranchych, 1988). Immunoblotting was performed as described previously (Pasloske *et al.*, 1988a).

Bacterial transformation

Bacterial transformation was conducted as previously described in Macdonald *et al.*, 1993 (Chapter II) with the following changes. The recipient *P. aeruginosa* strain was PAKp. The transformed bacteria were referred to as constucts with the name of the pilin preceeding it.

Bacteriophage sensitivity assay

The verified *P. aeruginosa* PAKp transformants were cultured in TSB to OD_{600} 0.5 and 50 µl was spread onto a Cb-TSB plate. Phage sensititvities were determined as previously described (Macdonald *et al.*, 1993; chapter II).

Electron microscopy

Transformants were grown on Cb-TSB overnight at 30°C. Phosphate buffered saline (PBS) was pipetted onto the tilted plate. The electron microscope grid was floated for 1 minute on the PBS that collected at the edge of the plate and then stained with 1% sodium phosphotungstate. The grids were examined with a Philips Model 410 transmission electron microscope with the assistance of Richard Sherburne (Department of Medical Microbiology and Infectious Diseases).

Secondary Structure Predictions

The secondary structure of the N-terminal region of the mutated pilin proteins was determined using the method of Garnier, Osugthorpe, and Robson (Garnier *et al.*, 1978) using PCGENE the nucleic acid and protein sequence analysis software system, IntelliGenetics Inc. The RNA secondary structure predictions were performed, with the assistance of Keith Robinson, using the program foldrna from the GCG (Genetics Computer Group) software manual version 7.3.1 1993 (Devereux, Haeberli, and Smithies 1984. Nuc. Acid Res. **12**: 387-395.). This program is based on the method of Zuker and Stiegler (1981).

Northern Blot Analysis

Total RNA was prepared from mid-log cultures grown in Cb-TSB. RNA was isolated by hot phenol extraction according to the method of Frost *et al.*, (1989). RNA was electrophoresed on 8% denaturing polyacrylamide gels and transferred to Zeta-probe membrane (Bio-Rad) using a Bio-Rad TransBlot apparatus, both with 0.5X TBE. The PAK pilin transcript was detected using a ^{32}P-labelled oligonucleotide encoding the C-terminal disulphide loop. The oligonucleotide was labelled with ^{32}P-ATP using T4 polynucleotide kinase as previously described (Sambrook *et al.*, 1989). Blots were prehybridized for 2-3 hours at 54°C in 2.5X SSC (0.37M NaCl, 0.037M sodium citrate), 5X Denhardt's solution (Sambrook *et al.*, 1989), 1.5% SDS, and 100 µg/ml of calf thymus DNA and *E. coli* strain W tRNA type XX (Sigma). The blots were hybridized overnight at 54°C, washed in 1X SSC (0.15M NaCl, 0.015M sodium

citrate) and 0.1% SDS for 30 minutes, and autoradiographed at -70°C on Kodak XAR film.

RESULTS

A glutamate to aspartate mutation was created in the fifth position of the pilin gene to determine whether a negative charge or glutamate specifically is required for recognition by the transmethylase activity of PilD. DNA sequencing analysis illustrated that the A to C base change had occurred to replace glutamate with aspartate in the fifth position (Fig. III.2). The mutant construct was tested for pili expression by its sensitivity or resistance to four pilus-specific phage. The Glu to Asp mutant was found to be completely resistant to all four phage (Table III.1). Lack of piliation is, however, not the sole reason for resistance to phage as lack of pilus retraction or phage adsorption will also yield phage resistance. Pili were present on the wild-type construct but not on the Glu to Asp construct by electron microscopy examination (Fig. III.3).

A previously studied mutation in the fifth position (Glu to Ala) did not express pili, but expressed pilin was detected through a Western immunoblot (Macdonald *et al.*,1993; chapter II). Pilin was detected by Western immunoblot for the wild-type and Glu to Ala constructs in both whole cell and cell fraction preparations (Fig. III.4 and Fig. III.5). Pilin was not detected in Western immunoblots of either whole cells (Fig. III.4) or cell fractions (Fig. III.5) of the Glu to Asp mutant construct. The substitution of aspartate for glutamate at position five in the mature pilin sequence profoundly affected the synthesis of pilin, which was unexpected from a conservative substitution. Pilin was either not synthesized or it was rapidly degraded, since it was not detected on western immunoblots of whole cells or cell fractions. These results are consistent with the phage resistance and the lack of pili when the construct was observed under the EM.

Secondary structure predictions may reveal potential anomalies in the protein structure possibly leading to degradation. Improperly folded proteins are cleaved by the degradative proteinases in the cell. The predicted structures (Fig. III.6) illustrated that there may be a disruption in the helical tendency of the first 21 amino acids, which is exhibited by all other fifth position mutations that are cleaved but not methylated (Pasloske and Paranchych, 1988; Pasloske *et al.*, 1989; Macdonald *et al.*, 1993). Structural anomalies that might cause the pilin protein to be degraded were not evident from the predictions. Since there was no pilin, the question was then asked as to whether *pilA*-specific mRNA is present in the cell.

Northern blot analysis was done with total cell RNA directly from the mutant construct in the pilin lacking background strain to accurately reflect the level of mRNA present in the same cells that did not express pilin. Wild-type and Glu to Ala pilin constructs were used as positive controls, a construct with the vector and no insert was the negative control. The pilin gene (both wild-type and with fifth position mutations) was present on a 1.2-kilobase fragment that contains the promoter region and the upstream regulatory site described by Pasloske *et al.*, (1988b). The 1.2-kilobase fragment was cloned into the plasmid vector pUCP18 in the opposite orientation to the lac promoter such that expression of the pilin gene is governed by its own promoter (Macdonald *et al.*, 1993). A *Sal I* site is located at the 3' end of the 1.2 Kb fragment which allowed the orientation of the insert to be determined by agarose gel electrophoresis. The plasmid pUCP is not a high copy number plasmid in *P. aeruginosa* (Schweizer, 1991). The northern blot showed that a pilin gene transcript was only detected in the wild-type construct and the Glu to Ala mutation (Fig. III.7).

DISCUSSION

The effect of the fifth position glu to asp substitution on pilin synthesis was both surprising and intriguing. Neither pili nor pilin were produced by the strain having

aspartate in place of glutamate at position five relative to the cleavage site. It was proposed that pilin was either synthesized at a very low level, or that it was rapidly degraded. Secondary structure predictions indicated a loss of α-helix potential in the N-terminal region similar to all other fifth position mutations which were cleaved but not methylated. These observations provided few, if any suggestions to explain the lack of pilin expression. Northern blot analysis revealed that mRNA for pilin could not be detected for the Glu to Asp mutant in contrast to the wild-type construct and Glu to Ala mutant. The amount of mRNA for a particular gene present in the cell depends on two factors: the rate of transcription and the half life of the mRNA. The absence of detectable pilin mRNA in the Glu to Asp mutant could be the result of extremely low levels of transcription, or rapid degradation of the message. Secondary structure predictions were conducted on the sequence of the entire transcript using the transcription initiation site previously mapped by Johnson *et al.*, (1986). The computer generated, secondary structure predictions indicated a shift of a mismatch from position 68 in the wild-type to position 71 in the Glu to Asp mutant (Fig. III.8). The A to C change which generated the Glu to Asp mutant was in the third position of the codon whereas the Glu to Ala and Glu to Lys mutations studied previously were base changes in the second and first positions respectively (Macdonald *et al.*, 1993). The calculated free energy of the PAK wild-type mRNA is -136.9 and the free energy of the Glu to Asp mRNA is -136.0 Kcal and the overall appearance of the two structures was almost identical. Although there is no obvious structural reason for the apparent lack of mRNA expression by the Glu to Asp mutant, predicted mRNA secondary structures are exactty that, predicted structures and may not accurately represent the *in vivo* structure. The possibility still exists that the Glu to Asp mutation altered the mRNA stability or interfered with the transcription of the pilin gene based on the proceeding discussion of other work.

Altering the stability of mRNA is recognized as an important means of modulating gene expression (Braverman, 1989). It provides a powerful means for bacteria to adapt to the changing external environment through differential gene expression. Bacterial mRNA turnover is thought to proceed by endonuclease attack and 3' exonuclease digestion. Polynucleotide phosphorylase and RNase II are the enzymes responsible for the degradation of mRNA to single nucleotides in *E. coli* (Belasco and Higgins, 1988). These two exonucleases possess processive 3' to 5' activity degrading single stranded RNA from the 3' end. There are two endonucleases, RNase III and RNase E, that have demonstrated a role in mRNA decay in *E. coli* (Belasco and Higgins, 1988). RNase III recognizes certain large stem-loop structures and cleaves one or both strands of the double-stranded RNA. RNase E recognizes a consensus sequence upstream of a stem-loop structure and cleaves single-stranded RNA. Similar exo and endonucleolytic enzymes may exist in *Pseudomonas aeruginosa* which degraded the pilin-specific mRNA. Stem-loop structures at the 3' ends of many mRNAs can function as barriers to 3' exonuclease digestion and molecules lacking these structures are extremely susceptible to exonucleolytic attack (Belasco and Higgins, 1988). RNA secondary structures that block 3' exonucleolytic activity seem to be sequence independent and there appears to be no accessory proteins required to fulfill this function. RNA degradation can occur directly by 3' exonucleases, or it may proceed by endonucleolytic cleavage of a stabilizing 3' stem-loop followed by exonucleolytic breakdown of the resultant new 3' end. Endonucleolytic attack at the 3' end of the Glu to Asp transcript is not likely unless the effects of the mutation is still evident at the 3' end. There are transcripts whose 5' ends decay first, like the *papBA, lac, trp,* and *bla* transcripts of *E. coli* (Baga *et al.,* 1988; Belasco *et al.,* 1986; Cannistraro and Kennell, 1985), which indicate the presence of as yet uncharacterized endonucleases. 5' endonucleolytic attack is a possible consequence of the Glu to Asp mutation since the mutation is 71 bases from the 5' end. Interestingly, the *papBA* genes

are involved in the biogenesis of adhesive pili in *E. coli*, *papA* being the major pilus subunit. RNA decay is primarily a reflection of the presence, efficacy, and accessibility of potential ribonuclease target sites. The selectivity of the degradation process appears to be determined by the interactions between internal RNA structures or sequences and endonucleases, although there may be a role for translation in RNA stability (Brawerman, 1989).

Analysis of deletion mutations in the leader region of *B. subtilis ermC* showed that stalling of a ribosome in the leader region protects the entire mRNA from degradation (Hue and Bechhofer, 1991). The stalled ribosome seems to block the initiation of decay from the 5' end of the transcript. A study of the Shine-Dalgarno sequence revealed that efficient ribosome binding stabilized the mRNA in the absence of translation, that is, ribosome binding via the Shine-Dalgarno sequence maintained the stability of the mRNA even in the presence of a poor initiation codon or a stop codon at the tenth codon (Wagner *et al.*, 1994). Here again, the ribosomal binding offers protection from a putative 5' RNase site although it is not clear why the 3' ends of these transcripts are not degraded. The A to C base change required to generate the Glu to Asp mutation may have created a recognition site for an uncharacterized repressor protein as operator sites have been found as far downstream as +12 (McClure, 1985). Ribosomes may not bind to the Glu to Asp mutant, and therefore no protection from degradation offered by bound ribosomes is possible.

RNA stability has not been well studied in *P. aeruginosa*, however, possible protective RNA structures and endonucleolytic cleavage were reported in the *arcDABC* operon encoding enzymes of the arginine deaminase pathway (Gamper *et al.*, 1992). Intergenic inverted repeats are located between the *arcB* and *arcC* genes in *P. aeruginosa*. For several bacterial operons it has been reported that intergenic inverted repeats act as partial transcription terminators and/or as barriers against 3' to 5' exonucleolytic mRNA degradation (Gamper *et al.*, 1992). In *E. coli*, mRNA

stability is critically dependent on endonucleolytic cleavages. The first evidence of endonucleolytic processing in *P. aeruginosa* is in transcripts of the *arcDABC* operon which were cleaved *in vivo* in the *arcD* region (Gamper *et al.*, 1992). These cleavage sites do not reveal any obvious similarity to sequences that are known to be recognized by the two major mRNA processing enzymes of *E. coli* , RNase III and RNase E. Computer analysis of the *arcD* mRNA suggested that the cleavage sites occur in unpaired mRNA regions, which shortens the half-life of the *arcD* mRNA significantly (Gamper *et al.*, 1992). When examining the proposed mRNA structure of the Glu to Asp mutant, the shift in the mismatch from position 68 to 71 creates a one base single-stranded region in the stem and could be a recognition site for an endonuclease.

RNase E plays an important role in the turnover of several mRNAs in *E. coli* (Nilsson and Uhlin, 1991). Presumably different transcripts are subject to decay by what may be loosely defined as different "degradation pathways" depending on which endonucleolytic activity is responsible for the rate-limiting step. Subsequent to initial attack(s) by endonucleolytic activities, the mRNA molecules are supposedly degraded in a processive manner by the concerted action of exonucleases in 3' to 5' direction (Nilsson and Uhlin, 1991). A number of sequences which are good substrates for RNase E were aligned (including the *papB-papA* intercistronic region of *E. coli*), and a comparison of the sequences flanking the putative cleavage sites revealed some similarities. The most striking similarity is a G-A/U-cut-A-U-U motif shared by four of the five, followed by three variable positions. The seventh nucleotide after the cleavage is a pyrimidine in all cases. Further analysis of confirmed and putative RNase E cleavage sites has established the following consensus sequence -A/G-A-U-U-A/U (Ehretsmann *et al.*, 1992). The predicted secondary structures in all cases showed a double-stranded region of different size and stability can be proposed to be formed one to nine base pairs downstream of the cleavage site, but the cleavage occurs in a

proposed single-stranded region (Nilsson and Uhlin, 1991). A study on the stability of RNA I molecules to RNase E mediated degradation indicated that the ability of a 5' stem-loop to stabilize RNA I is due to the resulting absence of unpaired nucleotides at the 5' end (Bouvet and Belasco, 1992). The addition of just 5 unpaired nucleotides upstream of the 5' stem-loop eliminated the stabilizing effect of the stem-loop (Bouvet and Belasco, 1992). These data suggest that RNase E preferentially cleaves mRNAs that contain several unpaired nucleotides at their 5' end. Mackie and Genereaux (1993) proposed that RNase E would recognize the consensus sequence described by Ehretmann *et al.* (1992) provide it occurred in an accessible, single-stranded region.

In *E coli*, RNase III has been shown to play a role in the decay of mRNA (Belasco and Higgins, 1988). Cleavage by RNase III occurs at double-stranded sites formed by complementary flanking sequences. The double-stranded RNA is cleaved in a staggered fashion yielding overhanging ends and there is evidence that separate reactions occur for each strand (King *et al.*, 1986). No primary or secondary structural features have been defined for RNase III recognition, though it appears that the tertiary structure of the cleavage site is essential in RNase III recognition (King *et al.*, 1986). The 5' region of the polynucleotide phosphorylase mRNA of *E. coli* contains an RNase III sensitive structure responsible for its decay (Brawerman, 1989) It is possible that the shift of the mismatch (from position 68 to 71 in the mRNA structure) in the Glu to Asp mutant, or the A to C base change necessary to create the mutant, could have generated an endonuclease sensitive structure or sequence recognized by an as yet uncharacterized enzyme.

The possibility of creating a termination sequence by making the A to C base change could not be discounted, but the sequence in this region does not show the characteristics of either a rho-dependent or rho-independent termination site. Simple terminators have a series of uridines preceded by a GC-rich region of dyad symmetry. Synthetic structures have confirmed that these self-complementary regions and a

stretch of uridines are sufficient for termination without additional factors (Platt, 1986). Rho-dependent terminators contain a common motif consisting of a high C over G region upstream of the 3' termination point of the transcript (Alifano *et al.*, 1991).

The lack of detectable pilin specific mRNA expression in the Glu to Asp mutant may be the result of either the formation of a nuclease sensitive site or the the presence of a repressor recognition site. It is clear from these observations, however, that glutamate is required in the fifth position of pilin for 100% processing, modification, and assembly into pili. As noted earlier, glutamate is conserved without exception in all type 4 pilins and pilin-like proteins. A negative charge may in fact be the requirement, but aspartate cannot be used because pilin will not be produced.

Table III.1. Phage Sensitivity of *P. aeruginosa* containing plasmids expressing wild-type and mutant pilin genes.

Bacterial strain	PO4	f116	B9	pf1
PAK wild-type	S	S	S	S
glu to ala	R	R	R	R
glu to asp	R	R	R	R

Note: S = sensitivity and R = resistance to the pilus-specific phage of which three (PO4, f116, B9) bind to the lateral surface of the pilus and one (pf1) binds to the pilus tip.

Figure III.1 Site-specific mutagenesis by the method of Kunkel *et al.*, 1987. Single-stranded DNA containing a small number of uracil (U) residues in place of thymine was prepared from the *E. coli* RZ1032 *dut*$^-$ *ung*$^-$ strain. A synthetic oligonucleotide containing the base changes to produce the Glu to Asp mutation was annealed to the template (the mismatch is represented by the discontinuity in the oligonucleotide). Double-stranded DNA was produced by treatment with T4 DNA polymerase and T4 DNA ligase in the presence of the 4 dNTPs. Introduction of the heteroduplex molecule into *E. coli* TG2 (*dut*$^+$ *ung*$^+$) allowed for strong selection of M13 phages with the Glu to Asp mutation because the uracil-containing DNA strand will be degaded by the *dut*$^+$ *ung*$^+$ strain.

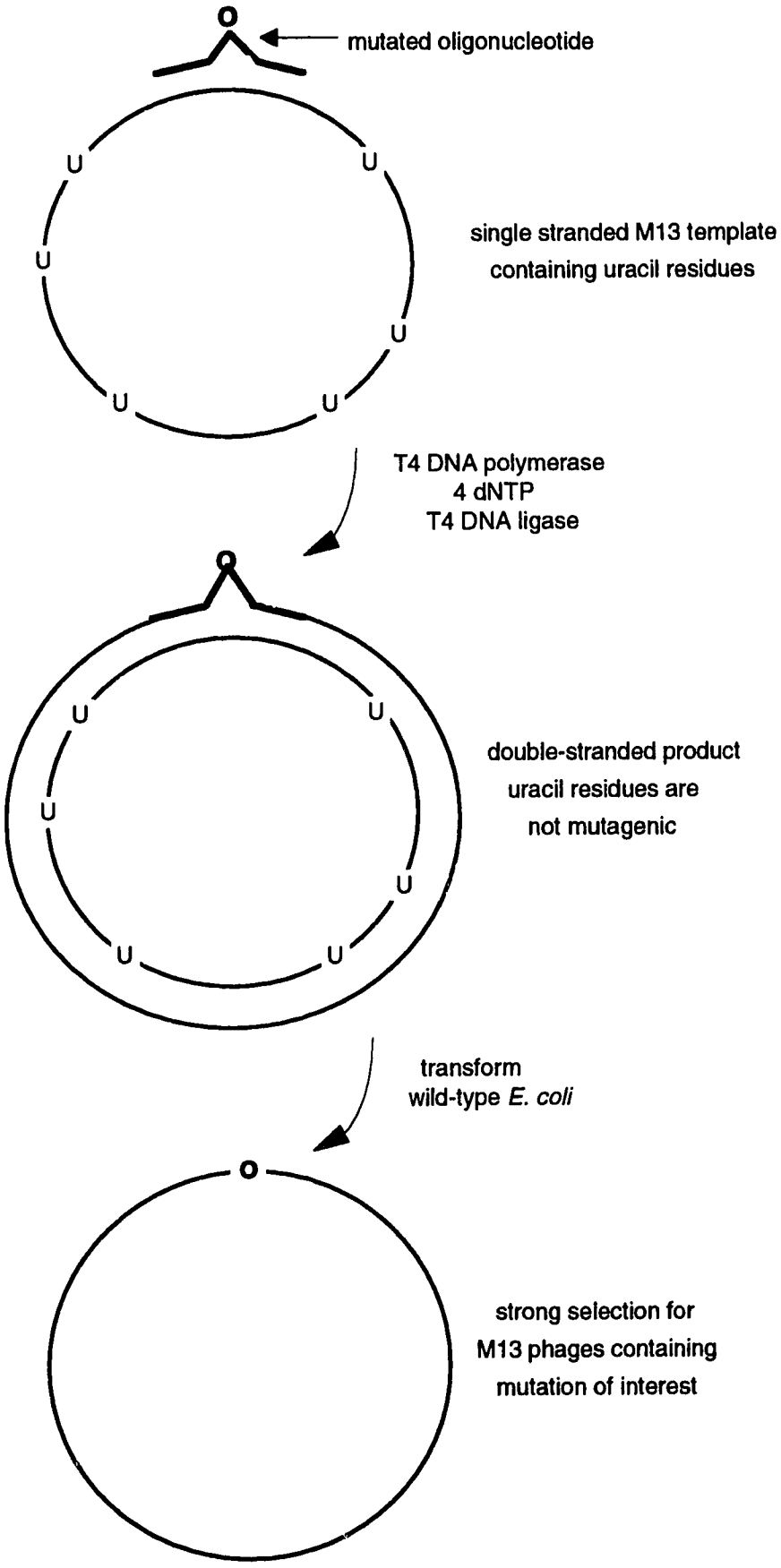

mutated oligonucleotide
single stranded M13 template
containing uracil residues
T4 DNA polymerase
4 dNTP
T4 DNA ligase
double-stranded product
uracil residues are
not mutagenic
transform
wild-type E. coli
strong selection for
M13 phages containing
mutation of interest

Figure III.2. Nucleotide and amino acid sequences of the N-terminus of the Glu to Asp mutant showing the base change A to C from the wild-type pilin. The sequence ladder illustrates that the A to C base change occurred. The lanes of the sequencing ladder are from left to right G, A, T, and C.

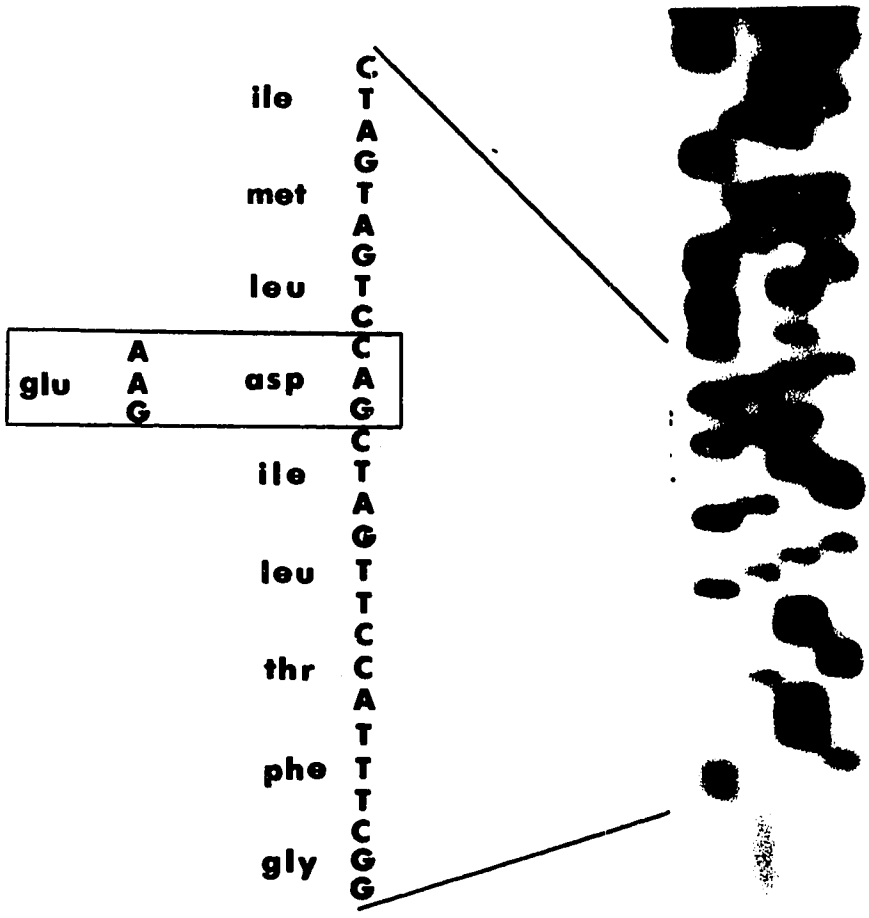

ile
met
leu
glu
A
A
G
asp
ile
leu
thr
phe
gly
C
T
A
G
T
A
G
T
C
C
A
G
C
T
A
G
T
T
C
C
A
T
T
T
C
G
G

Figure III.3. Electron micrographs of PAKp containing plasmids expressing no insert as a vector control (A), the Glu to Asp mutation (B), wild-type PAK pilin (C). Pili are present only on the PAK wild-type expressing bacteria and are shown as the fine filaments extending from the poles of the cell, whereas, flagella are the thicker wavy filaments.

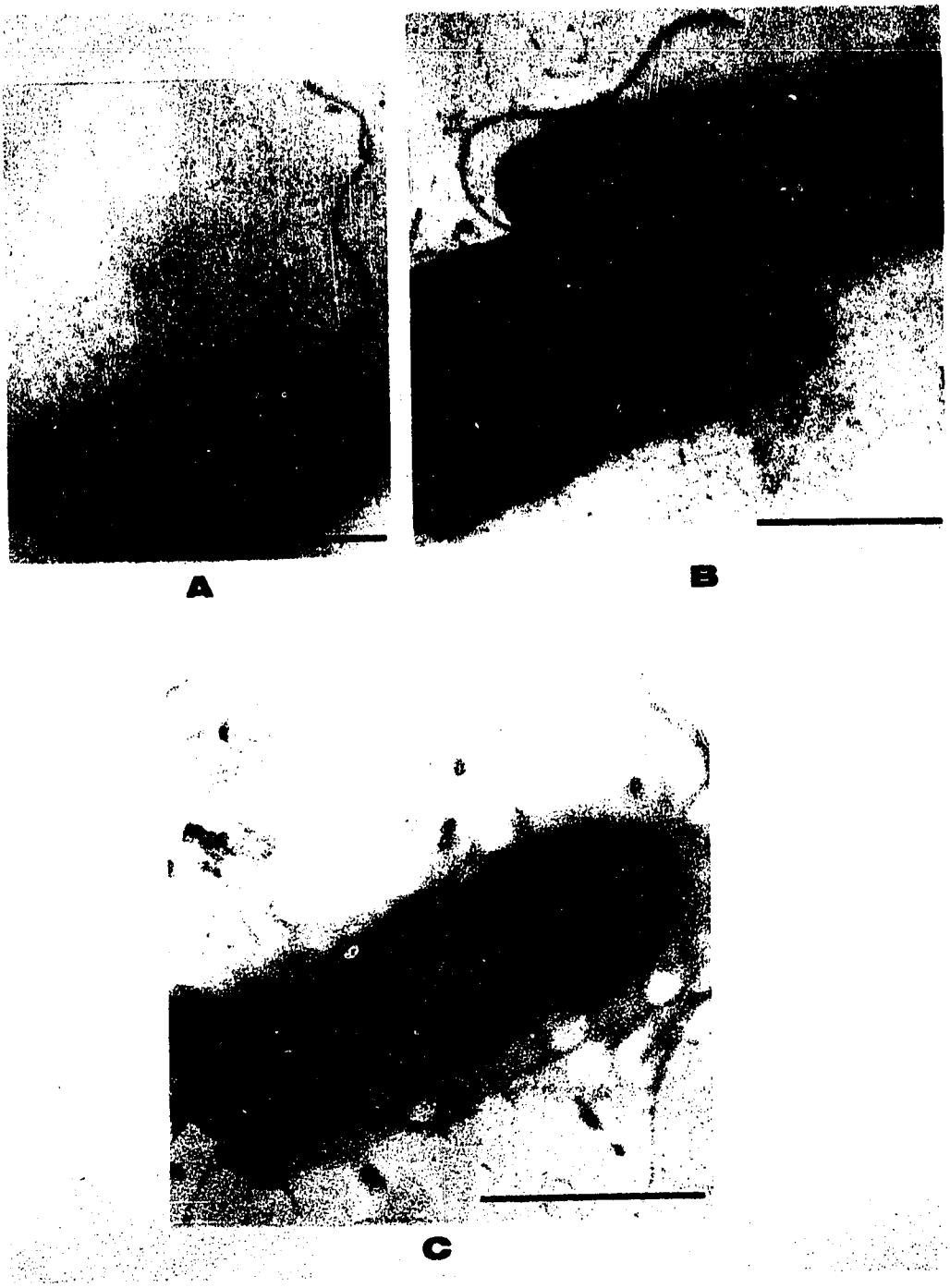

A
B
C

Figure III.4. SDS-PAGE (A) and Western immunoblots (B) of whole bacterial cells of PAKp containing plasmids expressing wild-type and mutant pilin genes. Equal amounts (by volume) of cells were subjected to 15% SDS-PAGE and transferred to nitrocellulose. Anti-PAK pilin antisera was used to detect the presence of pilin. The molecular weight standards are marked on the left hand side, and 4 μg of purified PAK pilin was used as a standard.

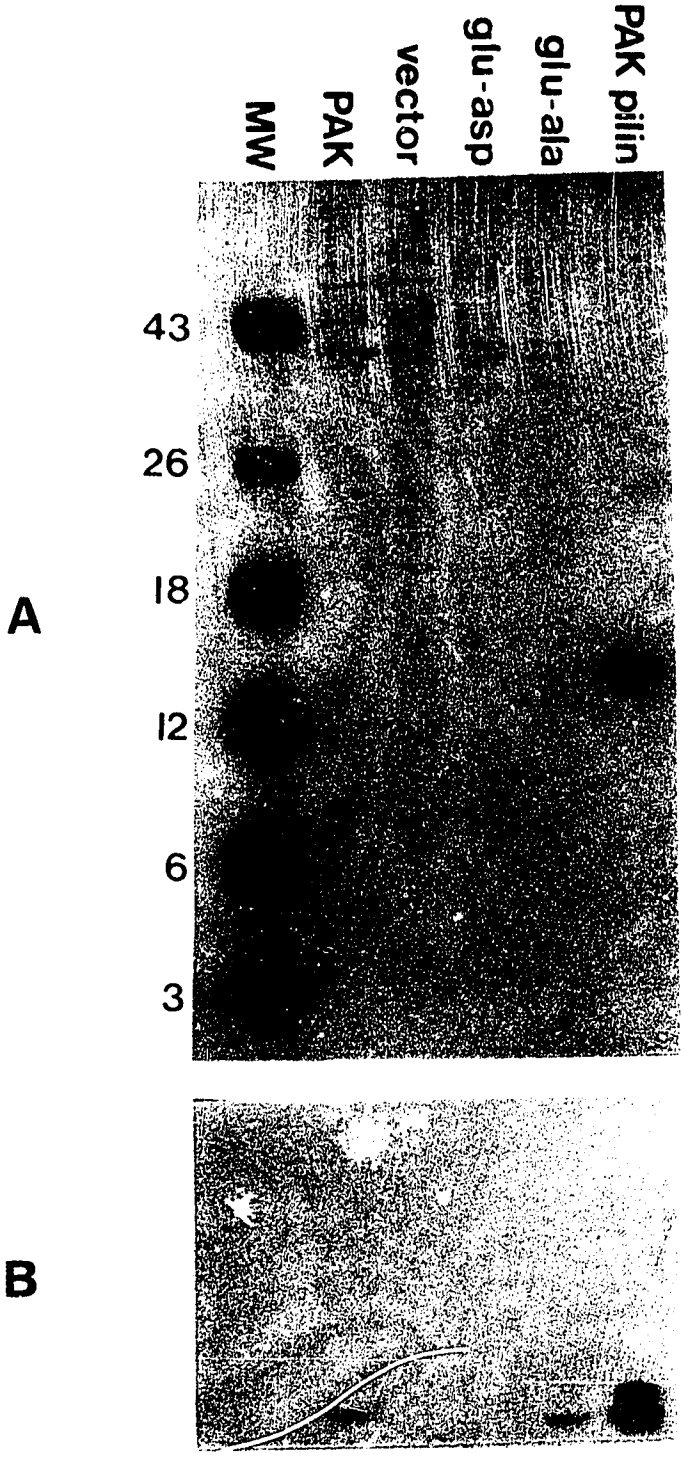
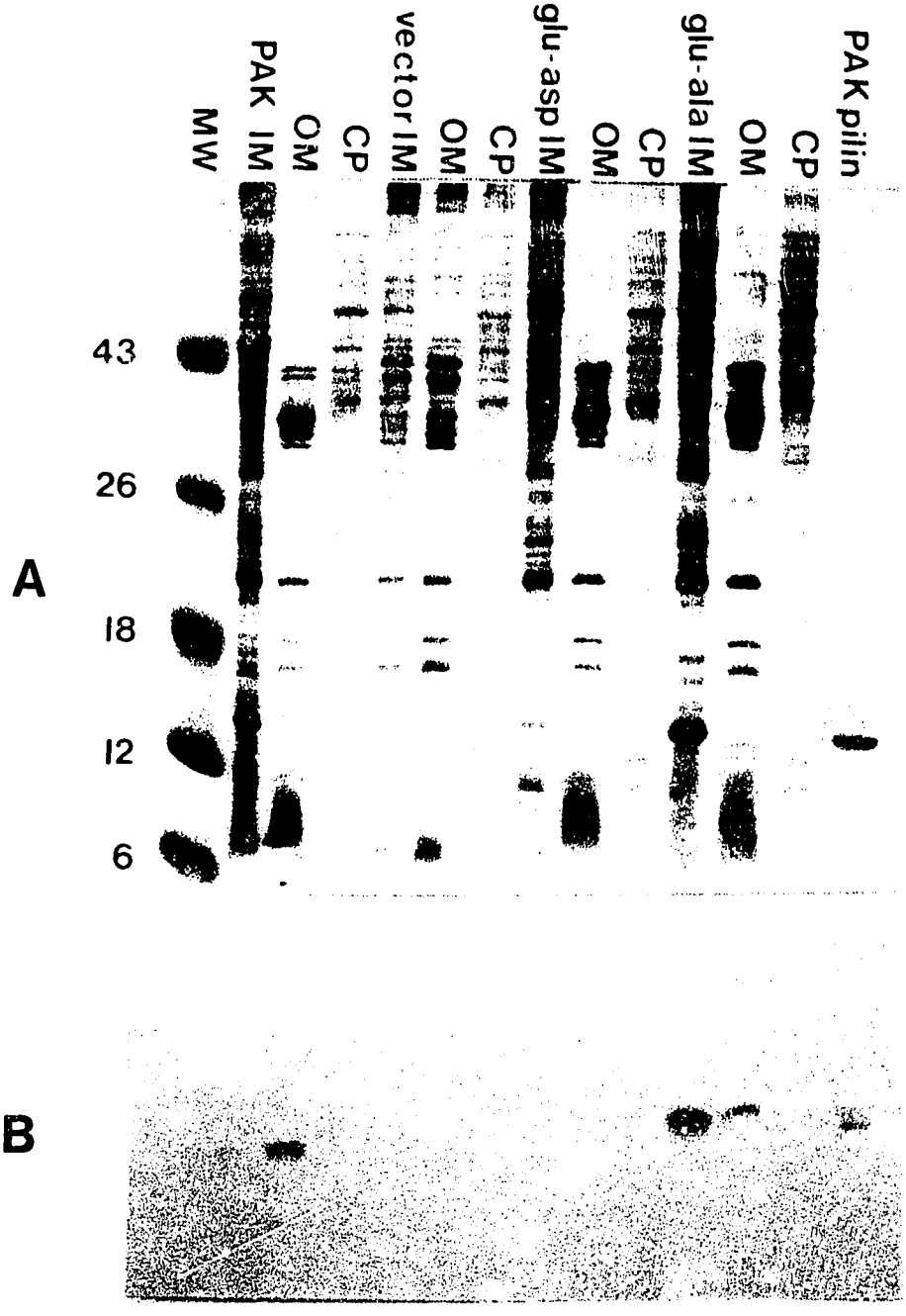

MW
PAK
vector
glu-asp
glu-ala
PAK pilin
43
26
18
12
6
3
A
B

Figure III.5. SDS-PAGE (A) and Western immunoblots (B) of cell fractions of PAKp containing plasmids expressing wild-type and mutant pilin genes. Equal amounts (by weight) of the fractions and 4 μg of purified PAK pilin used as a standard, were subjected to 15% SDS-PAGE and transferred to nitrocellulose. IM indicates inner membrane, OM indicates outer membrane, and C/P indicates cytoplasmic and periplasmic fraction. Anti-PAK pilin antisera was used to detect the presence of pilin. The molecular weight standards are marked on the left hand side.


A
B
MW
PAK IM
OM
CP
vector IM
OM
CP
glu-asp IM
OM
CP
glu-ala IM
OM
CP
PAK pilin
43
26
18
12
6

M	K	A	Q	K	G	F	T	L	I	E	L	M	I	V	V	A	I	I	G	I	pak wt
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
M	K	A	Q	K	G	F	T	L	I	D	L	M	I	V	V	A	I	I	G	I	glu-asp
X	X	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	X	X	X	
M	K	A	Q	K	G	F	T	L	I	K	L	M	I	V	V	A	I	I	G	I	glu-lys
X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	X	X	X	
M	K	A	Q	K	G	F	T	L	I	A	L	M	I	V	V	A	I	I	G	I	glu-ala
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	X	X	X	

Figure III.6. Secondary structure predictions for fifth position substitutions in the PAK pilin gene based on the method of Garnier *et al.*, 1978. The amino acid sequence is displayed using one letter code. The X indicates α-helix potential and the - indicates putative extended conformation. For each amino acid, an equation was computed for each of four conformational states (α-helix, extended, turn, random coil) which takes into account the influence of the 8 residues before and after the one being evaluated. Each amino acid was assigned the conformation whose equation yielded the greatest value.

Figure III.7. Northern blots of total RNA from PAKp containing plasmids expressing either wild-type PAK pilin (lane D), the glu to ala mutation (lane C), the glu to asp mutation (lane B), or no insert as a vector control (lane A). The migration of the molecular weight standard markers in Kb are labelled on the left hand side.

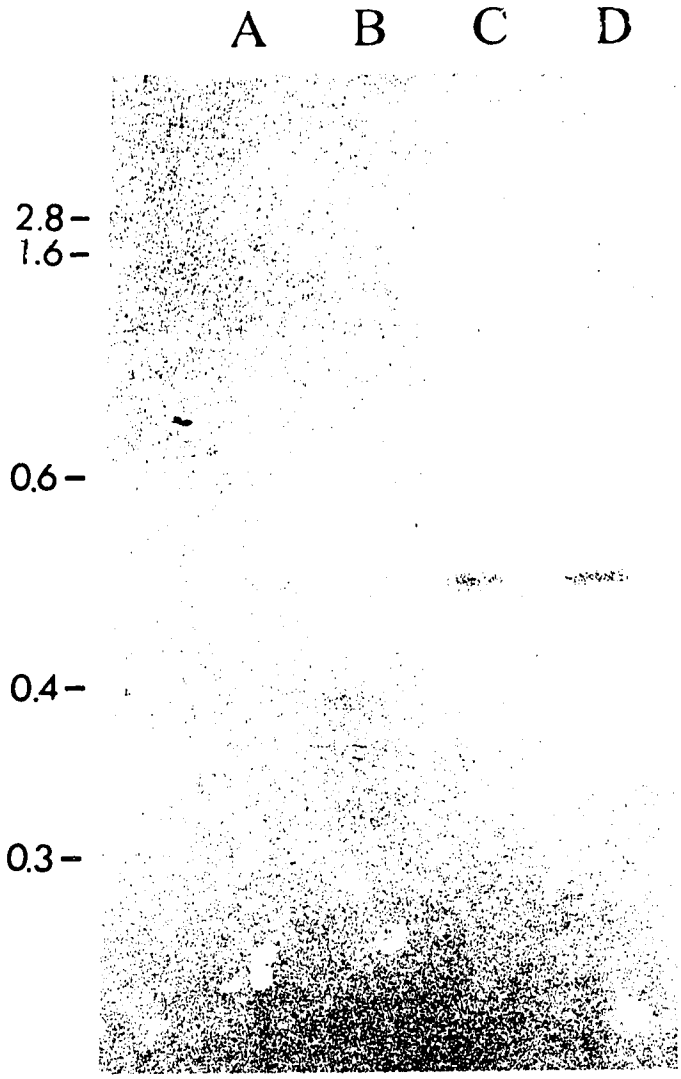
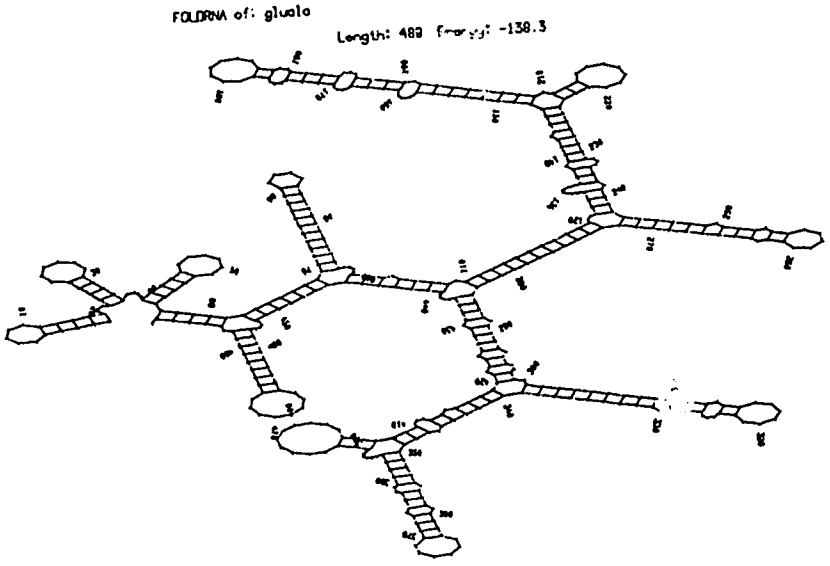
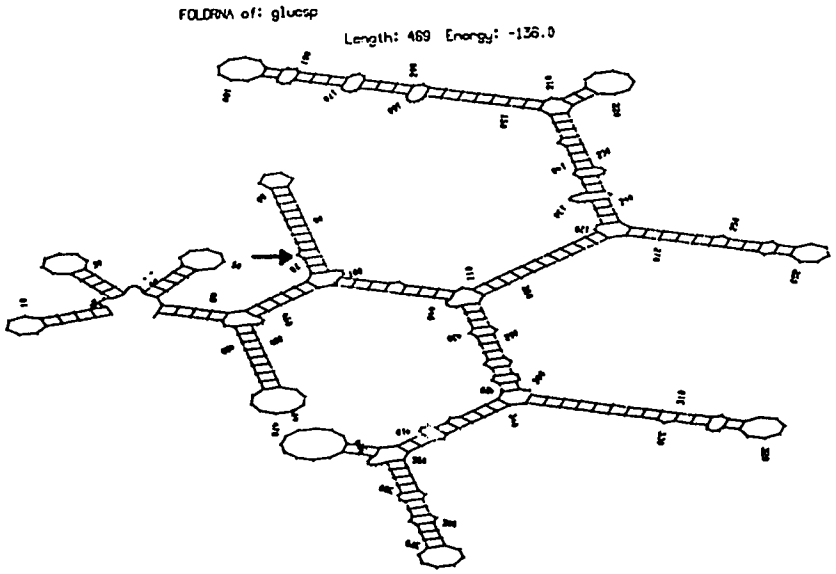

A
B
C
D
2.8
1.6
0.6
0.4
0.3

Figure III.8. Predicted Secondary Structures of wild-type and mutant constructs. (A) represents the structure for the glu to ala mutant, (B) represents the structure for the glu to asp mutant, and (C) represents the structure for PAK wild-type. Structures are of the full length transcript. The mismatch in the wild-type and the glu to asp mutant are indicated by arrows. The secondary structures were generated using foldrna and visualized using squiggles from the GCG software package (Devereux *et al.*, 1984).

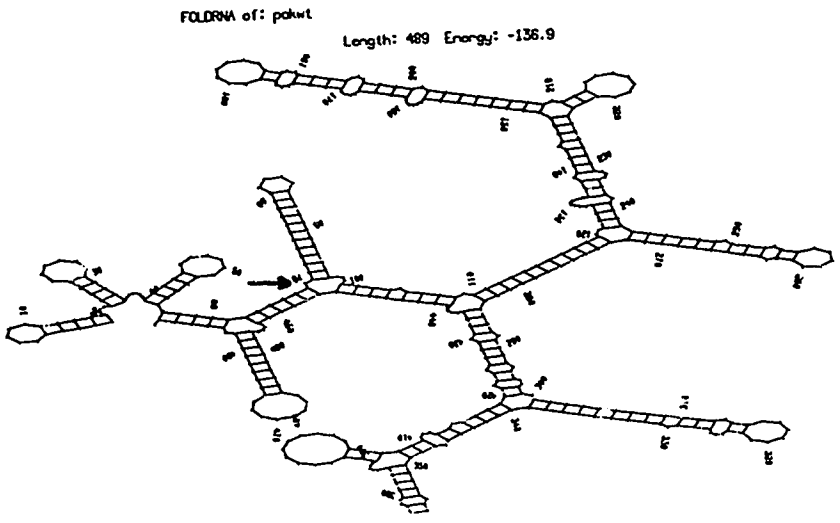
A


FOLDRNA of: gluala
Length: 489

B


FOLDRNA of: glucsp
Length: 489 Energy: -136.0

C


FOLDRNA of: pakwt
Length: 489 Energy: -136.9

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CHAPTER IV BINDING OF *PSEUDOMONAS AERUGINOSA* PILIN PROTOTYPES TO HUMAN LUNG PNEUMOCYTE CELLS AND TO PURIFIED GLYCOLIPIDS

INTRODUCTION

Adherence and attachment of bacteria to the host target cell are the essential primary events in the initiation of infection. The attachment sites for bacterial ligands have been shown to be cell surface carbohydrates, glycolipids [illegible]ar (Beachey, 1980; Mirelman, 1986). Carbohydrates cover the majority o[illegible] and mucosal surfaces and are likely to be the first contact for bacteria (Karlsson, 1989). Candidates for the epithelial cell surface receptor for *P. aeruginosa* are sialic acid-containing glycolipids, asialoGM1, asialoGM2, and lactosylceramide and glycoproteins (Baker *et al.*, 1990; Krivan *et al.*, 1988a,b; Doig *et al.*, 1989).

P. aeruginosa produces filamentous appendages termed pili at its poles. These are believed to promote the initial adherence of bacteria to the host cell surface (Paranchych *et al.*, 1986; Doig *et al.*, 1988,1990; Irvin *et al.*, 1989). Non-piliated mutants of *P. aer*[illegible] (PAK) are bound to A549 human lung pneumocyte cells at only 10-20% o[illegible]iated counterpart (Chi *et al.*, 1990). This demonstrated the importance of the pilus adhesin but did indic[illegible] presence of other adhesins (such as alginate and exoenzyme S). Epidemiological studies of cystic fibrosis lung infection found that primarily non-mucoid strains of *P. aeruginosa* initiate the infection (Rivera and Nicotra, 1982).

The binding domain of the pilus is thought to be in the semi-conserved disulphide loop region (Irvin, 1990; Lee *et al.*, 1989a). This region was shown to be exposed at the pilus tip (Lee *et al.*, 1994). A mutant strain of PAO was constructed in which the 9 C-terminal amino acids of pilin were replaced by 11 amino acids encoded by the flanking region of the *cat* gene (chloramphenicol [illegible] transferase) from the

mini-tn10*Cam* transposon (Farinha *et al.*, in press). The adherence of this strain to A549 cells was reduced by 90% and the LD_{50} in A.BYSnJ mice was the same as that of the PAK-*rpo*N strain which lacks both pili and several other adhesins. These findings suggested that the 9 terminal amino acids do not play a role in pili biogenesis, and that adhesins other than pili do not significantly contribute to cell surface attachment and virulence of *P. aeruginosa*.

There is amino acid sequence variation amongst the pilin prototypes in the C-terminal disulphide loop region. Antibodies raised against the oxidized C-terminal peptide PAK 128-144 were able to cross-react with PAO pili (Lee *et al.*, 1989b). Monoclonal antibodies (MAb) have been raised against PAK pili with one, MAb PK99H, being specific for the C-terminus of PAK only. The epitope of MAb PK34C was located in the C-terminal domain of pilin and partially encompassed the disulphide loop (Doig *et al.*, 1990). MAb PK34C was able to inhibit the binding of all *P. aeruginosa* strains, whereas MAb PK99H inhibited PAK and 492c only. Perhaps there are core binding residues that are conserved amongst all the strains and additional residues that are more strain specific. The aim of this study was to examine the binding characteristics and amino acid sequence differences of five pilin prototypes in the same background strain. A set of isogenic strains each containing one of five pilin prototype genes was created. Binding of pili, purified from the isogenic strains, to cell monolayers and to purified glycolipids or phospholipids was studied. An assay was developed to assess the binding characteristics of the five pili types to A549 human lung pneumocyte cells. The human lung pneumocyte A549 cell line was derived from a human alveolar cell carcinoma and maintains the characteristics of type II alveolar epithelial cells of the lung (Lieber *et al.*, 1976). A549 cells have a human karyotype and appear to have been derived from a single parent cell. The type II alveolar epithelial cell properties are maintained at both early and late passage levels. This made it an ideal host cell system for testing the binding of the pili prototypes. Thin layer

chromatography overlay assays were used to examine pili binding to purified putative glycolipids and phospholipids receptors.

METHODS

Media, Antibiotics, Bacterial strains and Plasmids

The media and antibiotics were previously described (Macdonald *et al.*, 1993; chapter II). *Escherichia coli* DH5α *hsdR recA lacZYAΔ80 lacZM15* (Bethesda Research Laboratories, Gaitherburg, Md.) was utilized for all DNA manipulations. *P. aeruginosa* PAKp, a laboratory strain derived from PAK that does not express any detectable pilin (Pasloske and Paranchych, 1988) (ATCC 25102) was used as the background strain for all constructs. The isogenic strains were maintained on tryptic soy broth (TSB) with 300 μg/ml carbenicillin. The vector used for all constructs was pUCP18 (Schweizer, 1991). MAb PK99H was a gift from Dr. R.T. Irvin (Department of Medical Microbiology and Infectious Diseases).

Pili Purification

The pili were purified by the method of Paranchych *et al.*, (1979) with the following changes. The bacteria were grown at 37°C in the presence of 300 μg/ml carbenicillin. Pili were purified to a single band visualized by SDS-PAGE. Purified pili were found to have the following concentrations as determined by the Lowry protein assay: PAK- 9.2 mg/ml, PAO- 1.28 mg/ml, KB7- 7.5 mg/ml, K122- 0.18 mg/ml.

Phage Sensitivity and Electron Microscopy

The phage sensitivity assay and electron microscopy were performed as described in Macdonald *et al.*, (1993; chapter II).

Recombinant DNA Techniques

All DNA manipulations were performed as outlined in Sambrook *et al.*, (1989). The set of isogenic strains were created in the same fashion as the plasmid pDM101 (Macdonald *et al.*, 1993; chapter II). All the pilin gene fragments were

HindIII except P1 which was a *HindIII BamH1* fragment and were cloned into the multiple cloning site of pUCP18 (Fig. IV.1), transformed into *E. coli* DH5α, and selected on TSB-Amp plates. Plasmid DNA was prepared from the transformants by the method of Birnboim and Doly (1979), and analysed by agarose gel electrophoresis to confirm that the correct fragment was present. Plasmid DNA (1 μg) from positive transformants was transferred to PAKp as previously described (Macdonald *et al.*, 1993; chapter II). The set of isogenic strains were designated in the following manner: the pilin prototype name is preceeded by KC which indicates that it is a construct in pUCP18 transformed into PAKp. The resulting isogenic strains were analysed by phage sensitivity to confirm the correct pili types were expressed, and were examined by electron microscopy to confim the presence of pili. The *P. aeruginosa* P1 strain (DU-565) was originally cultured from the sputum of a cystic fibrosis patient in the University of Minneapolis Hospital, Minneapolis (Speert *et al.*, 1984). The 3.5 - kilobase fragment containing the P1 pilin gene was cloned into the vector pUC19 (Pasloske *et al.*, 1988). The *P. aeruginosa* strain K122 was originally isolated from a cystic fibrosis patient in Toronto, and the 1.2-kilobase fragment containing its pilin gene was cloned into pUC19 (Pasloske *et al.*, 1988). The KB7 strain of *P. aeruginosa* was an isolate from a cystic fibrosis patient in 1984 that was obtained from Dr. Speert, University of British Columbia, Vancouver, British Columbia, Canada. The KB7 pilin gene was located on a 1.5-kilobase *HindIII* fragment that was cloned into pUC18 by P.A. Sastry, a research associate in Dr. Paranchych's laboratory.

Cell Culture

The A549 cell line ATCCV CCL 185 passage 77 was obtained from the American Type Culture Collection Rockville, Md. The cells were maintained in Waymouth MB752/1 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 1 mM sodium pyruvate and incubated at 37°C in 7.5% CO_2. Passage

84-90 were used for all experiments in this report. The cells were fed every 3-4 days and passaged every 7-10 days as described in Chi *et al.*, (1991).

Antisera Production and Immunoblots

Specific antisera for each pili type was produced in rabbits (Flemish giant X Lop ear) using the purified pili of the constructs. Rabbits were injected two times subscapularly and two times intramuscularly (0.5 ml per site) at three week intervals. The inoculum contained 0.5 ml Adjuvax (Alpha-Beta Technology, Worcester, Massachusetts, USA) plus 200 μg of pili in 0.5 ml PBS. Blood samples were taken from the ear artery. Immunoblot assays were conducted as outlined in Macdonald *et al.*, (1993; chapter II) to insure the specificity of the antisera.

Animal Infectivity Studies

The mouse strain used in this study, A.BY/SnJ, is a congenic strain developed Jackson Laboratory, Bar Harbor, Maine (Pennington and Williams, 1979). A breeding colony of A.BY/SnJ mice is maintained by our laboratory at the University of Alberta Biosciences Animal Services. Serological testing on a regular basis insured the mice were free of infectious agents. To determine the LD_{50} values for both the wild-type and the prototype constructs, male and female mice approximately eight weeks of age and weighing 20±2 grams were injected intraperitonealy with varying concentrations of bacteria. Mice were injected in groups of 10 per concentration per bacterial strain. The mice were monitored for 48 hours post injection for mortality and terminal illness. Mice exhibiting signs of terminal illness were euthanized. LD_{50} values were determined by plottting survivors at 48 hours versus bacterial concentration used and estimating the concentration of bacteria at which 50% survival was acheived.

Thin Layer Chromatography Overlays

Purified glycosphingolipids (GSL) and phospholipids (PL) were purchased from Sigma Chemical Co. (St. Louis, MO.). GSLs and PLs were separated on aluminum-backed silica gel Si60 HPTLC plates (E. Merck AG, Darmstadt, Germany)

using a 65:35:8 chloroform:methanol:water solvent and air dried. One plate was sprayed with 10% sulphuric acid in ethanol and charred to visualize the GSLs and PLs, while duplicate plates were tested for binding of pure pili. The remainder of the assay was conducted at room temperature with the plates inverted with the corners bent upward to support them. The plates were blocked with 3% gelatin in PBS for 2 hours with gentle agitation. The plates were then placed directly into a solution of pili in PBS (25 μg/ml) and incubated with gentle agitation for 2 hours. The plates were washed twice for 5 minutes in PBS-0.2% Tween 20 (PBST) and placed in a 1:500 dilution (1% gelatin in PBS with 0.1% Tween 20) of rabbit anti-pili antiserum and incubated with gentle agitation for 1 hour. The plates were washed twice for 5 minutes each with PBST, then were incubated for 1 hour in a 1:5000 dilution (1% gelatin in PBS with 0.1% Tween 20) of goat anti-rabbit alkaline phosphatase conjugate. The plates were washed two times with PBS and then placed in the substrate solution (4-Nitroblue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate supplied by Boehringer Mannheim, Laval, Quebec). The reaction was stopped after 8 minutes by submerging the plate in 0.1 M EDTA for 5 minutes and then the plates were air dried. The antisera were preabsorbed with the background strain (PAKp) to avoid detection of non-pilus adherence factors. Polyisobutylmethacrylate has been used in TLC overlay assays to prevent the loss of the silica gel during the procedure, to reduce non-specific binding, and to orient the carbohydrate moieties for ligand binding (Hansson *et al.*, 1985). In our hands, coating the plates with the plastic delayed the colour development in the final step of the assay, without decreasing the loss of silica or reducing the background, hence its use was discontinued in this study (Fig. IV.1).

Pili Binding and Inhibition of Pili Binding to Human Lung Cells

Ninety-six well cell culture plates were seeded at a density of 5 x 10^4 cells per well from a confluent 25 cm^2 flask of A549 human lung pneumocyte cells and incubated overnight at 37°C with 7.5% CO_2. Phosphate buffered saline with 1% fetal

calf serum and 0.25 mM $CaCl_2$ (PBSFC) was warmed to 37°C and maintained at that temperature for the duration of the assay. The plates were washed once with 100 µl/well of PBSFC by inversion of the plate. One hundred µl/well of PBSFC was added and the desired amount of pili was loaded into each well. For the inhibition assay, the pili were preincubated at 37°C with 35 nM MAb PK99H. The plates were incubated for one hour at 37°C in 7.5% CO_2. The plates were washed three times for 2 min by inversion and then loaded with 100 µl/well of a 1:100 dilution of rabbit anti-pili antiserum, and incubated for one hour at 37°C in 7.5% CO_2. The washing step was repeated and then 100 µl of a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugate was added to each well and the plate was incubated at 37°C in 7.5% CO_2 for 45 minutes. The washing step was repeated and then 100 µl of substrate solution (Sigma alkaline phosphatase substrate tablets, 1 tablet/5 ml 10% diethanolamine pH 9.8) was added to each well. After 30 minutes incubation under the previously stated conditions the plate was read at 405 nm in a Titretek ELISA reader. The amount of pili bound was calculated using the straight line equation derived from an ELISA of known quantities of pili and their respective 405 nm readings.

RESULTS

Characterization of the Isogenic Constructs

A set of isogenic constructs in a pilin-lacking background strain were created, each expressing one of five pilin prototypes. The pilin genes were cloned into the pUCP18 vector in the opposite orientation to the *lac* promoter such that they are transcribed only from their own promoter. The configuration of the plasmid constructs is shown in Figure IV.2. Each of the isogenic strains displayed the correct phage sensitivity pattern corresponding to its respective cloned pilin gene (Table IV.1). The isogenic strains were examined by electron microscopy and found to be piliated with

the exception of the KC-P1, which was poorly piliated much like its wild-type counterpart (Fig. IV.3). Pili were purified from the isogenic strains, with the exception of KC-P1, and used to generate pilin-specific antisera in rabbits. KC-P1 was so poorly piliated that purification of pili from this construct was not possible. An electron micrograph showed the purified pili preparations and it was noted that K122 pili tended to form bundles (Fig. IV.4). LD_{50} studies in A.BYS/J mice performed on both the isogenic strains and the wild-type strains showed that the isogenic strains had the same LD_{50} as the wild-type strains from which they were derived, with the exception of K122 (Fig. IV.5). The LD_{50} of K122 wild-type strain could not be ascertained, however, because the original K122 wild-type strain, the source of the isolated pilin gene used for the K122 construct, was unfortunately lost. The phage sensitivity pattern for KC-K122 was correct as specified by Pasloske *et al.*, (1988), and the antiserum produced against the purified pili from KC-K122 was specific for K122 only.

Development of the A549 Binding Assay

There was a need to devise a simple method to test the binding abilities of the different pili prototypes. Antisera specific for each pili prototype were prepared in rabbits using the pili purified from the isogenic strains. An ELISA type assay could be developed to quantitate the amount of pili bound to an immobilized epithelial cell line if the cell line was able to remain attached to a 96-well plate during the assay. S180, A549 and HepG2 cells were tested for their ability to stick to the bottom of plastic 96-well tissue culture plates with enough vigour to withstand several washing steps. The A549 human lung epithelial cells were the only cell line able to remain attached to the plates after the washes required for the ELISA-like attachment assay. Tween 20 could not be used for washing steps in this assay because it caused the detachment of the epithelial cell monolayers, and 1% fetal calf serum and 0.25 mM $CaCl_2$ were added to the PBS to maintain the integrity of the monolayer throughout the assay. The presence

of these two supplements did not affect the attachment of pili to the monolayer (Fig. IV.6). The A549 cell attachment assays were done in triplicate on different days and the data were averaged to account for well to well variation (4-7% variation was observed). Scatchard plots of the data were used to calculate the apparant association constant, K_a, and N, the average amount of pili bound per A549 cell at saturation (Fig. IV.7). The apparent association constants do not represent affinity, they are a measure of the comparative avidity of the different pili prototypes for the A549 cells. The number of receptors cannot be calculated because of the polydispersed size distribution of purified pili (Doig *et al.*, 1990). The value that was calculated represents the average amount of pili protein bound per A549 cell at saturation, which is a reflection of both the number of receptors and the size of pili. The K_a and the N values are used to compare one pili type to another. This assay proved to be an efficient method for assessing the binding ability of purified pili. The pili prototypes all bound to the A549 cells with the exception of P1. The K_a value of KB7 pili was 1.4 X the value for PAO pili which was 3.5 X greater than that of PAK and K122 pili (Table IV.2). There were notable differences in the average amount of pili bound per A549 cell (Table IV.2). KB7 had the greatest average amount of pili bound per cell, followed by PAO and PAK, and then K122 which averaged the least amount of pili bound per cell. The efficiency of binding was obtained by calculation of the percentage of pili present in the well that bound at saturation. The K_a values and N (the average amount of pili bound per A549 cell) correlated well with the binding efficiency to the A549 cells (Table IV.2). In other words, KB7 bound most efficiently, followed by PAO, PAK, and K122 which was the least efficient binder.

Inhibition of pili binding to A549 cells

The adherence domain of pili has been shown to be the C-terminal disulphide loop region (Doig *et al.*, 1989b). MAb PK99H, which binds to the disulphide loop region of PAK pili but not to PAO pili (Wong *et al.*, 1992), was used to determine if

blocking this region on the PAK pili could inhibit its binding to A549 cells. MAb PK99H was able to inhibit the binding of PAK pili and not PAO pili to A549 cells (Fig. IV.8). A second monoclonal antibody, MAb PK3B, which binds to the basal end of pili (Lee *et al.*, 1994), was not able to inhibit the binding of either PAK or PAO pili to A549 cells (Fig. IV.9).

Thin Layer Chromatography Overlay Assays

Thin layer chromatography overlay assays were used as a efficient method to detect binding of the pili prototypes to purified glycolipids (possible cell surface receptors). Both asialo and monosialo GM1 and GM2 were tested along with phosphotidylcholine, phosphotidylethanolamine, phosphotidylglycerol and lactosylceramide. AsialoGM1 was bound by all pili prototypes and asialoGM2 was bound by all pili prototypes except K122 (Table IV.3, Fig. IV.10). It is interesting to note that K122 pili was a poor binder and had lowest K_a for A549 cells. The three pili prototypes that adhered well to the A549 cells (KB7, PAO, PAK) also bound asialoGM2 in the TLC overlay (Fig. IV.10, Table IV.3). The band for phosphotidylethamolamine (PE) was visible 8-10 hours after the reaction was stopped with EDTA and the plates dried overnight. The presence of this band appears to be caused by lengthy exposure of PE to air. The phosphotidylethanolamine band was a brown colour not the purple colour that represented specific pili binding. A TLC plate with PE alone, placed in the substrate solution for 8 minutes, then in the 0.1M EDTA stop solution for 5 minutes, and left overnight yielded a brown PE band. The PE reaction was termed non-specific since it did not result from specific pili binding.

DISCUSSION

A set of isogenic strains, each containing one of five pilin prototype genes, were created to study the adherence properties of these five related but unique pili types. Phage sensitivity, electron microscopy, and LD_{50} studies in mice illustrated that

the isogenic strains behaved in a similar manner to their wild-type counterparts. Attachment assays of whole cells containing each of the plasmid constructs were not possible as the bacteria did not remain piliated during the assay because the plasmids containing the pilin genes were unstable in the assay conditions. Up to 50% of the bacteria lost the plasmid by the end of the assay as determined by lack of carbenicillin resistance. Pili were purified from each isogenic strain and an assay was developed to assess the attachment of the different pili prototypes to A549 human lung pneumocyte cells.

Examination of the amino acid sequences of the disulphide loop region of the pilin prototypes, which contains the adherence domain, illustrated the semi-conserved nature of this region (Fig. IV.11). Variation in this region may have evolved as a response to evade the host defences, but the adherence function of the pili appears to have been maintained since all four pilin prototypes were capable of binding to A549 cells. The 2 dimensional NMR spectroscopy solution structure of the peptide PAK(128-144) in the oxidized state revealed two β-turns (McInnes *et al.*, 1993). Residues 134-137 (DEQF) formed a type I β-turn and residues 139-142 (PKGC) formed a type II β-turn. Reverse or β-turns often involve Gly and Pro because of their rather special geometry (Creighton, 1984). Type II β-turns are found almost exclusively with Gly in the *i*+2 position (*i* being the first residue in the turn) to avoid the steric hindrance with the *i*+1 carbonyl oxygen (Schulz and Schirmer, 1979). The type I β-turn is compatible with any amino acid combination except Pro in position *i*+2 (Creighton, 1984). Preliminary NMR data revealed the presence of β-turns in the same positions in the KB7 (McInnes *et al.*, unpublished results), and PAO (Patricia Campbell, personal comunication) peptides. The structures of the peptides remain quite similar in spite of the sequence dissimilarity. Proteins are, however, surprisingly tolerant of amino acid substitutions (Bowie *et al.*, 1990). In studying tolerance in the *lac* repressor, these authors found that 50% of the substitutions were not

phenotypically visible. The amino acid sequence alignment of the disulphide loop region of four pilin prototypes (Fig. IV.11) illustrated four absolutely conserved residues (C129, D134, P139, C142 in PAK), four semi-conserved residues (S131, F137, K140, G141 in PAK), and six non-conserved residues (T130, D132, Q133, E135, Q136, I138 in PAK). Comparison of different sequences with similar messages can reveal key features important for the structure and function of the protein. Invariant residues are often directly involved in functions or structural aspects of protein. NMR data indicated that C129, C142, P139, and G141 are directly involved in structural aspects of the C-terminal region; C129 and C142 are required for the intrachain disulphide bridge, G141 and P139 are required for the type II β-turn (McInnes *et al.*, 1993). Substitution of the invariant D134 with alanine in the PAK C-terminal peptide did not affect binding of the peptide to A549 cells and it was an equally successful inhibitor of PAK pili binding to A549 cells as the native peptide (Wong *et al.*, submitted). The residues directly involved in the adherence function are likely semi or non-conserved amino acids.

An assay was developed for assessing the binding capacity of purified pili to A549 cells. The ability of this cell line to remain attached to the 96 well plate during the numerous washing steps was the technical basis of the assay. This ability was compromised if the monolayers were overgrown and had begun to form foci because a high density of cells were used to seed the plates. The cells of the monolayers began to round up slightly if the fetal calf serum and calcium chloride were not added to the PBS. Important features of the assay were, therefore, the cell density used to seed the plates and the supplements to the PBS solution used during all incubations. This ELISA-type assay system was suitable for quantitation of many samples simultaneously and also in the examination of the inhibitory effect of monoclonal antibodies. Adherence assays using the A549 cell line avoided the problems of heterogeneity, non-viability, and the presence of endogenous bacteria associated with

human buccal epithelial cells used in previous studies (Paranchych *et al.*, 1986; McEachran and Irvin, 1985).

MAb PK99H has been shown to bind specifically to the C-terminal region of PAK pili. As stated elsewhere, this region contains the adherence domain of the pilus (Doig *et al.*, 1989b). In fact, the epitope of MAb PK99H was deduced using amino acid replacement studies and found to be the sequence DEQFIPK within the disulphide loop of PAK pilin (Wong *et al.*, 1992). MAb PK99H has an extremely low affinity for peptides containing all or part of the corresponding sequences from PAO, K122, and KB7 pili. (Wong *et al.*, 1992). Immunoelectron microscopy studies using MAb PK99H demonstrated that the disulphide loop is exposed at the pilus tip (Lee *et al.*, 1994). PAK and PAO C-terminal disulphide bridged peptides were able to bind to asialoGM1 and were able to block the binding of whole pili to the glycolipid as did MAb PK99H (Lee *et al.*, 1994). These authors concluded that asialoGM1 binding is associated with the C-terminal disulphide loop region which is exposed at the pilus tip. MAb PK99H inhibited the binding of PAK pili to A549 cells, and PAK pili bound to asialoGM1 and asialoGM2 on the TLC plate. This suggested that the disulphide loop region may be responsible for pili binding to A549 cells and that one or both of the glycolipids could be the cell surface receptor for pili. When the C-terminal 9 amino acids of PAO pili were replaced with 11 non-specific amino acids from the flanking region of the *cat* (chloramphenicol acetyl transferase) gene, there was a significant reduction in binding of the bacteria to A549 cells and marked increase in the LD_{50} in the mouse infectivity assay (Farinha *et al.*, in press). This observation supports the hypothesis that the disulphide loop region is responsible for the adherence to A549 cells. MAb PK3B, which has its epitope in the basal end of pili (Lee *et al.*, 1994), did not inhibit the binding of PAK or PAO pili to A549 cells. MAb PK3B was not observed to inhibit PAK pili binding to asialoGM1 in agreement with the adherence domain residing in the disulphide loop region (Lee *et al.*, 1994). Previous studies

indicated that PAO pili have a higher avidity for asialoGM1 than PAK pili (Lee *et al.*, 1994). Whole cell binding to buccal epithelial cells showed that the affinity of PAO for the cell surface receptor was 10 times greater than PAK (Irvin *et al.*, 1990). The KB7 C-terminal peptide was a more successful inhibitor of PAK pili binding to A549 cells than the PAK peptide suggesting that the KB7 peptide had a slightly higher affinity for A549 cells (Wong *et al.*, submitted). The A549 cell attachment assay used in the present study showed that the apparent affinities of PAO and KB7 pili for A549 cells were 3.5 and 5-fold greater than that of PAK pili respectively (Table IV.2). Competition binding assays with buccal epithelial cells implied that affinity for the receptor was of greater significance than the number of receptors per cell in quantitating bacteria bound to the cell surface (Irvin *et al.*, 1990). The A549 cell binding data presented in this study indicated that both the avidity (K_a) and the amount of pili bound per A549 cell at saturation (N) influenced the binding efficiency. For example, the K_a of KB7 and PAO pili are almost identical, but KB7 pili was bound more efficiently because N was more than 10-fold greater for KB7 (Table IV.2). This concept is further illustrated by PAO and PAK pili in which N is identical, but PAO pili was bound more efficiently because the K_a is 3.5-fold greater than that of PAK pili (Table IV.2).

K122 pili exhibited the weakest binding to A549 cells and were also the only pili type that did not bind to asialoGM2 on the TLC. Electron microscopy studies found that K122 was the only pili that self-aggregated to form ropes or bundles of pili (Fig. IV.4). The aggregation of the pili may interfere with the binding to glycolipid receptors on the A549 cells. The poor binding ability of K122 to A549 cells could be the result of its low affinity, its lack of binding to asialoGM2, pili aggregation, or a combination of these effects.

The purified pili from the four isogenic strains bound to asialoGM1, and all but K122 bound to asialoGM2 on the TLC plates. Pili are likely the adhesin *P. aeruginosa*

utilized to bind asialoGM1 and asialoGM2 since these same glycolipids were bound by the bacteria in previous studies (Baker *et al.*, 1990; Krivan *et al.*, 1988a,b). AsialoGM1 and asialoGM2 may act as cell surface receptors or, alternatively, they may mimic the real receptors which may have a much stronger association constant. There is a distinct possibility that asialoGM1 is a receptor for pili on A549 cells and the human lung epithelial surface as it was found in significant amounts in human lung tissue (Krivan *et al.*, 1988b). Studies on the adherence of P-pilus-associated G-adhesins of *E. coli* to glycolipids on natural (erythrocytes) and artificial (TLC) surfaces indicated that receptors may be more conformationally restricted in natural membranes (Stromberg *et al.*, 1991). TLC allows multivalent presentation of receptors enabling detection of low affinity specificities lost by more traditional inhibition assays utilizing free oligosaccharides (Karlsson, 1989). There is no detection of binding, however, below a certain level because of the shearing forces of the washing steps. The different amino acid sequences of the pilin prototypes may interact with different epitopes on the receptors, which in a more fixed conformational state, could represent either more or less efficient binding of the pili to epithelial cells. All pili prototypes bound asialoGM1 immobilized on the TLC plate, but the accessibility of the specific epitopes on a receptor such as asialoGM1 for binding each prototype at the cell surface may account for differences in binding efficiencies to A549 cells. The differences in the molecular interactions of each pili type would appear as variations of the avidity or the average amount of pili bound per A549 cell. The conformation of the receptor in the epithelial cell membrane may be crucial for binding. The possibility cannot be dismissed that the diffferences in the N values may be the result of the presence of multiple receptors or epitopes with the same avidity. The K_a and N values may also be affected by fragmentation of the pili which may not have occurred to the same extent in all pili preparations. Fragmentation creates new tips on each pili fragment which do not necessarily have the same adherence capabilities as the distal tip of the pilus

yielding different proportions of ligands present in each pili preparation. Aggregation may influence pili binding to A549 cells, although it was not evident in pili other than K122.

As previously stated, conserved residues of the disulphide loop region (C129, C142, P139, G141) appear to be required for maintaining the disulphide bridge and the type II β-turn. The structural importance of the two conserved cysteines, P139, and G141 was also suggested by Wong *et al.*,(submitted). Of the three non-structural residues (S131, Q136, I138) deemed to be important for binding in peptide studies (Wong *et al.*, submitted), one is semi-conserved and two are non-conserved amongst pilin prototypes. The variability in this region appears to be partially in the binding domain itself. The residues present in the KB7 disulphide loop were perhaps the best combination for the orientation of the receptors in the A549 cell membranes.

TLC overlay procedures have included coating the chromatogram with a plastic polymer (usually polyisobutylmethacrylate) to prevent the loss of the silica gel during the numerous incubations and washes, and to orient the carbohydrate groups of the glycolipids. It has been suggested that the plastic may alter the conformation of the glycolipid and change its receptor characteristics (Yiu and Lingwood, 1992). We found that the use of the plastic coating only caused a delay in the development time of the colour reaction.

In conclusion, the disulphide loop region of pilin is responsible for the adherence of *P. aeruginosa* pili to A549 cells and to putative glycolipid receptors. Substantial amounts of asialoGM1 have been found in human lung tissue (Krivan *et al.*, 1988b), therefore it is possible that a receptor for pili on A549 cells and human lung epithelia may be asialoGM1. Alternately, asialoGM1 may mimic the real receptor. It is not known if asialoGM2 is present in significant amounts in lung tissue, however, the possibility still exists that asialoGM2 is also a pilus receptor. The different pili prototypes may not interact with the same epitopes on the cell surface receptors. The

accessibility of the specific epitopes on the receptors may play a role in the efficiency of binding of different pili prototypes. The semi-conservation of the C-terminal disulphide loop region may be an adaptation to the more restricted orientation of the receptor.

Table IV.1 Phage sensitivity of the five pilin prototype constructs

Construct	PO4	pf1	f116	B9
KC-VEC	R	R	R	R
KC-PAO	S	R	S	S
KC-PAK	S	S	S	S
KC-P1	S	R	S	S
KC-K122	R	R	R	S
KC-KB7	S	R	R	S

The KC prefix denotes that the strain is a PAKp construct containing the pilin prototype gene listed immediately after.

Table IV.2. The apparent association constants (K_a) and the average amount of pili bound per A549 cell (N) for four pili prototypes of *P. aeruginosa*

pilin type	Ka (μg/ml)	N (μg pili/A549)	Efficiency
KB7	2.00×10^{-2}	5.10×10^{-5}	.35%
PAO	1.44×10^{-2}	1.51×10^{-6}	.01%
PAK	4.20×10^{-3}	1.52×10^{-6}	.008%
K122	3.60×10^{-3}	2.22×10^{-7}	.002%

The K_a wascalculated from the slope of the Scatchard plot and yielded a value in μg/ml of pili protein. N was calculated from the y-intercept of the Scatchard plot and is the amount of pili bound to each A549 cell at saturation. Efficiency of binding was calculated as the percentage of pili bound at saturation.

Table IV.3 Binding of purified pili to phospholipids and glycolipids.

pili	a1	a2	m1	m2	Lact	PE	PC	PG
PAK	+	+	-	-	-	NS	-	-
PAO	+	+	-	-	-	NS	-	-
KB7	+	+	-	-	-	NS	-	-
K122	+	-	-	-	-	NS	-	-

Note: This table is a summary of the TLC overlay assay results for the purified pili prototypes. + indicates binding to the glycolipid, - indicates no binding, and NS indicates non-specific binding.

a1 = asialoGM1 a2 = asialoGM2
m1 = monosialoGM1 m2 = monosialoGM2
Lact = Lactosylceramide PE=phosphatidylethanolamine
PG = phosphatidylglycerol PC = phosphatidylcholine

Figure IV.1 The effect of coating the TLC plate with polyisobutylmethacrylate. Panel A is the sulphuric acid char of the glycolipids. Panel B is PAK pili binding to the purified glycolipids without polyisobutylmethacrylate. Panel C is PAK pili binding to the purified glycolipids with polyisobutylmethacrylate.

a1 = asialoGM1	a2 = asialoGM2
m1 = monosialoGM1	m2 = monosialoGM2
Lact = Lactosylceramide	PE=phosphatidylethanolamine
PG = phosphatidylglycerol	PC = phosphatidylcholine

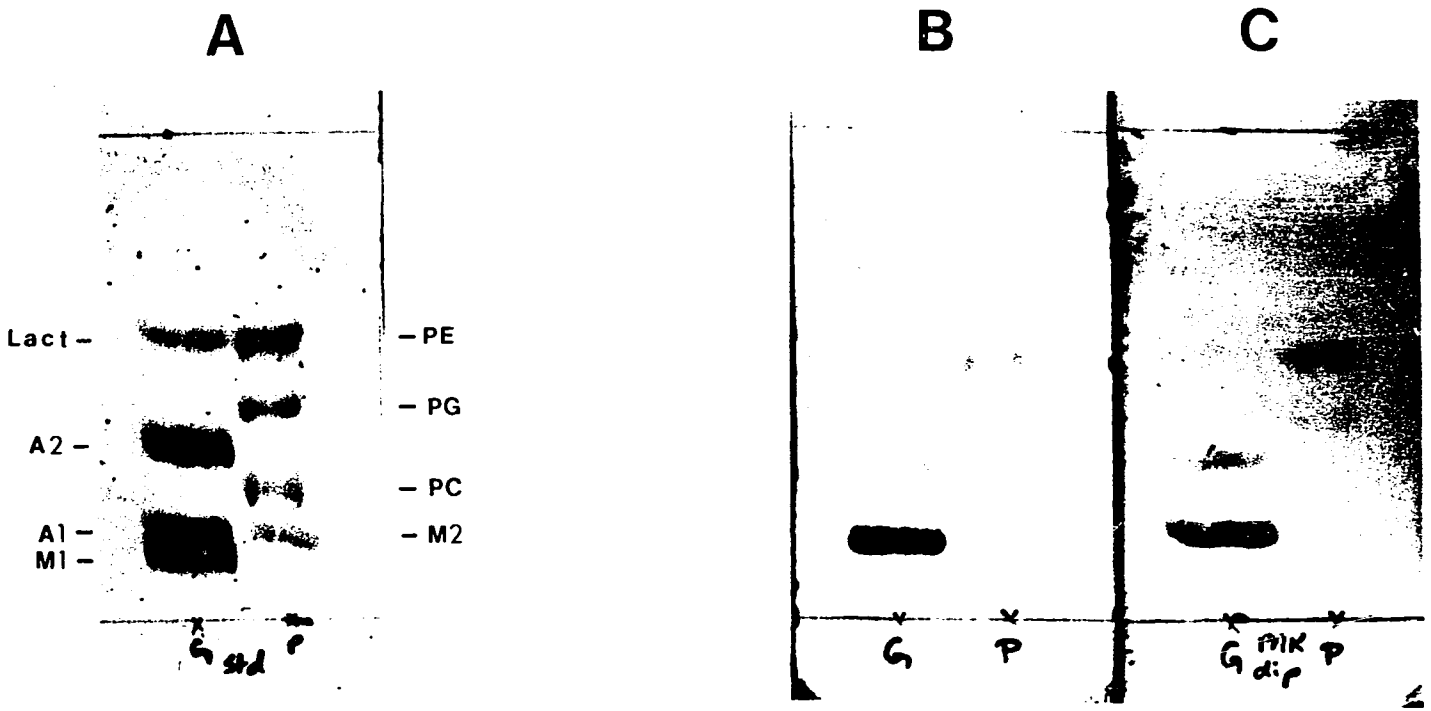

A
B
C
Lact –
A2 –
A1 –
M1 –
– PE
– PG
– PC
– M2
G std P
G P
G MIK dig P

Figure IV.2 Construction of the plasmids for the expression of pilin prototype genes. The pilin genes were ligated into the pUCP18 vector such that they were oriented in the opposite direction to the *lac* promoter. The arrows indicate the direction of transcription of their respective genes. SF is a stabilizing fragment which allows pUCP18 to replicate in *Pseudomonas*. Ori is the origin of replication and Hind III was the cloning site used for the construction. Amp encodes a β-lactamase gene and Pil A encodes the pilin prototype for each construct.

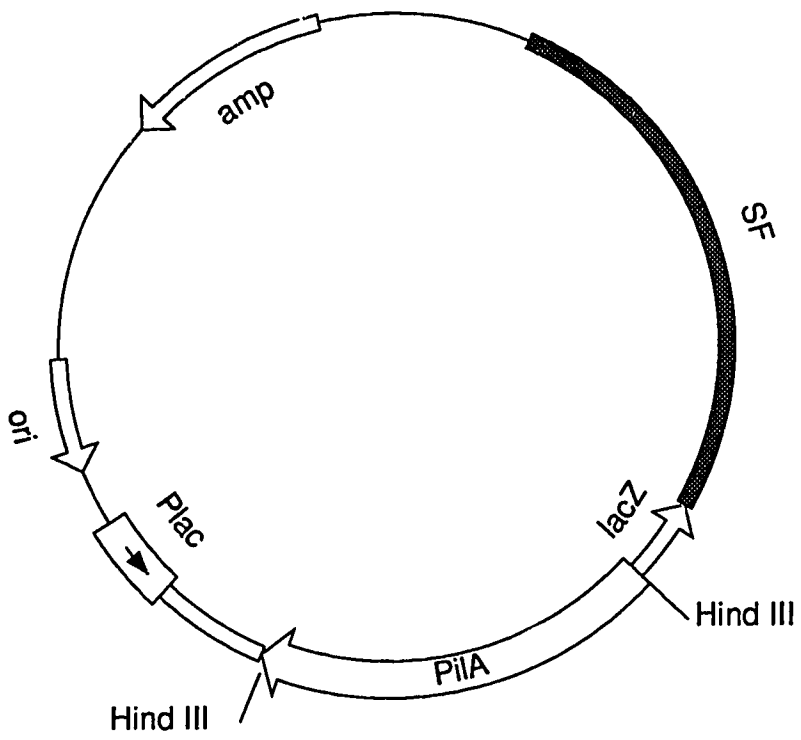

amp
SF
ori
Plac
lacZ
Hind III
PilA
Hind III

Figure IV.3. Electron micrographs of the background strain PAKp containing plasmids expressing the pilin prototypes PAO (A), KB7 (B), K122 (C), P1 (D), PAK (E), and the control vector (F). Pili are the fine straight filments extending from the poles of the bacteria and flagella are the thicker wavy filaments. The bars represent 1 μm.

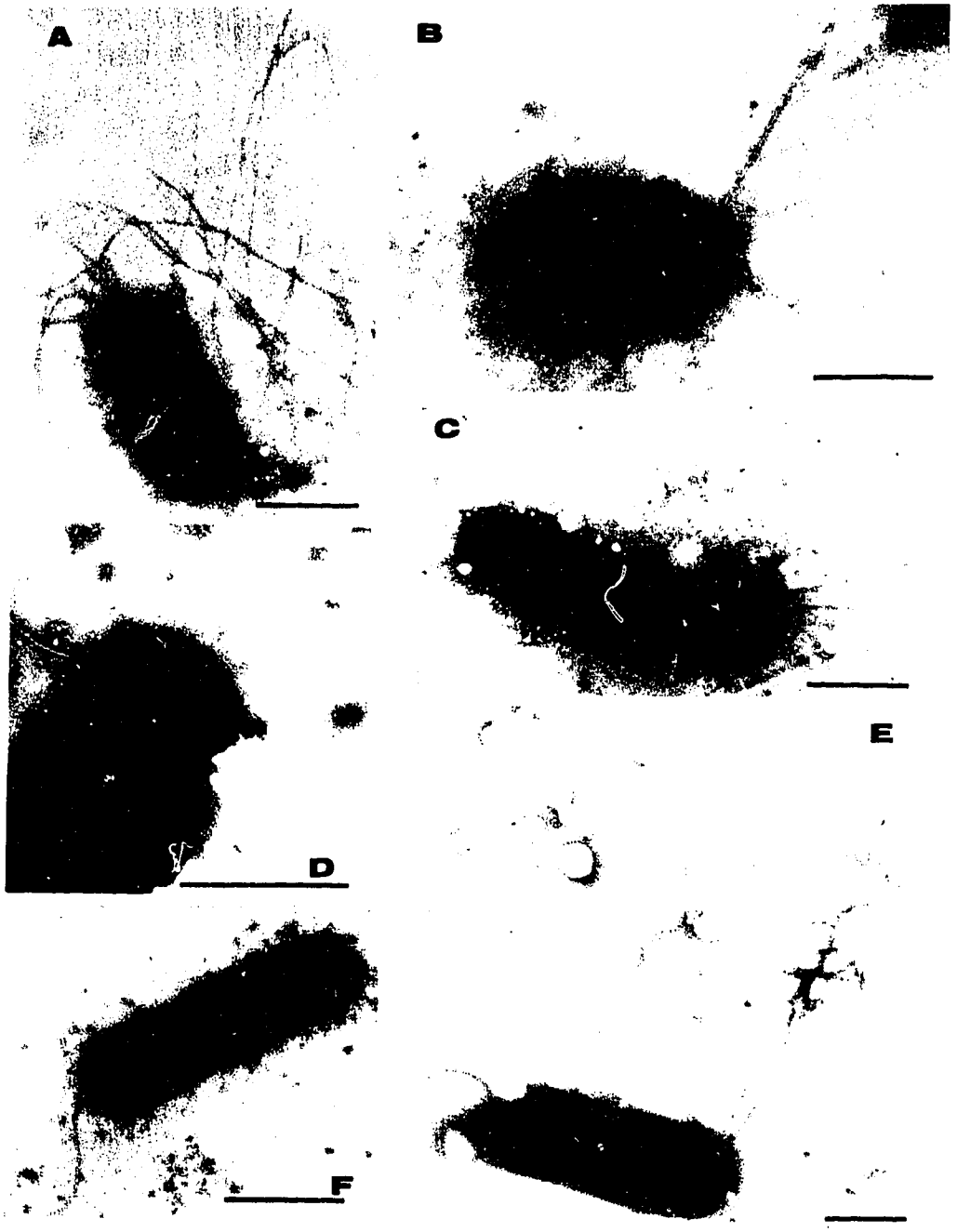

A
B
C
D
E
F

Figure IV.4. Electron micrographs of the purified pili from the pilin prototype constructs: K122 (A), PAO (B), PAK (C), KB7 (D). The bar represents 0.25 μm.

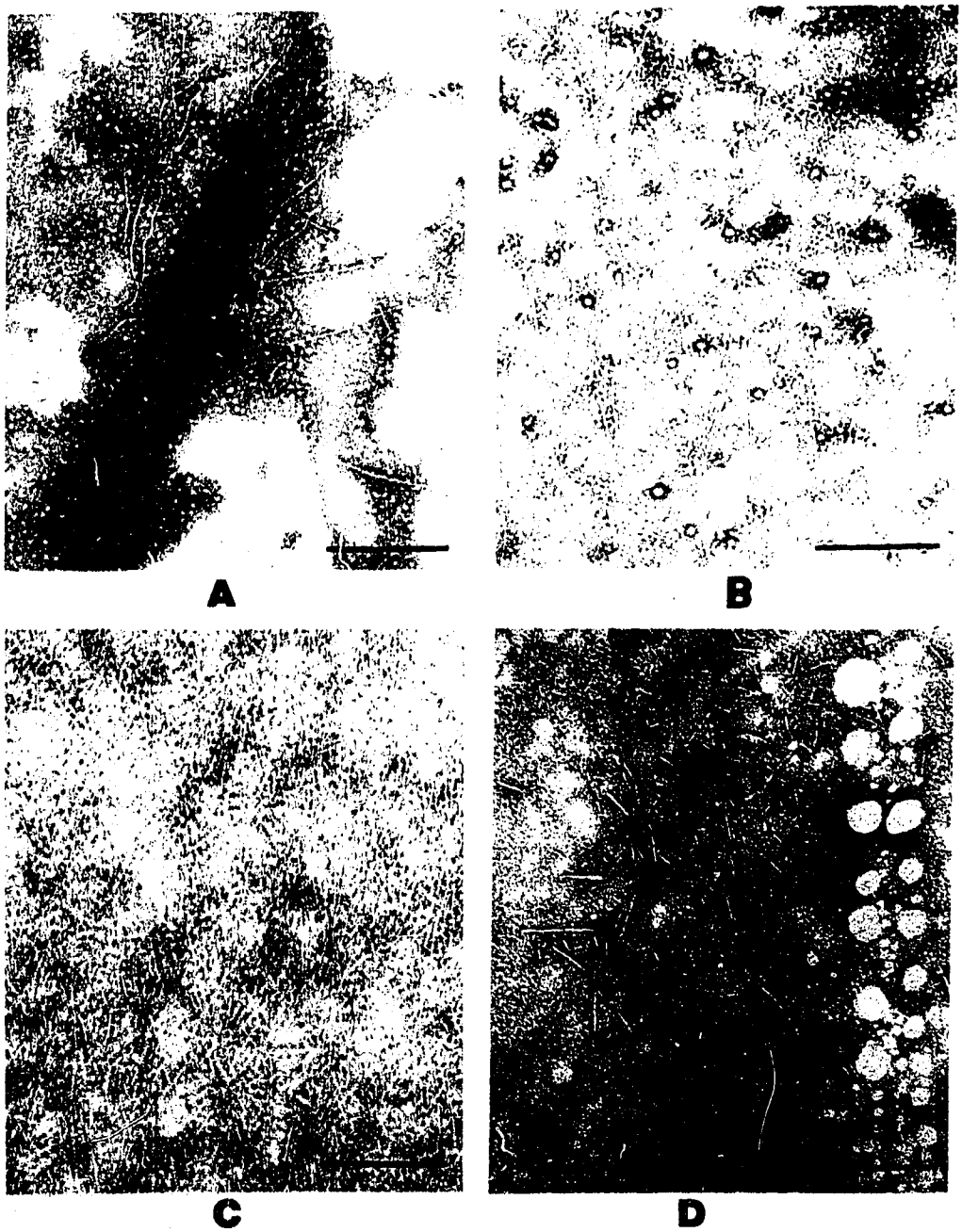
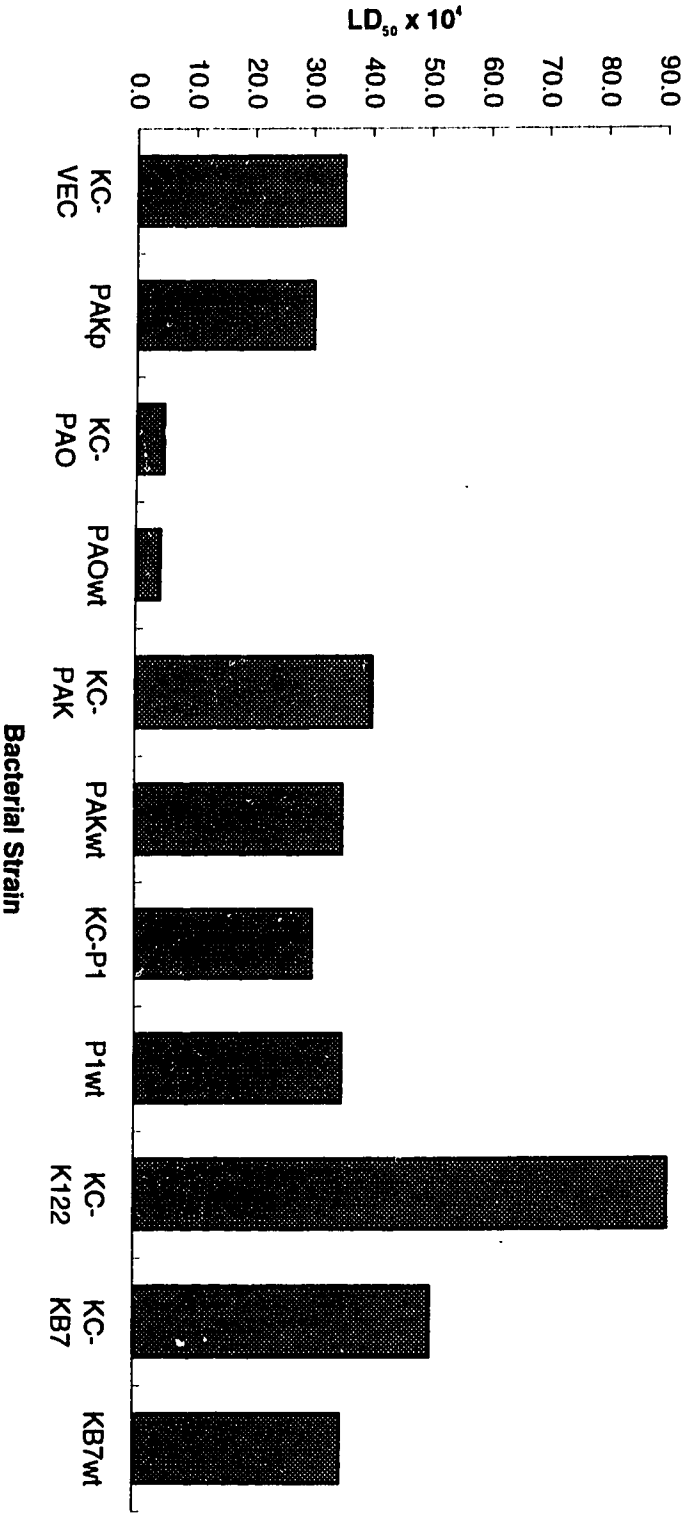
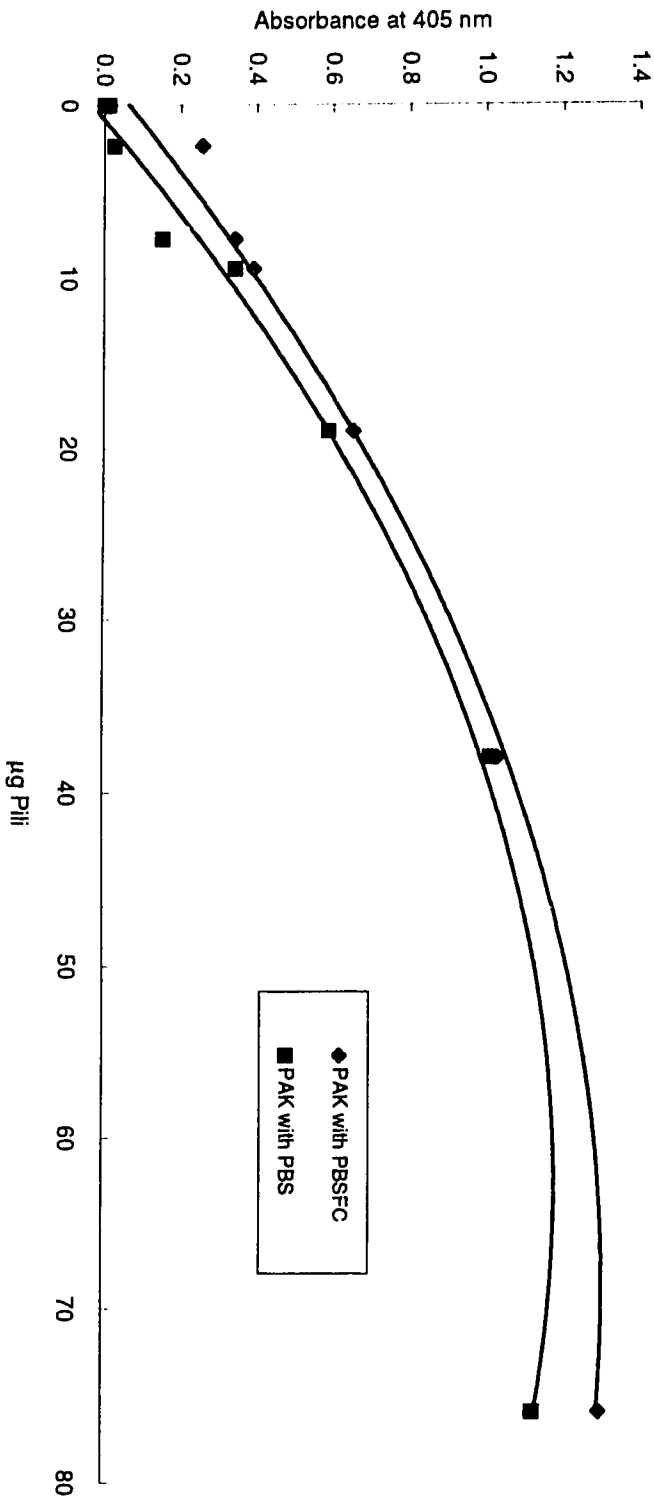

A
B
C
D

Figure IV.5. LD_{50} values in the A.BY/SnJ strain of mice of *P. aeruginosa* wild-type strains and the pilin-lacking background strain PAKp with plasmids expressing five pilin prototypes. Mice were injected in groups of 10 per concentration per bacterial strain. LD_{50} values were obtained by plotting survivors versus bacterial concentration.



LD_{50} Values in Mice for Wild Type *P. Aeruginosa* Strains and *P. aeruginosa* PAK Containing Plasmids

Figure IV.6. The effect of supplements on the binding of PAK pili to A549 cells. The absorbance at 405 nm obtained in the ELISA-like attachment assay was plotted against the amount of pili added. ■ PAK pili binding with PBS alone, ◆ PAK pili binding with PBS supplemented with 1% fetal calf serum and 0.25 mM $CaCl_2$.



Absorbance of PAK with PBSFC and PBS

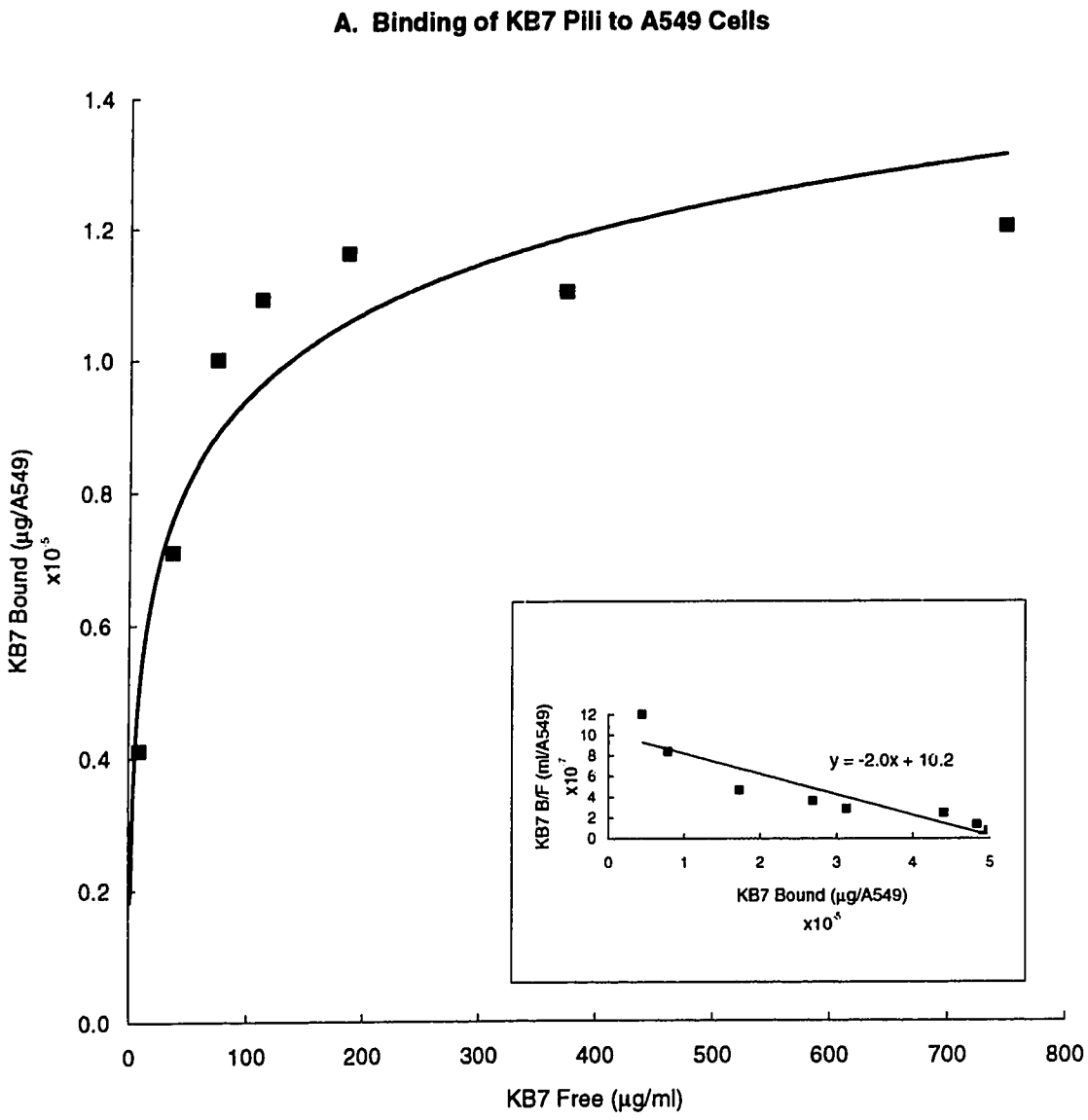
Figure IV.7. Binding of the pili prototypes to A549 human lung pneumocytes using the ELISA-like attachment assay. The large plot is bound versus free pili (pili not bound to A549 cells) and the insets are the Scatchard plots used to calculate the K_a and the average amount (N) of pili bound per A549 cell at saturation. The free pili, in this case, was the quantity of pili added to the well as the amount bound was very small and its absence from the added aliquot could not be measured. The y-intercept represents N/K_D and the slope represents $-1/K_D$. The K_a was calculated using the relationship $K_a = 1/K_D$ and the N value was calculated form the y-intercept using the calculated K_D value.

KB7 pili (A)

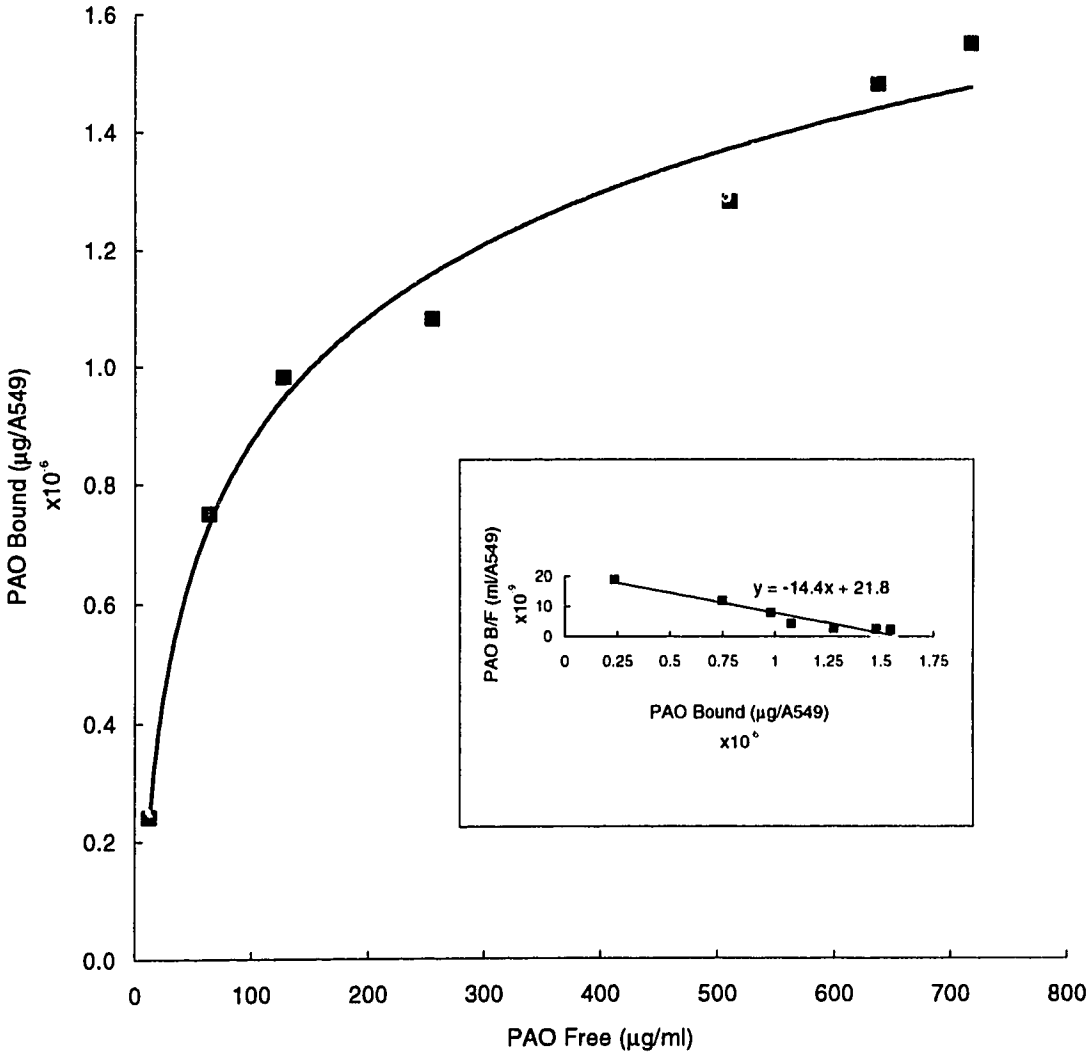
PAO pili (B)

PAK pili (C)

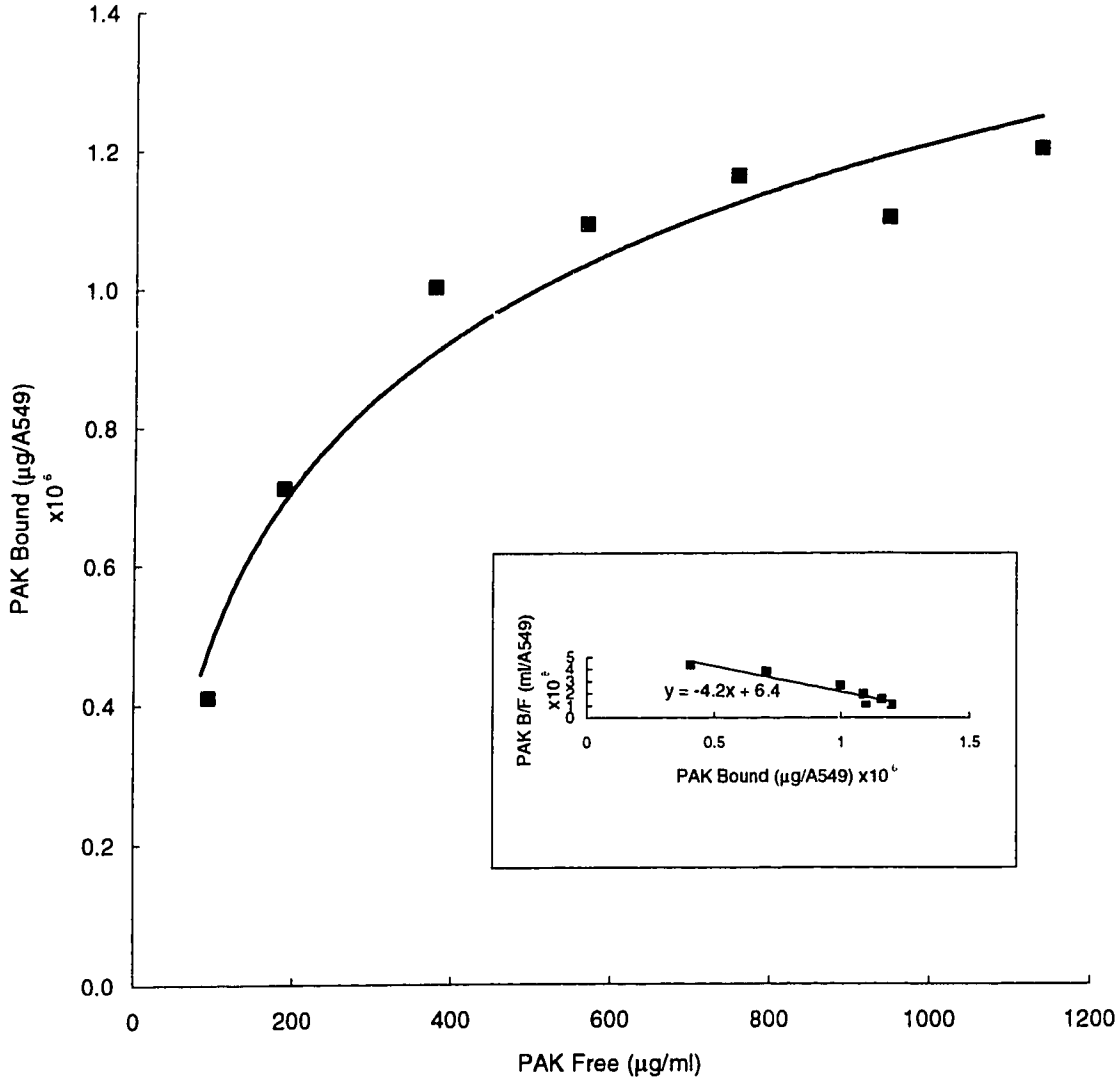
K122 pili (D)


A. Binding of KB7 Pili to A549 Cells
KB7 Bound (µg/A549)
x10⁻⁵
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
700
800
KB7 Free (µg/ml)
KB7 B/F (ml/A549)
x10⁻⁷
12
10
8
6
4
2
0
0
1
2
3
4
5
y = -2.0x + 10.2
KB7 Bound (µg/A549)
x10⁻⁵

B. Binding of PAO Pili to A549 Cells



C. Binding of PAK Pili to A549 Cells



D. Binding of K122 Pili to A549 Cells

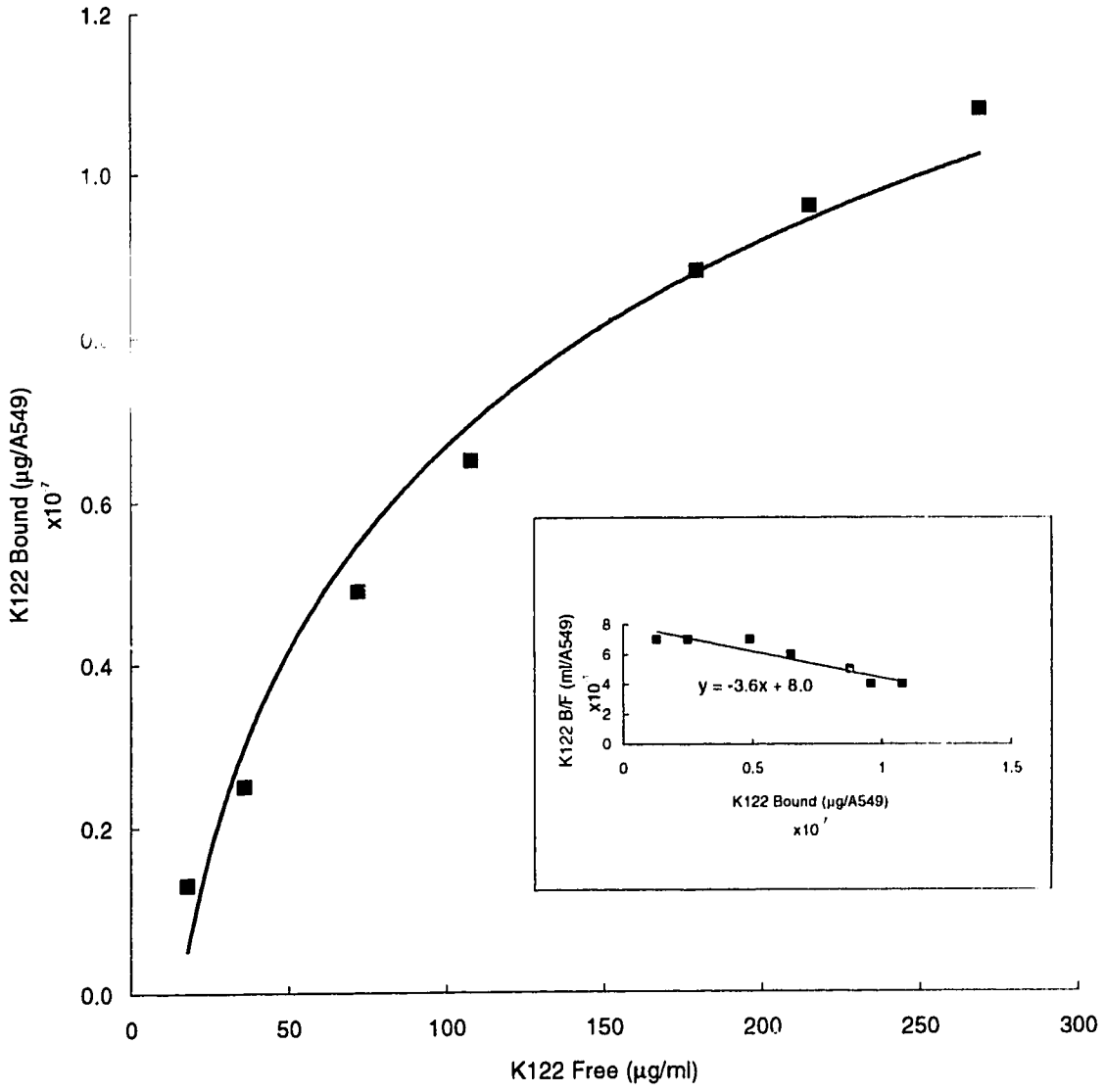
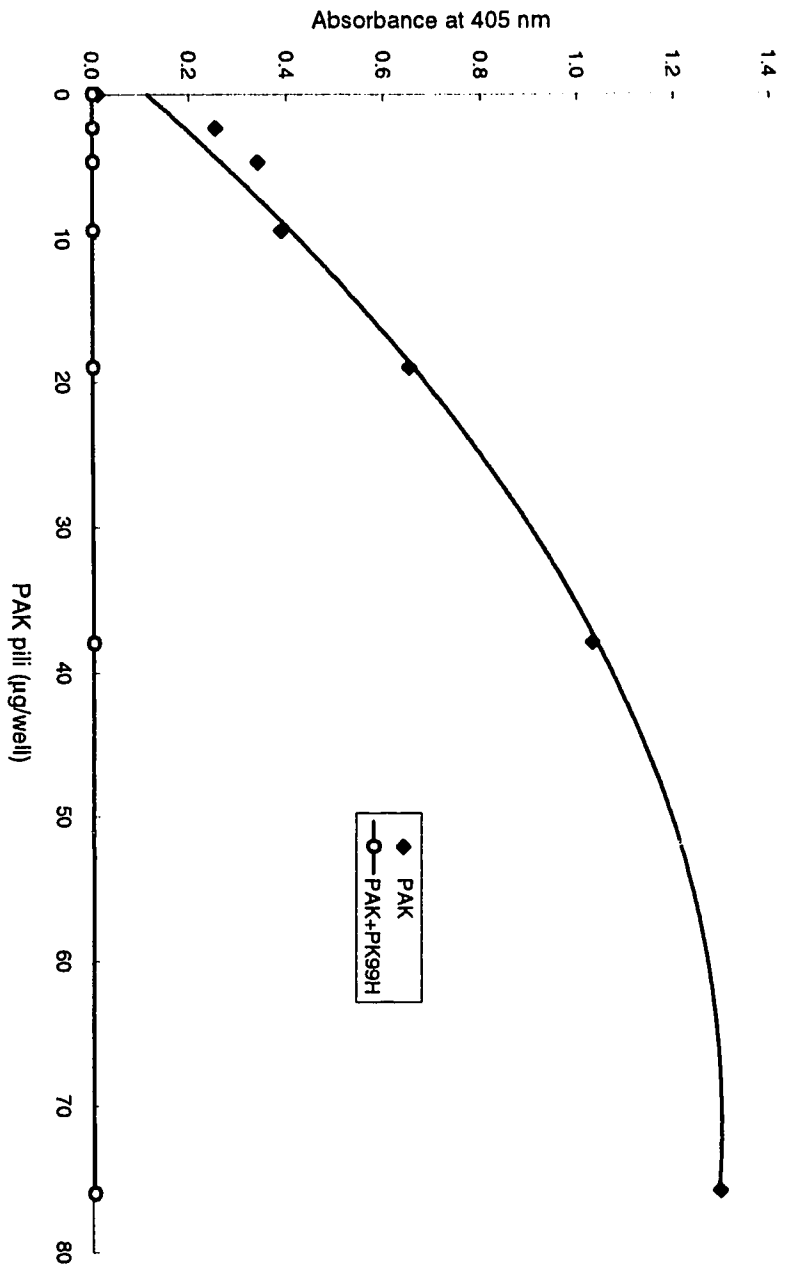


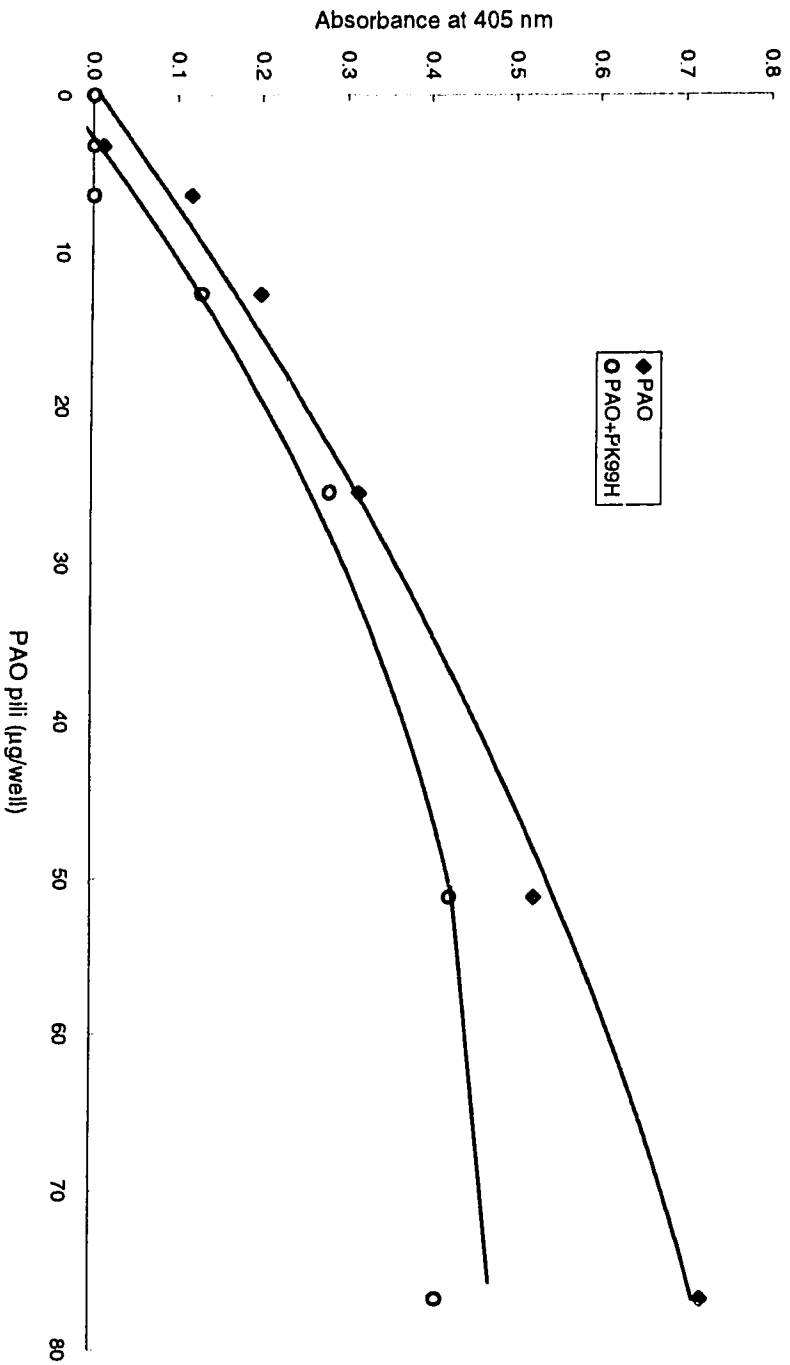
Figure IV.8. Inhibition of binding assay of PAK and PAO pili to A549 cells using the monclonal antibody PK99H. PK99H binds specifically to the sequence DEQFIPK in the C-terminal region of PAK (Wong *et al.*, 1992).

PAK pili (A)

PAO pili (B)



A. Effect of PK99H on Binding of PAK Pili to A549 Cells.

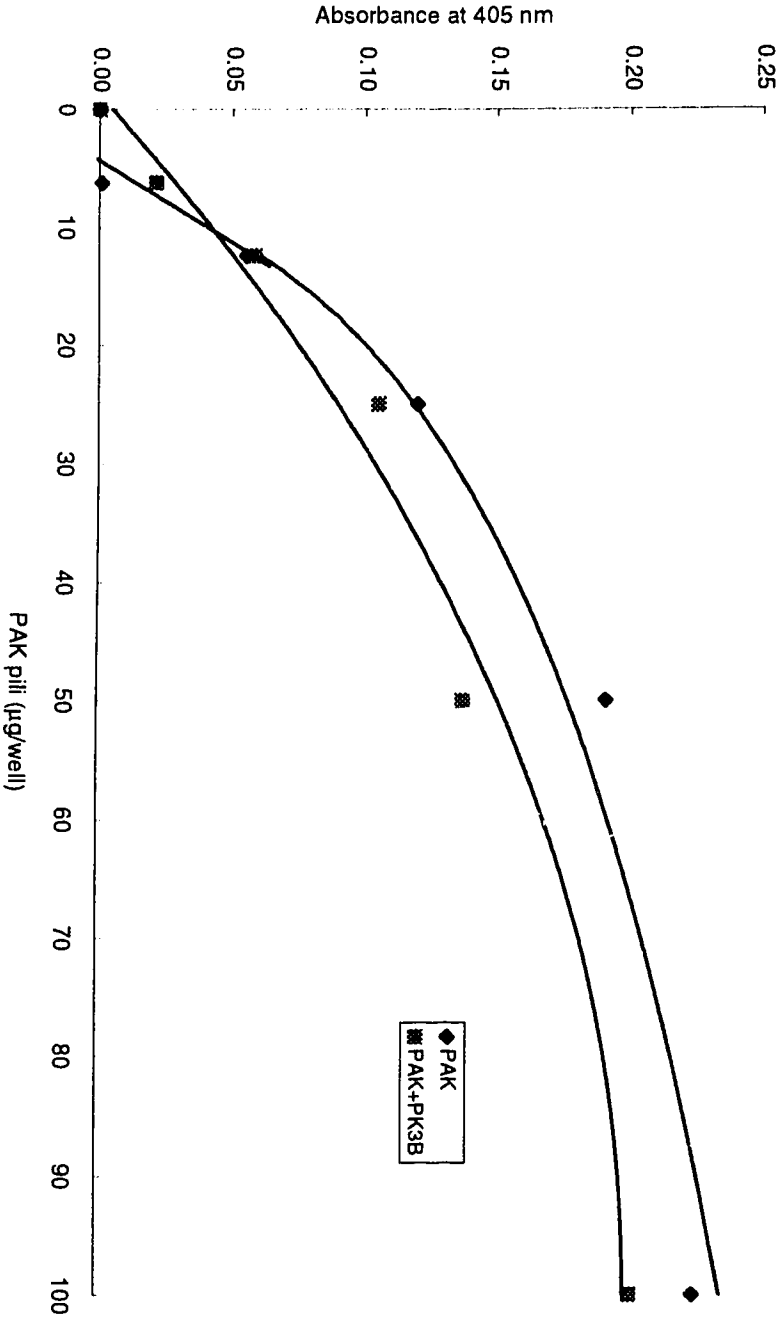


B. Effect of PK99H on Binding of PAO Pili to A549 Cells.

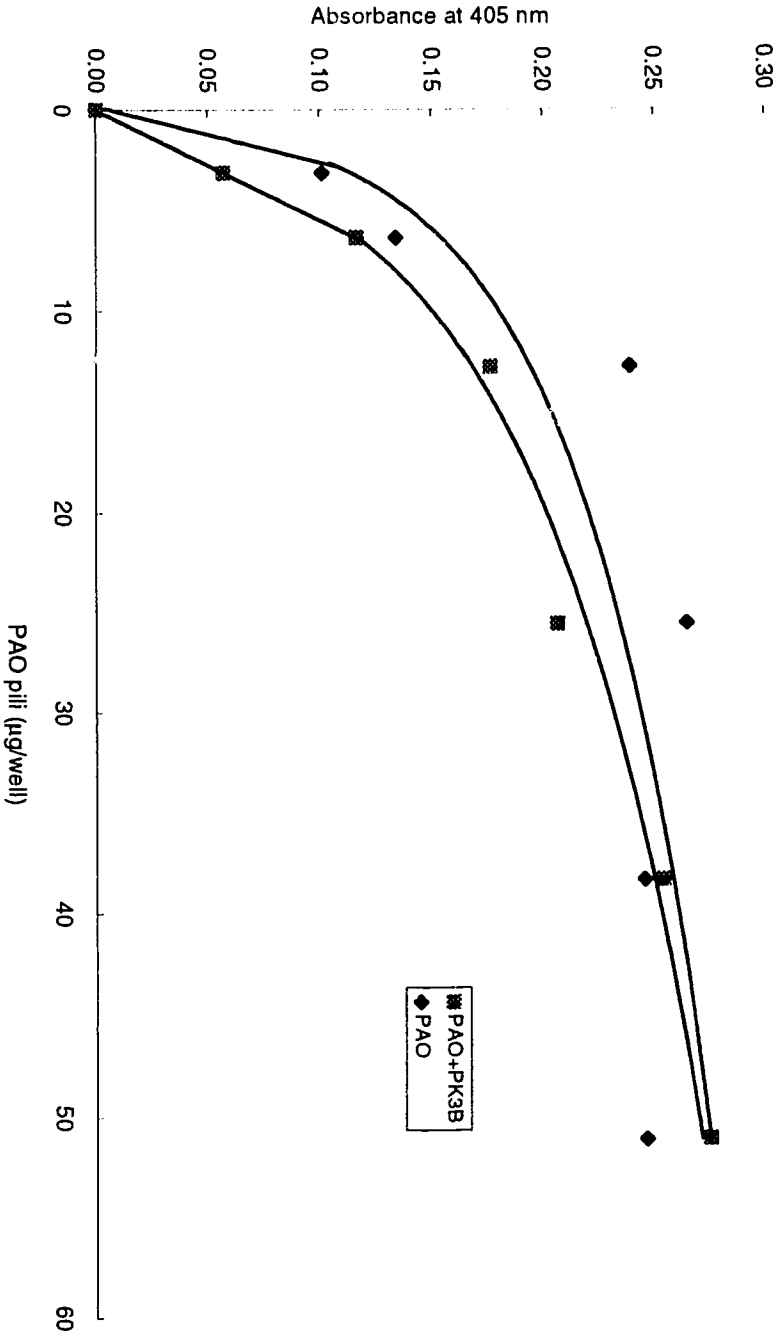
Figure IV.9. Inhibition assay of the binding of PAK and PAO to A549 cells using the monoclonal antibody PK3B. PK3B binds to the basal end of *P. aeruginosa* pili (Lee *et al.*, 1994).

PAK pili (A)

PAO pili (B)



A. Effect of PK3B on Binding of PAK Pili to A549 Cells.



B. Effect of PK3B on Binding of PAO Pili to A549 Cells.

Figure IV.10. TLC overlay assay of pili prototypes to purified glycolipids. Panel A is KB7 pili, panel B is K122 pili, panel C is the sulphuric acid char of the glycolipids, panel D is PAO pili, and panel E is PAK pili.

a1 = asialoGM1	a2 = asialoGM2
m1 = monosialoGM1	m2 = monosialoGM2
Lact = Lactosylceramide	PE=phosphatidylethanolamine
PG = phosphatidylglycerol	PC = phosphatidylcholine

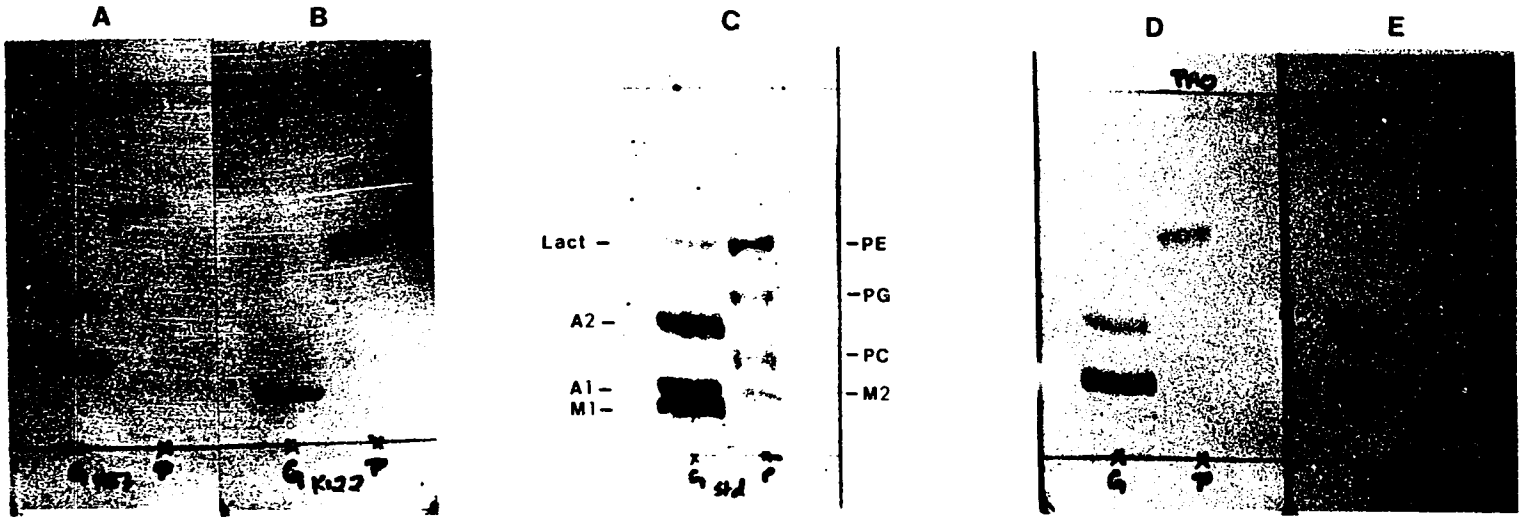

A
B
C
D
E
Lact –
A2 –
A1 –
M1 –
–PE
–PG
–PC
–M2

Figure IV.11. Amino acid sequences of The C-terminal disulphide loop regions of *P. aeruginosa* pilin prototypes. The bold residues are absolutely conserved. The boxed residues were found to be important for maintaining the disulphide bridge and the type II β-turn (Wong *et al.*, submitted). The ^ indicate residues that are semi-conserved, and the * indicate residues that were found to be important for PAK pili binding.

Amino Acid Sequences of C-terminal Regions of *P.aeruginosa* pilin types.

STRAIN	SEQUENCE															
	128			^				*	*	^	*		^	^		144
PAK	K	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	S	K
PAO	A	C	K	S	T	Q	D	P	M	F	T	P	K	G	C	D	N
K122	A	C	T	S	N	A	D	N	K	Y	L	P	K	T	C	Q	T
KB7	S	C	A	T	T	V	D	A	K	F	R	P	N	G	C	T	D

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CHAPTER V ANALYSIS OF AMINO ACID SUBSTITUTIONS IN THE C-TERMINAL DISULPHIDE LOOP OF *PSEUDOMONAS AERUGINOSA* PILIN

INTRODUCTION

Adherence to the host epithelial cell surface is considered a significant initial step in the process of infection. The pili of *Pseudomonas aeruginosa* have been shown to bind directly to many cell types (Chi *et al.*, 1991; Doig *et al.*, 1988; McEachran and Irvin, 1985; Zoutman *et al.*, 1991). Previous studies with monoclonal antibodies and synthetic peptides have shown that the adherence domain is located within the C-terminal disulphide loop region, and that this region is exposed only at the pilus tip (Lee *et al.*,1994). Pili have been shown to bind to the glycosphingolipids asialoGM1 and asialoGM2 using thin layer chromatography techniques. There are significant amounts of asialoGM1 present in human lung tissue, therefore it is possible that it is a cell surface receptor for pili. Four pili prototypes were shown to have different binding efficiencies to A549 human lung pneumocytes, and some variability in the affinity for the lung cells (chapter IV). The conformation of glycolipids was found to be more restricted in cell membranes than on artificial surfaces such as TLC plates (Stromberg *et al.*, 1991). The C-terminal region is semi-conserved amongst pilin prototypes, but residues shown to be important for binding to A549 cells in peptide studies were either semi or non-conserved (Wong *et al.*, submitted). The different pili types may not interact with the same specific epitope on the receptor, therefore the region responsible for adherence need not be conserved. The orientation of the receptor in the membrane may either mask or expose the specific epitope yielding differences in the avidity of each pili type for its epitope.

In this study, seven site-specific mutants were generated in which both conserved and non-conserved amino acids from the PAK disulphide loop were

substituted with those present in the PAO disulphide loop. There are three possible options to explain binding efficiency to A549 cells. First, the binding efficiency to A549 cells of the PAK pili harbouring the point mutations would by expected to increase if the individual amino acid is highly significant for PAO binding. Secondly, the binding efficiency may decrease if the residue is important for PAK pili binding and additional compensation mutations are necessary to exhibit the more efficient PAO pili binding phenotype. The third option is that the point mutation will have no effect on the pili binding because it may not be involved in adherence. An additional mutant was created with PAK pilin containing the entire PAO pilin disulphide loop sequence. This exchange mutant would be expected to exhibit increased binding efficiency to A549 cells if the disulphide loop is solely responsible for epithelial cell adherence. The binding properties of the mutants were assessed using the thin layer overlay assay and A549 cell attachment assay.

MATERIALS AND METHODS

Media, Antibiotics, Bacterial strains, and Plasmids

Media and antibiotics were used as previously described (Macdonald *et al.*, 1993; chapter II). The bacterial strains and plasmids were previously described (chapter IV). The phagemid vector pUC118 (Vieira and Messing, 1987) was used to generate the PAK/PAO exchange mutant.

Mutagenesis and Recombinant DNA Techniques

The nucleotide changes required to generate the five individual amino acid substitutions in the PAK pilin gene to those of PAO pilin are illustrated in Figure V.1. The two silent mutations introduced to generate unique restriction sites (*BsiWI* and *XhoI*) upstream and downstream of the disulphide loop region and the sequence of the oligonucleotides required for the exchange of the disulphide loop of PAK with that of PAO are shown in Figure V.1. The mutagenesis was performed according to the

method of Kunkel *et al.*, (1987). The oligonucleotides were purchased from the Department of Microbiology DNA Synthesis Laboratory, Edmonton, Alberta. RZ1032 (HfrKL16 PO/45 [*lysA* (61-62)] *dut 1, ung 1, thi 1, relA1* Zbd-279 :: Tn10, *supE44*) was the strain used to generate the uracil containing template DNA. The resultant plaques were screened by plaque hybridization (Sambrook *et al.*, 1989) using the mutagenic primer as the probe as outlined in chapter III, and verified by sequencing with the Sequenase kit (United States Biomedical Corporation, Cleveland, Ohio). The verified mutation was cloned into the *HindIII* site of pUCP18 as previously described (Macdonald *et al.*, 1993; chapter II). For the PAK/PAO exchange, the 1.2 kilobase fragment containing the two silent mutations was cloned into pUC118 and the presence of the PAK insert was verified by DNA sequencing. The two oligonucleotides (25 pmol/µl) containing the PAO disulphide loop sequence were heated to 95°C for 2 minutes and then allowed to anneal slowly over an eight hour period. The DNA was digested with *BsiWI* and *XhoI* and ligated with the annealed oligonucleotides at a ratio of 100 to 1 (Oligonucleotide to vector). The ligation was transformed into DH5α and the colonies were probed with a PAK C-terminal probe. DNA was made from 15 colonies that did not and 1 colony that did hybridize with the probe. Restriction endonuclease digestion and agarose gel electrophoresis indicated that the 1.2 kilobase *HindIII* fragment was present in 12 of the colonies that did not and the 1 colony that did hybridize with the probe. Southern blot analysis (Sambrook *et al.*, 1989) with a PAO C-terminal probe showed that 4 colonies hybridized with the PAO probe that did not hybridize with the PAK probe. DNA from these 4 colonies was sequenced and the 1.2 kilobase fragment was cloned into the vector pUCP18 and transformed into PAKp. All DNA manipulations were conducted according to the methods of Sambrook *et al.* (1989). Enzymes were supplied by Boehringer Mannheim (Laval, Quebec).

Pili Purification

The pili were purified as previously described (chapter IV). Purified pili were found to have the following concentrations as determined by the Lowry protein assay : PAK- 9.2 mg/ml, PAO- 1.28 mg/ml, T130K- 0.63 mg/ml, E135P- 1.21 mg/ml, Q136M- 1.9 mg/ml, F137L- 1.45 mg/ml, I138T- 0.60 mg/ml.

Bacteriophage sensitivity assay and Electron microscopy

The bacteriophage sensitivity assay and electron microscopy were conducted as outlined in Macdonald *et al.*, (1993; chapter II) with the noted changes. The grids were examined with a Philips Model 410 transmission electron microscope. The grids were examined with the assistance of Richard Sherburne in the Department of Medical Microbiology and Infectious Diseases.

Cell Culture and Immunoblots

The A549 human pneumocyte cell line was maintained and utilized as previously described in chapter IV. The immunoblot assays were conducted as outlined in the BIO-RAD Immuno-Blot Assay Kit Instruction Manual. Goat anti-rabbit and goat anti-mouse alkaline phosphatase conjugates were used.

Pili Binding to Human Lung Cells and Thin Layer Chromatography Overlays

A549 cell attachment assays were performed as outlined in chapter IV with the noted changes. PAK anti-pili antiserum was used to detect binding of all pili (wild-type and point mutants) to A549 cells. The TLC overlay assays were conducted as previously described in chapter IV.

RESULTS

Two types of site-specific mutants were generated in this study: one in which the five non-conserved amino acids in PAK pilin were changed individually to that in PAO, the second in which two conserved amino acids (F137 and P139) were substituted. A mutant in which the entire C-terminal disulphide loop of PAK was

substituted with the sequence of the C-terminal disulphide loop of PAO was engineered for this study. During the exchange of the PAO oligonucleotides for the PAK fragment 78 nucleotides were deleted upstream from the engineered *BsiWI* site. Several attempts were made to avoid the formation of this deletion by ligating the *BsiW1-HindIII* fragment containing the PAK C-terminal region with the PAO sequence, the *HindIII-BsiW1* fragment without the deletion from the M13mp18RF containing the PAK pilin gene containing the engineered *BsiW1* site, and *HindIII* digested vector. These efforts, however, resulted in the formation of either the same deletion or a much larger deletion.

Piliation of the mutant strains was assessed by pilus-specific phage sensitivity and electron microscopy. The phage sensitivity of the mutant strains is presented in Table V.1. Five of the site-specific mutant strains retained the PAK sensitivity pattern, the D132T mutant was sensitive to B9 only, and P139A and the PAK/PAO exchange mutants were resistant to all four phage. Pili were evident, by electron microscopy, on E135P, Q136M, I138T, T130K, and F137L mutant strains as seen in Figure V.2, and pili were purified from these constructs. P139A and the PAK/PAO exchange mutant were not piliated, which is in agreement with the resistance to all four pilus-specific phage. The pili on the D132T mutant were very short and present in such low numbers that purification was not possible.

The expression of pilin was examined by immunoblots of whole bacteria using anti-PAK antisera (Fig. V.3). A pilin band was present for all the mutants, although pilin was poorly expressed in the D132T and the P139A mutants. The PAK/PAO exchange mutant expressed a pilin band of 13 kDa, which corresponded to the molecular weight of 13088 Daltons calculated for the loss of 26 amino acids. Immunoblots using MAb PK99H, which is specific for the C-terminal sequence DEQFIPK of PAK, detected pilin bands in all mutants except D132T, P139A, and the PAK/PAO exchange mutant (Fig. V.3). No further work was possible with the

D132T, P139A, and PAK/PAO exchange mutants since pili could not be purified and the expression of pilin was very low.

A549 cell attachment assays were performed with pili from five of the point mutations to assess the effect of the amino acid changes on binding of PAK pili to A549 cells. The binding of T130K and F137L pili to A549 cells was not significantly different from the binding of PAK wild-type pili (Fig. V.4). There was a significant reduction in the binding of E135P, Q136M, and I138T pili to A549 cells compared to the PAK wild-type pili binding (Fig. V.4). It is interesting to note that these three residues are non-conserved amongst *P. aeruginosa* pilins.

TLC overlay assays were conducted to determine the effects of the point mutations on purified putative glycolipid receptor binding. TLC overlays (Fig. V.5, Table V.2) showed that asialoGM1 was bound by all the pili, but was bound less well by Q136M and extremely weakly by E135P. AsialoGM2 was bound by all pili except E135P, but was bound less well by I138T and Q136M. Thus, the three point mutants that exhibited reduced adherence to A549 cells (E135P, Q136M, I138T) also had altered binding to asialoGM1 and asialoGM2.

DISCUSSION

A series of site-specific point mutations in the C-terminal disulphide loop of PAK pilin were created to examine the role of individual amino acids in adhesion. Five mutations in which the amino acids present in the PAK prototype were changed to the amino acids present in the PAO prototype were constructed to determine if the mutated pili would yield the increased binding efficiency to A549 cells exhibited by PAO pili. Mutations in two conserved residues (F137 and P139) were constructed to assess their contribution to A549 cell and purified glycolipid binding. An exchange mutant of the PAO C-terminal disulphide loop for that of PAK was designed to determine if the disulphide loop was solely responsible for adherence and receptor

binding. Pili were purified from all but three mutant strains (D132T, P139A, and PAK/PAO exchange). P139A did not assemble any pili and D132T had such short and infrequent pili that they could not be purified. No pili were assembled by the PAK/PAO exchange mutant because of a 26 amino acid deletion that occurred from position 91 to position 117 in the central variable region of pilin. This suggested that the central variable region is necessary for pilus assembly. Five of the seven point mutants maintained the PAK phage sensitivity pattern which indicated that T130, E135, Q136, F137, and I138 were not critical residues involved in phage attachment. D132T was only sensitive to B9 phage, which implied that the only functional phage receptor present on the very short pili was for B9. This unusual phenotype may be the result of a defect in the pilus elongation process caused by the presence of the threonine residue in place of aspartate. The pilus was assembled incorrectly or the elongation process was short circuited resulting in the short pili with only the B9 receptor. Alternatively, the aspartate may be part of the receptor for the other three phage. This is less likely since PO4 and F116 are side binders and pf1 is a tip binder. A unique mutant in the *pilG* gene that was incapable of forming pili was still sensitive to pilus-specific phages B3 and F116L, but not D3112 (Darzins, 1993). It was suggested that the mutation caused a defect in the pilus elongation process such that only a preliminary structure consisting of an exposed pilus tip was formed thus restricting the access to certain phage (Darzins, 1993). Previous studies with MAb PK99H found that the epitope, which is within the C-terminal disulphide loop of PAK was exposed at the pilus tip only (Lee *et al.*, 1994). The D132T mutant is located two residues from the epitope of MAb PK99H which encompasses residues 134 to 140. Since the C-terminal region is not exposed on the lateral surface of the pilus, residues in this region may participate in subunit-subunit interactions in the assembly of pili. D132 may have a role in the subunit-subunit interactions and the change to threonine caused the assembly process to malfunction resulting in very short pili.

Immunoblots showed pilin bands of apparently the same molecular weight as PAK pilin for five mutants (T130K, E135P, Q136M, F137L, and I138T) which corresponded to their maintained PAK phage sensitivity pattern. The D132T and P139A mutants synthesized pilin at very low levels as seen by the immunoblot with PAK antiserum. The infrequent pili produced by the D132T mutant could have been the result of the very low level of pilin expression. The level of pilin expresion was greatly affected by the P139A mutation and pili were not assembled in this strain either. MAb PK99H did not react with the pilin from either D132T or P139A mutant strains. The P139A mutant is within the MAb PK99H epitope (DEQFI*P*K) whereas the D132T mutant is outside the epitope and was not expected to react with MAb PK99H. It was shown previously that the PAK C-terminal peptide with this same Pro to Ala substitution had decreased binding affinity for MAb PK99H (Wong *et al.*, 1992) in agreement with the immunoblot data presented here. The four other point mutations of residues within the epitope of MAb PK99H (D***EQFI***PK) all reacted with MAb PK99H. Substitutions in the PAK C-termninal peptide of Q136A and E135A were previously shown to have a small decrease in the affinity for MAb PK99H, but F137A and I138A substitutions had large decreases in their affinity for MAb PK99H (Wong *et al.*, 1992). A leucine substitution for phenylalanine is somewhat more conservative than alanine substitution. On a scale of amino acid similarity where 9 is identical and 0 is unrecognized, Phe to Leu rates a 7 and Phe to Ala rates 6 (Bacon and Anderson, 1986). On the same scale, Ile to Thr rates a 3 and Ile to Ala rates 4, therefore, by the indications of the amino acid similarity Ile to Thr is somewhat less conservative than Ile to Ala. The context of the entire pilin protein as opposed to a peptide may lessen the effect of a single amino acid substitution on recognition by MAb PK99H because of the presence of or interaction with neighbouring residues in the structure.

Attachment assays revealed that some substitutions affected the binding of the pili to A549 cells even though the phage sensitivity and pili expression appeared to be unaffected. The attachment of pili was not affected by the T130K and F137L mutations. T130 and F137 are likely not involved in pili binding to A549 cells as alanine substitutions of these residues in the PAK C-terminal peptide did not affect peptide binding to A549 cells either (Wong *et al.*, submitted). Superposition of the NMR structures of the C-terminal peptides of KB7 and PAK revealed that the F137 side chain was facing in different directions in the two structures (McInnes *et al.*, 1993), another indication that F137 may not be involved in adherence.

The reduction in binding of the I138T and Q136M to A549 cells was similarly observed in the binding of PAK C-terminal peptides with alanine substitutions in these same two positions (Wong *et al.*, submitted). The I138-P139 bond was observed to be the location of the *cis-trans* isomerization in the PAK C-terminal peptide which may be important for binding (McInnes *et al.*, in press). Alanine substitutions of I138 and P139 in the PAK peptide as well as R138 in the KB7 C-terminal peptide caused a loss of binding affinity for A549 cells (Wong *et al.*, submitted). A549 cell binding studies with I138T pili indicated that I138 is likely involved in PAK pili binding, however, it could not be determined whether the isomerization of the I138-P139 bond is significant. The fact that R138 was shown to be important for binding of the KB7 C-terminal peptide creates the possibility that the binding domain for A549 cells may be defined by position rather than residue. Substitution of Q136 with alanine in the PAK C-terminal peptide caused a reduction in peptide binding to A549 cells (Wong *et al.*, submitted) much like that of Q136M mutant pili. Q136 occupied the *i*+2 position of the type I β-turn in the PAK peptide and was predicted to tolerate an alanine substitution, but in reality it did not (Wong *et al.*, submitted), suggesting that it functions in a capacity other than the β-turn structure. The function of Q136 in binding

may utilize its hydrogen bonding properties and replacement with either alanine or methionine may disrupt the hydrogen bonding.

An alanine substitution of E135 in the PAK C-terminal peptide did not display reduced A549 cell adherence (Wong *et al.*, submitted) as did the E135P mutant pili. E135 occupied the *i*+1 position of the type I β-turn in the NMR structure of the PAK C-terminal peptide (McInnes *et al.*, 1993) and the alanine substitution may disrupt the structure necessary for binding. The E135A substitution in the PAK peptide, however, had only a small effect on its binding to A549 cells (Wong *et al.*, submitted). The E135P mutation removed a negative charge from the C-terminal disulphide loop which may perform a direct or indirect function in binding. The reduction in binding capability of the E135P mutant is possibly a manifestation of either a disruption of the type I β-turn in pilin structure or the removal of a negative charge.

TLC overlay assays revealed alterations in the receptor binding ability of some of the point mutations in PAK pili. The receptor binding ability of T130K and F137L pili were not significantly different from PAK wild-type, which is consistent with their unaffected binding to A549 cells. The three mutants found to bind less well to A549 cells (E135P, Q136M, andI138T) displayed diminished binding to either asialoGM1, asialoGM2 or both. The residues important for binding of PAK pili to A549 cells and to asialoGM1 and asialoGM2 appeared to be the E135, Q136, and I138. These three amino acids are non-conserved amongst pilin prototypes suggesting that the different pilin types do not bind to the receptor in the same fashion. There may be specific epitopes on the receptor for each pili type which interact with the non-conserved residues. Receptors were shown to be in a more restricted conformation in their natural membrane environment (Stromberg *et al.*, 1991). The accessibility of the specific epitopes in the membrane may determine the efficacy with which the pili binds to the receptor.

The binding characteristics of the point mutations in PAK pili to A549 cells did not resemble those of PAO pili as would be expected if the individual residues significantly contributed to the increased affinity of PAO for A549 cells. The reduction in A549 cell binding and altered glycolipid binding of E135, Q136 and I138 did, however, reveal that these residues play an important role in PAK pili adherence to A549 cells and to the putative receptors asialoGM1 and asialoGM2. The greater affinity of PAO pili for A549 cells may involve compensation mutations in all three or perhaps in all five positions that differ between the two prototypes. The residues that were shown to be important for PAK pili adherence (E135, Q136, I138) are all non-conserved in pilin prototypes. Q136 and I138 were also found in the adhesintope mapped by Wong *et al.* submitted. These results support the idea that each pili type may not interact with the receptors in the same fashion. The variation present in the adherence domain may be the result of the presence of specific epitopes for each pili type on the cell surface receptor. There appears to be common putative receptors, asialoGM1 and asilaoGM2, for *P. aeruginosa* pili. The adherence domain, however, appears to be composed of non-conserved residues in the C-terminal disulphide loop of pilin.

Table V.1. Phage sensitivity of eight pilin prototype mutants
The strains are defined in Fig V.1

Construct	PO4	pf1	f116	B9	pili by TEM
T130K	S	S	S	S	+
D132T	R	R	R	S	+/-
E135P	S	S	S	S	+
Q136M	S	S	S	S	+
F137L	S	S	S	S	+
I138T	S	S	S	S	+
P139A	R	R	R	R	-
PAK/PAO	R	R	R	R	-
PAKwt	S	S	S	S	+

Table V.2. Binding of purified pili to phospholipids and glycolipids.

pili	a1	a2	m1	m2	Lact	PE	PC	PG
T130K	+++	+++	-	-	-	NS	-	-
E135P	+	-	-	-	-	NS	-	-
Q136M	++	+	-	-	-	NS	-	-
F137L	+++	+++	-	-	-	NS	-	-
I138T	+++	+	-	-	-	NS	-	-

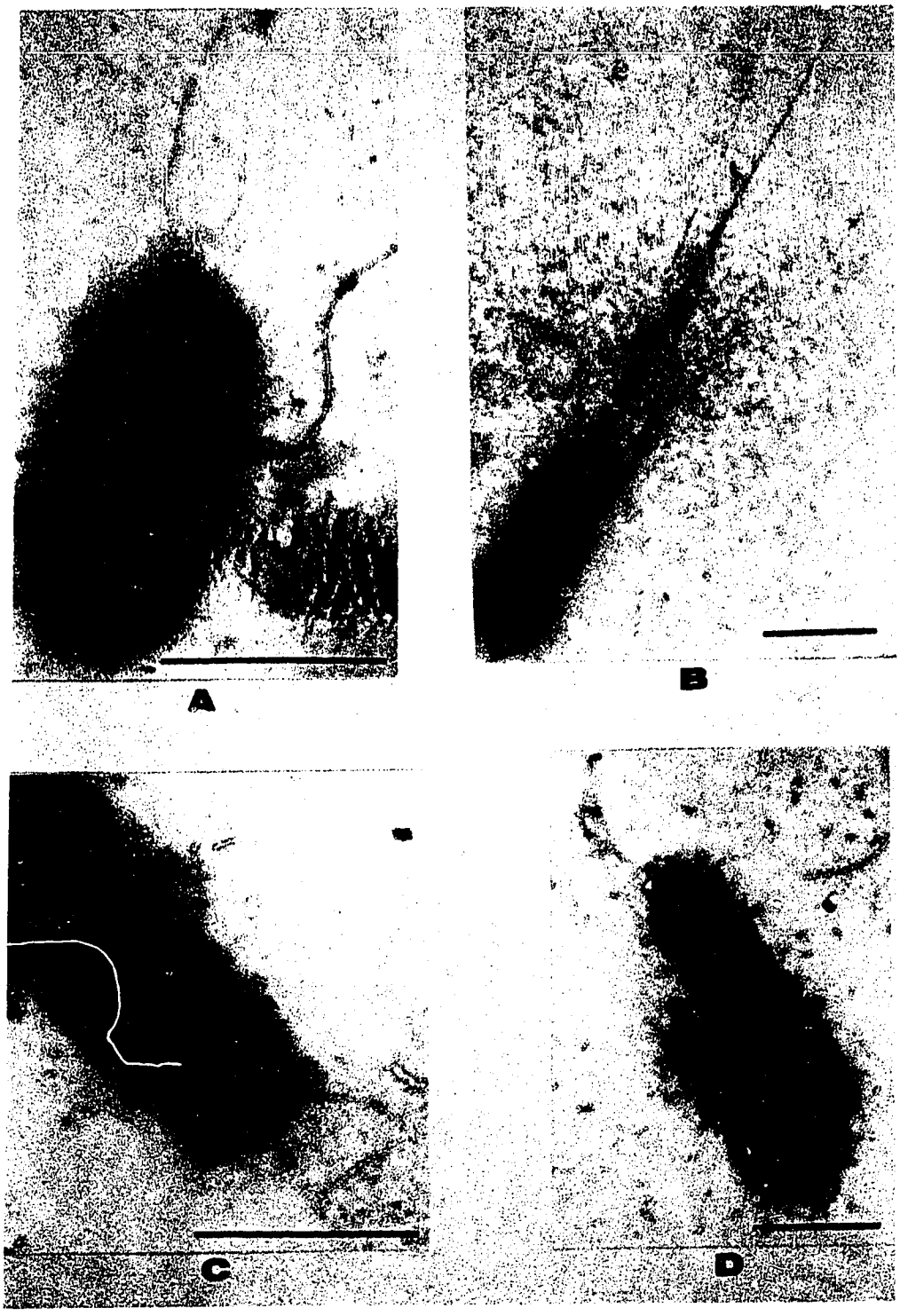
Note: NS indicates non-specific binding and the strength of binding is indicated by the number of + signs. The - indicates no binding.

a1 = asialoGM1 a2 = asialoGM2
m1 = monosialoGM1 m2 = monosialoGM2
Lact = Lactosylceramide PE=phosphatidylethanolamine
PG = phosphatidylglycerol PC = phosphatidylcholine

Figure V.1. The C-terminal region of the PAK/PAO exchange mutant. The two engineered restriction sites are labelled and their cut sites are marked on the sequence. The sequences of the two oligonucleotides used for the exchange are shown in bold. The nucleotide changes required for the point mutations are noted above the sequence affected and the amino acid changes are noted below the sequence.

BsiWI
Xho I
Arg Thr Ala Ala Asp Gly Leu Trp Lys Gly Cys Ser Arg End
Thr 130
Asp 132
Glu 135
Gln 136
Leu 137
Ile 138
Ala 139
Met Phe Thr Pro Lys Gly Cys Ser Arg
TTTT

Figure V.2. Electron micrographs of the background strain PAKp containing plasmids expressing the PAK pilin gene with point mutations in the disulphide loop region. Pili are the fine straight filments extending from the poles of the bacteria and flagella are the thicker wavy filaments. The bar represents 1 μm. E135P (A), I138T (B), T130K (C), Q136M (D), F137L (E), D132T (F).


A
B
C
D

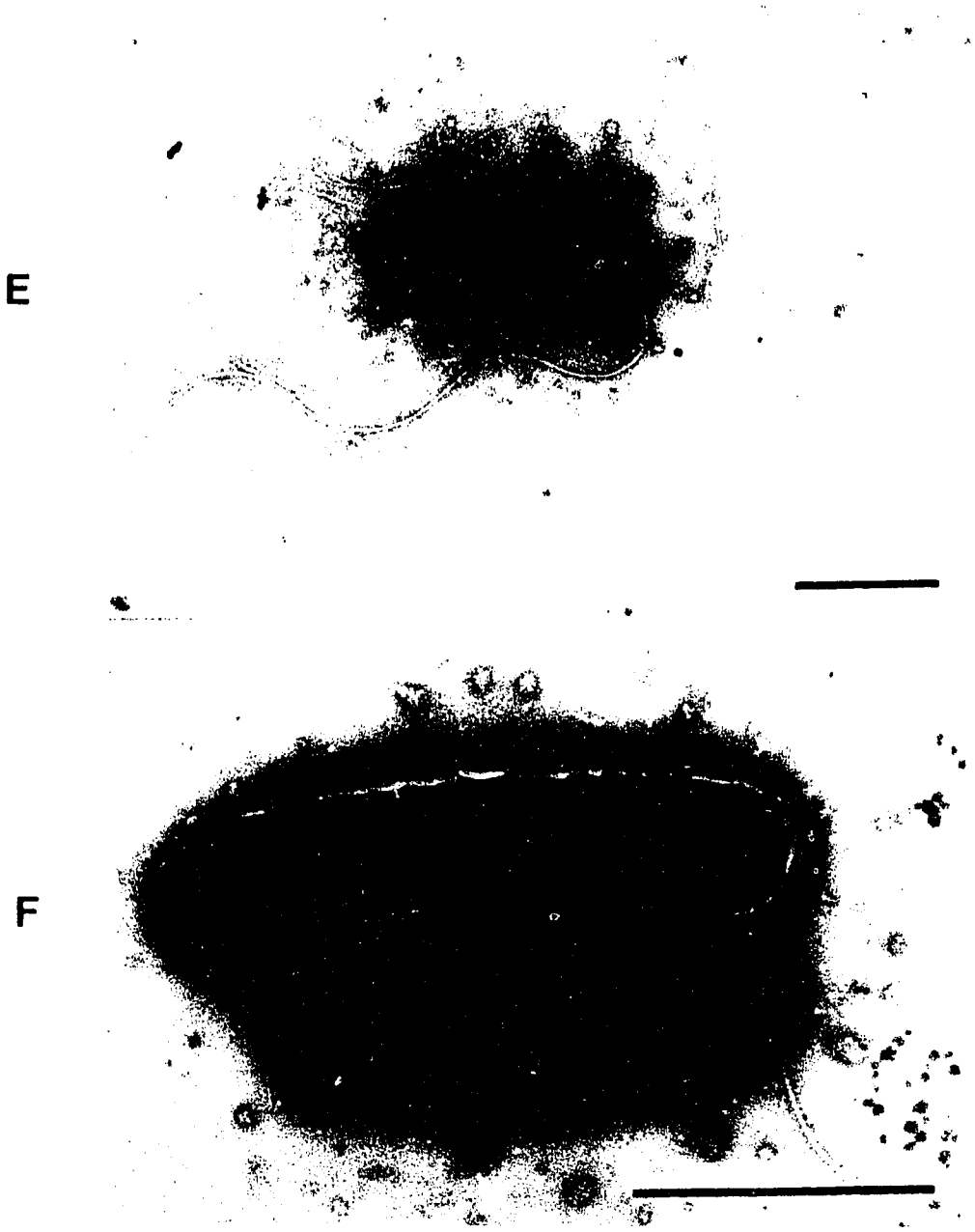

E
F

Figure V.3. SDS-PAGE gel stained with coomassie blue (A) and immunoblots (B, C) of whole bacterial cells of PAKp containing plasmids expressing PAK wild-type and mutant pilin genes. (B) anti-PAK pilin antisera was used to detect the presence of pilin in B. (C) immunoblot using the monoclonal antibody PK99H which is specific for the sequence DEQFIPK of PAK. The molecular weight standards are marked on the left hand side, the PAK wild-type strain was used as a standard.

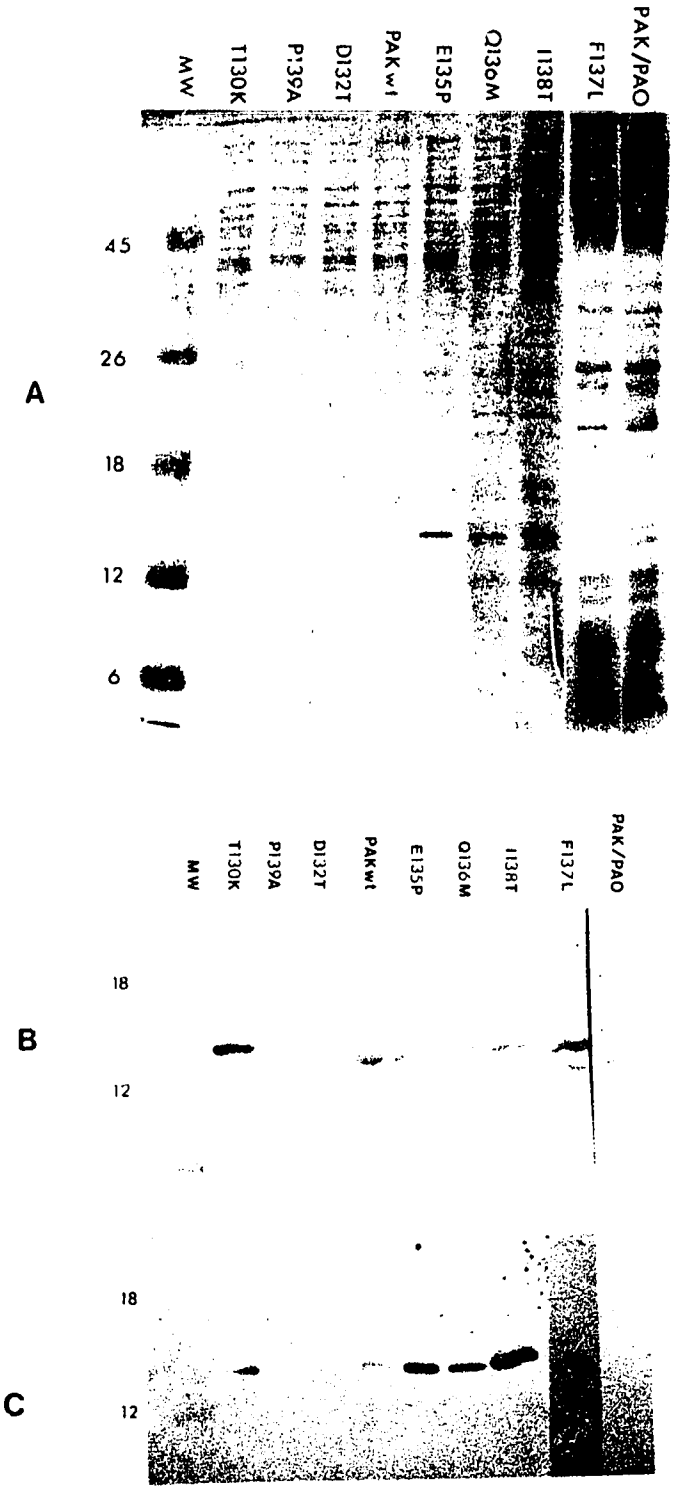
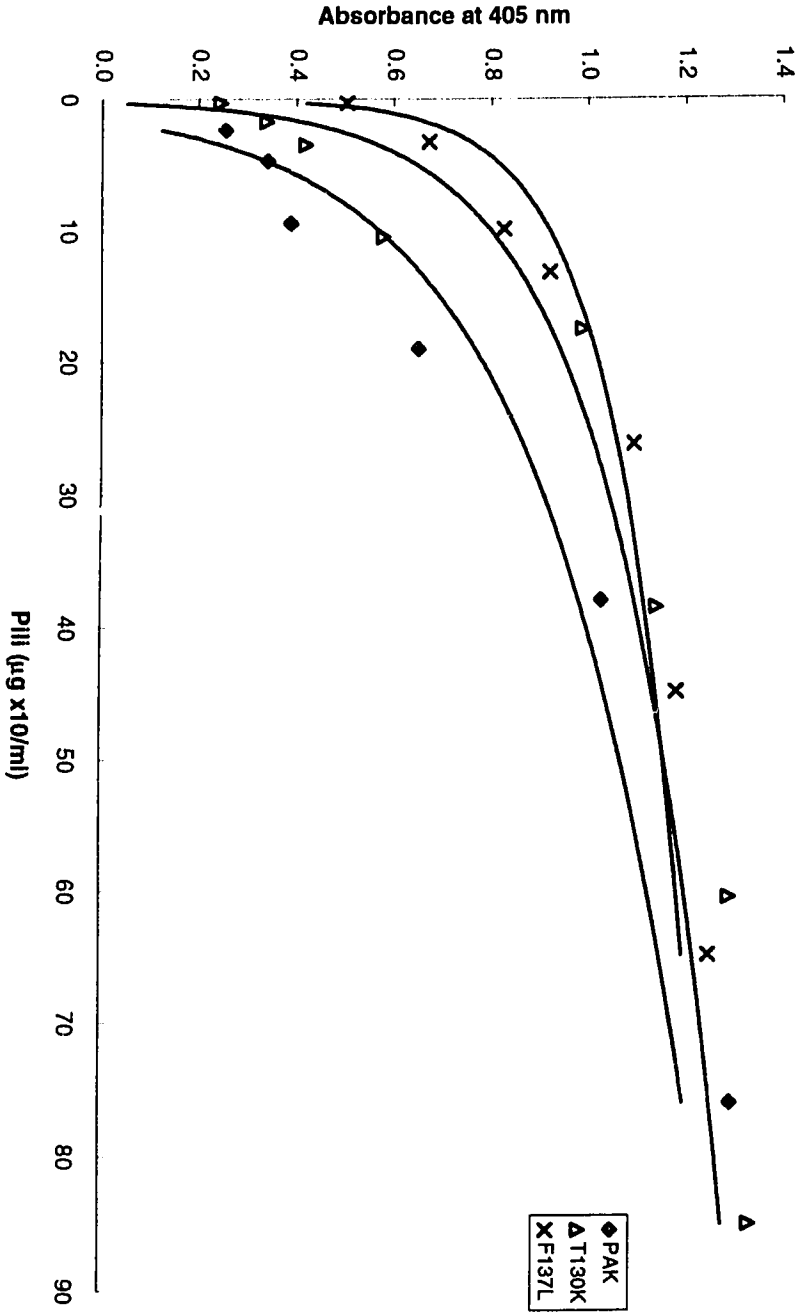
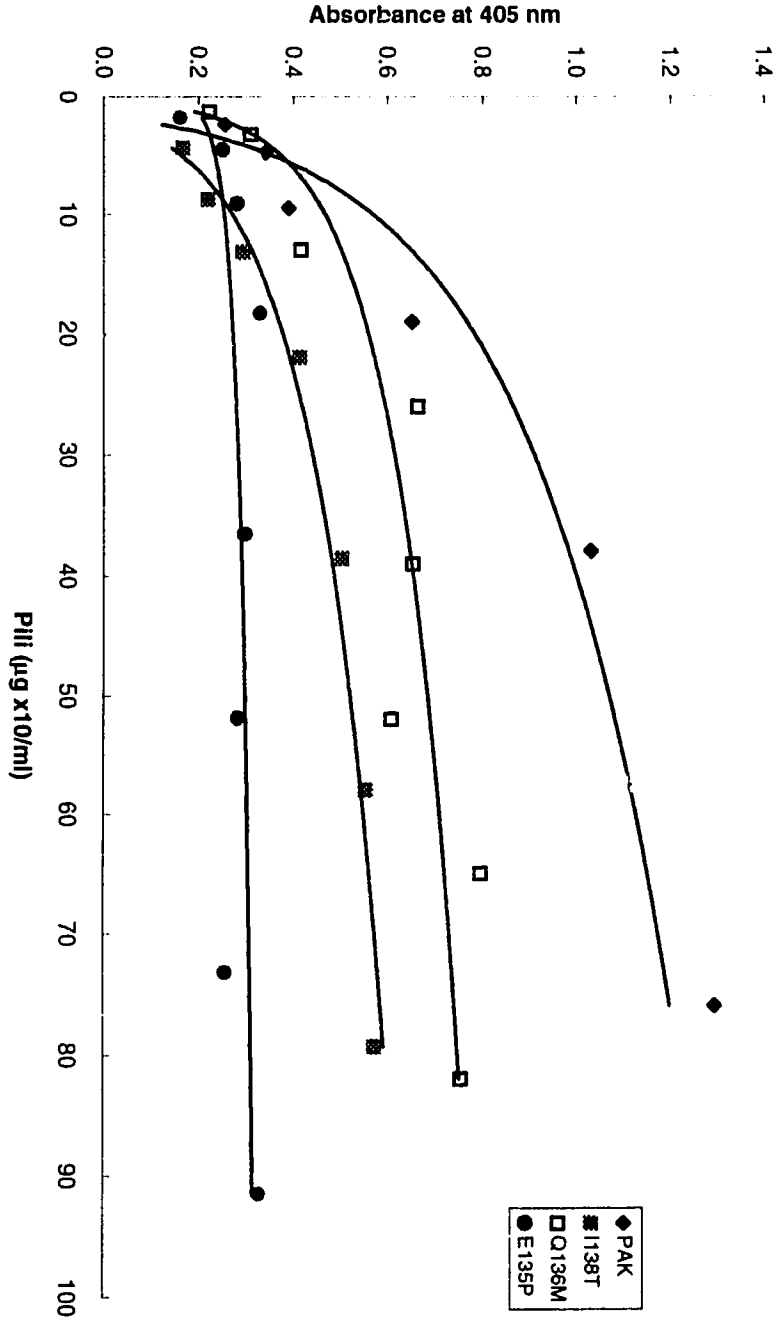

A
MW
T130K
P139A
D132T
PAKwt
E135P
Q136M
I138T
F137L
PAK/PAO
45
26
18
12
6
B
18
12
C
18
12

Figure V.4. Binding of the pili with point mutations to A549 human lung pneumocytes using the ELISA-like attachment assay. The absorbance at 405 nm is plotted against the concentration of free pili. A is the binding of ◆ PAK pili, Δ T130K, and X F137L. B is the binding of ◆ PAK pili, ▒ I138T, [] Q136M, and ● E135P.



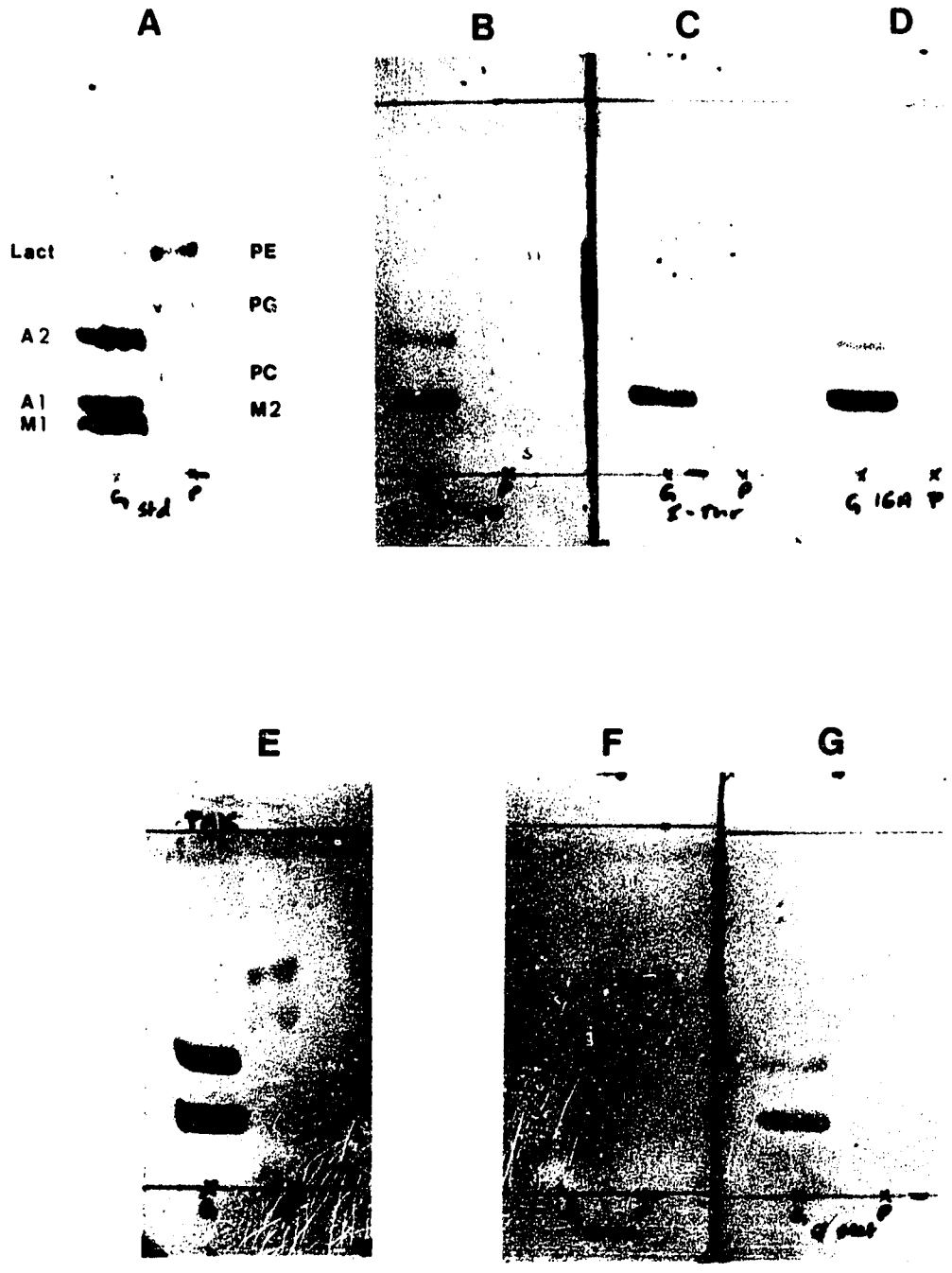
A. Binding of Pili with Point Mutations



B. Binding of Pili with Point Mutations

Figure V.5. TLC overlay assay of pili with point mutations to purified glycolipids. Panel A is T130K pili, panel B is I138T pili, panel C is F137L (16A) pili, panel D is the sulphuric acid char of the glycolipids and phospholipids, panel E is E135P, and panel F is Q136M.

a1 = asialoGM1	a2 = asialoGM2
m1 = monosialoGM1	m2 = monosialoGM2
Lact = Lactosylceramide	PE=phosphatidylethanolamine
PG = phosphatidylglycerol	PC = phosphatidylcholine


A
B
C
D
Lact
PE
PG
A2
PC
A1
M1
M2
E
F
G

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CHAPTER VI DISCUSSION

INTRODUCTION

Prior to the studies presented in this thesis, *P. aeruginosa* was known to produce extracellular filaments called pili from 15-kDa pilin subunits. *P. aeruginosa* pilins, of which there are at least eight unique but related types, belong to the type 4 family of pilins that share highly conserved hydrophobic N-termini (Paranchych *et al.*, 1990; Castric and Deal, 1994). Pilin precursors contain a six or seven amino acid leader which is cleaved by a unique leader peptidase, leaving the N-terminal Phe which becomes methylated. The Glu in the +5 position to the cleavage site appeared to be important for the transmethylation (Pasloske and Paranchych, 1988). Pili contribute to the virulence of *P. aeruginosa* by promoting adherence to epithelial cells (Paranchych *et al.*, 1986; Doig *et al.*, 1988).

Several different adhesins are available to *P. aeruginosa* to mediate attachment to the host cell surface: alginate (Ramphal and Pier, 1985), pili (Woods *et al.*, 1980), and more recently exoenzyme S (Baker *et al.*, 1991), and flagella (Saiman *et al.*, 1990). Purified whole pili and anti-pilus antiserum inhibited, but were not able to completely block adherence of *P. aeruginosa* bacteria to polymorphonuclear leukocytes and to human buccal epithelial cells (BEC's) (Paranchych *et al.*, 1986; Doig *et al.*, 1990). Adherence of a Pil$^-$ mutant was 80-90% less efficient than that of the isogenic Pil+ strain to A549 human lung pneumocyte cells (Chi *et al.*, 1991). The cause appeared to be very low levels of receptors (~6/cell) for the non-pilus adhesins as compared to ~74 receptors per cell for pili. The monoclonal antibody PK99H (specific for the PAK C-terminal disulphide loop) has provided immunoelectron microscopy evidence that the C-terminal disulphide loop region is exposed at the pilus tip and is responsible for receptor binding (Lee *et al.*, 1994). Recently, a mutant, PAO-MP, was engineered in which the C-terminal adherence domain was replaced with

non-specific amino acids (Farinha *et al.*, in press). There was a siginificant decrease in the binding of PAO-MP to A549 lung pneumocytes (4-12% of the binding of the wild-type PAO) and the LD_{50} in A.BY/SnJ mice was reduced to that of the Pil$^-$ strain and the RpoN$^-$ strain which lacks both pili, and non-pilus adhesins, and other RpoN dependent factors. The pilus adhesin accounted for 80-90% of the adherence to A549 cells, and is the only detectable adhesin employed in the mouse virulence assay. Other adhesins may have been at work in the mouse virulence assay, but there effects were not discernible. A Pil$^-$ and Fla$^-$ strain of PAO bound less well to bovine tracheal epithelial monolayers than a Pil$^-$ strain (Saiman *et al.*, 1990). There was no difference seen in the virulence of Pil$^-$ and RpoN$^-$ strains in the A.BY/SnJ mouse model (Farinha *et al.*, in press). Similarly, other studies have found piliated bacteria bound efficiently whereas nonpiliated bacteria bound poorly to mouse epidermal cells regardless of the state of flagellation (Sato and Okinaga, 1987). Flagella appear to be important for binding to monolayers, likely enabling the bacteria to swim to the fixed monolayer, but they do not improve the virulence *in vivo*. Pili are the major virulence-related adhesin. Understanding the molecular aspects of bacterial adhesion to host cell surfaces may provide alternate approaches to treatment and prevention of infection.

The process by which *P. aeruginosa* synthesizes and assembles pili, and implements them in the inititation of infection has yet to be unravelled. The study reported in this thesis examined both pili biogenesis and the function of pili in adherence to the epithelial cell surface. Site-specific mutants were created to examine the role of the fifth position glutamate and neighbouring residues in the recognition of pilin by the transmethylase of the bifunctional enzyme PilD. The adherence function of pili was investigated using two approaches. The first involved examining the adherence capabilities of a set of isogenic strains, each containing one five pilin prototype genes. The second was to study the adherence of point mutations in the C-terminal disulphide loop of PAK pilin. The discussion below attempts to integrate the results of these

studies with the work of others and examines the process of pilus biogenesis and the role of the C-terminal disulphide loop in adherence.

PILI BIOGENESIS

Pilin is synthesized in a precursor form containing a short leader sequence which is cleaved by a unique leader peptidase, PilD, leaving the N-terminal phenylalanine which becomes methylated by the same enzyme (Strom *et al.*, 1993b; Nunn and Lory, 1993). The proposed consensus sequence for processing by PilD is G-][-F-T-L-I-E-X-X-X-, where][is the cleavage site. Glycine was shown to be required in the -1 position, relative to the cleavage site, for recognition of pilin by the leader peptidase activity of PilD (Strom and Lory, 1991). Sequence alignments of type 4 pilins revealed that the -1 glycine is conserved in all type 4 pilins (Sohel *et al.*, 1993; Strom and Lory, 1992; Elleman, 1988). An N-terminal deletion of residues 4-7 (Δ4) in the mature pilin protein, however, resulted in only 10% cleavage and no methylation of the pilin subunits (Macdonald *et al.*, 1993; chapter II). The reduction in proteolytic cleavage of this deletion mutant suggested that N-terminal residues, other than the -1 glycine, may be included in the PilD recognition site. A second residue that was found to be absolutely conserved in all type 4 pilins, is the fifth position glutamate. Substitution of this glutamate with alanine, lysine, or valine resulted in a significant reduction in the degree of methylation of pilin monomers (Macdonald *et al.*, 1993; chapter II). Re-establishing the fifth position glutamate in a mutant with amino acids 4-7 (Δ4 Val to Glu) deleted restored the leader peptidase, but not the methylase function (Macdonald *et al.*, 1993; chapter II). These observations indicated that the fifth position glutamate is required for methylation, but other residues in the N-terminus of the mature pilin protein also participate in the methylase recognition site. It appears that the leader peptidase and the methylase recognition sites overlap in the N-terminus of pilin since the 4-7 (Δ4) deletion mutant was not cleaved and the Δ4 Val to Glu mutant was not methylated. PilD contains a relatively hydrophilic 80 amino acid

cytoplasmic domain containing the four active site cysteines (Strom *et al.*, 1993a). It is possible that the invariant fifth position glutamate could form hydrogen bonds with active-site cysteines in PilD and that substitution with hydrophobic or large basic amino acids did not allow this interaction. The apparent localization of one active site in the PilD is consistent with the overlap of the peptidase and methylase recognition sites in the N-terminal region of mature pilin.

The fifth position glutamate to lysine mutant strain was unable to assemble pili without the presence of wild-type methylated subunits, which indicated that methylation of at least some pilin subunits is a prerequisite for assembly (Pasloske *et al.*, 1989). Similarly, the Glu to Ala and the Δ4 Val to Glu mutants, in which the pilin subunits were not methylated, did not assemble pili (Macdonald *et al.*, 1993; chapter II). The purpose of methylation is not clear, but it is evident that methylation is pre-requisite for pilus assembly. The methyl group may either participate in the interaction of the pilin with other proteins or membrane components involved in the assembly process, or it could allow the pilin to be transported to the cell surface for assembly (Dupuy *et al.*, 1992). The conserved long hydrophobic segment may anchor the subunit in the inner membrane for the next assembly step necessitating its retention by the mature pilin .

A Glu to Asp mutation in the fifth position was generated to determine if the requirement in this position was for glutamate specifically or if a negatively charged amino acid was the all that was necessary. Replacement of the fifth position glutamate with aspartate resulted in a lack of pilin expression. The lack of pilin, in this case, was found to be caused by undetectable levels of *pilA*-specific mRNA expression (chapter III). The drastic effect of the Glu to Asp mutation was quite surprising for what is considered to be a conservative substitution. A pronounced feature of the study by Strom and Lory (1991) of amino acid substitutions in the N-terminal region of pilin was the extent of substitutions that had little or no effect on pili biogenesis. Levels of

mRNA in the cell are contolled by two factors, the rate of transcription initiation and the half-life of the mRNA. The lack of detectable *pilA*-specific mRNA expression in the Glu to Asp mutant strain may be the result of the formation of a nuclease sensitive site causing rapid degadation. Decay of mRNA has been well studied in *E. coli* but not in *P. aeruginosa*, although there is evidence of endonucleolytic cleavage in the *arcDABC* operon of *P. aeruginosa* (Gamper *et al.*, 1992). Alternately, a repressor recognition site may have been formed by the base change used to create the Glu to Asp mutation. Binding of the repressor to this newly created recognition site would inhibit the transcription of the pilin mRNA. It is clear from these observations that glutamate is required in the fifth position of pilin for 100% methylation and assembly into pili. Glutamate in the fifth position may fulfill the requirement for a negative charge as the only option since the aspartate codon in that position prevented the formation of pilin.

Two proteins, PilB and PilC participate in the assembly of pilin monomers into a helical array in the periplasm to form a pilus strand. PilB is a cytoplasmic protein containing a short ATP-binding site and PilC may be an integral membrane protein (Nunn *et al.*, 1990). Regions within the pilin protein itself may also be involved in pilus assembly. The hydrophobic region in the vicinity of Y24 and Y27 may play a role in subunit-subunit interactions as it is solvent inaccessible in the native pili, but not in pilin dimers (Watts *et al.*, 1983). A mutant with threonine substituted for aspartate (D132) was sensititive only to the pilus-specific phage B9 and produced very few, extremely short pili (chapter V). A defect in the pilus elongation process caused by the mutation may be responsible for this unusual phenotype. Immunoelectron microscopy revealed that the C-terminal disulphide loop was exposed at the pilus tip (Lee *et al.*, 1994). The disulphide loop region could be present at the subunit interface since it is only accessible at the tip of the pilus. Subunit-subunit interactions may involve D132 and the presence of threonine in its place caused a malfunction in pilus elongation

resulting in very short pili. The PAK pilin gene containing a P139A mutation did not form pili and expressed pilin very poorly (chapter V). Substitution of P139 also affected pili biogenesis, which suggested that this residue may play a role in the subunit:subunit interactions. P139 along with D132 may be involved either directly or indirectly in the assembly process.

PILI: THE GLUE STICK OF THE CELL

The attachment of bacteria to the host cell surface is the initial event in the infectious process and is considered to be an important virulence factor. Initial kinetic studies of *P. aeruginosa* strain 492c binding to trypsinized and untrypsinized human buccal epithelial cells (BEC) revealed the presence of high affinity-low copy and low affinity-high copy binding sites (McEachran and Irvin, 1985). The low affinity sites were trypsin sensitive and proposed to be putative receptors for capsular alginate. The high affinity sites were proposed to be binding sites for pili. Purified whole pili, anti-pilus antiserum, and a 23 amino acid C-terminal peptide were capable of inhibiting the binding of *P. aeruginosa* to BEC's and polymorphonucear leukocytes (Paranchych *et al.*, 1986). The results of these inhibition studies suggested that pili, more precisely the C-terminus of pili, were responsible for the adherence of *P. aeruginosa* to mammalian cells. *P. aeruginosa* was observed to bind to an array of structural proteins and enzymes, but no detectable binding was observed to a variety of carbohyrates present on mammalian glycoprotein and glycolipids (Paranchych *et al.*, 1985). It was thought that the pilus receptor may be a cell surface protein.

Equilibrium analysis of *P. aeruginsa* PAK binding to BEC's indicated that PAK bound to one receptor class (Doig *et al.*, 1988). Purified pili and anti-pilus Fab fragments competitively inhibited binding of PAK to BEC's indicating that pili and bacteria bound to the same receptor (Doig *et al.*, 1988; Lee *et al.*, 1989a). Synthetic peptides of 17 amino acids comprising the C-terminal disulphide loop in the oxidized and the reduced states both bound to BEC's and competitively inhibited PAK pili

binding showing that the same site as pili was bound (Irvin *et al.*, 1989). The oxidized peptide, however, had a higher affinity for BEC's than the reduced peptide. The presence of the intrachain disulphide bridge in the C-terminus of pili appears to be significant for epithelial cell binding. Using the computer program Surfaceplot, the C-terminal disulphide bridge region was proposed to be surface exposed (Lee *et al.*, 1989b). Antibodies to the oxidized PAK 128-144 C-terminal peptide were the only antibodies able to cross-react with PAO pili (Lee *et al.*, 1989a). The two cysteine residues are conserved in all pilin prototypes isolated to date and appear to be necessary for the maintenance of the binding conformation of pili. Monoclonal antibodies were produced against PAK pili. The epitopes of MAb PK34C and MAb PK99H were located in the C-terminal region (Doig *et al.*, 1990). MAb PK34C was able to block the binding of all *P. aeruginosa* strains to BEC's, whereas MAb PK99H was more strain specific and blocked only the PAK and 492c strains (Doig *et al.*, 1990).

Antibody and peptide studies of *P. aeruginosa* pili provided ample evidence that the C-terminal region of pilin contained the adhesion domain and that the presence of the intrachain disulphide bridge was important for the binding activity. In *P. aeruginosa* pilins, the N-terminus is highly conserved, the central region is the most variable of the molecule, and the C-terminal portion is semi-conserved. It was demonstrated, however, that pili from heterologous strains were able to prevent the adhesion of *P. aeruginosa* to injured mouse trachea, but only antibodies raised against the homologous pili were able to inhibit *P. aeruginosa* adherence (Ramphal *et al.*, 1984). The binding domain may be conserved within an antigenically active region of pilin. The different prototypes may have evolved as a mechanism to evade the immune system, but still maintaining the binding function of pili. Recently, immunoelectron microscopy studies demonstrated that the C-terminal disulphide loop was exposed only at the tip of the pilus and that receptor binding is a tip-associated event (Lee *et*

al., 1994). Identification of the pilus adherence domain would advance the understanding of the initial process of bacterial infection

Glycoconjugates are suspected to be the dominant receptor species because of their abundance on the cell surface. Carbohydrate chains make up the majority of cell surfaces especially on mucous membranes and are the primary collision contacts for microorganisms (Karlsson, 1989). Krivan and coworkers demonstrated that both mucoid and non-mucoid *P. aeruginosa* bind specifically to asialoGM1 and asialoGM2 (Krivan *et al.*, 1988a,b). Baker and collegues, on the other hand, found that *P. aeruginosa* bound to lactoslyceramide and sialic acid containing glycolipids as well as asialoGM1 and asialoGM2 (Baker *et al.*, 1990). *P. aeruginosa* was also reported to bind type 1 and type 2 disaccharides from breast milk (Ramphal *et al.*, 1991). The nature of the true pili receptor on the epithelial cell surface remains a mystery.

Two approaches were taken to define more clearly the adherence domain of *P. aeruginosa* pili, and the nature of the cell surface receptor. The first involved the construction of a set of isogenic strains each containing one of the pilin prototypes. The second involved making site-specific mutations in both conserved and non-conserved residues in the C-terminal disulphide loop region. Attachment to A549 human lung pneumocytes and binding to purified putative glycolipid receptors was the basis of the analysis.

An assay was developed to assess the binding capabilities of the pili form the isogenic strains and pili containing site-specific mutations. The assay involved culturing A549 human lung pneumocyte cell monolayers in 96 well plates, binding the pili to the monolayers, and detecting the presence of the pili by ELISA. The A549 cell attachment assay is a unique procedure designed to compare the binding of different pili. This assay was an improvement over previously employed assays using human buccal epithelial cells. Attachment assays using the A549 cells proved to be an

effective means to assess the binding capabilities of the purified pili prototypes and the pili containing site-specific muations.

Alignment of the pilin prototype sequences in the disulphide loop region revealed the semi-conserved nature of this region. In spite of the variation in this region, solution structures from NMR data of synthetic C-terminal peptides revealed that a type I β-turn (DEQF in PAK), and a type II β-turn (PKGC in PAK) were present in PAK, KB7, and PAO (McInnes *et al.*, 1993; McInnes *et al.*, unpublished results; Patricia Campbell, personal communication). Analysis of the binding capabilities of four pili prototypes found that KB7 was bound most efficiently followed by PAO, PAK and K122 (chapter IV). The sequence variation was indeed having an effect on attachment even though the structure was not affected. Whole cell binding to buccal epithelial cells showed that the affinity of PAO for the cell surface receptor was 10 times greater than PAK (Irvin *et al.*, 1990). The KB7 C-terminal peptide was a more successful inhibitor of PAK pili binding to A549 cells than the PAK peptide suggesting that the KB7 peptide had a slightly higher affinity for A549 cells (Wong *et al.*, submitted). The A549 cell attachment assay showed that the avidities of PAO and KB7 pili for A549 cells were 3.5-fold greater than that of PAK pili (chapter IV). The affinity for the receptor was found to be more significant than the number of receptors per cell in quantitating adherent bacteria in competition binding assays with buccal epithelial cells (Irvin *et al.*, 1990). The binding efficiency of purified pili to A549 cells was influenced by both the avidity and the amount of pili bound per A549 cell at saturation (chapter IV).

TLC overlay assays indicated that asialoGM1 and asialoGM2 were excellent receptors for *P. aeruginosa* pili. *P. aeruginosa* was previously shown to bind to asialoGM1 and asialoGM2 (Krivan *et al.*, 1988a,b; Baker *et al.*, 1990). The disulphide bridge was found to be exposed only at the pilus tip and C-terminal disulphide bridged peptides of both PAK and PAO were shown to bind to asialoGM1 (Lee *et al.*, 1994).

MAb PK99H, which is specific for PAK was also able to block the binding of PAK pili to asialoGM1 (Lee *et al.*, 1994). It was concluded from these observations, that the adhesin for *P. aeruginosa* is in the disulphide bridge of pilin which is exposed at the pilus tip. MAb PK99H inhibited the binding of PAK pili to A549 cells, and PAK pili bound to asialoGM1 and asialoGM2 on the TLC plate (chapter IV). This suggested that the disulphide loop region was also responsible for the pili binding to A549 cells and that one or both of the glycolipids could be the cell surface receptor for pili. The cell surface receptor may be asialoGM1, since a significant amount of this glycolipid was found in human lung tissues (Krivan *et al.*, 1988a). AsialoGM1 and asialoGM2 contain the common trisaccharide sequence GalNAcβ1-4Galβ1-4Glc which may represent the minimal sequence required for binding. Alternatively, the trisaccharide common to asialoGM1 and asialoGM2 may mimic or form part of the true receptor. TLC allows the receptors to be presented in a multivalent fashion enabling low affinity interactions to be detected (Karlsson, 1989).

The amino acid alignment showed four conserved (C129, D134, P139, C142 in PAK), four semi-conserved residues (S131, F137, K140, G141 in PAK), and six non-conserved residues (T130, D132, Q133, E135, Q136, I138 in PAK). Invariant residues are usually involved directly in the protein's function or are required for structure formation (Bowie *et al.*, 1990). C129 and C142 are required to maintain the disulphide bridge, which is a prominent structural feature of pilin. A synthetic peptide with these two cysteines replaced with alanines was not able to inhibit the binding of PAK pili to A549 cells (Wong *et al.*, submitted). A mutant in which the C-terminal disulphide loop of PAO was replaced with amino acids from the flanking sequence of the chloramphenicol acetyltransferase gene, did not contain a disulphide bridge and exhibited a significant reduction in binding of the bacteria to A549 cells (Farinha *et al.*, in press). These observations demonstrated the importance of the disulphide bridge structure for adherence. NMR structural studies indicated that P139 and G141 are

important to maintain the type II β-turn (McInnes *et al.*, 1993). Synthetic peptides with alanine substitutions of P139 and G141 were unable to inhibit PAK pili binding to A549 cells, which emphasized the structural importance of these residues (Wong *et al.*, submitted). Substitution of T130 and F137 had no effect on pili binding to A549 cells or to putative glycolipid receptors (chapter V). These results suggested that T130 and F137 were not involved in pilus-mediated adherence. When the structures of PAK and KB7 peptides were superimposed, the F137 side-chain was oriented in different directions in the two peptides (McInnes *et al.*, unpublished results). In addition, peptides with alanine substitutions of T130 and F137 inhibited PAK pili binding to A549 cells as well as the native PAK peptide (Wong *et al.*, submitted). The earlier noted results that suggested T130 and F137 were not involved in adherence are supported by these observations.

The three residues found to be important for PAK pili binding in this study (E135, Q136, I138) were non-conserved residues. Substitutions of these amino acids significantly reduced pili binding to A549 cells and to putative glycolipid receptors (chapter V). Peptide studies also found Q136 and I138 to be important residues as well as S131 which is semi-conserved (Wong *et al.*, submitted). Interestingly, the arginine in position 138 of the KB7 peptide was also found to participate in A549 cell binding (Wong *et al.*, submitted). Position 138 was shown to lie at the point between the two β-turns in the NMR structure of the C-terminal peptides (McInnes *et al.*, 1993). The residue in this position may form the apex of the pilus tip and may have primary contact with the receptor at the cell surface.

The accessibility and orientation of the receptor within the natural membrane may be a major factor in pili binding. Adherence studies on the P-pilus-associated G-adhesins of uropathogenic *E. coli*, revealed that receptors adopt more restricted conformations in natural membranes compared to artificial surfaces like TLC plates (Stromberg *et al.*, 1991). The differential binding of the three G-adhesins studied

resulted from differences in epitope presentation at the membrane (Stromberg *et al.*, 1991). The trisaccharide common to asialoGM1 and asialoGM2, GalNAcβ1-4Galβ1-4Glc, could adopt many conformations on the cell surface. The different saccharide orientations may expose or mask different epitopes on the receptor which may explain the variation in binding abilities of the different prototype sequences. The orientation of the saccharide units of cell surface glycolipids may affect their receptor function for bacterial adhesins. The semi-conserved nature of the C-terminal binding domain may be an adaptation to the more restricted conformational state of the receptor in the cell membrane. The KB7 pili prototype appears to have adapted the most successfully to the receptor orientation present in A549 lung cells.

CONCLUDING REMARKS

The study presented here examined structure and function of *Pseudomonas aeruginosa* pili. It is an important area and will expand the understanding of the pathogenic mechanisms of *P. aeruginosa* during the initial stage of infection, particularly in chronic lung infections of cystic fibrosis patients which are often fatal. In order to gain knowledge into the role played by various regions of pilin, site-specific changes were introduced into the fifth position of the N-terminus as well as to the C-terminal disulphide loop. Through this approach it was revealed that the fifth position glutamate was required for recognition of pilin by the methylase activity of the PilD enzyme. A unique assay was developed to assess the binding of purified pili to A549 human lung pneumocyte cells which contributed significantly to the understanding of the function of pili in adherence. Site-directed mutagenesis of the C-terminal disulphide loop revealed particular residues that were important for adherence of pili to A549 cells. Refinement of TLC overlay assays to assess the binding of pili to purified putative glycolipid receptors showed that possible receptors for *P. aeruginosa* pili were asialoGM1 and asialoGM2. The results discovered in this study have helped

to gain an understanding of the biology of pili structure and adhesive function on a molecular level.

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