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THE UNIVERSITY OF ALBERTA

Synthetic Peptide Approaches to Study the Adherence Binding Domain of the Pilin Protein of *Pseudomonas aeruginosa* Strain PAK

By

Wah Yau Wong

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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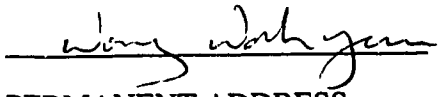

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Synthetic Peptide Approaches to Study the Adherence Binding Domain of the Pilin Protein of *Pseudomonas aeruginosa* Strain PAK submitted by Wah Yau Wong in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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For my wife

and

my parents

ABSTRACT

The 17-residue disulfide-bridged C-terminal region of the *Pseudomonas aeruginosa* pilin contains an important antigenic determinant and a human epithelial cell binding domain. The epitopes of four anti-pilus antibodies that display different cross-reactivities against *P. aeruginosa* strains PAK and PAO were studied by a single alanine replacement analysis and a representative combinatorial peptide library technique. All epitopes were identified within a 9-residue linear peptide sequence ^{132}DQDEQFIPK^{140}; however, the specificity of individual residues were different. Residues Asp^{134} and Gln^{136} were more important for the cross-reactive antibody, whereas Glu^{135} and Ile^{138} were more important for the strain-specific antibodies. To test the hypothesis that a more cross-reactive antibody response could be generated by synthetic peptides where the side-chains critical for recognition by the strain-specific antibodies are deleted, four PAK peptide analogs with residues Glu^{135}, Gln^{136}, Phe^{137}, and Ile^{138} substituted by alanine were employed to immunize rabbits. Antisera raised against the Glu^{135} and Ile^{138} analogs cross-reacted with both pili and peptides from PAK and PAO strains. Moreover, some of these antisera showed improved cross-reactivity. Interestingly, antisera raised against the Gln^{136} and Phe^{137} analogs only bound to peptide antigens, suggesting the importance of these side-chains in generating antipeptide antibodies that recognize the native protein.

On the other hand, six amino acid side-chains (Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141}, and Lys^{144}) located in the C-terminal binding domain of the PAK pilin were found to be important for A549 cell binding. Four of these side-chains (Gln^{136}, Ile^{138}, Pro^{139}, and Gly^{141}) were located in a structurally more rigid region. This suggests that the conformation of the peptide conferred by the intrachain disulfide bridge was also critical for receptor interactions. Furthermore, the 17-residue C-terminal sequence of the KB7 pilin strain bound as well to A549 cells as did the PAK peptide despite their

sequence dissimilarity. The results of the single alanine substituted analogs of both the KB7 and PAK sequences demonstrated that the majority of positions essential for binding were similar in both sequences even though many of these positions involved heterologous residues. These studies illustrate how a pathogen like *P. aeruginosa* compromises the need of antigenic variations of its pilus adhesin with the desire to maintain its adherence properties.

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-di-(3-ethylbenzthiazoline sulfonic acid)
ADP	Adenosine 5'-diphosphate
Amino acids:	
Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
Cys, C	Cysteine
Gln, Q	Glutamine
Glu, E	Glutamic acid
Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Nle	Norleucine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

Asialo-GM_1	Gangliotetraosyl ceramide
Asialo-GM_2	Gangliotriaosyl ceramide
ATCC	American Type Culture Collection
BB	Benzophenone
BCA	Bicinchoninic acid
Boc	*tert*-Butyloxycarbonyl
BOP	Benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate
BSA	Bovine serum albumin
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	N,N'-Dicyclohexyl urea
DIEA	N,N-Diisopropylethylamine
DME	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDT	1,2-Ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
EF-1	Elongation factor 1
EF-2	Elongation factor 2
EGTA	Ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
Fmoc	9-Fluorenylmethoxy-carbonyl
HAT	Hypoxanthine, aminopterin, thymidine

HBSS	Hank's balanced salt solution
HBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
I_{50}	Competitor concentration that causes 50% inhibition of binding
IgG	Immunoglobulin of the G subclass
IL-1	Interleukin-1
K_a	Apparent association constant
KLH	Keyhole limpet hemocyanin
K_N	K_a of the native peptide
K_{PAK}	K_a of the PAK strain
K_{PAO}	K_a of the PAO strain
K_S	K_a of the peptide analog
LD_{50}	Lethal dose that causes 50% mortality
LPS	Lipopolysaccharide
MAP	Multiple antigen peptide
MCPS	Multiple column peptide synthesizer
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-Ethylmaleimide
NMePhe	N-Methyl-phenylalanine
NMP	N-Methylpyrolidone
NMR	Nuclear magnetic resonance
NNIS	National Nosocomial Infections Surveillance

ODhbt	3-Hydroxy-2,3-dihydro-4-oxo-benzotriazine
OPfp	Pentafluorophenyl
PBS	Phosphate buffered saline
Pristane	2,6,10,14-Tetramethylpentadecane
RCPL	Representative combinatorial peptide libraries
SPPS	Solid phase peptide synthesis
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TnC	Troponin C
TNF	Tumor necrosis factor
TnI	Troponin I
Tris	Tris(hydroxymethyl)methyl-amine

CHAPTER I

INTRODUCTION

A. Taxonomy of *Pseudomonas aeruginosa*

1. *Pseudomonas*: The genus

The word *Pseudomonas* is constructed from the two Greek terms, *pseudes* (false) and *monas* (a unit or monad), to imply the meaning of "false monad". *Pseudomonas* is the major genus of the family *Pseudomonadaceae.* This family contains four genera: *Pseudomonas*, *Xanthomonas*, *Frateuria* and *Zoogloea* (for reviews in taxonomy see Palleroni, 1984; 1986). The genus *Pseudomonas* consists of a vast and heterogeneous group of rod-shaped, Gram negative, and polarly flagellated bacteria. One of the main features of these microorganisms is their ability to grow in simple organic media. Their sizes are generally in the range from 0.5-1.0 μm in diameter by 1.5-5.0 μm in length.

Pseudomonas are strictly aerobic microorganisms, with oxygen generally being used as the terminal electron acceptor. However, some species use nitrate or other substrates as alternate electron acceptors and grow anaerobically (Palleroni, 1984). For example, the species *Pseudomonas aeruginosa* can utilize arginine to undergo facultative anaerobic respiration (Vander Wauven *et al.*, 1984). *Pseudomonas* are chemoorganotrophic organisms, and are able to use a variety of organic compounds as the sole carbon and energy sources. Nevertheless, some species are facultative chemolithotrophs, and are able to grow autotrophically utilizing H_2 and/or CO as energy sources. In general, their growth temperature is between 4°C to 43°C while the optimum growth temperature is around 28°C. The simple nutritional requirement and versatility in adapting to the environment allow members of *Pseudomonas* to be distributed ubiquitously (primarily in soil and water).

Flagella and pili (fimbriae) can be found on the bacterial cells. Typically, *Pseudomonas* cells have polar flagella that facilitate their motility. In some species lateral flagella may also be formed, especially when they are growing on solid media. *Pseudomonas* sp. are rarely nonmotile, but occasionally non-motile strains of various species can be isolated. Pili or fimbriae have also been reported for a number of *Pseudomonas* species. They can be either polarly (e.g. *P. aeruginosa*) or peritrichously (e.g. *P. cepacia*) localized.

Many *Pseudomonas* species contain plasmids in their genetic make-up. These plasmid genes encode various properties and important functions for the bacteria (for review see Jacoby, 1979; 1986) such as: to act as chromosome donors; to allow the cell to use unusual carbon sources; and to provide drug-resistance capability to the cells. A total of thirteen different drug-resistance incompatibility groups have been classified (Jacoby, 1986). One of the characteristics of these drug-resistance plasmids is the production of ß-lactamases and this allows the microorganism to neutralize the effect of penicillin-based antibiotics. Surprisingly, penicillin-utilizing *Pseudomonas* strains have also been reported (Beckman and Lessis, 1980).

2. *Pseudomonas aeruginosa*: The species

Pseudomonas aeruginosa is the type species of the genus *Pseudomonas*. The first scientific record of *P. aeruginosa* appeared as early as 1872 (Schroeter, 1872), whereas the pure culture was isolated in 1882 by Gessard (1984). The term *aeruginosa* means "full of copper rust or verdigris" and thus refers to a greenish color, which reflects the pigment-producing capability of this microorganism. This is why *P. aeruginosa* was once known as *P. polycolor* (Clara, 1930), *P. pyocyaneus* (Migula, 1895) and *Bacterium aeruginosum* (Schroeter, 1872).

Pyoverdin and pyocyanin are the two most commonly found pigments in *P. aeruginosa* and other *Pseudomonas* species. Pyoverdin is a fluorescence pigment,

which is produced abundantly in media of low iron content, functions as an iron-siderophore or chelator (Ankenbauer *et al.*, 1985). It emits a blue-green fluorescence upon ultraviolet irradiation. Pyocyanin is a blue-color phenazine pigment. Like pyoverdin, it is water-soluble and diffuses freely into the medium (Wilson *et al.*, 1988). In addition to pyoverdin and pyocyanin, *P. aeruginosa* also produces a number of other pigments.

Different colony morphologies can be observed when *P. aeruginosa* are grown on solid medium (Clarke, 1990). There are two major colony types: the large and smooth colony, and the small and rough colony. Usually, most strains obtained from natural sources appear as smaller colonies, whereas those clinical isolates are generally larger and appear as smooth colonies in laboratory culture. After prolonged culture on high-carbon medium, most of the colonies will change to slime-producing colonies which are different from the mucoid colonies obtained from cystic fibrosis isolates.

P. aeruginosa have also been documented to produce bacteriocins (termed pyocins) with antibiotic activity. Bacteriocins are bacterial protein toxins that are lethal to other strains of bacteria. In *P. aeruginosa*, two types of bacteriocins are identified, the S-type and the R-type. The S-type is sensitive to proteases and has an amorphous appearance, whereas the R-type is resistant to proteases (Bradley, 1967). In addition, a substance like pyocyanin can interfere with bacterial respiration and causes an antibiotic effect on other bacteria (Ingram and Blackwood, 1970). These observations have once led to the proposal that *P. aeruginosa* could be used as a potent antimicrobial agent (Schoenthal, 1941).

P. aeruginosa is also characterized by having only one polar flagellum, as well as the polar pili that protruded at either end or both ends of the bacterial rod. They can grow at temperature as high as 42°C but not at 4°C; in general, their optimum growth temperature is 37°C.

B. *P. aeruginosa* Infections

1. *P. aeruginosa* as an opportunistic pathogen

Infections caused by *P. aeruginosa* were rarely known decades ago. Indeed, this organism has once been suggested to be a source of antimicrobial agent (Schoenthal, 1941) rather than a pathogen. As a result, the pathogenicity of this organism was doubted even though it was first reported as a pathogen by Charrin as early as 1889 (Forkner, 1960) and with the first clinical culture isolated from infected patients in 1896 (Stanley, 1947). Its true potential for causing life-threatening diseases was not recognized until 1959 (Finland *et al.*, 1959).

P. aeruginosa infects a wide range of animal and plant hosts. In humans, *P. aeruginosa* has a predilection for a specific group of the population, particularly those whose natural defense mechanisms are impaired, either immunocompromised or immunosuppressed. *Pseudomonas* infections are commonly found in patients with cancer, cystic fibrosis, burn wounds or a long history of hospitalization; however, it seldom infects healthy individuals. Therefore, the terms opportunistic pathogen and nosocomial (hospital-acquired) pathogen are designated for *P. aeruginosa*.

From 1976 to 1980, data from the National Nosocomial Infections Surveillance (NNIS) Study conducted by the U.S. Centers for Disease Control showed an overall increase in the rate of infections caused by *P. aeruginosa*, which accounted for 8% to 9% of nosocomial infections at all sites (Cross *et al.*, 1983). This figure increased to 11% for the period of 1985-1989 (Schaberg *et al.*, 1991), which accounted for 12% of urinary tract infections, 17% of lower respiratory infections, and 8% of surgical wound infections (Horan *et al.*, 1986; Schaberg *et al.*, 1991). During the 1984 to 1988 period, *P. aeruginosa* was ranked by NNIS from fourth to first as the most frequent pathogen of nosocomial septicemia (Horan *et al.*, 1988). It is also the most common organism causing bacteremia in burn patients (McManus, 1989). From 1980 to 1990,

P. aeruginosa continued to be the second most frequent Gram negative bacterium (after *Escherichia coli*) causing nosocomial infections (Rhame, 1980; Botzenhart and Döring, 1993). Furthermore, *P. aeruginosa* is also the most common ocular pathogen of all the Gram negative bacteria (Holland *et al.*, 1993).

The rapid increase in *P. aeruginosa* infections during the past 20 years could be accounted for by three factors:

(a) *Versatility and adaptability of the bacteria. P. aeruginosa* is a very versatile microorganism that can colonize many different kinds of surfaces, including stainless steel (Vanhaecke *et al.*, 1990). It can grow at extremely limited nutrient condition. Even in distilled water, *P. aeruginosa* can survive and multiply up to a maximum cell population of 10^7 cells/ml (Favero *et al.*, 1971). Furthermore, its ability to degrade organic compounds may also explain its high resistance to disinfectants such as quaternary ammonium compounds and chlorinated phenols (Adair *et al.*, 1969; Carlson *et al.*, 1972). The hospital environment is one of the common sites where this microorganism can easily be found. This is probably due to the effectiveness of the pathogen to be transmitted in a crowded area, as well as its high resistance to disinfectants. There have been reports of outbreaks of *P. aeruginosa* infections due to contamination of hospital equipment such as tracheotomy tubes, suction catheters, reservoir and jet nebulizers (Grieble *et al.*, 1970; Lowbury *et al.*, 1970; Whitby and Rampling, 1972).

(b) *Intrinsic resistance to antibiotics. P. aeruginosa* displays resistance to many conventional antibiotics such as ß-lactams, aminoglycosides and quinolones (for review see Bellido and Hancock, 1993). Two major mechanisms can be accounted for the antibiotic resistance. First, the permeability of the *P. aeruginosa* outer membrane to antibiotics is low (Angus *et al.*, 1982; Yoshimura and Nikaido, 1982). Alteration of the expression of certain outer membrane proteins (or porins) further reduces the uptake of antibiotics. Examples of the outer membrane proteins are OprD (Trias and

Nikaido, 1990), OprF (Hancock *et al.*, 1990; Piddock *et al.*, 1992) and OprG (Chamberland *et al.*, 1990). In addition, the self-promoted uptake pathway of aminoglycosides can also be reduced by non-enzymatic or impermeability-mediated mechanisms (Miller *et al.*, 1980; Bryan *et al.*, 1984). Second, *P. aeruginosa* carries both plasmid and chromosomal ß-lactamase genes (Sykes and Matthew, 1976; Jacoby, 1986), which neutralize the toxic effect of ß-lactam molecules. Plasmid-mediated resistance to aminoglycosides due to the production of aminoglycoside-modifying enzymes was also reported (Miller *et al.*, 1980; Phillips and Shannon, 1984). Furthermore, the selection pressure plays a key role in promoting antibiotic resistance in *P. aeruginosa*. The extensive use of antibiotics since the World War II and the introduction of broad-spectrum antibiotics have markedly increased the frequency of *P. aeruginosa* infections (Neu, 1992; Travis, 1994). It is generally accepted that during antibiotic treatment, the antibiotics used may disrupt the growth of competitive normal flora and at the same time select the drug-resistance *P. aeruginosa* strains (Hentges *et al.*, 1985).

(c) *Increase in the number of susceptible hosts.* *P. aeruginosa* infections are rarely observed in healthy persons with an intact defense mechanism. The advances in medical technology have greatly prolonged the life-expectancy of the elderly and patients with terminal diseases or fatal genetic illness, while the introduction of immunosuppression drugs further promoted *P. aeruginosa* infections. Consequently, there is a significant increase in the number of susceptible individuals in the population.

2. Acute and chronic infections

P. aeruginosa infections can occur at different locations of the body, notably on the mucosal surface. The most important mucosal routes for *P. aeruginosa* infections are the respiratory tract, the urinary tract, the gastrointestinal tract, and the cornea (Aksamit, 1993; Baker, 1993; Holland *et al.*, 1993). *P. aeruginosa* infections can

range from acute nosocomial pneumonia to the chronic respiratory infections of the cystic fibrosis patients. Chronic infections are believed to be mediated by mucoid phenotype (Lam *et al.*, 1980; Baltimore *et al.*, 1989), whereas acute infections and early colonization are caused by cells with the non-mucoid phenotype (Pier, 1985). The relationship between the mucoid and non-mucoid phenotypes has been investigated. Recently, Woods *et al.* (1991; Woods, 1993) have demonstrated the phenotypic conversion of the non-mucoid to a less virulent mucoid phenotype during *P. aeruginosa* infection in cystic fibrosis lung. The chronic infection of the cystic fibrosis patients are usually non-invasive and less toxemic (Costerton *et al.*, 1990), and the bacterial colony is usually protected from the host's immunity and antibiotic treatment by a mass of alginate (Govan and Harris, 1986; Costerton *et al.*, 1990). Costerton *et al.* (1987; 1990) have proposed a biofilm model to explain the persistence of the chronic infections in cystic fibrosis patients.

P. aeruginosa infections are responsible for a wide spectrum of morbidity (Table I.1). These range from the fatal bacteremia to less severe ear infections (Artenstein and Cross, 1993). The overall mortality rate is high, for example, *P. aeruginosa* alone causes a mortality rate of 30 to 60% just in the case of nosocomial pneumonia (Aksamit, 1993). In addition, *Pseudomonas* pneumonia is also the direct cause of death in the majority of the cystic fibrosis patients (Döring, 1993). This has prompted an extensive research on the pathogenesis of this microorganism. Recently, some excellent books (Campa *et al.*, 1993; Fick, 1993b) and reviews have been devoted in particular to the severe diseases caused by *P. aeruginosa*, for example, *Pseudomonas* pneumonia and bacteremia (Aksamit, 1993; Tonner *et al.*, 1993), and *Pseudomonas* ocular infections (Holland *et al.*, 1993).

Table I.1 Diseases caused by *Pseudomonas aeruginosa**

Site	Disease	Reference
Blood	Bacteremia	Flick and Cluff, 1976; Bodey *et al.*, 1985; Bisbe *et al.*, 1988.
Respiratory tract	Respiratory infections	Iannini *et al.*, 1974; Hughes, 1988.
	Bacteremic pneumonia	Pennington *et al.*, 1973; Iannini *et al.*, 1974.
	Non-bacteremic pneumonia	Tillotson and Lerner, 1968; Iannini *et al.*, 1974.
Eye	Ocular keratitis	Bodey *et al.*, 1983; Hassman and Sugar, 1983.
	Conjunctivitis	Rosenoff *et al.*, 1974.
	Dacryocystitis	Kreger, 1983.
	Orbital cellulitis	Kreger, 1983.
	Ophthalmia neonatorum	Traboulsi *et al.*, 1984.
	Endophthalmitis	Ayliffe *et al.*, 1966.
Urinary tract	Urinary tract infections	Strand *et al.*, 1982.
Wound	Surgical wound infections	Tong, 1972; Klein *et al.*, 1975.
	Burn wound infections	Pruitt *et al.*, 1983.
Gastrointestinal tract	Enterocolitis	Stone *et al.*, 1979.
	Stomach/small intestine ulcers	Stanley, 1947.
	Diarrhea in infants	Florman and Schifrin, 1950.
	Typhlitis (necrotizing entero-colitis)	Bodey *et al.*, 1983; Starnes *et al.*, 1986.
	Perianal & Perirectal abscess	Schimpff *et al.*, 1972.
Peritoneal	Dialysis infection: Peritonitis	Krothapalli *et al.*, 1982; Parrott *et al.*, 1982; Piraino *et al.*, 1987.
Skin	Skin infections	Hall *et al.*, 1968; Washburn *et al.*, 1976; Gustafson *et al.*, 1983.
Heart	Endocarditis	Saroff *et al.*, 1973; Cohen *et al.*, 1980.
Bone & joint	Osteomyelitis & Septic arthritis	Bodey *et al.*, 1983; Cross, 1985.
	Osteochondritis	Green and Bruno, 1980; Jacobs *et al.*, 1989.
Central nervous system	Meningitis	Chernik *et al.*, 1973; Bodey *et al.*, 1983; Fong and Tomkins, 1985.
	Brain abscess	Chernik *et al.*, 1973.
Ear	External otitis	Stanley, 1947; Hall *et al.*, 1968.
	Malignant external otitis	Chandler, 1968; Rubin and Yu, 1988.

*Data were obtained from a review by Artenstein and Cross (1993)

C. *P. aeruginosa* Virulence Factors

It is known that pathogenicity is multifactorial (Smith, 1977). In *P. aeruginosa*, a large number of virulence factors have been reported. These virulence factors are more or less associated with the pathogenicity of this microorganism (for reviews see Nicas and Iglewski, 1986; Holder, 1993). Accordingly, the *P. aeruginosa* virulence factors can be classified into five different categories: toxins, enzymes, adhesins, motility, and secondary metabolites (Table I.2).

1. Toxins

P. aeruginosa produce four different kinds of toxins, these include the endotoxin lipopolysaccharide (LPS), exotoxin A, exoenzyme S, and a cell-associated cytotoxin. LPS causes a wide variety of physiological and biochemical derangement of the host such as fever, enhanced muscle catabolism, hypoglycemia, and finally lethal shock. The endotoxic shock may be due to an acute inflammatory response towards LPS through the production of prostaglandin E2, thromboxane A2, tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Cryz *et al.*, 1984; Kropinski *et al.*, 1985). Furthermore, LPS also plays an important role in protecting the microorganism from being opsonized and phagocytosed by polymorphonuclear leukocytes (Engels *et al.*, 1985).

Exotoxin A is a lethal toxin secreted by *P. aeruginosa*. The presence of this toxic substance in the culture of *P. aeruginosa* had been noticed in the 1960s (Liu *et al.*, 1961; Liu, 1966). Exotoxin A is a 66.6 kD protein (Iglewski and Sadoff, 1979) that functions as a protein synthesis inhibitor by catalyzing the transfer of the ADP-ribosyl moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2). EF-2 is a translocation factor required for polypeptide chain synthesis in eukaryotic cells (Iglewski and Kabat, 1975; Pavlovskis *et al.*, 1978). The LD_{50} (the amount of protein

Table I.2 *Pseudomonas aeruginosa* virulence factors

Category	Virulence factors	References
Toxins	Lipopolysacharrides	Pitt, 1989.
	Exotoxin A	Liu, 1974; Pavlovskis *et al.*, 1978; Nicas and Iglewski, 1986; Vasil *et al.*, 1990; 1993.
	Leukocidin	Hayashi and Terawaki, 1991; Lutz *et al.*, 1991.
	Exoenzyme S	Baker *et al.*, 1991; Lingwood *et al.*, 1991; Ramphal *et al.*, 1991b.
Enzymes	Elastase	Galloway, 1991.
	Alkaline protease	Steuhl *et al.*, 1987.
	Phospholipases C	Meyers and Berk, 1990; Vasil *et al.*, 1991.
Adhesins	Pilus	Woods *et al.*, 1980b; Ramphal *et al.*, 1984; Doig *et al.*, 1988; Lee *et al.*, 1989a.
	Alginate	Doig *et al.*, 1987; Baker, 1990.
	Exoenzyme S	Baker *et al.*, 1991; Lingwood *et al.*, 1991; Ramphal *et al.*, 1991b.
Motility	Flagellum	Drake and Montie, 1988.
Secondary metabolites	Pyoverdin, pyochelin	Cox *et al.*, 1981; Abdallah *et al.*, 1989.
	Pyocyanin	Wilson *et al.*, 1987; Munro *et al.*, 1989.
	Rhamnolipids	Leisinger and Margraff, 1979; Döring *et al.*, 1987.

that kills 50% of the experimental animals) of exotoxin A was about 0.2 μg when injected intraperitoneally in mice and was more than 20,000 times as toxic as the endotoxin (Liu, 1974). It was considered as the most toxic substance produced by *P. aeruginosa* (Liu, 1974).

The exoenzyme S, like exotoxin A, acts as an ADP-ribosyltransferase that catalyzes the transfer of ADP-ribose from NAD to eukaryotic protein (Iglewski *et al.*, 1978). However, it employs a different mechanism from that of exotoxin A. It modifies a large number of proteins other than EF-2 in eukaryotic cells (Iglewski *et al.*, 1978). The physiological function of exoenzyme S is not known yet, but it has been suggested that exoenzyme S may modify elongation factor 1 (EF-1) (Iglewski *et al.*, 1978).

Certain strains of *P. aeruginosa* produce a cell-associated protein called leukocidin, which causes the lysis of lymphocytes and granulocytes but not red blood cells or platelets (Scharmann, 1976). The molecular weight of leukocidin is 44.7 K (Hirayama and Kato, 1983). The cytotoxic effect was proposed to be mediated by a calcium-dependent mechanism in which a change in phosphatidylinositol and polyphosphoinositides metabolism causes an accumulation of phosphatidic acid and results in an increase in intracellular Ca^{2+} (Hirayama and Kato, 1983), and finally induces cell lysis.

2. Enzymes

Several enzymes produced by *P. aeruginosa* were reported to be virulence-related factors. Examples are the alkaline protease, elastase and phospholipases C. Alkaline protease and elastase are the two major proteases produced by *P. aeruginosa* (for review see Nicas and Iglewski, 1986). The molecular weights of the two proteases are 48 K and 33 K, respectively (Morihara, 1963; Morihara *et al.*, 1965). Their major action is to degrade host proteins and tissues so as to reduce the host defense mechanisms such as IgG (Döring *et al.*, 1984; Holder and Wheeler, 1984), interferon-γ

(Horvat and Rarmely, 1988), complement component (Schultz and Miller, 1974), protease inhibitors (Moskowitz and Heinrich, 1971; Morihara *et al.*, 1979), and α1-antichymotrypsin (Catanese and Kress, 1984) and to increase invasiveness of the pathogen (Kawaharajo *et al.*, 1975). In addition, they also act to produce preferential growth substances in the local area to enhance the growth of the bacteria (Cicmanec and Holder, 1979). In general, elastase degrades a broader range of proteins than alkaline protease (Döring *et al.*, 1984). Recent studies have shown that a protein fragment secreted by *P. aeruginosa*, termed the *lasA* gene product, can enhance the proteolytic activity of elastase by acting directly upon the elastin substrate. The authors concluded that the LasA protein is a second elastase and thus a virulence factor (Galloway, 1991; Wolz *et al.*, 1991).

P. aeruginosa produces two types of phospholipases C, the hemolytic (phospholipase C-H) and the non-hemolytic (phospholipase C-N) proteins (Ostroff *et al.*, 1990). Both enzymes hydrolyze phosphatidylcholine into phosphorylcholine and diacylglycerol (Esselemann and Liu, 1961) and this action can cause skin lesions, hepatic necrosis and pulmonary edema *in vivo* (Liu, 1966).

3. Adhesins

In *P. aeruginosa*, at least four different adhesins that mediate the adherence of the pathogen to the host tissue have been identified. These adhesins include the pili (Woods *et al.*, 1980b; Doig *et al.*, 1988), alginate (Ramphal and Pier, 1985; Doig *et al.*, 1987), exoenzyme S (Baker *et al.*, 1991; Lingwood *et al.*, 1991), and the outer membrane proteins (Saiman *et al.*, 1990; Ramphal *et al.*, 1991b; Simpson *et al.*, 1992). They are important for the attachment of the bacteria to the host's mucosal surface and allow subsequent colonization. These adhesins have unique and important roles in *P. aeruginosa* infections, they will be discussed in a separate section.

4. Motility

P. aeruginosa employs its polar flagellum to facilitate locomotion (Holder *et al.*, 1982; Holder and Naglich, 1986; Drake and Montie, 1988). The importance of motility and chemotaxis to the pathogenicity of *P. aeruginosa* was first suggested by McManus *et al.* (1979) and further elaborated by Craven *et al.* (1981) that both mechanisms were required for the pathogenicity. The role of motility as a virulence factor in *P. aeruginosa* burn wound sepsis was studied using mutants with a deficiency in either flagellum construction (Fla^-) or chemotaxis (Mot^-), and such mutations caused a decrease in the invasiveness of the organisms (Drake and Montie, 1988). However, analysis of clinical isolates obtained from cystic fibrosis patients indicated that a large number of the isolates were of a Fla^- phenotype, which may reflect the selection pressure against the invasive Fla^+ phenotype during chronic infections (Luzar and Montie, 1985; Luzar *et al.*, 1985).

5. Secondary metabolites

Secondary metabolites such as pyoverdin, pyochelin, pyocyanin and rhamnolipid are also important for the pathogenicity of *P. aeruginosa.* Pyoverdin and pyochelin are potent iron-chelators or siderophores (Cox *et al.*, 1981; Ankenbauer *et al.*, 1985; Abdallah *et al.*, 1989). They are produced in iron-limiting media and function to chelate trace amounts of iron ions for the metabolism of the bacteria. On the bacterial membrane, there are specific ferripyoverdin receptors to collect the iron-siderophore complexes (Sokol, 1984; Meyer *et al.*, 1990; Poole *et al.*, 1991). With the help of elastase, pyoverdin can capture the iron released from the host's transferrin efficiently (Döring *et al.*, 1988). On the other hand, the phenazine pigment, pyocyanin, exerts its toxic effects on the beating of human nasal ciliated epithelial cells *in vitro* (Wilson *et al.*, 1987; Kanthakumar *et al.*, 1993), and on the tracheal mucus velocity *in vivo* (Munro *et al.*, 1989). This results in promoting the colonization of *P. aeruginosa* by

inhibiting the cleansing mechanism of the respiratory tract. Furthermore, the glycolipid rhamnolipid has a detergent-like activity that solubilizes phosphatides on the cell membrane (Kurioka and Liu, 1967). Like pyocyanin, rhamnolipid can suppress ciliary beating (Hingley *et al.*, 1986). It has been suggested that rhamnolipid may work together with phospholipase C to cause tissue damage (Liu, 1979).

D. Treatments for *P. aeruginosa* Infections

1. Antibiotic therapy

According to a recent report by Gibbons (1992), *P. aeruginosa* has been considered as one of the ten major drug-resistant microbes. Antibiotics resistance in *P. aeruginosa* is usually a combination of poor membrane permeability to antibiotics (Angus *et al.*, 1982; Yoshimura and Nikaido, 1982; Trias and Nikaido, 1990) and the presence of ß-lactamase (Sykes and Matthew, 1976) and aminoglycoside-inactivating enzymes (Finland, 1979; Milatovic and Braveny, 1987) that inactivate or modify the antibiotics. Since ß-lactams and aminoglycosides are the two major classes of antibiotics used to treat *Pseudomonas* infections, high resistance rates to these two antibiotics would necessitate the finding of new anti-*Pseudomonas* agents. Drug resistance in *P. aeruginosa* could also be attributed to an alginate coat produced by the mucoid strain, which further limits the accessibility of the antibiotics (Costerton *et al.*, 1990). As a result, complete eradication of *P. aeruginosa* using chemotherapy treatment is virtually never achieved once the organisms have colonized the susceptible host. In order to tackle these problems, a new generation of anti-*Pseudomonas* antibiotics such as fluoroquinolones were introduced. Nevertheless, resistance cases have also been reported (Wolfson and Hooper, 1989). The different studies on antibiotic therapy to *P. aeruginosa* infections are summarized in the excellent reviews by Michel (1988) and Bellido and Hancock (1993).

2. Immunotherapy

An effective way for dealing with the emergence of antibiotic resistance in *P. aeruginosa* would be immunoprophylaxis and immunotherapy. Different types of vaccines have been tried or are under development. These include lipopolysaccharides (Hanessian *et al.*, 1971; Jones *et al.*, 1978; MacIntyre *et al.*, 1986), polysaccharide (Pier and Thomas, 1983), polysaccharide conjugate (Cryz *et al.*, 1989), outer-membrane protein (Lam *et al.*, 1983; Matthews-Greer and Gilleland, 1987), mucoid exopolysaccharide (Pier *et al.*, 1990), flagella (Holder and Naglich, 1986; Rotering and Dorner, 1989), protease (Sezen *et al.*, 1975), elastase (Cryz *et al.*, 1983), exotoxin A (Cryz *et al.*, 1983; Lydick *et al.*, 1985), lipoprotein I (Finke *et al.*, 1991) and pili (Irvin and Paranchych, unpublished data). These vaccines have potential use for the elderly and other patient populations who are at risk to *P. aeruginosa* infections.

Nevertheless, the effectiveness of vaccination depends greatly on the physical condition of the recipients. In the case of immunosuppressed or critically ill patients, passive immunization seems to be the solution. Several *P. aeruginosa* specific immune serum globulin (Bjornson and Michael, 1972; Pollack, 1983) and monoclonal antibodies (Anderson *et al.*, 1989; Doig *et al.*, 1990; Pier *et al.*, 1990) are available. These antibodies have been shown to be effective in blocking the infection of *P. aeruginosa*. However, immunotherapy may be difficult for patients with chronic infections since these patients are not deficient in circulating antibodies to *P. aeruginosa* (Pedersen *et al.*, 1989). In this case, combined treatments with antibiotics may prove to be more efficacious.

E. *P. aeruginosa* Adhesins

1. Role of bacterial adherence to pathogenicity

Bacterial infections involve the interactions between the host tissue and the

pathogen, and has been envisioned as a three-step process, that is, adherence, colonization, and/or invasion (Ofek and Beachey, 1980b). It is generally agreed that bacterial adherence is an important initial event, whereby the pathogen first adheres to a tissue surface (for reviews see Gibbons, 1977; Jones, 1977; Ofek and Beachey, 1980a; 1980b). Hence, bacterial adherence may be considered as a prerequisite step in order for local bacterial proliferation to occur (Krogfelt, 1991). The adherence process is not just limited to pathogenic microorganisms. It is a general characteristic for most microorganisms, which include pathogenic and non-pathogenic (normal flora) bacteria. As a result, the pathogenic bacteria have to compete with the normal flora in order to adhere to specific sites on the host tissue (Arbuthnott and Smyth, 1979).

Since the overall surface charges of both eukaryotic and prokaryotic cells are negative, attractive forces must then be presented in order to overcome the repulsive force between the two different surfaces during the adherence process (Ofek and Beachey, 1980b). This could be attained by means of adhesins, which function to counteract the repulsive electrostatic forces by forming the bridges between the two interacting surfaces (Jones and Isaacson, 1983).

In overview, two important roles can be attributed to bacterial adherence: first, it allows the microorganism to resist the mechanical flushing actions of the host cleansing mechanism, for example, peristalsis and ciliary action; and second, it ensures further colonization of the host tissues. Thus, the failure of bacterial adherence to the host would result in the bacterium being removed from the local environment.

2. *P. aeruginosa* adhesins

Despite the fact that *P. aeruginosa* produces a wide variety of virulence factors, they rarely infect healthy individuals with intact immune systems. Indeed, it remains as an opportunistic pathogen and waits for the opportunity to adhere and initiate the infection process. It has been found that the ability of *P. aeruginosa* to persist in the

respiratory tract is correlated with the organism's ability to adhere to the upper respiratory epithelium (Johanson *et al.*, 1969; 1980; Woods *et al.*, 1980a). Therefore, adhesin-mediated adherence seems to be a prerequisite step that allows *P. aeruginosa* to be able to exert the actions of other virulence factors subsequently.

Various types of adhesins have been identified in *P. aeruginosa*, for instance, the pilus, alginate, exoenzyme S, and presumably the less characterized outer membrane proteins. *P. aeruginosa* pili are believed to be the primary adhesin that facilitates the initial adherence process. Characterization of the *P. aeruginosa* pili will be discussed in the next section.

Alginate or mucoid exopolysaccharide is a heteropolymer of D-mannuronic and L-guluronic acids (Linker and Jones, 1966). The occurrence of alginate from isolates of all chronic infection cases is believed to be related to the persistence of *P. aeruginosa* in long term infection. It has been shown that alginate bound to the low affinity receptors on buccal epithelial cells (McEachran and Irvin, 1985; Doig *et al.*, 1987). Alginate was identified as an adhesin because the binding of purified alginate and the mucoid strains of *P. aeruginosa* to human epithelial cells could be reduced by anti-alginate antibodies (Ramphal and Pier, 1985). Recently, the role of alginate as an adhesin has been confirmed by using alginase and anti-alginate monoclonal antibodies to inhibit the adherence of mucoid *P. aeruginosa* (Mai *et al.*, 1993). In addition, alginate also functions as a protective barrier that protects the bacterial colony against attack by viruses, and phagocytes as well as the host humoral defense system (Costerton *et al.*, 1978).

Exoenzyme S is a dual function protein, which acts as a toxin and an adhesin. Its adhesin role was recognized by the fact that pili or anti-pilus antibodies cannot completely inhibit the binding of whole bacteria to epithelial cells, and that purified exoenzyme S and monoclonal antibody to exoenzyme S virtually eliminated the binding (Baker *et al.*, 1991). Using immunogold electron microscopy, exoenzyme S was

found to locate on the bacterial surface (Baker *et al.*, 1991). Further, the binding specificity of exoenzyme S to glycosphingolipids has been shown to be similar to that of the pili and their peptide analogs (Baker *et al.*, 1991; Paranchych *et al.*, 1991). Thus, it was suggested that exoenzyme S and the pili may have similar binding domains, and this inference was supported by a recent report that an amino acid sequence in exoenzyme S is homologous to the C-terminal binding domain of several pilin strains (Woods, 1991).

P. aeruginosa also produces two cytoplasmic lectins, PA-I and PA-II, which are specific for D-galactose and D-mannose, respectively (Gilboa-Garber, 1986). The role of these lectins as adhesins has been doubted since they are mainly found in the cytoplasm and in the periplasmic space with a low exposure on the bacterial surface (Glick and Garber, 1983). However, antibodies produced against PA-I lectin were found to cause agglutination of the bacteria (Avichezer *et al.*, 1992), and thus led to the postulation that bacterial adhesion could be mediated by small amounts of lectins exposed on the cell surface or by lectins released following cell lysis (Gilboa-Garber, 1986; Baker, 1993). Recently, the amino acid sequence of the PA-I lectin has been determined (Avichezer *et al.*, 1992).

During the binding studies of the non-piliated *P. aeruginosa* mutants to epithelial cells, low level attachment was usually observed (Saiman *et al.*, 1990; Ramphal *et al.*, 1991b). Simpson and coworkers (1992) have reported the identification of two classes of non-pilus adhesins, which showed different binding specificity to cultured A549 cells and mucins. These observations have led to the suggestion that non-pilus adhesins such as the outer membrane proteins could also contribute to adherence.

F. *P. aeruginosa* Pilus

1. General characterization

Pilus is a proteinacous, filamentous surface appendage whose size is thinner than the flagellum and appears either polarly or peritrichously on the microorganism. In 1950, Houwink and van Iterson (1950) reported the observation of non-flagellar filamentous structures at the polar end of *P. aeruginosa*. They called this structure "filament" in order to differentiate it from the flagellum. A few years later, Duguid and coworkers (1955) named this filamentous structure as "fimbria" (Latin for thread, fiber or fringe), whereas Brinton (1959) introduced another term "pilus" (Latin for hair or fur) to describe the same structure. Although Ottow (1975) proposed the use of the term "fimbria" for the adhesive structure while reserving the designation "pilus" for the sexual (conjugative) appendage of the bacteria, both terms are still being used interchangeably. For simplicity purpose, only the term pilus is used in this thesis to refer to this filamentous structure.

The *P. aeruginosa* pili is polarly located, with a structure resembling a hollow tube of 5.2 nm in outer diameter, 1.2 nm in central channel diameter, and an average length of 2.5 μm (Bradley, 1972c; Folkhard *et al.*, 1981; Paranchych *et al.*, 1986). Unlike the type I pili or Pap pili that consist of multi-components, *P. aeruginosa* pili are composed of a single protein subunit termed pilin. The pilus is an assemble of pilin subunits in a helical array of five subunits per turn with a pitch of 4.1 nm (Folkhard *et al.*, 1981; Watts *et al.*, 1983a). The *P. aeruginosa* strain PAK pilin has an amino acid sequence of 144 residues with a molecular weight of 15 K (Sastry *et al.*, 1983; Watts *et al.*, 1983a). More than eight different prototypes of the pilin gene have been identified (Paranchych *et al.*, 1990; Castric and Deal, 1994) (Fig. I.1). Studies on the frequency distribution of these pilin genes among clinical and environmental isolates have led to an estimation that there could be as many as nine unique pilin types (Paranchych *et al.*,

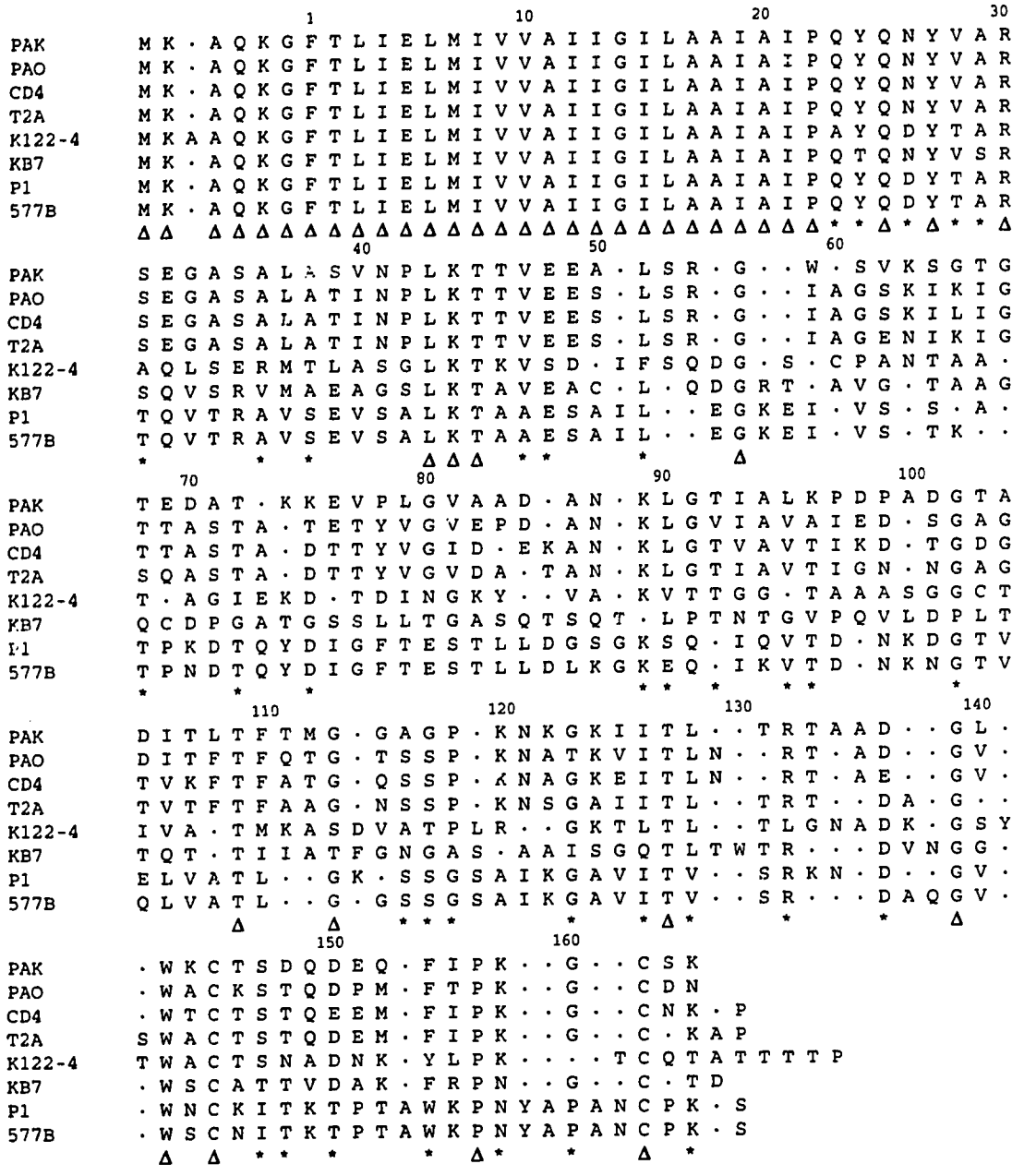

Figure I.1 Published amino acid sequences of various *P. aeruginosa* pilin strains: PAK (Sastry *et al.*, 1985a), PAO (Sastry *et al.*, 1985b), CD4 (Pasloske *et al.*, 1988a), T2A (Castric and Deal, 1994), K122-4 (Pasloske *et al.*, 1988b), KB7 (Paranchych *et al.*, 1990), P1 (Pasloske *et al.*, 1988b), and 577B (Castric and Deal, 1994). Conserved residues are indicated by triangle symbols (Δ) and semi-conserved residues are denoted by stars (*).

1990). Comparison of the predicted amino acid sequences of various pilin types suggested that the pilin can be roughly divided into three regions: a highly conserved N-terminal sequence, a semi-conserved C-terminal sequence with an intrachain disulfide bridge and a variable central region sequence (Paranchych and Frost, 1988; Paranchych *et al.*, 1990).

The N-terminal sequence of the *P. aeruginosa* pilin is highly homologous to the pilin sequences found in *Neisserie gonorrhoea*, *N. meningitidis*, *Dichelobacter nodosus*, *Moraxella bovis*, *M. nonliquefaciens*, and *Vibrio cholerae* (Elleman, 1988; Faast *et al.*, 1989). With the exception of *V. cholerae*, the pilins of the above species contain the modified amino acid N-methyl-phenylalanine as the first residue at the N-terminal sequence of the pilins (Frost *et al.*, 1978; Hermodsen *et al.*, 1978; McKern *et al.*, 1983; Paranchych, 1989) and are referred to as the N-methylphenylalanine (NMePhe) pili (Elleman *et al.*, 1986; Paranchych and Frost, 1988). This group of pili has also been designated as the type 4 pili based on morphological studies (Ottow, 1975; Mattick *et al.*, 1987). Recently, antigenicity studies have illustrated that antisera raised against the pili of *P. aeruginosa*, *M. bovis*, *N. gonorrhoeae*, *B. nodosus*, and *V. cholerae* shared some degrees of crossreactivity among the pili from these species (Patel *et al.*, 1991).

P. aeruginosa pili have several biological roles. They mediate the adherence of nonmucoid *Pseudomonas* strains to human epithelial cells (Woods *et al.*, 1980b; Ramphal *et al.*, 1984; Doig *et al.*, 1988). They also serve as receptor sites for pilus-specific bacteriophages (Bradley, 1973; Bradley and Pitt, 1974), and are responsible for the twitching motility of *P. aeruginosa* lacking flagella (Lautrop, 1965; Henrichsen, 1972; Bradley, 1980). In addition, *P. aeruginosa* pili are retractable (Bradley, 1972a; 1972b) and the retractability of the pili is correlated with the twitching motility of the bacteria as well as the susceptibility of the bacteria to the pilus-specific bacteriophages (Bradley, 1974a; 1980). Also, they are potent immunogens (Watts *et al.*, 1983b) and

are important for nonopsonic phagocytosis (Kelly *et al.*, 1989; Mork and Hancock, 1993).

2. Pilus-mediated adherence of *P. aeruginosa*: A model

P. aeruginosa employ pili to facilitate their adherence to various human epithelial cells, which include buccal epithelial cells (Woods *et al.*, 1980b; Doig *et al.*, 1988), tracheal epithelial cells (Ramphal *et al.*, 1984), corneal epithelial cells (Reichert *et al.*, 1983; Rudner *et al.*, 1992), epidermal cells (Sato and Okinaga, 1987), and culture pneumocyte cells (Chi *et al.*, 1991). The sequence of events that occur during the adherence process are depicted in Figure I.2. Unlike the Pap pilus in *E. coli*, which requires a pilus-associated protein with an adhesin function (Hultgren *et al.*, 1991), *P. aeruginosa* pilin itself functions as the adhesin (Irvin *et al.*, 1989; Irvin, 1993). Recent studies have shown that the pilus-mediated adherence of *P. aeruginosa* is a tip-associated event where the adherence-binding domain is exposed at the tip of the pilus (Lee *et al.*, 1994). The bound pili then serve as a bridge to counteract the repulsive forces between the two cell surfaces and mediate the initial attachment of the bacterium (step A and B, Fig. I.2). Upon the binding of the pili to the cell receptors, other adhesins such as exoenzyme S on the bacterial surface may be allowed to interact with the epithelial cell receptors and result in tighter attachment of the microorganism (step C, Fig. I.2). It is possible that the retractile mechanism of the pili may draw the bacterial cell closer to the epithelial surface and allow multiple interactions between the two surfaces to take place. The non-retractile mutants were reported to be less adherent than wild-type strains (Bradley and Pitt, 1974; Baker, 1993).

During chronic infection, phenotypic conversion of the non-mucoid *Pseudomonas* strain into the mucoid strain is most likely to happen (Fegan *et al.*, 1990; Woods *et al.*, 1991; 1993). The mucoid strain produces alginate that protects the bacterial cell from being swept away by the host defense mechanism, as well as to provide additional

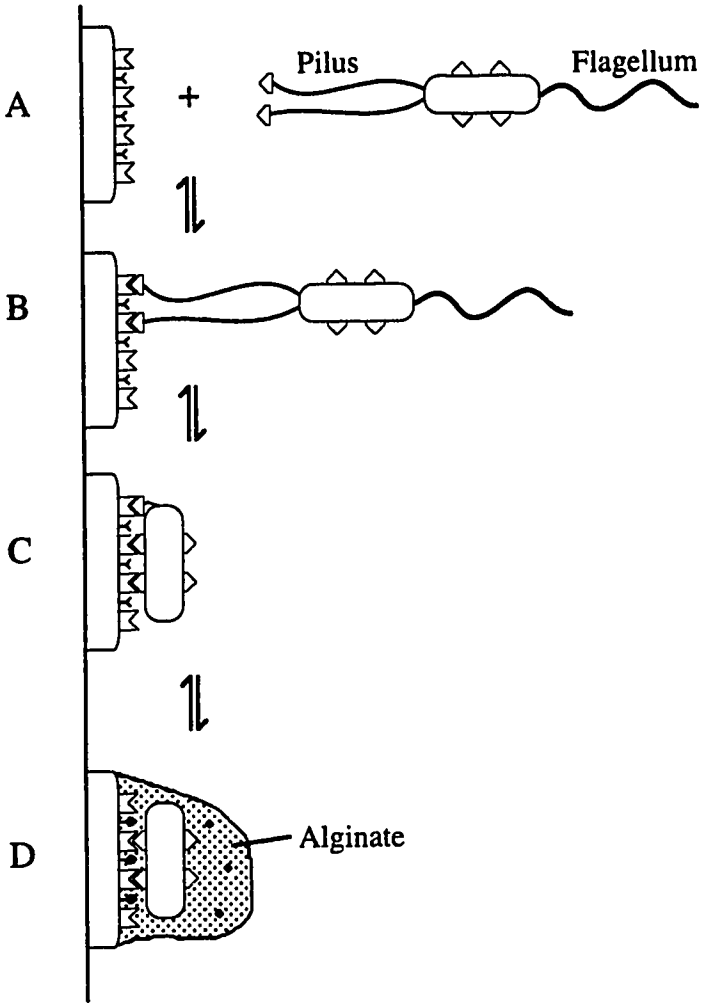

Figure I.2 A schematic model for the pilus-mediated adherence of *P. aeruginosa* to epithelium. (A-B) Pilus-mediated receptor interactions. (C) Pilus retraction and interactions between the two cell surfaces via adhesins on the outer membrane of the bacterium. (D) Phenotypic conversion of the non-mucoid strain into the mucoid phenotype during chronic infection. Alginate is produced to secure the attachment of the colony. ◁ is either the pilus adhesin or outer membrane adhesin such as exoenzyme S, and ◺ is their corresponding receptor; ◆ is the adhesin component present in the alginate, and ⊰ is the proposed low-affinity receptor for alginate binding. This figure is adapted from Baker and Svanborg-Edén, 1989; Costerton et. al., 1990; and Baker, 1993.

adhesion through the binding to the proposed low-affinity receptors on the epithelial surface (step D, Fig. I.2) (McEachran and Irvin, 1985). The mucoid colony continues to persist and proliferate.

3. Biogenesis and assembly of pili

The biogenesis of pili in *P. aeruginosa* requires the expression of several genes (Table I.3). These genes are designated as *pilA*, *pilB*, *pilC*, *pilD*, *pilQ*, *pilR*, *pilS* and *rpoN* (also called *glnF* and *ntrA*). The gene *pilA* encodes the type 4 structural pilin protein of *P. aeruginosa* and its transcription is under the control of RpoN (Ishimoto and Lory, 1989), an alternate sigma factor known as σ^{54}, as well as two regulatory genes *pilR* and *pilS* (Ishimoto and Lory, 1992). RpoN is also important for the transcription of flagellin gene (Totten *et al.*, 1990), and other non-pilus mucin-binding adhesins on the surface of the bacteria (Ramphal *et al.*, 1991b). A common transcriptional activation mechanism has been noted in several genes regulated by RpoN (Ronson *et al.*, 1987). This type of regulatory system consists of a two-component signal-transducing system in which a sensory protein responds to an environmental stimulus and transmits the signal by modifying a regulatory protein that binds to the DNA sites and finally activates gene transcription. PilR and PilS are two-component regulatory elements found in the regulation of the *pilA* gene. Signal transduction is performed utilizing a phosphotransfer mechanism (Stock *et al.*, 1989; Hobbs *et al.*, 1993), in which PilS is autophosphorylated and then the phosphate is transferred to the transcriptional activator, PilR, that coordinately functions with RpoN to activate *pilA* transcription (Ishimoto and Lory, 1992). Three accessory genes, *pilB*, *pilC* and *pilD*, are also important for the biogenesis and assembly of *P. aeruginosa* pili. Both PilB and PilC have been found to be important for pilus assembly, however, their exact activity is still unclear (Nunn *et al.*, 1990). PilD is a bifunctional enzyme that exhibits two major functions: first, it acts as an endopeptidase that recognizes and

Table I.3 Genes required for the biogenesis, assembly and functionality of the *P. aeruginosa* pili

Gene	Functions	References
pilA	Pilin structural gene	Johnson *et al.*, 1986.
pilB	Pilus assembly	Nunn *et al.*, 1990.
pilC	Pilus assembly	Nunn *et al.*, 1990.
pilD	Prepilin leader peptidase	Nunn *et al.*, 1990; 1991; Strom and Lory, 1992.
	Methyltransferase	Strom *et al.*, 1993a; 1993b.
	Protein-excretion apparatus processing	Nunn and Lory, 1992; Strom *et al.*, 1991.
pilG	Pilus biosynthesis and twitching motility	Darzins, 1993.
pilH	Twitching motility	Darzins, 1994.
pilI	Pilus biosynthesis and twitching motility	Darzins, 1994.
pilJ	Pilus biosynthesis and twitching motility	Darzins, 1994.
pilQ	Pilus assembly	Martin *et al.*, 1993.
pilR	Regulatory gene for pilA	Hobbs *et al.*, 1993; Ishimoto and Lory, 1992.
pilS	Regulatory gene for pilA	Hobbs *et al.*, 1993.
pilT	Twitching motility	Whitchurch *et al.*, 1991.
rpoN	Encoding the σ^{54} subunit of RNA polymerase	Ishimoto and Lory, 1989; Totten *et al.*, 1990.

cleaves the leader peptides of the *P. aeruginosa* prepilin (Nunn and Lory, 1991; Strom and Lory, 1992). It also acts on other proteins with type 4 pilin-like amino termini. Recently, it has been reported that *pilD* was important for the proper functioning of extracellular protein excretion since an accumulation of proteins such as alkaline phosphatase, phospholipase C, elastase and exotoxin A were observed in the periplasmic space of the *pilD* mutant (Strom *et al.*, 1991; Nunn and Lory, 1992). Second, it catalyzes the N-methylation of the N-terminal phenylalanine residue of the prepilin right after the removal of the leader sequence, and S-adenosyl-L-methionine is believed to be the methyl donor (Strom *et al.*, 1993a; 1993b). Recently, a new pilin gene called *pilQ* was identified (Martin *et al.*, 1993). This gene encodes a 77 kD protein that is important for the assembly of pilus in *P. aeruginosa*. It was proposed by Martin and coworkers (1993) that PilQ could possibly serve as a "porthole" on the outer membrane, which allows the assembling pilus to pass through the outer membrane. Another five pilus genes, *pilG* (Darzins, 1993), *pilH*, *pilI*, *pilJ* (Darzins, 1994), and *pilT* (Whitchurch *et al.*, 1991) were reported to be related to the twitching motility phenotype of the *Pseudomonas aeruginosa* pilus. The *pilG*, *pilH*, *pilI* and *pilJ* gene products were suggested to be part of a signal-transduction network that controls pilus biosynthesis and twitching motility (Darzins, 1993; 1994), whereas pilT is presumably required for pilus retraction (Whitchurch *et al.*, 1991).

4. Immunological properties of *P. aeruginosa* pili

The antigenic determinants of the pilin protein have been studied by means of arginine-specific cleavage (Watts *et al.*, 1983b; Sastry *et al.*, 1985a). There are three arginine residues in the sequence and thus such a cleavage results in four fragments: cTI (1-30), cTII (31-53), cTIII (54-120) and cTIV (121-144) (Paranchych *et al.*, 1985). cTIII and cTIV were further cleaved with chymotrypsin, trypsin, pepsin and thermolytic protease into various subfragments (Sastry *et al.*, 1985b). Results from

direct ELISA and immunoblot experiments have identified four antigenic determinants in PAK pilin: one in the cTI fragment, two in the cTIII fragment and one in the cTIV fragment (Watts *et al.*, 1983b). Since only cTIV caused a significant effect on *P. aeruginosa* binding to buccal epithelial cells (Paranchych *et al.*, 1986; Doig *et al.*, 1988), this region was subjected to extensive studies. Lee *et al.* (1989a) have delineated the antigenic region of the PAK pilus from its primary sequence using the predictive algorithm developed by Parker *et al.* (1986), which employs the hydrophilicity, accessibility and mobility parameters to predict possible antigenic regions. The prediction results were confirmed by synthetic peptide immunization studies (Lee *et al.*, 1989a). The C-terminal disulfide-bridged 17-residue region of the PAK pilin was found to be important in raising antibodies that block the binding of both the bacteria and their pili to epithelial cells (Lee *et al.*, 1989b; 1990b; Doig *et al.*, 1990).

5. *P. aeruginosa* pilus receptors

Several recent investigations have identified various receptor candidates for the pilus-specific interactions. Examples include glycosphingolipids such as asialo-GM_1 and asialo-GM_2, sialic acid-containing glycosphingolipids and lactosylceramide (Krivan *et al.*, 1988a; Baker *et al.*, 1990), and glycoproteins (Doig *et al.*, 1989; Irvin *et al.*, 1989; Rudner *et al.*, 1992). The carbohydrate moieties of both glycosphingolipids and glycoproteins are believed to be involved in pilus binding. For example, the minimum disaccharide binding sequence of asialo-GM_1 and asialo-GM_2 was identified as ßGalNAc(1-4)ßGal (Krivan *et al.*, 1988b; Sheth *et al.*, 1994); whereas type 1 ßGal(1-3)ßGlcNAc and type 2 ßGal(1-4)ßGlcNAc disaccharides have been reported as the minimum binding sequence found in mucin glycoproteins (Ramphal *et al.*, 1991a).

G. Synthetic Peptide Approach to Study Biological Processes

1. History of synthetic peptides

In the first half of the last century, the term "albuminoids" was first used by F. Magendie to describe an important category of organic substances during his studies on animal nutrition (Wieland and Bodanszky, 1991). This category of substances was later known as proteins (Greek *proteios*, primary; or *protos*, first), which depict the importance of this substance to life. At the end of nineteenth century, it became well established that proteins are composed of α-amino acids. As enzymatic hydrolysis was used to study the proteins, another term known as peptones or peptides (Greek *pepsis*, digestion) appeared to describe small protein fragments produced by partial digestion. However, during that time, little was known about how the α-amino acids assemble to form the protein structure. Since the beginning of this century, tremendous efforts have been made in order to develop the chemistry required for peptide synthesis. Important events in the historical development of synthetic peptide chemistry are summarized in Table I.4, and have been discussed in several excellent books and reviews (Bodansky *et al.*, 1976; Eberle *et al.*, 1981; Bodanszky, 1988; Wieland and Bodanszky, 1991; Grant, 1992).

The first key development was the identification of peptide bonds in proteins. It was first suggested by E. Grimaux in 1882 that proteins were formed by amide-like linkages (Wieland and Bodanszky, 1991). In 1902, the peptide bond was first recognized by Hofmeister (1902) as a linkage between carboxyl and amino groups of the α-amino acids. The first peptide bond was formed unintentionally early in 1881 when T. Curtius tried to benzoylate glycine-silver with benzoylchloride to synthesize hippuric acid (benzoylglycine). To his surprise, besides hippuric acid, he obtained the peptide derivative benzoylglycylglycine as well (Curtius, 1881). Twenty years later, Fischer *et al.* (1901) reported the synthesis of the first dipeptide, glycylglycine, by

Table I.4 Chronology of Synthetic Peptide Chemistry

Year	Event	Reference
1881	Earliest modes of peptide bond formation	Curtius, 1881.
1901	Description of the first prototype glycylglycine peptide	Fischer and Fourneau, 1901.
1902	Recognition of peptide bond in proteins	Hofmeister, 1902; Fischer, 1906.
1907	Synthesis of an 18-residue peptide	Fischer, 1907.
1932	Introduction of the amino-protecting benzyloxycarboxyl group	Bergman and Zervas, 1932.
1951	Invention of alkyl-carbonic acid mixed anhydrides	Boissonnas, 1951; Wieland and Bernhard, 1951.
1953	Synthesis of the first peptide hormone, oxytocin	du Vigneaud *et al.*, 1953.
1953	Determination of the structure of insulin	Sanger and Tuppy, 1951; Sanger and Thompson, 1953.
1955	Development of active esters	Bodanszky, 1955; Schwyzer *et al.*, 1955.
1955	Introduction of dicyclohexylcarbodiimide	Sheehan and Hess, 1955.
1957	Acid labile blocking groups such as t-butyloxycarbonyl group	Carpino, 1957.
1958	Development of automated amino acid analysis	Spackman *et al.*, 1958.
1963	Solid phase peptide synthesis	Merrifield, 1963.
1967	Automatic amino acid sequencing	Edman and Begg, 1967.
1972	Fmoc (9-fluorenylmethyloxycarbonyl) chemistry	Carpino and Han, 1972.
1984	Combinatorial peptide library	Geysen *et al.*, 1984; Houghten, 1985.

partial HCl hydrolysis of the diketopiperazine of glycine. These two investigations established the basis for peptide synthesis. These early modes of peptide synthesis could only be performed on the carboxylic acid terminus of the amino acid. Without the knowledge of blocking groups, the longest peptide sequence that could be made at that time was only eighteen residues in length (Fischer, 1907).

A turning point came when Bergman (1932) created the N-terminal protecting group for the amino acids, which could be cleaved without damaging the peptide bond formed. These began a new epoch in peptide chemistry and the area became the fertile ground for the development of other easily removable and specific protecting groups, in particular, the acid-labile *tert*-butyloxycarbonyl group (Boc) by Carpino (1957) and by McKay and Albertson (1957). Later in 1972, an alternate alkaline-labile protecting group, the 9-fluorenylmethoxy-carbonyl group (Fmoc), was developed (Carpino and Han, 1972).

Another important event in peptide chemistry was the improvement in coupling methods. The discovery of amino-protecting group stimulated the search for better coupling methods. In 1951, different alkyl-carbonic acid mixed anhydrides were invented in order to enhance the formation rate of the peptide bond (Boissonnas, 1951; Wieland and Bernhard, 1951). These successful improvements led to the synthesis of the first peptide hormone, oxytocin (du Vigneaud *et al.*, 1953). The mixed anhydride approach stimulated the concept of active esters in peptide synthesis. In 1955, Schwyzer and coworkers (1955) described the use of cyanomethyl ester to increase the reaction rate. In the same year, Bodanszky (1955) observed enhanced aminolysis rates when using thiophenyl esters. Aside from the mixed anhydrides and active esters, coupling reagent dicyclohexylcarbodiimide (DCC) was a remarkable discovery in peptide chemistry (Sheehan and Hess, 1955). DCC is still widely used today. The impact of DCC on the development of peptide chemistry was stated by Wieland (1981) in his review, "This reagent [DCC] acquired the top position among the methods for

coupling longer segments, ...", and "Without the introduction of DCC, it is very possible that the solid-phase technique would not have been introduced by R.B. Merrifield in 1962".

The concept of solid phase peptide synthesis (SPPS) was inspired by the realization that simplicity, speed and automation of peptide synthesis could be achieved if the amino acid was linked to an insoluble polymer support on which stepwise coupling is performed. In 1963, Merrifield (1963) published a landmark paper describing this technique, and very soon, it became the pillar of today's peptide synthesis methodology. The procedure of the SPPS method will be discussed in detail in Chapter II (Materials and Methods). Since the advent of the SPPS, peptide synthesis has become a routine practice in many laboratories worldwide and has opened the way to widespread use of synthetic peptides in areas such as immunology, biophysics and structure-function studies of protein.

2. Synthetic peptides as biomimetics

Nature has selected proteins as the crucial macromolecules in most biological processes because of their unique physicochemical properties that meet the needs of diversity in the living system. Life processes such as signal transduction, defense mechanisms, and enzymatic reaction all require specific interactions between macromolecules. Proteins fulfill the need of this wide range of molecular recognitions by providing both functional and structural diversity. Since proteins play a key role in virtually all life processes, understanding protein structure will certainly be beneficial in development of therapeutics. However, structural complexity of proteins has slowed down the work on protein research despite the development of various physical and chemical techniques.

The advent of synthetic peptides has provided a new tool for protein chemists. Working with a synthetic peptide is much more manageable than with the whole protein

and thus a more promising approach. The early success in synthesizing the small biologically active peptide (Harington and Mead, 1935) and the peptide hormone oxytocin (du Vigneaud *et al.*, 1953) established the potential of the synthetic peptide approach in mimicking naturally occurring peptides. Wieland (1981) once described these particular events in his review: "Since 1935, when Harington's synthesis of glutathione opened the door to peptide synthesis a crack, and du Vigneaud *et al.* in 195 pushed the door wide open with their synthesis of oxytocin, several thousand peptides have been chemically prepared". Today, synthetic peptides are being used in various areas such as investigations of antigenicity and immunogenicity of proteins, development of enzyme inhibitors, and studies of ligand-receptor interactions and protein-protein interactions.

In immunological studies, one of the primary roles of synthetic peptides is to identify the antigenic epitopes on the protein molecule. This can be achieved by making all the possible overlapping peptides covering the highly antigenic sequences (Geysen *et al.*, 1986) and subsequent determination of their antibody binding efficiency by enzyme-linked immunosorbent assay (ELISA). This work can be simplified by constructing libraries of peptides by either biological (Cwirla *et al.*, 1990; Scott and Smith, 1990) or chemical (Geysen *et al.*, 1984; Houghten, 1985) means. The epitope sequences obtained can be used to elucidate the mechanism of immune response (Smith, 1989), to study antibody-antigen interactions (Rini *et al.*, 1992), or to develop synthetic peptide vaccines (Jocobs *et al.*, 1983; Brown, 1989). Since the immune system is designed to recognize millions of diversified antigens including the peptides, the use of synthetic peptides as probes to study the immunological events is a natural choice. The successes in using peptides to produce antibodies that cross-react with the native protein has established a role for synthetic peptides in vaccine development. Several synthetic peptide vaccines have been developed and have demonstrated promising results. Examples are the foot and mouth disease virus (Brown, 1989),

diphtheria (Audibert *et al.*, 1981), cholera (Jocobs *et al.*, 1983), malaria (Richman *et al.*, 1989), and hepatitis B (Manivel *et al.*, 1993).

The synthetic peptide approach has also been used to design potent inhibitors for certain enzymatic processes such as the serine protease thrombin inhibitor for the prevention of blood clots (Stone and Hofsteenge, 1986; Yao *et al.*, 1992) and HIV protease inhibitors (Cieplak and Kollman, 1993; Lambert *et al.*, 1993). Furthermore, studies on ligand-receptor interactions are also an active research area where synthetic peptides are being used, for instance, the interactions between fibrinogen and the platelet integrin (Bajt *et al.*, 1992; Du *et al.*, 1993), substance P antagonists (Schwyzer, 1992), oxytocin antagonists (Melin *et al.*, 1983), somatostatin analogs (Freidinger and Veber, 1984), and the nucleotide phosphate binding sites (Keane *et al.*, 1993). In addition, synthetic peptides were also used to examine the protein-protein interactions during muscle regulation (Van Eyk and Hodges, 1993).

H. Aims of Investigation

The prevalence of *P. aeruginosa* infections as important nosocomial diseases imposes increased mortality and health-care costs on our society. Extensive research on the pathogenesis of *P. aeruginosa* has been carried out since the pathogenic nature of this microorganism was recognized. As we have learned, *P. aeruginosa* cells employ adhesins to bind to mucosal surfaces, and these cells become very difficult to eradicate once they start to convert to the mucoid phenotype (Woods *et al.*, 1991; Woods, 1993). Hence, one of the most effective ways to prevent *P. aeruginosa* infections is by early intervention (Baker, 1993).

It has become clear that the *P. aeruginosa* pili play a major role for the initial attachment of the microorganism to its host. In this study, the *P. aeruginosa* pilus adhesin was chosen as the subject of investigation. The ultimate goal of this study was

to obtain information that will enable us to develop therapeutics against *P. aeruginosa* adherence. An overview of this goal is outlined in Figure I.3. In order to achieve this goal, two biological roles of *P. aeruginosa* pili were studied: the antigenicity and the adherence properties. To summarize, understanding the pilus-antibody interactions and the pilus-receptor binding process were the two directions that were pursued in this study.

Previous studies on the antigenicity of the *P. aeruginosa* strain PAK pili have identified an antigenic region that contains the adherence binding domain (Watts *et al.*, 1983b; Paranchych *et al.*, 1985; Sastry *et al.*, 1985b; 1986; Doig *et al.*, 1988; Lee *et al.*, 1989a). This region is located at the C-terminal disulfide-bridged 17-residue sequence of the PAK pilin. Both the 17-residue peptide and the antibodies raised against it can effectively block the binding of the bacteria or purified pili to human epithelial cells (Lee *et al.*, 1989b; 1990b). Therefore, a synthetic peptide approach was designed and performed to investigate the molecular basis of the antibody-antigen and pilus-receptor interactions of the *P. aeruginosa* pili.

On the immunologic side, determination of the antigenic epitopes on the adhesin peptide is invaluable to studies of antibody-antigen interactions as well as synthetic vaccine development. New screening techniques making use of combinatorial peptide libraries were examined since this technique can speed up the epitope identification process, and since reactive peptides other than the native sequence (mimotopes) (Geysen *et al.*, 1986) can be identified. On the receptor side, the amino acid residues on the C-terminal sequence of the PAK pilin that were critical for human epithelial cell binding were identified. These residues should reveal important information on the interactions between the pilus adhesin and the host receptors. Although the majority of the research was focused on the PAK C-terminal peptide, attempts were made to compare these results with those from other strains to further expand our understanding of cross-reactive antibody protection and synthetic peptide vaccine development.

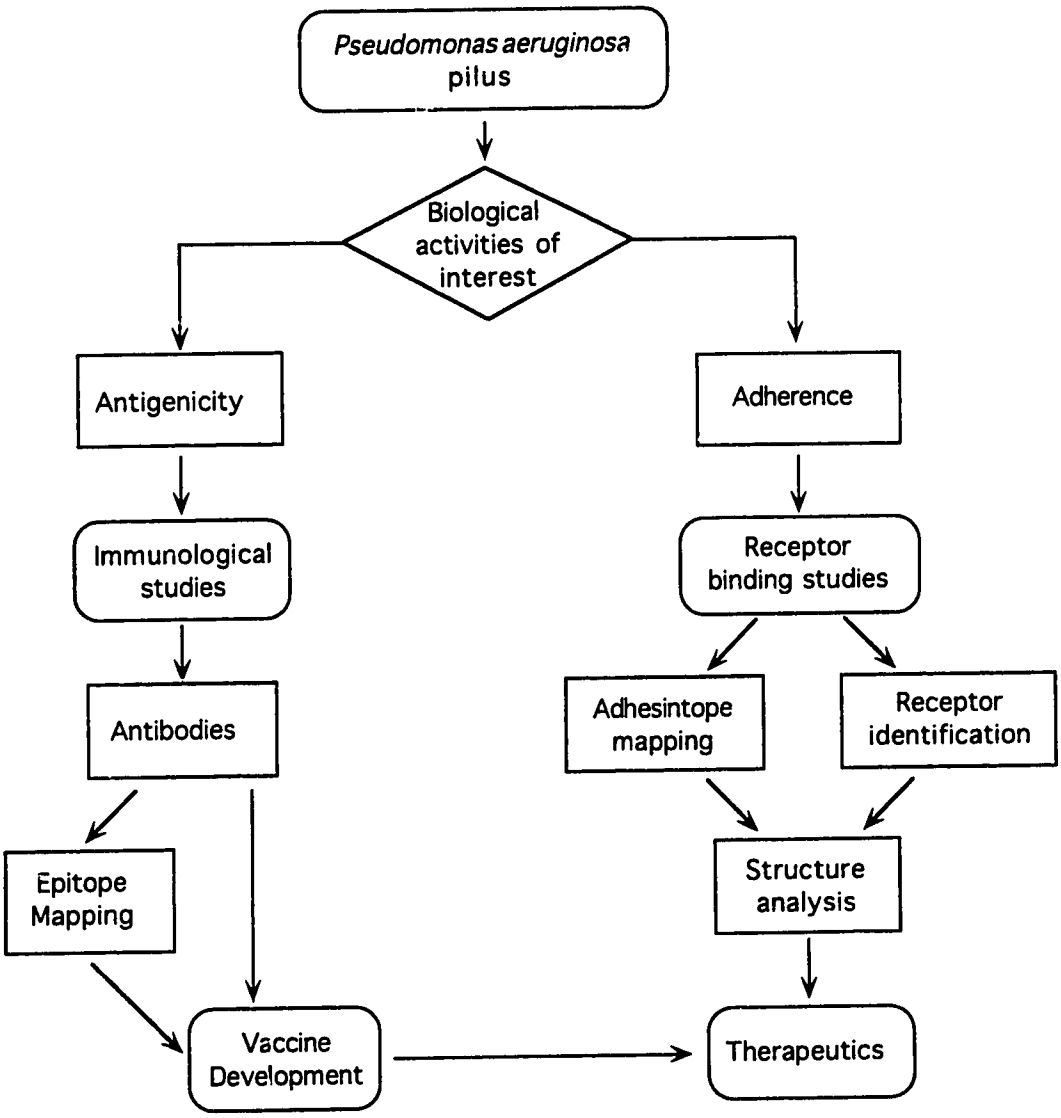

Figure I.3 A schematic diagram outlining the aim of investigation.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Chemicals and reagents

All chemicals and reagents were analytical grade unless otherwise stated. The chemicals and reagents used in this project are listed in Table II.1. All solutions were prepared in double distilled water. DIEA, DCM, and TFA were distilled prior to use.

2. *P. aeruginosa* pili

The bacterial pili used in this study were obtained from the *P. aeruginosa* strains PAK/2pfs, PAO/DB2, KB7, and K122-4. Strain PAK/2Pfs is a PAK multipiliated mutant that is resistant to bacteriophages Pf and PO4 (Bradley, 1974b), and strain PAO/DB2 is a multipiliated mutant obtained from a cross between strains PAO1264 (FP39) and PAO-2001/PP7 in which the non-retractile pilus colonies of PAO-2001/PP7 were selected (Sastry *et al.*, 1985a). All pili were kindly provided by W. Paranchych. Purification of pili was as previously described (Frost and Paranchych, 1977; Paranchych *et al.*, 1979). Briefly, pili were removed from the cells by blending and successively purified by precipitation with polyethylene glycol 6000 (1% w/v final concentration), precipitation with ammonium sulfate (10% w/v) and CsCl density gradient centrifugation. The primary structures of the pilin proteins have been determined and are shown in Figure I.1.

Table II.1 List of chemicals and reagents

Chemicals/Reagents	Suppliers
ABTS	Boehringer Mannheim, Germany
Acetic acid	BDH, Toronto, ON
Acetic anhydride	Aldrich Chemical, Milwaukee, Wisconsin
Acetonitrile	BDH, Toronto, ON
Ammonium bicarbonate, NH_4HCO_3	BDH, Toronto, ON
Ammonium sulfate, $(NH_4)_2SO_4$	Canadian Scientific Products, London, ON
Anisole	Fisher Scientific, Fair Lawn, NJ
Antibiotic-antimycotic solution	GIBCO-BRL, Gaithersburg, MD
Asialo-GM_1	Sigma Chemical, St. Louis, MO
BCA Protein Assay Reagent	Pierce, Rockford, IL
Benzene	J.T. Baker, Phillipsburg, NJ
Benzhydrylamine-HCl resin	Bachem, Torrance, CA
Benzoylbenzoic acid	Aldrich Chemical, Milwaukee, Wisconsin
Biotin	Sigma Chemical, St. Louis, MO
Biotinamidocaproate N-hydroxysuccinimide ester	Sigma Chemical, St. Louis, MO
Boc-amino acids	Bachem, Philadelphia, PA
Boc-lysine-PAM resin	Applied Biosystems, Foster city, CA
BSA	ICN Biomedicals, Aurora, OH
Calcium chloride, $CaCl_2 \cdot 2H_20$	J.T. Baker, Phillipsburg, NJ
Cesium bicarbonate, $CsHCO_3$	J.T. Baker, Phillipsburg, NJ
Chloromethyl resin	Bachem, Torrance, CA
DCC	Pierce Chemical, Rockford, IL
DCM	Anachemia, Rouses Point, NY
DIEA	Aldrich Chemical, Milwaukee, Wisconsin
Diethanolamine	Fisher Scientific, Fair Lawn, NJ
Diethyl ether	BDH, Toronto, ON
DME Medium	GIBCO-BRL, Gaithersburg, MD
DMF	General Intermediates of Canada, Edmonton
DMSO	BDH, Toronto, ON
DTT	ICN Biomedicals, Aurora, OH
EDT	Aldrich Chemical, Milwaukee, Wisconsin

Chemicals/Reagents	Suppliers
EDTA	BDH, Toronto, ON
EGTA	Sigma Chemical, St. Louis, MO
Ethanol	BDH, Toronto, ON
Fetal bovine serum	GIBCO-BRL, Gaithersburg, MD
Fmoc amino acids OPfp-esters	Novabiochem, La Jolla, CA
Freund's complete adjuvant	GIBCO-BRL, Gaithersburg, MD
Freund's incomplete adjuvant	GIBCO-BRL, Gaithersburg, MD
Gelatin	Bio-Rad Laboratories, Richmond, CA
D-Glucose	BDH, Toronto, ON
L-Glutamine	GIBCO-BRL, Gaithersburg, MD
Glycerol	BDH, Toronto, ON
Glycine	Schwarz/Mann Biotech, Cleveland, OH
Goat anti-mouse IgG-horseradish peroxidase	Jackson Laboratories, CA
Goat anti-rabbit IgG-horseradish peroxidase	Jackson Laboratories, CA
HAT supplement	GIBCO-BRL, Gaithersburg, MD
HBTU	Advanced ChemTech, Louisville, KY
HOBt	Bachem, Torrance, CA
Hydrochloric acid, HCl	BDH, Toronto, ON
Hydrogen fluoride, HF	Matheson Gas Products, Edmonton, AB
Hydrogen peroxide, H_2O_2	Fisher Scientific, Fair Lawn, NJ
KLH	Sigma Chemical, St. Louis, MO
Magnesium chloride, $MgCl_2 \cdot 6H_20$	BDH, Toronto, ON
Magnesium sulfate, $MgSO_4$	Fisher Scientific, Fair Lawn, NJ
Manganese chloride, $MnCl_2$	J.T. Baker, Phillipsburg, NJ
4-Methylmopholine	Aldrich Chemical, Milwaukee, Wisconsin
Methanol	BDH, Toronto, ON
NEM	Sigma Chemical, St. Louis, MO
Ninhydrin	Applied Biosystems, Foster City, CA
p-Nitrophenylphosphate	Sigma Chemical, St. Louis, MO
NMP	General Intermediates of Canada, Edmonton
Nonidet P-40	Sigma Chemical, St. Louis, MO
NovaSyn PR 500 resin	Novabiochem, La Jolla, CA
Phenol	GIBCO-BRL, Gaithersburg, MD
Phosphorus pentoxide, P_2O_5	BDH, Toronto, ON

Chemicals/Reagents	Suppliers
Picric acid	BDH, Toronto, ON
Potassium chloride, KCl	BDH, Toronto, ON
Potassium dihydrogen phosphate, KH_2PO_4	BDH, Toronto, ON
Pristane	Sigma Chemical, St. Louis, MO
Rink-AM resin	Aminotech, Nepean, ON
Sodium azide, NaN_3	J.T. Baker, Phillipsburg, NJ
Sodium bicarbonate, $NaHCO_3$	BDH, Toronto, ON
Sodium chloride, NaCl	BDH, Toronto, ON
Sodium citrate	Fisher Scientific, Fair Lawn, NJ
Sodium hydroxide, NaOH	BDH, Toronto, ON
Sodium phosphate dibasic, Na_2HPO_4	Fisher Scientific, Fair Lawn, NJ
Sodium pyruvate	GIBCO-BRL, Gaithersburg, MD
Streptavidin-alkaline phosphatase	GIBCO-BRL, Gaithersburg, MD
Substrate Amplification Kit	GIBCO-BRL, Gaithersburg, MD
Sulfuric acid, H_2SO_4	BDH, Toronto, ON
TFA	Aldrich Chemical, Milwaukee, Wisconsin
Thioanisole	Aldrich Chemical, Milwaukee, Wisconsin
Tris base	Schwarz/Mann Biotech, Cleveland, OH
Tween-20	Bio-Rad Laboratories, Richmond, CA
Urea	ICN Biomedicals, Aurora, OH
Waymouth MB 752/1 medium	GIBCO-BRL, Gaithersburg, MD

3. Peptide nomenclature

(a) *Pilin peptide analogs*

The sequences of all synthetic peptides used in this project are listed in Table II.2. The peptide analogs were named according to the pilin strain, the residues being substituted as well as their corresponding sequence found in the native protein. For instance, Ac(P139A)PAK(128-144)OH represents the PAK pilin synthetic peptide comprising the sequence from residues 128 to 144 with proline 139 substituted with an alanine. "Ac" denotes peptides with their N-termini acetylated, whereas "OH" indicates a peptide with a free α-carboxyl group at the C-terminus, and "NH_2" denotes a peptide with an amidated C-terminus.

(b) *Peptide libraries*

Three series of hexapeptide libraries were synthesized and their sequences were encoded as follows: Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, Ac-$X_1X_2O_3O_4X_5X_6$-NH_2 and Ac-$X_1X_2X_3X_4O_5O_6$-NH_2. The number in the sequence shows the relative position of each residue. Each series of the peptide libraries contained two specifically defined positions (O_nO_{n+1}) and positions that contained equimolar mixtures of the representative amino acids (X_n).

4. Animals

Female BALB/c mice, six to eight week old, were used for the production of monoclonal antibody PK99H in ascitic fluid. Female Flemish rabbits (a crossbreed between Flemish and the giant lobe ear Dutch rabbits), aged eight weeks, were used for the production of polyclonal antipeptide antibodies. Both types of animals were obtained from the animal research facility at the University of Alberta, Edmonton, Canada.

Table II.2 A summary of the synthetic peptide sequences used in this project

(A) Synthetic peptides used in Chapters III, IV, V, and VII

Peptide Name[a]	Sequence[b]
Strain PAK:	128 144
AcPAK(128-144)NH_2	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·K·G·C·S·K-NH_2
AcPAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(K144A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·K·G·C·S·**A**-OH
Ac(S143A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·K·G·C·**A**·K-OH
Ac(G141A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·K·**A**·C·S·K-OH
Ac(K140A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·P·**A**·G·C·S·K-OH
Ac(P139A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·I·**A**·K·G·C·S·K-OH
Ac(I138A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·**A**·P·K·G·C·S·K-OH
Ac(F137A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·**A**·I·P·K·G·C·S·K-OH
Ac(Q136A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·**A**·F·I·P·K·G·C·S·K-OH
Ac(E135A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·**A**·Q·F·I·P·K·G·C·S·K-OH
Ac(D134A)PAK(128-144)OH	Ac-K·C·T·S·D·Q·**A**·E·Q·F·I·P·K·G·C·S·K-OH
Ac(Q133A)PAK(128-144)OH	Ac-K·C·T·S·D·**A**·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(D132A)PAK(128-144)OH	Ac-K·C·T·S·**A**·Q·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(S131A)PAK(128-144)OH	Ac-K·C·T·**A**·D·Q·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(T130A)PAK(128-144)OH	Ac-K·C·**A**·S·D·Q·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(K128A)PAK(128-144)OH	Ac-**A**·C·T·S·D·Q·D·E·Q·F·I·P·K·G·C·S·K-OH
Ac(Q136Y)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·**Y**·F·I·P·K·G·C·S·K-OH
Ac(I138R)PAK(128-144)OH	Ac-K·C·T·S·D·Q·D·E·Q·F·**R**·P·K·G·C·S·K-OH

Peptide Name[a]	Sequence[b]
	128 132 137 144
Ac(C129A,C142A)PAK(128-144)OH	Ac-K·**A**·T·S·D·Q·D·E·Q·F·I·P·K·G·**A**·S·K-OH
AcPAK(137-144)OH	Ac-F·I·P·K·G·C·S·K-OH
AcPAK(137-140)NH_2	Ac-F·I·P·K-NH_2
AcPAK(136-140)NH_2	Ac-Q·F·I·P·K-NH_2
AcPAK(135-140)NH_2	Ac-E·Q·F·I·P·K-NH_2
AcPAK(135-139)NH_2	Ac-E·Q·F·I·P-NH_2
AcPAK(135-138)NH_2	Ac-E·Q·F·I-NH_2
AcPAK(135-137)NH_2	Ac-E·Q·F-NH_2
AcPAK(134-140)NH_2	Ac-D·E·Q·F·I·P·K-NH_2
AcPAK(134-139)NH_2	Ac-D·E·Q·F·I·P-NH_2
AcPAK(134-138)NH_2	Ac-D·E·Q·F·I-NH_2
AcPAK(134-137)NH_2	Ac-D·E·Q·F-NH_2
AcPAK(133-139)NH_2	Ac-Q·D·E·Q·F·I·P-NH_2
AcPAK(133-138)NH_2	Ac-Q·D·E·Q·F·I-NH_2
AcPAK(133-137)NH_2	Ac-Q·D·E·Q·F-NH_2
AcPAK(132-139)NH_2	Ac-D·Q·D·E·Q·F·I·P-NH_2
Ac(D132T)PAK(132-139)NH_2	Ac-**T**·Q·D·E·Q·F·I·P-NH_2
Ac(E135P)PAK(132-139)NH_2	Ac-D·Q·D·**P**·Q·F·I·P-NH_2
Ac(Q136M)PAK(132-139)NH_2	Ac-D·Q·D·E·**M**·F·I·P-NH_2
Ac(I138T)PAK(132-139)NH_2	Ac-D·Q·D·E·Q·F·**T**·P-NH_2
AcPAK(132-138)NH_2	Ac-D·Q·D·E·Q·F·I-NH_2
AcPAK(132-137)NH_2	Ac-D·Q·D·E·Q·F-NH_2

Peptide Name[a]	Sequence[b]
	134 140
AcPAK(132-136)NH_2	Ac-D·Q·D·E·Q-NH_2
Ac(K140A)PAK(134-140)NH_2	Ac-D·E·Q·F·I·P·**A**-NH_2
Ac(P139A)PAK(134-140)NH_2	Ac-D·E·Q·F·I·**A**·K-NH_2
Ac(I138A)PAK(134-140)NH_2	Ac-D·E·Q·F·**A**·P·K-NH_2
Ac(F137A)PAK(134-140)NH_2	Ac-D·E·Q·**A**·I·P·K-NH_2
Ac(Q136A)PAK(134-140)NH_2	Ac-D·E·**A**·F·I·P·K-NH_2
Ac(E135A)PAK(134-140)NH_2	Ac-D·**A**·Q·F·I·P·K-NH_2
Ac(D134A)PAK(134-140)NH_2	Ac-**A**·E·Q·F·I·P·K-NH_2
Ac(E135Q)PAK(134-140)NH_2	Ac-D·**Q**·Q·F·I·P·K-NH_2
Ac(D134N)PAK(134-140)NH_2	Ac-**N**·E·Q·F·I·P·K-NH_2
Ac(D134E)PAK(134-140)NH_2	Ac-**E**·E·Q·F·I·P·K-NH_2
Ac(D134E,E135Q,Q136A)PAK(134-140)NH_2	Ac-**E**·**Q**·**A**·F·I·P·K-NH_2
Ac(Q136C)PAK(134-140)NH_2	Ac-D·E·**C**·F·I·P·K-NH_2
Ac(Q136D)PAK(134-140)NH_2	Ac-D·E·**D**·F·I·P·K-NH_2
Ac(Q136E)PAK(134-140)NH_2	Ac-D·E·**E**·F·I·P·K-NH_2
Ac(Q136F)PAK(134-140)NH_2	Ac-D·E·**F**·F·I·P·K-NH_2
Ac(Q136G)PAK(134-140)NH_2	Ac-D·E·**G**·F·I·P·K-NH_2
Ac(Q136H)PAK(134-140)NH_2	Ac-D·E·**H**·F·I·P·K-NH_2
Ac(Q136I)PAK(134-140)NH_2	Ac-D·E·**I**·F·I·P·K-NH_2
Ac(Q136K)PAK(134-140)NH_2	Ac-D·E·**K**·F·I·P·K-NH_2
Ac(Q136L)PAK(134-140)NH_2	Ac-D·E·**L**·F·I·P·K-NH_2
Ac(Q136M)PAK(134-140)NH_2	Ac-D·E·**M**·F·I·P·K-NH_2

Peptide Name[a]	Sequence[b]
	128 134 140 144
Ac(Q136N)PAK(134-140)NH_2	Ac-D·E·**N**·F·I·P·K-NH_2
Ac(Q136P)PAK(134-140)NH_2	Ac-D·E·**P**·F·I·P·K-NH_2
Ac(Q136R)PAK(134-140)NH_2	Ac-D·E·**R**·F·I·P·K-NH_2
Ac(Q136S)PAK(134-140)NH_2	Ac-D·E·**S**·F·I·P·K-NH_2
Ac(Q136T)PAK(134-140)NH_2	Ac-D·E·**T**·F·I·P·K-NH_2
Ac(Q136V)PAK(134-140)NH_2	Ac-D·E·**V**·F·I·P·K-NH_2
Ac(Q136W)PAK(134-140)NH_2	Ac-D·E·**W**·F·I·P·K-NH_2
Ac(Q136Y)PAK(134-140)NH_2	Ac-D·E·**Y**·F·I·P·K-NH_2
Ac(I138T)PAK(134-140)NH_2	Ac-D·E·Q·F·**T**·P·K-NH_2
Ac(E135P)PAK(134-140)NH_2	Ac-D·**P**·Q·F·I·P·K-NH_2
Ac(E135P,I138T)PAK(134-140)NH_2	Ac-D·**P**·Q·F·**T**·P·K-NH_2
Ac(Q136M,I138T)PAK(134-140)NH_2	Ac-D·E·**M**·F·**T**·P·K-NH_2
Ac(E135P,Q136M)PAK(134-140)NH_2	Ac-D·**P**·**M**·F·I·P·K-NH_2
Ac(I138L)PAK(134-140)NH_2	Ac-D·E·Q·F·**L**·P·K-NH_2
Ac(F137Y)PAK(134-140)NH_2	Ac-D·E·Q·**Y**·I·P·K-NH_2
Ac(E135N)PAK(134-140)NH_2	Ac-D·**N**·Q·F·I·P·K-NH_2
Ac(K140N)PAK(134-140)NH_2	Ac-D·E·Q·F·I·P·**N**-NH_2
Ac(I138R)PAK(134-140)NH_2	Ac-D·E·Q·F·**R**·P·K-NH_2
Strain PAO:	
AcPAO(128-144)OH	Ac-A·C·K·S·T·Q·D·P·M·F·T·P·K·G·C·D·N-OH
AcPAO(134-140)NH_2	Ac-D·P·M·F·T·P·K-NH_2
AcPAO(132-139)NH_2	Ac-T·Q·D·P·M·F·T·P-NH_2

Peptide Name[a]	Sequence[b]
Strain CD4:	128 144
AcCD4(134-140)NH_2	Ac-E·E·M·F·I·P·K-NH_2
Strain K122-4:	
AcK122-4(134-140)NH_2	Ac-D·N·K·Y·L·P·K-NH_2
Strain KB7:	
AcKB7(128-144)OH	Ac-S·C·A·T·T·V·D·A·K·F·R·P·N·G·C·T·D-OH
Ac(T131A)KB7(128-144)OH	Ac-S·C·A·**A**·T·V·D·A·K·F·R·P·N·G·C·T·D-OH
Ac(V133A)KB7(128-144)OH	Ac-S·C·A·T·T·**A**·D·A·K·F·R·P·N·G·C·T·D-OH
Ac(K136A)KB7(128-144)OH	Ac-S·C·A·T·T·V·D·A·**A**·F·R·P·N·G·C·T·D-OH
Ac(F137A)KB7(128-144)OH	Ac-S·C·A·T·T·V·D·A·K·**A**·R·P·N·G·C·T·D-OH
Ac(R138A)KB7(128-144)OH	Ac-S·C·A·T·T·V·D·A·K·F·**A**·P·N·G·C·T·D-OH
Ac(D144A)KB7(128-144)OH	Ac-S·C·A·T·T·V·D·A·K·F·R·P·N·G·C·T·**A**-OH
AcKB7(134-140)NH_2	Ac-D·A·K·F·R·P·N-NH_2

(B) Synthetic peptides used in Chapter VI to verify the results of the peptide libraries

Sequence[b]	Sequence[b]	Sequence[b]	Sequence[b]
Ac-E·Q·F·I·P·K-NH_2	Ac-**Q**·**A**·F·I·**I**·**G**-NH_2	Ac-**G**·Q·F·I·P·**L**-NH_2	Ac-E·Q·F·I·P·**H**-NH_2
Ac-**Q**·Q·F·I·P·K-NH_2	Ac-**A**·**F**·F·I·**I**·**G**-NH_2	Ac-**D**·Q·F·I·P·K-NH_2	Ac-E·Q·F·I·P·**R**-NH_2
Ac-**Q**·**A**·F·I·P·K-NH_2	Ac-**G**·Q·F·I·**I**·**G**-NH_2	Ac-E·**N**·F·I·P·K-NH_2	Ac-**N**·Q·F·I·P·K-NH_2
Ac-**A**·**F**·F·I·P·K-NH_2	Ac-E·Q·F·I·P·**L**-NH_2	Ac-E·Q·**Y**·I·P·K-NH_2	Ac-**Q**·**N**·F·I·P·K-NH_2
Ac-**G**·Q·F·I·P·K-NH_2	Ac-**Q**·Q·F·I·P·**L**-NH_2	Ac-E·Q·**W**·I·P·K-NH_2	Ac-**N**·**A**·F·I·P·K-NH_2
Ac-E·Q·F·I·**I**·**G**-NH_2	Ac-**Q**·**A**·F·I·P·**L**-NH_2	Ac-E·Q·F·**M**·P·K-NH_2	Ac-**G**·**N**·F·I·P·K-NH_2
Ac-**Q**·Q·F·I·**I**·**G**-NH_2	Ac-**A**·**F**·F·I·P·**L**-NH_2	Ac-E·Q·F·**V**·P·K-NH_2	

Note: [a]Positions of the residues are corresponding to the sequence of the PAK strain.

[b]Residues different from the native sequence are highlighted in bold-face.

5. Cell line

The hybridoma cells used for the production of monoclonal antibody PK99H were kindly provided by R.T. Irvin.

The human lung carcinoma A549 cell line (ATCC CCL 185, batch F-8669) was obtained from the American Type Culture Collection (Rockville, Maryland). Passage 84 of this cell line was kindly provided by W. Paranchych. This cell line has been reported to possess the properties of type II alveolar epithelial cells (Lieber *et al.*, 1976), and thus represents a useful human epithelial cell model.

6. Buffers and culture media

(a) *Phosphate Buffered Saline (PBS)*

PBS was prepared by dissolving 8.00 g NaCl, 0.20 g KCl, 0.20 g KH_2PO_4 and 1.15 g Na_2HPO_4 in one liter of double distilled water. The pH of the solution was adjusted to 7.4, and the solution was filtered through a 0.22 μ membrane filter (Millipore, Bedford, MA) and autoclaved before used.

(b) *Hank's Balanced Salt Solution (HBSS)*

HBSS was composed of 8.00 g NaCl, 0.40 g KCl, 0.21 g $CaCl_2 \cdot 2H_20$, 0.05 g $MgSO_4$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.05 g Na_2HPO_4, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, and 1.00 g D-glucose in one litre of double distilled water. The pH of the solution was adjusted to 7.4, and the solution was filtered through a 0.22 μ membrane filter. Sodium azide (0.20 g/L) was added as a preservative.

(c) *Tris Buffered Saline (TBS), 50 mM*

TBS was prepared with 7.88 g Tris base and 8.77 g NaCl dissolved in double distilled water. The solution was adjusted to pH 7.5 with concentrated HCl and the volume was made up to one liter. The buffer was filtered through a 0.22 μ membrane

filter.

(d) *Culture media*

All tissue culture reagents were obtained from GIBCO-BRL (Life Technologies Inc., New York). The HAT medium was prepared by supplementing Dulbecco's modified Eagle's (DME) medium with 20% (v/v) fetal bovine serum, 1% (v/v) 200 mM L-glutamine, 1% (v/v) 100 mM sodium pyruvate, and HAT (100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymine).

The Waymouth MB 752/1 medium was supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). For medium used for assay, 10% (v/v) heat-inactivated fetal bovine serum (56°C for 30 min) was used instead.

B. Methods

1. Peptide synthesis

Conventional methods for peptide synthesis are generally divided into two main categories, namely, the solution-phase (classical method) and the solid-phase (SPPS) peptide synthesis. The major difference between these two methods is the presence of an insoluble polymeric support in the SPPS method so as to simplify the washing steps and to reduce manipulative losses. Today, most of the syntheses are based upon the SPPS method developed by Merrifield (1963) because of its speed, ease of handling, and the potential for automation. However, the classical solution-phase method is still valuable in large-scale synthesis and specialized applications. In this laboratory, peptide synthesis was performed following the general procedure for SPPS as described by Erickson and Merrifield (1976). A schematic diagram of th

procedure is shown in Fig. II.1

The general protocol for SPPS is carried out in the C→N direction and consists of four steps: anchoring, deprotection, coupling, and cleavage. The anchoring step involves the coupling of the first amino acid to the resin support. The type of resin used depends on whether a C-terminal amide or free acid is needed. In synthesis of peptide acids, the classical Merrifield (chloromethyl) resin (Gutte and Merrifield, 1971; Gisin, 1973) or the 4-(hydroxymethyl)phenylacetic acid (PAM) resin (Mitchell *et al.*, 1978) is used. The first amino acid is coupled to the resin via esterification with cesium salts (Gisin, 1973). When preparing peptide amides, a benzhydrylamine type resin is used, which allows the direct coupling of the first amino acid by means of its carboxyl group to the benzhydrylamine handler of the resin. Examples of this type of resins include the 4-methylbenzhydrylamine resin (Matsueda and Stewart, 1981) or the 4-(2',4'-dimethoxyphenylaminomethyl)phenoxy (Rink amide) resin (Rink, 1987).

The deprotection step involves the removal of the N^{α}-protecting group from the N^{α}-amino group of the peptide anchored on the resin. This step exposes the N^{α}-amino group of the peptide so as to facilitate subsequent peptide bond formation during the coupling step. Presently, two types of N^{α}-protecting groups are commonly used. They are the *tert*-butyloxycarbonyl (Boc) group (Barany and Merrifield, 1979) and the 9-fluorenylmethyloxycarbonyl (Fmoc) group (Carpino and Han, 1972). The Boc protecting group is stable to alkali but removed rapidly by acid such as TFA. A neutralization step with a tertiary amine is required following acidolysis. The Fmoc protecting group is base-labile and can be removed in the presence of piperidine.

The coupling step involves the formation of peptide bond between the N^{α}-protected amino acid and the peptide chain on the resin. Three major kinds of coupling approaches are currently available to ensure complete reaction. First, *in situ* reagents such as N,N'-dicyclohexylcarbodiimide (DCC) (Sheehan and Hess, 1955;

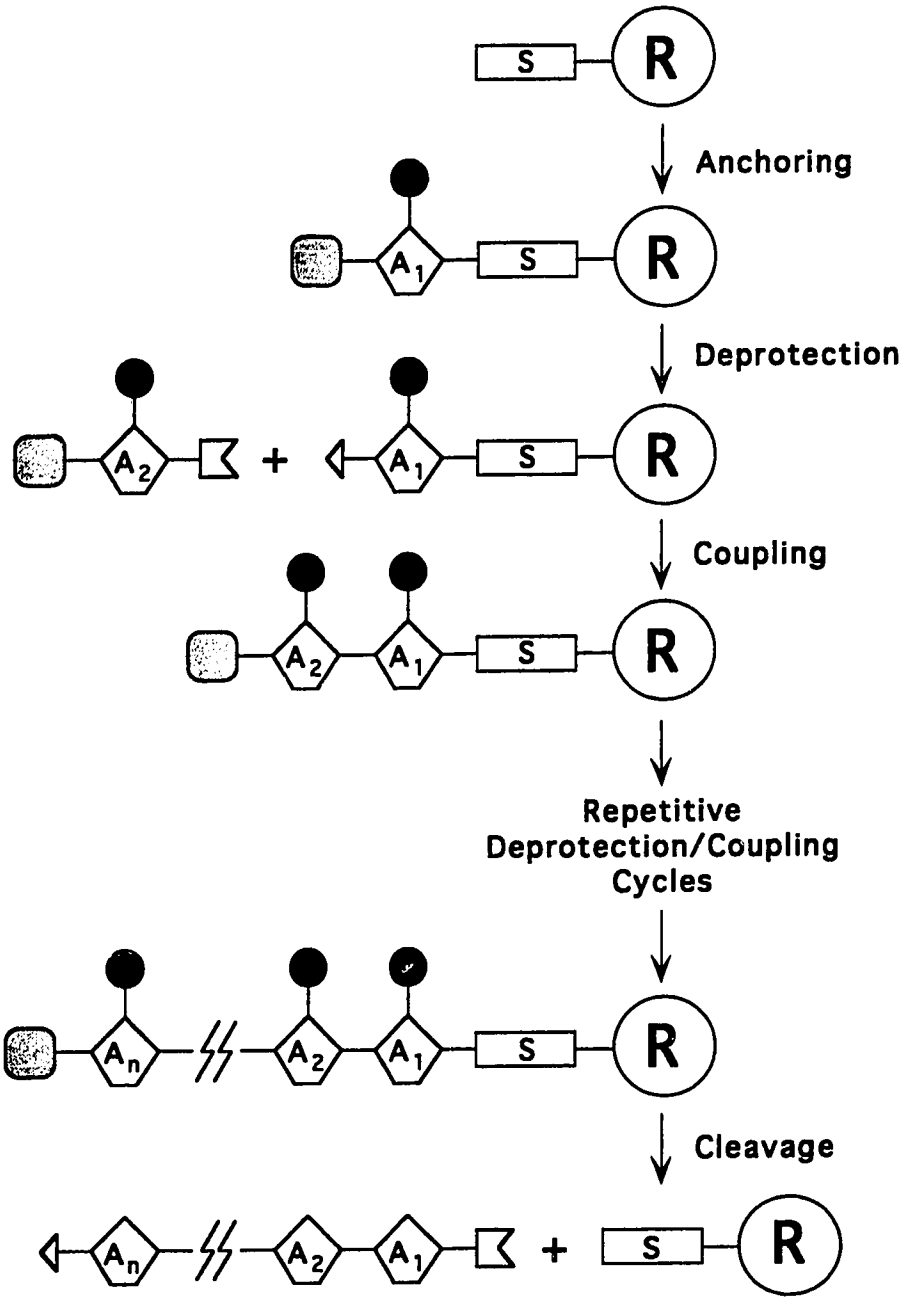

Figure II.1 A general procedure for stepwise solid-phase peptide synthesis. Where Ⓡ is the insoluble polymeric support; [S] is the spacer or handle between the peptide and the solid support; ◇ , ◇ ,..., ◇ are amino acid residues; ● is the side-chain protecting group; ▩ is the N-terminal protecting group; ⊏ is a free carboxyl group; and ◁ is a free amino group. This figure is adapted from Barany *et al.*, 1988 and Grant, 1992.

Rich and Gurwara, 1975), benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Hudson, 1988), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Fields *et al.*, 1991) can raise the reactivity of the carboxyl group of the N^{α}-protected amino acid and demonstrate efficient coupling in SPPS. Usually, HOBt is added to accelerate coupling as well as to suppress racemization and dehydration (Mojsov *et al.*, 1980). Second, the coupling reaction can be speeded up by the formation of symmetrical anhydrides in the presence of DCC and excess N^{α}-protected amino acids (Merrifield *et al.*, 1982; Yamashiro, 1987). The urea side-product is removed by filtration and DMF is used as the solvent for optimal coupling. This preformed symmetrical anhydride is highly reactive. Third, formation of active esters like pentafluorophenyl (OPfp), HOBt, and 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine (ODhbt). These esters are powerful acylating agents and highly reactive.

Repetitive cycles of deprotection and coupling allow further elongation of the peptide chain. Once the required resin-bound sequence has been synthesized, the peptide-resin is subjected to the cleavage step in order to remove the peptide from the resin as well as the side-chain protecting groups from the peptide. For Boc SPPS, hydrogen fluoride (HF) is usually used. A carbonium ion scavenger such as anisole is required. For peptides containing Cys residue, additional scavenger such as 1,2-ethanedithiol (EDT) is needed. On the other hand, in Fmoc SPPS peptides can be cleaved from the resin with moderate strength acid such as TFA.

(a) *Synthesis of pilin peptide analogs: Boc chemistry*

The pilin peptide analogs were synthesized using either a Beckman model 990 (Fullerton, CA) or an Applied Biosystems model 430A (Foster City, CA) peptide synthesizer. The Beckman peptide synthesizer was used for the large scale preparation of the core peptide, Boc-FIPKGCSK-resin, which allows subsequent

synthesis of the full length PAK peptide analogs using the Applied Biosystems peptide synthesizer. All amino acids used were protected at the N^{α}-position with the Boc group. The side-chain protecting groups used on the N^{α}-Boc-amino acids (in parentheses) are listed as follows: Benzyl (Asp, Glu, Ser, Thr, Tyr), 2-chlorobenzyloxycarbonyl (Lys), 4-toluenesulfonyl (Arg, His), formyl (Trp), 4-methylbenzyl (Cys) and sulfoxide (Met). Syntheses of peptide amides were carried out using co-poly (styrene, 1% divinylbenzene) benzhydrylamine-hydrochloride resin at a substitution of 0.92 mmol of amino groups/g of resin (Bachem, Torrance, CA). Syntheses of peptides with a C-terminal lysine and a free α-carboxyl group were synthesized on Boc-lysine-OCH_2-PAM resin (1% cross-linked) at a substitution level of 0.67 mmol Lys/g of resin (Applied Biosystems, Foster City, CA). While the syntheses of peptides with a different C-terminal residue (e.g. Ala) and a free α-carboxyl group were initiated by esterification of the cesium salts of Boc-amino acid to co-poly (styrene, 1% divinylbenzene) chloromethyl resin (Bachem, Torrance, CA) at a substitution level of 0.90 mmol of amino groups/g of resin. The cesium salts of the Boc-amino acids were prepared according to the method as described by Gisin (1973). Briefly, the Boc-amino acid was dissolved in 30 ml of ethanol and diluted with 10 ml of double distilled water. Cesium hydrogen carbonate was added in a ratio of 1 mole salt to 1.1 mole Boc-amino acid. The reaction mixture was reduced to dryness on a rotary evaporator and washed three times with 10 ml of benzene. The precipitate was dried over P_2O_5. A two molar excess of the cesium salt of the Boc-amino acid was used to esterify the chloromethyl resin by stirring the mixture in DMF (6 ml DMF/g resin) at 50°C for 20 hr. A small amount of the resin was dried and weighed. The resin was then hydrolysed with 100 µl of 12 N HCl and 100 µl of propionic acid *in vacuo* at 160°C for an hour and the amount of Boc-amino acid coupled on the resin was examined by means of amino acid analysis (see section 4b).

The coupling programs performed by the two synthesizers are listed in Table

II.3. All amino acids (three-fold excess) were double coupled using DCC generated preformed symmetrical anhydrides (with the exception of Asn, Gln, Arg and benzoylbenzoic acid, where HOBt active esters were used instead). In the case of the Beckman instrument, preformed symmetrical anhydride was prepared manually by mixing two molar equivalents of Boc-amino acid to DCC. The resultant symmetrical anhydride was filtered through glasswool to remove precipitated DCU and then added to the N^{α}-deprotected and neutralized peptide-resin. The mixture was allowed to react for one hour. Since only half of the Boc-amino acid in the symmetrical anhydride is coupled by this method, another molar equivalent of DCC was added and the reaction was continued for a further 30 min in order to push the reaction to completion. In the case of the Applied Biosystems instrument, the performed symmetrical anhydride and the active esters were prepared automatically.

Difficult couplings were monitored by either a quantitative ninhydrin test (Kaiser *et al.*, 1970), or picric acid titration (Hodges and Merrifield, 1975). Any couplings with coupling efficiency less than 99% were coupled a third time manually. Acetylation was performed on the instrument using acetic anhydride : DIEA : peptide resin in a molar ratio of 50 : 20 : 1 for 10 min and then 100 : 20 : 1 for 5 min in DCM. The completed peptides were dried and cleaved from the resins with anhydrous hydrogen fluoride (20 ml/g of peptide resin) in the presence of 10% (v/v) anisole and 1% (v/v) EDT as scavenging reagents for 1 hour at -4°C using type 1B HF-Reaction Apparatus (Peninsula Laboratories Inc., Belmont, CA). EDT was omitted for peptide containing a benzoylbenzoyl moiety since EDT will react with it. The solvent mixture was then removed under reduced pressure. The peptide-resin was washed three times with anhydrous diethyl ether (25 ml each), and the cleaved peptide was extracted from the resin with either neat or 30% (v/v) acetic acid (three times, 30 ml total) through a coarse sintered glass filter. The crude peptide solution was diluted with double distilled water, followed by lyophilization.

Table II.3 Coupling procedures used in the Beckman 990 and the Applied Biosystem 430A Synthesizer

	Beckman 990		Applied Biosystem 430A	
Step	**Reagents**	**Mix Time (min)**	**Reagents**	**Mix Time (min)**
Deprotection	DCM	2 x 0.5	33% TFA/DCM	1 x 1.3
	50% TFA/DCM	1 x 1	50% TFA/DCM	1 x 18
	50% TFA/DCM	1 x 20	DCM	3 x 1
	DCM	5 x 1		
	Isopropanol	3 x 2		
	DCM	3 x 1		
Neutralization	5% DIEA/DCM	3 x 2	10% DIEA/DMF	2 x 1
	DCM	4 x 1	DMF	5 x 1
Coupling	Boc-amino acid*	1 x 60	Boc-amino acid*	1 x 30
	DCC	1 x 30	DMF	3 x 0.75
	DCM	1 x 1		
	Isopropanol	3 x 5		
	DCM	6 x 1		
Neutralization	5% DIEA/DCM	2 x 2	10% DIEA/DMF	1 x 1
	DCM	6 x 1	DMF	1 x 1
			DCM	3 x 1
Second Coupling	Boc-amino acid*	1 x 60	Boc-amino acid*	1 x 30
	DCC	1 x 30	DMF	1 x 1
	DCM	1 x 1	DCM	5 x 1
	Isopropanol	4 x 1		
	DCM	4 x 1		

* in the form of symmetrical anhydride or HOBt active ester.

(b) *Preparation of peptide libraries: Fmoc chemistry*

The three series of peptide libraries (section 2b) were synthesized on NovaSyn PR 500 resin (0.3 mmol/g; Novabiochem, La Jolla, CA) for continuous flow synthesis of peptide amides, using a manual multiple column peptide synthesizer (MCPS) (see Fig. II.2). The physical layout and mechanical operation of the MCPS has been described in detail by Holm *et al.* (1989) and Meldal *et al.* (1993). Briefly, the instrument is a manually operated apparatus for parallel solid-phase peptide synthesis. It is constructed of a Teflon reaction block with a 96-well (8 x 12) configuration. Teflon filters (70 μ, 2 mm thick) are placed at the bottom of each of the 96 wells. Each well resembled a column in which a continuous flow synthesis is allowed. Two washers are installed for dispensing of solvent and deprotecting reagent, respectively, in a parallel manner through an eight port manifold. Nitrogen gas is used to maintain a positive pressure at the bottom of each well to prevent premature draining during the various steps of a protocol.

Resin (20 mg) were added to each well. Fmoc amino acid OPfp-esters (Novabiochem, la Jolla, CA) were used throughout the synthesis. The side-chain protecting groups used were: *tert*-Butyl (Glu, Ser) and Boc (Lys). For those defined positions (O_nO_{n+1}) in each peptide mixture, 0.3 M (10 folds excess) of the Fmoc amino acid OPfp-esters in DMF (200 μl/well) were used. Double couplings were performed for two hours and 30 min, respectively. In the case of X_n position, a mixture of 0.1 equivalent of each of the 10 Fmoc amino acid OPfp-esters (Ala, Glu, Gln, Gly, Ile, Leu, Lys, Phe, Pro, and Ser) in DMF were used and coupled for 2 hr, followed by a second coupling for an additional 2 hr.

Fmoc deprotection was carried out with 20% piperidine in DMF (2 times, 5 min each), and acetylation of the α-amino group was performed with excess acetic anhydride. The peptides were cleaved from the resin with 95% TFA/water (4 x 300 μl). The TFA solution was then evaporated to dryness using a Savant SpeedVac

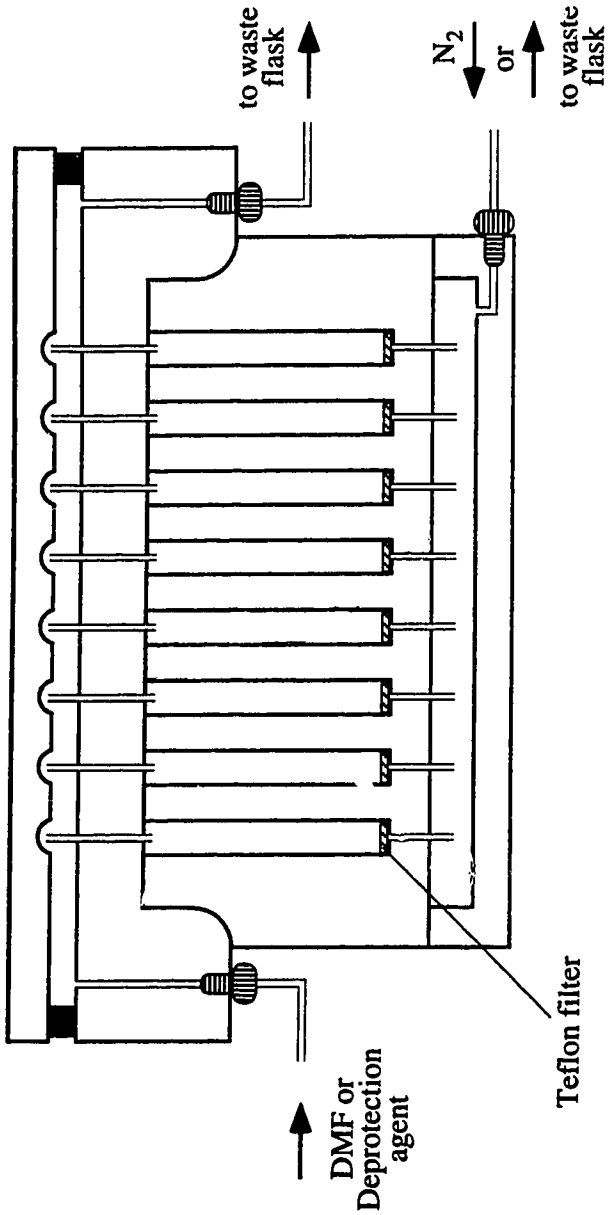

Figure II.2 Construction diagram for the multiple column peptide synthesizer. Figure is adapted from Meldal *et al.*, 1993.

(Savant Instruments Inc., Farmingdale, NY). The semi-solid products were washed three times with diethyl ether and then dried cautiously with a slow stream of nitrogen. The resultant peptides were dissolved in water and centrifuged, the supernatant was transferred into a cryo-tube (Nunc, Denmark). The process was repeated and the solutions were then lyophilized. The concentration of the peptide libraries were determined by amino acid analysis.

(c) *Synthesis of the heptapeptide analogs DEXFIPK*

Peptide analogs corresponding to the PAK epitope peptide sequence Ac-DEQFIPK-NH_2 with position 136 (Gln) replaced by all 20 naturally occurring amino acids were synthesized using the MCPS as described above. The Rink-AM resin (Aminotech, Nepean, ON) with a substitution of 0.48 mmol/g resin was used. The side-chain protecting groups for the Fmoc-amino acids were: 2,2,5,7,8-Pentamethylchroman-6-sulfonyl (Arg), triphenylmethyl (Asn, Cys and His), *tert*-butyl (Asp, Glu, Ser, Thr and Tyr) and Boc (Lys). Coupling was performed by first activating the Fmoc-amino acid with HBTU/HOBt in NMP with DIEA. The molar ratio of the mixture Fmoc-amino acid : HBTU : HOBt : DIEA : active sites on resin was 10 : 10 : 10 : 15 : 1. The Fmoc-amino acid was double coupled for 45 min each. Deprotection and acetylation were done as described previously. Most peptides were cleaved in 95% TFA/water. In peptide sequences containing Arg, Met, Trp or Tyr, Reagent K (82.5% TFA, 2.5% EDT, 5% phenol, 5% thioanisole and 5% water) was used instead. The organic impurities in the peptides were removed by washing three times with diethyl ether. The peptides were then dried and resuspended in water, followed by lyophilization.

2. Purification of the peptide analogs

The lyophilized crude peptides were dissolved in double distilled water, and

centrifuged at 14,000 RPM for two minutes using an eppendorf centrifuge 5414C (Fisher Scientific, Fairlawn, NJ) to remove particulates. The supernatant was then purified by reversed-phase high performance liquid chromatography (HPLC) using an Applied Biosystems 400 solvent delivery system and a 783A programmable absorbance detector connected to a Synchropak RP-4 (250 x 21.2 mm I.D.) reversed-phase column (Synchrom Inc., Lafayette, IN) operated at a flow rate of 5 ml/min. Similarly, smaller scale purification was performed on either a Spectra-Physics SP8700 solvent delivery system (San Jose, CA) and a SP8750 organizer module combined with a Hewlett Packard HP1040A detection system (Mississauga, ON), HP3390A integrator, and HP85 computer, or a Varian 5000 Vista series HPLC (Palo Alto, CA) coupled with a Waters Model M490 Programmable multi-wavelength detection system (Mississauga, ON) connected to a semi-preparative, C_{18}, RP-P Synchropak column (250 x 10 mm I.D.; Synchrom Inc., Linden, IN) or an analytical Synchropak RP-P column (250 x 4.6 mm I.D.) operated at a flow rate of 2 ml/min for the semi-preparative column and 1 ml/min for the analytical column. A linear AB gradient of 0.2% to 0.5% B/min (depending on the peptide) was used, where solvent A was 0.05% TFA in water, and solvent B was 0.05% TFA in acetonitrile. The absorbance was recorded at 210 nm. For peptides containing the benzophenone (BB) group, the absorbance at 260 nm was monitored as well. The fractions collected were then analyzed by a HP 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA) using the above solvent system at 2% B/min on a Zorbax Rx-C8 (150 x 2.1 mm I.D.) reversed-phase column (Rockland Technologies, Giberstville, PA).

3. Peptide oxidation

For those 17-residue peptides containing two cysteine residues in their sequence, the intrachain disulfide bridge was formed by air oxidation. The peptide solution (0.1 mg/ml) was stirred in 100 mM of NH_4HCO_3, pH 8.2, overnight at room temperature.

Completion of oxidation was examined by treating 100 µl of peptide solution with 10 µl of a 1 mg/ml aqueous solution of N-ethylmaleimide (NEM) (Lee *et al.*, 1990a). The HPLC chromatograms of NEM-treated and untreated peptides were compared. Only reduced peptide with free cysteine residues could react with NEM, and the modified peptide eluted off the column with a retention time much later than that of the oxidized or reduced peptide (Lee *et al.*, 1990a). The homogeneity of the oxidized peptides was examined by reversed phase HPLC. NH_4HCO_3 in the peptide solution was removed by lyophilization after the peptide solution was acidified with 30% (v/v) acetic acid.

4. Verification of synthesis

To ensure the quality of the synthetic peptides used in the assays, the homogeneity and authenticity of the purified peptides were examined by a series of analytic methods such as analytical HPLC, amino acid analysis, and mass spectrometry.

(a) *Analytical HPLC*

The homogeneity of the purified peptides was determined by analytical HPLC, which was performed on either a Zorbax Rx-C8 (150 x 2.1 mm I.D.) or an Aquapore RP-300, C_8 (220 x 4.6 mm I.D.) reversed-phase column (Brownlee Labs, Santa Clara, CA) connected to the HPLC instrument as mentioned in section 2. The flow rate was set at 1 ml/min with a linear AE gradient of 1% B/min, where solvent A was 0.05% TFA in water, and solvent B was 0.05% TFA in acetonitrile. The absorbance was recorded at 210 nm.

(b) *Amino acid analysis*

The amino acid composition of the purified peptides were examined by amino acid analysis (for review see Ozols, 1990). The peptides were dissolved in 100 µl of

6N HCl with 0.1% phenol in sealed, evacuated tubes and hydrolyzed at 110°C for 22 hours (Moore and Stein, 1963). The hydrolysates were dried and dissolved in citrate buffer (pH 2.2), and amino acid analyses were performed on either a Durrum Model D-500 high-pressure automatic analyzer (Durrum Instrument Corp., Palo Alto, CA) or a Beckman System 6300 high performance automatic analyzer (Beckman, Palo Alto, CA). These analyzers were equipped with a cation-exchange column by which the amino acids in the acid hydrolysate were analyzed and eluted with buffers of three different pH's: pH 3.28 for acidic amino acids, pH 4.25 for neutral amino acids, and pH 7.8 for basic amino acids. A post-column ninhydrin analysis was then performed and the modified amino acids were detected at two wavelengths — 570 and 440 (for the detection of proline) nm. The mean of the molar ratios of all accurately measured amino acids in the acid hydrolysate was used to calculate the concentration of the peptide.

(c) *Mass spectrometry*

The molecular weight of the peptides was determined with a BioIon 20 plasma desorption time of flight mass spectrometer (Uppsala, Sweden) to further ensure the authenticity of the purified peptide. The principles of the plasma desorption mass spectrometer were discussed in some excellent reviews (Macfarlane, 1983; Roepstorf *et al.*, 1988; 1990). Briefly, the HPLC purified peptide solution (1 mg/ml) was sampled by adsorption onto a nitrocellulose target matrix. The peptide sample on the matrix was then ionized by bombardment with α particles emitted from the ^{252}Cf source. The time the ionized peptide takes to traverse the flight tube is dependent on its mass to charge ratio, and thus the mass of the peptide molecule can be determined by measuring the time it reached the detector.

5. Preparation of peptide conjugates

Since small peptides are usually weak immunogens, it is necessary to couple them to protein carriers in order to enhance their immunogenicity (Butler and Beiser, 1973). The photoaffinity crosslinking method as described by Parker and Hodges (1985) with modifications by Lee *et al.* (1989b) was used to prepare peptide-protein carrier conjugates for the immunological study in this project. The photoreactive reagent used was benzoylbenzoic acid (Aldrich, Milwaukee, WI), which was coupled to the N-terminus of the synthetic peptide by the conventional SPPS procedure as described in previous section. The benzophenone photoaffinity probe is stable under acidic conditions and is compatible with the Boc chemistry used in the SPPS. In addition, the benzophenone group is inert until photolysis and so it permits well-controlled incorporation of the labeled peptides to the protein carriers. The benzophenone photoreacts non-specifically with primary, secondary, and tertiary carbons found in various side-chains of amino acids (Galardy *et al.*, 1974). The photochemical reaction is shown in Figure II.3. In this laboratory, the benzophenone photoaffinity crosslinking has successfully been used to study the interactions between the TnI peptide 104-115 and the protein, TnC (Van Eyk and Hodges, 1993; Ngai *et al.*, 1994).

Two glycine and a norleucine residues were coupled at the N-terminus of the peptide prior to the coupling of the benzoylbenzoic acid. The glycine residues were used as a spacer between the protein carrier and the peptide hapten. It has been shown that the presence of a spacer could increase the production of high-affinity antibodies (Hastings *et al.*, 1988). The norleucine residue was incorporated to enable the quantitation of the photoprobe-labeled peptides coupled to the protein carriers by means of amino acid analysis. The protein carriers used were keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Stock solutions of the KLH (100 mg/ml in 0.1 M of ammonium bicarbonate) and the BSA (500 mg/ml PBS) were

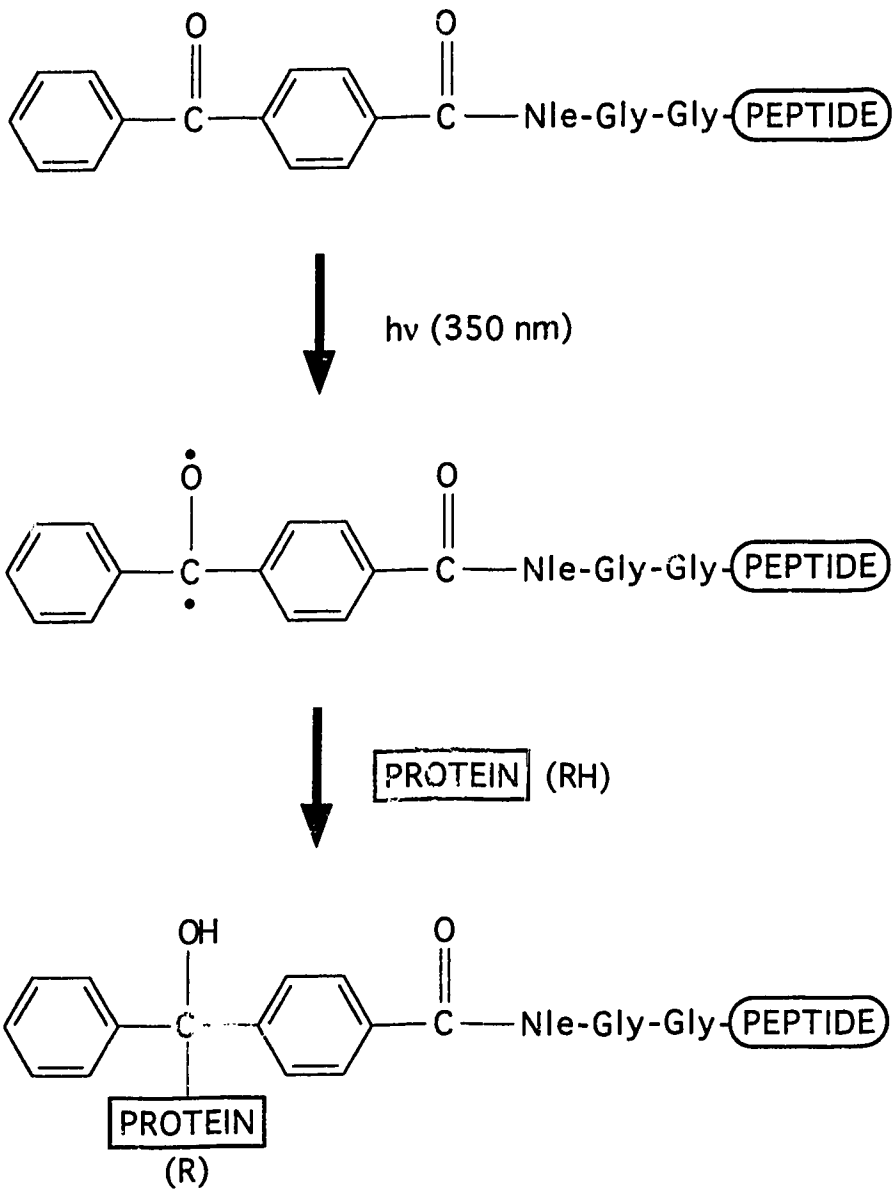

Figure II.3 Schematic diagram showing the photochemical crosslinking between a benzoylbenzoyl-labeled peptide and a protein carrier (RH). Figure is adapted from Van Eyk and Hodges, 1993.

prepared by stirring the proteins in the buffers at 4°C. Peptide (6 mg) were dissolved in 10 µl of PBS (more PBS may be needed for a less soluble peptide) in a 10 x 75 mm glass tube. The KLH solution (100 µl) or BSA solution (13.3 µl) was then added. The solution was irradiated at 350 nm in a RPR 208 preparative photochemical reactor (Rayonet, Southern New England Ultraviolet Co., Middletown, CT) for one hour at 4°C. The unconjugated peptide was removed by dialysis. The peptide-conjugate solution was mixed with 5 ml of 8 M urea and placed into dialysis tubing with a molecular weight cutoff of 12,000 to 14,000 (Spectra/Por, Spectrum, Houston, TX). The peptide-conjugate was subsequently dialyzed against 6 M urea, 1 M urea, 100 mM ammonium bicarbonate, and 10 mM ammonium bicarbonate at 4°C. The ammonium bicarbonate was then removed by lyophilization. Quantitation of peptides coupled to the protein carriers was accomplished using amino acid analysis in which the molar ratio of the norleucine represented the quantity of peptides coupled.

6. Immunization — preparation of antipeptide antisera

Prior to immunization, a small sample of blood (5 ml) was drawn from the vein of the rabbit's ear and used as preimmune sera. The peptide-KLH conjugates were dissolved in sterile PBS and mixed with equal volume of Freund's complete adjuvant (GIBCO-BRL Life Technologies Inc., New York) (Freund and McDermott, 1942). This mixture was then thoroughly mixed until a thick white emulsion formed. A two-site (subcutaneous and intramuscular) injection with 200 µl/site of the emulsion was performed on each rabbit. The amount of peptide-conjugate injected was 200 to 350 µg/rabbit, depending on the degree of incorporation of the peptide analog on KLH. Two booster injections were administered at two week intervals using the same amount of the peptide conjugate emulsified with Freund's incomplete adjuvant. Blood samples (5 ml each) were taken 10 days after the third injection. The antiserum titer was then determined by direct ELISA. Further booster injections and sera collections

were subsequently performed at 4 week intervals.

7. Production of monoclonal antibody in mouse ascites

Monoclonal antibody, PK99H, was prepared as described by Doig *et al.* (1990). PK99H is an IgG subclass 1 with κ light chains. The hybridoma cells stored in liquid nitrogen were thawed quickly in a 37°C water bath. The cells were then washed three times with Dulbecco's modified Eagle's (DME) medium and finally resuspended in DME medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT). The cells were allowed to grow at 37°C. In addition, BALB/c mice were primed intraperitoneally with 200 μl of pristane (2,6,10,14-tetramethylpentadecane; Sigma, St. Louis, MO) at least two weeks prior to ascites production (Brodeur *et al.*, 1984). Ascites tumors were produced by injecting 10^6 hybridoma cells intraperitoneally into the pristane-primed mice. The mice were monitored frequently. Seven to ten days later, ascites fluid was collected and the hybridoma cells were removed by centrifugation. Partial purification was accomplished with ammonium sulfate fractionation and subsequent dialysis against PBS, pH 7.4. Antibody was then further purified by HPLC on a protein G affinity column (ChromatoChem, Missoula, MT) by elution with 10 mM citrate buffer, pH 2.7. The eluant was dialyzed against PBS and stored as aliquots at -20°C.

8. Protein determination

The protein concentration of the *P. aeruginosa* pili or antibodies was determined colorimetrically by the Pierce Bicinchoninic Acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL). Briefly, BCA assay determines the amount of Cu^+, which was formed as a result of reduction of Cu^{2+} by amino acid residues such as cysteine (or cystine), tryptophan, tyrosine, and the peptide bond in the protein. A colorimetric reaction between the Cu^+ and BCA (Smitch *et al.*, 1985; Wiechelman *et al.*, 1988) is

measured at 562 nm and the resulting absorbance reading is converted to protein concentration when compared to a standard. Stock BSA solution (4 mg/ml) was used as the protein standard. A set of protein standards was prepared by serially diluting the BSA solution (2-fold dilution). The protein standard and the sample protein solution were pipetted to the wells of a microtiter plate (10 µl/well), followed by the addition of 190 µl/well of the BCA protein assay reagent. The plate was incubated at room temperature for 2 hours. The absorbance at 562 nm was measured and the reading of the protein standards was used to prepare a standard curve, from which the concentration of the protein sample could be determined.

9. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was carried out according to the principles of Voller *et al.* (1974) with modifications described previously (Worobec *et al.*, 1983; Lee *et al.*, 1989b). In general, the assay can be divided into four steps: coating, blocking, binding, and detection. The protocol is illustrated schematically in Figure II.4.

(i) *Coating* — Antigens or receptors can be immobilized onto the wells in the ELISA plate (Costar, Cambridge, MA; or DiaMed, Mississauga, ON) through adsorption to the polystyrene surfaces. Some of the examples employed in this project were the *P. aeruginosa* pili (0.2 µg/well), peptide-BSA conjugates (1 µg/well), ßGalNAc(1-4)ßGal-BSA conjugate (0.5 µg/well), and glycosphingolipids such as asialo-GM_1 (0.2 µg/well), where the amount of antigens or receptors used to coat the ELISA plates are shown in parentheses. For the pilus proteins, peptide conjugates, and disaccharide conjugates, the samples were prepared in 0.01 M carbonate buffer, pH 9.5 (coating buffer). The solution (100 µl/well) was transferred to an ELISA plate. The plate was then wrapped with the plastic wrap (Saran wrap) and incubated for 6 hours at room temperature. However, in the case of glycosphingolipids, coating

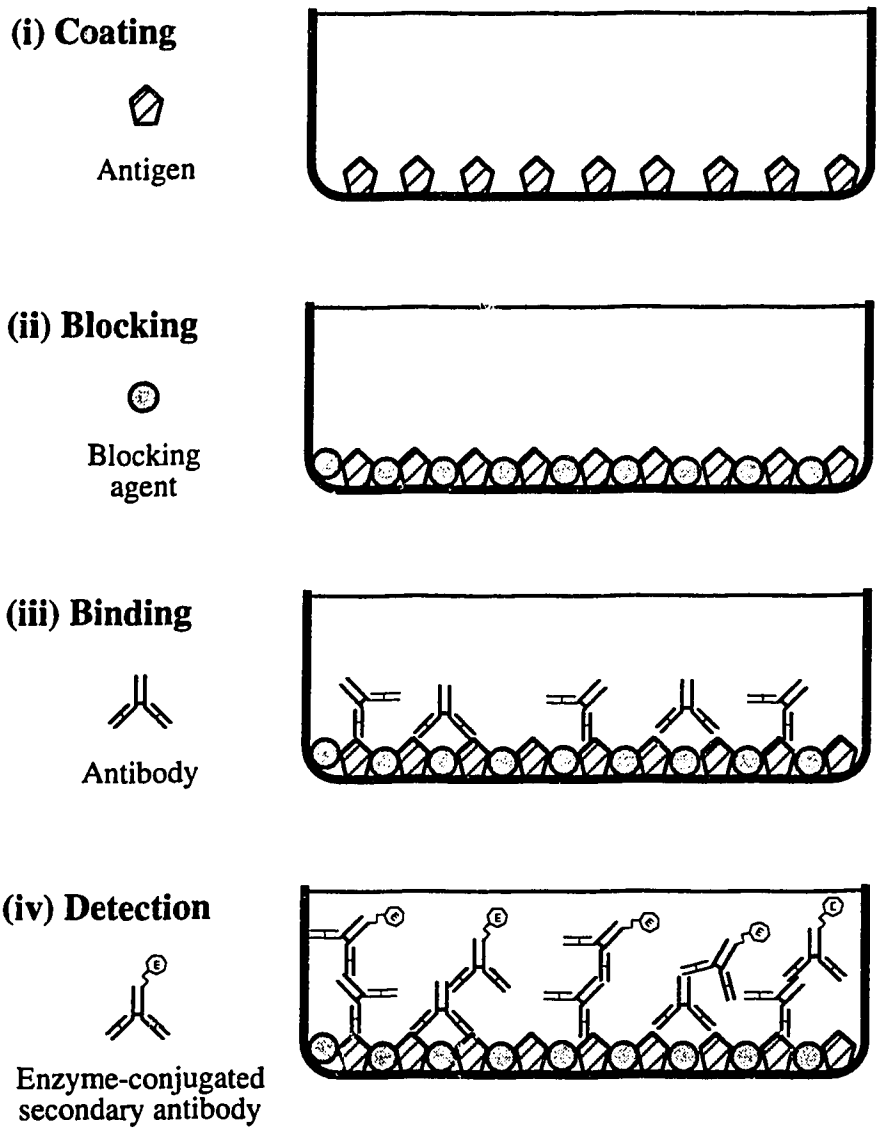

Figure II.4 Schematic diagram showing the four general steps found in an enzyme-linked immunosorbent assay (for details see text).

was performed according to the method of Karlsson and Stromberg (1987). Aliquots of 100 µl of asialo GM_1 solution (2 µg/ml in methanol) was pipetted to the wells of an ELISA plate. The samples were then allowed to evaporate to dryness in a fume hood.

(ii) *Blocking* — Followed the coating step, the plate was washed three times with 0.05% (w/v) BSA in PBS, pH 7.4 (Buffer A). Blocking solution (200 µl/well of 3% (w/v) BSA in PBS) was added to block the non-specific binding sites in the wells. The plate was wrapped and stored at 4°C overnight.

(iii) *Binding* — Two different types of assays can be performed at this step, namely, the direct ELISA and competitive ELISA. In a direct ELISA such as the determination of the endpoint titers for the antipeptide antisera, serial diluted samples of the antisera were prepared. The plate was washed twice with Buffer A and the antiserum solutions were then transferred to the corresponding wells (in triplicates). The plate was incubated at 37°C for 2 hours. In the case of competitive ELISA, the competitors (such as pilus proteins or synthetic peptides) at various concentrations were mixed with a fixed concentration of the binding species in Buffer A and preincubated for an hour at room temperature. For ELISA using the reduced 17-residue peptide, reducing conditions were maintained by including 0.5 mM dithiothreitol (DTT) in the solution. The binding species used in this project were: monoclonal antibody PK99H (295 µg protein/ml in stock solution, and final dilution in each well was 5 x 10^{-6} of the stock solution), antipeptide antisera (the final dilution for polyclonal antibodies 17-R1, 17-O1, and 17-O2 were 1.[illegible] 10^{-3}, and 1.25 x 10^{-3}, respectively; whereas the final dilution for other antipeptide antisera were: N-1 (2 x 10^{-5}), N-2 (10^{-5}), N-3 (4 x 10^{-5}), E135A-1 (10^{-5}), E135A-2 (10^{-5}), E135A-3 (10^{-5}), Q136A-1 (5 x 10^{-4}), Q136A-2 (5 x 10^{-4}), Q136A-3 (10^{-5}), F137A-1 (5 x 10^{-4}), F137A-2 (5 x 10^{-4}), F137A-3 (10^{-5}), I138A-1 (10^{-5}), I138A-2 (2 x 10^{-5}), and I138A-3 (10^{-5}), respectively), and biotinylated PAK and PAO 17-residue peptides (final concentrations were 2.6 and 2.9 µM, respectively). The plate was washed two times

with buffer A. Aliquots of 100 µl of the solution mixture were added to the corresponding wells (in triplicates) and incubated for two hours at 37°C. The plate was washed 5 times following the incubation.

(iv) *Detection* — Detection methods were dependent on the binding species and reporter groups used in the assay. When antibodies were used (such as PK99H and the antipeptide antisera), either goat anti-mouse (for PK99H) or anti-rabbit (for the rabbit antipeptide antisera) IgG immunoglobulin conjugated to horseradish peroxidase (Jackson Laboratories, CA) was used as the reporter group. The secondary IgG was diluted by 5,000-fold in Buffer A and 100 µl of the diluted solution was added to each well. While in the case of biotinylated peptides, streptavidin-alkaline phosphatase solution (1:3,000 dilution, 100 µl/well) was used as the reporter group. After the addition of the reporter groups to the ELISA plate, the mixture was incubated at 37°C for two hours. The plate was then washed five times with Buffer A and 125 µl of the substrate solution was added. For reporter groups using horseradish peroxidase, 1 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) in 10 mM sodium citrate buffer, pH 4.2, containing 0.03% (v/v) hydrogen peroxide was used. The reaction was stopped by the addition of 125 µl of 4 mM sodium azide. On the other hand, the substrate used for the alkaline phosphatase was 1 mg/ml *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine, pH 9.8. The reaction was quenched by 125 µl of 2 N NaOH. Finally, the absorbance at 405 nm was determined by using a Titertek Multiskan Plus MK II microplate reader (Flow Lab Inc., McLean, Virginia).

The percentage inhibition (%Inhibition) for each competitive assay was calculated by the following formula:

$$\%\text{Inhibition} = 100\% - \frac{A_{405}\ \text{competition}}{A_{405}\ \text{no competition}} \times 100\%$$

The competitive binding profile was plotted as %Inhibition vs Log(competitor

concentration). The I_{50} value (competitor concentration that causes 50% inhibition) was determined either manually or by using the software TableCurve (Jandel Scientific Inc., CA). The apparent association constant (K_a) of the monoclonal antibody for each peptide analog can be calculated by the formula $K_a = (I_{50})^{-1}$ as described by Nieto *et al.* (1984). Furthermore, by utilizing the K_a values of the peptide analogs (K_S) and the native sequence (K_N), one can determine the loss of binding free energy [$\Delta(\Delta G)$] of each peptide analog as compared with the native peptide through the following equation: $\Delta(\Delta G) = R \cdot T \cdot \ln(K_N/K_S)$ (Bhattacharyya and Brewer, 1988), where R is the gas constant (1.9872 cal $^\circ K^{-1}$ mol^{-1}) and T is the absolute temperature (298 °K).

10. Screening of the peptide library

The peptide libraries were screened by competitive ELISA. The microtiter plate was coated with the oxidized AcPAK(128-144)OH peptide (200 µM, 80 µl/well) in 0.01 M carbonate buffer, pH 9.5. The plate was incubated for 6 hr at room temperature and was subsequently blocked with 3% BSA in PBS at 4°C overnight. Peptide library solutions (8 mM, final concentration) were preincubated for an hour with the monoclonal antibody PK99H (250 µg protein/ml in stock solution, final dilution in each well was 2×10^{-5} of the stock solution) in 50 mM PBS containing 0.05% BSA, pH 7.4. The solution mixtures (80 µl) were transferred to corresponding wells and incubated for two hours at 37°C. The plates were washed five times with the incubation buffer. The amount of antibody bound was detected by goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Laboratories, CA) using ABTS as the substrate. The substrate solution was prepared in 10 mM sodium citrate buffer, pH 4.2, containing 0.03% (v/v) hydrogen peroxide. The reaction was stopped by 4 mM sodium azide, and the absorbance was measured at 405 nm.

The results obtained from the peptide libraries were verified by synthesizing

hexapeptides with sequences according to the combinations of the dipeptide sequences mapped from the three series of peptide libraries. The synthesis was performed following the general procedure for SPPS as described previously. Competitive ELISA was then performed to determine the ability of these hexapeptides to inhibit antibody binding to the peptide antigen.

11. Biotinylation of pilus protein and pilin peptides

In the whole cell binding assay, biotinylated probes were employed as the reporter groups to determine the amount of ligands bound. The biotin-avidin system was used because of its high specificity and affinity, and its well characterized thermodynamic and structural properties. In this project, streptavidin was used instead of avidin because it causes less non-specific binding. Streptavidin is an extracellular protein of *Streptomyces avidinii* and has a molecular weight of 60,000 (Chaiet and Wolf, 1964). Like avidin, streptavidin consists of 4 identical subunits, each subunit contains a high affinity binding site for biotin with a dissociation constant of about 10^{-15} M (Chaiet and Wolf, 1964). Recently, the adhesion force between the biotin and avidin molecules has been determined to be 160 piconewtons (Florin *et al.*, 1994).

(a) *Biotinylation of the PAK pili*

The PAK pili were biotinylated with a stock solution of 20 mg/ml of biotinamidocaproate N-hydroxysuccinimide ester (Sigma, St. Louis, MO) in dimethyl sulfoxide (DMSO). Limited biotinylation was attained by controlling the incubation time and rapid removal of excess biotinylation reagent after the reaction. The PAK pili (300 µl, 4.8 mg/ml) was pipetted into a dialysis tubing (with a MW cutoff of 12,000-14,000, 10 mm in diameter). A 10 µl aliquot of the stock biotinylation reagent was then added. The dialysis tubing was sealed and put into a 50 ml conical culture tube.

The reaction was allowed to proceed for 45 min at room temperature under constant shaking by a gyroshaker. Excess biotin ester was removed by extensive dialysis with four changes of PBS, pH 7.4, at 4°C overnight. The biological activities of the biotinylated PAK pili was checked by examining the ability of the biotinylated pili to bind to the PAK pilus-specific monoclonal antibody PK99H (data not shown) as well as to A549 cells.

(b) *Biotinylation of the PAK and PAO synthetic peptides*

Biotinylation was performed by manual coupling of activated biotin to the N-terminus of the peptide. Before the biotinylation, four glycine residues were coupled to the N-terminus of the peptide to function as a spacer. Biotin (Sigma, St. Louis, MO) was activated by 1 equivalent of 1 M HOBt/NMP and 1 equivalent of 1 M DCC/NMP, and stirred at room temperature for 30 min. The reaction mixture was then filtered with a sintered glass funnel. Peptide-resin with N-terminus protecting group deprotected was coupled with the activated biotin solution for 1 hour with mixing. DIEA (700 µl) was added and vortexed for 7 min. The solution was drained and washed with NMP. The coupling efficiency was checked by the ninhydrin test (Kaiser *et al.*, 1970). The biotinylated peptides were then cleaved from the resins, purified, oxidized and verified according to the procedures as previously described (sections 1 to 4). A control peptide, which corresponds to the sequence 18 to 28 of skeletal actin (Van Eyk *et al.*, 1991), was synthesized and biotinylated. The sequence of this biotinylated peptide is biotin-GGGGKAGFAGDDAPR-amide.

12. Cell culture

The A549 cells were grown in 25 cm^2 tissue culture flask (Corning, New York) in Waymouth MB 752/1 medium supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and antibiotic-antimycotic solution (100 U/ml penicillin, 100

μg/ml streptomycin and 0.25 μg/ml amphotericin B). All tissue culture reagents were obtained from GIBCO-BRL (Life Technologies Inc., New York). The culture was maintained at 37°C under an atmosphere of 5% CO_2 in a humidified incubator. The cells were passaged at 4- to 5-day intervals. The cell monolayer was detached by a 30-minute incubation in Hank's balanced salt solution (HBSS) containing 5 mM EGTA at 37°C. The harvested cells were then washed and centrifuged twice in plain medium and the final pellet was resuspended in medium with supplements. Passages 87 to 100 were used in the whole cell binding assays

13. Whole cell binding assay

A549 cells were suspended in Waymouth medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1 mM sodium pyruvate, and the antibiotic-antimycotic solution (assay medium). The cell density was adjusted to 4 x 10^5 cells/ml. A549 cells were seeded into a 96-well tissue culture plate (Corning, New York) by transferring a 100 μl aliquot of the cell suspension to each well. Another 100 μl/well of assay medium was added. The plate was incubated at 37°C overnight. After incubation, the medium in each well was removed manually by a multichannel pipette using electrophoresis pipette tips (Bio-Rad, Hercules, California). The plate was washed once with 200 μl/well of HBSS supplemented with 1 mM of $CaCl_2$, 2 mM of $MgCl_2$ and 0.05% (w/v) BSA (Buffer B). During each washing, the buffer was delivered slowly to the wells in order to minimize detachment of the cells. Biotinylated PAK pili (stock solution: 3 mg/ml, diluted 1:2,000) was mixed with serially diluted competing ligands (the peptide analogs) and the assay mixtures were added to the wells (75 μl/well). In binding assays where biotinylated peptides were employed, a concentration of 2 μM was mixed with serially diluted competing ligands. Following a 2-hour incubation period at 37°C, the plate was washed three times with 150 μl/well Buffer B. Streptavidin-alkaline phosphatase (GIBCO-BRL, Life

Technologies Inc., New York) was diluted 1:3,000 in HBSS containing 1% (w/v) BSA, and 75 µl of the solution was added to each well. The reaction was allowed to proceed at room temperature for one hour. The plate was then washed twice with 150 µl/well Buffer B and twice with 150 µl/well of 50 mM Tris buffered saline containing 0.05% (w/v) BSA, pH 7.5. Following the washes, 100 µl/well of *p*-nitrophenylphosphate (1 mg/ml in 10% (v/v) aqueous diethanolamine, pH 9.8) was added and the plate was incubated at room temperature for 2 hr. In the case of biotinylated peptide, a substrate amplification kit from GIBCO-BRL (Life Technologies Inc., New York) was employed in order to increase the sensitivity of the assay. The enzymatic reaction was stopped by addition of 100 µl of 2N NaOH (or 0.3 M H_2SO_4 in the case of biotinylated peptide) to each well. The absorbance at 405 nm (492 nm for biotinylated peptide) was determined.

14. ELISA substrate amplification system

The ELISA amplification system was purchased from GIBCO-BRL (Life Technologies Inc., New York) as a substrate kit. The methods have been described by Stanley *et al.* (1985) and Self (1985). This signal amplification method makes use of a secondary cyclic enzyme reaction to intensify the signal. The substrate in this system is NADPH, which is dephosphorylated by the bound streptavidin-alkaline phosphatase to NADH. NADH initiates a secondary enzyme system that comprises a redox cycle driven by diaphorase and alcohol dehydrogenase. In this cycle, NADH, in the presence of diaphorase, reduces a tetrazolium salt (INT-violet) to form an intensely colored formazan dye that can be measured at 492 nm. The oxidized NAD^+ is then regenerated by alcohol dehydrogenase in the presence of ethanol. The rate of the colored end product formation is directly proportional to the concentration of NADH originally produced by the bound streptavidin-alkaline phosphatase.

CHAPTER III

ELUCIDATION OF THE EPITOPE AND STRAIN-SPECIFICITY OF A MONOCLONAL ANTIBODY DIRECTED AGAINST THE PILIN PROTEIN ADHERENCE BINDING DOMAIN OF *PSEUDOMONAS AERUGINOSA* STRAIN PAK[1]

A. Introduction

Since the realization that *P. aeruginosa* is a potent opportunistic pathogen which causes serious morbidity and mortality, extensive research has been carried out in order to understand the pathogenicity of this microorganism, particularly its virulence-associated factors (Cryz *et al.*, 1984; Nicas and Iglewski, 1986; Döring *et al.*, 1987; Drake and Montie, 1988; Holder, 1993). One of the key components of the *P. aeruginosa* virulence factors was identified to be the pilus adhesin, whose critical role in mediating bacterial adherence to human epithelial cells has been well established (Woods *et al.*, 1980b; Ramphal *et al.*, 1984; Doig *et al.*, 1988). It is generally accepted that the pilus-mediated adherence is an initial step for the colonization of *P. aeruginosa* on the surface of the host (Pier, 1985; Irvin *et al.*, 1990).

Immunotherapy using pilus-specific antibodies seems to be a rational prophylaxis to treat *P. aeruginosa* infections by blocking its initial adherence process. Woods *et al.* (1980b) were the first to show that anti-pilus antibody can reduce the adherence of *P. aeruginosa* to buccal epithelial cells. Since then, similar results from various laboratories have been reported (Ramphal *et al.*, 1984; Doig *et al.*, 1988; Lee *et al.*, 1989a). Previous studies have demonstrated that PK99H, a monoclonal antibody

[1]A version of this chapter has been published: Wong, W.Y., R.T. Irvin, W. Paranchych, and R.S. Hodges (1992). *Protein Science* **1**: 1308-1318.

directed against the PAK pilus, bound specifically to the C-terminal region of the *P. aeruginosa* PAK pilin and blocked bacterial adherence to both human buccal and tracheal epithelial cells (Doig *et al.*, 1990). When different pilus strains were tested, only PAK pili were found to bind to PK99H (Doig *et al.*, 1990).

Antigenicity studies on the PAK pilin protein have been carried out earlier in this laboratory. Watts and coworkers (1983b) identified an antigenic determinant in the C-terminal region (residue 121 to 144) of the PAK pilin. In addition, studies using pilin protein fragments and synthetic peptides showed that the C-terminal region of PAK pilin contains an epithelial cell binding domain (Paranchych *et al.*, 1986; Irvin *et al.*, 1989). Hence, the objective of this study was to elucidate the antigenic determinant (or epitope) recognized by PK99H. Epitope mapping was performed in a similar approach employed by Geysen *et al.* (1984) and Hodges *et al.* (1988) in studying the antibody-antigen interactions. In this study, a series of single alanine-substituted peptide analogs corresponding to the C-terminal 17-residue sequence of PAK pili was synthesized. These peptides were then used to determine the epitope sequence by comparing their binding affinity to PK99H. In addition, the nature of the strain-specificity was addressed by examining the binding affinity of peptide sequences derived from other *P. aeruginosa* pilin strains.

B. Results

Monoclonal antibody PK99H has been shown to be strain-specific in that it binds to the pilus of *P. aeruginosa* strain PAK and does not bind to the heterologous strain PAO. It also binds to the C-terminal region of PAK pilin within the sequence 121-144 (Doig *et al.*, 1990). In this study, an antibody is designated to be strain-specific if its binding affinity for another strain is decreased by at least 1,000-fold. The relative binding affinity of the PAK and PAO peptides (AcPAK(128-144)OH and AcPAO(128-

144)OH, Table III.1) to PK99H was assessed by means of their ability to inhibit the binding of PK99H to PAK pili. As indicated in Figure III.1, these two peptides have different binding affinities to PK99H in the competitive ELISA assay. The apparent association constants (K_a) were calculated from the I_{50} values as described by Nieto *et al.* (1984). The I_{50} values (peptide concentration required for 50% inhibition of PK99H binding to PAK pili) for both peptides showed that a 1,500-fold higher concentration of PAO peptide was required to attain the same inhibitory effect as that of PAK peptide. The K_a's of PAK and PAO peptides were determined to be 4.0×10^6 M^{-1} and 2.63×10^3 M^{-1}, respectively (Table III.2). The loss of binding free energy, $\Delta(\Delta G)$, of PAO peptide was 4.32 kcal/mol compared with PAK peptide.

1. PK99H epitope

In order to locate the PK99H epitope, residues (excluding the two cysteines) in the PAK sequence (128-144) were substituted with alanine to prepare single Ala-substituted analogs (Fig. III.2). The effects of these substitutions on PK99H binding were examined. If a single alanine-substituted peptide showed a decrease in its ability to inhibit the binding of PK99H to PAK pili, the side-chain substituted was considered to be important for PK99H binding and contributed to the epitope.

The I_{50} value of each peptide analog was compared with the I_{50} value of the native peptide (I°_{50}), and was represented graphically by ($\log I_{50} - \log I^{\circ}_{50}$). A positive value indicated a loss of binding affinity of the peptide analog. In Figure III.2, the importance of the intrachain disulfide bond in PAK peptide for PK99H binding was also assessed. The I_{50} value of the reduced native peptide, AcPAK(128-144)OH, was similar to that of the oxidized peptide (2.2×10^5 pM and 2.5×10^5 pM, respectively, data not shown), which were comparable to values obtained in previous studies (Doig *et al.*, 1990). In addition, the absence of the disulfide bridge did not affect the determination of the PK99H epitope, since the reduced and oxidized peptide analogs showed similar

Table III.1 Amino acid sequences of the C-terminal regions from residues 128 to 144 of five different *P. aeruginosa* strains

Strain	Sequence[a]															
	128						134					140				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)
CD4	(T)	C	T	S	(T)	Q	(E)	L	(M)	F	I	P	K	G	C	(N)	K
K122-4	(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)

[a]The sequences of the five *P. aeruginosa* strains (PAK, PAO, CD4, K122-4 and KB-7) are taken from Paranchych *et al.* (1990). The circled residues denote sequence differences from strain PAK.

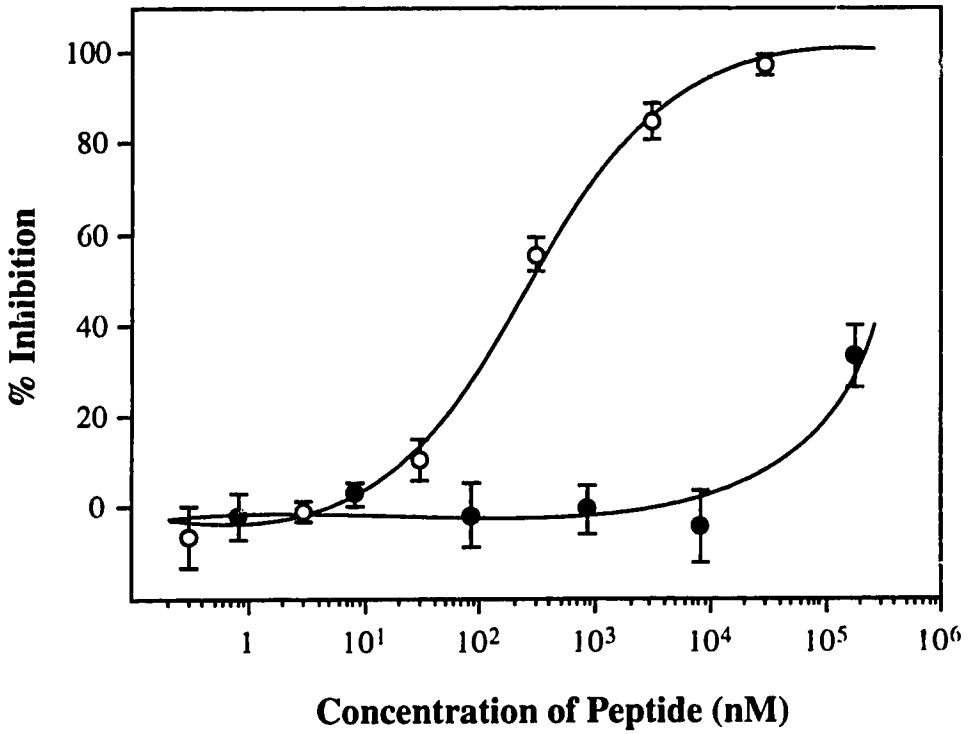

Figure III.1 Competitive ELISA profiles of both AcPAK(128-144)OH and AcPAO(128-144)OH synthetic peptides showing the competitive inhibition of monoclonal antibody PK99H binding to PAK pili by AcPAK(128-144)OH (O) and AcPAO(128-144)CH (●). The amino acid sequences of the two peptides are listed in Table III.1.

Table III.2 Thermodynamic analysis of the binding of synthetic peptides from various strains of *Pseudomonas aeruginosa* pilins to monoclonal antibody PK99H

Peptide Name[a]	K_a[b] (M^{-1})	K_N/K_S[c] ratio	$\Delta(\Delta G)$[d] (kcal/mol)
AcPAK(128-144)OH	4.00 x 10^6	1	
AcPAO(128-144)OH	2.63 x 10^3	1,500	4.32
AcPAK(134-140)NH_2	2.22 x 10^6	1	
AcPAO(134-140)NH_2	2.00 x 10^3	1,000	5.50
AcCD4(134-140)NH_2	1.18 x 10^5	19	1.73
AcKB7(134-140)NH_2	7.14 x 10^2	3,100	4.75
AcK122-4(134-140)NH_2	< 1.00 x 10^2	> 22,000	> 5.91

[a]The peptide sequences are listed in Table III.1.

[b]K_a is the apparent association constant of monoclonal antibody PK99H for the corresponding peptide analog, which is calculated by the formula: $K_a = 1/I_{50}$ (Nieto *et al.*, 1984).

[c]Where K_N and K_S stand for the apparent association constants (K_a) of the PAK peptides (K_N) (i.e. AcPAK(128-144)OH or AcPAK(134-140)NH_2) and peptides of other strains (K_S).

[d]$\Delta(\Delta G)$ is the relative loss of binding free energy of the synthetic peptide analogs to monoclonal antibody PK99H as compared with the native PAK peptide. It is calculated as described by Bhattacharyya and Brewer (1988): $\Delta(\Delta G) = RT\ln(K_N/K_S)$.

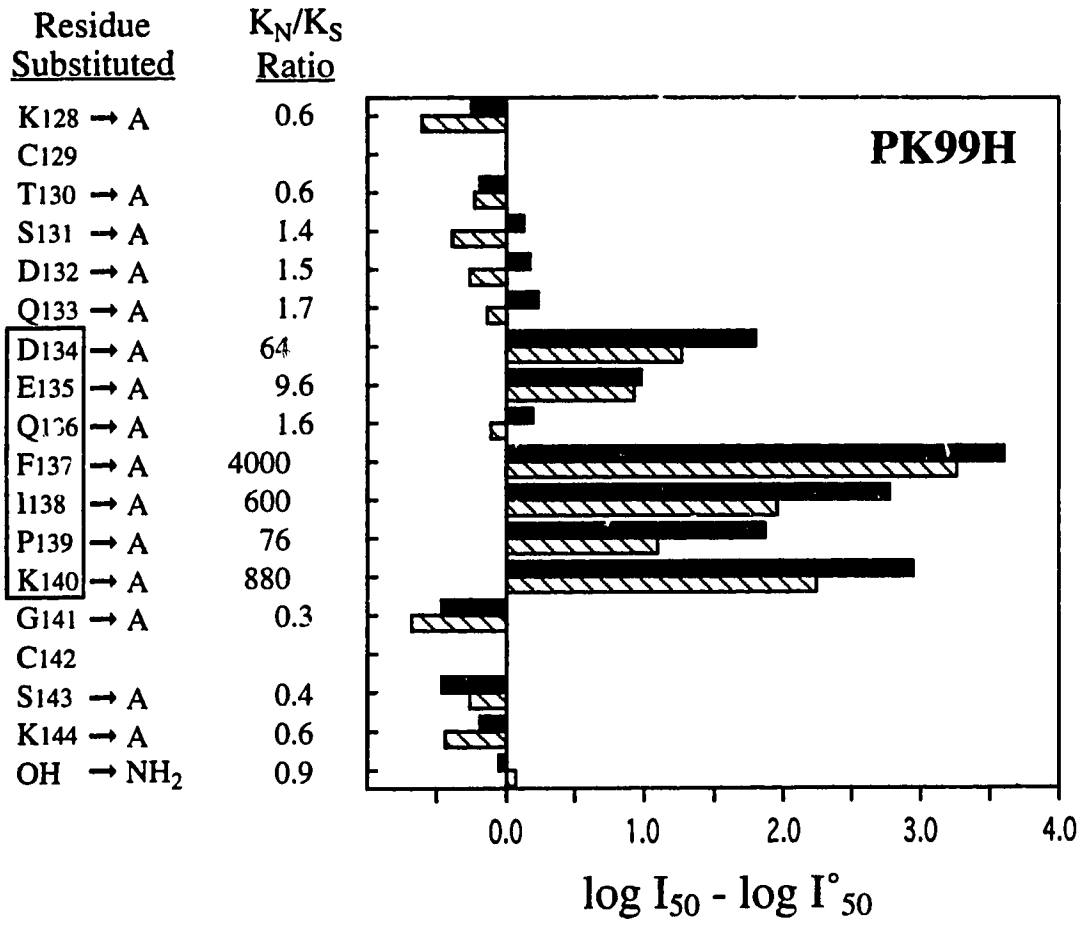

Figure III.2 Effects of single alanine substitution on the binding of AcPAK-(128-144)OH to monoclonal antibody PK99H as determined by competitive ELISA. The amino acid sequence of the peptide is listed from N- (top) to C- (bottom) terminus on the ordinate, where the position of alanine substitution in each peptide analog is denoted. OH→ NH_2 denotes peptide analog with an amide at its C-terminus. The K_N/K_S ratio indicates the relative loss of binding affinity to PK99H with respect to that of the native PAK peptide. A value greater than 1.0 denotes an x-fold decrease in binding affinity, whereas a value less than 1.0 denotes an x-fold enhancement in binding. $I°_{50}$ is the I_{50} of the native peptide. A positive value of log I_{50} - $I°_{50}$ indicates a loss of binding affinity of the peptide analog to PK99H with respect to the native peptide. The solid bars denote results obtained from the oxidized peptides, whereas the hatched bars are results from the reduced peptides. The boxed sequence defines the linear epitope for PK99H.

binding profiles. The only difference observed between reduced and oxidized peptides was a general decrease in the magnitude of the log I_{50} - log I°_{50} values for the reduced peptides (Fig. III.2). This Ala replacement analysis suggested that the minimum peptide sequence for maximum binding to PK99H was located in the linear sequence 134-140. Of this 7-residue sequence (region boxed in Fig. III.2), six side-chains were found to be important for PK99H binding (Asp^{134}, Glu^{135}, Phe^{137}, Ile^{138}, Pro^{139} and Lys^{140}) and showed decreases in binding affinity from 10 to 4,000 fold. These results suggested that a 7-residue peptide representing the epitope could bind to PK99H.

To determine whether the minimum peptide sequence required for antibody binding was the 7-residue epitope or a peptide shorter than seven residues, peptides of various lengths were synthesized (Fig. III.3). These peptides varied in length from four to eight residues and constituted all or part of the binding epitope as predicted from Figure III.2. Even though the four residues ^{137}Phe-Ile-Pro-Lys^{140} (FIPK) represented the four most important residues in the epitope with decreases in binding affinity ranging from 76- to 4,000-fold for the single alanine-substituted 17-residue oxidized peptides (Fig. III.2), it was demonstrated in Figure III.3 that this sequence alone (FIPK) was not sufficient to compete with PAK pili binding to PK99H. The peptide FIPK lost its ability to compete with PAK pili for PK99H binding (>10,000-fold decrease) as compared with the native AcPAK(128-144)OH peptide. As the peptide sequence of the core peptide FIPK was extended towards the C-terminus, binding affinity to PK99H remained poor (7,600-fold decrease). On the other hand, extension towards the N-terminus resulted in increased binding affinity. When Gln^{136} was added (QFIPK), an enhancement in binding affinity by at least 33-fold was observed. A further 20-fold enhancement was observed as the Glu^{135} was added (EQFIPK) and at least 8-fold as Asp^{134} was added to obtain the maximum affinity for PK99H. Thus, the PK99H epitope was again located to the sequence 134-140 (DEQFIPK). Interestingly, there was no significant change in binding affinity for this 7-residue peptide as compared

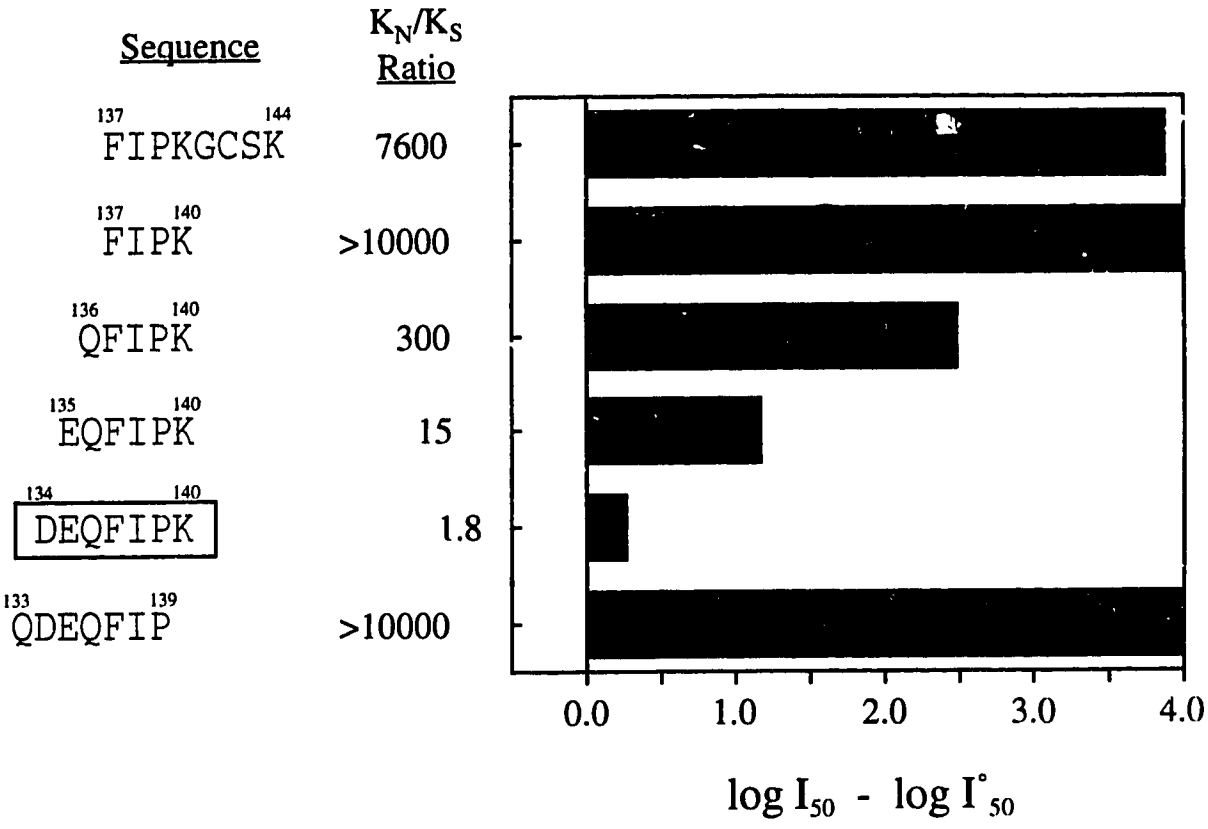

Figure III.3 Determination of the binding epitope for monoclonal antibody PK99H. Six truncated peptides with lengths ranging from residues 133 to 144 were synthesized. The change in binding affinity of each peptide analog to PK99H was assessed by comparing their I_{50} values with that of the native AcPAK(128-144)OH peptide. The boxed sequence defines the linear epitope of PK99H. All peptides are N^{α}-acetylated at the N-terminus and have an amide group at the C-terminus except for ^{137}FIPKGCSK^{140}, which has an α-carboxyl group at the C-terminus.

with the native oxidized 17-residue AcPAK(128-144)OH peptide. Within any linear epitope there is also the minimum sequence required to exhibit antibody binding (within 1,000-fold in binding affinity compared to the epitope peptide). This sequence consisted of residues 136-140 compared to the epitope sequence 134-140 (Fig. III.3).

2. Importance of individual side-chains in PK99H epitope

The importance of individual side-chains in the epitope was assessed by single alanine substitutions in the 7-residue and 17-residue peptides. Figure III.4 shows representative competitive binding curves of the competitive ELISA from two of the single alanine-substituted peptide analogs. together with the native epitope peptide. The change in the inhibition of the alanine-substituted peptide can be reflected by a corresponding shift of the binding curve. The results from Figures III.2 and III.5 suggested that the 7-residue peptide analogs have similar antibody binding characteristics to the 17-residue peptide analogs. In addition, it further confirmed that the disulfide bridge was not important to PK99H binding. Though the disulfide bond in the pilin protein controls the conformation of this region in the protein, the residues between the two cysteines involved in the disulfide bond can apparently still adapt to the binding pocket of PK99H as easily as the non-constrained linear 7-residue peptide.

I defined the side-chains within the epitope arbitrarily as one of three types: critical, important, and non-essential to antibody binding. A critical side-chain was one that on substitution by alanine (in a synthetic peptide representing the epitope only) decreased binding affinity greater than 1,000-fold as compared to the native sequence. On the other hand, if the decrease in binding affinity was less than 3-fold, the side-chain was considered as non-essential. Side-chains whose contribution fell between these two extremes were defined as important.

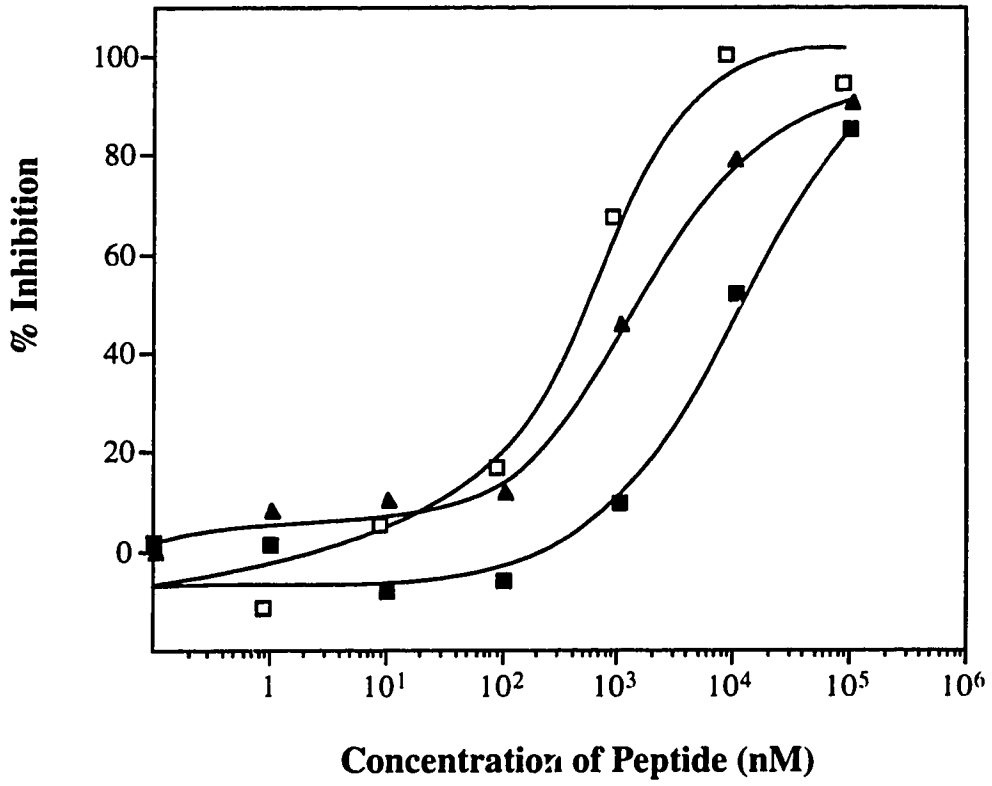

Figure III.4 Representative competitive ELISA profiles of some of the 7-residue peptide analogs showing inhibitory effects on PK99H binding to PAK pili by AcPAK(134-140)NH_2 (□), Ac(P139A)PAK(134-140)NH_2 (■), and Ac(E135A)PAK(134-140)NH_2 (▲). The amino acid sequences of the peptides can be found in the lengend of Figure III.5.

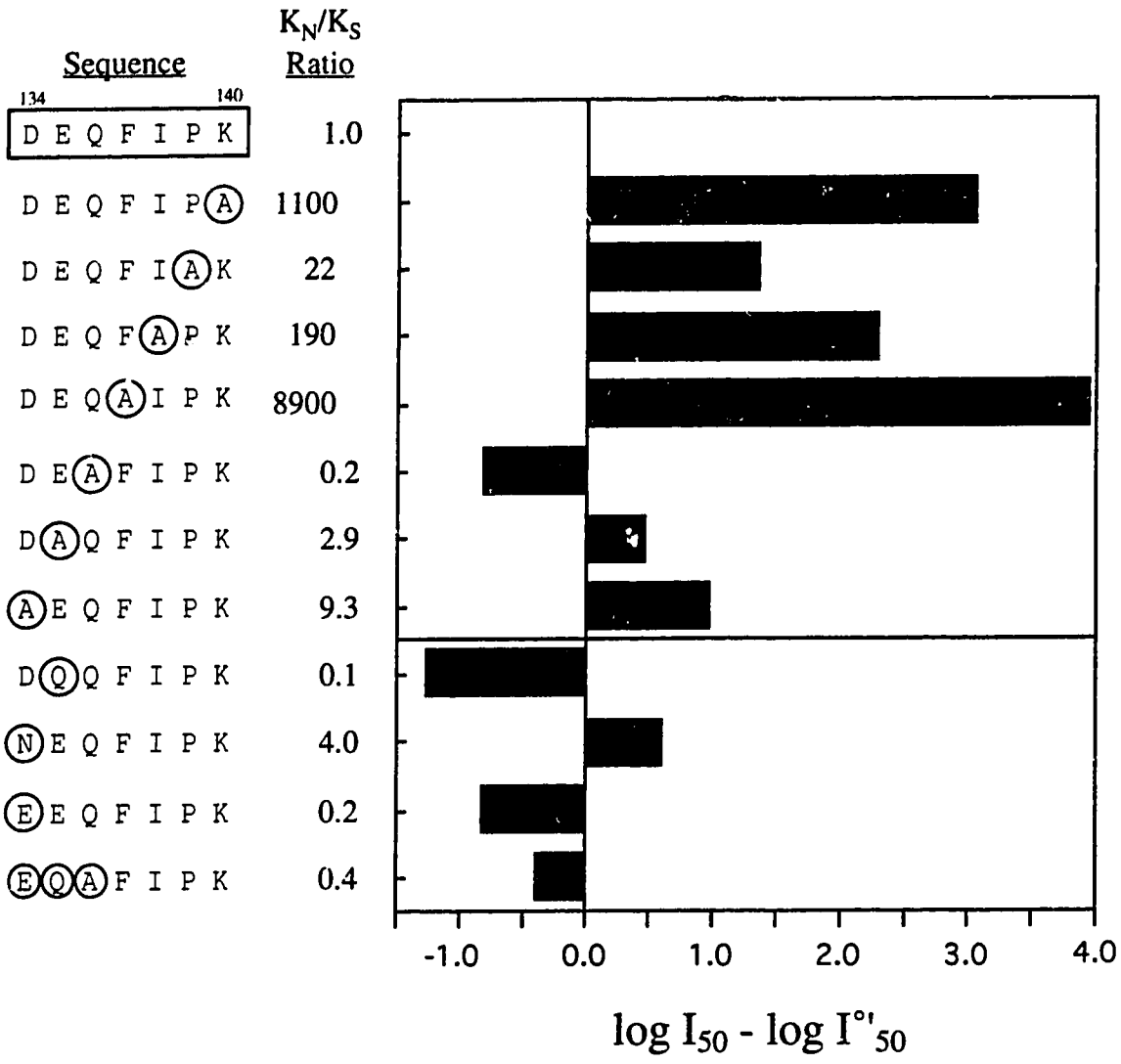

Figure III.5 Effects of amino acid substitution on the binding of the PK99H epitope sequence, AcPAK(134-140)NH_2 (boxed), to monoclonal antibody PK99H. The residues substituted are circled. $I^{\circ\prime}_{50}$ is the I_{50} value of the native epitope peptide sequence (boxed). All peptides are N^{α}-acetylated at the N-terminus and have an amide group at the C-terminus.

3. Charged side-chains

There are three charged residues in the epitope (Asp^{134}, Glu^{135} and Lys^{140}). The importance of the negative charges on Asp^{134} and Glu^{135} to antibody binding was investigated by the isosteric substitutions of Asn^{134} and Gln^{135}, respectively. Each individual negative charge was not critical for binding because the peptides NEQFIPK and DQQFIPK showed a 4-fold decrease and a 10-fold enhancement in binding affinity, respectively (Fig. III.5). However, a negative charge at either position could still play a role in binding. The isosteric side-chain substitutions were compared to the Ala substitutions. In both cases, an enhancement in binding affinity was observed (Asn^{134} showed a 2-fold enhancement in binding affinity compared to the Ala^{134} analog; Gln^{135} showed a 29-fold enhancement in binding affinity compared to the Ala^{135} analog). This suggests that hydrogen bonding may be more important for binding than the ionic interactions with the antibody binding site at these positions. In contrast, the positively charged residue, Lys^{140}, was one of the two critical residues in the epitope. Substitution (Figs. III.5 and III.7) and deletion (Fig. III.3) of this residue had a tremendous effect on binding affinity ($Lys^{140}\rightarrow$Ala, 1,100-fold decrease; $Lys^{140}\rightarrow$Asn, 2,000-fold decrease; AcPAK(133-139)NH_2 compared to AcPAK(134-140)NH_2, >10,000-fold decrease). Shi *et al.* (1984) have reported the importance of a lysine residue at the C-terminus of a peptide antigen in antibody binding. Hence, Lys^{140} may contribute an electrostatic attraction that increases the rate of antibody-antigen complex formation by facilitating the stability of an initial complex (Geysen *et al.*, 1987b).

4. Hydrophobic side-chains

Phe^{137} and Ile^{138} are the two major hydrophobic residues found in the epitope along with Pro^{139}. Substitution of Pro^{139} with Ala resulted in a 22-fold and 76-fold decrease in binding affinity for the 7- and 17-residue peptide, respectively (Figs. III.5

and III.2). By comparison, substitution of Ile^{138} with Ala showed a 190-fold and 600-fold decrease in binding affinity for the 7- and 17-residue peptides, respectively. Substitution of Phe^{137} with Ala caused a 8,900-fold and 4,000-fold decrease in binding affinity for the 7- and 17-residue peptides, respectively, making it a critical side-chain. The importance of these side-chains can be related to their relative hydrophobicities (Phe > Ile > Pro) (Parker *et al.*, 1986). Thus, it seems that hydrophobic interactions could be one of the major forces that determine the affinity of the antibody. Interestingly, on either side of the hydrophobic residues are charged residues that could also be important in ensuring that these hydrophobic residues are surface exposed and available for antibody (receptor) binding (Hodges *et al.*, 1988).

5. Importance of position 136 to PK99H binding

The PAK sequence contains Gln at position 136 and the side-chain was judged non-essential based upon the Ala substitution in the 7- and 17-residue peptides. Actually, there was a small enhancement of binding affinity in the case of PAK epitope sequence 134-140 (5-fold, Fig. III.5) and in the reduced sequence 128-144 (Fig. III.2). However, in the case of oxidized peptide (sequence 128 to 144), there was a very small decrease in binding affinity (1.6 fold, Fig. III.2). Interestingly, substitution with amino acids other than Ala at this position in the PAK sequence could dramatically affect binding to PK99H. The Gln^{136} to Lys substitution found in strains K122-4 and KB7 decreased binding affinity by 950 fold to PK99H (Fig. III.7). It is possible that Gln^{136} could be positioned in the binding interface with PK99H but that the side-chain's contribution to binding affinity was small. Thus, replacement of the side-chain by a smaller residue such as Ala would have little effect on binding affinity. On the other hand. the presence of Lys at this position, which is larger and carries a full positive charge, would seriously disrupt the binding site. Interestingly, Met^{136}, which is much closer in molecular size to Gln and is a neutral amino acid, caused a much

smaller disruption in binding affinity (14-fold decrease). It is also possible that the Gln residue at this position was important for controlling conformation of the folded peptide in the antibody binding site and was itself not part of the binding interface.

In order to distinguish these two possibilities, 20 heptapeptide analogs DEXFIPK, in which position X was substituted with all twenty naturally occurring amino acids, were synthesized. The peptides were synthesized employing the multiple column peptide synthesizer as described in Materials and Methods (Chapter II). The binding affinities of these peptides were determined by competitive ELISA, and the relative affinities of these peptide analogs as compared with the native sequence (DEQFIPK) are shown in Figure III.6. The results indicated that residue Gln^{136} could be replaced by Ala and Cys (3.3- and 2-fold enhancement in binding affinity), but it could not adapt to substitutions with charged side-chains (Asp, Glu, Lys and Arg substitution caused 82-, 220-, 1,000- and 1,400-fold decrease in binding affinity, respectively), bulky hydrophobic side-chains (Phe, Tyr and Trp caused 490-, 1,500- and 6,100-fold decrease, respectively), or complete removal of the side-chain (Gly, 580-fold decrease). Four residues such as Lys, Arg, Tyr, and Trp were the most incompatible residues (with 1,000-fold or more decrease in binding affinity). Preliminary NMR studies on the oxidized 17-residue peptide analog Ac(Q136Y)PAK(128-144)OH have shown that the bulky Tyr side-chain did not cause any significant conformational change to the peptide analog as compared to that of the native peptide (McInnes *et al.*, unpublished data). These results have not ruled out the possibility that Gln^{136} was part of the binding interface and participated in antibody-antigen interactions.

6. Enhancement of peptide binding to PK99H

As shown in Figures III.2, III.5 and III.7, the binding affinity of peptides to PK99H could be enhanced as compared to the native sequence. For the 17-residue peptides, whether in their oxidized or reduced forms, there were small enhancements of

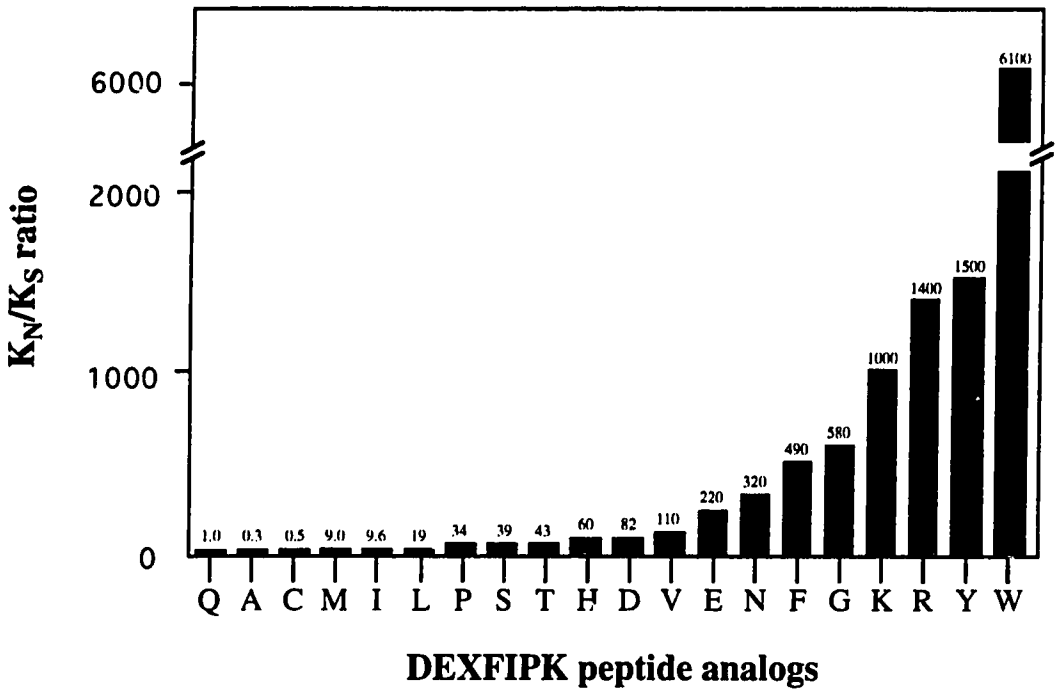

Figure III.6 The relative binding affinity of the 7-residue peptide analogs DEXFIPK to monoclonal antibody PK99H. Where X denotes residue substituted by all 20 naturally occurring amino acids. The first column "Q" thus represents the native PAK epitope sequence DEQFIPK for PK99H. All peptides are N^{α}-acetylated at the N-terminus and have an amidated C-terminus.

binding affinity for residues substituted outside the epitope of PK99H (up to 4.8-fold). Similarly, substitutions of certain residues inside the epitope showed enhancements (Asp^{134} to Glu, Glu^{135} to Gln and Gln^{136} to Ala resulted in a 5-, 10- and 5-fold increase in binding affinity, respectively, Fig. III.5). These results confirmed previous studies that even though an antibody is generated to a certain sequence, the antibody is not absolute in its specificity and changes in sequence can enhance as well as decrease binding affinity (Imanishi and Mäkelä, 1973; Reichlin and Noble, 1974). It has been well documented that large enhancements in binding can be achieved for both peptide substrates and inhibitors of enzymes, so it is not unreasonable to expect amino acid substitutions to enhance binding affinity of peptides to monoclonal antibodies. In order to test whether these enhancements were additive, a synthetic peptide containing the above three substitutions was prepared (EQAFIPK). This peptide also showed enhanced binding affinity to PK99H as compared to the native PAK sequence but the magnitude of the enhancement was not additive (about 2.5-fold rather than 20-fold if the effect was additive, Fig. III.5).

7. Strain specificity of PK99H

The C-terminal sequences of several heterologous *P. aeruginosa* pilin strains (Paranchych *et al.*, 1990) are shown in Table III.1. The 7-residue peptides (residues 134-140) containing the sequences of the strains PAO, CD4, KB7 and K122-4 were synthesized. Two of these strains, PAO (purified pili and whole cell extract) and K122-4 (whole cell extract), were tested previously and were not recognized by PK99H (Doig *et al.*, 1990). As the sequences of these four strains in the sequence 134-140 corresponding to the PK99H epitope are compared (Table III.1), PAO has three different residues as compared with PAK sequence, CD4 has two, K122-4 has four, and KB7 has four. Indeed, as shown in Table III.2, three of them, PAO, KB7 and K122-4, demonstrated the strain-specificity of PK99H with decreases in binding

affinity from 3,100- to >22,000-fold. PK99H reacted with CD4 where only a 19-fold decrease in affinity was observed. The loss of binding free energy, $\Delta(\Delta G)$, of such peptides varied from 1.73 kcal/mol (CD4) to greater than 5.91 kcal/mol (K122-4) with respect to the native 7-residue peptide AcPAK(134-140)NH_2 (Table III.2).

To understand which side-chains were accounting for strain-specificity in strains PAO, KB7 and K122-4 or the decrease in binding affinity of strain CD4 to PK99H, all different residues found in these strains were substituted one at a time in the PAK sequence (Fig. III.7). In the case of the PAO sequence, the decrease in binding affinity of PK99H was greater than 10,000-fold. However, no single substitution of the PAO residues in the PAK sequence were able to account for this specificity (Fig. III.7). The PAO residues Pro^{135}, Met^{136} and Thr^{138} as single mutants of the PAK sequence decreased affinity by 7.4-, 14- and 79-fold, respectively. Interestingly, the three double mutants decreased affinity in a synergistic rather than an additive manner. In fact, one of these double mutants (Pro^{135} Met^{136}), was able to account for the decrease in binding affinity of strain PAO to PK99H (>10,000-fold). This was unexpected when one considers that Pro^{135} or Met^{136} individually had only small effects on binding affinity. In contrast, when the Thr^{138} substitution, which had the largest effect on binding affinity as a single substitution in the PAK sequence (79-fold), was combined with either Pro^{135} or Met^{136} as a double mutant was unable to account for the >10,000-fold decrease in binding affinity caused by the triple mutant (PAO sequence) (Fig. III.7).

In the case of K122-4, which differed from the PAK sequence in four positions, two of the substitutions Leu^{138} for Ile and Asn^{135} for Glu did not cause significant decreases in binding affinity for PK99H as single mutants (1.4- and 4-fold decreases). The most significant substitution was Lys^{136} for Gln in the PAK sequence. This single amino acid change accounted for a 950-fold decrease in affinity. In similar fashion to K122-4, KB7 differed from the PAK sequence at four positions, one of

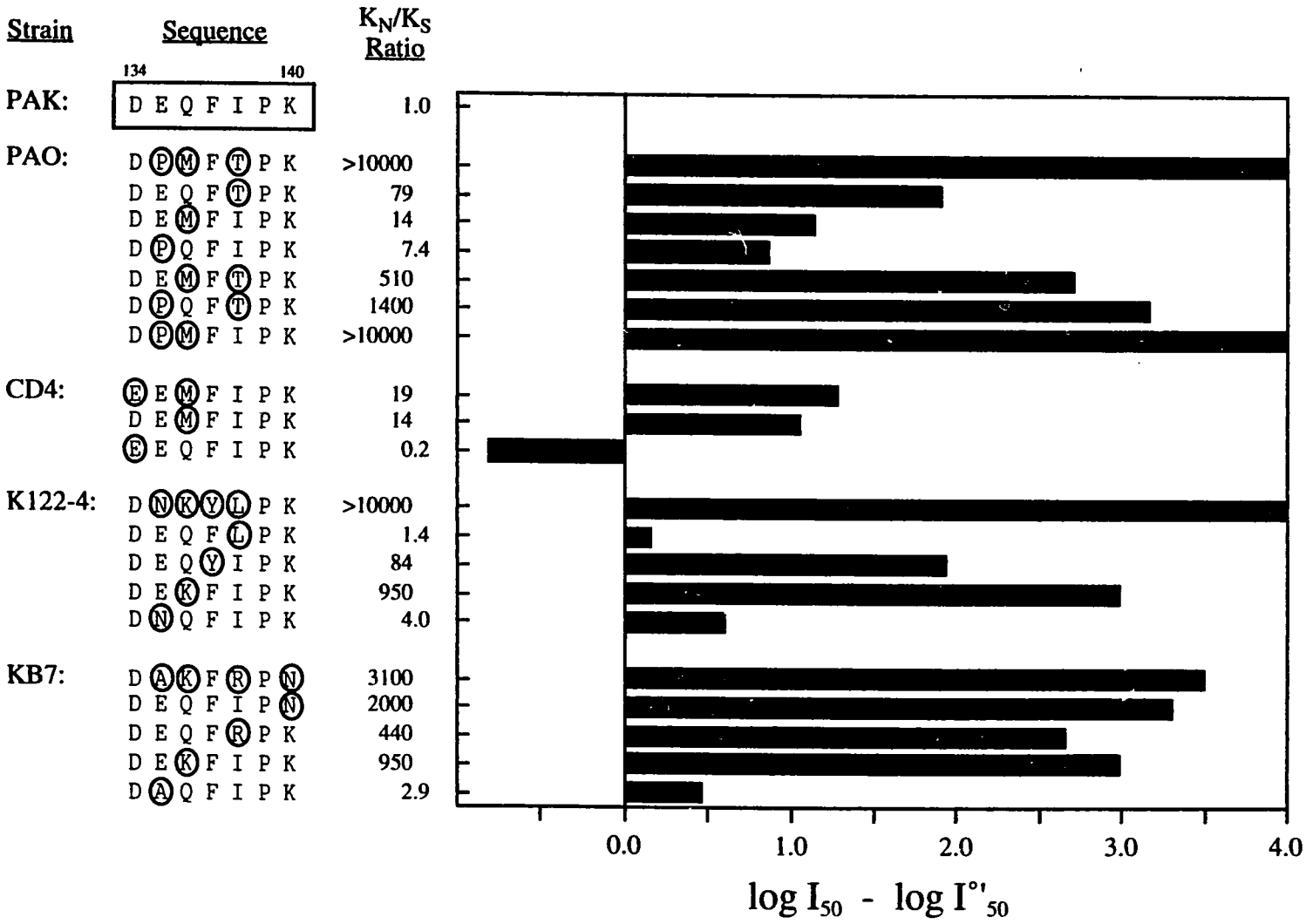

Figure III.7 Comparison of the binding affinity of synthetic peptide analogs from five *Pseudomonas aeruginosa* strains with sequence confined to PK99H epitope of PAK pilin (boxed). The native peptide sequence of each strain is indicated by the name of the strain on the legend of the ordinate. Circled residues denote different residues as compared with the native PAK sequence. All peptides are N^{α}-acetylated at the N-terminus and have an amide group at the C-terminus.

which (Ala^{135} for Glu) did not cause a significant decrease in binding affinity (2.9-fold). However, any of the single mutants—Lys^{136} for Gln, Arg^{138} for Ile, and Asn^{140} for Lys—resulted in large decreases in binding affinity (950-, 440- and 2,000-fold, respectively). By analogy with the PAO sequence, a combination of any of the two amino acid changes indicated above could readily account for the 3,100-fold decrease in affinity of KB7 sequence.

The CD4 sequence varies from the PAK sequence in two positions, Glu^{134} for Asp and Met^{136} for Gln. The Met^{136} substitution could, by itself, account for the decreased affinity of the CD4 sequence for PK99H. The Glu^{134} for Asp substitution was the only naturally occurring substitution that enhanced binding affinity over the native PAK sequence (5-fold).

C. Discussion

An important issue for the understanding of antigen-antibody interactions is the underlying principles that determine the binding affinity and specificity of an antibody. There are two kinds of epitopes, continuous and discontinuous epitopes (Atassi and Smith, 1978). The linear or continuous epitopes can be defined as the minimum linear sequence required for maximum antibody binding. The epitope can be mapped and the relative importance of individual side-chains can be determined by systematically walking a single alanine-substitution through a synthetic peptide known to contain the epitope (an Ala residue in the native sequence is replaced by a Gly residue). For example, the monoclonal antibody PK99H, used in this study has been shown to bind to or near the adherence binding domain of *Pseudomonas* PAK pilin (Doig *et al.*, 1990), which was located at the C-terminus of the pilin protein. The present data also showed that PK99H bound to the synthetic 17-residue C-terminal peptide in its native conformationally constrained form (a disulfide bond between cysteine residues 129 and

142) or in its reduced form (lacking the disulfide bond). However, the affinity constant for PAK pili binding to PK99H was not directly comparable with that of the 17-residue peptide due to the polydispersed size distribution of purified pili (Doig *et al.*, 1990). It is assumed that the deletion of a side-chain through an alanine substitution would allow the examination of the importance of the side-chain for antibody binding while having a minimum effect on the conformation of the peptide and its ability to fold into the antibody binding pocket.

In this study, this replacement approach suggested that the epitope was contained in the 7-residue sequence 134-140 (Fig. III.2). In fact, the antibody can bind strongly to PAK(134-140)NH_2 (the deduced epitope of PK99H) conjugated to BSA as observed in direct ELISA (data not shown). This result was further verified by preparing peptides of varying lengths at the N- and C-terminal regions of the suspected epitope (Fig. III.3). Using the proposed criteria, antibody binding was found to be influenced by six of seven side-chains involved in this epitope: two of these side-chains were classified as critical (Phe^{137} and Lys^{140}), four were important (Asp^{134}, Glu^{135}, Ile^{138} and Pro^{139}), and one was non-essential (Gln^{136}) (Fig. III.5). It is interesting that alteration of the lysine side-chain ($Lys^{140}\rightarrow Ala$) resulted in a 1,100-fold decrease in binding affinity (Fig. III.5) whereas omission of Lys^{140} resulted in a >10,000-fold decrease in affinity (Fig. III.3). Surprisingly, the minimum sequence required for antibody binding was 136-140, which contains a non-essential side-chain. Omission of Gln^{136} in the sequence 137-140 resulted in a >10,000-fold decrease in binding affinity (Fig. III.3). These results show the importance of the peptide backbone of residues Gln^{136} and Lys^{140} in antibody binding. Thus, it is important to distinguish between the side-chain and backbone requirements for antibody binding when discussing the importance of an amino acid residue. These results agree with those of previous workers; Hodges *et al.* (1988) showed that the four monoclonal antibodies directed to the cytoplasmic carboxyl terminus of bovine rhodopsin recognized linear

epitopes from 4 to 11 residues and the minimum sequences required for antibody binding, which were shorter than the epitope, always contained the critical side-chains. The number of critical side-chains in an epitope varied from three to four.

Geysen *et al.* (1987a) reported that they have not observed any epitopes in which more than five residues were contact residues. Their definition of contact residues would relate to the critical residues identified in this study, for which antibody binding was lost or significantly decreased (>1,000-fold) when the original critical residue was replaced with residues of dissimilar character. Recently, Xing *et al.* (1991) have also reported a minimum epitope sequence of five residues for three monoclonal anti-mucin antibodies (BC1, BC2 and BC3), and the number of critical residues found was four for BC1 and BC2, and one for BC3. In addition, the epitope length reported for other monoclonal antibodies falls into the range of 5 to 10 residues (Anderson *et al.*, 1988; Kövamees *et al.*, 1990; Scott *et al.*, 1990) and the minimum sequence found is between 5 and 7 residues (Anderson *et al.*, 1988).

It has been previously reported that antibody binding of small peptides (two to seven residues) is dominated by nonspecific ionic and hydrophobic interactions, and only peptides of 15 to 20 residues in length could undergo meaningful specific binding (Shi *et al.*, 1984; Berzofsky, 1985). In this study, there were three observations which support that the binding of the 7-residue epitope sequence to PK99H was specific. First, the ability of the 7-residue peptide to inhibit PK99H binding to PAK pili was very similar to that of the 17-residue peptide in either its oxidized or reduced form. In addition, the inhibition profiles of the single alanine-substituted 7-residue peptide analogs (Fig. III.5) were similar to those of the 17-residue peptide analogs (Fig. III.2). This observation showed that the binding of a conformationally constrained peptide such as the disulfide bridged 17-residue peptide could be mimicked by a small 7-residue peptide. Second, PK99H could react with PAK and CD4 peptides only, but had extremely low affinity for the PAO, K122-4, or KB7 peptides (Fig. III.7). Third,

the small peptide FIPK, which constituted the four most important side-chains for antibody binding did not compete with PAK pili binding to PK99H. These data strongly support the conclusion that the 7-residue peptide binds specifically to its monoclonal antibody. Worobec *et al.* (1985) showed that the binding of synthetic peptides to polyclonal anti-EDP208 pilus antibodies was enhanced as the peptide was shortened from 12 residues to the 5 residues which defined the epitope. Though these small peptides probably lack conformational properties (being free in solution), they can readily fold into a preformed antibody binding pocket and also maintain the sequence-specific and conformational properties when bound. This is important because large synthetic peptides containing a small linear epitope may not readily fold into the antibody binding pocket. Kodama *et al.* (1991) have reported a 5-residue epitope sequence responsible for the strain-specificity of the monoclonal antibody SF8/5E11 directed against the transmembrane protein of simian immunodeficiency virus of macaque monkey by site-specific mutagenesis. However, they failed to demonstrate specific reactivity of this monoclonal antibody to 25-residue peptides containing the epitope.

An antibody produced against an immunogen can be either cross-reactive or strain-specific to various heterologous strains depending on the nature of the mutations in the epitope sequence and the highly specific binding nature of antibody. In the present studies, not only has the strain-specific epitope recognized by PK99H (AcPAK(134-140)NH_2) been mapped, but also the importance of individual residues from other strains (Table III.1) that varied in sequence from the PAK epitope was determined. The strain-specificity of PK99H for strains PAO, K122-4 and KB-7 could be explained by the accumulated sequence changes in these strains. It was found that at least two amino acid changes in the epitope were required to decrease binding affinity and achieve strain-specificity with PK99H. Cross-reactivity of PK99H to CD4 could be explained by the fact that there was a single side-chain responsible for decreasing

binding affinity as compared to the PAK sequence. On the other hand, a single amino acid change in an epitope sequence could substantially decrease antibody binding to result in strain-specificity (substitution of the critical residues, Phe^{137} and Lys^{140}). Pathogens escape immune surveillance of the host (McKeating *et al.*, 1989) and survive under the pressure of natural selection through mutations of immunogenic regions. However, the pathogen must compromise this desire with the ability to maintain its adherence properties as in the case of this epitope, which is in the adherence binding domain.

CHAPTER IV

COMPARISON OF THE STRAIN-SPECIFICITY AND CROSS-REACTIVITY OF VARIOUS ANTI-PAK PILUS ANTIBODIES BASED ON EPITOPE ANALYSES[2]

A. Introduction

The C-terminal region of *P. aeruginosa* pilin contains an intrachain disulfide bridge and is semiconserved among different strains studied (Paranchych and Frost, 1988; Paranchych *et al.*, 1990). Previous studies on the antigenicity of the *P. aeruginosa* pilin have led to the finding of an important antigenic determinant, which was confined to the C-terminal region of the pilin protein (Watts *et al.*, 1983b; Sastry *et al.*, 1985b). This region was later shown to contain the epithelial cell binding domain as well (Paranchych *et al.*, 1986; Irvin *et al.*, 1989). The presence of these two distinct properties, antigenicity and receptor binding capability, in the C-terminus of the pilin protein reveals the physiological significance of this region to the survival of this opportunistic pathogen.

Recently, Lee and coworkers (1989a; 1990b) have conducted a series of experiments on the immunogenic properties of both PAK and PAO pili. The potential surface antigenic regions of these two pilin strains were predicted using the algorithm "Surface Plot" as developed by Parker *et al.* (1986). Eight surface exposed regions were predicted in both PAK and PAO pilins. Immunological studies using synthetic peptides corresponding to these sequences have shown that four anti-PAK and five anti-PAO peptide antisera were cross-reactive to these two strains (Lee *et al.*, 1990b).

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[2]A portion of this chapter has been submitted for publication: Wong, W.Y., H.B. Sheth, R.T. Irvin, and R.S. Hodges (1994). *Pediat. Pulmonol.*

Interestingly, peptides corresponding to the C-terminal 17-residue region (residues 128-144) of both PAK and PAO pilin strains could produce highly cross-reactive antibodies with high endpoint titers (Lee *et al.*, 1989a; 1990b).

Further immunological studies on both reduced and oxidized PAK 17-residue peptides have produced four antisera termed 17-R1, 17-R2, 17-O1 and 17-O2 (Lee *et al.*, 1989b). ELISA and immunoblot experiments showed that the purified antipeptide antibodies could bind to the native PAK pili. The Fab fragments prepared from these antibodies also showed inhibitory effects on the binding of PAK pili to buccal epithelial cells (Lee *et al.*, 1989a). Moreover, these antisera also demonstrated various degrees of cross-reactivity between the PAK and PAO strains. One of these antisera, 17-O1, was found to be the most cross-reactive; whereas the other three antisera showed weaker binding to the PAO strain and were considered to be more strain-specific. It has been suggested that the disulfide bridge found in the oxidized peptide was important in producing cross-reactive antibodies (Lee *et al.*, 1989b).

Since these four antibodies recognized the same 17-residue peptide and displayed different degrees of cross-reactivity, it is of interest to know whether these antibodies bind to the same epitope sequence. The single-alanine replacement analysis used to map the linear epitope of murine monoclonal antibody PK99H (Chapter III) was used to map the epitopes of these antipeptide antibodies. In this study, three of the four anti-PAK peptide antibodies, 17-R1, 17-O1, and 17-O2, were chosen for epitopic analysis. A comparison of the epitope sequences and binding affinities of each side-chain in the epitopes among the three antipeptide antibodies and the strain-specific monoclonal antibody PK99H was carried out. The assumption of this project was that a cross-reactive antibody may recognize a different epitope from that of a strain-specific antibody, and that comparison between these epitopes may eventually reveal important residues in the epitope sequence that could be used to characterize the cross-reactivity of an antibody.

B. Results

1. Binding affinity of antipeptide antibodies 17-R1, 17-O1 and 17-O2

The three antipeptide antibodies 17-R1, 17-O1, and 17-O2 were produced by Lee *et al.* (1989b) using either reduced (17-R) or oxidized (17-O) synthetic peptide, PAK(128-144)OH, corresponding to the C-terminal 17-residue region of the PAK pilin. These peptides were conjugated to KLH, which were then used to immunize rabbits. The antisera obtained from individual rabbits were named according to the immunogens and the rabbits used. For example, antisera 17-O1 and 17-O2 were obtained by immunizing two rabbits with oxidized KLH-PAK(128-144)OH peptide-conjugates. The endpoint dilutions of 17-R1, 17-O1 and 17-O2 as determined by direct ELISA were 3.5×10^{-6}, 4.5×10^{-6}, and 2.1×10^{-6}, respectively, when assayed against the corresponding peptide-BSA conjugates; and were 1.0×10^{-5}, 1.5×10^{-5}, and 2.0×10^{-5}, respectively, when assayed against the PAK pili (Lee *et al.*, 1989b). These antipeptide immunoglobulins were then purified on a Protein-A Sepharose affinity column. In this study, the antibodies used were prepared from lyophilized samples, which were dissolved in PBS containing 0.05% (w/v) BSA (Buffer A) as 1 mg/ml solutions. The working dilutions of these antibodies were determined by direct ELISA with PAK pili coated on the plate. The concentration at which the antibodies gave an OD_{405} close to 1 was chosen as the working dilution for subsequent competitive ELISA experiments. The final dilution factors determined for these antibodies were: 17-R1 (1:10,000), 17-O1 (1:1,000), and 17-O2 (1:800).

As shown in Figure IV.1, the binding of these three antibodies could be inhibited by either oxidized PAK peptide AcPAK(128-144)OH (Fig. IV.1, panel A) or native PAK pili (Fig. IV.1, panel B). All three antibodies, 17-R1, 17-O1, and 17-O2 had very similar binding affinities for PAK pili, as shown by their competitive binding profiles (Fig. IV.1, panel B), with apparent association constants (K_a) equal to 1.5,

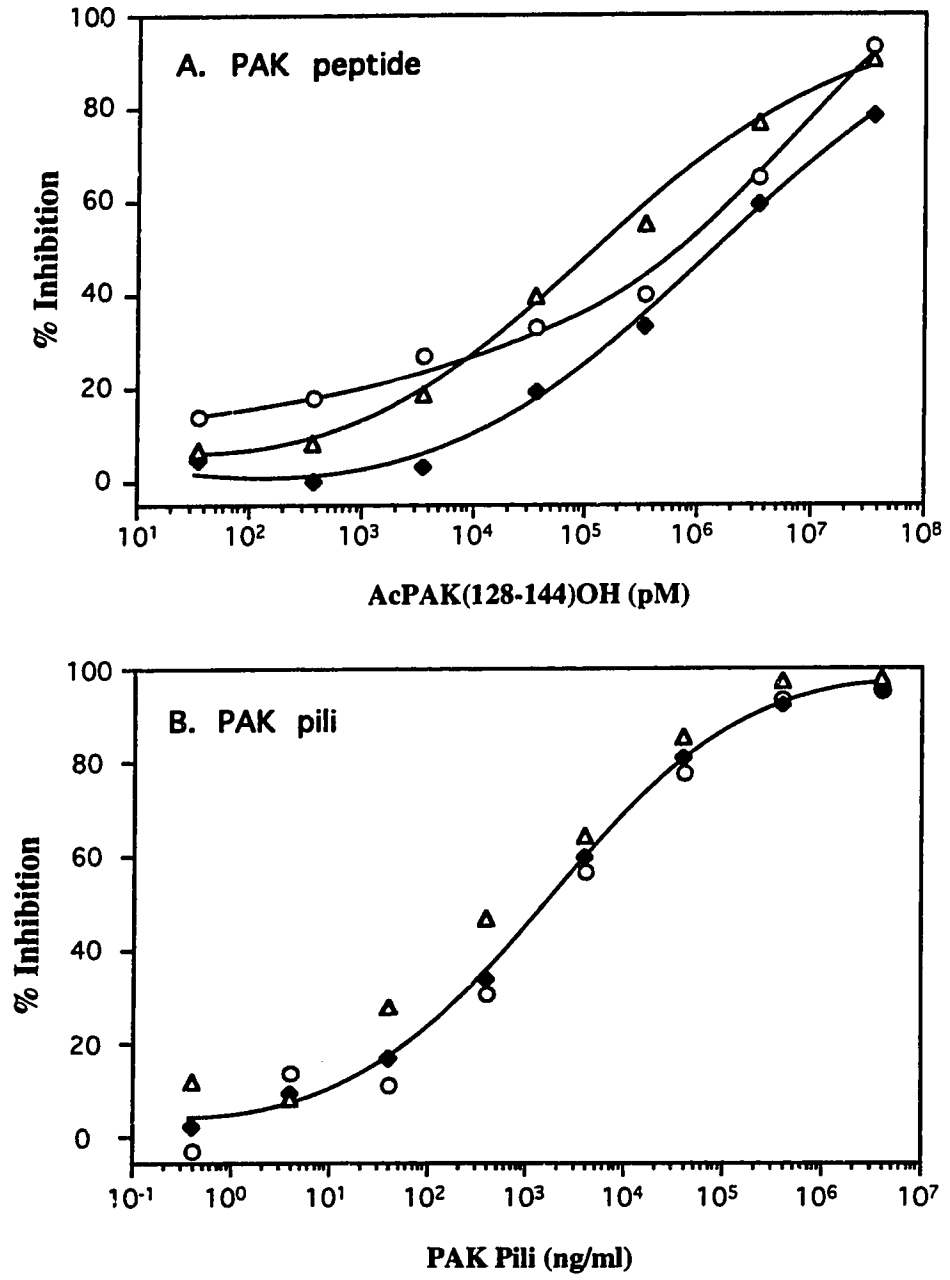

Figure IV.1 Competitive binding curves showing the inhibitory effects of (A) oxidized PAK peptide AcPAK(128-144)OH and (B) PAK pili on the binding of polycolonal antibodies 17-R1 (▲), 17-O1 (◆) or 17-O2 (o) to PAK pili coated on ELISA plates.

0.53, and 0.42 ml/μg, respectively (Table IV.1). In the case of PAK peptide AcPAK(128-144)OH, a larger difference was found in their K_a's, which were 5.0 x 10^6, 5.6 x 10^5, and 8.3 x 10^5 M^{-1} for 17-R1, 17-O1, and 17-O2, respectively (Table IV.1). The relative cross-reactivities among these three antibodies in binding pili or peptide from both PAK and PAO strains were also compared. A comparison of the K_a values from both PAK and PAO strains was performed to illustrate the relative cross-reactivity of these antibodies. In Table IV.1, the K_a's for the three antibodies binding to the pili and peptide from both strains are shown. In general, 17-R1 bound better to the PAK strain than did the other two antibodies. For PAK pili, 17-R1 bound 3- and 4-fold better than 17-O1 and 17-O2, respectively; whereas in the case of AcPAK(128-144)OH peptide, 17-R1 bound 9- and 6-fold better than 17-O1 and 17-O2, respectively. However, 17-R1 had a poorer binding affinity than that of 17-O1 and 17-O2 (8-fold and 1.5-fold, respectively) for PAO pili; but they had only a small difference in binding affinity to AcPAO(128-144)OH peptide. The K_{PAK}/K_{PAO} ratio was used to indicate the number of fold decrease in binding affinity of the three antibodies to PAK strain with respect to the PAO strain. According to the definition stated in Chapter III, an antibody is considered as strain-specific if its binding affinity to another strain is decreased by at least 1,000-fold. From Table IV.1, 17-R1 was definitely a strain-specific antibody since 17-R1 had the K_{PAK}/K_{PAO} ratios of 1,500 for the pili and 2,200 for the 17-residue peptides. In contrast, 17-O1 could be considered as a cross-reactive antibody because its K_{PAK}/K_{PAO} ratios were only 64 for pili and 220 for peptide. In addition, 17-O2 was a moderately cross-reactive antibody since its K_{PAK}/K_{PAO} ratios were 280 for the pili and 750 for the peptides. In summary, the order of cross-reactivity could be ranked as follows: 17-O1 > 17-O2 > 17-R1.

2. Epitope mapping

The PAK peptide sequences representing the binding epitopes for 17-R1, 17-O1,

Table IV.1 Binding affinities of antipeptide antibodies 17-R1, 17-O1 and 17-O2 to either synthetic peptides or pilus protein of *Pseudomonas aeruginosa* strains PAK and PAO, showing the relative cross-reactivity of these antibodies

	K_a[a]			K_{PAK}/K_{PAO}[b]		
	17-R1	17-O1	17-O2	17-R1	17-O1	17-O2
PAK pili	1.5	5.3×10^{-1}	4.2×10^{-1}			
PAO pili	1.0×10^{-3}	8.3×10^{-3}	1.5×10^{-3}	1,500	64	280
AcPAK(128-144)OH	5.0×10^{6}	5.6×10^{5}	8.3×10^{5}			
AcPAO(128-144)OH	2.3×10^{3}	2.6×10^{3}	1.1×10^{3}	2,200	220	750
AcPAK(132-139)NH_2	3.1×10^{5}	5.6×10^{4}	2.2×10^{5}			
AcPAO(132-139)NH_2	$< 1.0 \times 10^{2}$	1.1×10^{2}	1.2×10^{2}	> 3,100	510	1,800

[a] K_a is the apparent association constant of the corresponding pili or peptides, and the unit is either ml/μg or M^{-1}, respectively.

[b] K_{PAK} denotes the K_a of either PAK pili or peptides (AcPAK(128-144)OH and AcPAK(132-139)NH_2), whereas K_{PAO} indicates the K_a of either PAO pili or peptides (AcPAO(128-144)OH and AcPAO(132-139)NH_2). Hence, the K_{PAK}/K_{PAO} ratio defined the number of fold decrease in binding affinity of the PAO strain as compared with the PAK strain.

and 17-O2 were mapped using a similar approach to that utilized for monoclonal antibody PK99H (Chapter III). Briefly, a series of single alanine substituted peptide analogs corresponding to the C-terminal peptide AcPAK(128-144)OH were employed to identify residues that were important for antibody binding. The mapping results are shown in Figures IV.2a, IV.2b, and IV.2c for antibodies 17-R1, 17-O1, and 17-O2, respectively. The alanine replacement analysis of the three antibodies suggested that these three antipeptide antibodies had a very similar epitope sequence, which was located in the linear PAK sequence 132-139. Six residues from this sequence overlap the epitope sequence of monoclonal antibody PK99H, which comprises the PAK sequence 134-140 (Fig. III.2). The binding affinities of the reduced PAK peptide analogs to both 17-R1 and 17-O2 were similar to those of the oxidized peptides (data not shown), suggesting the intrachain disulfide bridge of these peptide analogs was not required for antibody binding. However, the binding affinity of the reduced peptide analogs to 17-O1 was not available since the maximum inhibition caused by the reduced peptides was only about 40% (data not shown).

Of the 8-residue epitope region determined (boxed in Figs. IV.2a, IV.2b, and IV.2c), eight side-chains (Asp^{132}, Gln^{133}, Asp^{134}, Glu^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139}), six side-chains (Asp^{132}, Gln^{133}, Asp^{134}, Gln^{136}, Phe^{137}, and Pro^{139}), and seven side-chains (Asp^{132}, Asp^{134}, Glu^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139}) were found to be important for 17-R1, 17-O1, and 17-O2 binding, respectively. The binding affinities of the antibodies for these alanine-substituted peptide analogs had decreased from 3.2- to more than 10,000-fold. These results suggested that the 8-residue peptide, AcPAK(132-139)NH_2, would also bind to these antibodies. In order to define the minimum binding epitope for these three antipeptide antibodies, the 8-residue peptide as well as other peptide analogs with truncated N- or C-terminal sequence were synthesized. The competitive ELISA results with these peptide analogs are shown in Table IV.2. All three antibodies bound to the 8-residue peptide

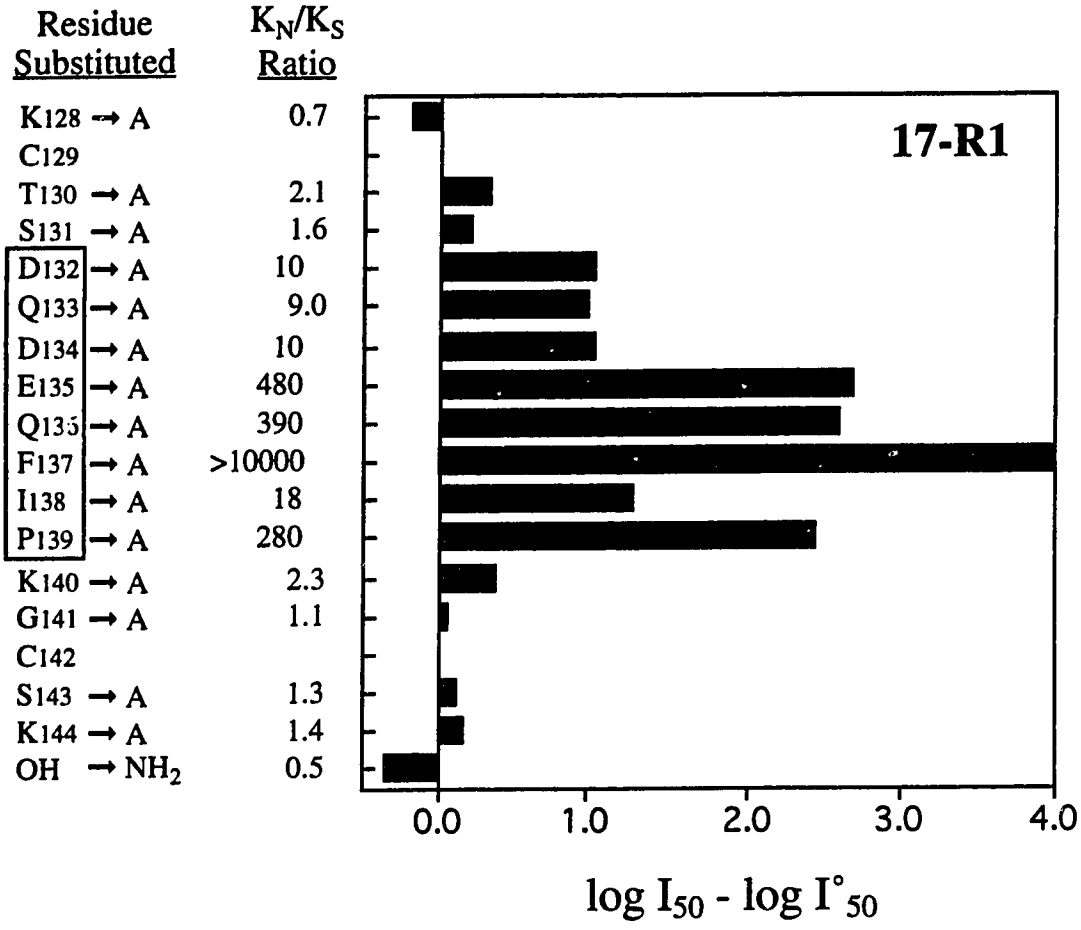

Figure IV.2a Effects of single alanine substitution on the binding of AcPAK(128-144)OH to antipeptide antiserum 17-R1 as determined by competitive ELISA. Please refer to the legend of Fig. III.2 for explanation of the legends on both axes. The boxed sequence defines the linear epitope for 17-R1.

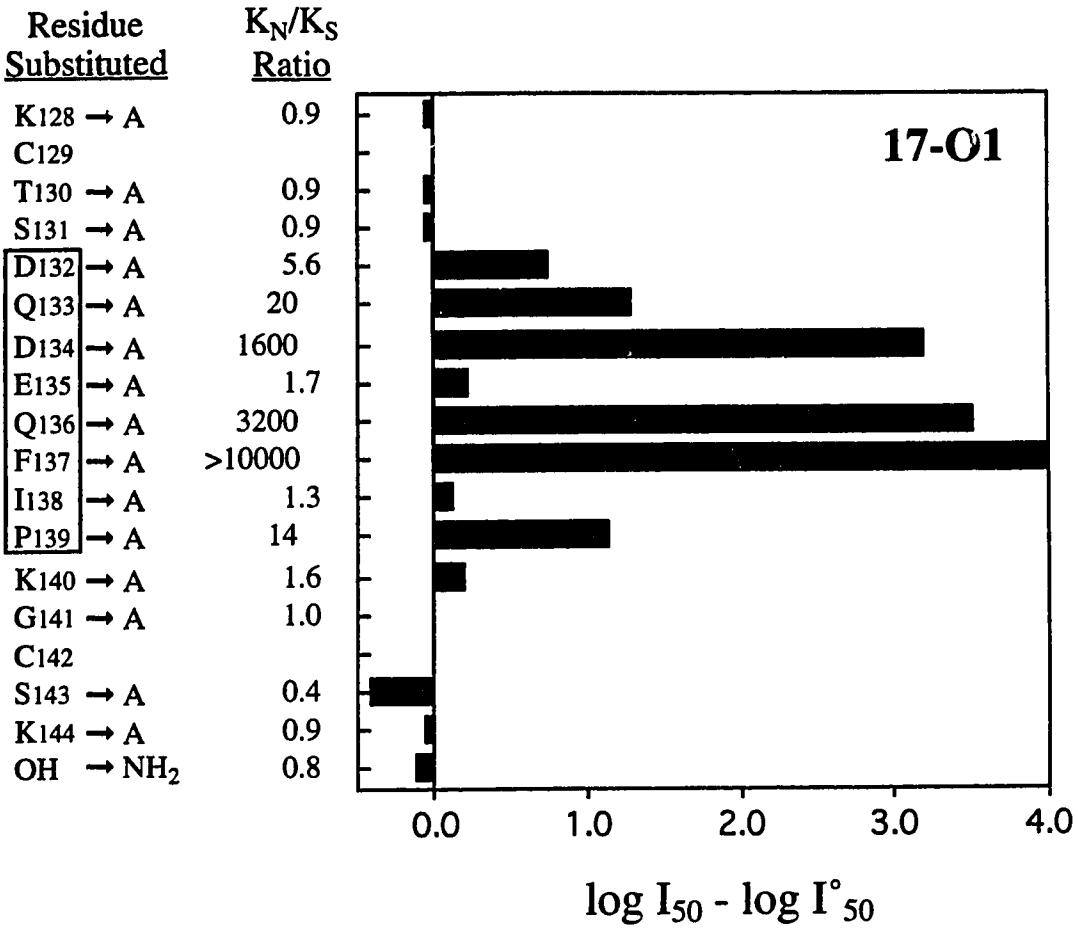

Figure IV.2b Effects of single alanine substitution on the binding of AcPAK(128-144)OH to antipeptide antiserum 17-O1 as determined by competitive ELISA. Please refer to the legend of Fig. III.2 for explanation of the legends on both axes. The boxed sequence defines the linear epitope for 17-O1.

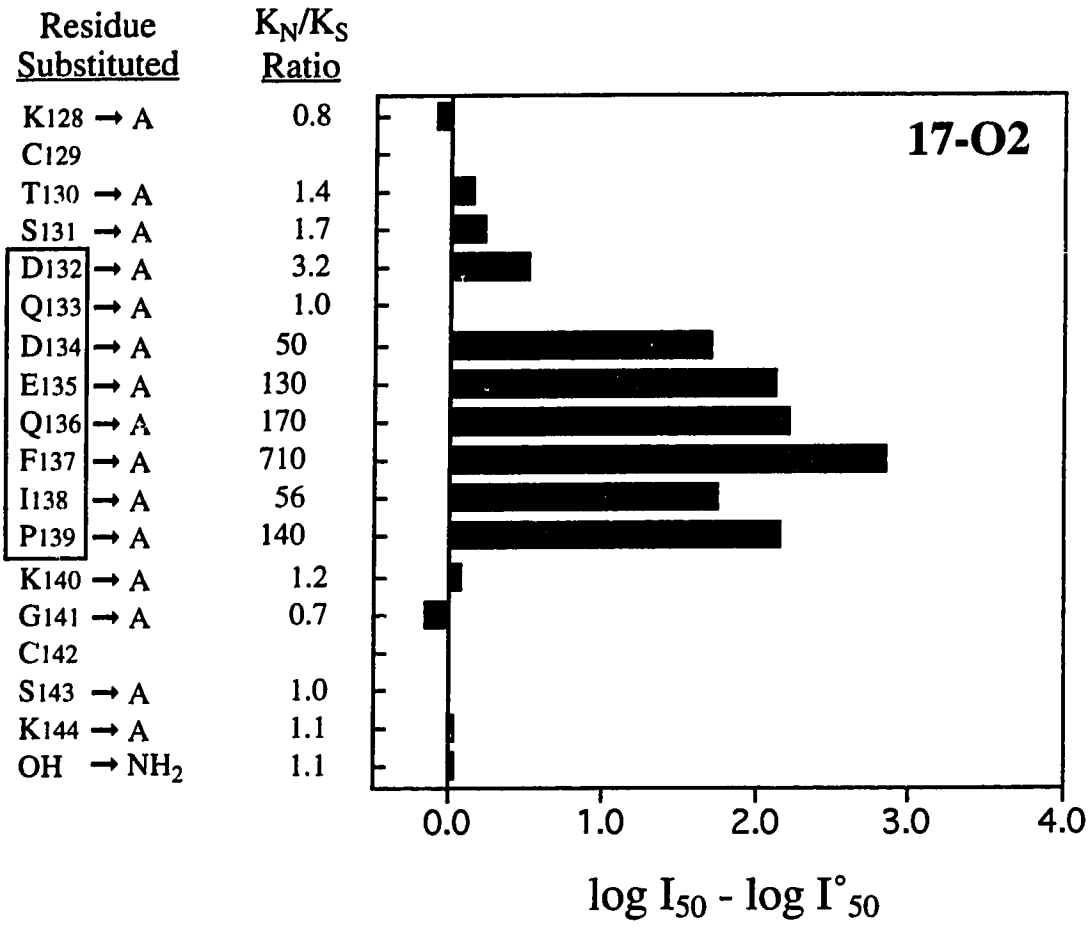

Figure IV.2c Effects of single alanine substitution on the binding of AcPAK(128-144)OH to antipeptide antiserum 17-O2 as determined by competitive ELISA. Please refer to the legend of Fig. III.2 for explanation of the legends on both axes. The boxed sequence defines the linear epitope for 17-O2.

Table IV.2 Elucidation of the epitope for antipeptide antisera 17-R1, 17-O1 and 17-O2 using PAK truncated peptides with sequence length ranged from residues 132 to 139

Sequence[a] (132 – 139)	Relative decrease in binding affinity		
	17-R1	17-O1	17-O2
Ac-D·Q·D·E·Q·F·I·P-NH_2	16[b] (1.0)[c]	10[b] (1.0)[c]	3.8[b] (1.0)[c]
Ac-D·Q·D·E·Q·F·I···-NH_2	150 (9.4)	23 (2.3)	45 (12)
Ac-D·Q·D·E·Q·F····-NH_2	1,900 (120)	28 (2.8)	190 (50)
Ac-D·Q·D·E·Q·····-NH_2	> 50,000 (> 3,100)	> 5,600 (> 560)	> 8,300 (> 2,200)
Ac···Q·D·E·Q·F·I·P-NH_2	210 (13)	21 (2.1)	5 (1.3)
Ac····D·E·Q·F·I·P-NH_2	340 (21)	110 (11)	1.2 (0.3)
Ac·····E·Q·F·I·P-NH_2	600 (38)	> 5,600 (> 560)	46 (12)
Ac···Q·D·E·Q·F·I···-NH_2	580 (36)	NA[d]	140 (37)
Ac····D·E·Q·F·I···-NH_2	1,000 (62)	NA	220 (58)
Ac·····E·Q·F·I···-NH_2	> 2,200 (> 130)	NA	450 (120)
Ac···Q·D·E·Q·F····-NH_2	3,800 (240)	93 (9.3)	890 (240)
Ac····D·E·Q·F····-NH_2	7,700 (480)	510 (51)	1,100 (280)
Ac·····E·Q·F····-NH_2	> 11,000 (> 690)	> 1,200 (> 120)	> 1,800 (> 490)

[a]The range was determined from the results of Figures IV.2a, IV.2b and IV.2c.

[b]As compared with the corresponding binding affinity of the native oxidized peptide AcPAK(128-144)OH.

[c]As compared with the corresponding binding affinity of the 8-residue peptide AcPAK(132-139)NH_2 (first row in this table).

[d]Data not available.

AcPAK(132-139)NH_2 with affinities close to those observed for the 17-residue peptide AcPAK(128-144)OH (Table IV.1). The fold decreases in binding affinity of these three antibodies as compared to the 17-residue peptides were 16-, 10-, and 3.8-fold for 17-R1, 17-O1, and 17-O2, respectively (Table IV.2). The minimum binding sequence for antibody 17-R1 seemed to be the pentapeptide Ac-EQFIP-NH_2. However, 17-O1 recognized a shorter epitope than 17-R1, which was Ac-DEQF-NH_2. In the case of 17-O2, it recognized a tetrapeptide sequence of Ac-EQFI-NH_2.

3. Recognition between the PAK and PAO sequence in the epitope region

Results from the competitive binding studies showed that the three antipeptide antibodies in general bound better to the PAK strain than to the PAO strain, with differences in binding affinity from 64- to 1,500-fold in the case of the pili and from 220- to 2,200-fold in the case of the peptides AcPAK(128-144)OH and AcPAO(128-144)OH (Table IV.1). When the two sequences of PAO and PAK were compared, there were four different residues found in the 132-139 region (Thr^{132}, Pro^{135}, Met^{136}, and Thr^{138} in the PAO sequence instead of Asp^{132}, Glu^{135}, Gln^{136}, and Ile^{138} in the PAK sequence) (Table IV.3). To understand which amino acid side-chain in the PAO sequence affected the binding of the antibodies, all different residues found in the PAO strain were substituted one at a time into the PAK epitope sequence AcPAK(132-139)NH_2. The binding affinity of these peptide analogs to the three antibodies were determined by competitive ELISA. As shown in Table IV.3, the PAO residues, Thr^{132}, Pro^{135}, Met^{136}, and Thr^{138}, as single mutants in the PAK sequence caused a decrease in binding affinity by 38-, 6,000-, 480-, and 45-fold in the case of 17-R1; by 17-, 1.7-, 940-, and 11-fold in 17-O1; and by 1.2-, 55-, 55-, and 15-fold in 17-O2, respectively. Hence, it was found that some of these single substitutions showed serious effects on the binding of the antipeptide antibodies, for example, Pro^{135} (6,000-fold) and Met^{136} (480-fold) for 17-R1 and Met^{136} (940-fold) for 17-O1. This

Table IV.3 Effects of substitution of residues from the PAO pilin strain on the binding of the PAK epitope sequence 132-139 to the three antipeptide antibodies 17-R1, 17-O1 and 17-O2

Sequence[a] (132 … 139)	Relative decrease in binding affinity: 17-R1	17-O1	17-O2
PAK: Ac- D Q D E Q F I P-NH_2	16[b] (1.0)[c]	10[b] (1.0)[c]	3.8[b] (1.0)[c]
Ac-Ⓣ Q D E Q F I P-NH_2	38 (2.4)	17 (1.7)	1.2 (0.32)
Ac- D Q D Ⓟ Q F I P-NH_2	6,000 (380)	1.7 (0.17)	55 (14)
Ac- D Q D E Ⓜ F I P-NH_2	480 (30)	940 (94)	55 (14)
Ac- D Q D E Q F Ⓣ P-NH_2	45 (2.8)	11 (1.1)	15 (3.9)
PAO: Ac-Ⓣ Q D Ⓟ Ⓜ F Ⓣ P-NH_2	> 50,000 (> 3,100)	5,100 (510)	7,000 (1,800)

[a]Those different residues found in the PAO sequence are circled. The native PAK sequence is shown in the first row, whereas the native PAO sequence is in the last row.

[b]As compared with the corresponding binding affinity of the native oxidized peptide AcPAK(128-144)OH.

[c]As compared with the corresponding binding affinity of the 8-residue peptide AcPAK(132-139)NH_2 (first row in this table).

could account for the loss in binding affinity of these antibodies to the PAO sequence. Interestingly, not much effect could been seen with 17-O2, in which maximum loss in binding affinity was only 55-fold for both the Pro^{135} and Met^{136} single substitution mutants. This may be due to the fact that 17-O2 was quite adaptable to single amino acid change. For instance, even the critical residue (Phe^{137}) identified by single alanine substituted mutant had only a 710-fold decrease in binding affinity (Fig. IV.2c), whereas in the case of 17-R1 and 17-O1 this particular substitution caused over 10,000-fold decrease in binding affinity (Fig. IV.2a and IV.2b).

Similar to the above-described study (Fig. III.7), none of the single substitution mutants could account for the large reduction in binding affinity for the PAO peptide AcPAO(132-139)NH_2 (Table IV.3, >50,000-, 5,100-, and 7,000-fold for 17-R1, 17-O1, and 17-O2, respectively). This agreed well with our previous finding that more than one substitution was required and that the effects were more than additive when compared to the results of single substitutions.

As the results of the PAK 8-residue single mutants (Table IV.3) were compared with those of the single-alanine substituted 17-residue peptide analogs (Figs. IV.2a, IV.2b, and IV.2c), some interesting observations could be drawn. For instance, certain substitutions with PAO residues in the PAK sequence (Table IV.3, data in parentheses) have an overall better binding infinity than those of the single-alanine substituted peptide analogs (Figs. IV.2a, IV.2b, and IV.2c). In other words, all three antipeptide antibodies in general were more tolerant to substitutions with residues from the PAO strain than substitutions caused by single alanine substitution. In the case of 17-R1, Glu^{135} and Gln^{136} were the two important residues in the PAK sequence that contributed to the epitope. Either of these two residues when substituted by an alanine induced a large decrease in the binding affinity ($Glu^{135}\rightarrow$Ala, 480-fold; and $Gln^{136}\rightarrow$Ala, 390-fold; Fig. IV.2a). However, replacement of these two residue with the PAO residues in the 8-residue PAK peptide resulted in a smaller reduction in

binding affinity (Glu^{135}→Pro, 380-fold; and Gln^{136}→Met, 30-fold; Table IV.3). Likewise, both 17-O1 and 17-O2 showed a similar pattern (17-O1: Gln^{136}→Ala, 3,200-fold compared to Gln^{136}→Met, 94-fold; and 17-O2: Glu^{135}→Ala, 130-fold; and Gln^{136}→Ala, 170-fold; compared to Glu^{135}→Pro, 14-fold; and Gln^{136}→Met, 14-fold; Table IV.3). The most noteworthy difference could be found in 17-O1 where substitution of residue Gln^{136} to Met (94-fold) was much more adaptable to antibody binding than an Ala substitution (3,200-fold) at this position (a 34-fold improvement). In addition, the Glu^{135}→Pro substitution caused a 5.9-fold enhancement in binding affinity of the 8-residue PAK peptide to 17-O1 as compared with that of the native sequence (Table IV.3). However, in the case of the other two antibodies (17-R1 and 17-O2), PAO substitutions into PAK sequence were not as adaptable. For example, the Glu^{135}→Pro substitution resulted in a 380-fold decrease in binding affinity for 17-R1 and a 14-fold decrease for 17-O2.

4. Comparison of the epitope sequences among antibodies PK99H, 17-R1, 17-O1, and 17-O2

In Chapter III, the epitope of monoclonal antibody PK99H was mapped in the PAK sequence 134-140, which comprises the heptapeptide DEQFIPK. The PK99H epitope was very similar to the consensus epitope of the three polyclonal antibodies 17-R1, 17-O1, and 17-O2. The polyclonal antibody epitopes were contained within a sequence of DQDEQFIP in the PAK sequence. Six residues were found in both the polyclonal and monoclonal epitopes. They were: Asp^{134}, Glu^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139} (Table IV.4). Two common features could be found in these two classes of epitopes. First, they required the side-chain of Phe^{137} for antibody binding since substitution of this side-chain with Ala caused a substantial loss of binding affinity of the peptide analog as compared with that of the native peptide. Second, the

Table IV.4 A summary of the relative binding affinity of nine single alanine substituted peptide analogs of the PAK C-terminal 17-residue sequence to antibodies PK99H, 17-R1, 17-O1, and 17-O2

Residue Substituted[b]	K_N/K_S ratio[a]			
	PK99H	17-R1	17-O2	17-O1[c]
$D_{132} \rightarrow A$	1.5	10	3.2	5.6
$Q_{133} \rightarrow A$	1.7	9.0	1.0	20
$D_{134} \rightarrow A$	64	10	50	1,600
$E_{135} \rightarrow A$	9.6	480	130	1.7
$Q_{136} \rightarrow A$	1.6	390	170	3,200
$F_{137} \rightarrow A$	4,000	> 10,000	710	>10,000
$I_{138} \rightarrow A$	600	18	56	1.3
$P_{139} \rightarrow A$	76	280	140	14
$K_{140} \rightarrow A$	800	2.3	1.2	1.6

[a]The values of the K_N/K_S ratio are extracted from Figures III.2 (PK99H), IV.2a (17-R1), IV.2b (17-O1), and IV.2c (17-O2).

[b]The sequence of the peptide analogs are shown in Table II.1. PAK peptide sequence 134-140 is the epitope recognized by PK99H (see Chapter III), while sequence 132-139 is the epitope for 17-R1, 17-O1, and 17-O2 (Figures. IV.2a, IV.2b and IV.2c). The overlapping sequence between the two epitopes is shaded.

[c]17-O1 is a cross-reactive antibody that binds to the pili and peptides from both PAK and PAO strains, whereas 17-O2 is considered as a moderate cross-reactive antibody, and both PK99H and 17-R1 are strain-specific antibodies that have over 1,000-fold better binding affinity to PAK strain than PAO strain (see Table IV.1 and Table III.2).

length of these two classes of epitopes were very close to each other, that is, 7 residues for the PK99H epitope and 8 residues for the epitope of the three polyclonal antibodies. Despite this similarity, these four antibodies varied in their ability to cross-react with pili and synthetic peptides from both the PAK and PAO strains. Previous studies have shown that PK99H is a strain-specific antibody that binds preferentially to strain PAK but not to strain PAO (Chapter III and Doig *et al.*, 1990). Moreover, 17-R1 showed a similar strain-specificity to PK99H. The binding affinity of these two antibodies to strain PAO was more than 1,000-fold weaker than to strain PAK (Table III.2 and Table IV.1). On the other hand, 17-O1 could be considered as a cross-reactive antibody because the difference in binding affinity between PAK and PAO strains was less than 220-fold (Table IV.1). In the case of 17-O2, it was intermediate between 17-R1 and 17-O1, and thus represented a moderately cross-reactive antibody. In addition, a summary of the binding affinity data from single-alanine replacement analysis (Table IV.4) showed that the different side-chains in the epitope could play a variable role in antibody binding since the extent to which the peptide analogs lost their binding affinity to the four antibodies was different. Therefore, the relative importance of individual residues found in the two epitope sequences can be assessed quantitatively.

5. Elucidation of the relationship between cross-reactivity and critical residues found in the epitopes

Since the four antibodies, PK99H, 17-R1, 17-O1, and 17-O2, displayed various degrees of cross-reactivity in binding to pili or pilin peptide from both strains PAK and PAO, it is of interest to know if there is any significant relationship between the critical residues found in the epitopes and the degree of cross-reactivity of these antibodies. A comparison was made by subtracting the binding affinity indices (K_N/K_S ratios) of the more strain-specific antibodies such as PK99H, 17-R1, and 17-O2 from those of the cross-reactive antibody 17-O1 (Fig. IV.3). This simple subtraction would eliminate

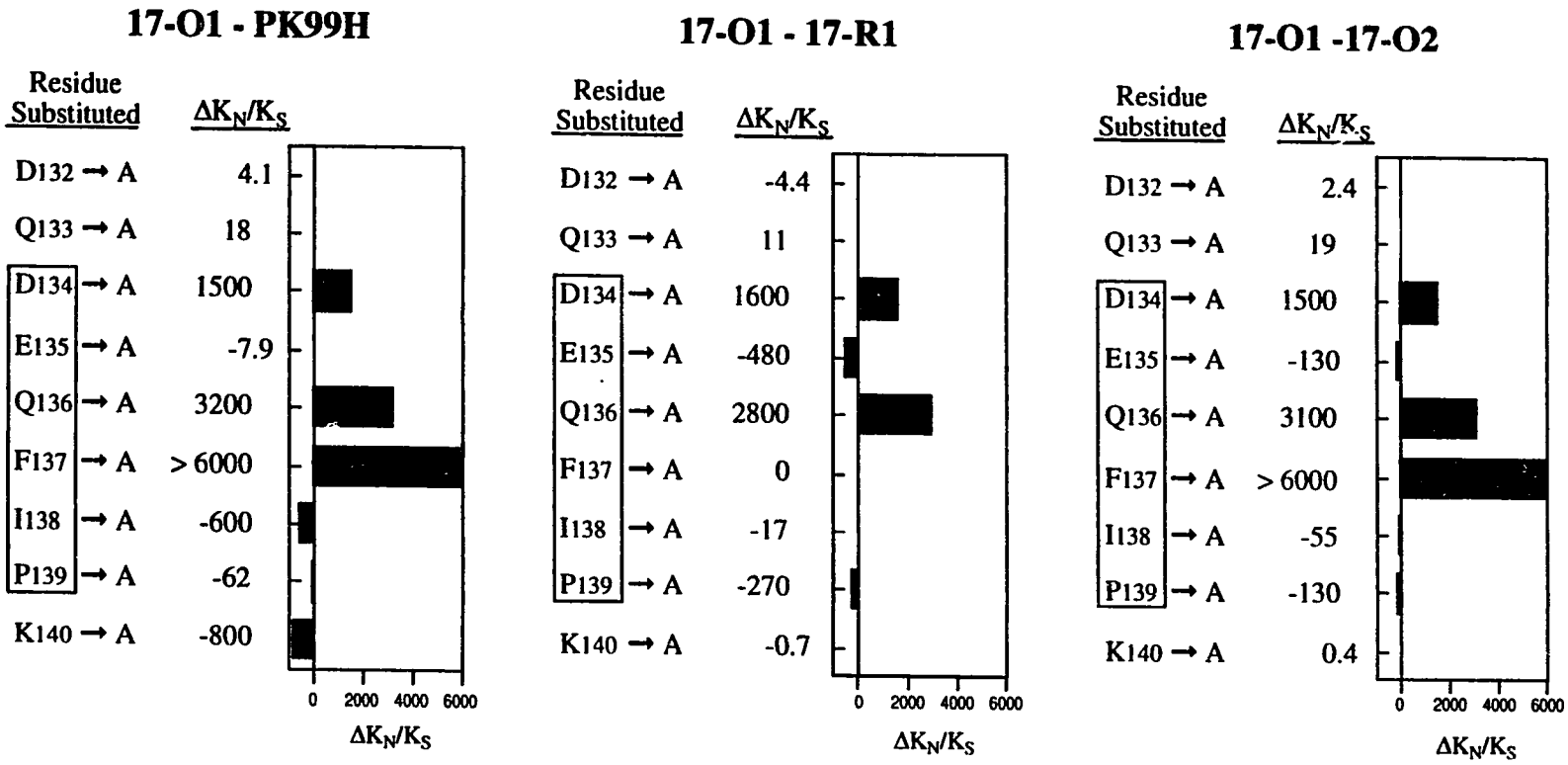

Figure IV.3 Determination of the residues in the epitope sequence that are important for recognition by the cross-reactive antibody 17-O1 as compared with the more strain-specific antibodies PK99H, 17-R1, and 17-O2. The K_N/K_S ratios were obtained from Table IV.4. Where $\Delta K_N/K_S$'s are calculated by subtracting the corresponding K_N/K_S ratios of the strain-specific antibodies from those of 17-O1. Positive $\Delta K_N/K_S$'s indicate amino acid residues that are important for being recognized by the cross-reactive antibody 17-O1; while negative $\Delta K_N/K_S$'s mean that the side-chain at this position is more important to the strain-specific antibody than to 17-O1. The overlapping sequence between the epitope of PK99H and the three antipeptide antibodies is boxed.

those residues that have a similar contribution to both cross-reactive and strain-specific antibodies, while retaining residues that were important for either cross-reactivity or strain-specificity. The resultant values ($\Delta K_N/K_S$) thus represent residues in the epitope sequence that were related to the degree of cross-reactivity of an antibody. A positive $\Delta K_N/K_S$ value indicated that the corresponding residue was more important to being recognized by the cross-reactive antibody 17-O1; whereas a negative $\Delta K_N/K_S$ value would mean that the residue was more important to the less cross-reactive (or more strain-specific) antibodies as compared with 17-O1.

The results of the comparison are shown in Figure IV.3. It is obvious that three single-alanine substituted peptide analogs showed significant positive $\Delta K_N/K_S$'s in the overlapping region of the two classes of epitopes. They are Asp^{134}, Gln^{136}, and Phe^{137} in the PAK sequence. Since Phe^{137} is a critical side-chain for binding to all four antibodies, this residue is considered equally important to both cross-reactive and strain-specific antibodies. As a result, only two residues, Asp^{134} and Gln^{136}, were considered to be important for binding of cross-reactive antibodies in antibody 17-O1 ($\Delta K_N/K_S$ = 1,500-1,600 for Asp^{134}, and 2,900-3,200 for Gln^{136}, Fig. IV.3). In contrast, three side-chains found in the PAK sequence (Glu^{135}, Ile^{138}, and Pro^{139}) showed negative $\Delta K_N/K_S$'s and thus were non-essential to 17-O1 but were important for the more strain-specific antibodies PK99H, 17-R1, and 17-O2.

C. Discussion

Antibody-antigen binding involve the interaction between an antigen and the combining site of an antibody. This type of interaction is often highly specific. However, in some instances specificity is lesser. Cross-reactive antibodies that recognize more than one antigen are occasionally produced. Landsteiner was among one of the earliest investigators who showed that antisera could display a great deal of

cross-reactivity (Landsteiner, 1936). The reason for production of antibodies that cross-react with different antigen species is as yet unclear. It seems likely that the combining site of the antibody and/or the antigen itself can adopt a new conformation that is complementary. The concept of the induced fit model to describe the flexibility of the antibody-antigen interactions has been recently illustrated by various structural analyses of the antibody-antigen complexes (Colman *et al.*, 1987; Bhat *et al.*, 1990; Rini *et al.*, 1992). Conformational change has been observed when a nonapeptide immunogen from the influenza virus hemagglutinin bound to the antibody combining site as determined by X-ray crystallography at 2.0 to 3.1 Å resolution. This conformational change allows the formation of a binding pocket that can accommodate the ß turn secondary structure of the peptide (Rini *et al.*, 1992). In fact, different shapes of antibody combining sites have been reported including deep pockets (Stanfield *et al.*, 1990) or shallow troughs (Rose *et al.*, 1990). In addition, a study on the three-dimensional structure of an antibody-antigen complex between lysozyme and the Fab fragment from a monoclonal antibody against lysozyme have shown that some imperfections in the form of holes exist in the antibody-antigen contacting surface. These authors suggested that these holes might provide extra binding pockets which allow heterologous antigens to bind (Amit *et al.*, 1986). In summary, the structural information suggests that antibody-antigen interactions could be less specific and selective than previously thought.

In the course of immunological studies of the *P. aeruginosa* pili, various cross-reactive antibodies have been reported. For example, the monoclonal antibodies PK41C and PK34C produced by Doig *et al.* (1990) cross-reacted with a large number of pilus strains, and the Fab fragments of PK34C could inhibit adhesion of different pilus strains to buccal epithelial cells. Furthermore, Saiman *et al.* (1989) reported that monoclonal antibodies produced against purified PA1244 pilin showed a broad cross-reactivity with different *P. aeruginosa* strains such as the PAK and PAO strains. The

epitope of one of these monoclonal antibodies, 6-45, has been mapped to the C-terminal region of the PA1244 pilin (Castric and Deal, 1994). In previous studies, Lee *et al.* (1990b) used the 17-residue peptide corresponding to the C-terminal region of the PAK pilin to produce an antipeptide antibody (17-O1) that cross-reacted with the pili and pilin peptides from both PAK and PAO strains.

In this study, the epitopes for the cross-reactive antipeptide antibody 17-O1 and two other antipeptide antibodies (17-R1 and 17-O2), with more strain-specific natures, were elucidated. All three antibodies recognized the peptide sequence DQDEQFIP. As this epitope was compared with that of the strain-specific monoclonal antibody PK99H (DEQFIPK), six overlapping residues (DEQFIP) were found between these two classes of epitopes. It is not a surprising phenomenon since Anderson *et al.* (1988) have reported a similar observation that the same epitope sequence was recognized by both strain-specific and cross-reactive antibodies.

The number of amino acid residues found in the epitope sequence of the three polyclonal antipeptide antibodies was eight. This agreed well with our previous discussion (Chapter III) that the length of most linear epitopes was in the range from 5 to 10 residues (Anderson *et al.*, 1988; Kövamees *et al.*, 1990; Scott *et al.*, 1990; Wong *et al.*, 1992). Our present results further confirm this observation. Recently, structural analyses of the antibody-antigen binding complexes by X-ray crystallography have revealed that the contact area between an antibody and its antigen is about 30 x 20 Å (Amit *et al.*, 1986). The average antibody-antigen contact area was also reported to be 16 to 20 Å in diameter (Barlow *et al.*, 1986). Since the length of a peptide bond is only 1.32 Å, it seems that the antibody-antigen contact area is far larger than an average epitope peptide, which contains only about 5 to 10 residues. However, the binding affinity of the smaller epitope peptide is usually large enough to compete with the whole protein for binding to the antibody combining site, as in the case of this study and from other studies (Lerner, 1982).

Despite the fact that all four antibody preparations studied bind to similar epitope sequences at the C-terminus of the PAK pilin, the importance of individual residues to antibody binding was varied. This implied that the degree of cross-reactivity of an antibody might be related to the binding affinity of individual side-chains in the same epitope. By comparing the relative binding affinity of the single amino acid substituted peptide analogs, some conclusions could be drawn. First, Phe^{137} was a critical residue found in the epitopes of both strain-specific and cross-reactive antibodies (Tables III.2, IV.2a, IV.2b, and IV.2c). Second, in addition to Phe^{137}, the cross-reactive antibody 17-O1 required Asp^{134} and Gln^{136} to be present in the epitope for binding to occur; whereas for the strain-specific antibodies, PK99H and 17-R1, three residues Glu^{135}, Ile^{138}, and Pro^{139} were required (Fig. IV.3). Third, the epitope of the cross-reactive antibody 17-O1 was more adaptable to mutations that altered the PAK sequence to corresponding amino acids in the PAO sequence. Among the three PAK to PAO substitutions (Glu^{135}→Pro, Gln^{136}→Met, and Ile^{138}→Thr) found in the overlapping epitope sequence 134-139 (for sequences see Table IV.3), 17-O1 was more tolerant to the two substitutions (Glu^{135}→Pro and Ile^{138}→Thr) than were the other antibodies. The decrease in binding affinity of these two PAO residues substitution analogs to 17-O1 was less than 1.1-fold (Table IV.3). Fourth, as the PAK sequence and the PAO sequence were compared in the overlapping epitope region 134-139, only one substitution (Gln^{136}→Met) was found to cause a large decrease in binding affinity to 17-O1 (Table IV.3). However, in the case of the other three antibody preparations, two substitutions (Glu^{135}→Pro and Gln^{136}→Met for 17-R1 and 17-O2; Gln^{136}→Pro and Ile^{138}→Thr for PK99H) were found important instead (Table IV.3 and Fig. III.7, respectively). This observation may explain why 17-O1 cross-reacted with both PAK and PAO strain better than the other three antibodies.

In conclusion, results from these studies have improved our understanding of antibody cross-reactivity and strain-specificity. The identification of specific residues

in the epitopes that were important for cross-reactive and strain-specific antibodies will permit the design and synthesis of synthetic vaccine candidates that induce the production of antisera with different specificity (to be discussed in Chapter V). The knowledge obtained from this study should enable us to design cross-protective vaccines that protect against a broad range of *P. aeruginosa* strains, and diagnostic reagents that can be used to distinguish different clinical isolates.

CHAPTER V

DESIGN OF SYNTHETIC PEPTIDE IMMUNOGENS THAT INDUCE THE PRODUCTION OF IMPROVED CROSS-REACTIVE ANTIBODIES[3]

A. Introduction

Many studies have demonstrated that synthetic peptides can induce antibodies that react with the native proteins (Lerner, 1982; 1984; Geysen *et al.*, 1985; Lee *et al.*, 1989b). With the advances in synthetic peptide chemistry, synthetic peptides have become an important source of synthetic haptens for immunological studies (Plaué *et al.*, 1990). In fact, synthetic peptides can induce antibodies that are directed to different parts of the protein surfaces (Lerner, 1982). During the course of immunological studies of the *P. aeruginosa* pilus adhesin, synthetic peptides have been used to mimic various antigenic regions of both PAK and PAO pilins to induce antisera that bind to the pilus proteins (Lee *et al.*, 1989b; 1990b). These studies have demonstrated the feasibility of using synthetic peptide haptens to protect against *P. aeruginosa* adherence. However, one of the problems encountered in designing an anti-*Pseudomonas* pilus vaccine is the presence of heterologous pilin strains. More than eight different pilin genes from *P. aeruginosa* have been identified and sequenced (Paranchych *et al.*, 1990; Castric and Deal, 1994). The observation that antibodies produced against the *P. aeruginosa* pili and synthetic pilin peptides cross-reacted with a number of pili strains could be a solution to this problem (Saiman *et al.*, 1989; Doig *et al.*, 1990; Lee *et al.*, 1990b).

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[3]A portion of this chapter has been submitted for publication: Wong, W.Y., H.B. Sheth, R.T. Irvin, and R.S. Hodges (1994). *Pediat. Pulmonol.*

Results described previously (see Chapter III) have shown that the cross-reactive antibody 17-O1 had different side-chain requirements for epitope recognition compared to other strain-specific antibodies. Residues that were important for recognition by either strain-specific or cross-reactive antibodies were identified. For example, the epitope of the cross-reactive antibody 17-O1 involved three critical residues, Asp^{134}, Gln^{136}, and Phe^{137} in the antibody-antigen interaction (substitution by Ala resulted in a decrease of binding affinity by more than 1,000-fold). However, the side-chain requirements for binding changed dramatically for the three strain-specific antibody preparations. For example, the importance of Asp^{134} decreased by 160-fold and Glu^{135} increased by 282-fold for the strain-specific antibody 17-R1 (Table IV.4).

Earlier studies on the hepatitis B surface antigen (HBsAg) have shown that peptides which differing at two residues in the sequence could produce antibodies that were specific for different subtypes of HBsAb (Gerin *et al.*, 1983). This is a good example showing that the specificity of an antibody can be altered simply by modifying the residues of the hapten sequence. Hence, in the case of *P. aeruginosa* pilin peptide, if the immune system recognizes the epitope as a hapten, it will then be possible to use the epitope sequence to induce antibodies with preferred specificity or cross-reactivity by manipulating individual residues presented in the epitope sequence. Indeed, a peptide conjugate comprising the PAK sequence DEQFIPK (an epitope for monoclonal antibody PK99H) conjugated to a protein carrier has been shown to be able to induce antisera that bound to both PAK native peptide and PAK pilus protein (Irvin *et al.*, unpublished data).

In this study, information obtained from a previous study (Chapter IV) was used to design peptide haptens that would have a greater chance of inducing cross-reactive antibodies. It is assumed that substitution of the non-essential residues in the epitope sequence obtained for a cross-reactivity antibody should allow the production of antisera with enhanced cross-reactivity; whereas substitution of residues that are

important for cross-reactive antibody recognition would induce more strain-specific antisera. To test this assumption, several PAK peptide analogs were synthesized and their abilities to induce cross-reactive antibodies were examined. Since the disulfide bridge has been shown to be important for the production of cross-reactive antibodies (Lee *et al.*, 1989b), the PAK peptide analogs employed in this immunological study were the 17-residue peptides with an intrachain disulfide bridge in their sequences.

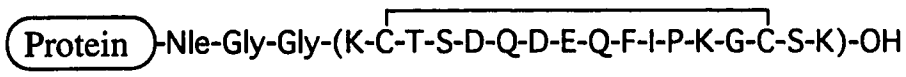
B. Results

1. Preparation of protein-peptide conjugates

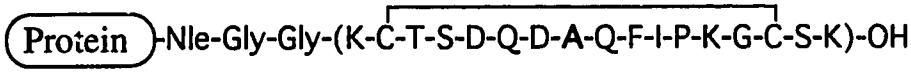
Previous studies showed that six side-chains in the PAK peptide sequence are important for recognition by either cross-reactive or strain-specific antibodies. These side-chains are Asp^{134}, Glu^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139} (Table IV.4 and Fig. IV.3). Phe^{137} was a critical residue for all antibodies studied, whereas residues Asp^{134} and Gln^{136} are essential only for the cross-reactive antibody 17-O1 but their importance for the strain-specific antibodies such as PK99H and 17-R1 were much less. In addition, residues Glu^{135}, Ile^{138}, and Pro^{139} were much more important for the strain-specific antibodies. Of these six residues, three of them (Glu^{135}, Gln^{136}, and Ile^{138}) are mutated to Pro^{135}, Met^{136}, and Thr^{138} in the PAO sequence. Therefore, these three heterologous residues became the residues of choice for studying the antibody cross-reactivity between the PAK and PAO strains.

Based on the above analysis, five KLH-peptide conjugates were synthesized (Fig. V.1). These peptide conjugates included the native PAK 17-residue peptide PAK(128-144)OH, and the peptide analogs (E135A)PAK(128-144)OH, (Q136A)PAK(128-144)OH, (F137A)PAK(128-144)OH, and (I138A)-PAK(128-144)OH. Their conjugation ratios as determined by amino acid analyses were 6.1, 5.8, 7.5, 8.0, and 4.3 peptides/KLH, respectively. The amount of peptide conjugates used in

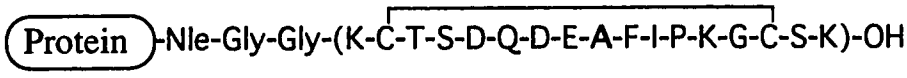
1. PAK(128-144)OH



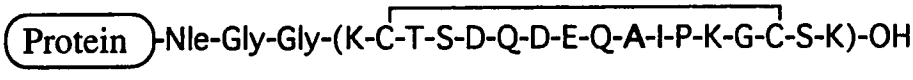
2. (E135A)PAK(128-144)OH



3. (Q136A)PAK(128-144)OH



4. (F137A)PAK(128-144)OH



5. (I138A)PAK(128-144)OH

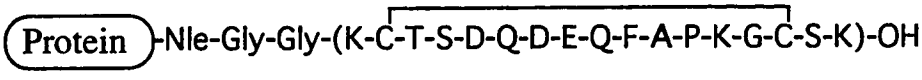


Figure V.1 Peptide conjugates used in rabbit immunization. The peptide analogs were conjugated to either KLH (for immunization) or BSA (for ELISA assay) (see Chapter II). Two Gly residues were added to the N-termini of the peptides to act as a spacer that does not display any side-chain and has excellent flexibility; the Nle (norleucine) residue was employed for peptide quantitation purposes. The intrachain disulfide bridge in each peptide sequence is shown.

immunizations was calculated according to their conjugation ratios. For each KLH-peptide conjugate, three rabbits were immunized. After three injections, the antisera were collected and characterized for antibody activities (see Chapter II for immunization protocol).

2. Determination of antiserum reactivity by direct ELISA

The endpoint titers of the antipeptide antisera were determined by direct ELISA (Fig. V.2). Good antibody titers were found for all fifteen antisera when assayed against their corresponding BSA-peptide conjugates. The endpoint titers as expressed by the reciprocal of the endpoint dilution were found in the range of 10^5 to 10^9. However, when the antisera were assessed utilizing the PAK pili, some interesting results were observed. Antisera raised in rabbits against the native PAK 17-residue peptide (antisera N-1 to N-3), the peptide analogs (E135A)PAK(128-144)OH (antisera E135A-1 to E135A-3), and (I138A)PAK(128-144)OH (antisera I138A-1 to I138A-3) demonstrated high titers against the PAK pili (in the range of 10^4 to 10^6), which titers were only about one order of magnitude lower than those measured for the BSA-peptide conjugate (in the range of 10^5 to 10^8). Interestingly, antisera raised against peptide analogs (Q136A)PAK(128-144)OH (antisera Q136A-1 to Q136A-3) and (F137A)PAK(128-144)OH (antisera F137A-1 to F137A-3) showed very weak binding to the PAK pili. The endpoint titers of these two series of antisera were in the range of 10^2 to 10^3, which was close to the titers of the pre-immune sera from the rabbits (about 10^3, data not shown), and was about five orders of magnitude lower than those tested with the BSA-peptide conjugates (in the range of 10^7 to 10^9, Fig. V.2).

The cross-reactivities of the antipeptide antisera to different *P. aeruginosa* pilus strains were also screened (Fig. V.3). Results from direct ELISA showed that antisera N-1 to N-3, E135A-1 to E135A-3, and I138A-1 to I138A-3 demonstrated various degrees of cross-reactivity among the PAK, PAO, KB7, and K122-4 pili. The

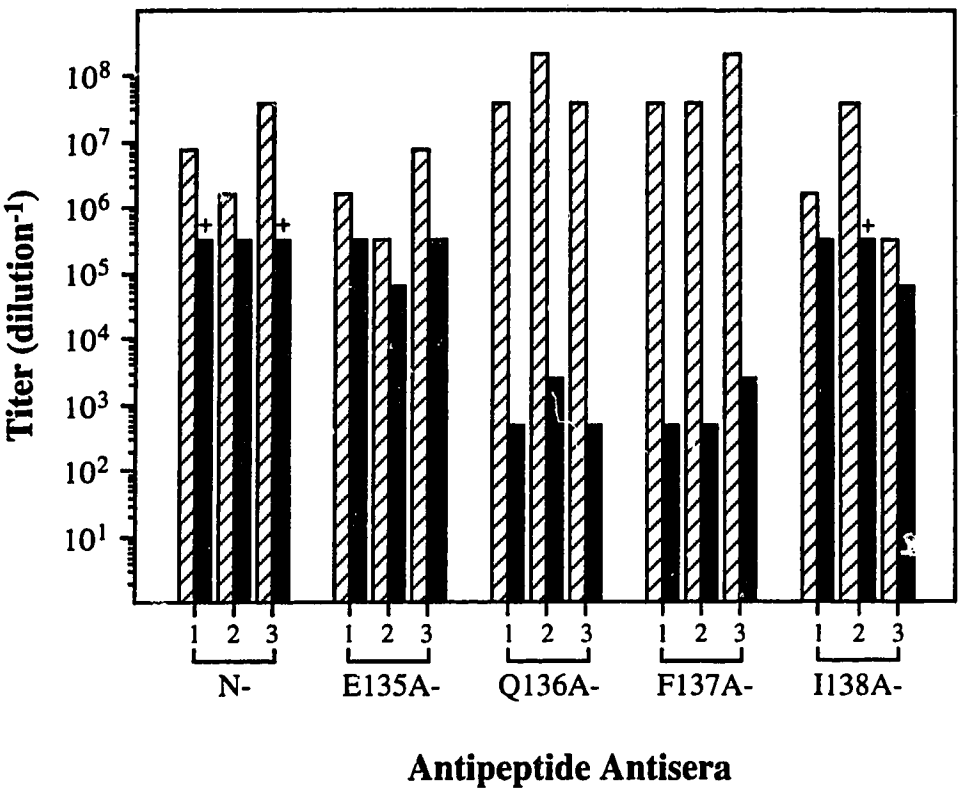
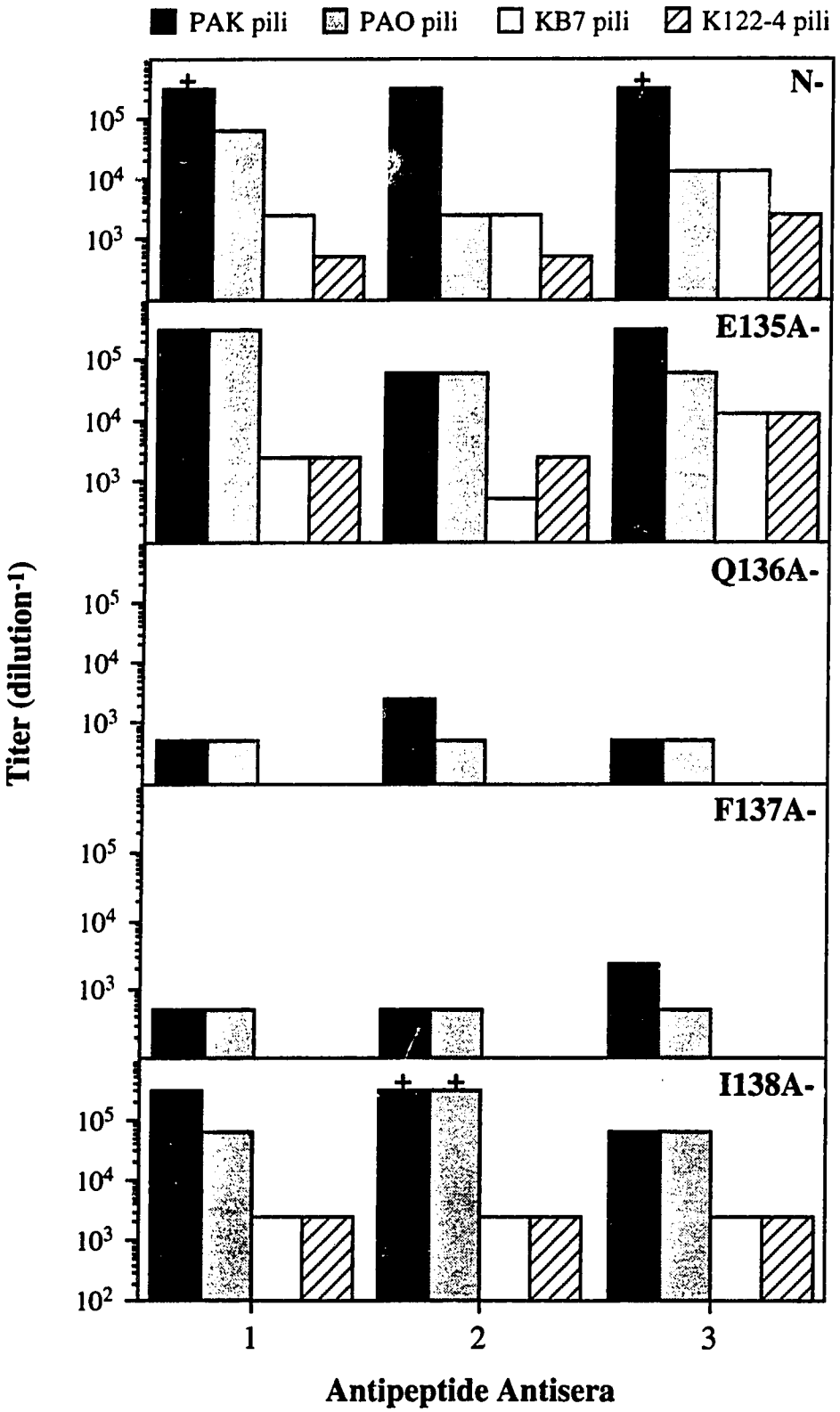


Figure V.2 Endpoint titers of anti-PAK C-terminal peptide antisera as determined by direct ELISA. For each KLH-peptide conjugate, three rabbits were immunized. The antipeptide antisera were named according to the residue substituted, and numbered from 1 to 3 to indicate the rabbit used. Where N-1 to N-3 are antisera raised against the native peptide conjugate, KLH-PAK(128-144)OH, the endpoint titers were determined by assaying against corresponding BSA-peptide conjugates (▨) and the PAK pili (■). The (+) symbol denotes a value greater than what is indicated.

Figure V.3 Endpoint titers of anti-PAK C-terminal peptide antisera as determined by direct ELISA on vaious *P. aeruginosa* pilus strains. Four strains of pili were assayed, they are the PAK pili (■), PAO pili (▩), KB7 pili (□), and K122-4 pili (▨). The KB7 and K122-4 data for Q136A- and F137A-antipeptide antisera are not available. The (+) symbol denotes value greater than what is indicated.


PAK pili
PAO pili
KB7 pili
K122-4 pili
N-
E135A-
Q136A-
F137A-
I138A-
Titer (dilution-1)
10^5
10^4
10^3
10^2
1
2
3
Antipeptide Antisera

endpoint titers were found in the range of 10^4 to 10^6 for PAK pili, 10^3 to 10^6 for PAO pili, and 10^2 to 10^4 for both KB7 and K122-4 pili. The highest titers could be found in assays with the PAK and PAO pili. In contrast, for other pili, reasonable titers could only be observed for antisera N-3 (KB7 pili: 1.3 x 10^4), and antisera E135A-3 (KB7 pili: 1.3 x 10^4 and K122-4 pili: 1.3 x 10^4). In the case of the Q136A- and F137A-series of antisera, very weak or insignificant binding were found to both PAK and PAO pili. Since these two series of antipeptide antisera reacted weakly with the pilus proteins, the direct ELISAs on other pilus strains were not performed.

In summary, three antisera N-1, N-3, and I138A-2 showed the highest endpoint titers when assayed with the PAK pili. I138A-2 and E135A-1 had high titers to both PAK and PAO strains. The most cross-reactive antiserum (as judged by the number of pilus strains reacted) was E135A-3, which cross-reacted with the PAK pili (3.1 x 10^5), PAO pili (6.3 x 10^4), KB7 pili (1.3 x 10^4), and K122-4 pili (1.3 x 10^4). The second most cross-reactive antiserum was N-3, which could cross-reacted with three pilus strains (PAK, PAO, and KB7). Moreover, all the N-, E135A-, and I138A- series of antisera (except N-2) demonstrated cross-reactivity between the PAK and PAO pili. However, the remaining antisera (Q136A- and F137A- series) did not show any significant binding to either PAK or PAO pili.

3. Evaluation of the binding affinity of the antipeptide antisera

The binding affinities of the antipeptide antisera were determined by competitive ELISA. The ELISA plate was coated with either the PAK pili (Table V.1) or BSA-PAK(128-144)OH peptide (Table V.2). The I_{50} for each competitive binding was determined and the apparent association constants (K_a's) were calculated (see Chapter II and legend of Table III.2 for details in calculation). Since the Q136A- and F137A-series of antisera bound weakly to the pilus protein, the binding affinities of these antisera to the pilus proteins were not examined. In Table V.1, the K_a's of the nine

Table V.1 Comparison of the apparent association constants of the anti-PAK peptide antisera for PAK and PAO pili binding

	K_a[a] (ml/μg)		
Antisera	PAK pili	PAO pili	K_{PAK}/K_{PAO}
N-1	4.9×10^{-1}	1.4×10^{-2}	35
N-2	1.7×10^{-1}	5.6×10^{-3}	30
N-3	1.2×10^{-1}	1.2×10^{-2}	10
E135A-1	2.7×10^{-1}	4.5×10^{-3}	60
E135A-2	6.6×10^{-2}	1.1×10^{-3}	60
E135A-3	2.8×10^{-1}	7.5×10^{-3}	37
I138A-1	2.0×10^{-1}	1.7×10^{-3}	120
I138A-2	4.0×10^{-1}	4.6×10^{-2}	8.7
I138A-3	3.1×10^{-2}	1.7×10^{-3}	18

[a]K_a is the apparent association constant of the antipeptide antiserum for the corresponding pilus proteins. Definition of K_a can be found in the legend of Table III.2. K_{PAK} and K_{PAO} stand for the K_a's of the PAK and PAO pili, respectively. The K_{PAK}/K_{PAO} ratio thus represents the number of folds increase in PAK pili binding as compared to PAO pili. The ELISA wells were coated with PAK pili.

Table V.2 Comparison of the apparent association constants of the anti-PAK peptide antisera for binding to synthetic peptides AcPAK(128-144)OH and AcPAO(128-144)OH

	K_a[a] (M^{-1})		
Antisera	PAK peptide	PAO peptide	K_{PAK}/K_{PAO}
N-1	5.7×10^5	1.0×10^3	570
N-2	4.0×10^4	$< 6.3 \times 10^2$	> 63
N-3	6.8×10^4	2.1×10^3	32
E135A-1	1.3×10^5	6.3×10^2	210
E135A-2	2.5×10^4	$< 6.3 \times 10^2$	> 40
E135A-3	3.6×10^4	2.0×10^3	18
I138A-1	1.5×10^5	$< 6.3 \times 10^2$	> 240
I138A-2	2.1×10^6	7.9×10^3	270
I138A-3	1.8×10^4	6.3×10^2	29

[a]K_{PAK} and K_{PAO} are the K_a's of the synthetic peptides AcPAK(128-144)OH and AcPAO(128-144)OH, respectively. See legend of Table V.1 for details. The ELISA wells were coated with BSA-PAK(128-144)OH (Chapter II).

antipeptide antisera (N-, E135A- and I138A- series) binding to both PAK and PAO pili are listed. Antisera N-1 and I138A-2 showed the highest binding affinities to the PAK pili (K_a's are 0.49 and 0.40 ml/μg, respectively), whereas E135A-2 and I138A-3 showed the lowest binding affinity to the PAK pili (K_a's were 6.6×10^{-2} and 3.1×10^{-2} ml/μg, respectively). In the case of PAO pili, antisera I138A-2, N-1, and N-3 showed the highest binding affinity (K_a's are 4.6×10^{-2}, 1.4×10^{-2}, and 1.2×10^{-2} ml/μg, respectively). As the K_a's of the PAK and PAO pili were compared, the resultant ratio, K_{PAK}/K_{PAO}, could be used to denote the preference of an antiserum binding to the two pilus strains. Among the nine antipeptide antisera assayed (Table V.1), all except I138A-1 had a K_{PAK}/K_{PAO} ratio less than 100. This illustrated that these antisera had cross-reactivity close to that of 17-O1 (K_{PAK}/K_{PAO} ratio was 64, Table IV.1). The best K_{PAK}/K_{PAO} ratio could be found for two of the antisera, N-3 and I138A-2, which had K_{PAK}/K_{PAO} ratios of 10 and 8.7, respectively. On the other hand, E135A-2, which was shown to display a broad range of specificity to the four different pilus strains, had the weakest binding affinity to both PAK ($K_a = 6.6 \times 10^{-2}$) and PAO ($K_a = 1.1 \times 10^{-3}$) pili, and the ratio was 60.

The binding affinities of all fifteen antipeptide antisera to both PAK and PAO C-terminal 17-residue peptides (AcPAK(128-144)OH and AcPAO(128-144)OH) were also determined. For comparison and clarity purpose, only the data for the N-, E135A- and I138A- series of antisera are shown (Table V.2). The K_a's of the antipeptide antisera were in the range of 1.8×10^4 to 2.1×10^6 M^{-1} for the PAK peptide and from less than 6.3×10^2 to 7.9×10^3 M^{-1} for the PAO peptide. E135A-3 showed the lowest K_{PAK}/K_{PAO} ratio, which meant that the antiserum bound 18-fold better to the PAK peptide than to the PAO peptide. Other antisera such as N-3 and I138A-3 also showed low K_{PAK}/K_{PAO} ratios (32 and 29, respectively). As the results from Table V.1 and Table V.2 were compared, the most noteworthy changes were found in antisera I138A-2, where the K_{PAK}/K_{PAO} ratio was increased from 8.7 (Table V.1) to 270 (Table V.2).

In general, the overall K_{PAK}/K_{PAO} ratios from Table V.2 were higher than those observed in Table V.1, which suggested that the antipeptide antisera cross-react better with the structurally more defined pilus proteins than with the flexible peptides.

4. Mapping of the epitope sequences for the antipeptide antisera

Five antipeptide antisera were chosen for epitopic analysis. These represented one of each type of antisera, that is: N-1, E135A-3, Q136A-2, F137A-3, and I138A-2. The epitopes for these antisera were mapped by a series of single alanine substituted peptide analogs corresponding to the PAK C-terminal 17-residue sequence. For antisera N-1, E135A-3, and I138A-2, competitions were performed between the peptide analogs and PAK pili coated on the ELISA plate. However, in the case of Q136A-2 and F137A-3, BSA-PAK(128-144)OH peptide conjugates were coated onto the ELISA plate instead because these two antisera bound poorly to the pilus proteins (Fig. V.3).

According to the previous definition (Chapter III) that a peptide analog with a loss in binding affinity (the K_N/K_S ratio) of greater than 3-fold would be considered to have an altered, important residue for antibody interactions, three linear epitopes were identified for antisera N-1, E135A-3, and I138A-2 (Table V.3). N-1 recognized a 8-residue epitope comprising the sequence DQDEQFIP (residues 132-139), which was also the epitope sequence recognized by the polyclonal antibodies 17-R1, 17-O1, and 17-O2 (Chapter IV). Interestingly, the epitope for E135A-3 was also an 8-residue sequence but with one position shifted towards the C-terminus as compared with that of the N-1. The sequence was QDEQFIPK (residues 133-140), which contained the epitope sequence recognized by monoclonal antibody PK99H (DEQFIPK, Chapter III). Finally, the epitope sequence identified for I138A-2 was a 7-residue peptide, which is similar to the epitope of PK99H and comprised the sequence DEQFIPK.

Although these three epitope sequences were very similar to each other, the

Table V.3 Epitopic analysis of three anti-PAK peptide antisera

	K_N/K_S Ratios of the Antipeptide Antisera[a]		
Peptide analogs	N-1	E135A-3	I138A-2
K128→A	6.0	0.4	0.1
T130→A	2.3	0.1	0.7
S131→A	2.7	0.2	0.8
D132→A	3.6	1.0	0.5
Q133→A	3.4	830	1.0
D134→A	11	1,200	8.4
E135→A	> 360	0.8	70
Q136→A	> 550	520	> 660
F137→A	> 770	> 4,800	> 920
I138→A	> 210	450	0.02
P139→A	> 92	390	29
K140→A	2.7	8.3	3.8
G141→A	1.7	1.1	0.1
S143→A	2.9	1.0	2.1
K144→A	1.8	0.9	1.4

[a] K_N/K_S is the ratio of the K_a of the native PAK peptide (AcPAK(128-144)OH) to that of the corresponding peptide analog as determined by competitive ELISA for each antipeptide antiserum. The boxed regions indicate possible epitope sequence for corresponding antiserum. The ELISA plate was coated with the PAK pili.

contribution of individual side-chains to antisera binding was quite different. For example, five residues were found essential for N-1 binding (Glu^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139}), whereas six residues were found essential for E135A-3 (Gln^{133}, Asp^{134}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139}), and only two residues (Gln^{136} and Phe^{137}) were essential for I138A-2. Among the three antisera studied, Gln^{136} and Phe^{137} were the two common residues that were essential for the antibody-antigen interactions. Surprisingly, the epitopes for both Q136A-2 and F137A-3 could not be mapped (data not shown) because the binding affinities of these two antisera to the peptide analogs were too close to the native PAK peptide, and therefore, not a single critical residue could be identified.

C. Discussion

Antibody cross-reactivity is a generally observed phenomenon and investigations have been carried out to understand cross-reactivity with different antigens. For example, a recent report by Carlin *et al* (1994) was devoted to examining the cross-reactivity of an anti-furosemide monoclonal antibody. In their studies, molecular modeling was used to correlate the structural and electronic properties of the hapten with antibody binding. In this way the region on the furosemide hapten recognized by four monoclonal antibodies was identified. Both ELISA data and conformation data were combined to predict the cross-reactivity of the anti-furosemide monoclonal antibody to furosemide and related compounds.

In this project, the cross-reactivity of the anti-PAK pilin antibodies was elucidated by means of competitive ELISA studies to reveal the contribution of individual side-chains in the epitope to antibody interactions. The data obtained was then correlated with the cross-reactivity of the antibodies (Chapter IV). As a result, two residues in the PAK C-terminal antigenic region (Asp^{134} and Gln^{136}) were identified to be important

for recognition by cross-reactive antibody; whereas two other residues (Glu^{135} and Ile^{138}) were more important for recognition by strain-specific antibodies. In this study, a further step was taken in order to examine whether the above information could assist us in designing immunogens that induce antibodies with predetermined specificity or cross-reactivity.

Therefore, four groups of KLH-peptide conjugates were employed to examine this possibility. (i) The native peptide PAK(128-144)OH. It was used as a control. (ii) Peptide analogs with either residue Glu^{135} or Ile^{138} replaced by an Ala. These two residues were judged unimportant for recognition by cross-reactive antibody but were important for binding to strain-specific antibody. It was proposed that substitution of these two residues could allow the immune system to elicit antibodies with comparable cross-reactivity. (iii) Peptide analog with Gln^{136} substituted by an Ala. This residue was found to be essential for recognition by cross-reactive antibody. Therefore, removal of this side-chain could possibly reduce the ability of the immune system to produce cross-reactive antibodies. (iv) Peptide analog with Phe^{137} substituted by an Ala. This residue was essential for all antibodies studied and was thus another control for this study.

1. Production of cross-reactive antibodies

The three rabbits immunized with the native PAK peptide conjugate all produced cross-reactive antibodies which recognized both synthetic peptide and pilus protein from the PAK and PAO strains. This result was similar to that observed previously (Lee *et al.*, 1989b). The epitope mapped for antisera N-1 was also similar to the previous study, in which the epitope was found to be an 8-residue peptide containing the sequence DQDEQFIP. Five of the eight side-chains were found essential for antibody binding. These were the residues Asp^{135}, Gln^{136}, Phe^{137}, Ile^{138}, and Pro^{139}. Similarly, results obtained from both direct and competitive ELISA have

shown that the E135A- and I138A- series of antisera could induce the immune system to produce cross-reactive antisera in rabbits. One of the best examples was I138A-2, which binds very well to both peptides and pili from the PAK and PAO strains. Furthermore, from each series of antipeptide antisera, antisera that cross-react well with pilus proteins and peptides from both PAK and PAO strains could be identified. Examples were antisera N-3, E135A-3, and I138A-3. Additionally, the best cross-reactive antisera for the pilus proteins and the pilin peptides were I138A-2 (Table V.1) and E135A-3 (Table V.2), respectively.

As the binding affinities (the apparent associated constants, K_a) of the N-, E135A-, and I138A- series of antisera were averaged and compared to each other, a similar order of cross-reactivity could be observed for both synthetic peptides and pilus protein. For example, from Table V.1 the mean binding affinities for PAK pili were in order: N- > I138A- ≈ E135A- (the mean K_a's were 0.26, 0.21, and 0.21 ml/μg, respectively); whereas for the PAO pili, the order was: I138A- > N- > E135A (the mean K_a's were 1.6×10^{-2}, 1.1×10^{-2}, and 4.4×10^{-3} ml/μg, respectively). Similarly, from Table V.2 the mean K_a values for PAK and PAO peptide binding were compared. For PAK peptide, the mean binding affinities were in order: I138A- > N- > E135A- (the mean K_a's were 7.6×10^5, 2.3×10^5, and 6.4×10^4 M^{-1}, respectively); whereas for PAO peptide, the order was the same as that of the PAK peptide (with mean K_a's of 3.1×10^3, 1.2×10^3, and 1.1×10^3 M^{-1}, respectively). In addition, the cross-reactivities were in order: I138A- > N- > E135A- (the mean K_{PAK}/K_{PAO} ratios were 13, 24, and 48, respectively) for the pilus proteins; and were in order: E135A- > N- > I138A- (the mean K_{PAK}/K_{PAO} ratios were 58, 190, and 250, respectively) for the pilin peptides. It was of interest to observe that the I138A- series of the antisera showed the highest binding affinities for the PAO pili, PAK peptide and PAO peptide. This series of antisera also demonstrated the highest cross-reactivity between the PAK and PAO pili. However, the degree of cross-reactivity dropped when binding to the pilin

peptides was assessed. In contrast, the E135A- series of antisera showed the highest degree of cross-reactivity to the pilin peptides. It is possible that the combining sites of the I138A- series of antisera were in general more selective for the structurally more rigid pilus protein; whereas the E135A- series of antisera could more readily adjust to the flexible peptide antigen.

Results from epitope mapping (Table V.3) illustrated some consistency with previous study. First, Phe^{137} was again a critical residue for all epitopes mapped. Second, Gln^{136} was also essential for recognition by the three cross-reactive antisera. However, Asp^{134} was only critical for antiserum E135A-3 (1,200-fold decrease in binding affinity for peptide Ac(D134A)PAK(128-144)OH). This agreed with the previous study that both Asp^{134} and Gln^{136} were important for binding to 17-O1. On the other hand, Glu^{135} and Ile^{138} might not be as important for strain-specific antibodies as was proposed above since these two residues were also found to be important for the cross-reactive antibodies. For instance, N-1 (Glu^{135} and Ile^{138} showed > 360-fold and > 210-fold decrease in binding affinity, respectively) and E135A-3 (Ile^{138} showed a 450-fold decrease in binding affinity). As a comparison, the epitopic analyses of the strain-specific antibodies PK99H and 17-R1 indicated that Ile^{138} (600-fold decrease in binding affinity) was important for PK99H, whereas Glu^{135} (480-fold decrease in binding affinity) was important for 17-R1 (Table IV.4).

2. Antisera with restricted specificity

The antipeptide antisera produced by the two peptide analogs (Q136A)PAK(128-144)OH and (F137A)PAK(128-144)OH showed some interesting results. In terms of specificity, these two series of antisera were specific for the peptide antigen only, but bound poorly to the pilus protein (Fig. V.2). Epitope mapping of two of the antisera selected from these two series of antisera was unsuccessful since the binding affinity of the single Ala substituted peptide analogs to these antisera were very close to those of

the native peptides. These results suggested that these two polyclonal antisera could display a broad range of specificity over the single Ala mutants of the PAK peptide probably due to a broad polyclonal response. It is also possible that the combining site of these antisera could accommodate changes due to single side-chain deletion by an Ala.

The Phe^{137} residue is a very critical residues for all antibodies studied to date. The contribution of this hydrophobic side-chain to antibody-antigen interactions had led to the postulate that this residue may also be very important for antibody production. However, the result obtained was quite surprising since deletion of this side-chain did not affect antibody production, but instead, altered the specificity of the antisera in such a way that they only recognized the flexible peptides but not the native pilus proteins. It seems likely that the presence of Phe^{137} and Gln^{136} in the peptide hapten is important to direct the host immune response to selectively produce antisera with broader specificity such as recognition of a more rigid protein structure. In addition, Gln^{136} may also be important in controlling the production of antisera with cross-reactivity between the PAK and PAO strains since the Q136A- series of antisera are specific to the PAK pilin peptide only (data not shown). These observations provide important information on how to manipulate the amino acid residues in a peptide hapten that can in turn affect the specificity of the antisera produced.

3. Conclusion

Four residues in the PAK C-terminal antigenic region have been identified to be important for inducing antibodies with different specificity. Among these four residues, Glu^{135} and Ile^{138} were previously shown to be important for the epitopes that are recognized by the strain-specific antibodies. Deletion of the side-chains of these two residues in the PAK sequence did not affect the production of cross-reactive antibodies in rabbits, but instead caused some improvement in the cross-reactivity of

these antisera. On the other hand, Gln^{136} and Phe^{137} were residues that were important for maintaining the capability of the immune system to produce antibodies with specificity to the structurally more rigid intact proteins. In addition, Gln^{136}, a critical residue found in the epitope for all cross-reactive antibodies, was also important for inducing cross-reactive antisera in rabbits. These results confirm the opinion expressed by Lerner (1982), "By using peptide immunization, one can generate antibody specificities which cannot be obtained in any other way."

This study demonstrates the feasibility that the specificity of the antisera can be manipulated through the modifications of the epitope sequence. In the immunological study of the *P. aeruginosa* PAK and PAO pili, both strain-specific and cross-reactive antisera could be selected and produced by altering the side-chains of the Glu^{135}, Gln^{136}, Phe^{137}, and Ile^{138} residues. This knowledge is important for us to design and produce highly specific antibodies that will be useful for clinical diagnosis, as well as vaccine candidates that cross-protect against a broad range of *P. aeruginosa* strains. To achieve this goal, antibody epitopes of other *P. aeruginosa* pilin strains should be studied and compared with the results of present study.

CHAPTER VI

REPRESENTATIVE COMBINATORIAL PEPTIDE LIBRARIES: AN APPROACH TO REDUCE BOTH SYNTHESIS AND SCREENING EFFORTS IN ANTIBODY BINDING DIVERSITY STUDY[4]

A. Introduction

The combinatorial peptide library is a powerful tool in which a vast number of diverse peptides are produced and screened to identify peptide sequences that may be used clinically or used for the development of pharmaceutical compounds. Present uses of peptide libraries include the mapping of B-cell (Houghten *et al.*, 1991; 1992; Blake and Litzi-Davis, 1992) and T-cell (Schumacher *et al.*, 1992) epitopes, elucidation of ligand/receptor interactions (Gausepohl *et al.*, 1992; Hortin *et al.*, 1992; Houghten *et al.*, 1992), and the identification of antimicrobial peptides (Houghten *et al.*, 1991; 1992).

Two major technological advancements have made the routine use of combinatorial peptide libraries possible. Firstly, major advances in solid-phase peptide synthesis (SPPS) including the development of Fmoc SPPS and the increased availability of a variety of solid supports, linkers, side-chain protecting groups and improved solvation conditions (Fields and Noble, 1990), coupled with improvements in cleavage methods (King *et al.*, 1990) allow for the successful synthesis of a wide range of peptide sequences under identical conditions. Secondly, recent developments in parallel peptide synthesis methods have greatly improved synthesis efficiency and made the synthesis of a large array of peptides feasible. A number of parallel methods have been

[4]A version of this chapter has been accepted for publication: Wong, W.Y., H.B. Sheth, A. Holm, R.S. Hodges, and R.T. Irvin (1994). *Methods (A Companion to Methods in Enzymology).*

reported. These include the pin approach reported by Geysen *et al.* (1984; 1985), the simultaneous multiple peptide synthesis developed by Houghten *et al.* (1985; 1986), and the light-directed parallel chemical synthesis described by Fodor *et al.* (1991). In this project, a multiple column peptide synthesizer was employed for parallel synthesis of peptides (Holm and Meldal, 1989; Meldal *et al.*, 1993). This instrument consists of a Teflon reaction block containing a 96 well reactor in which the solid supports are loaded.

Peptide diversity of a library constructed with all 20 naturally occurring amino acids is extensive even at short peptide lengths and usually involves millions of peptides. Physical constraints in synthesis and screening thus arise and impose serious limitations on the length of peptide that may be examined due to the concentration of a single peptide sequence in a combinatorial library. In addition, difficulties in the coupling of certain amino acids, and potential side-chain modifications during the deprotection and cleavage steps of the synthesis may impact significantly on the composition and utility of a peptide library.

To further simplify both synthesis and library screening procedures, a novel approach using representative amino acids instead of all 20 naturally occurring amino acids was investigated. Representative combinatorial hexapeptide libraries were made employing a set of ten amino acids that displayed the basic physico-chemical properties associated with naturally occurring amino acid side-chains. This approach can greatly reduce the number of peptide mixtures that must be synthesized, minimizing the amount of labor in synthesizing and screening the libraries, and allows for higher concentrations of single peptide sequences and for the synthesis of libraries of increased peptide length.

Three series of hexapeptide libraries were synthesized in which two positions were defined. The two defined residues in the peptide pools are used to identify important positionally different dipeptide sequences, which contribute part of the peptide

sequences that interact with a given receptor. These three series of peptide libraries were then used to investigate the binding diversity of the murine monoclonal antibody PK99H, which was raised against the strain PAK pili of the opportunistic pathogen *Pseudomonas aeruginosa*, that binds specifically to the C-terminal receptor binding domain of the PAK pilin. The epitope recognized by PK99H has been mapped using single alanine substituted peptides. This epitope was well characterized and found to consist of the heptapeptide sequence DEQFIPK (Chapter III and Wong *et al.*, 1992) and thus served as a model system to validate the representative peptide library.

B. Results and Discussion

1. The optimal peptide length in a peptide library

To determine the optimal peptide length in a combinatorial peptide library, four representative combinatorial peptide libraries with peptide length of 4, 5, 6, and 7 residues were synthesized. The sequences of these peptide libraries were Ac-$X_1X_2X_3X_4$-NH_2, Ac-$X_1X_2X_3X_4X_5$-NH_2, Ac-$X_1X_2X_3X_4X_5X_6$-NH_2, and Ac-$X_1X_2X_3X_4X_5X_6X_7$-NH_2, respectively (see next section for explanation). The I_{50} values of these peptide libraries in inhibiting monoclonal antibody PK99H binding to the oxidized AcPAK(128-144)OH peptide were determined (Fig. VI.1). The I_{50} values obtained were peptide-length dependent, that is, the longer the peptide length in the library, the lower was the I_{50} value. A peptide library with peptide length shorter than 5 residues would result in a dramatic increase in the I_{50} value and thus a loss in the antibody binding capability (I_{50} = 23 mM for the tetrapeptide library). The I_{50} values for the penta-, hexa-, and hepta- peptide libraries were 2.6, 1.2, and 0.58 mM, respectively. These results suggest that the optimum peptide length in a peptide library for antibody binding analysis should be more than four residues. It also agreed well with previous studies that the length of an antibody epitope is in the range of 5 to 10

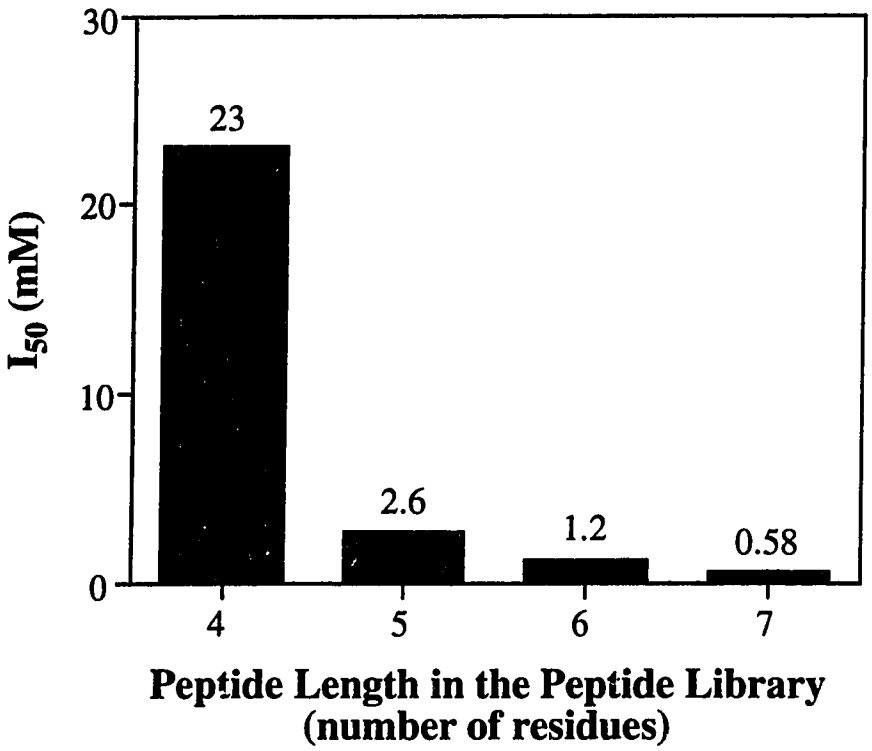


Figure VI.1 Determination of the optimal peptide length in a peptide library.

residues (Anderson *et al.*, 1988; Kövamees *et al.*, 1990; Scott *et al.*, 1990; Wong *et al.*, 1992) and that the minimu· . binding sequence is between 5 and 7 residues (Anderson *et al.*, 1988). In the present study, a peptide length of six residues was employed in the representative combinatorial peptide libraries.

2. The representative combinatorial peptide libraries

The representative combinatorial peptide libraries (RCPL) were composed of six-residue synthetic peptides with acetylated N-termini and amidated C-termini. Three series of hexapeptide libraries were used in this study. Their sequences were encoded as follows: Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, Ac-$X_1X_2O_3O_4X_5X_6$-NH_2 and Ac-$X_1X_2X_3X_4O_5O_6$-NH_2, where "Ac" denotes the acetylated N-terminal group and "NH_2" indicates the C-terminal amide. The numbers in the sequence show the relative position of each residue. Each series of the peptide libraries had two positions specifically defined as indicated by "O_nO_{n+1}" in the sequence, whereas "X_n" denoted a position containing equimolar mixtures of the representative amino acids. Each "O" and "X" was chosen from the following 10 representative amino acids (with the amino acids being represented in brackets): Ala, Glu (Asp), Phe (Trp, Tyr), Gly, Ile (Val, Met), Lys (His, Arg), Leu (Met), Pro, Gln (Asn) and Ser (Thr). No representative amino acid was selected for Cys. Each series of the peptide libraries thus represented 100 (10^2) hexapeptide mixtures with each mixture containing 10,000 (10^4) different peptides and being defined by the 100 possible dipeptide sequences (O_nO_{n+1}).

3. Importance of buffer strength of the incubation medium

Due to the presence of high concentrations of peptide mixtures in the incubation medium, the peptides themselves would behave as dominant acid-base species in the buffering system. It was found that conventional 10 mM PBS did not have sufficient buffering strength to maintain a physiological pH in the assay system. The hexapeptide

libraries, Ac-$X_1X_2O_3O_4X_5X_6$-NH_2, were incubated with monoclonal antibody PK99H in either conventional 10 mM PBS (Fig. VI.2) or 50 mM PBS (Fig. VI.3, panel B). False positive inhibition results were observed especially in peptide mixtures containing defined Glu in its sequence (Fig. VI.2). This effect could be circumvented by increasing the buffer strength of the medium to 50 mM (Fig. VI.3, panel B), where only FI showed an inhibition of over 50%.

Subsequent pH determination of the incubation mixtures indicated that the presence of Glu in either one or both of the two defined positions in the peptide mixtures caused a significant decrease in pH. The pH of the EE mixtures in 10 mM PBS was found to be 4, whereas the pH of the EO or OE mixtures dropped to about 5. This is mainly caused by the deprotonation of the acidic side-chain of the Glu residue, which has a pKa of 4.3. An increase in buffering strength from 10 mM to 50 mM PBS could maintain the pH close to 7 in both cases (Table VI.1).

4. Epitope mapping

The peptide libraries were screened by competitive ELISA. The ability of the libraries to inhibit the interaction of monoclonal antibody PK99H to the coated peptide was determined. From the ELISA results (Fig. VI.3), a cutoff at 50% inhibition was used to highlight the significant "hits" in order to locate those residues important for antibody binding. In libraries Ac-$O_1O_2X_3X_4X_5X_6$-NH_2 (Fig. VI.3, panel A), nine possible hits were observed, which were in the order of EQ, QQ, QA, AF, GQ, SL, EF, GL and QL. In libraries Ac-$X_1X_2O_3O_4X_5X_6$-NH_2 (Fig. VI.3, panel B), only one hit was observed, which was FI. In libraries Ac-$X_1X_2X_3X_4O_5O_6$-NH_2 (Fig. VI.3, panel C), five hits were observed, which were PK, IG, PL, LG and IL. The three best dipeptide sequences mapped by these three series of peptide libraries were EQ, FI and PK, respectively. These indeed constituted the native PAK epitope sequence EQFIPK (see Chapter III).

Figure VI.2 Effect of buffering strength of the incubation medium on the screening results. The representative combinatorial peptide libraries Ac-$X_1X_2O_3O_4X_5X_6$-NH_2 was screened using 10 mM PBS, pH 7.4, as the incubation medium. The sequence of the two defined residues, O_3O_4, are listed on the y-axis. A cutoff at 50% inhibition was used to highlight the significant hits. The sequences FI, SQ and all dipeptide sequences containing Glu (E), except EK, were identified as positive hits under this assay condition (see Fig. VI.3, panel B, for comparison). Subsequent pH determination indicated that the buffering system was seriously affected by Glu-containing peptide mixtures.

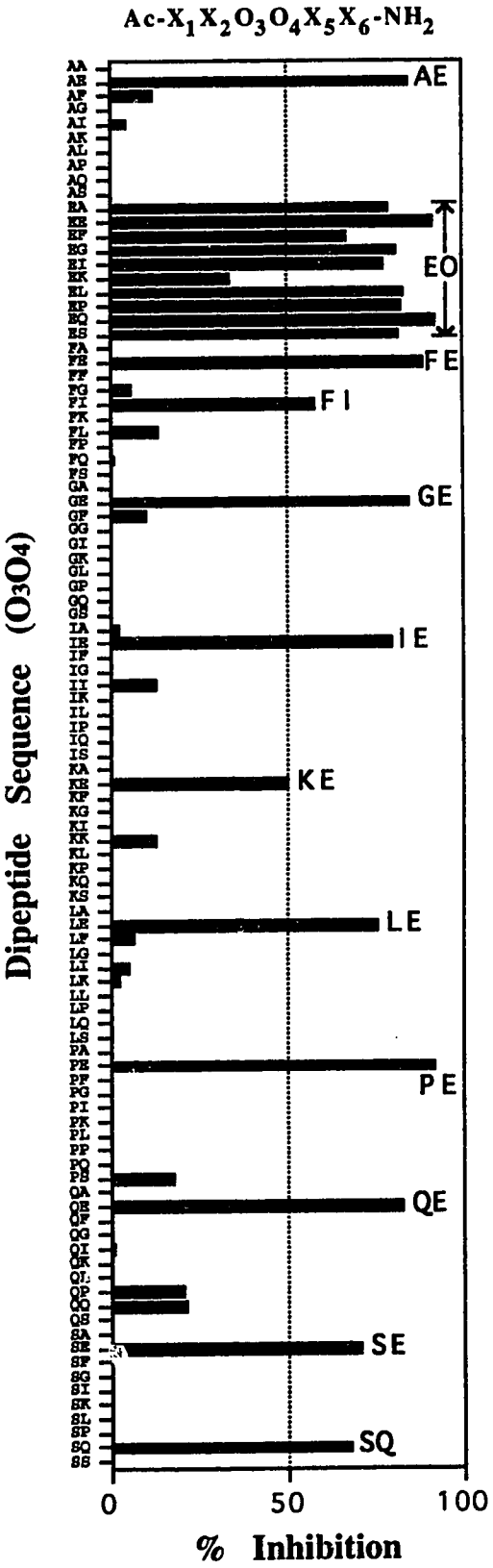
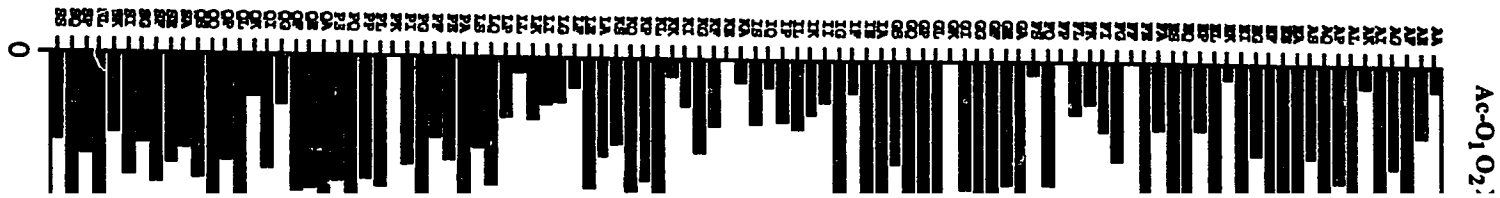

Ac-X1X2O3O4X5X6-NH2
Dipeptide Sequence (O3O4)
AE
EO
FE
FI
GE
IE
KE
LE
PE
QE
SE
SQ
0
50
100
% Inhibition

Figure VI.3 Screening of the three series of representative combinatorial peptide libraries using 50 mM PBS, pH 7.4, as the incubation medium. The sequence of the two defined residues, O_nO_{n+1}, in each series are listed on the y-axis. A cutoff at 50% inhibition was used to indicate positive hits. In panel A (Ac-$O_1O_2X_3X_4X_5X_6$-NH_2), 9 hits were observed, they were AF, EF, EQ, GL, GQ, QA, QL, QQ and SL. In panel B (Ac-$X_1X_2O_3O_4X_5X_6$-NH_2), only FI was identified. While in panel C (Ac-$X_1X_2X_3X_4O_5O_6$-NH_2), 5 hits were observed, which were IG, IL, LG, PK and PL.


Dipeptide Sequence (OnOn+1)
0
Ac-O1O2

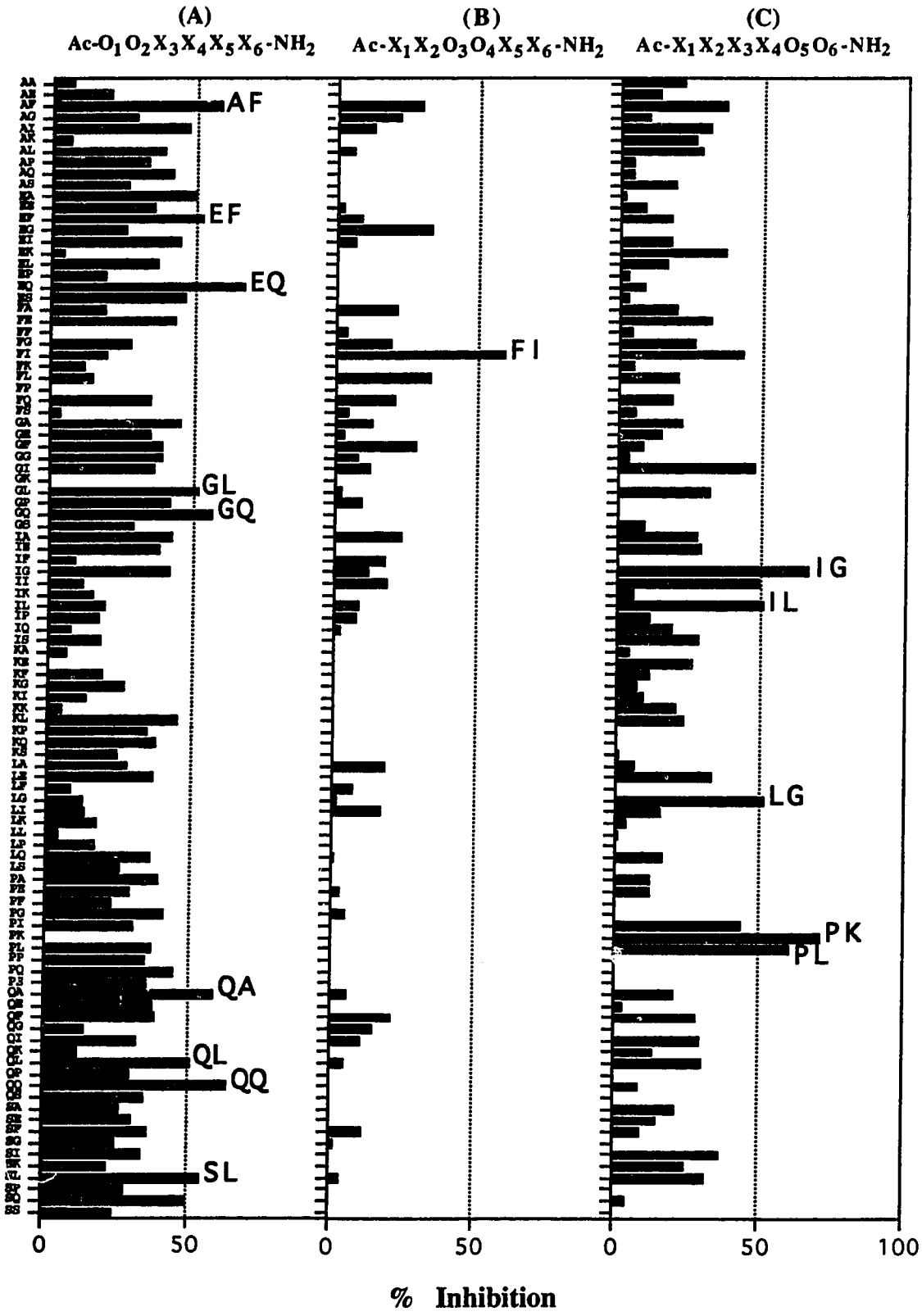

(A)
Ac-O1O2X3X4X5X6-NH2
(B)
Ac-X1X2O3O4X5X6-NH2
(C)
Ac-X1X2X3X4O5O6-NH2
AF
EF
EQ
GL
GQ
QA
QL
QQ
SL
FI
IG
IL
LG
PK
PL
0
50
0
50
0
50
100
% Inhibition

Table VI.1 The buffering effects of the hexapeptide libraries on the 10 mM and 50 mM PBS

Peptide Library	pH	
$X_1X_2O_3O_4X_5X_6$	10 mM PBS	50 mM PBS
EE	4	6.5 - 7
EG	4	7
FI	7.5	7.5
QE	4	7
QS	6	7

5. Verification of the screening results

In order to verify the ELISA results, synthetic hexapeptides based on a combination of the best hits from each series of libraries were made. The sequences of these hexapeptides are shown in Table VI.2. The inhibitory effects of these peptides on antibody binding to the peptide antigen were examined. In Table VI.2, among the 15 hexapeptides synthesized, only 6 were soluble in aqueous medium and could be used for I_{50} determination. The native sequence, EQFIPK, was one of the six hexapeptides mapped and found to have an I_{50} values of 1.3 μM. Two other sequences, QQFIPK and QAFIPK, had I_{50} values similar to that of the native sequence, 1.3 μM and 2.0 μM respectively. The sequences GQFIPK and EQFIIG showed a 8-fold and 35-fold increase in I_{50} values and thus displayed weaker binding affinity for the antibody as compared with the native peptide. Only one peptide, AFFIPK, showed far weaker inhibitory effect (I_{50} = 1,100 μM). This suggested that the first two residues mapped (AF) were not in combination with the last four residues (FIPK) to give the positive result shown in Figure VI.3, panel A.

6. Examination of the representative nature of the libraries

To assess whether the RCPL can represent a range of other sequences, twelve hexapeptides were synthesized according to the substitution of the corresponding "represented" residues in the four best mapped sequences as shown in Table VI.2. The sequences of these "represented" hexapeptides are listed in Table VI.3. Their I_{50} values were determined accordingly. Out of the 12 hexapeptides tested, six peptides (DQFIPK, EQWIPK, EQFMPK, EQFVPK, NQFIPK and NAFIPK) had I_{50} values within an order of magnitude of the native sequence EQFIPK (2.3, 0.9, 1.1, 3.4, 12 and 14 μM, respectively). Two of these peptides, EQWIPK and EQFMPK, had marginally lower I_{50} values (0.9 and 1.1 μM) than that of the native peptide (1.3 μM), and thus display a higher affinity for PK99H than the native sequence. The remaining

Table VI.2 Verification of the screening results by synthesizing hexapeptides corresponding to a combination of the three sets of dipeptide sequences as deduced from Figure VI.3

Sequence[a]	I_{50} (μM)[b]	Sequence[a]	I_{50} (μM)[b]	Sequence[a]	I_{50} (μM)[b]
EQFIPK	1.3	EQFIIG	45	EQFIPL	n/a
QQFIPK	1.3	QQFIIG	n/a	QQFIPL	n/a
QAFIPK	2.0	QAFIIG	n/a	QAFIPL	n/a
AFFIPK	1,100	AFFIIG	n/a	AFFIPL	n/a
GQFIPK	10	GQFIIG	n/a	GQFIPL	n/a

[a] The peptides were synthesized according to the five best hits (EQ, QQ, QA, AF and GQ) from Figure VI.3 (panel A), the only hit (FI) from Figure VI.3 (panel B), and the three best hits (PK, IG and PL) from Figure VI.3 (panel C). All peptides are N^{α}-acetylated and have an amide group at their C-termini.

[b] The I_{50} was determined by competitive ELISA; n/a denotes unavailability of the data due to the insolubility of the peptides.

Table VI.3 Examination of the representative nature of the peptide libraries

Sequence[a]	I_{50} (μM)	Sequence[a]	I_{50} (μM)
EQFIPK	1.3	QQFIPK	1.3
DQFIPK	2.3	NQFIPK	12
ENFIPK	190	QNFIPK	990
EQYIPK	190		
EQWIPK	0.9	QAFIPK	2.0
EQFMPK	1.1	NAFIPK	14
EQFVPK	3.4		
EQFIPH	620	GQFIPK	10
EQFIPR	>730	GNFIPK	>840

[a]The peptides were synthesized according to the four best mapped sequences (boxed) as shown in Table VI.2. The "represented" residues are circled. All peptides are N^{α}-acetylated and have an amide group at their C-termini.

hexapeptides, ENFIPK and EQYIPK, show a 150-fold weaker binding than the native peptide, and EQFIPH, EQFIPR, QNFIPK and GNFIPK, had a much higher I_{50} values (> 400-fold different) than that of the native sequence, indicating that these residues could not be substituted for their representative amino acids.

These results demonstrate the utility of representative peptide libraries and some potential limitations of this system. In this model system, three residues of the hexapeptide allowed for the substitution of representative residues. These three positions were position 1 (Glu, Gln), 3 (Phe) and 4 (Ile). Substitution of these residues by their corresponding "represented" residues did not substantially alter their binding affinity to PK99H. That is, Glu and Gln could represent Asp and Asn, respectively, in the first position, Phe could represent Trp in the third position, and Ile could represent Met and Val in the fourth position. However, the representative system had limitations too, especially with residues whose side-chain contribution to the antibody-antigen interactions did not merely depend on their physico-chemical properties. For example, in the case of Gln at the second position (this residue located at position 136 in the native PAK pilin sequence), the side-chain of this residue was not important for antibody interactions as determined by single alanine replacement analysis (Wong *et al.*, 1992), but substitutions with amino acids other than Ala or Cys in the 7-residue peptide could dramatically affect its binding to PK99H (Fig. III.6).

Interestingly, both His and Arg failed to substitute the Lys at the C-terminus of the hexapeptide sequence. As His (pKa = 6.0) was not charged at pH 7.4, it is reasonable that His could not mimic the charged Lys side-chain. However, substituting with Arg at this position also prevented peptide binding to the antibody. This may be due to steric limitations as Arg has a more bulky side-chain with extra hydrogen bond donors that may interfere with the antibody binding. Furthermore, the shorter and less hydrophobic hydrocarbon chain before the charged group in the Arg side-chain may reveal the specificity of the antibody in recognizing Lys at this position.

7. Applying the representative combinatorial peptide libraries

From the screening and verification results, a high degree of success could be obtained when using the RCPL to map the epitope of monoclonal antibody PK99H. A total of 11 high affinity epitope sequences were identified. This illustrated the feasibility of using the representative approach to perform epitope mapping. However, after the dipeptide sequences are identified from the three series of RCPL, all combinations of the three sets of dipeptide sequences must be synthesized and tested in order to confirm the results as not all predicted sequences are effective or soluble enough for standard assays.

Another consideration is the reliability of the representative features of the libraries. From Table VI.3, one third of the synthesized "represented" peptides (4 out of 12) showed extremely weak binding (Table VI.3, I_{50} values show > 600-fold decrease) to the antibody. The number of weak binding sequences was insignificant if one considers the net effectiveness of the RCPL approach since the RCPL allowed for the identification of residues that were not found in the native PAK sequence. Therefore, the RCPL was a quite successful approach for the examination of ligand-receptor interactions.

In summary, the RCPL approach can minimize synthesis difficulties and tremendously reduce the number of peptides in the libraries. As shown in Table VI.4, a representative library can reduce the number of peptide pools in a two position defined library by 4-fold (from 400 pools to 100 pools) as compared to the standard peptide library. Furthermore, the relative number of peptides in each pool will be reduced by 16-fold (from 160,000 to 10,000 peptides) in a hexapeptide library; and the difference will be even more if an octapeptide library is used (64-fold, from 64 million to 1 million peptides per pool). This reduction in peptide pools and peptide numbers not only simplifies the synthesis procedure but also increases the screening sensitivity.

Table VI.4 Peptide composition in a peptide library: Comparison of a representative peptide library to a standard peptide library

	Standard Library	**Representative Library**
No. of different amino acids used in the library	20	10
Dipeptide-defined hexapeptide (OOXXXX)	20^2 (400) pools of 20^4 (160,000) peptides/pool	10^2 (100) pools of 10^4 (10,000) peptides/pool
Dipeptide-defined heptapeptide (OOXXXXX)	20^2 (400) pools of 20^5 (3.2 million) peptides/pool	10^2 (100) pools of 10^5 (100,000) peptides/pool
Dipeptide-defined octapepide (OOXXXXXX)	20^2 (400) pools of 20^6 (64 million) peptides/pool	10^2 (100)pools of 10^6 (1 million) peptides/pool

8. Conclusion

In this study, a representative combinatorial peptide library technique that reduced both synthesis and screening efforts was developed. The epitopes on the native PAK pilin sequence were mapped successfully by three series of two-position defined hexapeptide libraries. In addition to the native sequence, other sequences that were not previously recognized were also mapped by the representative approach. These results are very interesting, because PK99H has been considered as a strain-specific antibody that binds preferentially to the PAK pilus strain. However, data from the RCPL showed that antibody-antigen interactions allow certain degrees of flexibility even for an antibody as specific as PK99H. Furthermore, these results illustrated the feasibility of using the representative peptide library to study the binding diversity of an antibody.

Screening conditions, such as ionic strength of the assay medium, were found to be critical parameters for the effective use of the combinatorial peptide libraries. Since high concentrations of peptide mixtures containing Glu can seriously disturb the pH if 10 mM PBS is used, it is suggested that a 50 mM PBS should be used or the peptide libraries should be neutralized with NH_4HCO_3 prior to use.

CHAPTER VII

STRUCTURE-FUNCTION ANALYSIS OF THE ADHERENCE-BINDING DOMAIN ON THE PILIN OF *PSEUDOMONAS AERUGINOSA* STRAIN PAK BINDING TO HUMAN EPITHELIAL CELL RECEPTOR

A. Introduction

Adherence is a critical initial event of pathogenesis (Beachey, 1981). The opportunistic pathogen *Pseudomonas aeruginosa* employs several distinct adhesins to mediate attachment to host mucosal epithelial cells (Prince, 1992; Irvin, 1993), for instance, pili (Woods *et al.*, 1980b; Doig *et al.*, 1988), alginate (Ramphal and Pier, 1985; Doig *et al.*, 1987), exoenzyme S (Baker *et al.*, 1991; Lingwood *et al.*, 1991), and outer membrane proteins (Saiman *et al.*, 1990; Ramphal *et al.*, 1991b). Among these adhesins, the pilus plays unique role in the adherence process and is believed to be responsible for the initial attachment of the microorganism to its host (Pier, 1985; Irvin, 1993). Woods and coworkers (1980b) were the first to suggest the importance of pili to *P. aeruginosa* adherence in the upper respiratory tract. During the past decade, investigations on the role of *P. aeruginosa* pili in interacting with different cell types such as buccal epithelial cells (Doig *et al.*, 1988), tracheal epithelial cells (Ramphal *et al.*, 1984), epidermal cells (Sato and Okinaga, 1987), corneal epithelial cells (Rudner *et al.*, 1992), and A549 pneumocyte cells (Chi *et al.*, 1991), have further illustrated the importance of pilus adhesin in mediating the adherence process.

The *P. aeruginosa* pili are polarly distributed and are composed of a 13-17 kD protein subunit termed pilin (Pasloske *et al.*, 1988b; Paranchych *et al.*, 1990). More than eight pilin genes have been described (Paranchych *et al.*, 1990; Castric and Deal,

1994), of which only a single copy of the pilin structural gene is encoded in the bacterial chromosome. The amino acid sequence of one of the best studied pilin strains, the PAK pilin, has 144 amino acid residues (Sastry *et al.*, 1985a) that contains an adherence binding domain at its C-terminus (Paranchych *et al.*, 1985; Irvin *et al.*, 1989). This region contains a consensus intrachain disulfide bridge formed by Cys^{129} and Cys^{142}. The C-terminal region of the PAK pilin is only exposed at the tip of the pilus where receptor binding is taken place (Lee *et al.*, 1994). Synthetic peptides containing the C-terminal domain competitively inhibit pilus binding to human epithelial cells (Irvin *et al.*, 1989).

As the C-terminal domain of *P. aeruginosa* pilin plays a significant role in infection, it is of interest to establish the structure-activity relationship of the binding domain of the pilus adhesin. Although we begin to understand more about the identity of *P. aeruginosa* adhesins and receptors, very little is known about the structural basis of adhesin-receptor interactions. In this study, a similar approach to epitope mapping was applied to identify amino acid residues that contribute significantly to adhesin function (referred to as the "adhesintope") on the C-terminal region of the PAK pilin to A549 human pneumocyte cells. Recently, McInnes *et al.* (1993) reported the solution structure of the C-terminal 17-residue PAK peptide as determined by 2-dimensional NMR spectroscopy. This provides us with significant structural information and sheds light on how the pilin peptide interacts with its antibody as well as its receptor. In this report, the pilin adhesintope residues in relation to the available NMR structure are discussed.

B. Results

1. Binding of PAK pili and PAK synthetic peptide to A549 cells

The C-terminal region of the PAK pilin has been shown to contain the adherence

binding domain that binds to epithelial cells (Paranchych *et al.*, 1985; Irvin *et al.*, 1989). The adherence of *P. aeruginosa* to epithelial cells can be blocked by either peptides derived from the C-terminal sequence of the PAK pilin or by antibodies raised against this region (Irvin *et al.*, 1989; Doig *et al.*, 1990). In this study, the highly specific interaction between biotin and streptavidin was used as the reporter group to investigate the binding of PAK pili to A549 cells, a cell line which has been shown to contain receptor sites specific for PAK pili (Chi *et al.*, 1991). The biotinylated probes of both PAK pili and the C-terminal 17-residue synthetic peptide of the PAK pilin were made. The ability of these biotinylated probes to bind to A549 cells was determined. In Figure VII.1 (panels A and B), both biotinylated PAK pili and peptide bound to A549 cells in a concentration-dependent manner, whereas a control biotinylated peptide, a 12-residue peptide derived from the skeletal actin sequence, showed no binding to A549 cells even at high concentration (Fig. VII.1, panel B). The signal obtained with the biotinylated PAK pili was considerably higher than that of the biotinylated peptide, which suggested that the PAK pilus might have been biotinylated at more than one site, thus allowing for an amplification of the signal.

Both biotinylated PAK pili and biotinylated PAK peptide binding to A549 cells was inhibited by the oxidized C-terminal PAK peptide, AcPAK(128-144)OH (Fig. VII.2). These results illustrated that both biotinylated PAK pili and biotinylated PAK peptide bind to A549 cells specifically. A substrate amplification system (see Chapter II) was used to enhance the signal of the biotinylated PAK peptide in the competitive binding assay as the biotinylated peptide gave a weaker signal than the biotinylated PAK pili.

2. Adhesintope mapping of *P. aeruginosa* PAK pilin

The residues that contributed to receptor binding of the PAK pilus (the adhesintope) were determined in a competitive binding assay employing a series of single alanine substituted peptide analogs corresponding to the C-terminal 17-residue region of PAK

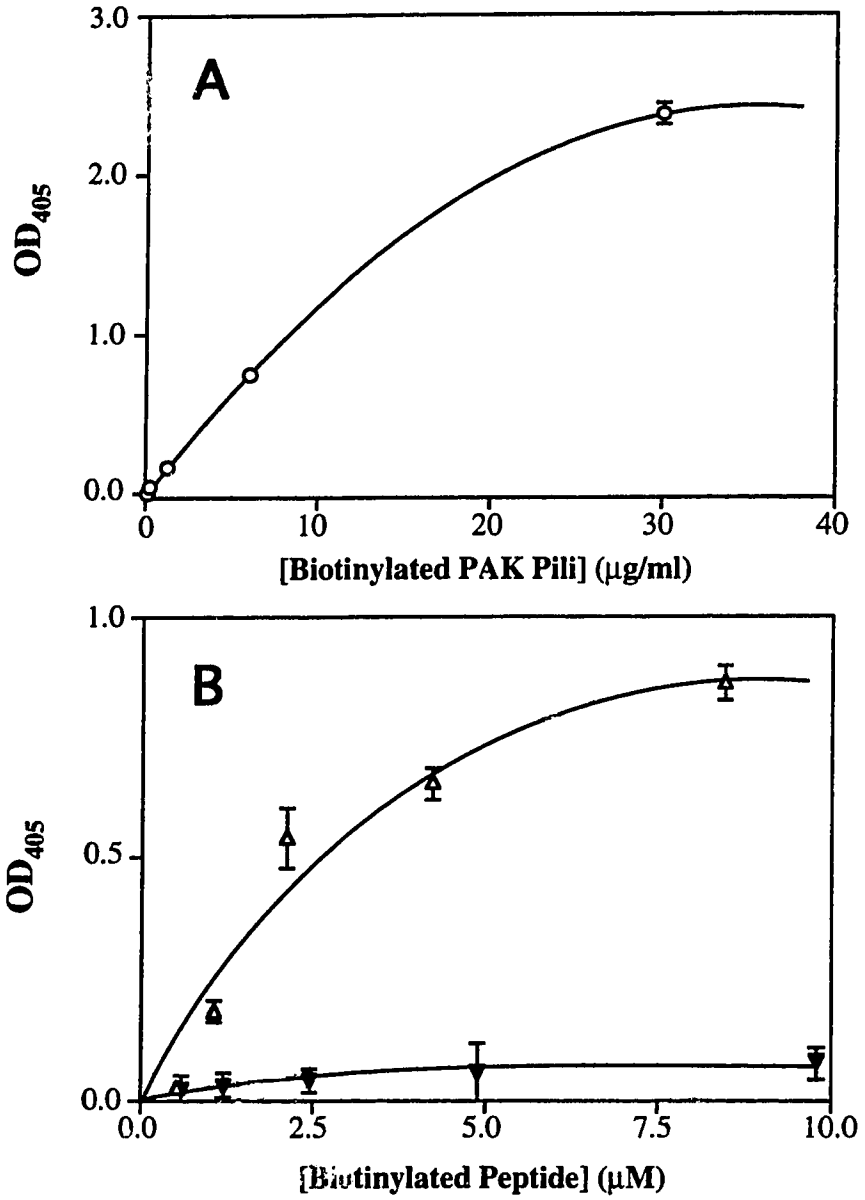


Figure VII.1 Direct binding of biotinylated PAK pili (O, panel A) and biotinylated PAK(128-144)OH peptide (Δ, panel B) to A549 cells. A biotinylated synthetic peptide corresponding to sequence 18-28 of the actin protein (▼, panel B) was used as a negative control. The amino acid sequences of the peptides can be found in Chapter II.

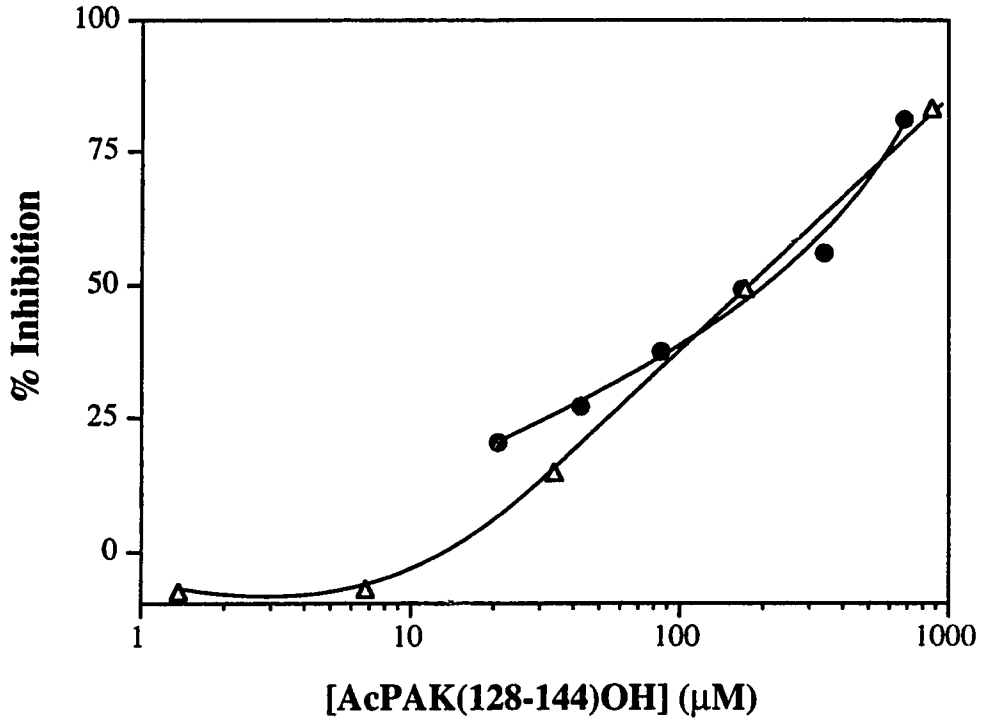


Figure VII.2 Competitive binding profiles showing the inhibitory effects of the oxidized synthetic peptide AcPAK(128-144)OH on the binding of both biotinylated PAK pili (Δ) and biotinylated PAK(128-144)OH peptide (●) to A549 cells.

pilin. Each residue (except the two cysteines) was substituted one at a time with an alanine along the 17-residue peptide sequence (see Table VII.1). The ability of these peptide analogs to inhibit the binding of the biotinylated PAK pili to A549 cells was then determined. If the peptide analog showed a significant decrease in its ability to inhibit the binding of biotinylated PAK pili to A549 cells, the side-chain substituted was considered to be important for receptor interaction and part of the adhesintope.

The change in the inhibitory effects of the alanine-substituted peptides were reflected by corresponding shifts of the binding curves. The relative importance of individual residues along the PAK 128-144 region that contributed to A549 cell binding was shown by their respective I_{50} values as deduced from the competitive binding curves. The I_{50} value was the concentration of peptide required to reduce the binding of biotinylated PAK pili to A549 cells by 50%. A summary of the I_{50} values of all oxidized PAK 17-residue peptide analogs is shown in Table VII.1. A comparison of the I_{50} values with respect to the native peptide revealed that six peptide analogs had I_{50} values greater than three fold of that of the native peptide (I_{50}=170 μM). The I_{50} values of these six PAK peptide analogs were: (S131A), > 750 μM; (Q136A), > 940 μM; (I138A), > 1,200 μM; (P139A), > 1,300 μM; (G141A), > 1,300 μM; and (K144A), > 980 μM (Table VII.1).

3. Importance of the intrachain disulfide bridge

The two cysteine residues (residue 129 and 142, respectively) located at the C terminal region of the *P. aeruginosa* pilin are highly conserved among different strains (Paranchych *et al.*, 1990). Previously, the significance of the intrachain disulfide bridge has been addressed by comparing the direct binding of the oxidized and reduced peptides to buccal epithelial cells (Irvin *et al.*, 1989). In this study, the importance of the disulfide bridge was further confirmed by synthesizing a PAK peptide analog with both cysteine residues substituted by alanine, and its ability to compete the binding of

Table VII.1 Identification of the A549 cell adherence binding domain on the PAK pilin by single alanine replacement analysis

PAK Peptide analogs[a]	I_{50} (μM)[b]
Native	170 ± 13
K128A	200 ± 56
T130A	150 ± 7
S131A	> 750
D132A	160 ± 89
Q133A	220 ± 30
D134A	90 ± 27
E135A	350 ± 32
Q136A	> 940
F137A	49 ± 21
I138A	> 1,200
P139A	> 1,300
K140A	140 ± 80
G141A	> 1,300
S143A	100 ± 62
K144A	> 980

[a]The peptide analogs are labeled according to the residue being substituted (first letter) by alanine (last letter) and the sequence position (middle number). "Native" denotes the native PAK 17-residue oxidized peptide AcPAK(128-144)OH (see Chapter II). The shaded regions denote important residues for A549 cell binding, i.e. the adhesintope.

[b]Values represent the mean concentration of the peptide analogs required to inhibit biotinylated PAK pili binding to A549 cells by 50% ± standard deviation. Data are means of two triplicate assays.

biotinylated PAK pili was examined. A much higher concentration of this peptide analog (> 990 μM; Table VII.2) was required to inhibit the binding of biotinylated PAK pili to A549 cells as compared with the native peptide (170 μM). Thus, the disulfide bridge contributes significantly to the adhesin function of the PAK peptide confirming our previous observations (Irvin *et al.*, 1989).

4. Inhibition of A549 cell binding by strain KB7

The C-terminal region of *P. aeruginosa* strain KB7 shows some homology with that from strain PAK (Table V. ?) Six consensus residues are found in both peptide sequences; two of these residues are the highly conserved cysteine residues. Interestingly, of these six homologous residues, four were found in the adhesintope sequence as deduced from the PAK peptide (Pro^{139}, Gly^{141}, Cys^{129} and Cys^{144}). However, the remaining four residues found in the PAK adhesintope sequence (Ser^{131}, Gln^{136}, Ile^{138}, and Lys^{144}) were mutated to Thr^{131}, Lys^{136}, Arg^{138}, and Asp^{144}, respectively in the KB7 sequence (Fig. VII.3). Despite the heterogeneity in its amino acid sequence, the KB7 peptide was shown to be as effective as peptide in the inhibition of biotinylated PAK pili binding to A549 cells (I_{50}=1. . for KB7 peptide nd 170 μM for PAK peptide). However, KB7 peptide analogs with single-alanine substitution at any of the four heterologous adhesintope residues showed a tremendous decrease in binding to A549 cells. The I_{50} values of these four KB7 peptide analogs were: (T131A), > 700 μM; (K136A), > 820 μM; (R138A), > 840 μM; and (D144A), > 910 μM (Table VII.2). These results suggest that these four residues also contribute to the adhesintope of the KB7 pilin peptide. A comparison of the adhesintopes between the PAK and KB7 strains is shown in Figure VII.3.

Table VII.2 Comparison of the binding affinity of synthetic peptide analogs from *P. aeruginosa* strain PAK and KB7

Peptide[a]	Sequence[b]	I_{50} (μM)[c]
	128 144	
PAK native	KCTSDQDEQFIPKGCSK Δ Δ Δ ΔΔ ΔΔ Δ	170
KB7 native	SCATTVDAKFRPNGCTD	120
KB7 (T131A)	SCA**A**TVDAKFRPNGCTD	> 700
KB7 (V133A)	SCATT**A**DAKFRPNGCTD	830
KB7 (K136A)	SCATTVDA**A**FRPNGCTD	> 820
KB7 (F137A)	SCATTVDAK**A**RPNGCTD	77
KB7 (R138A)	SCATTVDAKF**A**PNGCTD	> 840
KB7 (D144A)	SCATTVDAKFRPNGCT**A**	> 910
PAK (I138R)	KCTSDQDEQF**R**PKGCSK	> 720
PAK (C129A, C142A)	K**A**TSDQDEQFIPKG**A**SK	> 99(

[a]PAK native and KB7 native are the synthetic peptides corresponding to the native C-terminal 17-residue sequence of the *P. aeruginosa* PAK and KB7 pilin proteins. See legend of Table VII.1 for peptide nomenclature.

[b]The sequence of the two native peptides are taken from Paranchych *et al.* (1990). The triangle (Δ) indicates residues in the PAK adhesintope as determined from Table VII.1 and the disulfide bridge replacement analog, PAK (C129A, C142A), in this table; the bold letters denote substituted amino acid residues; the consensus residues between the two strains are underlined.

[c]Values indicate the mean concentration of the peptides required to inhibit the binding of biotinylated PAK pili to A549 cells by 50%. Data are means of two triplicate assays.

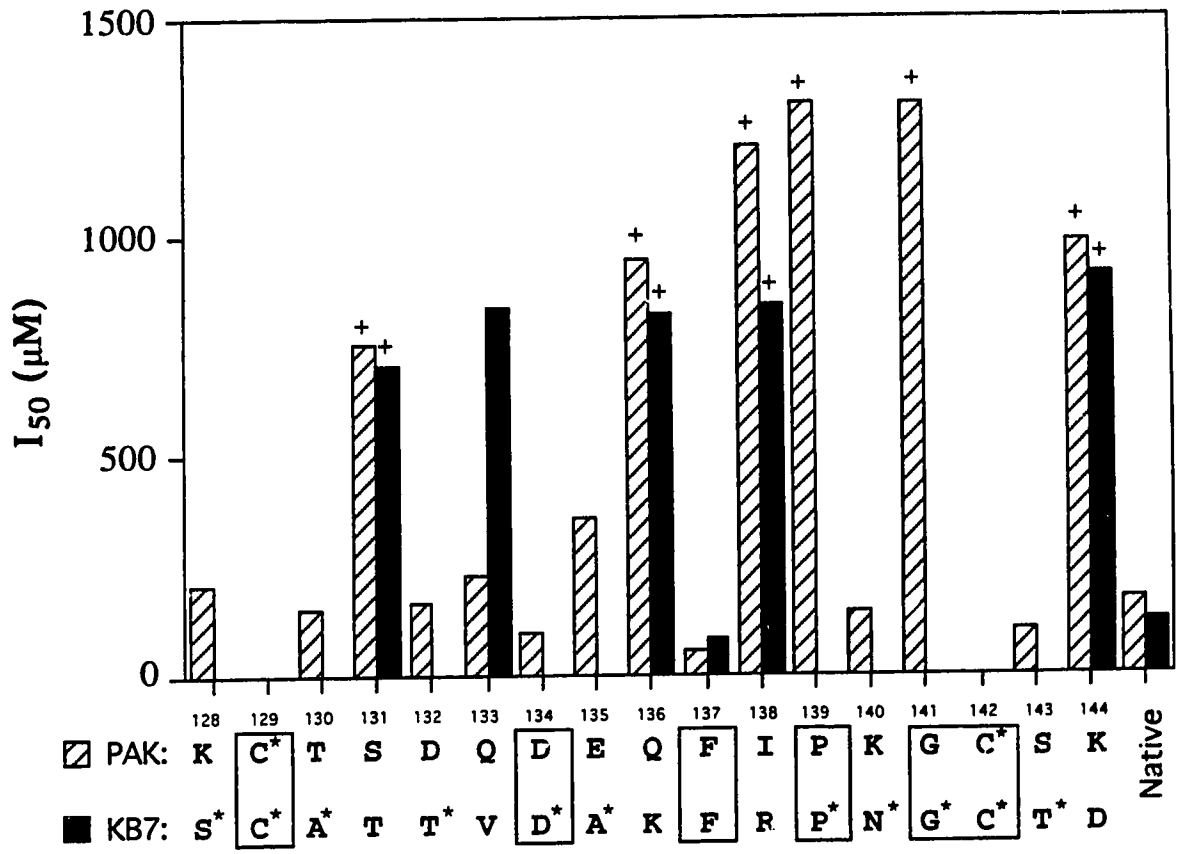


Figure VII.3 Diagram showing a summary of the results from Tables VII.1 and VII.2. The (+) symbol denotes value greater than what is indicated, whereas the (*) symbol means data not available. The consensus residues in both sequences are boxed.

5. Effects of hydrophobic side-chains on adhesin-receptor interactions

An unexpected observation obtained from the adhesintope mapping result was the non-essential phenylalanine residue at position 137. This hydrophobic residue is highly conserved among different *P. aeruginosa* strains (Paranchych *et al.*, 1990), and was found to be very critical for antibody binding (Chapter III). However, our data from whole cell binding studies showed that Phe^{137} was not involved in pilus-cell receptor interactions since substitution of Phe^{137} by an alanine did not result in any decrease in the binding affinity (Table VII.1). Another hydrophobic residue, Ile^{138}, contributed to the interactions between the pilin adhesintope and the cell-surface receptors. However, this residue was mutated to a positively charged residue, arginine, in the KB7 sequence. Thus, it became important to determine if Arg^{138} contributed to the adhesintope in KB7 or if the conserved Phe^{137} adjacent to Ile^{138} in PAK sequence provided the hydrophobic interactions in the KB7 adhesintope. That is, the loss of the Ile side-chain could be compensated for by a shift of the hydrophobic interaction to Phe^{137} in the KB7 peptide. To prove this, three peptide analogs, Ac(F137A)KB7(128-144)OH, Ac(R138A)KB7(128-144)OH and Ac(I138R)PAK-(128-144)OH were made and their binding affinities were examined (Table VII.2). It was striking that Ac(F137A)KB7(128-144)OH bound as well as the native KB7 peptide to A549 cells (I_{50} values of 77 μM and 120 μM, respectively). Moreover, the KB7 17-residue sequence contained another hydrophobic residue (valine) at position 133. Removal of this hydrophobic side-chain by alanine replacement caused a significant decrease in binding affinity (I_{50} = 830 μM for Ac(V133A)KB7(128-144)OH, Table VII.2) to A549 cells. It seems that Val^{133}, instead of Phe^{137}, may have contributed to the hydrophobic interaction between the KB7 peptide and the cell receptors. In fact, NMR data suggested that the side-chains of Val^{133} and Phe^{137} are in close proximity, and the hydrophobic interaction between these two side-chains may be important to the conformation of the KB7 peptide (McInnes *et al.*, in preparation).

In addition, the Arg^{138} in the KB7 peptide was found to be important for receptor binding since substitution of this residue by an Ala greatly reduced binding to A549 cells (I_{50} of Ac(R138A)KB7(128-144)OH was > 840 μM). Further, the Arg^{138} was unique to the KB7 sequence and substitution of Arg in the PAK sequence, Ac(I138R)PAK(128-144)OH, decreased binding affinity to A549 cells (I_{50} > 720 μM, Table VII.2).

C. Discussion

During the past decade, extensive research has been undertaken to study pilus-mediated adherence of *P. aeruginosa* to human respiratory epithelial cells. Different receptor candidates have been reported for the pilus-specific interactions. For instance, glycosphingolipids such as asialo-GM_1 and asialo-GM_2, sialic acid containing glycosphingolipids and lactosylceramide (Krivan *et al.*, 1988a; Baker *et al.*, 1990; Lee *et al.*, 1994; Sheth *et al.*, 1994), and glycoproteins (Doig *et al.*, 1989; Irvin *et al.*, 1989). The carbohydrate moieties of both glycoproteins and glycosphingolipids are believed to be involved in pilus binding. For example, pilus-specific disaccharides such as ßGalNAc(1-4)ßGal on asialo-GM_1 and asialo-GM_2 (Krivan *et al.*, 1988a; Sheth *et al.*, 1994); ßGal(1-3)ßGlcNAc and ßGal(1-4)ßGlcNAc (Ramphal *et al.*, 1991a) have been reported. Recently, the C-terminal region of the PAK pilin has been shown to contain the adherence binding domain, which is located at the tip of the PAK pilin, and that receptor binding is a tip-associated event (Lee *et al.*, 1994). Furthermore, the NMR solution structure of the C-terminal peptide of the PAK pilin is also available (McInnes *et al.*, 1993; in press).

1. Contribution of individual residues to the adhesintope

Six side-chains and the disulfide bridge (Cys^{129} and Cys^{142}) located in the C-

terminal region of the PAK pilin were found to be critical for mediating binding to A549 cells, and were considered to contribute to the adhesintope responsible for receptor interactions. These six side-chains were Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141} and Lys^{144}, which are distributed throughout the C-terminal region of PAK pilin. Five of these residues are located inside the disulfide loop. This suggests that a conformational determinant was required for receptor interactions. Interestingly, of these six residues, three of them (Gln^{136}, Ile^{138}, Pro^{139}) are found in the epitope sequence (DEQFIPK) of monoclonal antibody PK99H, which recognizes specifically AcPAK(128-144)OH (Chapter III). This suggests that PK99H blocks AcPAK(128-144)OH binding to the epithelial cells by interacting with part of the adhesintope sequence recognized by the cell receptors. Because of the diversity of these residues, different types of interactions could be involved, including hydrogen bonds (Ser^{131}, Gln^{136}), hydrophobic interactions (Ile^{138}, Pro^{139}), ionic interactions (Lys^{144}), and residues involved in conformational control (Pro^{139}, Gly^{141} and the two cysteines involved in the disulfide bridge, Cys^{129} and Cys^{142}). In the case of the KB7 pilin peptide, at least five residues were found to be important for the KB7 adhesintope, namely, Thr^{131}, Val^{133}, Lys^{136}, Arg^{138}, and Asp^{144}. These residues can also be involved in various interactions such as hydrogen bonds (Thr^{131}), hydrophobic interaction (Val^{133}), and ionic interactions (Lys^{136}, Arg^{138}, and Asp^{144}). The amino acid residues involved in A549 receptor binding are summarized in Figure VII.4.

Besides Thr^{131} in the KB7 sequence, the side-chain of Arg^{138} may also contribute to hydrogen bonding to the cell receptor. The importance of the arginine side-chain as a specific hydrogen bond donor has been observed in the crystal structure of a zinc finger-DNA complex (Pavletich and Pabo, 1991). This may compensate for the substitution of Gln^{136} to a lysine in the KB7 sequence (Table VII.2). In addition, hydrophobic interactions were required in the pilin-A549 cell receptor recognition. Surprisingly, Val^{133} rather than Phe^{137} in the KB7 peptide apparently acted as the

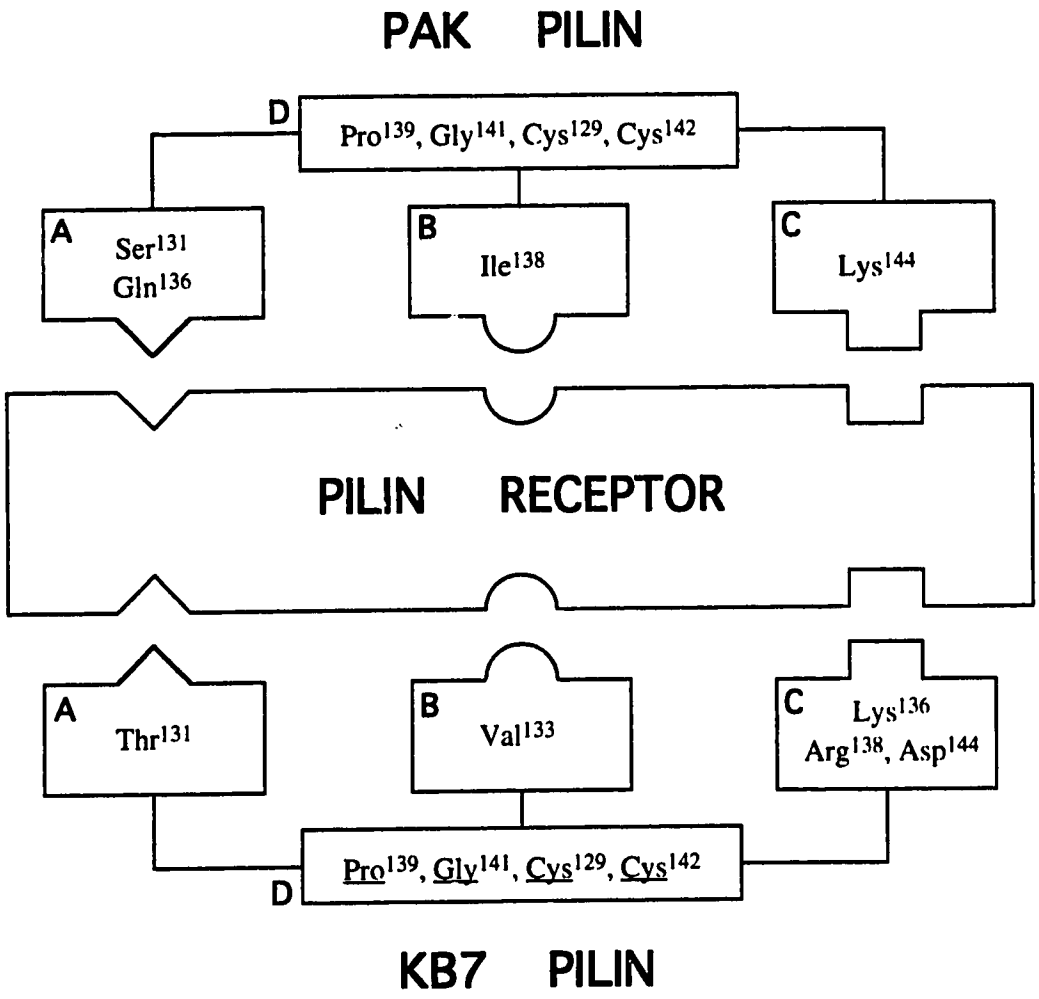


Figure VII.4 Schematic diagram showing residues that are important for the binding of both PAK and KB7 C-terminal 17-residue pilin peptides to A549 cell surface receptors. Amino acid residues involved in receptor interactions are grouped, for example, (A) polar; (B) hydrophobic; (C) ionic; and (D) conformational control residues. The proposed adhesintope residues in the KB7 pilin are underlined.

major contributor to the hydrophobic interaction that was apparently contributed by Ile^{138} in the PAK adhesintope.

2. Structure-function relationship: *cis/trans* conformation

Recently, the solution structure of the *trans* isomer of the oxidized PAK C-terminal 17-residue pilin peptide has been deduced by 2-D ^1H NMR spectroscopy (McInnes *et al.*, 1993; in press). The PAK peptide appears to have two isomers, the *cis* and *trans* configurations, due to the isomerization of the Ile^{138}-Pro^{139} amide bond (McInnes *et al.*, in press). In the oxidized form, the *trans* isomer is dominant over the *cis* by a ratio of 3:1 at 5°C. In the reduced form, the *trans/cis* ratio is 14:1. Therefore, formation of the intramolecular disulfide bridge between Cys^{129} and Cys^{144} shifts the equilibrium in solution towards the *cis* isomer. It has been proposed that the *trans* conformation is more favorable for antibody interaction (McInnes *et al.*, in press) since the reduced PAK peptide and truncated peptide without the two cysteine residues bind with equal affinity as the oxidized peptide to monoclonal antibody PK99H (Chapter III). In contrast, the *cis* isomer seems to be important to receptor binding, as the reduced peptide has a lower binding affinity as compared with the oxidized peptide (Irvin *et al.*, 1989). In Table VII.2, it was evident that a PAK peptide analog with both cysteine residues substituted showed a large decrease in binding affinity to A549 cells. In addition, substitution of Pro^{139} by an Ala in the PAK peptide has been shown to cause a loss of the *cis* configuration (McInnes *et al.*, in press), and this effect can be seen with Ac(P139A)PAK(128-144)OH that it lost its binding affinity to A549 cells (Table VII.1). Preliminary NMR studies on the binding of oxidized AcPAK(128-144)OH peptide to the pilin receptor candidate ßGalNAc(1-4)ßGal-BSA conjugate have shown that the ratio of the *cis* isomer of the PAK peptide increased upon titration with the receptor conjugate (Campbell *et al.*, unpublished observations). These results agreed well with the hypothesis that the *cis* conformation was important for receptor

interaction.

From the NMR studies, five residues show a large change in the amide proton chemical shift between the *trans* and *cis* conformations. These were Thr^{130}, Gln^{136}, Phe^{137}, Ile^{138} and Gly^{141} (Table VII.3). Three of them, Gln^{136}, Ile^{138} and Gly^{141}, also constituted part of the PAK adhesintope sequence (Tables VII.1 and VII.3). The large chemical shift difference observed for Thr^{130} between the *cis* and *trans* isomers is mainly due to the hydrogen bond contributed by Ser^{131} in the *cis* isomer, which is not present in the *trans* isomer. Thr^{130} did not appear to be involved in binding since substitution of this residue by an Ala did not change the binding affinity of the peptide (Table VII.1). Similarly, the change in chemical shift found in Gln^{136} is also due to the putative hydrogen bond that occurs in the *cis* conformation, but not in the *trans*. Since the PAK peptide is assumed to bind as a *cis* isomer, one could suggest that the hydrogen-bonding properties of Ser^{131} and Gln^{136} were important in the binding process. Therefore, this may explain why these two residues were important to the adhesintope.

Phe^{137} is an interesting residue. It was a very critical residue for antibody-antigen interaction but was non-essential for receptor binding. The loss of the hydrogen bond to Phe^{137} in the *cis* isomer (Table VII.3) indicated that there is a change in the local environment of the Phe side-chain in the *cis* conformation. Indeed, preliminary NMR studies on KB7 peptide have shown that the orientation of the side-chain of Phe^{137} in KB7 peptide is different from that of the PAK peptide. Since the Phe side-chain was not involved in binding, the change in conformation and hydrogen bonding would not affect the binding of the peptide analog to A549 cells.

The Ile^{138} is also a noteworthy residue, the difference in the amide proton chemical shift of Ile^{138} between the *cis* and *trans* isomers is mainly due to the proximity of this residue to Pro^{139}, and thus Ile^{138} is affected by the isomerization at the amide bond of Ile^{138}-Pro^{139}. Hydrophobic interaction is the major contribution of this side-chain.

Table VII.3 A summary of the change in chemical shift and hydrogen-bonding properties of the amino acid residues in the *cis* and *trans* isomers of AcPAK(128-144)OH

		131						136		138	139		141			144
PAK sequence		K	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	S	K
Change in chemical shift ($\Delta\delta$)[a]		·	·	Δ	·	·	·	·	·	Δ	Δ	Δ	·	·	Δ	·	·	·
H-bond protection from exchange with solvent[b]:	*trans*	·	·	·	·	·	·	·	·	·	p	·	·	·	·	p	·	·
	cis	·	·	·	p	·	·	·	p	p	·	·	·	·	·	p	·	·
PAK adhesintope[c]		·	·	·	*	·	·	·	·	*	·	*	*	·	*	·	·	*

[a]Residues for which $|\Delta\delta| > 0.2$ ppm as compared between the *cis* and *trans* isomers of AcPAK(128-144)OH at 5.0°C (McInnes *et al.*, in press). Residues with significant changes are denoted by 'Δ'.

[b]As deduced from the temperature coefficient ($-\Delta\delta/\Delta T$ ppb/°K) measurements of the amide proton (McInnes *et al.*, in press). Where $-\Delta\delta/\Delta T < 5$ ppb/°K indicates protected amide (Dyson *et al.*, 1985). Residues with possible hydrogen-bonding properties are denoted by 'p'.

[c]Residues involved in A549 cell binding (Table VII.1) are denoted by '*'.

3. Structure-function relationship: secondary structure

As the solution structure of the *trans* isomer of oxidized AcPAK(128-144)OH was generated from the NMR data, two different types of ß-turns were observed (McInnes *et al.*, 1993). A type I ß-turn was identified from residues 134 to 137 corresponding to the sequence DEQF, whereas a type II ß-turn was found from residues 139 to 142 with a sequence PKGC. These two ß-turns thus form a structurally more rigid region in the flexible peptide. Interestingly, the epitope sequence for PK99H and four residues of the PAK adhesintope are located in this rigid region.

The C-terminal region of KB7 pilin is only semi-conserved as compared to the PAK sequence; and along the 17-residue region, only six residues (including the two cysteines) are conserved. In spite of this, the secondary structure of the KB7 peptide is quite similar to that of PAK as revealed by preliminary NMR studies McInnes *et al.* (unpublished results). It was found that the KB7 peptide, like the PAK peptide, has two ß-turn structures in sequences 134 to 137 and 139 to 142, respectively. However, when the two ß-turns were aligned to those of the PAK peptide, the side-chain of Phe^{137} in KB7 peptide was differently orientated (McInnes *et al.*, unpublished data). The dissimilarity of the Phe^{137} in the solution structure of these two pilin strains is in close agreement with our result that Phe^{137} was not involved in binding in either of the peptides to A549 cells (Tables VII.1 and VII.2).

The importance of the secondary structure involved in A549 cells binding was examined. As mentioned before, four out of the six amino acid residues found in the PAK adhesintope sequence are located in the a structurally more rigid region of the flexible peptide. Two of these residues, Pro^{139} and Gly^{141}, are located in the i and i+2 position of the type II ß-turn. Gly^{141} at position i+2 is a very important residue in constructing the type II ß-turn, since substitution of this residue by an Ala will disrupt the formation of a type II ß-turn. Similarly, substitution of Pro^{139} by an Ala at position i will also lower the chance of a type II ß-turn formation (Wilmot and Thornton, 1988).

Therefore, it is possible that the type II ß-turn is an important structure motif required for receptor binding, and that both Gly^{141} and Pro^{139} are important for A549 cell binding because these two residues are required to maintain the secondary structure of a type II ß-turn.

In contrast, the type I ß-turn may not be as important as the type II ß-turn for A549 cell binding. There was only one PAK adhesintope residue found in the type I ß-turn region. Gln^{136} was found at the i+2 position of the type I ß-turn. However, the type I ß-turn can tolerate a substitution to an Ala at this position according to the prediction parameters developed by Wilmot and Thornton (1988). It is plausible that the substitution of Gln^{136} disrupted the hydrogen-bonding property of this residue rather than directly affecting the secondary structure of the peptide, since the amide proton of Gln^{136} showed a lowered temperature coefficient (McInnes *et al.*, 1993).

4. Compensation mutation of the KB7 adhesintope sequence

In summary, the interactions between the C-terminal peptide of the PAK pilin and the A549 cells required a conformational adhesintope, which contained the amino acid residues Ser^{131}, Gln^{136}, Ile^{138}, Pro^{139}, Gly^{141}, Lys^{144} and the disulfide bridge (Fig. VII.4). Two structural requirements, such as a *cis* conformation and the presence of a type II ß-turn, are proposed. These two structural requirements require the presence of an intrachain disulfide bridge between Cys^{129} and Cys^{142}, and the residues Pro^{139} and Gly^{141}. Other residues, such as Ser^{131} and Gln^{136}, contribute to hydrogen bond formation, whereas Ile^{138} involves hydrophobic interaction and Lys^{144} provides a positive charge for receptor interaction. Similarly, at least five residues were found important for the KB7 pilin peptide to bind to A549 cells, these residues are Thr^{131}, Val^{133}, Lys^{136}, Arg^{138}, and Asp^{144} (Fig. VII.3). As these residues were compared with those of the PAK adhesintope, it seems likely that a "compensation mutation" could occur in the KB7 sequence, in which different mutations work together to

maintain the functionality of the adherence binding domain. Multiple mutations such as Ser^{131}→Thr, Gln^{133}→Val, Gln^{136}→Lys, Ile^{138}→Arg, and Lys^{144}-→Asp are required in order for the KB7 pilin to maintain the side-chain or structural requirements for receptor interactions; while a single substitution is not permissible as in the case of Ac(I138R)PAK(128-144)OH (Table VII.2). These observations have very significant implications for the studies of bacterial virulence as well as their immune intervention.

It is of interest to note that sequence homology is not necessarily a prediction of the adhesintope. Aside from the Pro^{139} and Gly^{141} residues, Phe^{137} is also a conserved residue found in the *P. aeruginosa* pilin sequences (Paranchych *et al.* 1990); however, Phe^{137} is not involved in receptor interaction. Furthermore, the epitope deduced for specific anti-pilus antibody was also apparently not an adhesintope mimic. When the PK99H epitope was compared with the PAK adhesintope, only two common residues, Ile^{138} and Pro^{139}, were found to be critical in both cases.

In conclusion, the amino acid residues required for both PAK and KB7 pilins binding to A549 cell were elucidated. This information sheds light on how the pilus adhesin comes to interact with the epithelial cell receptors, and thus enables the proposal of a possible structure-function relationship between the *P. aeruginosa* pilus and its receptors. In the long run, this knowledge could assist us in designing therapeutics and cross-protective vaccines against *P. aeruginosa* infections.

CHAPTER VIII

GENERAL DISCUSSION AND PROSPECTS

A. General Discussion

R.B. Fick, Jr. made an incisive introduction on the opportunistic nature of *P. aeruginosa* in a recent book he edited: "Despite the possession of a potent armamentarium of injurious exoproducts, *P. aeruginosa* remains an opportunist. It, as with the wolf-like carnivore, is a pathogen characterized by hemorrhage and necrosis, yet like the cowardly hog-like part of the hyena, *Pseudomonas* frequently colonizes the human host, waiting for an opportunity to spring." (Fick, 1993a). As it was discussed in Chapter I, *P. aeruginosa* is equipped with an extensive range of virulence factors, from the highly toxic exotoxin A to the pilus adhesin. It is thus not difficult to appreciate the pathogenic potential of this microorganism although it is an opportunistic pathogen. *P. aeruginosa* infections are most likely to occur in specific groups of high-risk patients. The selective targeting of specific high-risk patients by *P. aeruginosa* implies mechanisms that prevent disease. Immunological methods would appear to be very effective at limiting infections as this microorganism is seldom isolated from healthy individuals with a sound defense mechanism.

One possible preventive approach is to interfere with the initial pathogenic process. It is now generally accepted that the pilus-mediated adherence forms an initial step for *P. aeruginosa* infection. The role of the *P. aeruginosa* pili as an adhesin has been studied extensively in this laboratory (Paranchych *et al.*, 1985; 1986; Doig *et al.*, 1988; Irvin *et al.*, 1989; 1990; Lee *et al.*, 1994; Sheth *et al.*, 1994) and others (Woods *et al.*, 1980b; Reichert *et al.*, 1983; Ramphal *et al.*, 1984; Sato and Okinaga, 1987; Sato *et al.*, 1988; Saiman *et al.*, 1990; Chi *et al.*, 1991; Rudner *et al.*, 1992). The adherence

binding domain was found to be located at the C-terminal 17-residue region of the pilin protein (Paranchych *et al.*, 1986; Irvin *et al.*, 1989) which is exposed at the tip of the pilus (Lee *et al.*, 1994). Two important properties have been identified for this peptide region, namely, the antigenic and receptor binding properties. These two properties are indeed the major focus of this thesis. A synthetic peptide approach was employed to elucidate the possible epitope sequences recognized by the *P. aeruginosa* pilus-specific antibodies and the adhesintope sequence required for receptor binding, in the hope that information obtained from these studies would be useful in the design and development of vaccines and therapeutics to prevent *P. aeruginosa* infections.

1. The host-pathogen relationship

The host immune system evolved to recognize a broad range of antigens, especially those repetitively encountered. In response to this, the pathogen usually undergoes mutations in order to escape from the surveillance of the immune system. However, at the same time the pathogen has to maintain the biological functions of the mutated structures. In the case of *P. aeruginosa*, it is obvious that antigenic variation occurs in order to diversify the antigenicity of the pilin proteins. Various pilin genes have been identified and each *P. aeruginosa* chromosome carries only a single copy of these genes (Paranchych *et al.*, 1990). In *P. aeruginosa*, most of the antigenic regions are located at the variable central regions and the semi-conserved C-terminal regions of the pilin proteins (Watts *et al.*, 1983b; Lee *et al.*, 1989a; 1990b). Indeed, the adherence binding domain on the PAK pilin is an important antigenic site (Watts *et al.*, 1983b; 1989a; Lee *et al.*, 1989b; 1990b; Doig *et al.*, 1990). From these observations, it is easy to understand that variation of the antigenic sites in the pilin proteins is important to the survival of *P. aeruginosa* since the pilin proteins must expose their adherence binding domain in order to interact with the receptor sites on the host surface but at the same time this region becomes an important antigenic target for the host immune response.

To deal with this dilemma, *P. aeruginosa* has had to develop a set of tactics to balance out the effects of antigenicity and adherence capability. These tactics are illustrated from the results of the present studies.

The receptor binding study in Chapter VII showed that the KB7 pilin peptide binds as well as the PAK peptide to A549 cells despite their sequences were only semi-conserved (about one third of the residues from the two sequences were homologous). This clearly showed that mutation of the *P. aeruginosa* sequence from strain PAK to strain KB7 did not affect their adherence properties. In fact, NMR studies showed that these two peptides have a similar backbone structure (McInnes *et al.*, in preparation). These results suggest that *P. aeruginosa* makes use of antigenic variation to alter its pilin sequence so as to diversify its antigenicity and avoid being monitored by the immune system; however, the alterations in the pilin sequence have been subject to selective pressure in order to maintain both structural and functional properties of the pilin for adherence purposes. A similar finding has been observed when the binding of PAO peptide and the PAK peptide to receptor candidates such as asialo-GM_1 (Lee *et al.*, 1994) and ßGalNAc(1-4)ßGal-BSA conjugate (Sheth *et al.*, 1994) was compared. In both cases, the PAO strain bound to the receptor with a higher affinity than that of the PAK strain despite their sequence variation. It seems that the way by which *P. aeruginosa* chooses to alter its pilin sequence is a favorable event. As a result, the microorganism is more likely to escape from the surveillance of the immune system without any sacrifice in its adherence properties.

On the other hand, the host immune system has its method of dealing with the mutational changes of the *P. aeruginosa* pilins. Antibody cross-reactivity was observed for some anti-*Pseudomonas* pilus antibodies (Lee *et al.*, 1989b; 1990b; Saiman *et al.*, 1989; Doig *et al.*, 1990). In general, an immune response against the semi-conserved receptor-binding domain on the C-terminal region of *P. aeruginosa* pilin will generate strain-specific antibodies. As it was shown previously, even a

highly strain-specific monoclonal antibody like PK99H allowed a certain degree of binding diversity with a number of peptide analogs of closely related peptide sequences (Chapter VI). Furthermore, different anti-peptide antisera were produced and most of them could cross-react with both PAK and PAO pilus strains (Chapter IV and V). The immunological studies reported here showed that certain substitutions such as $Glu^{135}\rightarrow Ala$ and $Ile^{138}\rightarrow Ala$ in the PAK pilin sequence favored the production of cross-reactive antibodies, whereas other substitutions such as $Gln^{136}\rightarrow Ala$ and $Phe^{137}\rightarrow Ala$ caused the immune system to produce peptide-specific antibodies that did not recognize the native protein. From these observations, an interesting scenario between *P. aeruginosa* and its host can be depicted: The pathogen tries to escape from the host immune system by altering its antigenic sites on the pilin proteins with the compromise that both structure and side-chain requirements for adhesion are maintained. However, due to the diversity and flexibility of the immune response, mutational events in the pilin proteins are subject to extensive selective pressure.

2. Sequence homology is not necessarily an indication of receptor binding

Sequence comparison is generally used to pinpoint biologically important residues. In the case of *P. aeruginosa*, comparison of the sequences of the adherence binding domains from eight different pilin strains (Fig. I.1) identified 3 conserved and 7 semi-conserved residues. The conserved residues are Cys^{129}, Pro^{139} and Cys^{142}, whereas the semi-conserved residues are Ser^{131}, Thr^{132}, Asp^{134}, Phe^{137}, Lys^{140}, Gly^{141}, and Lys^{144} (the positions of the residues are relative to the PAK sequence). Therefore, by conventional sequence homology comparison methods, the predicted adhesintope should be constructed from the above residues. However, the adhesintope mapping results (Chapter VII) showed that only six of the above residues were found in the PAK adhesintope, that is, Cys^{129}, Pro^{139}, Cys^{142}, Ser^{131}, Gly^{141}, and Lys^{144}. Interestingly, Asp^{134}, Phe^{137} and Lys^{140} were found unimportant for receptor

interactions. The residue Phe[137] has been shown to be a very critical residue for antibody binding but was found to be unimportant in receptor binding. In addition, two other residues (Gln[136] and Ile[138]) present in the PAK adhesintope were not predicted by the sequence comparison. These two positions were found important for both PAK and KB7 pilin peptide binding to the A549 cell receptors. Therefore, these results suggest that the presence of conserved residues in the pilin protein sequence is not necessarily an indicator of their presence in the adhesintope.

3. The antibody combining site is not a good receptor mimic

The antibody epitope on the PAK pilin was found to be a linear sequence that consisted of residues 134-140 for the mouse anti-pilus monoclonal antibody PK99H or residues 132-139 for the rabbit anti-peptide antibodies. In contrast, the adhesintope sequence contained residues that were distributed throughout the 17-residue peptide sequence 128-144. Some distinct differences between the epitope and the adhesintope could be observed. For example, Phe[137] was a very critical residue for binding in all of the antibodies studied; however, it was not required for receptor binding. Additionally, other residues such as Ser[131] and Lys[144], which were important for receptor recognition, were non-essential for antibody binding. Therefore, knowing the epitope sequence recognized by an anti-adhesion antibody does not mean the same sequence will be recognized by the receptor.

4. Residues important for receptor binding are not necessary important for the construction of an ideal cross-protective immunogen

When designing an anti-adhesin vaccine, one may think that the adhesintope recognized by the receptors will be a good vaccine candidate. This is probably true in most cases; however, when a cross-protective vaccine is required, other considerations should be taken into account. From the present immunological results (Chapter IV and

V), two residues, Gln^{136} and Phe^{137}, were found to be important for the peptide immunogen to generate a protective antibodies. Peptide analogs with either of these two residues substituted by an Ala led to the production of peptide-specific antisera that did not react with the native pilus protein. Interestingly, Phe^{137} was not required for binding to A549 cell receptors. On the other hand, the side-chain of Ile^{138}, which was critical for receptor binding, was found to be unimportant for the production of cross-reactive antibody. This residue can be mutated to an Ala without affecting the production of cross-reactive antibodies. It is obvious that the important residues found in the adhesintope may not be useful for design of a synthetic peptide vaccine that generates cross-reactive antibodies.

5. Epitope mapping provides important information for vaccine development

In the present project, epitopic analysis provided a great deal of information related to antibody-antigen interactions as well as vaccine development. Critical residues that were important for the epitope to interact with strain-specific antibodies (PK99H and 17-R1) and cross-reactive antibodies (17-O2) were identified. These residues were shown to be useful in the design of peptide immunogens that caused the immune system to produce antibodies with predetermined specificity (Chapter V). It has been suggested that the important amino acid residues found in the epitope may influence antigen processing and presentation (Horsfall *et al.*, 1991) and may affect the clonal selection process as well (Fish *et al.*, 1991). Therefore, the ability to identify the epitopes in the immune response should have important implications for immune intervention and disease diagnosis.

B. Prospects

This project can be considered as a continuation of many previous studies concerning *P. aeruginosa* pili, which ranged from the isolation and purification of the pili to the identification of the adherence binding domain. Each piece of information is the result of hard-work and intelligence from many investigators. The ultimate goal of these studies is to pursue the development of methods that can be used to interfere with the pilus-mediated adherence of *P. aeruginosa* and infection. As mentioned in Chapter I, the objective of the present studies was to use a synthetic peptide approaches to elucidate the antigenicity and adherence properties of the adherence binding domain of *P. aeruginosa* pilin, hoping that these studies would enable us to design therapeutics such as cross-protective vaccines to protect against a broad range of *P. aeruginosa* infections. Presently, the knowledge obtained is still far from sufficient for this task, and we can foresee that more research is required in order to achieve this long range goal.

1. Receptor studies

(a) Isolation of the pilus-receptor on A549 cells

Both glycoproteins and glycolipids have been suggested as receptor candidates for *P. aeruginosa* binding. Irvin and coworkers (Doig *et al.*, 1989; Irvin *et al.*, 1989; Rudner *et al.*, 1992) have identified glycoproteins with pilus-binding activity, whereas others (Krivan *et al.*, 1988a; Baker *et al.*, 1990; Ramphal *et al.*, 1991a) have suggested glycolipids to be the potential receptors. In the present studies, it was found that both biotinylated PAK pili and synthetic peptide, corresponding to the 17-residue adherence binding domain of the PAK pilin, bound very well to A549 cells (Chapter VII). Future studies should be performed on the purification of the pilus receptor by a streptavidin-biotinylated PAK peptide affinity column, the bound receptors could be eluted using

the glycine-HCl buffer at pH 2.7. Recently, a preliminary test using a similar affinity column to purify asialo-GM_1 (a glycolipid candidate for the *P. aeruginosa* pili) was demonstrated reasonably successful (Lee, unpublished data).

(b) Studies of the ßGalNAc(1-4)ßGal carbohydrate receptor candidate

Recently, the pilus adhesins of *P. aeruginosa* strains PAK and PAO have been reported to bind specifically to the glycolipid asialo-GM_1 (Lee *et al.*, 1994) and the minimal carbohydrate receptor sequence ßGalNAc(1-4)ßGal as deduced from asialo-GM_1 (Sheth *et al.*, 1994). Both biotinylated PAK and PAO pilin peptides bound to asialo-GM_1 (Fig. VIII.1 panel A) and ßGalNAc(1-4)ßGal-BSA conjugate (Fig. VIII.1 panel B) in a concentration dependent manner, whereas the PAO peptides showed a higher avidity than the PAK peptides in both cases. In addition, free pilin peptides (PAK and PAO) also inhibited the binding of biotinylated ßGalNAc(1-4)ßGal to the corresponding immobilized peptides, and the reverse was also true for free pilin peptides which inhibit the binding of biotinylated peptides to the immobilizing ßGalNAc(1-4)ßGal-BSA (Sheth *et al.*, 1994). These studies have identified the minimal receptor sequence ßGalNAc(1-4)ßGal for the *P. aeruginosa* pili. Structural analysis of this minimal receptor sequence by means of 2-D NMR is now in progress. Both the bound and unbound structure will be studied and compared, the results obtained will be valuable in expanding the scope of our understanding of the interactions between the carbohydrate receptor and the peptide ligand.

(c) Mapping of the adhesintopes on other pilin strains

In this study, the adhesintopes of two of the *P. aeruginosa* pilin strains (PAK and KB7) were elucidated. In the long run, the adhesintopes of other pilin strains should be mapped as well, particularly those clinically more important strains such as P1. The P1 strain is an interesting subject to study because its primary sequence is quite different from that of PAK and KB7 strains. There are five more residues (17 instead

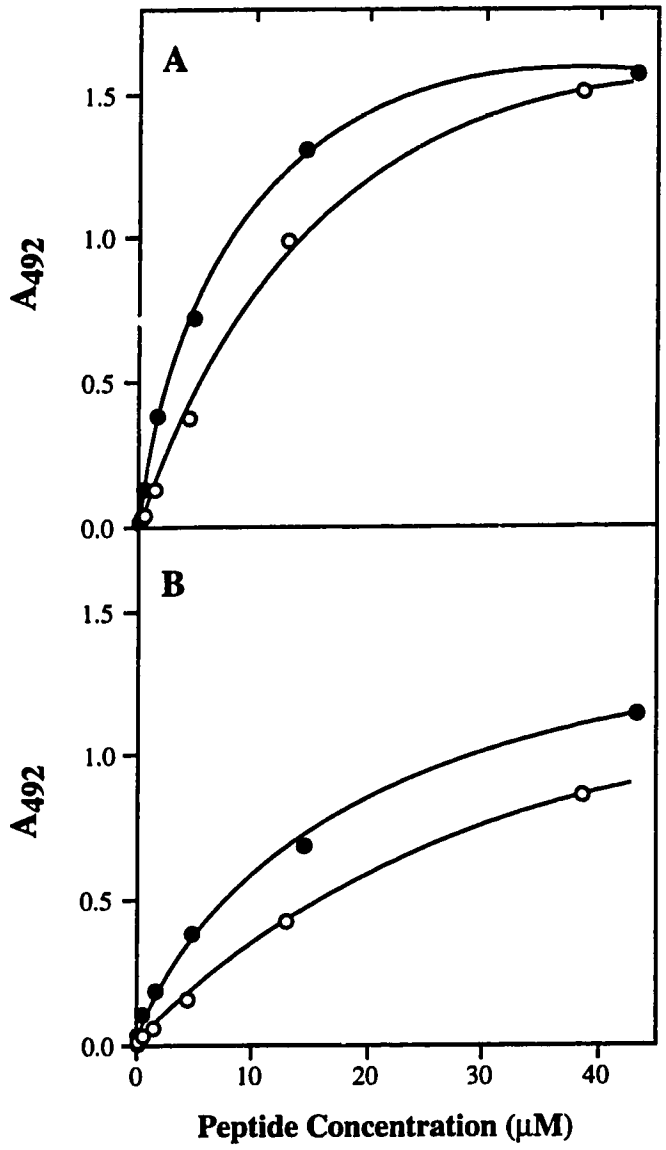


Figure VIII.1 Binding of biotinylated PAK(128-144)OH (O) and biotinylated PAO(128-144)OH (●) to asialo-GM_1 (panel A) and ßGalNAc(1-4)ßGal-BSA conjugate (panel B) coated on ELISA plate. The binding was monitored using streptavidin-alkaline phosphatase. The signals were amplified with an ELISA amplification kit (see Chapter II). The absorbance at 492 nm was measured.

of 12 as in PAK and KB7) found inside the intrachain disulfide bridge of the P1 pilin. Indeed, a recent report by Castric and Deal (1994) has suggested that the *P. aeruginosa* pili should be classified into two major groups based on their primary structures, where strain P1 belongs to group I and strain PAK belongs to group II. The adhesintope mapping result would eventually illustrate how these two different groups of pili interact with the receptors.

2. Peptide libraries

The representative combinatorial peptide libraries were demonstrated to be powerful tools in identifying antibody epitopes (Chapter VI). However, in this study a relatively large quantity of each of the two-position defined hexapeptide libraries was required to perform the competitive binding assay. In order to increase the sensitivity of the assay, three-position defined hexapeptide libraries (Ac-$O_1O_2O_3X_4X_5X_6$-NH_2) consisting of 1,000 hexapeptide mixtures are now being synthesized. This series of peptide libraries is expected to be more effective because the relative numbers of peptide sequences in each mixture are reduced (1,000 peptide sequences instead of 10,000 as in the two-position defined libraries).

In addition, a new prototype of the multiple column peptide synthesizer was designed and constructed, and is now being tested by D. Husband at the Protein Engineering Network Centre of Excellence, University of Alberta. The new instrument has a 10 x 10 layout and can be used to synthesize 100 peptides simultaneously. Further, the size of each well is increased so as to increase the productivity of the syntheses.

In future studies, peptide libraries will be used to identify not only the antibody epitopes, but also the adhesintopes as well.

3. Immunological studies

Pilus adhesins are excellent vaccine targets due to their crucial biological function. The ultimate goal of the present project is to develop a synthetic vaccine candidate for *P. aeruginosa* infections. A synthetic approach will be adopted because this can avoid the problems of large scale culture, pili purification and characterization, and the presence of contaminants such as lipopolysaccharide and other virulence products from the bacteria. To be considered as an effective synthetic peptide vaccine, two requirements are needed: First, the synthetic vaccine must possess a high level of immunogenicity; and second, it must induce antibodies that cross-react extensively with different strains of the pathogen (Van Regenmortel, 1989).

(a) Immunogenicity

Usually small peptides are not potent immunogens, and thus immunization with small peptides often requires the presence of a protein carrier in order to enhance the immunogenicity. The function of the protein carrier is to provide the necessary T-cell epitopes to stimulate the maturation and proliferation of T-helper cells, which subsequently stimulate the proliferation of the selected B-cell clones. Therefore, an effective immunogen should contain both B-cell and T-cell epitopes (Berzofsky, 1985). Protein carriers such as diphtheria or tetanus toxoids that are approved by the FDA should be tested with the peptide immunogens. Alternatively, a total synthetic vaccine that contains only the T-cell and B-cell epitope peptides for *P. aeruginosa* PAK pili is now under examination. These vaccines can be designed with different configurations which includes the multiple antigen peptide (MAP) approach as described by Tam (1989). It is anticipated that the T-cell epitope is able to stimulate the B-cell response against the synthetic peptides without utilizing a carrier protein. In addition, the total synthetic vaccine could also enhance the safety aspect as compared with the live or attenuated vaccines.

(b) Cross-reactivity

An unexpected number of monoclonal and polyclonal antibodies that cross-react with various *P. aeruginosa* pilus strains have been produced. One of them, termed PAK-13, is now under investigation. Interestingly, another monoclonal antibody 18B-41-13 raised against a *Bordetella pertussis* cell surface antigen was found to inhibit both *B. pertussis* and *P. aeruginosa* adherence to human buccal epithelial cells. This antibody was also found to bind to both the *P. aeruginosa* pilin and the pilin peptide. Animal challenge studies have shown that 18B-41-13 could confer protection to the A.BY/SnJ mice against *P. aeruginosa* infections. Purification and further characterization of these antibodies are now being carried out by H. Sheth. The epitope of these antibodies will be mapped by using the single-alanine substituted PAK peptide analogs, with the hope that the mapping results will provide more useful information on how the antibody cross-reacts with a broad range of antigens. In addition, structural analysis of various pilin C-terminal peptides such as P1, PAO and KB7, and their bound structures with the Fab's of both PAK-13 and 18B-41-13 are in progress. Comparison of these structures should allow us to engineer peptide immunogens that can be used to produce effective cross-reactive antibodies.

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[5]Publications arising from this thesis are printed in bold-face.

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