INFECTION AND IMMUNITY, Sept. 1990, p. 2727–2732
0019-9567/90/092727-06$02.00/0

Vol. 58, No. 9

Cross-Reactive and Strain-Specific Antipeptide Antibodies to *Pseudomonas aeruginosa* PAK and PAO Pili

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Received 1 February 1990/Accepted 27 May 1990

Antipeptide antibodies were raised against synthetic peptides corresponding to the amino acid sequences of eight surface predicted regions of the pilin proteins from *Pseudomonas aeruginosa* PAK and PAO. Four of the anti-PAK peptide antisera cross-reacted with strain PAO pili, while five anti-PAO peptide antisera cross-reacted with strain PAK pili. Only one region of the two pilin proteins (region 88-97) provided strain-specific antibodies when either strain PAK or strain PAO region 88-97 peptides were used to generate antipeptide antibodies. Our results clearly showed that cross-reactive and strain-specific antibodies cannot be based solely on the degree of homology in the aligned protein sequences. The majority of synthetic peptides bound to their homologous antipilus antiserum, suggesting that linear sequences play a significant role in the immunogenic response of native pili.

The pili of *Pseudomonas aeruginosa* are flexible, filamentous structures consisting of a polymer of a single protein subunit called pilin. These pili protrude from the ends of the rod-shaped organisms and can produce a unique form of locomotion called twitching mobility (11). In addition, they can act as receptors for a number of pilus-specific bacteriophages (2, 3) and are believed to mediate the adherence of the organisms to surface receptors of human epithelial cells (5, 19). The importance of pili as virulence factors has been demonstrated previously (22, 29). The pilin molecules having a molecular mass of 15,000 from a number of *P. aeruginosa* strains have been sequenced (21, 23). The pilin subunits are assembled in a helical array having five subunits per turn, with a pitch of 4.1 nm (7, 27). The N terminus of each pilin consists of a methylated phenylalanine (8, 12), followed by a highly conserved, hydrophobic region of about 30 amino acid residues (17). The central region of pilin is hypervariable, and the C-terminal region is semiconserved and contains a disulfide bridge.

The objective of this study was to examine the immunogenicity and antigenicity of two major pilin strains of *P. aeruginosa* (strains PAK and PAO) by using antipeptide antibodies to synthetic peptides which represent surface regions of the pilin proteins and antipilus antibody that binds to these synthetic peptides. The information which we obtained should improve our understanding of the requirements for generating strain-specific versus cross-reactive antibodies. The strain-specific antibodies could be useful as diagnostic reagents in *P. aeruginosa* infections. Cross-reactive antibodies that can block adherence of both homologous and heterologous *P. aeruginosa* strains could be useful in vaccine development.

The antigenic determinants of pili from *P. aeruginosa* PAK were determined previously by using enzymatically cleaved fragments (28) and antipeptide antibodies raised against peptide sequences corresponding to strain PAK pilin (13). Regions 82-110 and 128-144 of strain PAK pilin were shown to be antigenic in direct enzyme-linked immunosorbent assays (ELISAs) in which an anti-PAK pilus polyclonal serum was used. When anti-PAO pilus antiserum was assayed for cross-reactivity with strain PAK pilin fragments, only the N-terminal regions of the molecule were positive (28). However, we have shown previously that the C-terminal region of *Pseudomonas* pilin can give rise to cross-reactive antipeptide antibodies (14). Antipeptide antibodies raised against synthetic strain PAK region 128-144 peptide (intact disulfide bridge) were able to bind strain PAO pili in direct ELISAs and in immunoblot assays (14). Thus, while antiserum to the complete protein may not be able to give rise to cross-reacting antibodies because of the inherent immunogenicity of the intact protein molecule, it is possible to use synthetic peptides to raise cross-reacting antibodies. In this study we used pili from strains PAK and PAO because both purified pili and antipilus antisera are available for comparisons between whole pilus sera and antipeptide antisera.

MATERIALS AND METHODS

Selection of peptides based on surface prediction. The surface prediction computer program Surfaceplot (S.P.I. Synthetic Peptides Inc., University of Alberta, Edmonton, Alberta, Canada), which incorporated three different parameters (hydrophilicity, accessibility, and mobility), was used in the selection of synthetic peptides (20) which represented potential surface regions of *P. aeruginosa* pilin.

Peptide synthesis. The peptides which we synthesized are shown in Fig. 1. The synthesis of these peptides has been described previously (13). Each peptide to be used for immunization had a benzoylbenzoyl-norleucine moiety attached to its N terminus for photochemical cross-linking to protein carriers and for determination of the ratio of peptides to carrier. Peptides were conjugated to keyhole limpet hemocyanin for immunization, and antipeptide antibodies were screened with bovine serum albumin (BSA)-peptide conjugates. The photoreactive conjugation procedure which we used has been described previously (13).

Immunization. Eight-week-old female Flemish rabbits (two rabbits per peptide) were immunized with 500-μg portions of peptide-keyhole limpet hemocyanin conjugates dissolved in 700-μl portions of a 1:1 mixture of Freund complete adjuvant and phosphate buffered saline. The injections were given subscapularly (two injections, 200 μl each)

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Predicted Surface Regions	Peptide Region Synthesized	Amino Acid Sequences
23-29, 31-33	PAX 22-33	P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
23-34	PAX 22-33	P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
42-47	PAX 41-49	N-P-L-K-T-T-V-E-E-NH_2
42-51	PAX 41-49	N-P-L-K-T-T-V-E-E-NH_2
58-70	PAK 58-70	K-S-G-T-G-T-E-D-A-T-K-K-E-NH_2
55-71	PAO 58-70	S-K-I-K-I-G-T-T-A-S-T-A-T-NH_2
77-78, 80-81	PAK 74-83	G-V-A-A-D-A-N-K-L-G-NH_2
78-81	PAO 75-84	G-V-E-P-D-A-N-K-L-G-NH_2
89-96	PAK 88-97	K-P-D-P-A-D-G-T-A-D-NH_2
92-96	PAO 88-97	V-A-I-E-D-S-G-A-G-D-NH_2
106-113	PAK 105-114	G-G-A-G-P-K-N-K-G-K-NH_2
105-112	PAO 105-114	G-T-S-S-P-K-N-A-T-K-NH_2
120-122	PAK 117-125	T-L-T-R-T-A-A-D-G-NH_2
120-122	PAO 117-125	T-L-N-R-T-A-D-G-V-NH_2
130-137, 141	PAK 128-144	K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
130-137, 139-140	PAO 128-144	A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH

FIG. 1. Amino acid sequences of the synthetic peptides corresponding to predicted surface regions of strain PAK and PAO pilins. The boxes indicate differences in the amino acid sequences of strain PAK and PAO pilins. In addition to the sequences shown, the following residues were added to the N-terminal ends of the peptides: BB-Nle (BB is the photoaffinity probe benzoyl benzoic acid, which was used to couple the peptide to the protein carrier, and Nle is norleucine).

and intramuscularly (one injection, 300 μl). Booster injections were given 2 weeks later with the same quantity of peptide conjugate in Freund incomplete adjuvant. Rabbits were bled 2 weeks later, and antibody titers were determined by using ELISAs. The antisera from the two rabbits for each peptide were not pooled, but the sera with the highest titers were used to generate the data described below.

Pilus preparation. The *P. aeruginosa* strains used were strains PAK/Pfs and PAO/DB2. Strain PAK and PAO pili were purified as described previously (18).

ELISAs. The ELISAs were performed by using the principles of Voller and co-workers (25). The wells on the ELISA plates were coated with either a 5-μg/ml solution of pili or peptide-BSA conjugates. The preparations were incubated for 2 to 2.5 h with dilutions of the antibody at room temperature. Readings of the alkaline phosphatase reaction on *p*-nitrophenylphosphate were taken at 405 nm with a Multitek microplate reader.

RESULTS AND DISCUSSION

The prediction algorithms of Parker et al. (20) generated a surface profile of the strain PAO pilin which is similar to that of the strain PAK pilin (13), with eight potential surface regions. This observation corresponded to the reports of other workers, who found that secondary-structure predictions did not reveal significant differences (16, 23). Watts et al. (26) reported that strain PAO and PAK pilins have identical circular dichroism spectra. We made synthetic peptides encompassing the predicted surface regions of both pilins; these were coupled to keyhole limpet hemocyanin and used to raise antipeptide antisera in rabbits. The amino acid sequences of the synthetic peptides are shown in Fig. 1. The strain PAK series of peptides has been synthesized previously (13). Two of the eight surface regions have identical sequences in both pilins (regions 22-33 and 41-49). The corresponding antisera, which were prepared previously (13), were designated PAX 22-33 and PAX 41-49 to indicate the sequence identity between strains. The other surface regions exhibited varying degrees of sequence homology (Fig. 1).

Good anti-PAO peptide antibody titers were obtained against the BSA-peptide conjugates (generally 10^{-5}) (Table 1). Six of the eight antipeptide antisera bound to strain PAO pili with titers that were 10- to 100-fold lower than the titers against their corresponding BSA-peptide conjugates. This was expected, as antibodies raised against peptides generally do not bind as well to the native protein. It is possible that some contribution to the higher titers observed with the BSA-peptide conjugates compared with native pili could have resulted from some antibody recognition of the linker and the norleucine residue. Interestingly, the strain PAO region 128-144 peptide produced the highest endpoint titer antibodies against strain PAO pili (1.8×10^{-5}) (Table 1). This may have been due to the longer length of the peptide (17 amino acid residues) or to a more rigid and stable

TABLE 1. Endpoint titers of anti-PAO peptide antisera reacted against BSA-peptide conjugates and strain PAO pili in direct ELISAs

Antipeptide antiserum against strain PAO pilin region:	Endpoint titers	
	BSA-peptide conjugates	Strain PAO pili
22–33	2.0×10^{-5}	1.1×10^{-3}
41–49	1.6×10^{-5}	1.4×10^{-3}
58–70	2.0×10^{-4}	6.3×10^{-4}
75–84	1.5×10^{-5}	1.6×10^{-4}
88–97	1.0×10^{-4}	1.0×10^{-3}
105–114	4.2×10^{-5}	
117–125	4.2×10^{-5}	
128–144	3.2×10^{-6}	1.8×10^{-5}

TABLE 2. Cross-reactivities of anti-PAK and anti-PAO peptide antisera with heterologous pili from strains PAK and PAO as determined by direct ELISAs

Antiserum	Cross-reactivity with[a]:	
	Strain PAO pili	Strain PAK pili
Anti-PAK peptide antisera		
Anti-PAK 22–33	+ (1.1×10^{-3})	
Anti-PAK 41–49	+ (1.4×10^{-3})	
Anti-PAK 58–70	++ (1.7×10^{-4})	
Anti-PAK 74–83	− ($\sim 10^{-2}$)[b]	
Anti-PAK 88–97	−	
Anti-PAK 105–114	−	
Anti-PAK 117–125	−	
Anti-PAK 128–144	+++ (2.5×10^{-5})	
Anti-PAO peptide antisera		
Anti-PAO 22–33		+ (2.9×10^{-3})
Anti-PAO 41–49		+ (1.4×10^{-3})
Anti-PAO 58–70		++ (5.3×10^{-4})
Anti-PAO 75–84		+ (2.0×10^{-3})
Anti-PAO 88–97		− ($\sim 10^{-2}$)
Anti-PAO 105–114		−[b]
Anti-PAO 117–125		− ($\sim 10^{-2}$)[b]
Anti-PAO 128–144		++ (2.4×10^{-4})

[a] −, Endpoint titer of 10^{-2} or no binding of antipeptide antisera; +, ++, and +++, progressively higher endpoint titers and stronger binding. The numbers in parentheses are the titers determined by the ELISAs.
[b] Antipeptide antiserum did not bind to homologous or heterologous pili.

structure as a result of the disulfide bridge between residues 129 and 142 (Fig. 1). The resulting conformation of the disulfide-bridged peptide may have closely resembled the conformation of the same region in the intact protein, thus giving rise to the high antibody titer. Similar results were obtained with the strain PAK region 128-144 peptide (14). The importance of a stable conformation to the antigenicity of a peptide resulting from a covalent disulfide bridge has been illustrated in cases such as lysozyme (1), hepatitis-B surface antigen (6), and human choriomic gonadotropin (24). However, the integrity of the disulfide bridge is not critical for raising good titer antibodies that bind to pili in this region (14).

Cross-reactivities of anti-PAK and anti-PAO peptide antisera with heterologous strain PAK and PAO pili. Of the six anti-PAO peptide antisera that bound to strain PAO pili (Table 1), five cross-reacted with strain PAK pili (Table 2). Similarly, of the seven anti-PAK peptide antisera that bound to strain PAK pili (13), four cross-reacted with strain PAO pili (Table 2). As expected, anti-PAX 22-33 and anti-PAX 41-49 antisera bound to strain PAK and PAO pili (Table 2) because of the identical sequences in the two strains. However, anti-PAK and anti-PAO 58-70 antisera gave rise to unexpected cross-reactivities since little sequence homology was observed in the aligned protein sequences of the two pilin proteins. Only 1 of 13 residues was identical (Fig. 2A). To explain this cross-reactivity, we screened for sequence homology of region 58-70 with the complete sequence of the heterologous variant protein (Fig. 2B and C). Two tripeptide regions of strain PAK pilin were found to be identical to the strain PAO pilin sequence; residues 61 to 63 (T-G-T) and 66 to 68 (A-T-K) of strain PAK pilin are identical to regions 104-106 and 112-114 of strain PAO pilin (Fig. 2B). These two regions are in close proximity to each other and could account for the ability of anti-PAK 58-70 antiserum to cross-react with strain PAO pili. Strain PAK region 58-70 also exhibited homology with regions 61-73 and 71-84 of strain PAO pili. These homologies involved 5 identical residues out of 13.

The homology of strain PAK region 58-70 with strain PAO region 61-73 involved the shifting of the sequence by only three residues. The other two possibilities involved the insertion of spaces to achieve maximum sequence homology. The latter possibilities would suggest that cross-reactivities result from critical residues that are brought into close contact via folding of the polypeptide backbone to give rise to a discontinuous epitope. Similarly, strain PAO region 58-70 exhibited sequence homology with strain PAK region 55-67 which involved a shift of three residues (five identical residues out of 13) (Fig. 2C). Maximum sequence homology was observed between strain PAO region 58-70 and strain PAK region 111-123 (6 identical residues out of 13) (Fig. 2C). These results suggest that a comparison of aligned protein sequences of different pilin strains would not give any indication of cross-reactive antibodies raised to this region. A careful sequence search of the region of interest and the whole cross-reacting protein is necessary to predict potential cross-reactivity.

Anti-PAK 128-144 antiserum has been shown to cross-react with strain PAO pili (14). We found that anti-PAO 128-144 antiserum cross-reacted very well with strain PAK pili. This region is semiconserved (9 of 17 amino acid residues), and the disulfide bridge may induce a structural configuration to enable good recognition by heterologous antipeptide antibodies. Interestingly, this region provided the highest titer of cross-reacting antibodies (Table 2). The cross-reactivity observed when an antiserum raised against a synthetic peptide is assayed against another variant of the same protein is not unusual because of sequence homology. Recently, it was demonstrated that antibodies raised against a peptide corresponding to a region of subunit A of pertussis toxin was able to bind to subunit A of cholera toxin because of some sequence homology (7 of 12 amino acid residues) between the two proteins (4). Detection of cross-reactivity between tetanus and botulinum toxins when an antipeptide antibody was used has also been reported (10).

Anti-PAO 75-84 antiserum was able to cross-react with strain PAK pili; however anti-PAK 74-83 antiserum did not bind to strain PAK pili, nor was it able to cross-react with strain PAO pili even though the two peptides differed by only two amino acid residues (Fig. 1). The surface profile plot showed that this region of strain PAO pilin has a much higher surface potential than the corresponding strain PAK pilin region. The two amino acid substitutions of Ala-Ala (strain PAK region 76-77) to Glu-Pro in strain PAO region 77-78 (Fig. 1) had a pronounced effect on the surface accessibility of this region. The Glu substitution increased the hydrophilicity, and the Pro substitution enhanced the accessibility of this region. Interestingly, the increased surface potential predicted by Surfaceplot (20) seemed to be related to the ability of the peptide to generate antipeptide antibodies which not only bound the homologous protein but also cross-reacted with the heterologous protein.

Strain-specific antipeptide antibodies. Obviously, sequence homology cannot guarantee cross-reactivity. Anti-PAK 105-114 and anti-PAK 117-125 antisera failed to cross-react with strain PAO pili even though these regions have 50 to 55% identity (Fig. 2D and E). Regions that gave rise to strain-specific antibodies were strain PAK regions 88-97, 105-144, and 117-125 and strain PAO region 88-97 (Table 2). Region 88-97 was expected to give rise to strain-specific antibodies because of the lack of sequence homology between two pilins (2 of 10 residues were identical) (Fig. 1). A search for

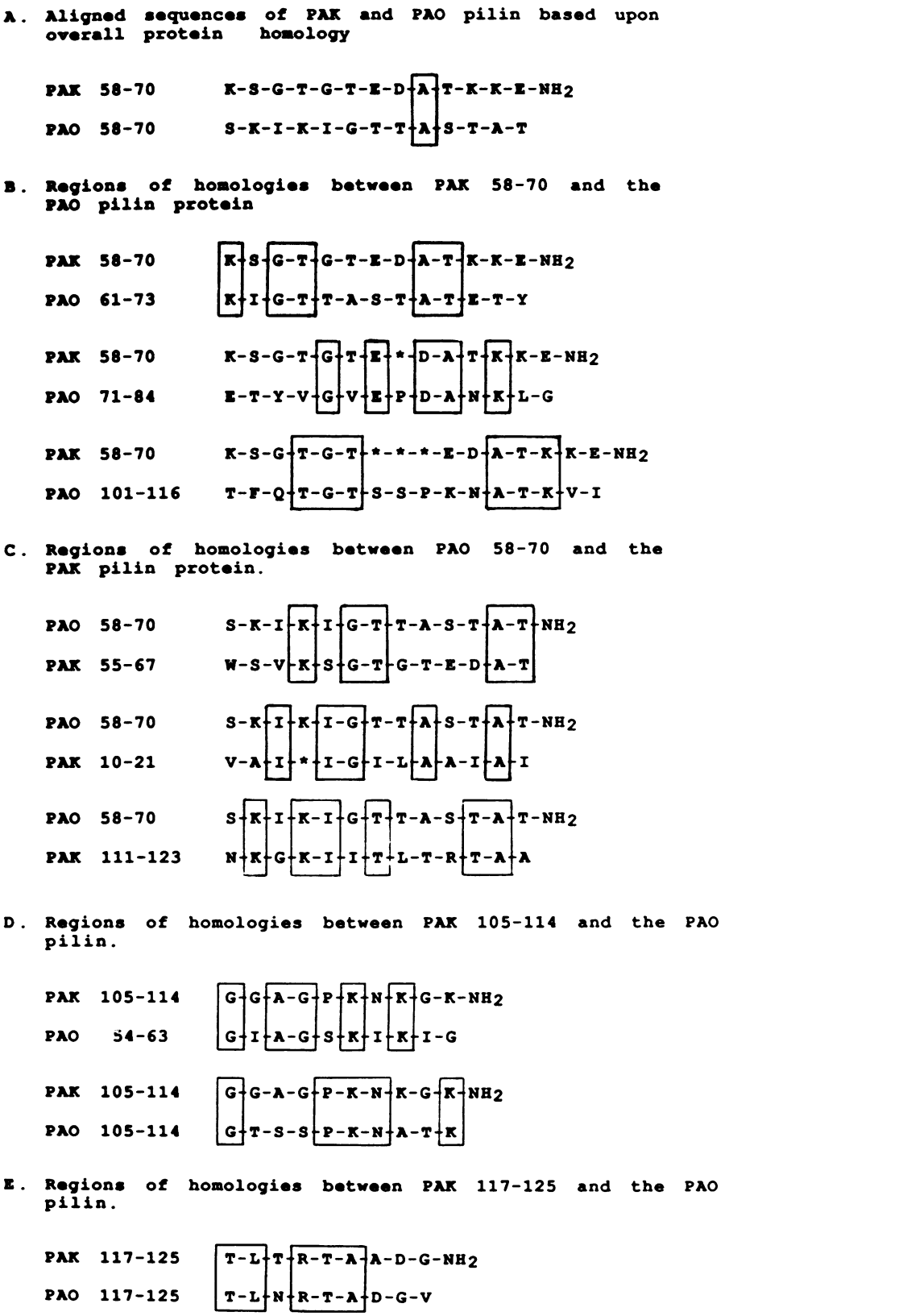

FIG. 2. Can sequence homologies explain cross-reactivities? Sequence homology to region 58-70 of strain PAK was examined in other regions of the strain PAK protein (internal homologies) and for homology in the protein sequence of the heterologous strain PAO pilin (external homologies). A similar search was carried out with strain PAO region 58-70 and strain PAK regions 105-114 and 117-125. The search was carried out by using a modification of a commercially available program, Sequence Search (S.P.I. Synthetic Peptides Inc., University of Alberta, Edmonton, Alberta, Canada). This program searches for short peptide homologies ranging from 25 to 100% to any selected protein.

TABLE 3. Antigenicities of corresponding peptide regions of strain PAK and PAO pilins when they were reacted against their homologous whole-pilus antisera

Peptide region	Antigenicity[a]
Strain PAK peptides	
22–33	−
41–49	++ (1.3×10^{-5})
58–70	+ (4.0×10^{-5})
74–83	+++ (2.8×10^{-6})
88–97	−
105–114	++ (2.0×10^{-5})
117–125	+ (2.7×10^{-3})
128–144	++ (4.3×10^{-5})
Strain PAOpeptides	
22–33	+ (4.0×10^{-4})
41–49	+ (2.3×10^{-4})
58–70	+ (5.2×10^{-4})
75–84	+ (9.2×10^{-4})
88–97	− (3.0×10^{-2})
105–114	+ (4.3×10^{-4})
117–125	+ (4.3×10^{-4})
128–144	++ (4.2×10^{-5})

[a] −, Endpoint titer of 10^{-2} or no binding of the peptide to antisera; +, ++, +++, progressively higher endpoint titers and stronger binding. The antigenicities of the peptides were determined by using direct ELISAs in which the wells of a microplate were coated with the BSA-peptide conjugates and reacted with antisera raised against whole pili. The numbers in parentheses are the titers determined by the ELISAs.

maximum homology between this region and any other region of the two protein sequences showed a A-D-G sequence in strain PAK region 92-94 and strain PAO region 122-124 and a G-A-G sequence in strain PAO region 94-96 and strain PAK region 106-108. It seems reasonable that a minimum number of identical residues is required for cross-reactivity and that two or three identical residues are not sufficient to create cross-reactivity.

Linear immunogenic determinants. The binding of whole-pilus strain PAK and PAO antisera of the synthetic peptides was assayed by using a direct ELISA with BSA-peptide conjugates (Table 3). Anti-PAO pilus antiserum bound to seven of eight synthetic peptides, anti-PAK pilus antiserum bound to six of eight synthetic peptides. Region 88-97 failed to bind either antiserum. Interestingly, it is this region to which antipeptide antibodies provided strain specificity. Although antibodies raised against the whole protein may also recognize conformational epitopes, our results clearly showed that linear sequences are immunogenic with native pili. These results are in contrast with those of Green et al. (9), who found that antibodies to the intact hemaglutinin did not react with any of 20 synthetic peptides corresponding to amino acid sequences of hemaglutinin, yet antibodies to 18 of these 20 peptides reacted with hemaglutinin. We assayed the whole-pilus antisera against heterologous pilin peptides and found little cross-reactivity except for anti-PAX 41-49 antiserum (data not shown). As suggested by Lerner (15), peptide immunization can generate antibody specificity (both cross-reactive and strain specific) that cannot be obtained in other ways.

In conclusion, our results indicate the complexity of the immune system and the difficulty of predicting regions of a protein molecule which could give rise to strain-specific and cross-reactive antibodies. An explanation for cross-reactivity or specificity in the presence of a reasonable degree of sequence homology remains an important area of investigation in molecular immunology. Our results also suggest that it may be possible to raise strain-specific antipeptide antisera which could be useful in diagnosing the presence of *P. aeruginosa* in infections and cross-reactive antisera which may be useful in synthetic vaccine development. We previously demonstrated the importance of the disulfide-bridged region of strain PAK for adherence (13, 14, 19). The results of this study showed that anti-PAO 128-144 antiserum can also cross-react with strain PAK pili, suggesting that this region is a good candidate for raising cross-reactive antiserum to block *Pseudomonas* adherence.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. A studentship from the Alberta Heritage Foundation for Medical Research to K.K.L. is gratefully acknowledged.

We thank P. A. Sastry for preparing strain PAK and PAO pili and acknowledge the technical assistance of Liz Newton and Wah Yau Wong. We thank Dawn Oare and Vicki Luxton for typing the manuscript.

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