Differentiation of *Pseudomonas aeruginosa* Pili Based on Sequence and B-Cell Epitope Analyses

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The nucleotide sequences of three previously undescribed *Pseudomonas aeruginosa* pilin structural genes are presented. Comparisons of deduced pilin primary structure and flanking DNA sequence allowed placement of these and six previously published sequences into one of two groups. Epitope mapping, using overlapping immobilized peptides representing the pilin primary structure, with antipillin monoclonal antibodies revealed several B-cell determinants grouped near the carboxyl terminus of *P. aeruginosa* 1244 pilin. One determinant was found to reside near the pilin constant region. These determinants were found associated with the pil of 31 of 95 *P. aeruginosa* clinical isolates.

Pseudomonas aeruginosa*, a major opportunistic pathogen (1), is capable of producing multiple virulence factors. However, a key component of this bacterium’s pathogenicity is adhesion mediated by pili (27, 35), thin protein fibers extending from the cell pole. Closely similar fibers, referred to collectively as the type 4 pili (22), are produced by other bacteria, including species of the genera *Dichelobacter* (formerly *Bacteroides*) (9), *Eikenella* (28), *Kingsella* (33), *Moraxella* (18), *Neisseria* (19), and *Vibrio* (10). The primary role of the pil of *P. aeruginosa* is in adhesion, in which they, in addition to functioning as virulence factors, facilitate a form of motility called twitching (3) and act as bacteriophage receptors (2). These pili are composed of a 15,000- to 16,000-molecular-weight monomeric subunit, pilin, which is synthesized with a six- or seven-residue leader peptide. Concomitant removal of this peptide and N-terminal pilin methylation occur shortly after synthesis.

While the first approximately 30 amino acid residues of type 4 pili are highly conserved, the remainder of the protein sequence is moderately to highly variable (24). *Neisseria gonorrhoeae* pili show a high degree of variation, which is the result of an exchange between expressed and silent pilin chromosomal structural genes (20). *Dichelobacter nodosus*, on the other hand, contains a single pilin gene which has evolved into a number of relatively stable types (9). *Moraxella bovis* has two relatively stable pilin genes which are alternately expressed (17). A predictable result of this structural variation is a high degree of antigenic heterogeneity among pil of a given species.

*P. aeruginosa* has a single copy of the pilin structural gene (25), and no evidence has been presented to suggest that this gene undergoes genetic variation. While a number of pilA genes have been sequenced (5, 14, 25, 26), the emergence of dominant types has not been seen. This report presents three previously undescribed pilA sequences and proposes the existence of at least two major *P. aeruginosa* pilin groups. B-cell pilus determinants, as defined by reaction with anti-pilus monoclonal antibodies (MAbs), are described. Distribution of strains carrying these epitopes suggests that this pilin type is common among clinical *P. aeruginosa* isolates.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The *P. aeruginosa* strains used were from the following sources: T2A and 577B, C. C. Brinton, University of Pittsburgh; 1244 and PA103, J. C. Sadoff, Walter Reed Army Institute of Research; 9-D2, S. D. Kominos, Mercy Hospital, Pittsburgh, Pa.; PAK and PAO, N. R. Baker, Ohio State University. Cultures were grown in LB broth at 37°C with vigorous shaking, unless otherwise indicated.

The 95 clinical isolates were provided by A. Cross, Walter Reed Army Institute of Research. These strains were grown in 3 ml of LB broth in 15-ml tubes overnight at 75 rpm and 37°C, conditions favorable for pilation. Pili were separated from cells by vortexing (six 15-s treatments), followed by centrifugation for 15 min at 12,000 × g. The supernatant fluids were tested by immunoblot as described previously (5), using MAbs directed against *P. aeruginosa* pil. Negative controls lacked primary or secondary antibody. Pilin-specific MAbs, a gift from J. C. Sadoff, were prepared as described previously (19).

**Cloning and sequencing.** The *P. aeruginosa* T2A pilA gene was cloned by PCR amplification, using primers based on the *P. aeruginosa* PA103 (14) sequence. Strain 577B pilA DNA was likewise obtained with primers based on the *P. aeruginosa* 1244 (5) sequence. The *P. aeruginosa* 9-D2 pilA gene was isolated as a cosmid clone by a procedure described earlier (5). Upon subcloning, the fragment containing the pilA gene was moved into M13 virus, in both orientations, as was the PCR-derived DNA described above. Sequencing of these DNAs employed the Sanger dyeoxy method (30) and used universal and synthetic sequencing primers.

**Peptide synthesis.** Continuous overlapping peptides were synthesized by the Geysen pin method (11), with blocks of derivatized pins purchased from Cambridge Research Biochemicals, Inc. (Wilmington, Del.) and with equipment and software previously described (4). Fmoc-amino acid pentafluorophenyl esters were purchased from Peninsula Laboratories (Belmont, Calif.) and used without further treatment or analysis. The activating agent, 1-hydroxybenzotriazole monohydrate, was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Solvents were reagent grade from Fisher Scientific (Springfield, N.J.). Peptides were synthesized as 12-mers and remained linked to the resin. Peptide composition was confirmed by amino acid analysis of one control pin per plate. Pins
strains 9-D2 and 577B that had 154 residues. Each sequence contained a likely loop region flanked by cysteine residues. Codon usage of these genes (results not shown) was consistent with that seen in other P. aeruginosa pilA genes (34).

**Pilin comparisons.** Alignment of the strain 9-D2, 577B, and T2A pilins with published pilin sequences (Fig. 2) showed high homology within the constant region (positions 1 through 36) but generally poor homology over the rest of the protein. However, closer examination indicated that these proteins may be placed into one of two homology groups on the basis of sequence alignment outside the constant region. The pilins of strains 577B, 1244, P1, and 9-D2 (group I) were nearly identical. Pilins of strains CD, PA103, PAO, T2A, and PAK (group II), while more heterogeneous, shared distinct and characteristic similarities (e.g., the ANKLG region beginning at position 86) not seen with the group I sequences.

Group I pilins were larger by either four or six residues, due primarily to the larger disulfide loop regions. The hydrophobicity plots of group I pilins were similar (results not shown), as would be expected from primary structure homology. While group II hydrophobicity plots showed no similarities to those of group I (except over the constant region), they did demonstrate distinctly conserved, similar patterns. For example, regions 75 to 80, 85 to 95, 100 to 110, 115 to 125, 130 to 135, and 140 to 145 were primarily hydrophobic, while regions 80, 85 to 95, 100 to 110, 115 to 125, 130 to 135, 140 to 145 and the carboxyl terminus were mostly hydrophilic.

The G+C content of the pilA genes further served to distinguish these two groups. The group I genes (577B, 51.0%; 1244, 52.2%; P1, 51.9%; 9-D2, 51.7%) differed in nucleotide composition when compared with those of group II (CD, 46.8%; PA103, 46.8%; PAO, 48.9%; T2A, 50.4%; PAK, 48.6%).

**DNA sequences flanking pilA.** Figure 3A showed that while upstream homology within groups I and II is high (100% for group I and 94% for group II), similarity between groups dropped to 54% and was limited to regions proximal to the start codon (−1 to −35), the promoter region (−45 to −65), and a further upstream region (−130 to −171). Although the pilA promoter sequences were nearly identical, there was an imperfect symmetrical structure encompassing only the group I promoter (Fig. 3B). The axes of symmetry were found near the promoter center. Such a structure is frequently considered the site of action of a regulatory protein.

With the exception of a 10-base deletion in strain 577B (Fig. 3C), group I sequences downstream from the pilA gene displayed a high degree of similarity. Virtually no homology was seen among the group II sequences until position 70, at which point they became nearly identical. It was within this group II region that the 5′ end of a tRNA^Gln_1 gene (12) could be seen. No tRNA gene could be found in the analogous group I region; however, structures characteristic of this group, including a potential transcriptional termination loop (positions 34 to 57 in strain 1244), a potential ribosome-binding site (positions 66 to 69), and the start codon for an adjacent gene (positions 77 to 79), were seen.

**Characterization of group I pilin B-cell epitopes.** It can be expected that many of the differences in pilin primary structure will be reflected as antigenic differences of native pili. With this in mind, group-specific antigenicity patterns were studied utilizing MABS (6-45, 219-6, and 5-44) directed against P. aeruginosa 1244 pilin, which reacted, as determined by both dot blot and Western blot (immunoblot) of whole cells, with strains were typical type 4 pilins possessing six-residue leader sequences (23). Strain T2A prepilin was composed of 150 residues, while those from strains 9-D2 and 577B had 154 residues. Each sequence contained a likely loop region flanked by cysteine residues. Codon usage of these genes (results not shown) was consistent with that seen in other P. aeruginosa pilA genes (34).
from group I (1244, 577B, and 9D-2) but not with those from group II (PA103, T2A, PAO, and PAK).

The locations of the linear epitopes recognized by these MAbs were determined by using the capture ELISA of Geyser et al. (11) in which continuous overlapping peptides representing the *P. aeruginosa* 1244 pilin primary structure were employed. Results (Fig. 4) indicated a strong response from each MAb with discrete linear epitopes from distinct regions of the pilin primary structure. While MAB 219-6 recognized a single region, 6-45 and 5-44 produced a more complex response, reacting with more than one determinant. All three MAbs reacted with group I-specific sequences located in the C-terminal region (Fig. 5), particularly the disulfide loop domain, which has previously been shown to contain major pilin epitopes (16, 32). The 6-45 and 5-44 epitopes of this region appeared to be identical. MAB 5-44 may be distinguished from 6-45 in that it strongly reacted with an N-proximal epitope bordering on the pilin constant region, which contains several residues in common with the group II pilins. A fourth and a fifth MAb gave reaction patterns identical to those of MAbs 6-45 and 219-6, respectively.

The group I specificity of these MAbs allowed them to be used as probes for determining distribution of these pilus groups within a clinical isolate population. When pilus preparations from 95 isolates were tested, those from 31 isolates reacted with one or more of the three MAbs (Table 1), indicating that these epitopes were well represented within this population. The patterns seen suggested that variation in epitope arrangement occurs. Only 11 of 31 of the MAb-positive preparations gave the same reaction pattern as strain 1244 pilin, with the remainder reacting with one or two of the MAbs.

Testing the pilus preparations with three other MAbs, raised against strain 1244 pilin but not utilized in epitope mapping, increased the total number of reacting strains to 41 of 95. Three additional MAbs, raised against pilin from *P. aeruginosa* 653A, which are serologically related to 1244 pilin, increased the total number of reacting strains to 53 of 95.

**DISCUSSION**

Evidence presented in this paper indicated that a majority of *P. aeruginosa* pilins may be placed in one of two homology groups on the basis of pilin primary structure comparison. The high degree of constancy among sequences within each of the groups suggests a relative stability as opposed to the extreme variability seen in *N. gonorrhoeae* pilins (9). For example, homology of the pilin variable region, determined with clustered amino acids (N = Q, D = E, G = T = A, S, M = V = I = L = F), showed that group I sequences were 88.1% identical, while group II pilins had a 65% similarity. By comparison, homology between groups was 28.7%While the pilins examined formed distinctive homology groups, it must be noted that pilin sequences from *P. aeruginosa* K122-4 (25) and KB7 (24) showed minimal variable region similarity to either each other or to either group I or II, suggesting that other *P. aeruginosa* pilin types exist.

The lack of homology in the group I and II flanking regions represented a fundamental difference in organization between the genes of these two pilin types. While the group II *pilA* genes were followed by a tRNA³*Thr* gene, which was followed by a potential bidirectional transcriptional stop, the group I pilin structural gene was followed by a loop region, a ribosome-binding site, and a large open reading frame (4a). Considering these differences, it would be interesting to determine whether group I and II *pilA* genes reside at the same chromosomal locus and whether group I *pilA* genes are adjacent to auxiliary genes, pilB, -C, and -D (21). Altogether, the comparisons of

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**FIG. 2.** Comparison of *P. aeruginosa* pilin primary structures deduced from Fig. 1 with published pilin sequences from the following strains: P1 (26), 1244 (5), PA103, PAK, and PAO (14), and CD (26). Boxed residues show regions of homology. Shaded areas indicate exceptions to the group majority.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>577B</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1244</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-D2</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>CN</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2A</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
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</tr>
<tr>
<td>PAK</td>
<td>M KA K G F T I L H V I V A I G I L A A I P O Q Y V T A R Q V T R A V S E V S A L K TA A K S I L</td>
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*Vol. 62, 1994 P. AERUGINOSA PILIN CLASSIFICATION 373*
these groups raise questions as to the evolutionary origin of these genes and suggest acquisition of the pilin genes by horizontal transfer. It has previously been noted that pilins from *M. bovis* and *P. aeruginosa* showed structural similarities (5, 6). What becomes obvious in the present work is that the similarity was primarily with the group I pilins. Homology determination of the pilin variable region, using the clustered amino acids described above, showed that group I and II pilins were 28.7% identical and the *P. aeruginosa* group II and *M. bovis* beta pilins (18) were 25.6% identical, while the *P. aeruginosa* group I pilins and *M. bovis* beta pilin value was 56.3%, suggesting that these two pilin genes had a recent common ancestor.

The distinctive and homogenous sequence structure of the pilin groups suggested a group-specific antigenic response. Work done by Siliapigni-Fusco (31) showed that a number of *P. aeruginosa* pilins could be divided into two serological families, of which strains 1244 and 577B were members of one and strains T2A and PA103 belonged to the other. The present work has shown that the group I-specific epitopes mapped primarily in the region of the pilin C terminus. Major pilus B-cell determinants of *P. aeruginosa* PAK and PAO lie within the disulfide loop of this region, which also contains the host cell adherence site (7, 8, 13, 15). One of the MAbs used in the present study, 6-45, was effective in blocking adherence of *P. aeruginosa* 1244 cells to bovine tracheal cells (29), indicating that a group I host adherence site also resides in this loop region. This MAb reacted with pili from a relatively large number of clinical isolates (23 of 95), suggesting a role for this serum in treatment of infections typed as group I. Furthermore, since the epitope structure is known, the use of peptides derived from the epitope sequence as an active vaccine component must be considered.

MAb 5-44 recognized an epitope bordering the pilin conserved region, indicating that this portion of the primary structure exists at the pilus surface. This MAb also reacted with C-terminal determinants recognized by MAb 6-45, indicating that (i) these epitope components are in close proximity on the pilus surface and (ii) MAb 5-44 may also block host cell adhesion, a point to be examined in future work. While MAb 5-44 was specific for undenatured group I pili, Western blots showed that this serum also reacted with all group II pilins tested (results not shown). These results suggest that this epitope, which probably contains residues 49, 50, 51 (LKT), and 54 (E), is present in undenatured group II pili but in a sterically inappropriate form, possibly buried within the protein or on an inaccessible surface. This cross-reaction in the absence of any homology in the C-terminal region suggests that the latter epitope components are not required for antibody binding.

A surprising feature of this work was the discovery that group I pilin determinants are relatively common among clinical isolates, indicating a conservation of major surface antigens, which are subject to extreme variation in other species. This becomes more interesting when it is clear that the results obtained are probably an underestimation due to the inherent specificity of MAbs. Future studies with polyclonal antisera should clarify this point. This lowered antigenic variability could be the result of lessened selective pressure placed on *P. aeruginosa*, primarily an environmental microbe, compared with that placed on *N. gonorrhoeae*, an obligate pathogen.
The MAbS tested produced variable reaction patterns among the clinical isolates. This suggests that all epitopes were not present in each positive pilus fiber or that portions of certain epitopes were not available for antibody binding. Whatever the reason, it is clear that variation exists within the group I pilins. Further sequence analysis will be required to clarify this point.

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