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Identification of Pilin Pools in the Membranes of *Pseudomonas aeruginosa*

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The proteins of purified inner and outer membranes obtained from *Pseudomonas aeruginosa* strains PAK and PAK/2Pfs were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and treated with antiserum raised against pure pili. Bound antipilus antibodies were visualized by reaction with ^{125}I-labeled protein A from *Staphylococcus aureus*. The results showed that there are pools of pilin in both the inner and outer membranes of *P. aeruginosa* and that the pool size in the multipiliated strain is comparable with that of the wild-type strain.

Pseudomonas aeruginosa strain PAK possesses polar pili, each consisting of a single protein subunit, pilin, with a molecular weight of approximately 18,000 (3). The pilin subunits are helically arrayed with four subunits in each turn of 4.1-nm pitch, and thus, the pilus is similar in quaternary structure to the pilus-specific *Pseudomonas* bacteriophage Pf1 (2). Although little is known about the assembly process for pili, it is likely by analogy with filamentous phage assembly that pili exist in a membrane-bound state at some stage of their synthesis or assembly (15). For this reason, it was of interest to look for pools of pilin in isolated inner and outer membrane fractions of *P. aeruginosa*. Both a wild-type strain, *P. aeruginosa* PAK and a multipiliated mutant strain, PAK/2Pfs, were examined.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* PAK strains were wild-type PAK and PAK/2Pfs, a multipiliated mutant that has been described by Frost et al. (3), who showed that the pili produced by the two strains are chemically and immunologically indistinguishable. Both strains were originally a gift from D. E. Bradley, Memorial University, St. John's, Newfoundland, Canada.

Media and buffers. L broth consisted of 1% tryptone, 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), and 1% NaCl, pH 7.2. Low-sulfate MOPS (morpholinepropanesulfonic acid) medium has been described (10). Toluene scintillation fluid contained 0.05% POPOP [1,4-bis-(5-phenyloxazolyl)benzene] and 0.6% PPO (2,5-diphenyloxazole; New England Nuclear Corp., Boston, Mass.) in toluene.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Lugtenberg et al. (8). Gel samples were solubilized for 10 s at 100°C; the brief heating was important in preventing heat-dependent aggregation of pilin. Duplicate sets of each membrane fraction and pilin standard were applied to each gel. After electrophoresis, one half of the gel was stained with Coomassie blue, and the proteins from the other half were transferred to nitrocellulose paper. Transfer of the proteins was carried out electrophoretically with an Electroblot system purchased from E-C Apparatus, St. Petersburg, Fla. The transfer conditions of Towbin et al. (14) were used. Transfer was carried out for 4 h at 0.2 mA per gel (8 by 15 by 0.15 cm). After transfer, the amount of pilin remaining in the gel was determined by Coomassie blue staining.

Immunological detection of pilin bound to nitrocellulose. Pilin bound to nitrocellulose was detected with antiserum raised against pure pili. The bound antiserum was then detected with ^{125}I-labeled protein A from *Staphylococcus aureus*, obtained from New England Nuclear Corp. at a specific activity of 85 to 90 μCi/μg. A 1-μCi sample of ^{125}I-labeled protein A was added to 50 ml of buffer for each five-sample gel transferred. The buffers and reaction times were those described by Towbin (14).

Preparation of antiserum. Antiserum against pure pili was obtained by injecting New Zealand white rabbits intravenously with 500 μg of pure pili in standard saline citrate buffer. A total of three injections 3 days apart were administered, and antiserum was collected 4 weeks after the first injection.

Pilus purification. Pilus purification has been described (13). For preparation of ^{35}S-labeled pili, 1 ml of a standing overnight culture of *P. aeruginosa* PAK/2Pfs was diluted into 500 ml of low-sulfate MOPS medium with 0.5% (wt/vol) glucose added as a carbon source. The cells were grown for 6 h (to 2×10^8 cells per ml), after which 5 mCi of $Na_2{}^{35}SO_4$ (specific activity, 933 mCi/mmol; New England Nuclear Corp.) was added. The cells were grown a further 13 h to 2×10^9 cells per ml. Pili were removed by blending for 2 min at 2,000 rpm in a Sorvall Omni-Mixer, and the cells were then removed by centrifugation at 8,000 × *g*. The remainder of the purification has been described previously (13). The specific activity of the

resulting pili was 2×10^7 cpm/mg. A total of 4 mg of pure pili was obtained.

Separation of inner and outer membranes. Inner and outer membranes were separated by the method of Hancock and Nikaido (5) with the modification that the starting material was 4 liters of strain PAK (or PAK/2Pfs) grown in L broth with shaking for 12 h. Briefly, the cells were harvested by centrifugation at 8,000 × *g* and were washed by suspension in 800 ml of 30 mM Tris-hydrochloride, pH 8.0, followed by recentrifugation.

The washed cells were then suspended in 20 ml of 20% (wt/vol) sucrose in Tris to which 1 mg of pancreatic DNase and 1 mg of pancreatic RNase had been added. The cell suspension was then passed two or three times through a pressure cell at 15,000 lb/in^2. Subsequently, 2 mg of hen egg white lysozyme was added, followed 10 min later by 3.5 mg of the protease inhibitor phenylmethylsulfonyl fluoride. Unbroken cells were removed by centrifugation at 10,000 × *g*, and the supernatants were applied to sucrose gradients consisting of 1 ml of 70% sucrose in Tris, 6 ml of 15% sucrose in Tris, and 6 ml of the sample. After centrifugation at 187,000 × *g* for 1 h in a Beckman ultracentrifuge with an SW41 rotor, the bottom 2 ml was collected and applied to a four-step sucrose gradient as described by Hancock and Nikaido (5). The four fractions obtained were washed by pelleting at 177,000 × *g* for 2 h in a Beckman 60 Ti rotor.

For experiments in which ^{35}S-labeled pili were added to monitor the efficiency of removal, the labeled pili were added to the cells in 50 ml of Tris buffer before the second centrifugation. The amount of starting culture was 250 ml in experiments with labeled pili.

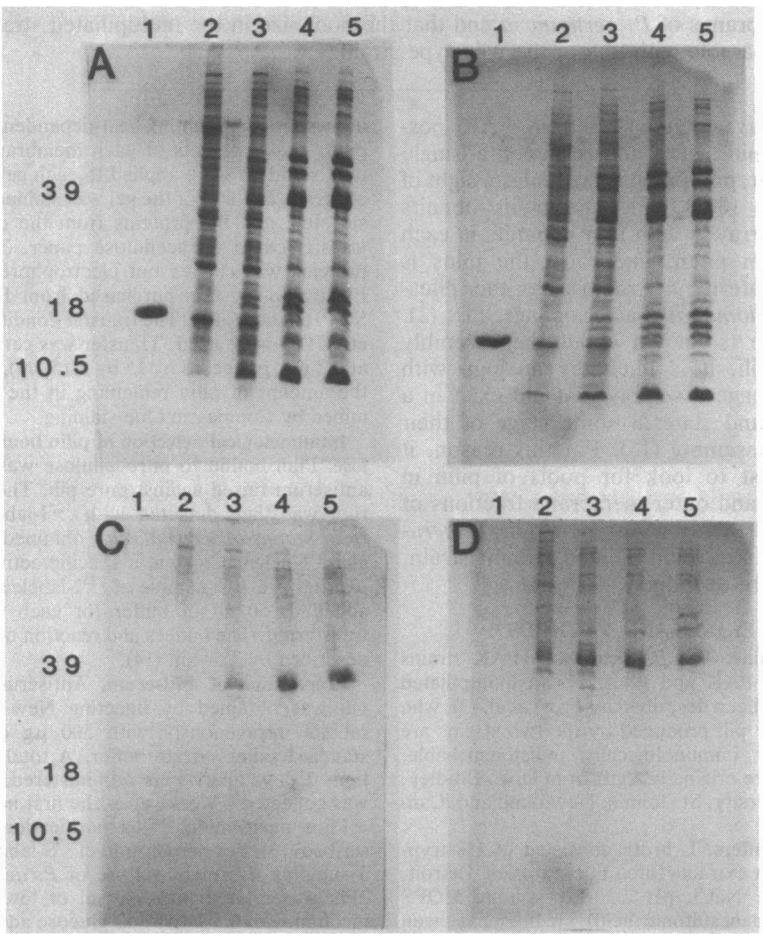

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose of *P. aeruginosa* membranes. (A and B) Coomassie blue-stained 15% sodium dodecyl sulfate-polyacrylamide gels of membrane fractions from *P. aeruginosa* strains PAK (A) and PAK/2Pfs (B). Lane 1 in each case contains 10 μg of pure PAK pilin; lanes 2–5 show the IM, M, OM2, and OM1 fractions, respectively. Each lane received 90 μg of membrane protein. The molecular weight markers are the major porin of *P. aeruginosa* outer membrane at M_r = 39,000 (4), pilin at M_r = 18,000 (3), and the lipoprotein of *P. aeruginosa* outer membrane reported at M_r = 9,000–12,000 (4) but shown here as 10,500. (C and D) Coomassie blue staining patterns obtained after the proteins had been transferred to nitrocellulose paper for 4 h. Samples in each lane are identical to those in (A) and (B).

TABLE 1. Characterization of isolated membrane fractions

Fraction	Protein (mg)[a]		KDO (nmol/mg of membrane protein)		Succinate dehydrogenase activity[b]	
	PAK	PAK/2Pfs	PAK	PAK/2Pfs	PAK	PAK/2Pfs
OM1	24	24	141	217	7	6
OM2	5	9	90	63	8	17
M	5	6	42	55	57	83
IM	12	8	30	9	223	255

[a] Total yield from 4 liters of cells.

[b] Expressed as micromoles of DCPIP reduced per minute per milligram of membrane protein.

Assays. Protein was determined by the method of Lowry et al. (7). KDO (2-keto-3-deoxyoctonic acid) was assayed by the method of Osborn et al. (11). Pure KDO (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Succinate dehydrogenase activity was measured by the method of Kasahara and Auraku (6) with DCPIP (dichlorophenylindophenol; Sigma Chemical Co.) and phenazinometosulfate as the electron acceptors. ^{35}S-labeled samples were counted on filters in toluene scintillation fluid.

RESULTS AND DISCUSSION

Membranes from *P. aeruginosa* strains PAK and PAK/2Pfs were isolated by the method of Hancock and Nikaido (5). Four membrane fractions were obtained in each case: OM1, OM2, M, and IM in the nomenclature of Hancock and Nikaido. The purity of the membrane fractions was assessed with the enzyme succinate dehydrogenase as an inner membrane marker and the lipopolysaccharide sugar KDO as an outer membrane marker. The results are summarized for the wild-type and multipiliated strains in Table 1. It may be seen that OM1 is highly purified outer membrane and IM is highly purified inner membrane, in agreement with the findings of Hancock and Nikaido (5).

Figure 1A and B shows the Coomassie blue staining patterns obtained from each membrane fraction of the two strains. The large number of proteins present makes it difficult to assess from Coomassie blue staining alone whether or not there was a band migrating at the same position as the pilin standard. Within 4 h of transfer, the removal of lower-molecular-weight proteins from the gel was virtually quantitative (Fig. 1C and D); transfer of the higher-molecular-weight bands was somewhat less efficient.

Figure 2 shows the autoradiograms obtained after treatment of the nitrocellulose transfers with pilus-specific antibodies and ^{125}I-labeled protein A as described above. The autoradiograms shown in Fig. 2 correspond to the gels shown in Fig. 1. In lane 1, the more intense band is the pilin monomer at M_r = 18,000. A small amount of pilin dimer (M_r = 36,000) is visible by the ^{125}I detection method, although the dimer is not apparent in the Coomassie blue-stained gels.

A protein that reacts with pilus-specific antibodies and migrates at the same position as pilin is present in all four membrane fractions from each strain. We therefore concluded that this represents pilin in the membrane of *P. aeruginosa*. With both the PAK and PAK/2Pfs strains, there appears to be a slight gradient in pool size, with a larger amount associated with the inner membrane. Comparison of the intensity of the pilin standard in lane 1 with the band in lane 2 suggests that the inner membrane of strain PAK possesses 3 to 6 μg of pilin per 100 μg of inner membrane protein. In the case of strain 2Pfs, the pool size is, if anything, smaller. Thus, increased piliation does not necessitate a larger pool size, and it may deplete the pool slightly.

Examination of the cells by electron microscopy at various stages of membrane isolation (data not shown) showed that a large amount of pili was lost during centrifugation and washing. The rest were completely sheared from the cell into small fragments after being run two times through the pressure cell. To demonstrate that mature pili that have been sheared from the cell surface do not contaminate the membrane preparations, ^{35}S-labeled whole pili were added during an early stage of membrane isolation, and their fate was followed throughout the separation procedure.

Table 2 shows the fate of ^{35}S-labeled pili that were added to 250-ml cultures of strains PAK and PAK/2Pfs after they had been centrifuged and suspended in 50 ml of Tris buffer. Of the 2 × 10^6 cpm added, more than 98% remained in the

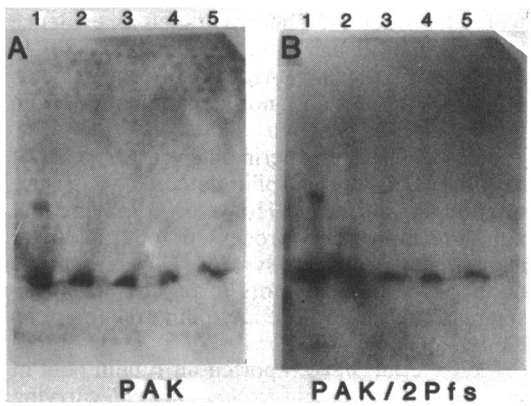

FIG. 2. Autoradiograms of nitrocellulose transfers. The proteins on the gels in Fig. 1 were transferred to nitrocellulose and treated with pilus-specific antibodies and ^{125}I-labeled protein A as described in the text. Lanes 2–5 are as described in the legend to Fig. 1.

TABLE 2. Fate of added ^{35}S-labeled pili during membrane isolation

Step (see text)	Data for strain: PAK			2Pfs		
	Total cpm[a]	^{35}S-labeled pili (μg)[b]	% Recovery	Total cpm[a]	^{35}S-labeled pili (μg)[b]	% Recovery
Cells in Tris	2.37×10^6	172		2.35×10^6	171	
Cell supernatant	2.37×10^6	172	100	2.00×10^6	146	85.1
Cell pellet in 20% sucrose	3.87×10^4	3	1.6	1.77×10^5	13	7.5
Bottom 2 ml of first sucrose gradient	1.0×10^3	0.07	0.4	1.5×10^3	0.1	
Fractions 7–11[c] of first sucrose gradient	3.40×10^4	2.5	1.43	1.71×10^5	12.4	7.3
Washed membranes	0	<0.01	<0.005	0	<0.01	0.00

[a] A 100-μl portion of each sample was counted in duplicate at each stage of the membrane separation and multiplied by the total volume to obtain total cpm, after subtracting background.

[b] Specific activity, 13.75×10^6 cpm/mg.

[c] Twelve 1-ml fractions were obtained; the crude membranes were in fractions 1 and 2.

supernatant of the *P. aeruginosa* PAK cells, whereas 85% remained in the supernatant of the multipiliated strain. In the case of strain PAK, very few pili were left on the cell surface at this stage.

However, with the multipiliated strain several pili per cell remained, and these may have been responsible for trapping the labeled pili and producing higher counts associated with the cell pellet than were found with the wild-type strain. Fractionation of the first sucrose gradient showed a large radioactive peak near the top of the gradient (free pili), whereas the crude membranes, obtained from the bottom 2 ml of the gradient, had very few counts associated with them.

Upon separation of the four membrane fractions on a second sucrose gradient followed by a washing step to remove sucrose, negligible amounts of radioactivity were found associated with each fraction. This was taken as evidence that the pilin observed in the membranes of *P. aeruginosa* PAK and PAK/2Pfs represents pools of pilin subunits and not contamination from mature extracellular pili.

In none of these experiments was a species of high-molecular-weight pilin detected by the use of antipilus antiserum. However, it may be that pilin precursors are processed too rapidly to allow detection in our system. Thus, it may be necessary to find mutants in processing or assembly functions before any pilin precursor can be detected.

Moore et al. have reported an F pilin pool in the inner membrane of *Escherichia coli* carrying an F plasmid (9). They calculated that the pool size represents 4 to 5% of the membrane protein, which is comparable with our findings for *Pseudomonas* pilin. This is somewhat surprising since one normally finds no more than two F pili per cell in *E. coli*, whereas *P. aeruginosa* PAK and PAK/2Pfs are much more heavily piliated.

Our experiments suggest that one of the first steps in pilus assembly is the accumulation of pilin in the inner membrane, as in filamentous phage assembly. Pulse-chase experiments on filamentous phage assembly have shown that phage is never found in the outer membrane but appears rapidly in the growth medium (15). In the case of pilin, it is possible that pilin is assembled at the cytoplasmic surface also and that the pili protrude through the outer membrane, as was observed through electron microscopy by Bayer (1). The fate of these so-called Bayer junctions in our membrane preparations is unclear, although they are most likely found in the impure fractions M and OM2. Alternatively, pilin may proceed to the outer membrane and be assembled at the cell surface. Pulse-chase experiments analogous to those of Smilowitz et al. (15) will be necessary to distinguish between these possibilities.

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