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Composition and Molecular Weight of Pili Purified from *Pseudomonas aeruginosa* K

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Pseudomonas aeruginosa strain K (PAK) bears polar pili that promote infection by at least six bacteriophages. Moreover, a recently isolated mutant of strain K (PAK/2PfS) is many times more piliated than the wild-type strain and facilitates the preparation of large amounts of pure pili for biochemical studies. The present investigation was carried out to establish the structural relatedness of PAK and PAK/2PfS pili and to determine their biochemical composition. A purification procedure is described for PAK and PAK/2PfS pili that yields about 8 mg of pure pili per 100 g (wet weight) of PAK/2PfS cells and 0.8 mg of pure pili per 100 g (wet weight) of PAK cells. PAK and PAK/2PfS pili were found to be free from phosphate, carbohydrate, and lipid and to contain a single polypeptide subunit of 17,800 daltons. Isopycnic centrifugation studies revealed that PAK and PAK/2PfS pili have the same buoyant density in sucrose (1.221) and CsCl (1.295). Both types of pili banded at pH 3.9 when subjected to isoelectric focusing. Amino acid analyses showed that PAK and PAK/2PfS pili have identical amino acid compositions, whereas microimmunodiffusion studies revealed that the two types of pili are immunologically indistinguishable. It was concluded that PAK and PAK/2PfS pili are identical and that the mutation responsible for producing the multipiliated state in PAK/2PfS is probably located outside the structural gene for PAK pili.

Bacterial pili are filamentous appendages that are considerably thinner than flagella and of variable length. A number of morphological types have been identified, the best known being the type 1 pili of *Escherichia coli* (8) and pili specified by conjugative plasmids of the F, I, and P types found in a variety of gram-negative bacteria (32). A third class of pili, designated PSA pili (7), grow from the poles of many species of *Pseudomonas*. These polar pili are flexible filaments, having a diameter of 6 nm and an average length of 2,500 nm (5, 31).

Although the genetic origin of PSA pili is unknown, and there is no evidence that they are involved in conjugational processes, they bear certain similarities to conjugative pili such as the F-type pili of *E. coli*. For example, they act as receptors for a number of pilus-dependent bacteriophages, including both RNA-containing (4, 13) and DNA-containing filamentous forms (27). Polar pili also act as receptors for a number of bacteriophages with long noncontractile tails (7). Since both polar pili and F pili apparently mediate phage infection through some type of pilus retraction mechanism (5, 24, 25), it is of considerable interest to compare the physical and biochemical properties of these two pilus types.

As a first step in this direction, we have begun studying pili from *P. aeruginosa* K (PAK). This *P. aeruginosa* strain bears polar pili that promote infection by at least six bacteriophages (7). Moreover, Bradley (6) has isolated a multipiliated mutant of strain K, PAK/2PfS, which is apparently resistant to pilus-dependent phage infection by virtue of being unable to retract its pili. Since the PAK/2PfS mutant is many times more piliated than the wild-type strain, it facilitates the preparation of large amounts of pure pili for biochemical studies.

The present investigation was carried out to establish the structural relatedness of PAK and PAK/2PfS pili and to determine their biochemical composition. We describe a procedure for the purification of PAK and PAK/2PfS pili to greater than 99% purity and present details on a number of observations relating to their physical and chemical properties.

MATERIALS AND METHODS

Bacteria. PAK is the host for phages Pf and PO4. *P. aeruginosa* K/2PfS (PAK/2PfS) is a multipiliated mutant of strain K that is resistant to phages Pf and PO4 (6). Both strains were kindly provided by D. Bradley, Memorial University, St. John's, New-

foundland. *E. coli* HB11/*Flac* was obtained from C. C. Brinton, University of Pittsburgh.

Media, buffers, and reagents. PAK and PAK/2PfS were grown in 3% Trypticase soy broth (TSB; purchased from BBL) in liquid culture. Growth of bacteria on solid medium involved spreading 10 ml of a late-log-phase culture on 350 ml of TSB agar (3% TSB-1.5% agar [Difco]) in aluminum trays (27 by 38 by 1.25 cm). The trays were covered with a fitted piece of aluminum and incubated overnight at 37°C. 37°C.

Standard saline citrate (SSC) buffer contained 0.15 M sodium chloride and 0.15 M sodium citrate adjusted to pH 7.0 with NaOH.

All reagents were prepared from analytical-grade chemicals in double-distilled water unless otherwise specified. Ultrapure sucrose and dithiothreitol were used as supplied by Schwarz/Mann. CsCl was obtained from Gallard-Schlesinger. Ampholytes were obtained from LKB Produkter, Bromma, Sweden. Sodium dodecyl sulfate (SDS), technical grade, was obtained from Matheson, Coleman and Bell. Chemicals used for polyacrylamide gel electrophoresis were obtained from Eastman Organic Chemical Co. Acrylamide was recrystallized from $CHCl_3$, whereas N,N'-methylenebisacrylamide was recrystallized from acetone. Carbonic anhydrase was obtained from Worthington Biochemicals Corp. Myoglobin, chymotrypsinogen and lysozyme were supplied by Schwarz/Mann. Bacteriophage R17 was purified as described by Krahn et al. (19).

Electron microscopy. Electron microscopic examination of pili involved staining the pilus preparations with 2% (wt/vol) sodium phosphotungstate (pH 7.0) and viewing the preparations in a Philips EM300 transmission electron microscope.

Protein estimation. Routine protein estimations were performed by the method of Lowry et al. (21).

Isoelectric focusing. Isoelectric focusing was performed by the method of Vesterberg and Svensson (29) as modified by Beard et al. (3). The ampholyte solution used was of pH range 3 to 10. The pH of the collected fractions (1.0 ml) was determined directly with a Radiometer 26 pH meter. Absorbance at 280 nm was determined with a Beckman DBG spectrophotometer.

Isopycnic centrifugation in CsCl and sucrose. The buoyant density of pili in CsCl was determined by adding 1.5 g of CsCl to 4.5 ml of SSC buffer containing approximately 4 mg of purified pili and spinning the gradient in an SW50.1 rotor in a Beckman L2-65B ultracentrifuge at 35,000 rpm for 20 h at 5°C. The refractive index (n_D) measurements of the CsCl gradient fractions were performed at 25°C and then corrected to a density at 5°C, using a graph that related $\rho^{5°C}$ to $n_D^{25°C}$ (constructed from data obtained from the International Critical Tables).

To determine the buoyant density of pili in sucrose, a discontinuous sucrose gradient containing layers of 3.0 ml of 70, 60, 50, 40, and 30% sucrose in SSC buffer was layered with 1.5 ml of pilus solution (4.0 mg/ml). The gradient was centrifuged for 72 h in an SW27.1 rotor at 22,000 rpm and 5°C. After the gradient dripped in 0.5-ml fractions, the refractive index was read at 25°C. The buoyant density of pili in sucrose at 5°C was determined by comparing the n_D^{25} to a standard curve constructed in this laboratory relating the n_D^{25} to sucrose concentration (and density) at 5°C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed at room temperature, using the method of Weber and Osborn (30). The gels (10 cm in length by 0.5 cm in diameter) consisted of 12.5% acrylamide, 0.34% N,N'-methylenebisacrylamide, 0.07% ammonium persulfate, 0.02% N,N,N',N'-tetramethylethylenediamine, and 0.1% SDS in 0.5 M sodium phosphate (pH 7.2). A total of 10 to 25 μg of protein was dissolved in 50 μl of 0.2% SDS and 0.02% dithiothreitol and then heated in a boiling-water bath for 5 min. Fifty microliters of 60% glycerol containing 0.005% bromophenol blue was added to the cooled samples, after which the samples were layered onto the gels and run at 5 mA/tube in 0.05 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. The run was terminated when the bromophenol blue band was 1 cm from the bottom of the tube. The gels were stained by a modification of the method of Fairbanks et al. (12), using a solution composed of 10% isopropanol, 10% acetic acid, and 0.005% (wt/vol) Coomassie brilliant blue for 24 h, followed by a solution composed of 25% isopropanol, 10% acetic acid, and 0.005% Coomassie brilliant blue for another 24 h. The gels were destained and stored in 10% acetic acid.

Acid and base hydrolysis of pili. Routine acid hydrolysis was performed on 1-mg amounts of salt-free lyophilized pili. The samples were hydrolyzed in 6 N HCl containing 0.1% phenol for 22 h at 110°C in evacuated, sealed tubes. After hydrolysis, the tubes were opened and then lyophilized to dryness over NaOH pellets.

Alkaline hydrolysis was by the method of Hirs (17). Pili samples (1 mg of salt-free lyophilized material) were placed in Pyrex tubes containing 0.15 ml of 1.35 N NaOH and autoclaved for 20 min at 15 lb/in^2. The samples were then cooled and acidified with 0.25 ml of glacial acetic acid.

Amino acid analysis. Amino acid analyses were carried out by the method of Moore (23), using a Beckman 121 amino acid analyzer. The values reported are averaged values for 24-, 48-, and 72-h hydrolysis periods. The values for serine and threonine were estimated by extrapolating to zero time.

The value for tryptophan was determined by hydrolyzing protein with *p*-toluene sulfonic acid by the method of Liu and Chang (20). The value for cysteine was estimated as cysteic acid after oxidizing the protein with performic acid by the method of Hirs (16). Methionine content was estimated as methionine sulfone in the oxidized protein.

Carbohydrate analysis. Total carbohydrate expressed as glucose equivalents was estimated by using the anthrone assay (2), and phenol-sulfuric acid assay was used for glycoprotein (18). The assays were performed on intact and acid- and base-hydrolyzed protein. The lower level of sensitivity of the assays was 5 μg of glucose. The presence of pentoses was checked with the orcinol reagent (2) and expressed as ribose equivalents.

Acid- and base-hydrolyzed pili samples were also examined by paper chromatography. After hydroly-

sis, samples (derived from 1 mg of protein) were dried at 110°C, dissolved in 0.1 ml of 50% ethanol, and then spotted onto Whatman 1MM paper (23 by 56 cm) along with 20 μg of the following standards: glucose, galactose, fructose, glucosamine, galactosamine, *N*-acetylglucosamine, ribose, fucose, rhamnose, xylose, sucrose, lactose, and 3,6-dideoxyglucose. After the paper preequilibrated in the chromatography chamber for 2 h, the solvent (ethyl acetate-pyridine-water [12:5:4]) was introduced into the trough and the chromatogram was run for 16 h at room temperature. After drying, the paper was developed with the $AgNO_3$-NaOH spray of Trevelyan et al. (28). This involved spraying the chromatogram with a solution containing 1 ml of saturated $AgNO_3$ in 199 ml of acetone plus 5 ml of water. After the $AgNO_3$-acetone spray was allowed to dry, the chromatogram was again sprayed with a solution containing 2 g of NaOH per 100 ml of methanol. Upon gentle heating, the carbohydrates appeared as black spots on a buff background. The sensitivity for reducing sugars was 1 μg.

Duplicate chromatograms were also sprayed with anisaldehyde-sulfuric acid (26). This spray gives characteristic colors for a wide range of sugars and has a sensitivity of 1 μg. The chromatogram is sprayed with a solution containing 90 ml of ethanol (95%), 5 ml of anisaldehyde, 5 ml of concentrated sulfuric acid, and 1.0 ml of glacial acetic acid. The chromatogram is heated at 90 to 100°C for 5 to 10 min.

SDS-polyacrylamide gels containing 10 to 50 μg of pili were stained for glycoprotein, using the periodic acid-Schiff reaction as described by Clark (10). The gels were first soaked in 7.5% acetic acid (room temperature) for 1 h and then in 0.2% aqueous periodic acid (4°C) for 45 min. After this, the gels were placed in reduced, acidified basic fuchsin (0.5% [wt/vol]) for 2 h at 4°C and then destained at room temperature in 10% acetic acid. Gels containing rabbit serum glycoproteins were run simultaneously for control purposes, and duplicate gels were always stained with Coomassie brilliant blue to establish coincidence of protein bands.

Phosphate analysis. To assay total organic phosphate, 1- to 2-mg samples of protein were hydrolyzed in acid or base as described above. The hydrolysates were neutralized, and the phosphorus was estimated by the method of Fiske and Subbarow (14). SDS-polyacrylamide gels were stained for phosphoprotein by the method of Cutting and Roth (11). This method can detect 1 nmol of phosphate or 1 phosphate residue per 300 amino acid residues. α-Casein was run as a phosphoprotein control to test the sensitivity of the procedure.

Antisera preparation. To ensure that absolutely pure pili were used in the preparation of anti-pilus antiserum, PAK and PAK/2PfS pili were subjected to a final purification step of SDS-polyacrylamide gel electrophoresis. The pilin band was located in the preparative gels by visualizing the location of prestained pili in a separate gel. The prestained pili had been treated with SDS and Remazol brilliant blue (15) and had been shown previously to migrate in SDS gels with the same mobility as unstained pili.

Preparative gels were prepared by the method of Weber and Osborn (30), which was similar to the procedure for SDS-polyacrylamide gels described above, except for the following differences. The gels were 1 cm in diameter and 6 cm in length and could accommodate a 0.5-ml sample containing 350 μg of SDS-treated pili. The gel buffer was 0.01 M sodium phosphate, pH 7.2. Pili that had been heated in 0.1% SDS as described above were layered onto four gels while a fifth gel was layered with 25 μg of SDS-treated, prestained pili. After the pilin band had migrated approximately 50% of the gel distance and separated from minor contaminants, the run was terminated and the section of the gel containing the pilin band was excised. The gel slices, containing 1.2 mg of protein in all, were thoroughly pulverized by passing through an 18-gauge needle and then suspended in 3 ml of gel buffer plus 3 ml of complete Freund adjuvant.

One third of the preparation was injected subcutaneously in the dorsal area of each of three New Zealand rabbits, each rabbit receiving 400 μg of pili per injection. Six rabbits were injected, three receiving PAK pili and three receiving PAK/2PfS pili. The injections were repeated three more times at approximately 6-week intervals. At 5 months, the rabbits were bled from the ear, using gentle suction. The resultant serum was stored at −20°C.

Microimmunodiffusion analysis. Double gel diffusion analysis was carried out on glass plates (5 by 5 cm) on which rested a plastic template supported by an 8-pound (ca. 3.63-kg)-test monofilament fishing line. The template contained six wells encircling a center well, each well capable of holding a 10-μl sample. A layer of hot 1% agarose (Induboise A37, Industrie Biologique Française) in 0.1 M sodium phosphate (pH 7.2) and 0.15 M NaCl was pipetted into the space between the glass plate and the template. After the agarose had cooled, the wells were sucked clean of excess agarose with a Pasteur pipette. The microdiffusion plates were stored in a water-saturated atmosphere at 5°C.

The serum was applied undiluted to the center well, and the antigens were placed in the surrounding wells. The plates were incubated at room temperature for 4 days in a water-saturated atmosphere. The templates were then gently removed, and the layer of agarose resting on the glass plate was rinsed for 2 days in cold (5°C) saline and 1 day in cold (5°C) distilled water. The plates were then stained in 0.005% Coomassie brilliant blue in 10% isopropanol-10% acetic acid at 5°C until the blue bands appeared. The background color was removed by destaining the plates in ice-cold 10% acetic acid. To preserve the plates, the agarose was allowed to dry to the surface of the glass.

Purification of F pili from *E. coli* HB11/F*lac*. F pili from *E. coli* HB11/F*lac* were prepared and purified essentially as described by Minkley et al. (22), except that our yields were much lower and we were only able to obtain approximately 20 to 30 μg of pure F pili.

RESULTS

Choice of producer strain. As shown in the electron micrographs (Fig. 1), the wild-type *P.*

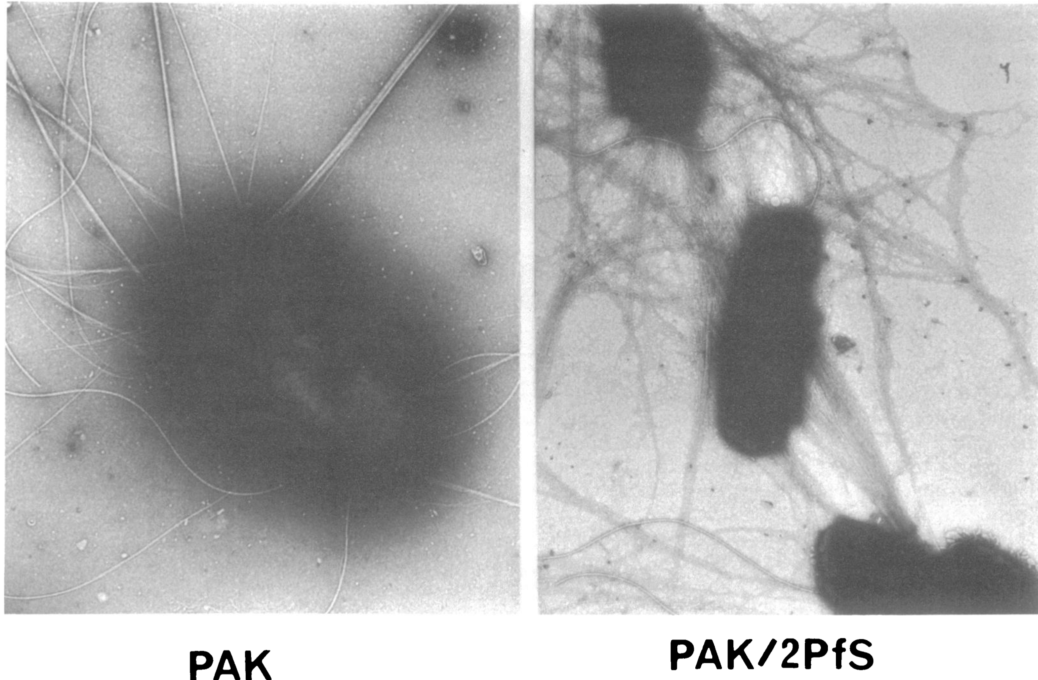

FIG. 1. *Electron micrographs of negatively stained P. aeruginosa strain K. PAK, Wild-type strain (×35,000); PAK/2PfS, multipiliated mutant derived from PAK (×19,920).*

aeruginosa strain K is itself a reasonably good producer of polar pili, whereas the mutant (PAK/2PfS) is extremely heavily piliated. PAK cells apparently do not produce the equivalent of the type 1 pili on *E. coli* (8), although numerous flagella are produced. The latter were successfully separated from polar pili with a simple $(NH_4)_2SO_4$ fractionation step.

Pili purification procedure. PAK or PAK/2PfS bacteria were grown on solid medium in large pans as described above and then harvested by scraping the surface of the agar and suspending the cells from 36 trays (~160 g, wet weight) in 500 ml of sucrose-SSC buffer. The cells were then stirred with a magnetic stirrer at 5°C for a minimum of 24 h, after which they were passed through a sieve to remove the bits of agar and then blended for 2 min in the cold in 200-ml portions at 2,000 rpm with a Sorvall Omnimixer. The cells were removed from the solution by centrifugation at 10,000 × *g* for 15 min, after which the supernatant solution was dialyzed for 72 h in 2-foot (ca. 61-cm) lengths of dialysis tubing against constantly running tap water at 15 to 17°C in a cold room. Ammonium sulfate was then added to the dialysate to 50% saturation, and the solution was allowed to stand overnight at 5°C, during which time the protein precipitate floated to the top. Most of the clear portion of the solution was removed by syphoning, and the precipitate was pelleted by centrifuging for 1 h at 27,000 × *g* in a Sorvall centrifuge. The precipitate was dissolved in approximately 200 ml of SSC buffer and then clarified by centrifugation at 3,000 × *g* for 10 min. It was important at this stage to insure that no pili were sedimented along with the cellular debris. If a white opalescent pellet formed, the pellet and supernatant solutions were recombined and diluted with 50-ml portions of SSC buffer until only cellular debris was pelleted in the low-speed centrifugation step.

To remove flagella, the principle contaminant of the pilus preparation, the supernatant solution containing 1 to 2 absorbancy units at 280 nm per ml was brought to approximately 20% saturation with ammonium sulfate (10% [wt/vol]) and kept at 5°C for 2 h. The pili precipitated out, whereas the flagella remained in solution. It should be noted that a 20%-saturated solution of ammonium sulfate removes pili readily at this stage of the purification procedure, but a 50%-saturated solution is required to precipitate pili out of the crude culture medium. The reasons for this are not entirely clear, but may be related to the fact that the pili at this stage are usually in large aggre-

gates, whereas they are in a more dissociated state and less concentrated in the growth medium. Since the ratio of PAK/2PfS pili to flagella was much greater than that of PAK pili to flagella, PAK/2PfS pilus preparations required only one removal of flagella, whereas PAK required two cycles of ammonium sulfate precipitation.

The pili were redissolved in SSC buffer at a concentration of approximately 1 mg/ml, and 15 ml was placed on a 20-ml discontinuous sucrose gradient consisting of 5-ml portions of 40, 50, 60, and 70% sucrose in SSC buffer. The gradients were centrifuged for 20 h at 5°C in a Beckman L2-65B ultracentrifuge, using an SW27.1 rotor at 20,000 rpm. The cellulose nitrate tube was punctured with a 20-gauge needle just below the prominent band of pilin material. After removal from the gradient, the pilus suspension was dialyzed against SSC buffer to remove sucrose and then rebanded in CsCl as described above. The pilin band was again retrieved with a syringe, dialyzed against SSC buffer to remove CsCl, and then stored in a frozen state at −20°C.

When salt-free pili were required for certain chemical studies, the pili were pelleted by centrifuging for 1 h at 27,000 × *g* and redissolved in distilled, deionized water. After two cycles of centrifugation, the pilus suspension was further dialyzed for 24 h against deionized water and then lyophilized. The dried powder was stored at −20°C.

Criteria of purity. The purity of the pili at various stages of the purification procedure was monitored by means of electron microscopy and SDS-discontinuous gel electrophoresis. Figure 2 shows electron micrographs of a PAK/2PfS pilus preparation before and after the removal of flagella with ammonium sulfate treatment, whereas Fig. 3 shows typical gel patterns before and after the removal of flagella and after subjecting the pilus preparation to CsCl density gradient centrifugation. It is evident from Fig. 2 and 3 that a high degree of pilus purity was achieved with the foregoing purification procedure. When polyacrylamide gels were overloaded with pili (30 μg), a second faint band with a relative mobility corresponding to a molecular weight of 32,000 consistently appeared above the pilin band. To determine whether this material represented a minor protein component of pili or a dimer of pilin, approximately 10 μg was extracted from each of 10 preparative SDS-polyacrylamide gels as described above. The eluted protein was treated with 0.05 N acetic acid in acetone to remove SDS, hydrolyzed as described above, and subjected to an amino acid compositional analysis, using a Durrum D500 amino acid analyzer. The analy-

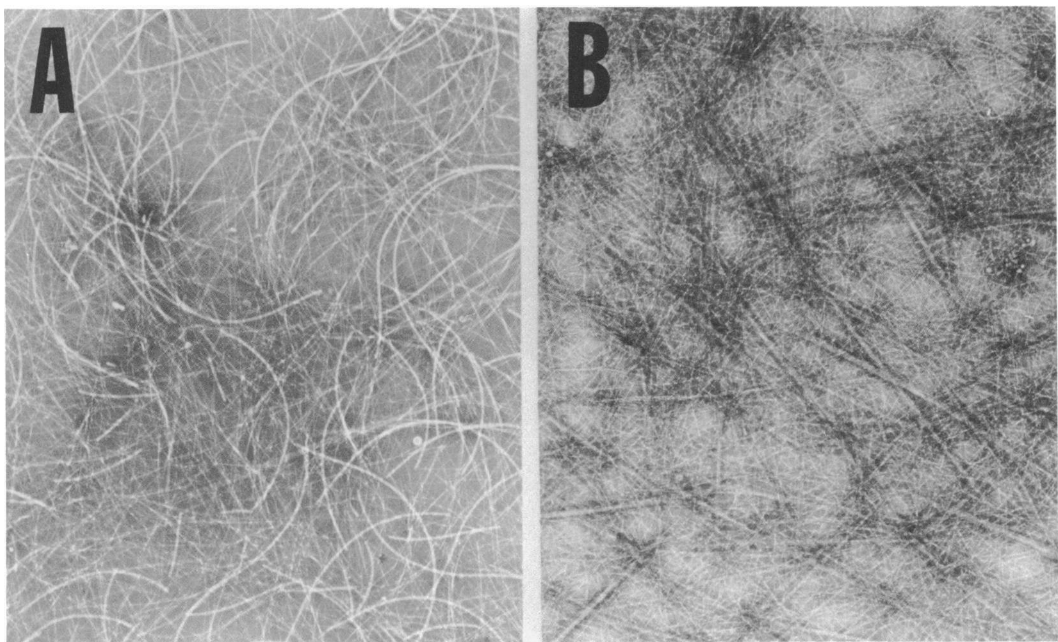

FIG. 2. *Electron micrographs of a negatively stained PAK/2PfS pilus preparation before and after removal of flagella by treatment with 10% (wt/vol) ammonium sulfate. (A) Before treatment with ammonium sulfate (×43,000); (B) after ammonium sulfate treatment (×23,034).*

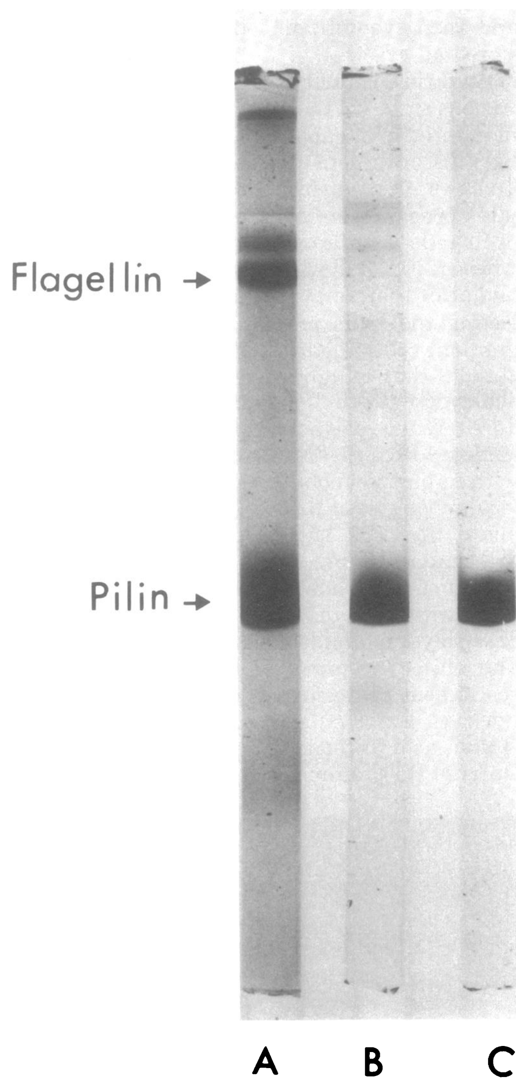

FIG. 3. *SDS-polyacrylamide gel electrophoresis of a PAK/2PfS pilus preparation at various stages of purification. (A) Before 10% (wt/vol) ammonium sulfate precipitation; (B) after 10% (wt/vol) ammonium sulfate precipitation; (C) after CsCl density gradient centrifugation.*

ses showed that the 32,000-molecular-weight component had the same amino acid composition as pilin (data not shown). It was therefore concluded that the 32,000-molecular-weight band was a dimer of pilin.

It should be noted that the amino acid composition analyses yielded nearly integral values for the moles of amino acid per mole of pilin Moreover, the N-terminal amino acid analysis showed only one amino acid residue per mole of protein (data not shown). These observations provide an additional indication of the high level of purity of the pilus preparations. Purified pilus solutions (suspended in water) were found to have an absorbance at 280 nm of 0.78/mg per ml.

Buoyant density in cesium chloride and sucrose. To determine the buoyant density of pili in CsCl, gradients containing either PAK or PAK/2PfS pili were run as described above. The gradient was dripped out in 10-drop fractions, after which the index of refraction was determined on every second fraction, using a Bausch & Lomb refractometer. The refractive index readings were converted to density values at 5°C as described above. SSC buffer (1.0 ml) was added to each fraction, and the absorbance at 280 nm was determined in a Beckman DBG spectrophotometer. Figure 4A shows that the density in CsCl of pili from both PAK and PAK/2PfS strains was 1.295.

The buoyant density of PAK or PAK/2PfS pili in sucrose was determined as described above. After dripping the gradient into 0.5-ml fractions, the index of refraction was determined on every second fraction and converted

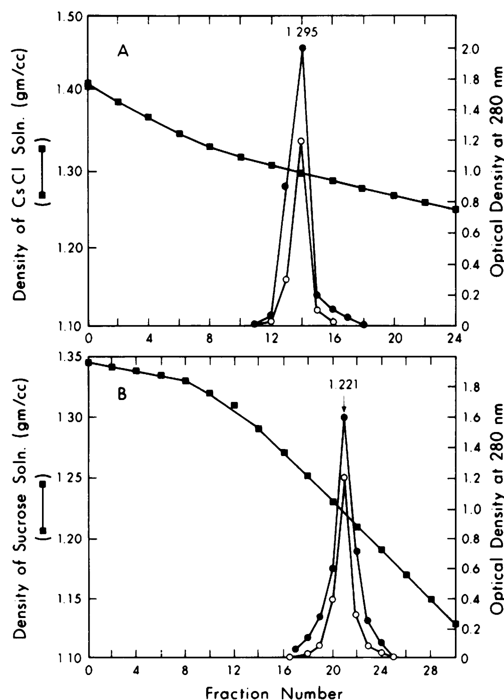

FIG. 4. *Isopycnic centrifugation of purified PAK and PAK/2PfS pili in CsCl and sucrose density gradients. (A) Absorbance at 280 nm of PAK and PAK/2PfS pili in CsCl gradient; (B) absorbance at 280 nm of PAK and PAK/2PfS pili in sucrose gradient. Symbols: ■, density at 5°C; ○, absorbance of PAK pili; ●, absorbance of PAK/2PfS pili.*

to density values as described above. The absorbance at 280 nm was recorded on every other fraction, and the resulting distribution of absorbance versus density is shown in Fig. 4B. It may be seen that the density in sucrose of both PAK and PAK/2PfS pili was 1.221.

Isoelectric focusing. Isoelectric focusing of purified pilus material was performed as described above. The results of the experiment are shown in Fig. 5, where it may be seen that the pI of intact PAK or PAK/2PfS pili was found to be 3.9. It should be noted that problems of pilus precipitation were encountered if ampholytes of a pH range narrower than 3 to 10 were employed. The pI of the pili remained unchanged if the polarity of the electrodes were reversed, indicating that precipitation effects were negligible.

Molecular weight determination of pilin subunits. The molecular weight of pilin subunits was estimated by using SDS-gel electrophoresis as described by Weber and Osborn (30). Pili were dissociated to pilin subunits by boiling for 5 min in 0.2% SDS. As mentioned above, a small amount of pilin was always found in dimer form, even after prolonged heating with higher concentrations of SDS. A comparison of the relative mobility of pili with that of a number of proteins of known molecular weight is shown in Fig. 6. It may be seen that a plot of the relative mobilities of the various proteins versus the log of the molecular weights yielded a molecular weight value for PAK/2PfS pilin of 17,800 ± 300. The same molecular weight value was also obtained for PAK pili (data not shown). The molecular weight value obtained for PAK pili is somewhat larger than the molecular weight value of 11,400 reported for F pili (22), but agrees well with the value of 17,500 for PAK pili obtained on the basis of amino acid compositional data. The latter are summarized in Table 1.

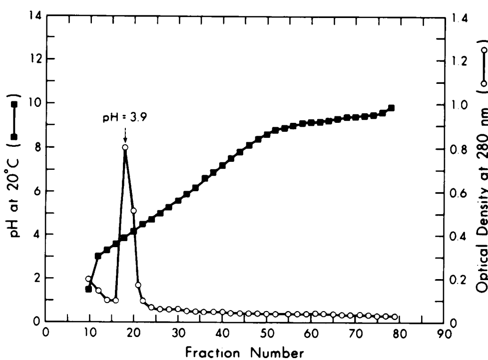

FIG. 5. *Isoelectric focusing of purified PAK/2PfS pili, using LKB ampholyte with a pH range of 3 to 10.*

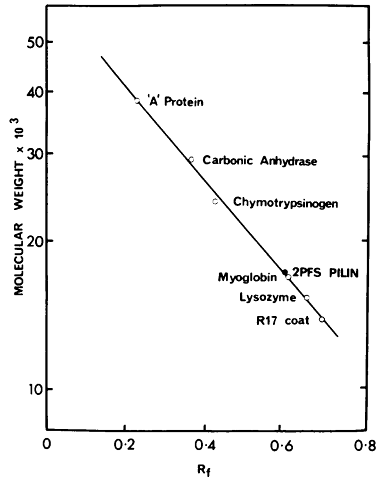

FIG. 6. *Determination of the molecular weight of PAK/2PfS pili by the SDS-polyacrylamide gel electrophoretic method. R_f = migration (in centimeters) of protein relative to migration (in centimeters) of bromophenol blue.*

TABLE 1. *Amino acid compositions of PAK and PAK/2PfS pili*[a]

Amino acid	No. of residues/17,800 daltons	
	PAK	PAK/2PfS
Lys	14.7	14.8
His	0	0
Arg	3.4	4.0
Asx	14.9	14.9
Thr	14.7	15.0
Ser	10.0	9.9
Glx	14.7	14.9
Pro	9.4	9.7
Gly	18.2	17.7
Ala	24.1	23.8
1/2 Cys	ND[b]	3.9
Val	9.2	9.0
Met	ND	2.1
Ile	11.8	11.7
Leu	14.4	14.0
Tyr	2.3	2.2
Phe	2.3	2.3
Trp	1.9	2.0

[a] The samples were hydrolyzed in vacuo at 110°C in 6 N HCl containing 0.1% phenol for 24, 48, and 72 h (see text). The analyses are normalized to a total of 173 residues.

[b] ND, Not determined.

Amino acid compositional analysis of PAK and PAK/2PfS pili. It may be seen in Table 1 that PAK and PAK/2PfS pilin subunits do not contain histidine and that they contain a relatively large number of proline residues. The latter suggests a low level of α-helix content in the pilus protein. It is of interest to compare the

percentage of hydrophobic residues in PAK or PAK/2PfS pili with that in F or type 1 pili. As shown in Table 2, PAK and type 1 pili contain a relatively large proportion of nonpolar amino acids, i.e., about 43%, but the proportion of nonpolar amino acids in F pili is higher still at 53%. This may account for our experience that PAK pili are much easier to dissociate from each other and from cell debris than are F pili. Consequently, PAK pili are considerably easier to purify than are F pili. This is also apparently true for type 1 pili (8).

Immunological relatedness of PAK and PAK/2PfS pili. It is evident from the amino acid composition data in Table 1 that the pilin subunits of PAK and PAK/2PfS pili are probably identical or closely related polypeptides. However, the possibility existed that PAK and PAK/2PfS pili might be antigenically dissimilar. The immunological relatedness of these two types of pili was therefore examined by performing microimmunodiffusion analyses on the purified pili and the corresponding antisera. The results of the experiment are shown in Fig. 7.

It should be noted that, although the antisera were prepared by challenging rabbits with pilus preparations suspended in pulverized SDS-polyacrylamide gel material (see above), similar precipitin bands were formed with either intact or highly denatured pili. Moreover, the specificity of the antiserum towards PAK/2PfS pili is shown by the absence of any reaction with purified F-type pili (Fig. 7A). Figure 7B shows reactions of identity between anti-PAK antiserum and purified PAK and PAK/2PfS pili. Similar results were obtained when anti-PAK/2PfS antiserum was used (data not shown). It was concluded that PAK and PAK/2PfS are antigenically similar and that the multipiliated state of the PAK/2PfS strain is due to the overproduction of PAK polar pili rather than to the formation of a new type of surface structure.

TABLE 2. *Polar and nonpolar amino acid residues of PAK, F, and type 1 pili*

Amino acid character	% PAK pilin[a] (173)[d]	F pilin[b] (124)[d]	Type 1 pilin[c] (163)[d]
Acidic (Asx, Glx)	17.3	9.7	20.3
Basic (Lys, His, Arg)	11.0	8.0	4.9
Uncharged polar (Ser, Thr, Tyr, Gly, Cys)	28.3	29.0	31.3
Nonpolar (Ala, Leu, Ile, Val, Pro, Phe, Trp, Met)	43.4	53.2	43.6

[a] Calculated from the data shown in Table 1.
[b] Calculated from the data reported by Brinton (9).
[c] Calculated from data reported by Brinton (8).
[d] Total amino acid residues per pilin subunit.

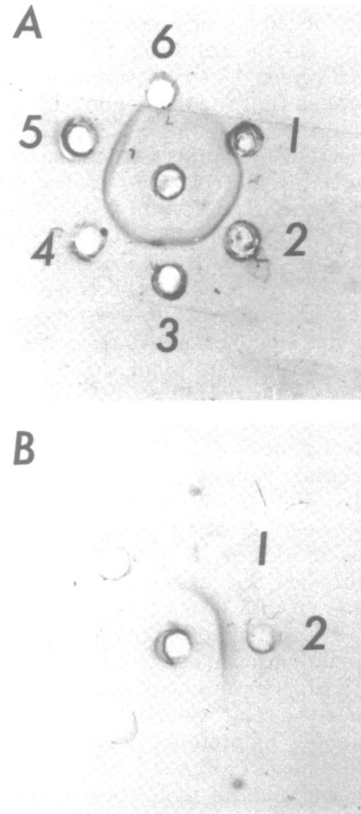

FIG. 7. *Gel diffusion pattern of anti-PAK and anti-PAK/2PfS serum versus PAK and PAK/2PfS pili. (A) Center well contained 2 μl of undiluted anti-PAK/2PfS serum. Contents of outer wells: (1) 10^7 PAK/2PfS cells; (2) 10 μg of intact PAK/2PfS pili; (3) 10 μg of PAK/2PfS pili in 0.1% SDS; (4) 10 μg of PAK/2PfS pili in 10 M urea; (5) 10 μg of PAK/2PfS pili in 0.1 N NaOH; (6) 10 μg of purified intact F pili prepared from E. coli HB11/Flac. (B) Center well contained 2 μl of undiluted anti-PAK serum. Contents of outer wells: (1) 10 μg of PAK pili in 0.1% SDS; (2) 10 μg of PAK/2PfS pili in 0.1% SDS.*

Phosphate analysis. Since Brinton (9) has reported that F pili contain two phosphate residues and one residue of D-glucose covalently linked to each pilin subunit, phosphate and sugar analyses were also performed on PAK and PAK/2PfS pili.

To assay for total organic phosphate, separate 1- to 2-mg samples of pure pili were hydrolyzed in acid and base as described above, neu-

tralized, and subjected to the Fiske and Subbarow (14) method of phosphate estimation. Neither acid nor alkaline hydrolysis was found to release any detectable phosphate under conditions in which 1 mol of phosphate per mol of polypeptide would have been detected easily.

Similarly, no phosphate was detected in association with pilin bands in SDS-polyacrylamide gels. Pure pili were subjected to SDS-polyacrylamide gel electrophoresis as described above and then stained for phosphoprotein, using methyl green as reported by Cutting and Roth (11). Since this method is capable of detecting 1 nmol of phosphate, 20 μg of pili would be expected to produce a positively stained band if pilin monomers contained 1 mol of phosphate per mol of protein. Although 50 μg of pili was subjected to this procedure, no positive reaction for phosphate could be identified. It was concluded from the foregoing observations that the PAK and PAK/2PfS pili do not contain covalently bound phosphate residues.

Carbohydrate analysis. Separate 1-mg samples of purified pili were hydrolyzed in acid and base as described above and then dried at 110°C. The residues were dissolved in 0.1 ml of 50% ethanol and subjected to paper chromatography as described above. A ratio of 1 glucose equivalent of carbohydrate per molecule of pilin should yield approximately 10 μg of carbohydrate per mg of pili. This amount would easily be detected with the $AgNO_3$-NaOH or the anisaldehyde-H_2SO_4 spray systems employed on the chromatograms. No carbohydrate was detected in PAK or PAK/2PfS pili after acid or alkaline hydrolysis at protein concentrations that would have allowed the detection of one glucose residue per 10 pilin molecules.

Acid and alkaline hydrolysates of purified pili were also tested for carbohydrate, using anthrone reagent (2), orcinol reagent (2), and the phenol-sulfuric acid assay for glycoproteins (18). No carbohydrate was detected with any of these procedures at protein concentrations that would have given positive results at a level of one glucose equivalent per two molecules of pilin.

Finally, 50-μg amounts of purified pili were subjected to SDS-polyacrylamide gel electrophoresis and then stained by the periodic acid-Schiff reaction (10) for glycoprotein. No positively staining bands containing amino sugars were detected in the gels.

It was concluded from the foregoing studies that PAK and PAK/2PfS pili do not contain carbohydrate residues.

Lipid analysis. The possible existence of lipid material in PAK or PAK/2PfS pili was tested for by staining SDS-polyacrylamide gels of purified pili (50 μg) with saturated oil red O in 60% ethanol for 18 to 24 h (1). The gels were destained in 50% ethanol and rehydrated. Both PAK and PAK/2PfS pili were found to be negative with respect to the oil red O staining process, indicating that the *P. aeruginosa* pili are probably lipid-free.

DISCUSSION

Progress in the characterization of F-type pili has been hampered by the very small yields of pili usually obtained with available purification procedures. This is partly due to the fact that F pili have a highly hydrophobic character and tend to adhere tenaciously to each other and to cell debris. Most attempts to dissociate them from contaminating cell material lead to significant losses of pili. An additional difficulty encountered in pilus purification is the lack of a simple and sensitive assay procedure with which to quantitate pilus levels at various stages of the purification procedure.

The most serious drawback to working with F pili, however, is attributable to the insufficiency of starting material. For example, if a culture of F-piliated *E. coli* is grown to a density of 5×10^8 cells/ml (in our hands, the level of piliation decreases significantly at cell densities greater than 5×10^8/ml), the culture is found to contain approximately 1.5 pili/cell (Moore and Paranchych, unpublished data). Based on an average pilus length of approximately 1,000 nm, it can be calculated that each pilus contains about 500 pilin subunits and weighs 10^{-17} g. Thus, even a 100% recovery would only yield 7.5 μg of pure pili per liter of starting culture. Since a complete characterization of F pili, including an amino acid sequence analysis and to establish that each F pilin subtein, it is evident that such an undertaking would involve the processing of 100,000 liters of culture.

Minkley et al. (22) have apparently found growth conditions in which male strains of *E. coli* produce significantly more than one to two F pili per cell, since they obtain approximately 5 mg of pili from 24 liters of culture. This procedure has allowed Brinton (9) to purify sufficient F pili to perform an amino acid compositional analysis and to establish that each F pili subunit is covalently bonded to one molecule of phosphate and two molecules of D-glucose. The molecular weight of the F pilin subunit is 11,400 (22), while the isoelectric pH of intact F pili is 4.15 (9).

Brinton (8) has also devised a purification

procedure for type 1 pili of *E. coli*. These pili, which are found in large numbers on both female and male strains of *E. coli*, are 7 nm in diameter and approximately 1,000 nm in length. The pilin subunit has a molecular weight of 17,000 and lacks the amino acids tryptophan and methionine. Chemical analysis showed no detectable amounts of carbohydrate, lipid, or nucleic acid (8).

With the intent of comparing the structure of yet another class of pili with those of types 1 and F, we have developed a purification procedure for PAK and PAK/2PfS pili. The method involves growing the bacteria on solid medium in large pans, suspending the cells in SSC buffer containing 15% (wt/vol) sucrose, and blending the cells to shear off the pili. After the cells are removed by low-speed centrifugation and the sucrose is dialyzed away, the pili are subjected to one or two cycles of $(NH_4)_2SO_4$ precipitation, followed by centrifugation in sucrose and CsCl density gradients. This procedure yields about 8 mg of pure pili per 100 g (wet weight) of PAK/2PfS cells and 0.8 mg of pure pili per 100 g (wet weight) of PAK cells.

As far as we can determine, PAK and PAK/2PfS pili contain a single polypeptide subunit of molecular weight 17,800 and appear to be free from moieties such as phosphate, carbohydrate, or lipid. Isopycnic centrifugation studies revealed that PAK and PAK/2PfS pili have the same buoyant density in sucrose (1.221) and CsCl (1.295) density gradients. Moreover, both types of pili were found to have an isoelectric pH of 3.9 as determined by isoelectric focusing. Of particular interest were the findings that the amino acid compositions of PAK and PAK/2PfS are the same and that the two types of pili appear to be serologically identical as determined by microimmunodiffusion studies.

On the basis of the foregoing, it was concluded that there are no chemical or physical differences between PAK and PAK/2PfS pili. The mutation responsible for producing the multipiliated state in PAK/2PfS is probably located in a gene other than the structural gene for PAK pilin. Presumably, the product of this gene is involved in regulating the level of piliation, perhaps by facilitating the pilus retraction process in some manner.

It is of interest that the structure of PAK pili appears to be more closely related to that of type 1 pili than that of F pili. Whereas both type 1 and PAK pili are free from phosphate and carbohydrate components, F pili contain phosphate and D-glucose. Moreover, the molecular weights of type 1 and PAK pili are both in the 17,000 range, whereas that of F pili is significantly less at 11,400. Finally, the subunits of type 1 and PAK pili both contain approximately 43% hydrophobic amino acid residues, whereas F pilin subunits are significantly more hydrophobic, with 53% of the amino acid residues having nonpolar side chains. The foregoing observations are somewhat surprising in view of the fact that PAK pili appear to be more closely related functionally to F pili than to type 1 pili. That is to say, both F and PAK pili function as receptors for specific bacteriophages, whereas type 1 pili have not yet been shown to have a receptor function. However, since the function of type 1 pili is not yet known and PAK pili may have functions in addition to that of mediating phage infection, it may yet turn out that type 1 and PAK pili have functional similarities. With a view to providing further information on the structure and function of PAK pili, an amino acid sequence analysis of PAK/2PfS pili is presently being performed in this laboratory and will be reported in a future communication.

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