PURIFICATION AND CHARACTERIZATION OF *HAEMOPHILUS INFLUENZAE* PILI, AND THEIR STRUCTURAL AND SEROLOGICAL RELATEDNESS TO *ESCHERICHIA COLI* P AND MANNOSE-SENSITIVE PILI

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Haemophilus influenzae type b is the most common cause of bacterial meningitis in childhood, and despite the availability of potent antibiotics, this pathogen continues to produce significant morbidity and mortality (1). The polyribosylribitol phosphate (PRP)[1] capsule of *H. influenzae* type b has been shown to be an important virulence factor in invasive haemophilus infection (2, 3), and there have been major efforts in recent years to develop an effective vaccine incorporating PRP antigen (4, 5). Although the pathogenesis and prevention of systemic haemophilus infection have been investigated intensively, mucosal colonization and penetration have received considerably less attention and are not well understood. As a result, immunization strategies directed against events preceding bloodstream invasion have not been actively pursued.

Many gram-negative bacteria possess filamentous surface appendages known as pili or fimbriae (6, 7). These protein structures may have a number of important functions in bacterial pathogenicity (8). One of the best documented roles for pili is mediation of bacterial adherence to epithelial cell surfaces, a logical first step in the pathogenesis of infection (9). Purified pili have been shown to be good immunogens, and studies in experimental animal models, as well as in natural hosts, have shown them to be potentially important vaccine components (10–12).

Recently (13–16), clinical isolates of *H. influenzae* have been shown to express pili. Furthermore, only the piliated phase of *H. influenzae* adheres strongly to isolated human oropharyngeal cells (13, 14). In this study, we report a rapid, straightforward purification procedure for *H. influenzae* pili, along with studies on their chemical, physical, and serological properties.

[1] *Abbreviations used in this paper:* BHI, brain heart infusion; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; MS, mannose-sensitive; PRP, polyribosylribitol phosphate; Pth, phenythiohydantoin; SAS, saturated ammonium sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Methods

Bacterial Strains. Seven *H. influenzae* type b strains and one *H. influenzae* nontypable strain were used in this study. Strains A02 and A14 have been described previously (14). Strain A46 was provided by Dr. S. Kaplan (Baylor College of Medicine, Houston, TX) and strain A52 was provided by Dr. D. Granoff (St. Louis Children's Hospital, St. Louis, MO). The remaining type b strains (A16, A01, A43) were supplied by the Bacteriology Laboratory, Children's Hospital, Boston, MA. All of the type b strains were clinical isolates from throat, blood, or cerebrospinal fluid cultures. Strain A03, the nontypable strain, was provided by Dr. J. Gilsdorf, (University of Minnesota Medical School, Minneapolis, MN).

Four *E. coli* strains were used in this study. *E. coli* strain C94 (07:K1:H−) is a cerebrospinal fluid isolate from a human neonate with meningitis and has been described previously (17). *E. coli* strain H10407 (078:K80:H11) is an isolate from an adult with diarrhea (18); it was obtained from Dr. Edgar Boedeker (Walter Reed Army Institute of Research, Washington, D.C.). Both strains produce non–mannose-sensitive and mannose-sensitive (MS) pili.

E. coli strain 3669 (02, K nontypable), a urine isolate from a girl with acute pyelonephritis, was kindly provided by Dr. C. Svanborg-Eden (University of Goteborg, Sweden). *E. coli* strain J96 (04, K6) is also a pyelonephritis isolate (19). When grown for 18 h on solid medium at 37°C, these strains exhibit only P-binding specificity (20).

Bacterial Growth and Pilus Purification. All *H. influenzae* strains were grown on brain heart infusion (BHI) agar (Difco Laboratories, Inc., Detroit, MI) supplemented with 10 μg/ml hemin, 10 μg/ml L-histidine, and 2 μg/ml β-nicotinamide adenine dinucleotide (all from Sigma Chemical Co., St. Louis, MO), at 37°C in 5% CO_2/95% air.

For pilus purification, each *H. influenzae* isolate was enriched for the piliated phase by selective absorption to human type O red blood cells as described previously (14). Piliated bacteria were then cultured onto fresh BHI agar and the overnight growth from each plate was suspended in 2% casamino acids (Difco Laboratories, Inc.,) and used to inoculate two 33 × 223 cm Pyrex baking dishes containing BHI agar. After incubation for 18 h, the growth was harvested by scraping the agar surface with a glass rod, suspending the organism in 15 ml of buffer, and then detaching and purifying the pili as described below (See Results).

E. coli strains C94 and H10407 were selectively grown for the piliated phases, and MS pili were purified as described previously (17). *E. coli* P pili were prepared from recombinant strain HU849, a construct derived from J96 DNA that expresses P pili, but not MS pili, and mediates P-specific hemagglutination (19). Organisms were grown on trypticase soy agar (Difco Laboratories, Inc.) for 24 h at 37°C, harvested into ice-cold 0.005 M Tris-HCl buffer, pH 8.3, (T buffer), and homogenized (4,000 rpm) for 30 min at 4°C in a Sorvall Omnimixer (Sorvall Biomedical Div. DuPont Co., Wilmington, DE). The sheared bacteria were then removed by centrifugation at 12,000 *g* for 30 min. Pilus filaments were precipitated in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl, by the addition of $MgCl_2$ to 0.1 M (TSM buffer), collected by centrifugation at 12,000 *g* for 45 min, and the pellet dissolved in T buffer. Insoluble contaminants were removed by centrifugation at 12,000 *g* for 60 min and the pili precipitated from the supernatant in TSM buffer. After six successive cycles of solubilization and crystalization by exposure to T and TSM buffer, respectively, the pili were judged to be pure by electron microscopy, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and amino-terminal amino acid sequence analysis (20).

Pilus Solution Solubility Studies. The solubility of intact *H. influenzae* pilus rods was determined as a function of percent saturated ammonium sulfate (SAS) and pH by solution optical density measurements as previously described (21). A wavelength of 400 nm was selected to obtain optimal light scattering from pilus aggregation without the effects of specific protein absorption. For SAS aggregation studies, pilus suspensions were adjusted to constant molarity Tris-HCl (0.05 M, pH 9.5) (Sigma Chemical Co.). For pH solubility studies, the pili were dialyzed overnight at 4°C against a constant ionic strength (μ = 0.15) sodium phosphate buffer (Mallinckrodt Inc., St. Louis, MO) at pH values ranging from ~2 to 12.

Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed in 1-mm-thick slab gels (gel concentration, 15%) by the method of Laemmli (22). Standard proteins were bovine serum albumin, egg albumin, typsinogen, β-lactoglobulin, and lysozyme (Sigma Chemical Co.).

Gels were stained with 0.25% Coomassie Brilliant Blue solution (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) in methanol/acetic acid/distilled water (5:5:1). To obtain an estimate of the percentage of total Coomassie Blue–stainable protein present in each gel band, we scanned gels with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Identification of the Major Protein Product in Pilus Preparation. The major protein band in SDS-PAGE profiles was identified as pili by immunolabeling pilus rods with antibody produced against this major band. A total of 1.5 mg of protein from a purified pilus preparation was electrophoresed in a 15% SDS-polyacrylamide gel under standard Laemmli conditions as described above. After electrophoresis, a portion of the gel was stained with Coomassie Blue as described above, and the remainder of the gel was placed in a 0.2 M KCl (Mallinckrodt Inc.) solution at 4°C. All Coomassie Blue–staining protein bands could also be identified as discrete white precipitates in the fresh unstained portion of the gel within 5 min of soaking in the cold KCl solution. The major (25,000 mol wt) band was cut from the unstained gel and emulsified in 0.001 M phosphate-buffered saline. The band preparation was then mixed 1:1 with Freund's incomplete adjuvant (Difco Laboratories, Inc.) and used to immunize rabbits by subcutaneous injection as described below. Each rabbit received three injections, each containing ~0.5 mg of protein. After the final injection, antisera were titered by enzyme-linked immunosorbent assay (ELISA) and used for pilus immunoelectron microscopy as described below.

Amino Acid Analysis. The amino acid composition of pure *H. influenzae* pili was determined by hydrolysis in 4 N methane–sulfonic acid (23) in evacuated, sealed tubes at 115°C for 22, 48, and 108 h. The values for serine and threonine were corrected for destruction during hydrolysis by extrapolation to 0 time. The values for leucine, isoleucine, and valine were corrected for slow hydrolysis of the peptide bond by extrapolation to infinite time. Half-cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the methane-sulfonic acid hydrolysate.

Sequence Analysis. Automated Edman degradation was performed with a Beckman 890C sequencer (Beckman Instruments, Inc., Palo Alto, CA), using a modified Quadral program (Beckman Instruments) in combination with polybrene. Thiazolinone derivatives of amino acids were converted to phenythiohydantoin (Pth) derivatives with aqueous 1.0 N HCl at 80°C for 10 min. Pth amino acids were identified by high performance liquid chromatography (HPLC) with a Hewlett Packard 1084B HPLC (Hewlett-Packard Co., Palo Alto, CA), using DuPont Zorbex ODS columns. They were then confirmed by gas chromatography and/or thin-layer chromatography.

Electron Microscopy. Bacterial suspensions and purified pilus preparations were routinely prepared for electron microscopy (EM) by the agar filtration, negative staining method (24) as modified by To (25). This method sandwiches all particulate material in a suspension between two colloidin membranes, thus permitting accurate evaluations of the degree of piliation in a bacterial culture, or the presence of particulate impurities in a pilus preparation, without the artifact of selective absorption encountered by simple adhesion methods.

To measure pilus diameters and lengths, EM specimens were prepared by adhesion/negative staining using tobacco mosaic virus stacked double discs as internal size markers, as previously described (21). In all cases, pilus preparations were in 0.05 M Tris-HCl (Sigma Chemical Co.), pH 9.5. All EM specimens were examined at 60–80 kV in a JEOL 100S electron microscope (JEOL USA, Cranford, NJ).

Serological Studies. Antisera were prepared against purified pili by subcutaneous immunizations of young virgin white female New Zealand rabbits (Pine Acre Rabbitry, Norton, MA). Three sequential subcutaneous injections (at 1 wk intervals), of 0.5 mg pili

mixed with an equal volume of Freund's incomplete adjuvant, were given, each 1–2 ml vol.

Preimmune sera and antisera were titered against purified pilus preparations using an ELISA. Purified pili (0.1 mg/ml in 50 mM $NaCO_3$, pH 9.5) were added to polyvinyl chloride microtiter plates (Dynatech Laboratories, Alexandria, VA) and incubated at 4°C overnight. The wells were then blocked with 1% gelatin in 50 mM $NaCO_3$ and incubated at 4°C overnight. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Miles Laboratories, Elkhart, IN) was added for 3 h at 37°C and then the substrate *O*-phenylenediamine was placed in each well. The reaction with substrate was stopped after 20 min with 3.5 M HCl. Optical density was then determined at 490 nm on an enzyme immunoassay (EIA) reader (Bio-Tek Instruments, Inc., Burlington, VT).

Cross-reactivities between antiserum against purified A02 pili and heterologous *H. influenzae* pili were also examined by whole-cell agglutination of purified bacteria. 50 μl of an antiserum dilution was placed on a glass microscope slide, and a loopful of piliated bacteria was vigorously mixed into the drop. The bacteria-antiserum mixture was gently swirled at 24°C on a Lab-line Junior Orbital Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) for 10 min, and examined in a dark-field microscope at 10× and 20× to determine agglutination. Twofold dilutions of antiserum were used to determine the agglutination titer. Antiserum produced against a native A02 pilus preparation showing very high protein purity, as well as antiserum produced against the major A02 pilus protein band cut from an SDS-polyacrylamide gel were used; the latter minimized the possible presence of antibody against nonpilus bacterial antigens. Preimmune sera and an isogenic nonpiliated subpopulation of each strain were used for controls except as noted in the text.

Cross-reactivity was also tested between *H. influenzae* pili and *E. coli* MS and P pili by ELISA, whole piliated cell agglutination and immunoelectron microscopy (21). For these studies, antisera were produced against purified pili from *E. coli* strains C94, H10407, and HU849.

Results

Pilus Purification

An initial crude pilus preparation was obtained from *H. influenzae* strain A02 by harvesting bacteria with 0.1 M Tris-HCl, pH 8.5, blending with a Sorvall Omnimixer (¾ maximal setting), and separating detached pili from bacteria by centrifugation at 8,000 *g* for 20 min at 4°C. Saturated ammonium sulfate (SAS) was then added to aliquots of the resulting supernatant at 24°C to achieve solutions ranging from 5 to 45% saturation. The resulting solutions were examined by dark-field microscopy for pilus aggregates, which were evident at SAS concentrations of 35% saturation. The aliquots were pooled, adjusted to 45% SAS, and centrifuged at 8,000 *g* for 60 min at 4°C. The pilus pellet obtained was resuspended in 0.05 M Tris, pH 7.0, and the pH was adjusted until pilus aggregates were no longer visible by dark-field microscopy (pH 8.5). The solution was centrifuged at 23,000 *g* for 60 minutes at 4°C and the supernatant was adjusted to 45% SAS. Pilus aggregates were again removed from solution by centrifugation at 8,000 *g* (60 min, 4°C), and the pellet was resuspended in 0.05 M Tris, pH 8.5. Although yield and purity were not optimal with this preparation, the final purification product gave a strongly staining Coomassie Blue protein band at 25,000 mol wt by SDS-PAGE, and EM revealed intact pilus rods with little particulate contamination. This preparation was sufficiently pure to determine optimal solution conditions for pilus solubilization and aggregation.

Optimal pilus purification conditions were determined by observing pilus

aggregation measured by solution optical density as a function of solvent pH and ionic strength (Figs. 1, 2). Maximal aggregation occurred at pH 5.0 or at an SAS concentration of ~45% saturation (ionic strength, 5.5). An identical ionic strength dependence was found with NaCl (data not shown). At low and moderate ionic strengths, pilus aggregates were completely dispersed at pH >9.0 and <3.0.

Based on the solubility results, the purification scheme shown in Fig. 3 was used for routine purification of *H. influenzae* pili. The initial wash step followed by cycles of pH precipitation and solubilization coupled with differential centrifugation produced optimal yield and purity. However, several minor bands could be detected in most pilus preparations (Fig. 4*a*). Two of these bands persisted, even with an added ammonium sulfate purification step. These bands also had molecular weights that corresponded closely to pilus subunit multimers (49,000 and 73,000). That these bands were in fact pilus subunit multimers was confirmed by immunoblotting on nitrocellulose paper using antiserum raised against the 25,000 mol wt major band cut from an SDS-polyacrylamide gel. This antiserum completely coated intact pilus rods on the homologous A02 strain, as determined by immunoelectron microscopy, confirming that the major SDS-PAGE band

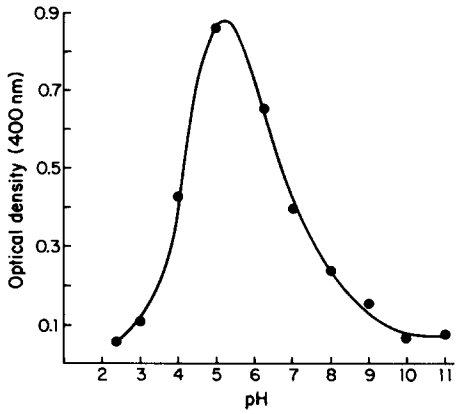

FIGURE 1. The solubility of purified *H. influenzae* pili as a function of pH at constant ionic strength (0.15). Pili exist predominantly as single dispersed rods above pH 9.0 and below 3.0. Peak precipitation occurs at pH 5.0.

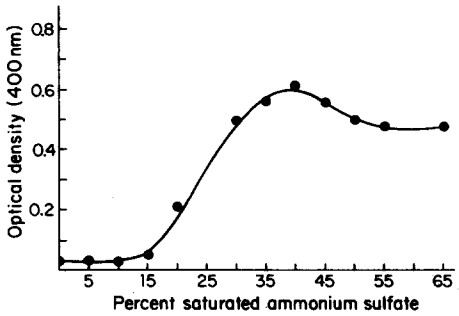

FIGURE 2. The solubility of purified *H. influenzae* pili as a function of percent saturated ammonium sulfate at constant pH (9.5). Peak pilus aggregation occurs at a percent saturation of >35%.

I	Growth from 20-h culture harvested in 0.1 M Tris-HCl, pH 8.0
II	8,000 *g*, 4°C, 20 min
III	Pellet resuspended in 0.1 M Tris-HCl, pH 8.0
IV	Suspension blended (Sorvall Omnimixer ¾-maximal setting)
V	8,000 *g*, 4°C, 20 min
VI	Supernatant centrifuged, 23,000 *g*, 4°C, 60 min
VII	Supernatant dialyzed overnight against 0.01 M phosphate-buffered saline, pH 5.0
VIII	8,000 *g*, 4°C, 20 min
IX	Pellet resuspended in 0.05 M Tris-HCl, pH 10.5
X	23,000 *g*, 4°C, 60 min
XI	Steps 7–10, repeated twice
XII	Supernatant made 45% saturated ammonium sulfate
XIII	8,000 *g*, 4°C, 60 min
XIV	Pellet resuspended in final buffer

FIGURE 3. Purification scheme proposed for *H. influenzae* pili.

contains pili (Fig. 5*c*). By immunoblotting, the 49,000 and 73,000 mol wt minor bands stained with an antiserum dilution of 1:2000 (data not shown). The intensities of the remaining minor bands were reduced, but not completely eliminated, on Coomassie Blue–stained gels, by a single added 45% SAS extraction (Fig. 4*a*).

Although the pH is not optimal for separation of bacteria from detached pili in the initial blending/centrifugation (steps 3–5), pilus preparations were found to contain lower quantities of protein impurities with the lower pH buffer (8.0). The initial buffer wash (steps 1 and 2) also decreased the amount of detectable protein impurities. Using the purification procedure outlined in Fig. 3, most pilus preparations showed >90% purity as estimated by SDS-PAGE (Fig. 4*a*); occasionally a pilus preparation showed only a single 25,000 mol wt band (Fig. 4a).

Chemical and Physical Characterization of Purified H. influenzae Pili

Subunit molecular weight. Fig. 4, a and b, shows a Coomassie Blue–stained SDS-PAGE profile and the corresponding log molecular weight vs. relative mobility graph of a highly purified preparation of *H. influenzae* pili from strain A02. A single band is observed giving an apparent subunit (pilin) molecular weight of 25,000. Because of its high purity, this preparation was used for all subsequent chemical and physical characterizations.

Electron microscopy. Purified *H. influenzae* pili consist of thin flexible rods

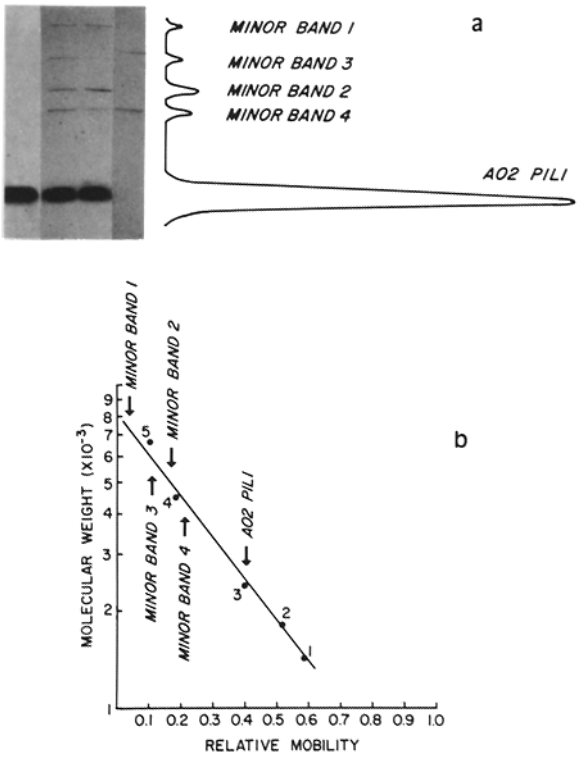

FIGURE 4. SDS-PAGE of purified *H. influenzae* pili from strain A02. (*a*, *left* to *right*) Pilus preparation showing only a single Coomassie Blue–staining protein band; pilus preparation before saturated ammonium sulfate (SAS) extraction showing four minor bands in addition to the major protein band; pilus preparation after SAS extraction; SAS supernatant after removal by centrifugation of precipitated pili (Fig. 3, step 13); a portion of two of the minor protein contaminants (minor bands 3 and 4) can be seen in the discarded supernatant; a densitometer scan of the final pilus purification product (third gel lane from the left) indicating that >90% of the protein detected is present in a single major band. (*b*) Log molecular weight vs. relative mobility plot. The major protein band has a molecular weight of 25,000, and minor protein bands 1 and 2 have molecular weights that correspond closely to multimers of the major band (49,000 and 73,000, respectively).

when stained with uranyl acetate and examined by transmission EM (Fig. 5*b*). Using tobacco mosaic virus stacked double discs as internal size markers, the purified pili have a diameter of 4.5–6.0 nm. Their lengths vary from 0.4 to 1.5 μm. Purified *E. coli* P pili have similar diameters (5.0–7.0 nm) with lengths varying from 0.5 to 1.0 μm (26).

Amino acid analysis and sequence. Purified *H. influenzae* pili were subjected to amino acid analysis and the results compared with the published amino acid compositions of gonococcal pili (27) and MS and P *E. coli* pili (20). The most striking difference between *H. influenzae* pili and *E. coli* and gonococcal pili is in the number of cysteine residues. *H. influenzae* pilin have four cysteine residues (Table I), whereas *E. coli* P and MS pili and gonococcal pili each have two cysteine residues per subunit, and these exist in a disulfide bond. This difference may be significant in terms of the tertiary structures of the pilus subunits.

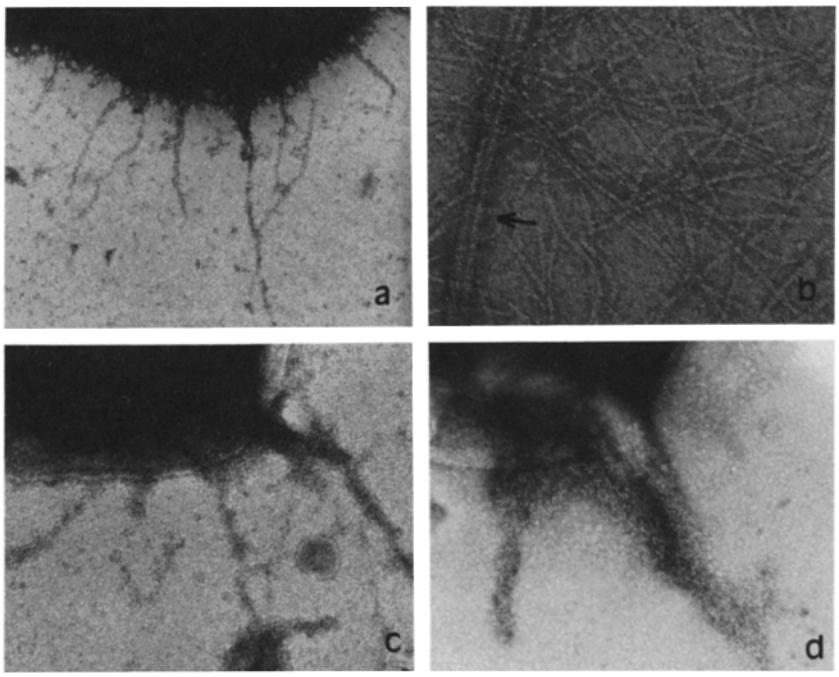

FIGURE 5. EM of piliated *H. influenzae* and purified pili. (*a*) The piliated phase of strain A02. × 30,000. (*b*) Purified A02 pili with tobacco mosaic virus (TMV) stacked double discs (arrow) as an internal size standard. The disc center-to-center repeating distance along the long axis is 4.5 nm. The pilus diameter measures 4.5–6.0 nm. (*c*) Immunolabeling of A02 pili with antiserum raised against the major (25,000 mol wt) protein band cut from an electrophoresis gel. The pili appear thickened and fuzzy (compared with *a*), and multiple pili are cross-linked into large aggregates extending from the cell surface. Antiserum dilution, 1:500. × 18,000. (*d*) Immunolabeling of A03 pili with antiserum raised against purified *E.coli* HU849 P pili. Complete coating of pilus rods with antibody is seen as described in *c*. Antiserum dilution, 1:40. × 18,000.

The amino-terminal amino acid sequence of *H. influenzae* pili was determined through residue 40 (Table II) and compared with the sequences of *E. coli* MS and P pili (20) and gonococcal pili (27). When the cysteine residues of *H. influenzae* and *E. coli* MS and P pili are aligned, 11 (27.5%) and 10 (25%) of the first 40 residues of P and MS pili, respectively, are homologous with the *Haemophilus* pilus sequence. This is comparable to the number of homologous residues between P and MS *E. coli* pili (12 of 40, or 30%) in this region, but substantially different than the number of homologous residues between gonococcal and *Haemophilus* pili (3 of 40, 7.5%).

Serological Analysis of H. influenzae Pili. Subcutaneous injections with highly purified A02 pili produced a high titer homologous antiserum in rabbits (average titer, 400,000) as determined by ELISA. Using this antiserum, studies on heterologous cross-reactivity were initiated with purified pili from three randomly selected clinical isolates of *H. influenzae* (Table III). Pili from one of the strains (A46) showed very high serological cross-reactivity. The remaining two strains showed significant cross-reactivity with heterologous titers of 12 and 16% of the homologous A02 titer. Similar results were obtained using antiserum

TABLE I

Amino Acid Composition of H. influenzae, E. coli Type I and P, and N. gonorrhoeae

Amino acid	Integral number: residues per subunit*			
	H. influenzae	*E. coli* P‡	*E. coli* type I‡	*N. gonorrhoeae*‡
Ala	20	17	35	18
Val	21	17	14	15
Leu	15	9	14	11
Ile	9	6	5	9
Pro	8	5	2	6
Phe	10	7	8	1
Trp	ND§	ND	ND	3
Met¶	3	1	ND	2
Asx	41	19	18	17
Glx	25	13	16	14
Lys	21	10	4	15
Arg	2	ND	2	5
His	6	2	2	2
Gly	17	21	21	11
Ser	6	11	9	11
Thr	20	12	20	11
½ Cys¶	4	2	2	2
Tyr	8	2	2	5

* Based on molecular weights of 25,000 (*H. influenzae* pili), 17,500 (P and *N. gonorrhoeae*), and 18,000 (type I pili) estimated by SDS-PAGE or calculated from the sequence (19, 28).

‡ Literature citations reporting the indicated amino acid compositions are as follows: P (28); type I (20); *N. gonorrhoeae* (27).

§ No amino acid was detected.

¶ For HI and type I pili, cysteine was analyzed as cysteic acid and methionine as methionine sulfone after performic acid oxidation (23). For P and *N. gonorrhoeae*, cysteine and methionine were calculated from the amino acid sequence.

produced against the major A02 pilus protein band cut from an SDS-polyacrylamide gel (data not shown). Thus, it is unlikely that cross-reactivity resulted from antibodies produced against nondetectable contaminating nonpilus antigens which might be present in purified pilus preparations. No activity was detected in rabbit preimmune serum.

No serological cross-reactivity was detected between the mannose-sensitive (MS) pili from *E. coli* H10407 and C94, and the *H. influenzae* pili tested (Table III). However, low reproducible titers were observed when antisera against purified *E. coli* P pili was tested against purified A02 and A46 pili (Table III). Similarly, low cross-reactivity was detected between purified *E. coli* HU849 pili and A02 antisera (including antiserum produced against the major pilus band cut from an SDS-polyacrylamide gel). No activity was detected in preimmune sera corresponding to all the antisera tested.

The serological relatedness between A02 pili and the pili from four additional randomly selected *H. influenzae* strains (including one nontypable strain) was examined by whole piliated bacterial cell agglutination (Table IV). This tech-

TABLE II

*Amino-Terminal Amino Acid Sequence of Pili Proteins**

Species	Reference		Residue Number																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
H. influenzae			Ser	Ile	Asn	Thr	Glu‡	Thr	Ser	Gly	Lys	Val	Thr	Phe	Phe	Gly	Lys	Val	Val	Glu	Asn	Thr
E. coli (P pili)	(20)	Ala	Pro	Thr	Ile	Pro	Gln	Gly	Gln	Gly	Lys	Val	Thr	Phe	Asn	Gly	Thr	Val	Val	Asp	Ala	Pro
E. coli (type I)	(20)		Ala	Ala	Thr	Thr	Val	Asn	Gly	Gly	Thr	Val	His	Phe	Lys	Gly	Glu	Val	Val	Asn	Ala	Ala
N. gonorrhoeae	(27)	Me§	Phe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala	Ile	Val	Gly	Ile	Leu	Ala	Ala	Val	Ala
			21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
H. influenzae			(Cys)‖	Lys	Val	Lys	Thr	Glu	(His)	Lys	Asn	Leu	Ser	Val	Val	Leu	(Asn)	(Asp)	(Thr)	Gly	Lys	(Asn)
E. coli (P Pili)			Cys	Ser	Ile	Ser	Gln	Lys	Ser	Ala	Asp	Gln	Ser	Ile	Asp	Phe	Gly	Gln	Leu	Ser	Lys	Ser
E. coli (Type I)			Cys	Ala	Val	Asp	Ala	Gly	Thr	Val	Asp	Gln	Thr	Val	Gln	Leu	Gly	Gln	Val	Arg	Thr	Ala
N. gonorrhoeae			Leu	Pro	Ala	Tyr	Gln	Asp	Tyr	Thr	Ala	Arg	Ala	Gln	Val	Ser	Glu	Ala	Ile	Leu	Leu	Ala

* The *H. influenzae* and the two *E. coli* sequences were aligned according to the position of Cys_{21}.

§ MePhe, *N*-methylphenylalanine.

‡ Underlined residues are conserved.

‖ Residue identified by reverse phase, high pressure liquid chromatography of Pth derivative only.

TABLE III

Log-Normal Titers for H. influenzae (HI) and E. coli (EC) Pilus Antisera by ELISA

Pili	Antiserum				
	A02 (HI)	HU849 P (EC)	3669 P (EC)	H10407 MS (EC)	C94 MS (EC)
A02 (HI)	400,000	4,000	2,000	<200	<200
A14 (HI)	64,000	ND	ND	<200	<200
A16 (HI)	48,500	ND	ND	<200	<200
A46 (HI)	400,000	2,000	2,000	<200	<200
HU849 P (EC)	3,000	128,000	ND	<200	<200
H10407 MS (EC)	<200	<200	ND	256,000	8,000
C94 MS (EC)	<200	<200	ND	4,000	256,000

TABLE IV

Log-normal titers for H. influenzae (HI) and E. coli (EC) pilus antisera by whole piliated cell agglutination

Pili	Antiserum				
	A02 (HI)	HU849 P (EC)	3669 P (EC)	H10407 MS (EC)	C94 MS (EC)
A01 (HI)	1,500	<10	<10	<10	<10
A02 (HI)	64,000	2,000	2,000	<10	<10
A03 (HI)	2,000	8,000	2,000	<10	<10
A14 (HI)	8,000	1,000	1,000	<10	<10
A16 (HI)	11,000	<200	<200	<10	<10
A43 (HI)	6,000	<10	<10	<10	<10
A46 (HI)	64,000	2,000	2,000	<10	<10
A52 (HI)	64,000	2,000	1,000	<10	<10
J96 P (EC)*	2,000	32,000	<50	<10	<10
3669 P (EC)	1,000	<50	32,000	<10	<10
H10407 MS (EC)	<10	<10	<10	64,000	<50
C94 MS (EC)	<10	<10	<10	<50	64,000

* J96 is the parent strain used for production of HU849 recombinant strain.

nique detected serological relatedness identical to that obtained by ELISA when strains A14, A16, and A46 were tested. Of the additional strains tested, the pili from one (A52) showed very high serological relatedness with A02 pili. Thus, of a total of eight *H. influenzae* strains examined, three (A02, A46, A52) showed very high serological relatedness. The remaining strains all showed some degree of serological relatedness with cross-reactivities ranging from 2 to 17%. No activity was detected with nonpiliated isogenic variants (controls) obtained for seven of the eight strains examined. No activity was detected in preimmune sera corresponding to the A02 antisera tested. Identical serological relatedness was detected using antiserum produced against highly purified A02 pili, or the major A02 pilus band cut from an SDS-polyacrylamide gel.

Whole piliated cell agglutination with antisera against purified *E. coli* P pili showed cross-reactivity with five of the eight *H. influenzae* strains tested (Table IV). In each case no activity was detected with preimmune control sera. The highest titer was seen between the nontypable strain A03 and *E. coli* HU849 P pilus antiserum. Strain A03 is stably piliated, so an isogenic nonpiliated variant was not available to help control for the possible presence of cross-reactive

antibodies against nonpilus cell surface antigens. However, no agglutinating activity was detected with the nonpiliated variants of the other seven *H. influenzae* strains. Furthermore, immunoelectron microscopy clearly demonstrated complete coating of A03 pili by *E. coli* HU849 P pilus antiserum (Fig. 5*d*). No serological relatedness was observed between *E. coli* MS pili and *H. influenzae* pili.

Discussion

Haemophilus influenzae pili were purified and found to consist of multiple copies of a single protein subunit having an apparent molecular weight of 25,000. The purification procedure involves simple manipulations of solvent ionic strength and pH coupled with differential centrifugation. Pilus preparations of >90% estimated protein purity were obtained with some preparations having no detectable contaminating protein bands. Purified pili consisted of intact rods that measured 4.5–6.0 nm diam and had variable lengths of 0.4–1.5 μm.

The amino acid compositions and N-terminal amino acid sequences of *H. influenzae* pili, *E. coli* MS, and P pili and gonococcal pili were compared in this study (Tables I, II). A remarkable segment of conserved sequence was found for *H. influenzae* pili and *E. coli* P pili between positions 8 and 20, where 9 of the 14 residues (64%) are identical. Indeed, the pattern of sequence homology previously recognized between *E. coli* P and MS pili (19) in this region (-Gly-X - Val-X -Phe-X -Gly-X -Val-Val-Asx-Ala-Xs-Cys-, where the intervening residues (X) tend to be charged or polar uncharged) is also evident between *H. influenzae* pili and the two *E. coli* pilus proteins.

These considerations suggest that these pili may be descendents of a common ancestral gene. The shared antigenicity between P pili and some *H. influenzae* pili support this proposal and might indicate that these two pilus proteins diverged more recently and are therefore more generally homologous than either are to MS pili. However, the presence of four cysteine residues per *H. influenzae* subunit (Table I) and two per *E. coli* MS and P pilus subunit suggests that significant differences in primary structure may occur beyond the area of known sequence, or that the region of the *H. influenzae* gene encoding a disulfide loop may have been duplicated.

Purified A02 pili were found to be highly antigenic, and varying degrees of serological relatedness were found between purified A02 pili and the purified pili from the three heterologous *H. influenzae* type b strains tested (Table III). Cross-reactivity was also found when A02 pilus antiserum was tested against six additional heterologous type b strains and one nontypable *H. influenzae* isolate by whole piliated cell agglutination (Table IV). Thus, it seems likely that some degree of cross-reactivity will be found among the pili from many *H. influenzae* strains. Also, there may be a limited number of major antigenic groups among *H. influenzae* pili, since three strains (A02, A46, A52) were found to have very high serological identity in this limited study. This is in striking contrast to gonococcal pili, for which no serological identity has been found among the major antigenic determinants of pili from heterologous strains (10, 21).

Significant cross-reactivity was found between *H. influenzae* pili and *E. coli* P pili. This correlates with the high degree of sequence homology between the

pili; however, more extensive serological analysis will need to be carried out to determine if *H. influenzae* and *E. coli* P pili belong to the same antigenic family, or if there is some divergence of the major antigenic determinants.

The proposed purification scheme (Fig. 1) is rapid and simple, using inexpensive reagents. It yields pilus preparations of sufficient purity for many physical and chemical characterization studies. Furthermore, preparations are likely to be adequate for accurate serological analysis since identical serological results were obtained either with antiserum produced against a complete purified pilus suspension or antiserum produced against the major pilus protein band cut from an SDS-polyacrylamide gel. Using this purification procedure, a more extensive serological analysis of pili from many clinical isolates of *H. influenzae* is currently underway.

Summary

Haemophilus influenzae pili were purified, and their physical and serological properties were examined. The solution properties of the pili were determined, and then a purification scheme involving repeated cycles of precipitation and solubilization was developed. The purified pili from one type b isolate (A02) were found to consist of multiple copies of a 25,000 mol wt subunit. Amino-terminal sequence analysis of A02 pili was carried out to 40 amino acid residues, and a remarkable degree of sequence homology was found with *E. coli* P and mannose-sensitive (MS) pili (27.5 and 25% homology, respectively). Purified A02 pili were found to be highly immunogenic, and serological analysis by enzyme-linked immunosorbent assay and whole piliated cell agglutination revealed significant cross-reactivity between A02 pilus antiserum and the pili of seven other *H. influenzae* strains tested (heterologous titers = 2–100% of the homologous titer). Cross-reactivity was also observed between the *H. infleunzae* pili (five of eight strains tested) and the P pili from *E. coli* strains HU849 and 3669; no cross-reactivity was detected with MS pili from *E. coli* strain H10407 and C94. The structural similarities between *H. influenzae* and *E. coli* P and MS pili suggest a common gene ancestry.

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