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Characterization of *Haemophilus influenzae* Type b Fimbriae

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We confirmed that the fimbriae of *Haemophilus influenzae* type b conferred hemagglutinating activity (HA) towards human erythrocytes, and erythrocytes of certain other species. Most (17/25) cerobrospinal fluid isolates lacked detectable HA on direct testing, but selective enrichment for fimbriation (f^+) indicated that 22 of 25 strains could produce these surface structures. HA was unchanged from pH 4.5 to 9.5 and was not inhibited by mannose or certain other simple sugars. The HA titer of a suspension of three f^+ strains was slightly decreased at 50°C; HA was lost by heating at 60°C for 3 min. Growth on a variety of solid and liquid media and under differing degrees of oxygenation did not change the HA titer of a suspension of three f^+ strains. Fimbriation was not lost on repeated subculture. Wild-type fimbriated strains, and those derived by transformation, did not contain detectable plasmid DNA. Transformation of a strain lacking fimbriae to f^+ was associated with the appearance of an outer membrane protein of 24 kilodaltons. This protein was purified from one strain to homogeneity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis by selective detergent solubilization and ammonium sulfate fractionation. Colonization capacity was equivalent with an isogenic untypable strain lacking or possessing fimbriae. Fimbriae of type b *H. influenzae* possess characteristics similar to those structures on other gram-negative bacteria; their role in cell physiology or pathogenesis of invasive disease is unknown.

Fimbriae are nonflagellar surface appendages which do not participate in the transfer of bacterial or viral nucleic acids (7). There may be several hundred peritrichous strands per cell. Fimbriae of *Escherichia coli* mediate adherence to mammalian cells. Often the adherence is associated with hemagglutinating activity (HA), facilitating the screening and categorization of strains. Certain fimbriae mediate HA which is inhibited by mannose (1).

Fimbriated isolates of fastidious invasive human pathogens, such as *Neisseria meningitidis*, frequently lose these structures after in vitro passage (18); this occurs without a change in colonial morphology (18). These structures mediate adherence to buccal epithelial cells which is not inhibited by mannose. The presence of fimbriae on *Neisseria gonorrhoeae* correlates with colonial opacity. Both adherence to human cells and resistance to phagocytosis have been postulated as virulence mechanisms associated with the fimbriae of *N. gonorrhoeae* (21, 24, 28).

Kahn and Gromkova (10) described a piliated *Haemophilus parainfluenzae*: this strain was originally isolated from the upper respiratory tract of a patient and was originally thought to be a *Haemophilus influenzae*. The pili were 8 by 500 nm, and 50 to 100 were present on each cell. Kahn and Gromkova found a correlation between colonial morphology and the percentage of piliated cells, but piliation status per se did not determine colonial morphology. Both piliated and nonpiliated *H. parainfluenzae* were capable of agglutinating human erythrocytes.

Kilian noted that conjunctival isolates of *H. influenzae* had HA (12). Scott and Old confirmed this observation and also studied additional unencapsulated respiratory tract isolates (25). They characterized the HA as (i) greatest at 4°C and eluting at 20°C, (ii) stable to heating at 56°C for 30 min, and (iii) lost with subculture.

Capsulated strains of *H. influenzae* with fimbriae have been recently reported (9, 23). A fimbriated strain of *H. influenzae* type b isolated from the nasopharynx of a child with meningitis was more adherent to human buccal epithelial cells compared with the cerebrospinal fluid (CSF) isolate (9). Other investigators found nasopharyngeal isolates to be f^+, whereas the CSF isolate was f^-. Fimbriated organisms were selected from f^- CSF isolates by enrichment for cells which adhere to erythrocytes (23).

The purposes of our investigations were to further characterize the hemagglutination assay, define the genetic basis of fimbrial expression, purify and partially characterize these structures, and use isogenic transformants to investigate the role of fimbriae in nasopharyngeal colonization.

MATERIALS AND METHODS

***H. influenzae* strains.** Nasopharyngeal type b isolates from healthy children were kindly donated by David Scheifele (Vancouver, British Columbia, Canada) and Sarah Sell (Vanderbilt University, Nashville, Tenn.). CSF isolates were randomly selected from strains received from Peter Wright (Vanderbilt University), Richard Jacobs (University of Arkansas, Little Rock, Ark.), Adnan Dajani (Children's Hospital of Michigan, Detroit), George Ray (University of Arizona Health Sciences Center, Tucson), Melvin Marks (Oklahoma Children's Hospital, Oklahoma City), David Bruckner (UCLA Hospital and Clinics, Los Angeles, Calif.), Joel Ward (Harbor-UCLA Medical Center, Torrance, Calif.), Donald Goldmann (Children's Hospital and Medical Center, Boston, Mass.), and Robert Daum (Tulane University, New Orleans, La.). Strains AO1 and AO2 reported by Guerina et al. (9) were provided by Janet Gilsdorf (Ann Arbor, Mich.). The other CSF isolates were obtained from children treated at Children's Hospital and Medical Center,

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TABLE 1. *H. influenzae* strains used in this study

Strain	Capsule type[a]	Resistance[b]	Reference
MAP	u	Sm, Ery, Spc	5
Ela[c]	b	Sm	27
Rd/pDM2	u	Amp, Tc	17
C54	b	s	23
C47	b	s	23
AO1[d]	b	s	9
AO2[d]	b	s	9

[a] u, Untypable.
[b] s, Sensitive to ampicillin (Amp), chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), erythromycin (Ery), and spectinomycin (Spc).
[c] Strain E_1 described by Smith et al. (27) was grown to the mid-log phase in 1972, divided into samples, and frozen at −70°C. A portion of the stock was thawed immediately before use.
[d] CSF isolate from the same patient who carried the fimbriated nasopharyngeal strain.

Boston, Mass. (1970 to 1978) or at Childrens Orthopedic Hospital and Medical Center, Seattle, Wash. (1978 to 1984). The strains from the first or second subculture were verified as *H. influenzae* and stored as previously described (27). Reference strains are described in Table 1.

Media and growth conditions. Brain heart infusion broth was supplemented with hemin and β-NAD^+ (sBHI) to grow *H. influenzae* strains (31). In certain experiments it was solidified with 2.5% agar (31). Media of defined composition (4) were used for broth cultures or solidified with 2% agarose (Sigma Chemical Co., St. Louis, Mo.) where indicated. In certain experiments the organisms were grown on GC-chocolate agar (Prepared Media, Renton, Wash.). Inoculated plates were incubated at 37°C in 5% carbon dioxide for 18 to 24 h. Standard broth conditions consisted of shaking at 200 rpm in room air at 37°C; for static growth a single colony was inoculated into 10 ml of sBHI broth in a loosely capped tube (12 by 70 mm) and placed upright in air in a 37°C incubator. Anaerobic growth conditions were achieved with a GasPak (BBL Microbiology Systems, Cockeysville, Md.). Biotyping was performed by the Minitek (BBL Microbiology Systems) method (3).

Erythrocytes. Human O^+, AB^-, A^+, and B^+ blood was sterilely collected in a heparinized syringe from laboratory personnel of known blood type. Animal erythrocytes (Prepared Media) and human erythrocytes were diluted with 5 volumes of phosphate-buffered saline (PBS) and harvested by centrifugation at 400 × *g* at 4°C for 10 min. All erythrocytes were washed at least five times in 5 volumes of PBS and resuspended in the same media at a final concentration of 3% (vol/vol).

Chemicals. Inorganic salts were purchased in purest commercial form from Mallinckrodt Inc., St. Louis, Mo. Urea was obtained from Bethesda Research Laboratories, Bethesda, Md. Acrylamide was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and all other reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories, Richmond, Calif. Lyophilized antisera directed against *H. influenzae* capsular types a through f were purchased from Difco Laboratories, Detroit, Mich., reconstituted, and used in slide agglutination testing as recommended.

Hemagglutination assay. PBS (50 μl) containing ca. 10^{10} CFU/ml was mixed with 50 μl of erythrocytes, and the suspension was oscillated at 40 to 50 cpm at room temperature in a humidified environment; human O^+ erythrocytes were used except as indicated. After oscillation for 5 min, readily apparent HA was designated as macro HA, whereas weak HA detected only by inspection of the suspension with an inverted microscope was defined as micro HA. In certain experiments the organisms were geometrically diluted with PBS; 50 μl of the diluted bacterial suspension was added to 50 μl of erythrocytes, and HA was determined as above.

Enrichment of fimbriated organisms. *H. influenzae* which produced fimbriae were selected by two methods. (i) Organisms from a standard sBHI agar plate (streaked for isolation to verify purity) were suspended in 9 ml of PBS, and 1 ml of a 3% suspension of human O^+ erythrocytes was added. The suspension was centrifuged at 40 × *g* for 10 min at room temperature, and the pellet was resuspended in 20 ml of PBS; the pellet was then washed six times by repeated resuspension and centrifugation as above. The final pellet was cultured on sBHI agar. (ii) Fimbriated organisms (f^+) were separated from non-fimbriated organisms (f^-) by isopycnic density gradient centrifugation in Percoll as described by Giesa et al. (8). The density of the f^- (1.080 g/cm^3) and f^+ strains (1.040 g/cm^3) was determined by comparing their banding position to that of reference beads of known density (Pharmacia Fine Chemicals, Piscataway, N.J.). Separation of fimbriated (f^+) and non-fimbriated (f^-) *H. influenzae* by isopycnic density gradient centrifugation was confirmed by mixing 10^8 CFU of E_{1a} f^+ (obtained by erythrocytic enrichment of strain E1 (27) and 10^8 CFU of Rd/pDM2 f^- and plating on selective media. The presence or absence of fimbriae on these strains was confirmed by electron microscopy. Direct testing of the organisms banding at 1.040 g/cm^3 after washing the aspirated band in PBS yielded macro HA. Quantitative culture of the resultant high- and low-density bands on selective antibiotic-containing media demonstrated efficient separation of f^+ and f^- strains.

The procedure adopted to ascertain whether an f^- strain was capable of producing fimbriae consisted of a single adsorption to O^+ human erythrocytes with pelleting at 40 × *g* and overnight culture of the washed erythrocyte pellet on sBHI agar; this culture was harvested by resuspending the organism in 2 ml of PBS and tested for HA. The remainder of the suspension was banded on a preformed Percoll gradient. The less dense band was then subcultured on sBHI agar, again banded on Percoll, and then tested for HA.

Isolation and analysis of outer membranes. Outer membrane protein fractions (OMPs) were prepared by lithium extraction of organisms grown to the mid-log phase as previously described (T. L. Stull, K. Mack, J. E. Haas, J. Smit, and A. L. Smith, submitted for publication). This technique also extracted fimbriae as determined by the presence of an additional major protein band of ca. 24,000 daltons in OMP preparations, evident by SDS-PAGE analysis of strains enriched for fimbriation; fimbrial presence was confirmed by electron microscopy. SDS-PAGE was performed as described by Laemmli (15). Each well contained ca. 7 μg of Lowry-reactive protein (22). Protein bands were visualized by staining with silver as described previously (Stull, submitted for publication).

Electron microscopy. Negative staining was performed as described by McGee (18). Negatively stained samples were examined and photographed in a Zeiss EM9 electron microscope.

Animal experiments. Two groups of Sprague-Dawley weanling rats (Charles River Laboratories, Wilmington, Mass.) were intranasally inoculated with ca. 10^5 CFU of an f^- strain (MAP, Table 1) and a transformant of MAP which was fimbriated (MAP/C54). Each group of animals was housed in isolated cages with separate, filtered ventilation. Two rats from each group were sacrificed on the days

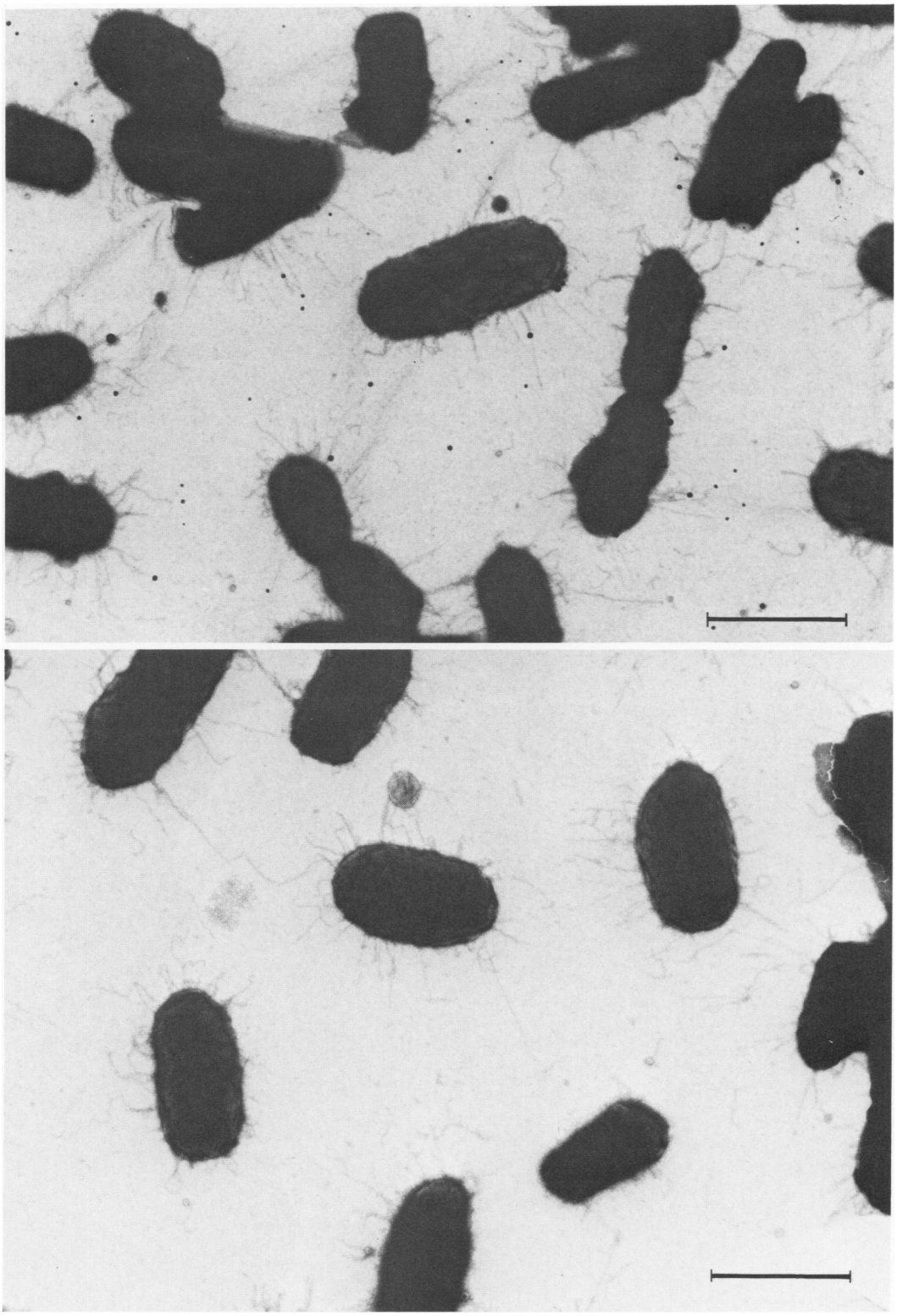

FIG. 1. Negatively stained electron photomicrograph of strains C54 (A) and E1a f^+ (B). Horizontal bars, 10 μm.

specified. Nasopharyngeal swabs and blood (0.1 ml obtained by cardiac puncture) were cultured anaerobically on sBHI agar containing 20 μg of erythromycin per ml.

Transformation. Transformation was performed as previously described (17). The f^+ transformant was selected by erythrocyte absorption and Percoll banding as described above. Selected f^+ strains were lysed, and the DNA was analyzed by agarose gel electrophoresis by the method of Meyers et al. (20).

Fimbriae purification. E1a f^+ (15 to 20 g [wet weight]) was

harvested from solid media; alternatively 10 g (wet weight) of cells was recovered from 2 liters of an overnight culture in sBHI broth. The cells were suspended in a solution consisting of 0.6 M ethanolamine (pH 10) containing 1 mM phenylmethylsulfonyl flouride; 1 g was suspended in 10 ml. The suspension was Omnimixed at 45°C for 15 min at a setting of 2.5, and the mixture was chilled to 4°C. After centrifugation at 20,000 × *g* for 15 min at 4°C, the supernatant (designated S1) was added to an equal volume of a 3% solution of deoxycholate containing 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA and buffered to pH 9.0 with 50 mM glycine. The emulsion was sonified at 15 W for 30 s at room temperature and shaken at 200 rpm at 37°C for 30 min. OMPs were recovered by pelleting at 300,000 × *g* for 1 h at 0 to 4°C (designated P1). The pellet was resuspended in 25 mM Tris-HCl (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (1/50 the volume of the ethanolamine extract) and sonified at 15 W for 30 s at 4°C. The solution was dialyzed against two changes of 500 volumes of 25 mM sodium phosphate (pH 7.2) for 24 h at 4°C. Solid $(NH_4)_2SO_4$ was added to yield a final concentration of 20% (wt/vol) at 4°C; the suspension was centrifuged at 20,000 × *g* for 10 min. Additional $(NH_4)_2SO_4$ was added to the supernatant (S2) to yield a concentration of 40% (wt/vol). The pellet (P3) was collected by centrifugation at 20,000 × *g* for 10 min at 4°C and dissolved in 25 mM sodium phosphate buffer (pH 7.2) at one-half the volume used to suspend the P1 fraction. The P3 fraction was dialyzed overnight against two changes of 500 volumes of 12% (wt/vol) mannose in 25 mM sodium phosphate buffer (pH 7.2) and centrifuged at 30,000 × *g* for 20 min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant (S4) to produce a final concentration of 20% $(NH_4)_2SO_4$. The supernatant (S5) was recovered after pelleting the insoluble material by centrifugation at 20,000 × *g* for 10 min at 4°C. After adjusting the $(NH_4)_2SO_2$ concentration to 40%, fimbriae which were homogeneous on SDS-PAGE (fraction P6) were collected by centrifugation at 20,000 × *g* for 10 min (see Fig. 6 and 7).

RESULTS

Morphology. Four strains (three type b and one untypable) which were fimbriated on primary isolation were examined by electron microscopy after negative staining. An average of 16 fimbriae were detected on each strain. They were 4.7 to 18.0 nm in diameter, the larger value derived from measuring the base, and 209 to 453 nm in length (Fig. 1). A hollow core was evident (Fig. 2). Fimbriae from one strain could not be morphologically distinguished from those present on other strains (Fig. 1A and B).

HA of isolates. Strain MAP was enriched for potential fimbriation by six cycles of adsorption to erythrocytes and Percoll banding. Electron microscopic examination of the band with a density of 1.080 g/cm^3 obtained on the sixth

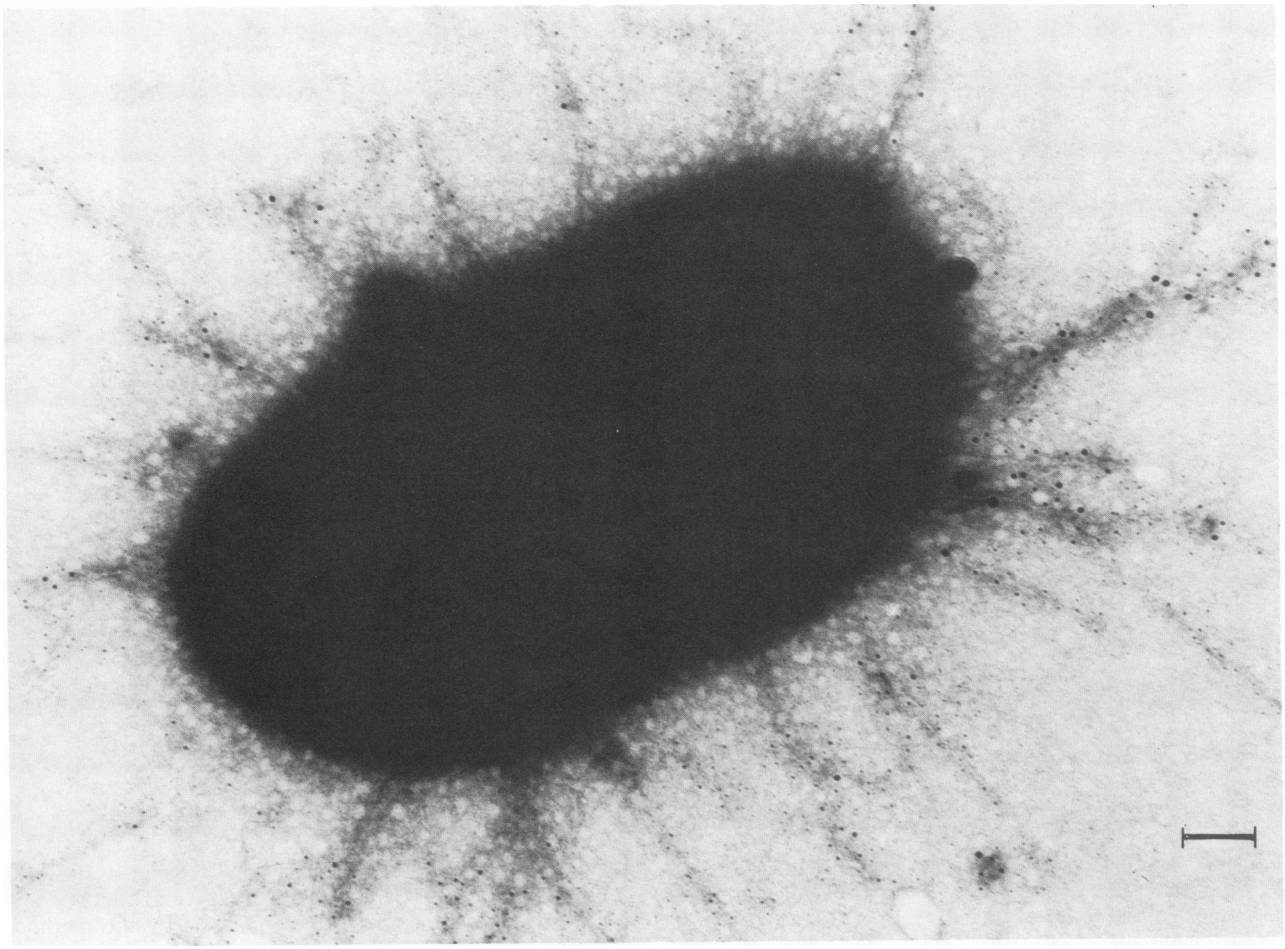

FIG. 2. Negatively stained electron photomicrograph of strain C54. Horizontal bar, 1 μm. Hollow cores are evident.

TABLE 2. HA of *H. influenzae* isolates according to isolation site

Patient source (no. of isolates)	Site[a]	Type[b]	HA		
			None	Micro	Macro
Healthy children (9)	NP	b	7	2	0
Day-care contacts (4)	NP	b	4	0	0
Cystic fibrosis (6)	Sputum	u	4	2	0
Alaskan children (25)	CSF	b	25	0	0
United States meningitis (25)	CSF	b	17	7	1
United States epiglottitis (10)	Blood	b	6	4	0
Healthy children (12)	NP	u	9	1	1

[a] NP, Nasopharynx.
[b] u, Untypable.

cycle did not reveal fimbriated organisms; there was no detectable HA with these bacteria on direct testing or after overnight subculture on sBHI agar. Table 2 shows HA of *H. influenzae* strains according to the anatomic site of isolation. Most anatomic sites had isolates with micro HA; none of the strains isolated from the CSF of Alaskan children had macro HA.

Table 3 shows HA of 25 CSF isolates randomly selected from various regions of the continental United States: all of these strains were biotype I and serotype type b. Approximately one-third of these strains had micro HA, and only one strain showed macro HA on initial subculture. After selective enrichment for cells adhering to erythrocytes of those strains initially lacking detectable HA, 8 (36%) strains had macro HA and 3 (14%) had micro HA. Isopycnic centrifugation of these strains on Percoll yielded low density bands with every strain. HA testing of subcultures of these bands increased the number of isolates with macro HA to 18; 4 (18%) previously lacking detectable HA had detectable micro HA after isopycnic banding. Thus, 22 of 25 CSF isolates of type b *H. influenzae* had detectable fimbriae after selective enrichment.

Correlation of HA with morphology on electron microscopy and OMP profile on SDS-PAGE. Strains MAP, E1a, C47, and C54 were used to correlate HA with the morphology of negatively stained organisms on electron microscopy. Micro HA was found with strain C47, but strain C54 had macro HA. Approximately 1% of the cells of strains E1a and C47 had fimbriae. Several hundred cells of strain C54 were examined, and all had surface fimbriae. Six CSF isolates which did not have detectable HA on initial testing but possessed macro HA after selective enrichment were examined by electron microscopy. All had surface appendages which were morphologically similar to those described in Fig. 1. Fimbriae could not be detected on any cells of strain MAP (Fig. 3): several hundred were examined in parallel with a known f^+ strain (C54).

TABLE 3. HA of *H. influenzae* b isolated from the CSF of United States children

Stage of isolate	HA		
	None	Micro	Macro
Initial isolate	17	7	1
After erythrocyte adsorption	13	4	8
After isopycnic banding	3	4	18

The protein profile of the major OMPs of strain C47 was identical to that of C54 except for the increased amount of a band with an apparent molecular weight of 24,000 (Fig. 4).

Effects of culture conditions. Since *N. meningitidis* and *N. gonorrhoeae* do not express fimbriae after repeated subculture, we investigated HA of strains C54 and E1a f^+ after repeated passage on Catlin agarose, sBHI agar, and GC-chocolate agar. C54 and E1a f^+ maintained macro HA at dilutions of up to 1:128, starting with 10^8 CFU/ml after three passages on GC-chocolate or sBHI agar, or on Catlin agarose, whether grown in an aerobic or anaerobic atmosphere. Macro HA of C54 and E1a f^+ also was maintained after 30 aerobic subcultures on sBHI and chocolate agar with overnight incubation at 37°C. Colonies were examined on sBHI agar by using both reflected and transmitted light; no apparent colonial morphology was correlated with the presence or absence of HA. Additional enrichment of E1a f^+ (with erythrocytic adsorption and Percoll banding) yielded cells which had a coarse granular appearance on sBHI agar; this is the only instance in which colonial morphology correlated with the presence of fimbriae.

Effect of growth in serum. Since type b *H. influenzae* acquire resistance to the bactericidal activity of convalescent rat serum when preincubated in rat or human serum (26), we studied the effect of growth in serum on the HA titers of fimbriated strains. Enriched strain E1a (f^+) grown to a density of 10^8 CFU/ml and tested in PBS at the same density yielded an HA titer of 1:128. A 10-μl volume of a 1:10 dilution in PBS (ca. 10^5 CFU) at the same density was added to 3 ml of sBHI broth, and an identical amount was added to 3 ml of serum from one of the authors (T.L.S.) which had been heated to 56°C for 30 min. After 4 h of incubation at 37°C with shaking at 200 rpm, the bacterial density in the broth was 2×10^8 CFU/ml and in the serum was 8×10^7 CFU/ml. Each suspension had an HA titer of 1:128.

Effect of growth in broth. *N. gonorrhoeae* lose piliation in liquid culture and on certain solid media. We therefore compared static versus shaking liquid culture of f^+ *H. influenzae*. The HA titers of strains E1a, C54, AO2, and MAP/C54 were determined after growth for 18 h in sBHI broth at 37°C with shaking at 200 rpm, and with stationary incubation. The HA titer of a sample removed from the top of the static broth after 18 h of growth was 1:16 with each organism. The HA titer of a sample removed from the bottom of the static broth of each was 1:32. No pellicle was observed with any of these strains after growth in static broth. In parallel flasks containing sBHI broth incubated with and without shaking there was no difference in the HA titer of each strain grown under each condition.

Characterization of HA. C54, E1a f^+, MAP f^- (no detectable HA), and C691 (CSF isolate, micro HA) were tested for HA at 4, 25, and 37°C. The titer of the HA was the same for each strain at each temperature.

Three strains (E1a f^+, C54, and AO2) with macro HA were grown overnight on sBHI agar and serially diluted in PBS to 2×10^9 CFU/ml. They then were tested at 4, 25, 35, 40, 45, and 50°C with the assay temperature increased in a stepwise fashion in a humidified environment. After examination at 50°C for 5 min, the temperature was then reduced to 25°C, and the HA titer again was evaluated with human O^+ erythrocytes. Table 4 shows the HA titer of each strain at each temperature. The titer remained constant for each strain until the temperature of the assay mixture reached

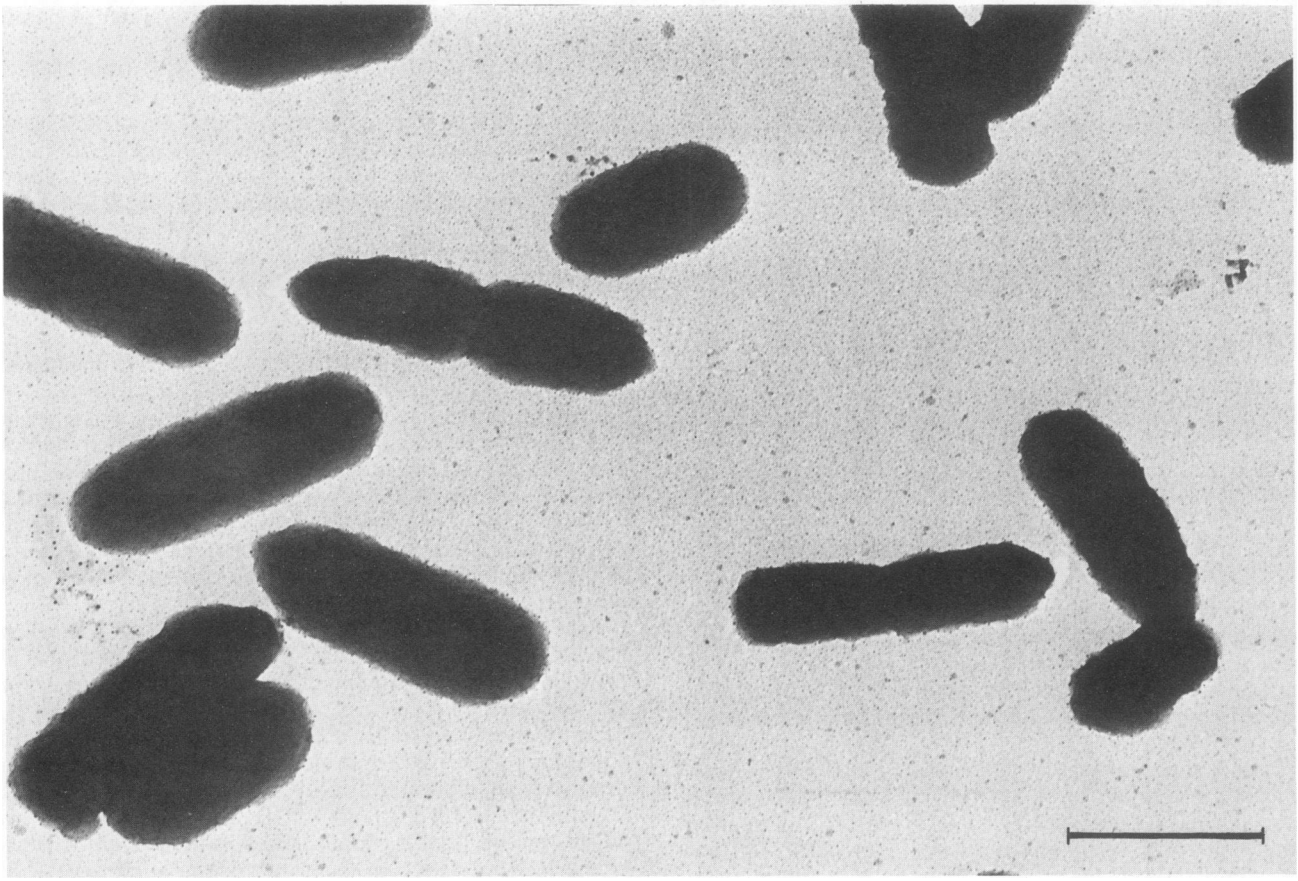

FIG. 3. Negatively stained electron photomicrograph of strain MAP after blind passage through erythrocyte absorption and isopycnic banding in Percoll. Horizontal bar, 10 μm.

50°C; at this temperature the titer decreased. When the assay system was cooled to room temperature, the HA titer again increased to slightly more than the original value (Table 4). Increasing the temperature to 60°C for 3 min destroyed detectable HA except in the undiluted bacterial suspension. The density of viable bacteria in the suspension decreased from 2×10^9 to 3×10^6 CFU with heating at 60°C for 3 min.

The effect of pH on HA was investigated with strains MAP (f^-) and E1a f^+ and MAP/C54 (the latter having macro HA). The assays were performed as described above except that the erythrocytes were suspended in normal saline containing 10 mM Tris-acetate at pH 4.5, 5.5, 6.5, 7.5, 8.5, or 9.5 at 25°C. Each strain had the same HA titer at each pH examined.

Inhibition of HA. D-Mannose, D-glucose, D-Galactose, α-D-fucose, L-Rhamnose, melibiose, D-raffinose, Ribitol, α-mannitol, myoinositol, α-methyl D-mannoside, dextran sulfate, L-alanine, L-ascorbic acid, choline chloride, or urea was added to PBS at a concentration of 2%. HA of strains C54, E1a f^+, and AO2 was assayed as described. None of the compounds produced detectable inhibition of HA. Strain MAP (f^-) did not have detectable HA with or without the above reagents in the hemagglutinating assay.

Erythrocyte specificity. Sixteen of the strains with macro HA after selective enrichment with human O^+ erythrocytes were tested with human AB^-, A^+, B^+, and guinea pig, horse, sheep, chicken, and rabbit erythrocytes. All strains which had macro HA with human O^+ had micro or macro HA against human AB^-, A^+, and B^+ erythrocytes. The strains with macro HA with human O^+ erythrocytes had a variable HA^+ pattern with animal erythrocytes (Table 5). Adsorption on nonhuman erythrocytes, subculturing the adherent cells, and retesting the species-specific HA pattern did not broaden or narrow the erythrocyte specificity of any strain.

Genetic basis of piliation. Six randomly selected CSF isolates which were selected for f^+ (as described) possessed macro HA; these as well as strains C54 and AO1 were examined for plasmid DNA. None had detectable extrachromosomal DNA.

Whole cell DNA from strain C54 was transformed into strain MAP with selection based on erythrocyte absorption and Percoll banding. The resulting strain (MAP/C54) had fimbriae on electron microscopy (Fig. 5). This transformant, which had macro HA, had an additional protein band on SDS-PAGE with a molecular weight of 24,000. This band has the same relative mobility of a protein present in strain C54 but absent in the CSF isolate from the same patient strain C47 (Fig. 4). The fimbriated transformant, MAP/C54, did not have a detectable type b or d capsule when tested for agglutination with specific antisera. This procedure for selective enrichment for fimbriation utilizes hemadsorption, described by Connor and Loeb (6), and the observation that fimbriated strains are less dense than nonfimbriated strains (8).

Effect of fimbriation status on colonizing capacity. Serial nasopharyngeal cultures were performed at 2-day intervals after intranasal inoculation of MAP/C54 (f^+) into weanling

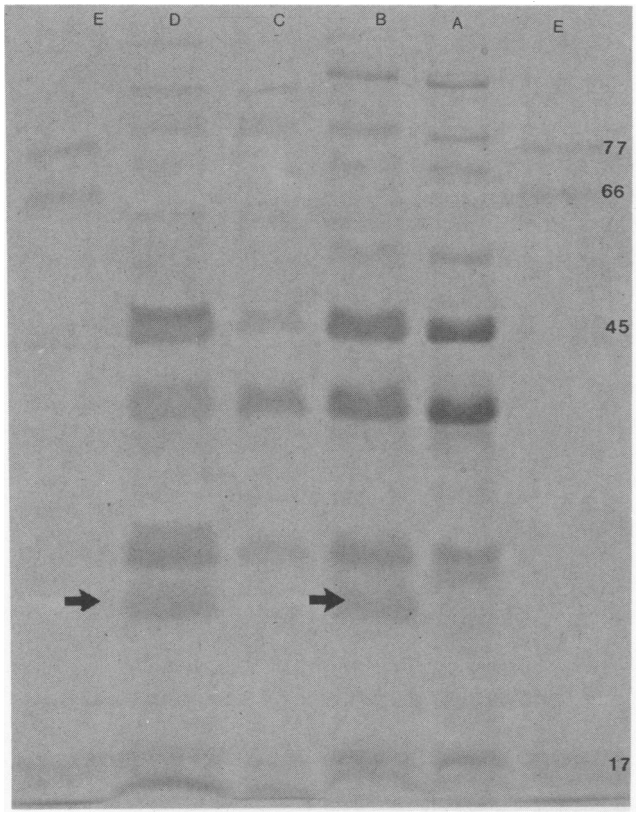

FIG. 4. SDS-PAGE in 10% acrylamide of outer membrane proteins of strains C47 (lane A), C54 (lane B), MAP (lane C), and MAP/C54 (lane D) stained with Coomassie blue. The arrows indicate the new protein band. Lanes E contain molecular weight standards consisting of ovotransferrin (77,000), bovine serum albumin (66,000), and myoglobin (17,000).

rats. All blood cultures obtained simultaneously were sterile. During the 14-day period of observation, there was no difference in the estimated density or duration of colonization of animals inoculated with organisms which expressed fimbriae compared to inoculation with the isogeneic strain (MAP) lacking these structures. All animals in each group were colonized. The observation period was concluded 14 days after inoculation.

Purification of fimbriae. Purified fimbriae from E1a f^+ were obtained by ammonium sulfate precipitation and differential solubilization. Growth from 100 plates yielded ca. 1 mg of purified fimbrial protein. Each step of purification was monitored by SDS-PAGE (Fig. 6), and final purification was verified by (i) the presence of a single band on SDS-PAGE and (ii) electron microscopy (Fig. 7). The approximate molecular weight of the fimbrial subunit was 24,000.

TABLE 4. Effect of temperature on hemagglutination titer with O^+ erythrocytes

Strain	Hemagglutination titer at following temp (°C)					
	4	25	37	45	50	25
C54	64	64	64	64	32	128
E1a f^+	64	64	64	64	32	128
AO2	64	64	64	64	32	64

TABLE 5. Patterns of hemagglutination with isolates from different sites[a]

Site	GMRS	GMRH	GMR	GM	G	MRSH	R
NP[b]	2	1		2	2	1	
CSF			1	3	3		
Sputum				1	1		1

[a] All isolates were selected for fimbriation as described in the text. Erythrocytes agglutinated were species of: G, guinea pig; M, human; R, rabbit; S, sheep; H, horse.
[b] NP, Nasopharynx.

DISCUSSION

In this discussion, we have used the term fimbriae as defined by DuGuid et al. (7): fimbriae are nonflagellar bacterial appendages not involved in the transfer of bacterial or viral nucleic acids. Describing these surface structures on *H. influenzae* as fimbriae is provisional: it may be replaced as new knowledge of the genetics and functions of these surface structures is acquired.

The agglutination of human erythrocytes by *H. influenzae* correlates with the presence of fimbriae demonstrated by electron microscopy and the presence of a 24-kilodalton (KDal) OMP on analysis by SDS-PAGE. This HA is relatively specific for human erythrocytes. An occasional strain had micro HA with erythrocytes of other species; however, no strain had greater HA with erythrocytes of other species than it did with human erythrocytes. This is similar to the findings of Koransky et al. (13) for *N. gonorrhoeae*, although opposite results also have been reported for this species (24). With type b *H. influenzae*, HA is constant over a pH range of 4.5 to 9.5. Virulent piliated *N. gonorrhoeae* show the same phenomenon. In contrast, adherence to human buccal epithelial cells is pH dependent, being maximum at pH 6.5 (30). Experiments are in progress which define the interaction of fimbriated type b *H. influenzae* with human respiratory epithelial cells.

Scott and Old reported mannose-resistant, temperature-sensitive HA of *H. influenzae* isolated from the nasopharynx and trachea (25). Our data extend these observations to *H. influenzae* type b strains isolated from blood and CSF. In addition to mannose, we were unable to inhibit HA with a wide variety of simple organic reagents. It is possible that more complex compounds such as gangliosides may inhibit the HA of *H. influenzae*, as has been demonstrated in *N. gonorrhoeae*. The reduction of HA of untypable *H. influenzae* with increasing temperature reported by Scott and Old has also been extended to invasive strains of *H. influenzae*. We confirmed the decreasing HA with increasing temperature and the recovery of HA with subsequent cooling. We were unable to confirm in type b strains the finding that the HA of *H. influenzae* was stable to heating at 60°C.

In *N. gonorrhoeae*, a 1.54- to 3.08-megadalton fragment of chromosomal DNA encodes for pili (19). In contrast, the genes encoding for an adherence factor in enterotoxigenic *E. coli* reside on a plasmid. *H. influenzae* type b fimbriae could be transferred to an f^- recipient by transformation with whole cell DNA. Neither wild-type f^+ strains, the f^+ transformant (MAP/C54), nor the donor (C54) contained detectable plasmid DNA. This suggests that the genes for fimbriation are chromosomal in location; however, the integration of a plasmid mediating fimbriation in the chromosome is also a possibility.

The role of fimbriae in pathogenesis of invasive disease due to *H. influenzae* is not understood. Kaplan et al. (11) isolated 50 type b *H. influenzae* from the nasopharynx of

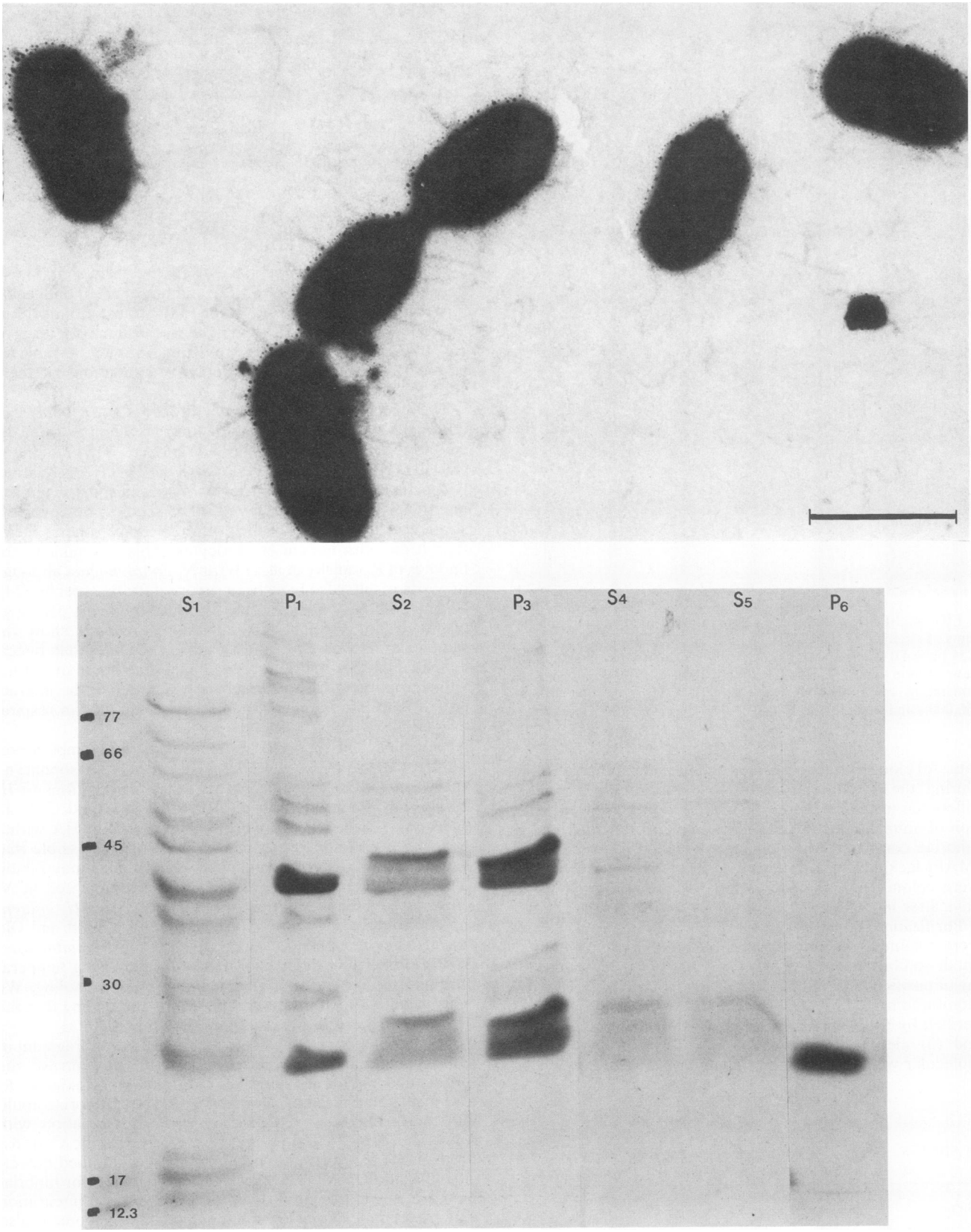

FIG. 6. Electrophoresis of 20 μg of Lowry reactive protein in 10% acrylamide. SDS-PAGE was performed as described in the text with the proteins visualized by Coomassie staining. Each lane heading refers to a fraction obtained during purification (see the text). The apparent molecular weight was calculated from the relative mobility of the following proteins: ovotransferrin (77,000), bovine serum albumin (66,250), ovalbumin (45,000), carbonic anhydrase (30,000), β-myoglobulin (17,000), and cytochrome *c* (12,300).

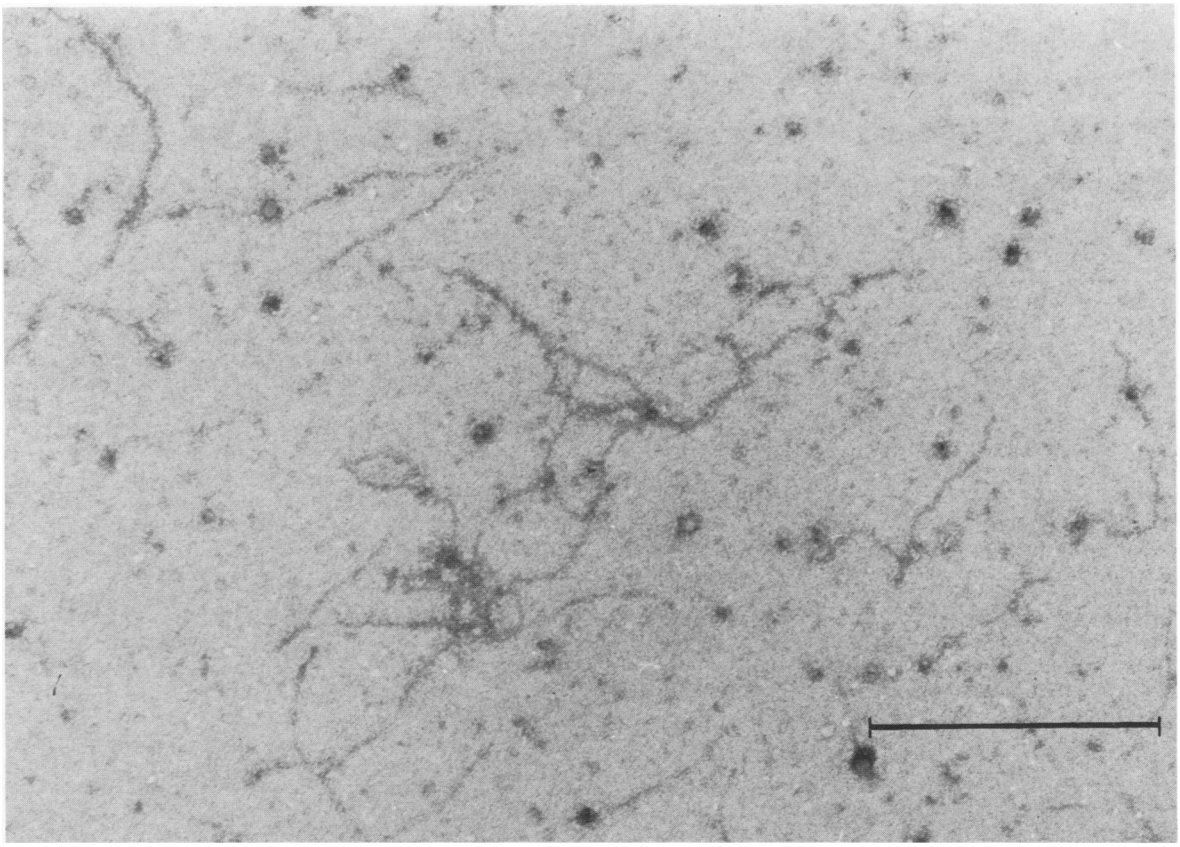

FIG. 7. Negatively stained electron photomicrograph of fimbriae homogenous on SDS-PAGE (pellet P6). See the text for purification scheme. Horizontal bar, 10 μm.

children admitted with *H. influenzae* type b meningitis. Only three of these isolates were piliated, as determined by electron microscopy, and adherent in vitro. We found that no type b's isolated from the nasopharynx (13 examined) were heavily fimbriated. One of fifty CSF isolates (combining the Alaskan and continental United States isolates) had macro HA. Kaplan et al. (11) reported that all 50 CSF isolates they examined by electron microscopy had "low levels of piliation." We found that none of the 13 type b strains isolated from nasopharynx had macro HA; however, we did not perform a systematic survey of strains isolated from respiratory mucosa. Fimbriae are not uniformly present in type b strains isolated from either the nasopharynx or CSF, although 86% of the CSF isolates we examined had the genetic information to express fimbriae: selective enrichment techniques yielded f^+ cells. This indicates that a majority of type b strains have the potential to produce fimbriae.

One potential function of fimbriae is to mediate adherence to respiratory epithelial cells (16). Kaplan et al. (11) compared the adherence of three pairs of type b strains: one from the CSF was HA^-, whereas the nasopharyngeal isolate was HA^+. Adherence in vitro to human buccal epithelial cells occurred only with the piliated, HA^+ strains. Surprisingly, there was no difference in virulence in the infant rat model, as defined as bacteremia incidence, between the HA^- and HA^+ strains (11). However, the conclusion that piliation does not contribute to virulence may not be valid, as piliated *H. influenzae* do not adhere to rat buccal epithelial cells (11). Thus the role of piliation in virulence needs to be tested in an animal model which has the appropriate receptor for *H. influenzae* type b fimbriae.

We have purified fimbriae from a type b *H. influenzae* by solubilizing OMPs in ethanolamine, by disaggregating the suspension with deoxycholate, and by ammonium sulfate fractionation. Tramont et al. (29) used 0.6 M ethanolamine at pH 10.5 to prevent aggregation of gonococcal pili after mechanical shearing. We compared shearing in a variety of buffers and with several suspending agents: urea, guanidinium, and LiCl. We obtained a greater yield of the 24KDa1, fimbrial protein with ethanolamine, but the procedure also extracted other proteins of the outer membrane. Deoxycholate was used by Korhonen et al. (14) to solubilize type 1 *E. coli* pili: the deoxycholate-insoluble fraction lacked the pilus protein. By SDS-PAGE examination of the fractions soluble and insoluble in deoxycholate, we found that the putative fimbrial protein (24 KDa1) and a 39-KDa1 protein were relatively enriched in the insoluble fraction (data not shown). We therefore used the deoxycholate-insoluble material for additional purification steps. The same approach was used in the ammonium sulfate fractionation: examination of the proteins insoluble in 0 to 20%, 10 to 20%, 20 to 30%, and 30 to 40% saturation indicated that the fraction insoluble in 20 to 40% saturation was enriched for the 24-KDa1 protein. Similar to the findings of Altman et al. (2) with the K99 pili of *E. coli*, we found that the *H. influenzae* fimbrial protein was relatively insoluble in 12% mannose. The yield, however, was increased by adding to the mannose solution ammonium sulfate to a final saturation of 40%. We also observed that when a high concentration of purified fimbrial protein was

electrophoresed in 9% SDS–PAGE two bands would appear; their apparent molecular weight was 20,500 and 24,500, respectively. A similar finding has been observed with gonococcal pili (T. Buchanan, personal communication). The reason for this observation is not available.

Future studies will define the role of fimbriae in the pathogenesis of disease due to *H. influenzae* type b.

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