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Purification and Characterization of a Pilin Specific for Brazilian Purpuric Fever-Associated *Haemophilus influenzae* Biogroup Aegyptius (*H. aegyptius*) Strains

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Brazilian purpuric fever (BPF) is a recently described fatal pediatric disease caused by systemic infection with *Haemophilus influenzae* biogroup aegyptius. Previous studies have shown that all *H. influenzae* biogroup aegyptius strains isolated from BPF cases and case contacts share several unique phenotypic and genotypic characteristics that differentiate them from other *H. influenzae* biogroup aegyptius strains isolated from conjunctivitis cases in Brazil. One key characteristic of this BPF clone is reactivity in a BPF-specific monoclonal antibody enzyme-linked immunosorbent assay. We have purified and partially characterized a pilin, referred to as the 25-kilodalton (kDa) protein. Aggregates of this protein contain a heat-labile epitope which is recognized by a monoclonal antibody used in the BPF-specific enzyme-linked immunosorbent assay. The protein has a molecular weight of approximately 25,000, is insoluble in most detergents, and fractionates with outer membrane vesicles after LiCl extraction. Biochemical analysis of the 25-kDa protein shows it to have an amino acid composition similar but not identical to that of the *H. influenzae* type b pilin. The sequence of 20 N-terminal amino acids of the 25-kDa protein shows almost complete homology with the N terminus of the *H. influenzae* type b pilin and the types 1 and P pilins of *Escherichia coli*. Transmission electron microscopic analysis of the purified protein shows the presence of filamentous structures similar in morphology to those of *H. influenzae* pili. Reactivity between the 25-kDa protein and the BPF-specific monoclonal antibody is demonstrated by Western blotting (immunoblotting) and colloidal gold-enhanced immunoelectron microscopy. Hemadsorption analysis shows that expression of this protein is associated with increases in piliated cells and enhanced binding of these cells to human erythrocytes. These studies indicate that expression of the 25-kDa protein is a characteristic unique to the BPF clone and suggest that this protein plays a role in the pathogenesis of BPF.

Brazilian purpuric fever (BPF) is a newly described fatal pediatric disease caused by systemic infection with *Haemophilus influenzae* biogroup aegyptius (4, 5, 8, 9). Except for two cases identified in Australia (27, 29), all known cases of BPF have occurred in the Sao Paulo and Parana states of Brazil. Recently, Brenner et al. (6) reported the results of a comprehensive investigation of *H. influenzae* biogroup aegyptius isolates obtained from children with conjunctivitis and children with BPF in Sao Paulo State during outbreaks in 1985 and 1986. This study concluded that all cases of BPF of Brazilian origin were caused by a unique *H. influenzae* biogroup aegyptius clone, termed the BPF clone, which has the following features: (i) a 24-megadalton plasmid with an identical restriction endonuclease profile, (ii) a characteristic whole-cell sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile, (iii) a characteristic isoenzyme electrophoretic profile, (iv) the ability to agglutinate in rabbit antisera made by immunization with a BPF case strain, (v) a positive enzyme-linked immunosorbent assay (ELISA) reaction in an assay that uses monoclonal antibodies made by immunization with a BPF case strain, (vi) one of two characteristic rRNA hybridization patterns, and usually (vii) resistance to sulfamethoxazole-trimethoprim.

An incidental finding of this study was the identification of a protein with an apparent molecular weight of 25,000 that displayed an unusual charcoal gray color in silver-stained whole-cell SDS-PAGE profiles of BPF clone strains. This protein was initially detected in 59% (27 of 46) of BPF clone isolates versus 8% (3 of 36) of non-BPF clone *H. influenzae* biogroup aegyptius strains of Brazilian origin. Interestingly, the three non-BPF clone strains in which the 25-kilodalton (kDa) protein was detected exhibited some of the BPF clone characteristics and were epidemiologically associated with BPF. They contained a 24-megadalton plasmid with a restriction pattern similar but not identical to that of the BPF-associated plasmid, and all three gave positive results in the BPF-specific agglutination assays and ELISAs. No other non-BPF clone strains exhibited these characteristics. These observations led us to investigate the 25-kDa protein as a potential virulence factor for BPF or as a diagnostic marker for the BPF clone.

In this study we describe the purification of the 25-kDa protein along with biochemical characterization and transmission electron microscopic analysis, which indicate it to be a pilin similar in composition and morphology to other

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Haemophilus pilins. We also show that aggregates of this protein contain a heat-labile epitope recognized by the monoclonal antibody B5E8 that is the basis for BPF-specific ELISAs and latex agglutination assays (3, 6).

MATERIALS AND METHODS

Bacterial strains. Strain F3031 is a BPF clone strain isolated from the blood of a BPF patient. Strain F3049 is a BPF clone strain isolated from the conjunctiva of a patient with conjunctivitis who lived in a town with concurrent BPF. These strains were obtained from the Special Pathogens Reference Laboratory, Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Ga. Strain 34b is a hemagglutination-positive variant of strain F3049. Permanent stock cultures were maintained in brain heart infusion broth supplemented with 50% glycerol at −70°C. Plate cultures were obtained by inoculating chocolate agar plates (BBL Diagnostic Systems, Cockeysville, Md.) and incubating them at 37°C for 18 to 24 h in a candle-extinction jar. For broth culture, bacteria were removed from the plate cultures with sterile Dacron swabs and suspended in 5 ml of sterile phosphate-buffered saline (pH 7.2). These suspensions were inoculated into 2-liter flasks containing 1 liter of brain heart infusion broth supplemented with 1.5 μg of nicotine adenine dinucleotide per ml and 5.0 μg of hemin per ml. The broth culture flasks were incubated overnight at 37°C with gentle shaking.

Purification of 25-kDa protein. The 25-kDa protein was purified from strain F3031 outer membrane vesicles extracted from 20-liter broth cultures by the LiCl method of McDade and Johnston (28) as modified by Gulig et al. (18). The purification protocol is a modification of a method reported by Munson and Granoff (31) for the purification of the *H. influenzae* outer membrane protein P5. Unless otherwise indicated, all chemicals used in the purification procedure were obtained from the Sigma Chemical Co., St. Louis, Mo. The outer membrane extraction was conducted in 0.015 M MES–0.3 M LiCl (pH 6.0), and membrane vesicles were isolated by differential centrifugation [MES is 2-(*N*-morpholino)ethanesulfonic acid; Research Organics Inc., Cleveland, Ohio]. Outer membrane vesicles suspended in phosphate-buffered saline were added to 4 volumes of 4% sodium *N*-lauroyl sarcosine (sarcosyl) and incubated for 2 h at room temperature. Sarcosyl-insoluble outer membrane vesicles containing the 25-kDa protein were removed by centrifugation at 100,000 × *g* for 4 h (type 35 rotor; Beckman Instruments, Inc., Palo Alto, Calif.). The resulting pellet was suspended in 1% octylglucoside (Calbiochem-Behring Corp., La Jolla, Calif.)–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–5 mM EDTA (pH 8.0) (buffer A) at a detergent-to-protein ratio of >20:1, homogenized in a Potter-Elvehjem homogenizer, and incubated for 1 h at room temperature. The octylglucoside-insoluble material that contained the 25-kDa protein was removed by centrifugation at 100,000 × *g* for 2 h (Beckman 75-Ti rotor), homogenized, and resolubilized in buffer A under the same conditions. The resulting octylglucoside-insoluble pellet was suspended in 1% SDS–10 mM HEPES–0.5 M NaCl–0.1% 2-mercaptoethanol (pH 8.0) (buffer B) at a detergent-to-protein ratio of >20:1 and incubated for 1 h at 80°C. The SDS-insoluble material was pelleted at 100,000 × *g* for 2 h at 25°C, homogenized in buffer B, and resolubilized under the same conditions. The resulting pellet, referred to as purified 25-kDa protein, produced a single 25,000-Da band on silver-stained SDS-PAGE gels. This material was used as an immunogen for producing rabbit anti-25-kDa-protein antiserum and analyzed for amino acid content and transmission electron microscopic morphology. Samples for transmission electron microscopy were washed twice in phosphate-buffered saline. Samples for biochemical analysis were solubilized by incubation in buffer B for 5 min at 100°C. Amino acid sequencing was performed on samples dialyzed in 0.5% SDS–0.1 mM dithiothreitol. Amino acid composition analysis was performed on samples dialyzed in 0.01% SDS–50% acetic acid.

Immunologic reagents and Western blots (immunoblots). Polyclonal antiserum directed against the 25-kDa protein was made by immunizing a rabbit subcutaneously with 250 μg of purified 25-kDa protein in phosphate-buffered saline mixed with an equal volume of Freund complete adjuvant. The animal was boosted twice intramuscularly at 21-day intervals with 150 μg of purified protein in Freund incomplete adjuvant. Sera were collected immediately before immunization and 7 days after each booster dose. The BPF-specific monoclonal antibody B5E8 (immunoglobulin G2b) is produced by a murine-hybridoma line derived by fusing X63-Ag 8.653 myeloma cells with spleen cells from BALB/c mice immunized with Formalin-killed cells of strain F3031. Mice were immunized intraperitoneally with 10^8 cells 32 days before fusion. Booster doses of 10^8 cells were given intraperitoneally at 18 days and intravascularly at 1, 2, 3, and 4 days before fusion. The fusion was performed by the method of Claflin and Williams (10) as modified by Helsel et al. (19). Hybridoma supernatants were initially screened for specificity by ELISA against whole-cell lysates of strain F3031 and two noncase *H. influenzae* biogroup aegyptius strains. Specificity for the BPF clone was further established by extensive testing of *H. influenzae* biogroup aegyptius strains obtained during outbreaks of the disease in Brazil (6). Antibody subclass was determined by ELISA, using subclass-specific peroxidase-labeled antibodies (Southern Biotechnology, Birmingham, Ala.). Mouse immunoglobulin G antibodies were purified by affinity chromatography, using protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.) as described by the manufacturer. The monoclonal antibody PEC-1 (41) was obtained from the Immunodiagnostics Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control. This antibody, which is directed against a staphylococcal protein, was used as a negative control for experiments involving B5E8.

Western blotting was performed by the method of Towbin et al. (38) as modified by Cohen et al. (11). Nonspecific binding was blocked by the casein buffer described by Kenna et al. (22), and antibody binding was detected with horseradish peroxidase-conjugated protein A (Bio-Rad Laboratories, Richmond, Calif.) as described by the manufacturer. Prelabeled molecular weight standards (Amersham Corp., Arlington Heights, Ill.) were used for reference on blots.

SDS-PAGE. Separating gels (12.5%) with a 5% stacking gel were used for SDS-PAGE as described by Laemmli (24) with the modifications of Swanson (35, 36). The solubilization buffer consisted of 4% SDS (BDH Chemicals Ltd., Poole, United Kingdom), 8% 2-mercaptoethanol, and 20% glycerol in 1.25 M Tris hydrochloride (pH 6.8). Samples were electrophoresed at 30 mA of constant current for 1.5 h and then fixed overnight in 50% methanol–10% acetic acid. The method of Hitchcock was used for two-dimensional SDS-PAGE experiments (20). Outer membrane vesicles,

extracted from strain F3031 as described above, were incubated in solubilization buffer at 40°C for 1 h and electrophoresed in an 8.5% gel at 30 mA of constant current for 1 h. A slice of the gel containing the separated proteins was then placed in solubilization buffer and boiled for 5 min. After being boiled, the piece was placed at the top of a 12.5% gel and oriented perpendicularly to the direction of the current. Samples consisting of boiled F3031 outer membrane vesicles and molecular weight standards (Bio-Rad) were run on the side of the second-dimension gel for reference. This gel was then electrophoresed at 30 mA of constant current for 1.5 h. All gels were silver stained by the method of Morrissey (30).

Enrichment for hemadsorption. Hemadsorbing (HA^+) colonies were detected by the colony filter hemadsorption method of Connor and Loeb (12), a procedure used to detect colonies of *H. influenzae* type b expressing pili. Briefly, cultures were plated for isolation on chocolate agar, incubated overnight at 37°C in a candle-extinction jar, and then transferred to nitrocellulose. The membranes were blocked with 1% bovine serum albumin and incubated in a 1% solution of freshly obtained human erythrocytes. HA^+ colonies, which appeared red, were selected for further enrichment until a >99% HA^+ variant was obtained.

Electron microscopy. Analysis of the purified 25-kDa protein and whole bacteria was performed by the methods of Stephens et al. (33). Briefly, purified protein or organisms were examined by transmission electron microscopy after being transferred to copper electron microscopic grids and negatively stained with 1% phosphotungstic acid.

For colloidal gold-enhanced immunoelectron microscopy, specimens were glutaraldehyde fixed to copper electron microscopic grids and then incubated at 37°C with B5E8. As a negative control, specimens were reacted with PEC-1. Bovine serum albumin was used to block nonspecific antibody binding. The grids were then stained with gold-protein A conjugates (Auroprobe EM Protein A G15; Janssen Life Science Products, Olen, Belgium).

Analytical methods. Protein concentration was determined by the method of Markwell et al. (26), using a commercially available kit (Pierce Chemical Co., Rockford, Ill.). A bovine serum albumin reference was used to produce a standard curve. Amino acid composition was determined by high-performance liquid chromatography, using the Waters PICOTAG system. The protein was hydrolyzed for 24 h at 110°C in 6 N HCl before composition analysis. N-terminal amino acid sequencing was performed, using the Applied Biosystems GP-407 automated protein sequencer.

RESULTS

25-kDa protein purification. By using the purification protocol outlined above, a yield of 150 to 200 μg of purified protein could be obtained from 20 g (wet weight) of stationary-phase F3031 cells. The protein exhibits a characteristic charcoal gray color on silver-stained SDS-PAGE gels and could be readily detected in both whole-cell and outer membrane profiles. Figure 1A illustrates the effect of solubilization temperature on detection of the 25-kDa protein by SDS-PAGE. Purified protein was solubilized at 100 and 30°C before electrophoresis. The 25-kDa protein is visualized only when solubilized at 100°C. To determine whether this finding resulted from changes in silver-staining characteristics or in electrophoretic mobility, we performed two-dimensional SDS-PAGE (Fig. 1B). This experiment showed a protein that migrated with a high apparent molecular weight after solubilization at 30°C in the first dimension and a molecular

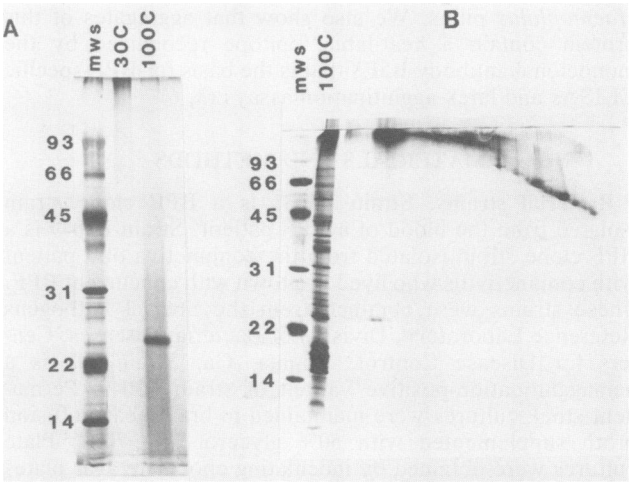

FIG. 1. Effect of heat on electrophoretic mobility of the 25-kDa protein. (A) Silver-stained 12.5% SDS-PAGE gel of purified protein solubilized at 30°C (lane 30C) and 100°C (lane 100C). (B) Two-dimensional silver-stained SDS-PAGE gel of BPF strain F3031 outer membrane vesicles. Vesicles solubilized at 100°C (lane 100C) are shown. The remainder of the gel represents outer membrane vesicles solubilized at 30°C, separated by 8% SDS-PAGE, and then solubilized at 100°C (see text for details). Molecular sizes (mws) are presented in kilodaltons.

weight of approximately 25,000 after high-temperature solubilization in the second dimension. This protein has silver-staining and electrophoretic characteristics identical to those of the 25-kDa protein included in the F3031 outer membrane sample solubilized at 100°C (Fig. 1B, lane 2). These results suggest that the native 25-kDa protein exists in a heat-labile complex with a molecular weight of >200,000.

A transmission electron micrograph of the purified protein is shown (see Fig. 6A). Filamentous structures approximately 2 nm in diameter are observed. These structures are morphologically similar to those of purified pili of *H. influenzae* type b (17, 34, 39).

Biochemical analysis. The amino acid composition of the 25-kDa protein is listed in Table 1. Asparagine and glutamine are converted to the corresponding acids during acid hydrolysis of the protein, so an estimate of amide content could not be made. In addition, cysteine and tryptophan residues were not quantitated. Hydrophobic amino acids, including glycine, alanine, proline, valine, methionine, isoleucine, leucine, and phenylalanine, make up approximately 56% of the protein, which may explain its resistance to solubilization in aqueous solutions. Charged amino acids such as aspartate and glutamate make up at most 25% of the protein. Also included in this table is the amino acid composition of the *H. influenzae* type b pilin as reported by Guerina et al. (17). Although these proteins are similar in overall composition, differences exist in aspartate/asparagine, glutamate/glutamine, serine, and glycine content. N-terminal amino acid sequence data for the 25-kDa protein are compared with the published N-terminal sequence of the *H. influenzae* type b pilin (17) in Fig. 2. From N-terminal positions 3 through 20, these sequences are identical, except for position 4 which is an alanine in the 25-kDa protein and a threonine in the *H. influenzae* type b pilin. Positions 1 and 2 of the 25-kDa protein could not be determined, presumably because of the presence of residual Tris buffer in the sample. This buffer contains primary amine groups which may react with the amino acid labeling reagent.

TABLE 1. Amino acid composition of the *H. influenzae* biogroup aegyptius 25-kDa protein and the *H. influenzae* type b pilin[a]

Amino acid	Approx no. of residues/molecule in:	
	25-kDa protein	*H. influenzae* type b pilin
Aspartate/asparagine	13	41
Glutamate/glutamine	8	25
Serine	25	6
Glycine	34	17
Histidine	3	6
Arginine	2	2
Threonine	22	20
Alanine	28	20
Proline	7	8
Tyrosine	9	8
Valine	24	21
Methionine	7	3
Cysteine	ND[b]	4
Isoleucine	18	9
Leucine	16	15
Phenylalanine	6	10
Lysine	26	21
Tryptophan	ND	ND

[a] As reported by Guerina et al. (17).
[b] ND, Not determined.

Hemadsorption activity. Strain F3049 is a BPF clone strain which is negative for the 25-kDa protein when analyzed by whole-cell SDS-PAGE. Using the colony filter hemadsorption assay for detecting HA^+ colonies, we found approximately 0.1% of F3049 colonies to be HA^+. By subculturing the HA^+ colonies and reselecting HA^+ variants, we could isolate a variant, designated 34b, which gives rise to >99% HA^+ colonies. Western blotting (Fig. 3) of strains F3049 and 34b with polyclonal antiserum raised against purified pilin showed expression of the 25-kDa protein in the HA^+ variant but not in the HA^- variant. Strain F3031 (Fig. 3, lane 1) was included as a 25-kDa-positive reference. The bands observed at 40, 70, and 80 kDa represent antibodies also detected in the preimmune serum of the animal. An equivalent silver-stained SDS-PAGE gel of these variants showed the presence of the 25-kDa protein in 34b but not in F3049 (data not shown). This represented the only observable difference in SDS-PAGE profiles between the two variants. We have attempted to apply the colony filter hemadsorption assay to non-BPF clone strains to determine whether these strains also contain 25-kDa-positive subpopulations. However, these strains produced virtually 100% HA^+ colonies in the absence of 25-kDa-protein expression, suggesting that they have other mechanisms of erythrocyte binding.

Transmission electron microscopy was used to determine whether morphologic differences exist between the 25-kDa-negative strain F3049 and its 25-kDa-positive variant 34b. Representative views are presented in Fig. 4. Strain F3049 (Fig. 4A) is essentially unpiliated, although rare (<1%) piliated forms were observed. Strain 34b (Fig. 4B) is piliated. The piliated cells contained between 5 and 15 observable pili per cell. The pili were morphologically similar to *H. influenzae* type b pili as previously described (17, 34, 39), with pilus diameters ranging from 2 to 5 nm and lengths ranging from 50 to 250 nm.

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Position    1   2   3   4   5   6   7   8   9  10  11  12  13
Hae 25Kd  XXX XXX ASN ALA GLU THR SER GLY LYS VAL THR PHE PHE
Hfl pilin SER ILE ASN THR GLU THR SER GLY LYS VAL THR PHE PHE

Position   14  15  16  17  18  19  20
Hae 25Kd  GLY LYS VAL VAL GLU ASN THR
Hfl pilin GLY LYS VAL VAL GLU ASN THR
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FIG. 2. N-terminal amino acid sequence of the 25-kDa protein (Hae 25Kd) and *H. influenzae* type b (Hfl) pilin (17). XXX, Not determined.

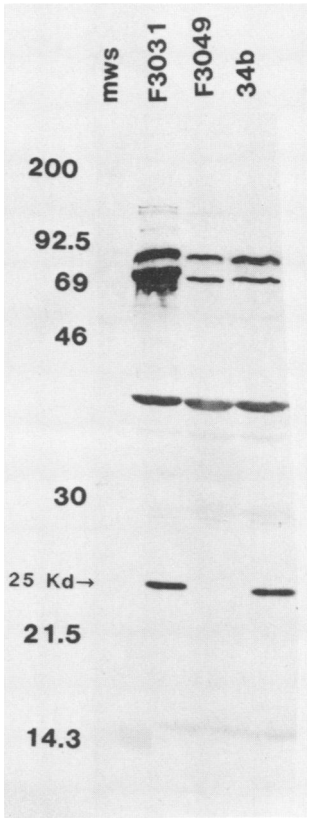

FIG. 3. Association between 25-kDa-protein expression and erythrocyte binding. Western blots of BPF clone strains F3031 (25-kDa positive), F3049 (25-kDa negative), and 34b, a 25-kDa-positive hemagglutinating variant of F3049. Blots were developed with rabbit anti-25-kDa-protein antiserum. Molecular sizes (mws) are expressed in kilodaltons.

Reactivity of the 25-kDa protein with B5E8. When purified 25-kDa protein and strain F3031 were reacted with monoclonal antibody B5E8 in Western blotting experiments, no reactivity was observed when the antigens were solubilized at 100°C (Fig. 5). When the antigens were solubilized at 30°C, however, high-molecular-weight complexes were observed, suggesting that the antibody recognizes a heat-labile epitope present in aggregates of the 25-kDa protein.

The recognition of the 25-kDa protein by B5E8 was further investigated, using colloidal gold-enhanced immunoelectron microscopy (Fig. 6). Native 25-kDa protein self assembled into filamentous structures 2 to 5 nm in diameter and approximately 150 to 200 nm in length (Fig. 6A). When exposed to B5E8, these structures formed aggregates 10 to 20 nm in diameter (Fig. 6B). Specific antibodies binding to these aggregates were detected with colloidal gold-protein A conjugate (Fig. 6C). Similar results were obtained when whole-cell samples of strains F3031 and 34b were examined (data not shown). Control grids, which were developed with either no antibody or the unrelated monoclonal antibody

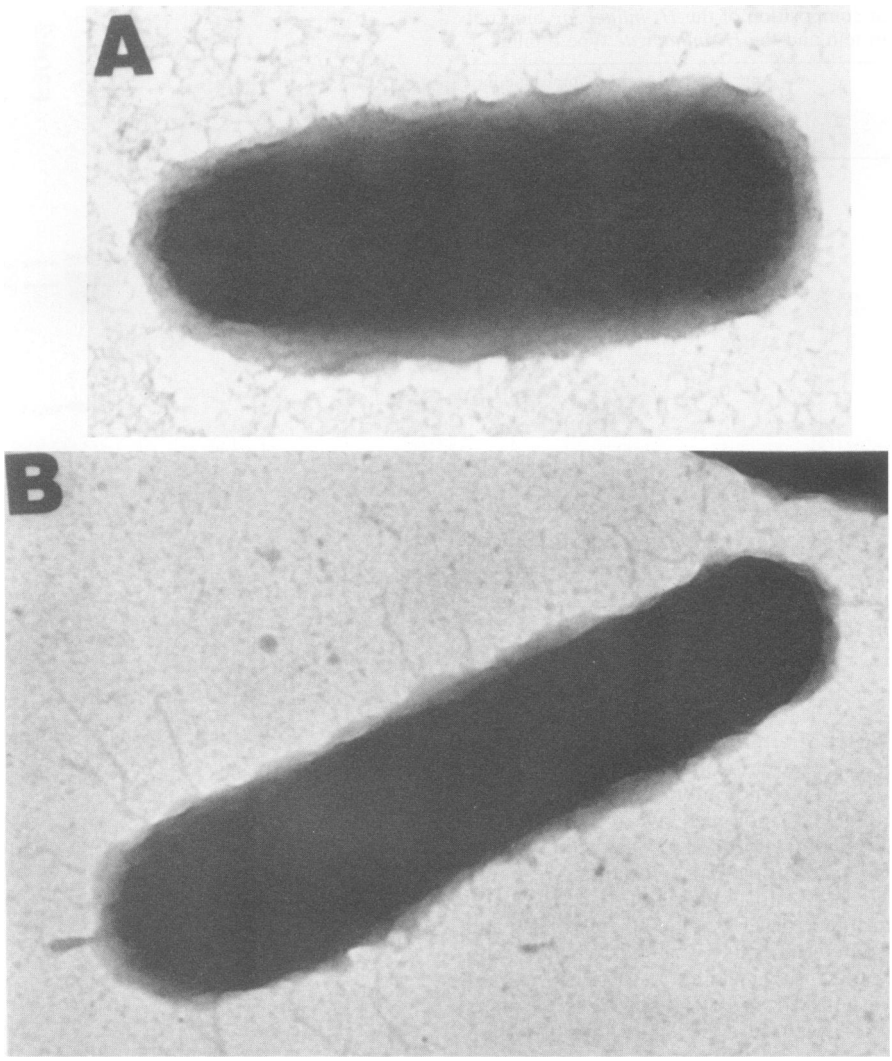

FIG. 4. Association between 25-kDa protein expression and piliation of BPF clone strains. (A) Transmission electron micrograph of 25-kDa-negative BPF clone strain F3049; (B) transmission electron micrograph of 25-kDa-positive variant 34b. Magnification, ×80,000.

PEC-1, showed no evidence of either pilin aggregation or protein A-colloidal gold binding.

DISCUSSION

This paper describes the purification and characterization of a BPF-associated *H. influenzae* biogroup aegyptius 25-kDa protein. This protein plays a key role in the detection of BPF clone strains. Taken together, these studies indicate that the 25-kDa protein is a pilin. The SDS-PAGE experiments show this protein to exist in either a high-molecular-weight aggregate or a dissociated subunit form. Before heat treatment, the protein molecules are assembled into polymeric structures resembling *Haemophilus* pili by electron microscopy (17, 34, 39). These structures are resistant to dissociation by many detergents and reducing agents, such as 2-mercaptoethanol, suggesting that the polymeric form is stabilized by hydrophobic interactions. Similar findings have been reported for pili of *H. influenzae* type b (17) and other species, including *Pseudomonas aeruginosa* (40) and *Escherichia coli* (14). These interactions between adjacent subunits probably involve specific amino acid sequences which are hydrophobic in nature and highly conserved among species (14, 16). Recently, Abraham and Beachey illustrated the structural importance of these conserved regions by using a 13-residue synthetic peptide corresponding to N-terminal amino acids 13 through 35 of the *E. coli* type 1 pilin to assemble aggregates in vitro which were recognized by pilin-specific monoclonal antibodies (1).

The amino acid composition of the 25-kDa protein appears to be unique, although it shares many similarities with the *H. influenzae* type b pilin (17) and the *E. coli* type 1 and P pilins (2, 32). N-terminal amino acid sequencing shows the 25-kDa protein to have a striking degree of homology with the N terminus of the *H. influenzae* type b pilin (17) and also to share a highly conserved region from N-terminal positions 8 through 17 with *E. coli* type 1 and P pilins (32). As with its counterpart in *H. influenzae* type b, the 25-kDa protein is probably a major structural subunit of pili.

The ability to select a piliated 25-kDa-positive subpopulation from a largely nonpiliated 25-kDa-negative BPF clone strain by hemadsorption demonstrates an association between the expression of this protein and the ability to bind

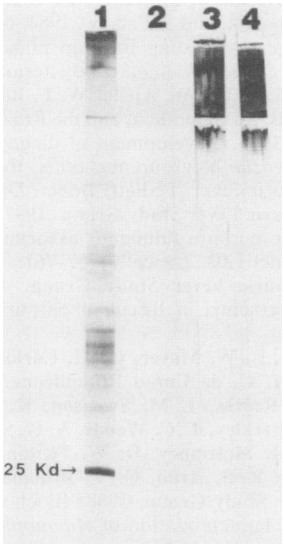

FIG. 5. Western blot of anti-25-kDa-protein rabbit antiserum and BPF-specific monoclonal antibody B5E8 against the 25-kDa protein solubilized at 30 and 100°C. Lanes 1 and 2, protein solubilized at 100°C; lanes 3 and 4, protein solubilized at 30°C. Lanes 1 and 3 were developed with anti-25-kDa antiserum, and lanes 2 and 4 were developed with B5E8.

human erythrocytes. On the basis of our data, it is not possible to determine whether the erythrocyte adhesin is the 25-kDa protein itself or some minor component of the pilus complex coexpressed with this protein. We have applied the colony filter hemadsorption assay to numerous other 25-kDA-negative BPF clone strains and have observed the same results as those obtained with strain F3049. These findings suggest that all BPF clone strains either express the 25-kDa protein or contain small 25-kDa-positive subpopulations which may be expanded in response to environmental stimuli. The rate of transition from the HA^- phenotype to the HA^+ phenotype observed in this study (10^{-3} per cell per generation) is similar to that reported for pili of *H. influenzae* type b (M. M. Farley, D. S. Stephens, S. L. Kaplan, and E. O. Mason, Jr., Abstr. Clin. Res. 1988, p. 20) and *E. coli* (13). This suggests that, as reported for pili of other species (14), expression of the 25-kDa protein is a function of some mechanism of phase variation.

The fact that this protein is only observed in plasmid-bearing strains suggests that either the protein is encoded by the plasmid or that the plasmid in some way regulates the expression of the protein. A striking example of this association was observed in a series of *H. influenzae* biogroup aegyptius strains obtained from children with unconfirmed BPF in the Brazilian town of Pradopolis (27). These strains express some, but not all, of the BPF clone characteristics. They agglutinate in the BPF-specific polyclonal antiserum

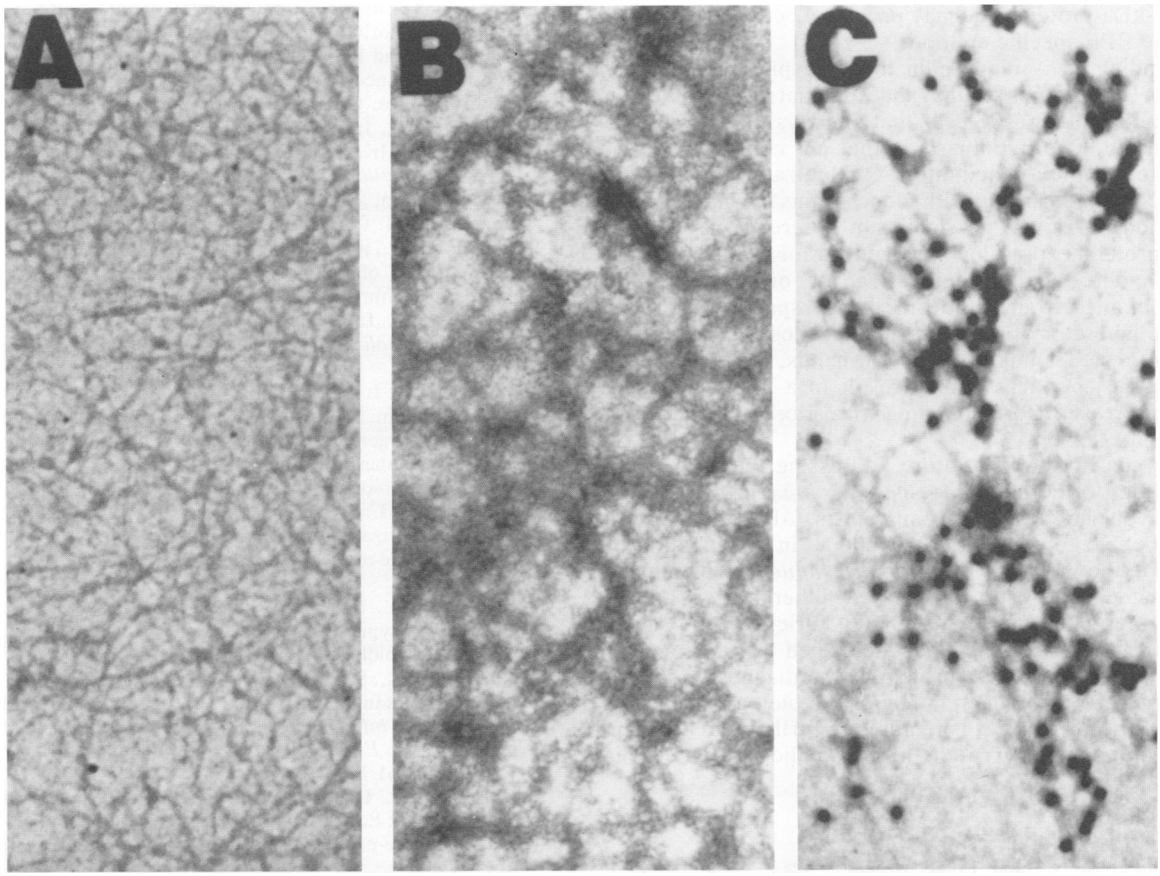

FIG. 6. Colloidal gold-enhanced immunoelectron micrographs of purified 25-kDa protein reacted with the BPF-specific monoclonal antibody B5E8 and 15 nm of colloidal gold-protein A conjugate. (A) Unreacted protein; (B) protein reacted with B5E8; (C) protein reacted with B5E8 and then with colloidal gold-protein A conjugate. Magnification, ×100,000.

and produce BPF-specific SDS-PAGE, isoenzyme electrophoretic, and rDNA hybridization patterns. However, they fail to react in the BPF-specific ELISA, and they do not contain plasmids. Analyses of these strains by colony filter hemadsorption, SDS-PAGE, and reactivity with anti-25-kDa-protein antiserum have failed to detect 25-kDa-positive subpopulations. Previous studies of other species have identified regulatory genes that, under environmental influence, control expression of virulence determinants by release of *trans*-acting factors (7, 23, 37). In *Vibrio cholerae*, for example, pili and cholera toxin are coordinately regulated by a single gene, *toxR* (37). These regulating genes can be carried on plasmids and activate chromosomal genes after acquisition of the plasmid.

The experiments described in this paper show conclusively that the monoclonal antibody B5E8 recognizes a heat-labile epitope present on aggregates of the 25-kDa protein. This antibody forms the basis of ELISAs (3, 6) and latex agglutination tests (3) that have been developed to detect BPF clone strains. Western blotting of purified 25-kDa protein with B5E8 suggests that the monoclonal antibody recognizes an epitope present on assembled pili but absent on the dissociated monomeric subunits. Anti-pilin antibodies with similar reactivity patterns have been reported for type 1 pili of *E. coli* (1, 15). The epitope recognized by B5E8 may be either a quaternary structure formed by amino acids of adjacent subunits or a tertiary structure composed of amino acids from a single subunit that is stabilized by the interactions of adjacent subunits. Molecular cloning, nucleotide sequence analysis, and peptide mapping of the 25-kDa-protein gene may provide a better understanding of the BPF-specific epitope.

The finding of a unique pilin that is expressed preferentially in BPF clone strains and associated with human erythrocyte binding suggests that it plays a role in BPF pathogenesis. One hypothesis is that pili composed of 25-kDa-protein subunits facilitate the colonization of mucosal surfaces. Of the Brazilian BPF clone strains in which the site of isolation is known, no significant difference is found in 25-kDa-protein expression between strains obtained from mucosal sources, i.e, conjunctiva, or oropharynx, and strains obtained from blood or cerebral spinal fluid. However, the laboratory passage history of these strains before they were tested is unknown. In our hands, multiple laboratory passages of these strains result in a loss of 25-kDa-protein expression corresponding with a loss in hemadsorption activity.

A growing body of literature addressing the role of pili with respect to the pathogenesis of *H. influenzae* has been reported, but no clear consensus exists. Although piliation does not seem to confer any advantage in colonization or invasion in the infant rat model for *H. influenzae* meningitis (21), piliated forms show increased adherence to human nasopharyngeal cells in culture (25; Farley et al., Abstr. Clin. Res. 1988). The studies described here show the importance of the 25-kDa protein in the diagnosis of BPF; however, a greater understanding of the biology of pili and human epithelial surfaces is required to further elucidate the role, if any, of this protein in the pathogenesis of this disease.

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