INFECTION AND IMMUNITY, Feb. 1991, p. 600–608
0019-9567/91/020600-09$02.00/0
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Vol. 59, No. 2

Contribution of a 28-Kilodalton Membrane Protein to the Virulence of *Haemophilus influenzae*

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Received 21 March 1990/Accepted 14 November 1990

A *Haemophilus influenzae* b (Hib) membrane protein with a molecular mass of 28 kDa bound polyclonal antisera raised against a highly purified Hib fimbrial subunit. We cloned the gene encoding this protein and found that the gene was expressed in *Escherichia coli*. DNA sequence analysis identified an 843-bp open reading frame which predicted a 26.78-kDa protein with an amino-terminal signal sequence and a mature protein with 70% similarity to the 28-kDa lipoprotein of *E. coli* (F. Yu, S. Inouye, and M. Inouye, J. Biol. Chem. 261:2284, 1986). Colony blot hybridization analysis with an intergenic probe of the cloned gene demonstrated that 29 of 32 *H. influenzae* strains hybridize with this gene. Insertion of a chloramphenicol acetyltransferase gene into the open reading frame inactivated expression of the 28-kDa protein in *E. coli*. Isogenic Hib strains were derived by marker exchange mutagenesis to generate mutants which no longer expressed the 28-kDa protein as recognized with Western immunoblot analysis. There was no difference in the rate of nasopharyngeal colonization of infant rats or monkeys by the isogenic mutants which lacked the 28-kDa protein compared with colonization by the wild-type strain. In contrast, the frequency of invasion and density of bacteremia in infant rats caused by the isogenic mutants were reduced relative to those caused by the wild-type Hib strain. We conclude that this 28-kDa outer membrane protein aids transepithelial invasion of type b strains but is not essential.

Invasive *Haemophilus influenzae* type b (Hib) disease is an important health problem. It is the most common cause of bacterial meningitis in the United States and is an important agent causing septic arthritis, pneumonia, and bacteremia in children (7). Infants rarely produce antibodies to polyribosyl-ribose-phosphate (PRP) after an invasive Hib infection, and vaccines consisting solely of the carbohydrate capsule of Hib are not reliably immunogenic in children less than 18 months of age, the target population at the greatest risk. An alternative vaccine approach is to make the type b carbohydrate capsule, PRP, T dependent by coupling it to a protein. Current conjugate vaccines consist of PRP-tetanus toxoid (37), PRP oligosaccharide coupled to CRM (a mutant diphtheria toxin) (1), PRP-diphtheria toxoid mixture (22), or a meningococcal outer membrane protein (OMP) coupled to PRP (25). Resultant carbohydrate-protein conjugates have been administered to infants as young as 2 months and have been shown to be immunogenic (1, 22, 25, 37). Since antibodies directed against Hib OMP are protective in animal models of Hib invasive disease (24), a conjugate vaccine composed of a *Haemophilus* OMP and the capsular carbohydrate might afford additional protection.

No single OMP on wild-type strains is consistently antigenic after bacteremia and meningitis; however, antibodies to a variety of OMPs are found after bacteremic Hib infections (13, 17). This has led to the search for a highly conserved, antibody-accessible OMP or OMP epitope as a candidate for conjugating to PRP. Candidate OMPs from Hib (8) have included the major outer membrane porin, a 39-kDa protein, subsequently called P2 (3) or "b" (27); a 46-kDa OMP identified as P1 (3) or "a" (27); and a 16-kDa protein associated with lipid, identified as P6 (3) or "g" (27), the last presumably analogous to the Braun transmembrane lipoprotein of *Escherichia coli* (11).

The ideal OMP from Hib to be used as a vaccinogen should be highly conserved. In studies of the immunogenicity of whole bacteria in rabbits, our associates found that a 28-kDa membrane protein is present in a variety of haemophili, including nontypeable *H. influenzae* as well as *Haemophilus parainfluenzae*, and appears to be antigenically conserved (12). Antibodies directed against a 28-kDa protein present in LiCl-EDTA extracts have been identified in the sera of patients convalescing from Hib meningitis in several studies (13, 17). We found that rabbit polyclonal antibody raised to the purified pili of Hib cross-reacted with a 28-kDa protein in the same strain. Since other pilus-associated proteins have a role in adherence (28), we sought to examine the role of this protein in virulence for infant rats and monkeys.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *H. influenzae* strains used in these experiments are described in Table 1. Growth media used for *H. influenzae* consisted of brain heart infusion (BHI) broth supplemented with hemin hydrochloride (10 μg/ml) and β-NAD (10 μg/ml) (BHI) (39). Solid medium was prepared from the same broth but containing 1.5 g of agar per 100 ml; streptomycin at 500 μg/ml was added when strain E1a and its derivatives were studied. *E. coli* HB101 and DH5α were used for cloning experiments. *E. coli* strains were grown on L agar containing the appropriate antibiotics. A *Hae*II fragment from plasmid pACYC 184 (6) was used as the source of the chloramphenicol acetyltransferase (CAT) gene. The cosmid vector pHC79 (19) and plasmid vector pUC 18/19 derivatives (43) were used for subcloning experiments.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
E. coli		
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	BRL[a]
DH5α	F^- *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^-) *relA1* Δ(*lacZYA-argF*) *U169* φ80 d *lacZ*ΔM15 *supE44* λ^-	BRL
Hib		
E1a	Fm^-	40
R1369	Fm^+ derived from E1a	41
R1010	Fm^+ (called A02 in original publication)	16
C54	Fm^+	33

[a] BRL, Bethesda Research Laboratories.

Immunization. Pili were purified from strain R1369 as described previously (41). This preparation yielded a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. One milligram of Lowry reactive protein was suspended with an equal volume of complete Freund adjuvant and inoculated subcutaneously into weanling New Zealand White rabbits. Prior to immunization, blood was collected from an ear vein and the serum was harvested. Two weeks following the subcutaneous inoculation, 500 μg of subunit protein per ml was suspended in phosphate-buffered saline (PBS) and infused intravenously. One month following this booster dose, blood was collected and the serum was harvested.

Serum was adsorbed three times with boiled HB101 and tested for reactivity with purified pili by enzyme-linked immunosorbent assay (ELISA) with 1 μg of protein per well of the microtiter tray. The ELISA was performed by the method of Chandler et al. with alkaline phosphatase-conjugated goat rabbit immunoglobulin G for detection (5).

Female Swiss-Webster mice, 6 to 8 weeks old, were immunized by subcutaneous injection of 30 μg of purified fimbrial subunit protein in complete Freund adjuvant. Thirty days later, each mouse received a booster dose of the same amount of purified protein in PBS by intraperitoneal injection. Thirty days later, blood was obtained from each mouse and tested for antifimbrial antibodies by ELISA. The ELISA was performed in the manner described above except that goat anti-mouse immunoglobulin G was the secondary antibody. Because of prior work in this laboratory, we used ascitic fluid derived from immunized mice as a source of antibody. Ascites was established in mice with ELISA titers against the subunit protein of >1:4,000 by intraperitoneal injection of 180/TG sarcoma cells (35). Ten days later, ascitic fluid was obtained by paracentesis and centrifuged at 10,000 × *g* for 10 min; the ascitic-fluid supernatant was used as a source of antibodies against the pili.

Western blot analysis. Western immunoblotting was performed as follows. After SDS-PAGE by the method of Laemmli (23) in 10% acrylamide, the sample was electrophoretically transferred to Immobilon (Millipore Corp., Bedford, Mass.), nitrocellulose, or nylon-backed nitrocellulose (Nitroplus 200; MSI, Inc., Westboro, Mass.). The transfer buffer consisted of 25 mM Tris, 150 mM glycine, and 20% methanol adjusted to pH 8.2. Molecular size standards radiolabeled with ^{14}C were also electrophoretically separated and transferred to estimate M_rs. Membranes were stained with Ponceau S to determine the locations of relevant bands after electrophoretic transfer and before Western blotting. The membrane was then immersed in a blocking solution consisting of 3% bovine serum albumin (BSA) in TS buffer (50 mM Tris chloride, pH 7.5, 150 mM NaCl) for 60 min at room temperature. The membranes were immersed for 60 min at room temperature in rabbit or mouse antifimbrial antibody diluted 1:100 in the blocking solution. The membranes were subsequently washed in TS containing 0.1% SDS at room temperature for 10 min, and then 5-min washes in TS were performed five times. After immersion in the above-described blocking solution for 30 min, each membrane was exposed to 100,000 dpm of ^{125}I-protein A in the blocking solution for 60 min to detect bands which had absorbed the rabbit antibodies. Membranes were dried and autoradiographed for ~18 h. Membranes which had been reacted with mouse antisera were subjected to the detection system which used alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Bio-Rad, Richmond, Calif.) as a secondary antibody. After reacting with the secondary antibody, the membrane was washed twice for 5 min in Tris-buffered saline containing 0.5% Tween at room temperature and then washed once for 5 min in Tris-buffered saline. Each membrane was then immersed in a developing solution consisting of 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium in 0.1 M carbonate buffer containing 1 mM magnesium, pH 9.8, as recommended by the manufacturer.

Colony immunoblot. *E. coli* colonies which expressed *H. influenzae* antigens binding antipilus antisera were detected by a colony immunoblot procedure. Ampicillin-resistant colonies were transferred to nitrocellulose, lysed by treatment with 0.5 N sodium hydroxide for 5 min, washed with 5 M Tris chloride at pH 7.5 for 5 min, washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0), and fixed with 70% ethanol for 5 min at room temperature. The membrane was dried and incubated at 80°C for 1 h. The membrane was then exposed to 3% BSA in Tris-buffered saline for 1 h. Postimmunization rabbit antiserum at dilutions from 1:25 to 1:200 in TS containing 3% BSA were applied to the membrane for 1 h at room temperature. The membrane was then washed with 0.1% SDS in TS for 10 min and four times with TS for 5 min each, all at room temperature. The membrane was then exposed to ^{125}I-protein A in TS for 1 h at room temperature (10^5 cpm added to each membrane). It was then washed repeatedly with TS containing 3% BSA at room temperature, dried, and autoradiographed.

Cloning. High-molecular-weight DNA was prepared from *H. influenzae* R1369 by the method of Hull et al. (21). DNA was then partially digested with *Sau*3A and fractionated on a sodium chloride gradient as described elsewhere (38). Fragments ranging in size from 20 to 40 kb were then ligated to *Bam*HI-cleaved pHC79 (19). DNA from this ligation was packaged with a commercial bacteriophage lambda packaging extract (Stratagene) and used to transduce *E. coli* HB101. Ampicillin-resistant transfectants were screened by colony immunoblot for reactivity with rabbit antisera prepared against purified Hib pili. Western blot analysis was used to confirm expression of a 28-kDa protein reactive with the antifimbrial antisera.

Deletions of recombinant DNA in pHC79 were created by digestion with restriction endonuclease (Bethesda Research Laboratories, Gaithersburg, Md., and New England BioLabs, Beverly, Mass.), followed by religation with T4 DNA ligase. This resulted in a recombinant plasmid containing a 10.9-kb *Ava*I fragment expressing the 28-kDa protein; this

plasmid was designated pMCC1. The location of the 28-kDa gene was determined by mapping λTn*5* insertions (λ *b*221 *rex*::Tn*5 c*I857, *O*am8, *P*am29 [9]). When the location of the gene encoding the 28-kDa protein was determined, a 5.2-kb *Cvn*I-*Sst*I fragment was subcloned into pUC18; the recombinant plasmid was designated pARS1. Deletions in pARS1 were created with *E. coli* exonuclease III and cloned into bacteriophage M13 derivatives for sequence analysis (43).

DNA sequence analysis. DNA sequence analysis was completed for both strands by the method of Sanger (34). The Klenow fragment of *E. coli* polymerase (Boehringer Mannheim, Indianapolis, Ind.) or avian myeloblastosis virus reverse transcriptase was used for sequencing with [^{35}S]5′ α-thio-dATP purchased from New England Nuclear Corp. (Boston, Mass.) (29). The universal primer for M13 was purchased from New England BioLabs, while other primers were based on derived sequences; 12- to 20-mers were synthesized on a DNA synthesizer (Biosearch BioSystems). Sequence data was analyzed on a VAX/VMS computer using the University of Wisconsin Genetics Computer Group program (10).

DNA probe preparation and hybridizations. Appropriate restriction fragments were radiolabeled with α-^{32}P-dATP by random hexamer priming (14). For colony hybridization, *H. influenzae* strains were plated on sBHI and grown overnight at 37°C in 5% CO_2 and the colonies were transferred to nitrocellulose. The membrane was air dried, immersed in a Tris-saline, dried, and prehybridized with salmon sperm DNA in 50% formamide (15). The radiolabeled probe was added, and the membrane was washed at high stringency and autoradiographed.

For Southern blots, DNA was prepared from total *H. influenzae* strains by the method of Hull et al. (21). DNA was subjected to *Eco*RI digestion and agarose gel electrophoresis on a 0.7% gel. The DNA was transferred overnight to nylon-backed nitrocellulose as suggested by the manufacturer. The membranes were then hybridized to probes labeled with digoxigenin-dUTP (Boehringer Mannheim) by random hexamer priming for 6 h at 68°C (38). DNA fragments which hybridized to the probes were detected by reacting the blots with sheep antidigoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim), by using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium.

Construction of 28-kDa mutants. pARS1 (see below) was digested with *Eco*RI, and the ends were filled by a Klenow fragment-mediated reaction. The 1.4-kb *Hae*II fragment from pACYC 184 which contains the CAT gene was ligated into this site, and the resulting plasmid was transformed into HB101. CAT-producing strains were selected by a rapid screening method (2); several had detectable CAT activities and encoded chloramphenicol resistance (MIC, >20 μg/ml). The recombinant plasmid was purified by cesium chloride ethidium gradient centrifugation, digested to completion with *Pst*I, and transformed into Hib strains. Transformation was accomplished by the competence procedure of Herriot et al. (18). Transformants were selected on sBHI agar containing chloramphenicol at 2 μg/ml. Transformation of R1369 yielded strain 5-9; transformation of Ela yielded strain 5-4.

Analytical methods. Protein concentrations were measured by a modification of the method of Lowry (32). SDS-PAGE was performed on 10% modified Laemmli gels (23) as performed previously (41).

Virulence assays. Adherence was assessed in vitro with primate (*Macaca nemestrina*) respiratory epithelial cell cultures. These monolayers were used for assessment of the mean residence time by a kinetic method (39).

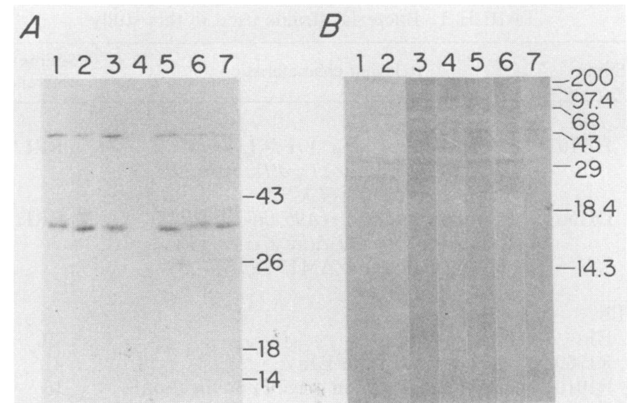

FIG. 1. Autoradiograph of Western blot developed with ^{125}I-staphylococcus A. The primary antibody was raised against the purified 22-kDa subunit protein in rabbits. Lane 1, 5 μg of purified R1369 (22-kDa) fimbrial subunit; lanes 2 through 7, supernatants (12 to 20 μg) of lysates of strains R1369, R906, HB101, R1010, C54, and E1a, respectively. Panel B contains preimmune sera, while panel A contains postimmunization sera. Positions of molecular weight standards (in thousands) are indicated at the right.

Adherence in vivo was tested by the following manner: 10^8 CFU of each bacterial strain was separately inoculated intranasally into individual infant *M. nemestrina* of either sex between the ages of 1 and 3 days (36). Twenty-four hours after intranasal inoculation and daily for 7 days, a Calgi swab was inserted into the right nostril, removed, and agitated in 1 ml of sterile PBS containing 0.1% gelatin (PBS-G); 0.1 ml of this and serial 10-fold dilutions were plated on sBHI agar containing streptomycin (500 μg/ml) and incubated overnight, and *H. influenzae* colonies were enumerated.

Similar experiments were performed with 5-day-old COBS/CD outbred Sprague-Dawley rats by the technique previously described (31). However, with rats, 10 μl containing 10^5 CFU was inoculated into each nostril. At 1 day after inoculation and daily for 5 days, a Calgi swab was inserted into the right nostril and streaked on sBHI agar containing streptomycin at 500 μg/ml. In a separate series of experiments, the invasion potential in 5-day-old infant rats was assessed by obtaining blood from the external jugular vein 48 h after intranasal (31) or intraperitoneal (40) inoculation. Quantitative blood cultures were performed by plating undiluted blood as well as 1:10 and 1:100 dilutions in PBS-G on sBHI agar containing streptomycin. In selected animals, cerebrospinal fluid was obtained for culture (31).

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence is M59804.

RESULTS

Antigenicity. After recognizing that rabbit antisera raised by immunization with the R1369 pili reacted on dot blots with a variety of capsulated and untypeable *H. influenzae* strains, we examined the specificity of the reaction by Western blot. Antiserum raised against purified pili failed to detect the 22-kDa pilin subunit after separation on denaturing gels, although a protein of approximately 28 kDa was detected in the purified pilus preparation and Hib lysates which were examined (Fig. 1). Because of the apparent cross-reactivity, we sought to determine whether this pro-

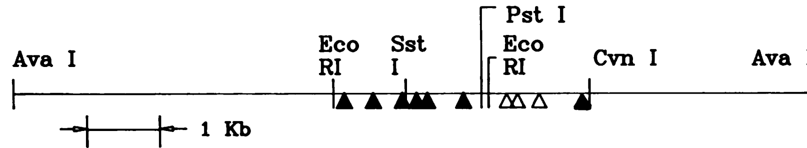

FIG. 2. Restriction map of pMCC1 depicting the effect of Tn*5* insertions on the expression of the 28-kDa protein. λTn*5* insertions were mapped by Southern analysis in the presence (▲) or absence (△) of the immunoreactive 28-kDa protein, as determined by Western immunoblotting with mouse pilus antisera.

tein was related to colonization and invasion. The reactivity at the top of the blot is apparently a trimer of the subunit, as prolonged boiling prior to SDS-PAGE yielded a single band with Coomassie blue and antisera. This protein was of a greater apparent mass than the fimbrial subunit.

Cloning. A cosmid library of DNA from Hib strain R1369 was screened by colony immunoblot with rabbit antiserum raised against R1369 pili; 8 of 1,000 clones screened were reactive with the antisera. Proteins from these eight clones were subjected to Western blot analysis. One colony was found to produce a 28-kDa protein which bound immunoglobulin in postimmunization antisera (data not shown). Plasmid DNA was purified from this strain, which harbored a recombinant plasmid, designated pKKW1, and contained a 40-kb insert chromosomal DNA from strain R1369. This plasmid was digested with *Ava*I, religated to itself, and transformed into HB101.

Two transformants were reactive by colony blot. One, designated pMCC1, expressed a protein of approximately 28 kDa in mass by Western blot analysis. This protein bound with mouse and rabbit polyclonal antibodies raised against purified pili. Mouse polyclonal antibody was used to confirm the results obtained with rabbit antisera (data not shown). A restriction map of pMCC1 was derived, and the location of the gene for the 28-kDa protein was determined by mapping λTn*5* insertions (Fig. 2), which inactivated production of the immunologically reactive 28-kDa protein by using mouse ascitic fluid. An *Sst*I-*Cvn*I fragment from pMCC1 was blunt ended with the Klenow fragment, cloned into the *Sma*I site of alkaline phosphatase-treated pUC18, and identified as pARS1 (Fig. 3). This plasmid was found to encode for production of the immunoreactive 28-kDa protein by Western blot analysis with mouse polyclonal antibody. Both strands of this DNA fragment were sequenced and, an 843-bp open reading frame extending from nucleotide 88 to the TAA stop codon at nucleotide 907 was found (Fig. 4). Seven nucleotides 5′ to the initiator methionine is AAGG, a putative ribosome-binding site. Comparison of the predicted translation product of the open reading frame with other proteins logged in the GenBank data base revealed significant homology with the *E. coli* 28-kDa lipoprotein (44). Thirty-two bases upstream of the start codon is the sequence TTAAAC. This is similar to the *E. coli* consensus promoter TTGACA and identical to the putative promoter for the *E. coli* 28-kDa lipoprotein NlpA gene (44). We prepared from pARSI a *Pst*I-*Nde*I fragment internal to the coding region for the 28-kDa protein and used it as a probe for homologous sequences. This fragment was labeled with digoxigenin-

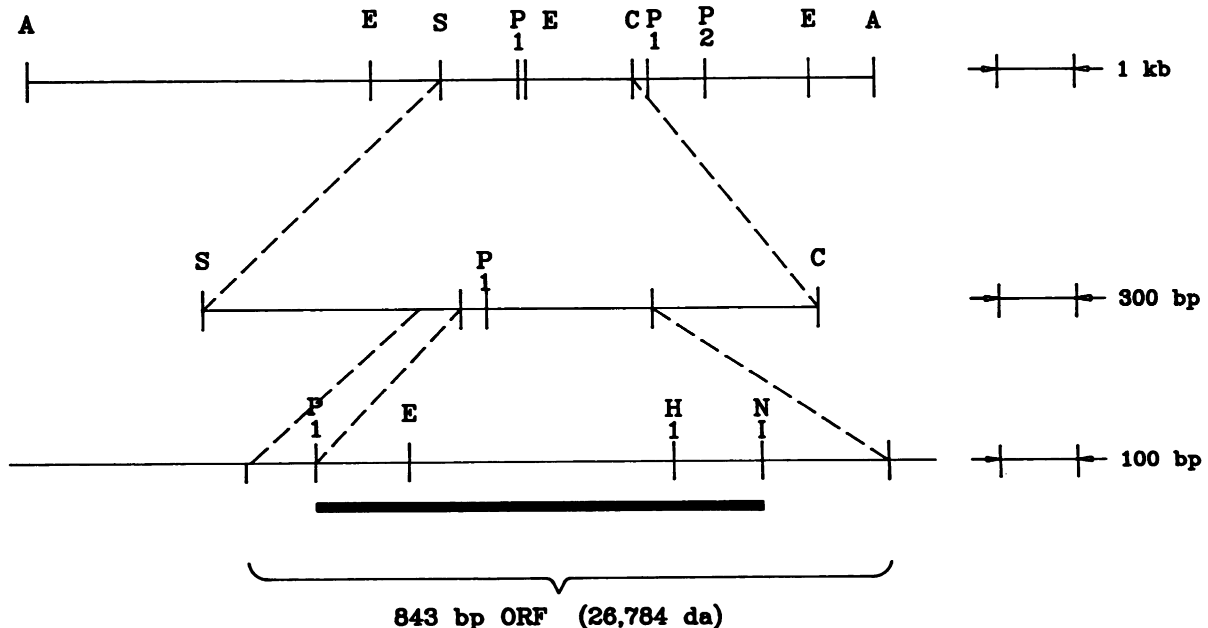

FIG. 3. Derivation of pARSI (bottom) from pKKWI (top) and pMCCI (middle). The solid bar indicates the *Pst*I-*Nde*I probe used in colony hybridization. Abbreviations: A, *Ava*I; E, *Eco*RI; S, *Sst*I; P1, *Pst*I; P2, *Pvu*II; C, *Cvn*I; H1, *Hpa*I; NI, *Nde*I; ORF, open reading frame.

```
TAG CCA AAA  ACT CGG  CGA  TAC ACT  GGC TAA  AAA AGT  GGA TCA  TCG  TTA ATT AAA CGC AAC
                       SD
TCT TAA CTT AAA CAG G AA GG A AAT CCT ATG AAA TTA AAA CAA CTT TTT GCA ATC  ACT GCA  ATC
                                      Met                                    P
GCA TCA GCT CTC GTT TTA ACA GGC TGT AAA GAA GAC AAA AAA CCT GAA GCT GCA GCA GCA CCG CTT

AAA ATC AAA GTA GGC GTG ATG TCT GGC CCT GAG CAT CAA GTT GCA GAA ATT GCA GCA AAA GTC GCT

AAA GAA AAA TAT GGT TTA GAC GTT CAA TTC GTT GAA TTC AAT GAC TAC GCA TTA CCA AAT GAA GCT

GTA TCT AAA GGT GAT TTA GAT GCA AAC GCA ATG CAA CAT AAA CCT TAT TTA GAT GAA GAT GCA AAA

GCG AAA AAT TTA AAT AAC TTA GTT ATC GTG GGT AAT ACT TTC GTC TAT CCA TTA GCG GGT TAT TCT

AAA AAA ATC AAA AAT GTG AAT GAA TTA CAA GAC GGT GCT AAA GTT GTT GTT CCT AAC GAT CCA ACA

AAC CGT GGC CGT GCA TTA ATT CTT CTT GAG AAA CAA GGT TTA ATC AAA TTA AAA GAT GCA AAT AAC

CTT CTT TCA ACT GTA TTA GAT ATT GTT GAA AAT CCG AAA AAA TTA AAC ATC ACT GAA GTA GAT ACT

TCT GTT GCG GCA CGC GCA TTA GAC GAC GTT GAT TTA GCT GTA GTA AAC AAT ACT TAT GCG GGT CAA
                                                                         N
GTA GGC TTA AAT GCT CAA GAT GAC GGT GTA TTT GTA GAA GAT AAA GAT TCG CCA TAT GTG AAC ATT

ATC GTT TCT CGT ACC GAT AAC AAA GAC AGC AAA GCT GTT CAA GAT TTC GTA AAA TCT TAC CAA ACA

GAA GAA GTT TAC CAA GAA GCT CAA AAA CAC TTT AAA  GAT  GGT  GTT  GTA  AAA  GGT  TGG  TAA
                                                                              *
TTT CTA CCG CAC TTT AAT TAA TTA CTA AAT CCC TTG CTC TGC AAG GAT TTT CTT ATC TAA GCT CTG

AAT GTT TTA AAG AAT ATT GAT AGC TAA AAC TAA TTA GAT AAT ACA GAA ATA TGA AAT TAT CTT TAA

TTG ATA AAA AAT AAA TAC TTT TAC
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FIG. 4. Derived nucleotide sequence of 28-kDa-protein-encoding gene. The putative promoter is underlined, and the ribosome-binding site is indicated by SD. The N terminus of the protein is indicated by Met under the start codon, while the termination of translation is indicated by an asterisk under the last codon. P, The point of cleavage by *Pst*I; N, the site of cleavage by *Nde*I.

dUTP and used to probe the *Eco*RI-restricted DNA of strains R1369, R1010, C54, and Ela (Fig. 5). These strains all contain a 3.2-kb fragment which hybridized to the intragenic probe.

Conservation of the 28-kDa protein. An intragenic probe (Fig. 3) for the 28-kDa protein was also used in dot blot assays to determine whether this gene is highly conserved in *H. influenzae*. Table 2 depicts the strains hybridizing and those not hybridizing with this probe, indicating that the genes encoding for the 28-kDa protein are highly conserved in *H. influenzae*.

Mutagenesis of the 28-kDa gene. A *Hae*II fragment from pACYC 184 encoding CAT was inserted into the *Eco*RI site of pARSI. The resulting plasmid, pSLM1, encoded chloram-

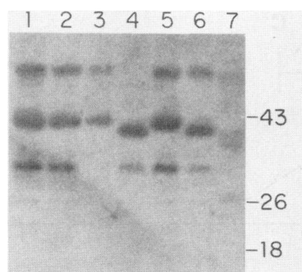

FIG. 5. Autoradiograph of Western blot developed with ^{125}I-protein A. The antibody was raised against the purified pili in rabbits. A loop of cells was lysed in 2% SDS and boiled for 5 min in mercaptoethanol prior to electrophoresis. Lane 1, Strain E1a; lane 2, strain R1369; lane 3, strain 5-9; lane 4, strain R906; lane 5, strain R1010; lane 6, strain C54; lane 7, *E. coli* HB101. Molecular mass markers (in kilodaltons) are on the right.

TABLE 2. Hybridization of intragenic 28-kDa probe with selected *H. influenzae* strains of various capsulation statuses

Strains(s)	Type	Location/yr
Hybridizing		
C1, C9, C140, C240, C513, C545	b	Boston, Mass./1970–1978
C890, C1011	b	Seattle, Wash./1982
R1005, R1009	b	Ann Arbor, Mich./1982
R1023, R0128	b	Chicago, Ill./1982
R1076, R1077	b	Toronto, Ontario, Canada/1982
GL34	b	New York, N.Y./1941
C2766, C2768	b	Seattle, Wash./1989
R384	u	Omaha, Neb./1979
R385, R1198	b	Pittsburg, Pa./1979
R458 (Goodgal)	u (4)	
R539 (ATCC 9006)	a	
R540 (ATCC 9007)	c	
R541 (ATCC 9008)	d	
R542 (ATCC 8142)	e	
R842 (A8)	u (30)	
R858	b	Los Angeles, Calif./1982
R925	b	Tucson, Ariz./1982
R935	b	Oklahoma City, Okla./1982
R949	b	Detroit, Mich./1982
R954	b	Athens, Greece/1982
R982	b	New Orleans, La./1982
Not hybridizing		
GL 45	b	New York, N.Y./1943
R543 (ATCC 9833), R1197	b	Pittsburgh, Pa./1982

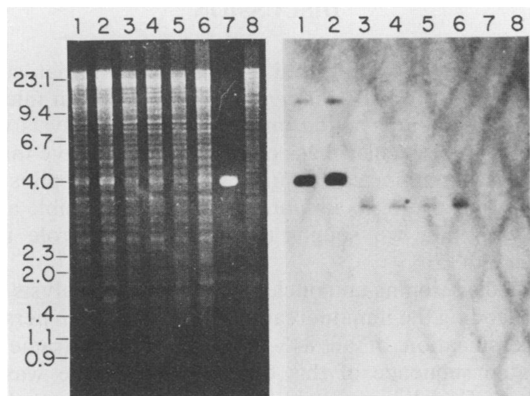

FIG. 6. Southern analysis of *Eco*RI-restricted whole-cell DNA (left panel) with the *Pst*I-*Nde*I fragment derived from pARSI (right panel). Lanes 1, HB101(pSLM 5-9); lanes 2, HB101(pSLM 5-4); lanes 3 to 6, *H. influenzae* R1369, Ela, R1010, and C54, respectively; lane 7, purified undigested pACYC 184 DNA; lane 8, restricted DNA from *E. coli* HB101. Size markers (in kilobases) are on the left.

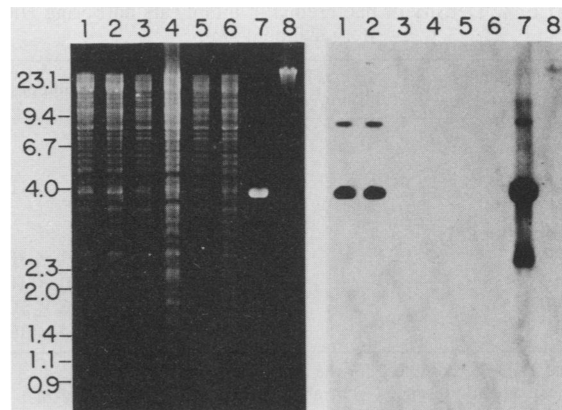

FIG. 7. Southern analysis of *Eco*RI-restricted whole-cell DNA (left panel) with the 1.4-kb *Hae*II fragment of pACYC 184 as a probe (right panel). Lanes 8, 1, and 2, *E. coli* HB101, HB101(pSLM 5-9), and HB101(pSLM 5-4), respectively; lanes 3 to 6, *H. influenzae* R1369, E1, R1010, and C54, respectively; lane 7, purified undigested pACYC 184 DNA; lane 8, unrestricted DNA from *E. coli* HB101. Size markers (in kilobases) are on the left.

phenicol resistance but failed to encode the production of the 28-kDa immunoreactive protein, according to Western blot analysis with mouse ascitic fluid. The insertionally inactivated gene was introduced into *H. influenzae* by transformation of strain Ela with *Pst*I-digested pSLM1. A chloramphenicol-resistant transformant of Ela selected for further study was designated strain 5-4. Strain 5-4 failed to produce the 28-kDa immunoreactive protein on Western blot analysis (Fig. 5). We also observed on certain Western blots rabbit sera bound to an *H. influenzae* protein present in whole cells with a relative mobility of 38 to 41 kDa. This band was not seen if cell lysate supernatants were analyzed by Western blot with the same sera. Transformation of strain R1369 with *Pst*I-digested pSLM1 yielded similar transformants. One, designated 5-9, lacked the 28-kDa protein (Fig. 5) but remained fimbriated on electron microscopic examination (data not shown). Southern blot analysis using the 28-kDa protein gene probe described above demonstrated an increase in the size of the 3.2-kb *Eco*RI fragment containing the 28-kDa-protein-encoding gene to 4.1 kb in strains 5-4 and 5-9 (Fig. 6). To confirm that the increase in the size of the fragment in strains 5-4 and 5-9 was due to the presence of the CAT gene, a 1.3-kb probe was prepared from pACYC 184. This probe consisted of a *Hae*II fragment from pACYC 184 and contained one *Eco*RI site and all of the CAT structural gene. When *Eco*RI digests of these strains were probed with the CAT gene, homologous sequences were seen only in strains 5-4 and 5-9 and were present on 4.1 kb fragments. The larger fragments of strains 5-4 and 5-9 seen hybridizing with the probes in Fig. 6 and 7 were due to the presence of the *Eco*RI site in the CAT gene. The non-CAT-producing strains did not hybridize with the CAT probe.

Virulence. We found no difference in the adherence of strains lacking or possessing the 28-kDa protein as observed by an in vitro kinetic method (39). In replicate experiments, the mean residence time of strain R1369 in perfused respiratory epithelial monolayer cultures was 19.23 ± 3.28 min ($n = 5$), while the time of strain 5-9 was 18.65 ± 2.67 min ($n = 4$). Three infant monkeys yielded strain 5-9 on daily cultures of the nasopharynx for 1 week. The bacterial density remained constant at approximately 10^7 CFU per swab.

To determine whether the 28-kDa protein is important in virulence in infant rats, 40 pups randomized among four litters were inoculated intranasally with 10^5 CFU of strain Ela or 5-4. All 40 animals had *H. influenzae* detected in their nasopharynxes from 48 to 120 h after inoculation. Intranasal inoculation of hyperpiliated Hib in infant rats, whether the strain possessed (strain R1369) or lacked (strain 5-9) the 28-kDa protein, was associated with a low frequency of bacteremia. In a separate series of experiments, 5-day-old infant rats were inoculated intranasally with *H. influenzae*; 48 h later, the rats were anesthetized with CO_2 and blood cultures were obtained from the transected external jugular vein. For one group of intranasally challenged animals, cerebrospinal fluid was also cultured. The results are depicted in Table 3. After intranasal challenge, strain 5-4 was

TABLE 3. Comparative virulence of strain Ela and its derivatives

Route (CFU) and strain[a]	No. of bacteremic animals/no. of animals incubated	No. of animals with meningitis/no. of animals inoculated
Intranasal (10^8)		
Ela	39/73[c,d]	26/40[e]
R1369	2/40[c]	2/40
5-9	2/50	1/50
5-4	10/33[d]	10/33[e]
Intraperitoneal (10^4)		
Ela	10/10	ND[b]
R1369	10/10	ND
5-9	18/20	ND
5-4	26/27	ND

[a] Strain R1369 is a piliated derivative of Ela, a type b cerebrospinal fluid isolate. Strain 5-9 was constructed by transformation of R1369 with pARS1, which was insertionally inactivated by a CAT cassette. Strain 5-4 was constructed by transformation of Ela with the same linearized plasmid.
[b] ND, Cerebrospinal fluid not cultured.
[c] For these values, $P < 0.001$.
[d] For these values, $P = 0.03$.
[e] For these values, $P = 0.047$.

TABLE 4. Density of bacteremia in infant rats harboring Hib 48 h after inoculation

Route of challenge and strain[a]	28-kDa protein[b]	No. of animals	CFU/ml of blood
Intranasal			
Ela	+	20	2.9×10^3
5-4	−	10	2.4×10^3
Intraperitoneal			
Ela	+	20	1.2×10^{4}[c]
5-4	−	10	5.0×10^{2}[c]
R1369	+	10	1.6×10^{4}[d]
5-9	−	10	3.4×10^{2}[d]

[a] See Table 3, footnote *a* for the origins of the strains.
[b] Indicates the presence (+) or absence (−) of the 28-kDa protein.
[c] For these values, $P < 0.01$.
[d] For these values, $P < 0.01$.

significantly compromised in its ability to produce bacteremia and meningitis in comparison with its parent strain Ela. Strain R1369, the hyperpiliated derivative of Ela, was drastically reduced in virulence. The few animals that did become bacteremic with R1369 and 5-9 yielded from their blood organisms that were nonpiliated (data not shown). This indicates that the hyperpiliation phenotype interfered with the ability of the organisms to invade respiratory epithelium. Strain 5-9, the mutant of R1369 unable to express the 28-kDa protein, was indistinguishable from its parent strain in this model. When the four strains were inoculated intraperitoneally, all were able to produce bacteremias, suggesting that the primary contribution to virulence of the 28-kDa protein involves the initial process of invasion. However, when the densities of bacteremia produced by the different strains were compared following intraperitoneal challenge, the 28-kDa-protein-producing mutants 5-4 and 5-9 were recovered in significantly lower numbers than their parent strains Ela and R1369 (Table 4). There was no detectable difference in the in vitro growth rates in sBHI among the four strains. This suggests that the 28-kDa protein plays some role in the intravascular survival of Hib.

DISCUSSION

Polyclonal antisera raised against the fimbrial subunit protein of a type b *H. influenzae* strain raised in mice and rabbits prominently bound on Western blot analysis with a protein with molecular mass of 28 kDa. We believe that this protein was a minor contaminant of our purified pilus preparation. Because of its immunogenicity and possible association with pili, we sought to investigate its role in the virulence of Hib.

Molecular cloning and nucleotide sequence analysis of the gene encoding the immunoreactive 28-kDa protein permitted the identification of an 843-bp open reading frame. The translated sequence of this open reading frame was 70% similar to *E. coli* lipoprotein 28 (44) (Fig. 8), a cytoplasmic membrane protein of *E. coli*. The Hib 28-kDa immunoreactive protein may be identical to Hib P5 as described by Barenkamp and coworkers (3), "e" as described by Loeb and Smith (26), and D* as described by van Alphen et al. (42). It is not clear whether this protein is identical to another Hib 27-kDa protein which has been cloned in *E. coli* (20). That protein, cloned with a monoclonal antibody (7B2), was found to be expressed on the surface of *E. coli* HB101 but was unstable in that host. We found that strains expressing the 28-kDa protein, in comparison with those having the gene insertionally inactivated, were more virulent, as determined by the ability to cause bacteremia after intranasal inoculation of infant rats. The role of this protein in virulence involves the initial invasion of the mucosal epithelium, since animals inoculated intraperitoneally with either the wild type or the mutant were equally likely to develop bacteremia.

Since membrane proteins in Hib may be antigenic and antibodies to these may be protective even though they are not essential virulence factors, we studied the conservation of the gene encoding for the 28-kDa protein in a variety of *H. influenzae* isolates. We found (Table 2) that the majority of Hib and certain typeable but non-b *Haemophilus* strains hybridize with the intragenic probe derived from the 28-kDa-protein-encoding gene. These data suggest that this protein may be a candidate immunogen. Additional experiments are

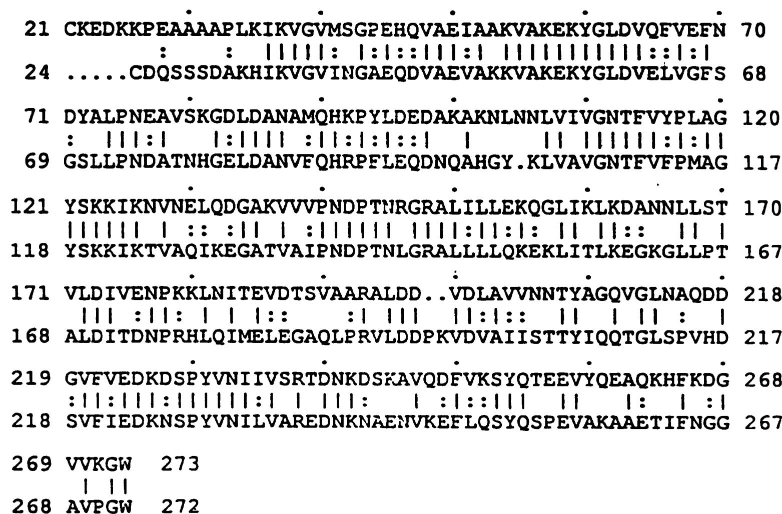

FIG. 8. Comparison of the translated amino acid sequences of 28-kDa *H. influenzae* protein (top rows) and lipoprotein 28 of *E. coli* (bottom rows) (44). Vertical bars indicate identical amino acids; two vertical dots indicate conservative substitutions; a single dot indicates that the sequence is not available.

necessary to determine whether antibodies directed against this protein confer protection against invasive Hib disease.

ACKNOWLEDGMENT

This study was supported by Public Health Service grant AI 20625 from the National Institute of Allergy and Infectious Diseases.

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