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Expression of the Heat-Modifiable Major Outer Membrane Protein of *Haemophilus influenzae* Type b Is Unrelated to Virulence

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The heat-modifiable major outer membrane protein (P1) of *Haemophilus influenzae* type b (Hib) has been shown to be both exposed on the cell surface and capable of inducing the synthesis of antibodies protective against experimental Hib disease. Chemical mutagenesis of a recombinant plasmid containing the Hib gene encoding P1 resulted in inactivation of P1 expression by this plasmid. The mutated P1 gene was transformed into Hib to obtain an isogenic mutant lacking only the ability to synthesize this surface protein. In addition, the P1 gene was inserted into a plasmid shuttle vector and used to construct a recombinant Hib strain that overexpressed the P1 protein. Lack of P1 expression did not affect the ability of Hib to grow in vitro. Neither the absence nor the overproduction of P1 affected expression of capsular polysaccharide and lipooligosaccharide by Hib. The P1-negative mutant and the P1-overexpressing strain were both as susceptible to the bactericidal activity of pooled normal human serum as was the wild-type parent strain, while the P1-negative mutant was as resistant to the bactericidal activity of normal infant rat serum as was the wild-type parent strain. The P1-negative mutant was no less virulent than was the wild-type parent strain in an animal model system, such that both the numbers of animals infected by this mutant and the mean magnitudes of the resultant bacteremias were essentially identical to those obtained with challenge by the wild-type parent strain. Similarly, overexpression of P1 did not detectably affect the virulence of Hib. These data indicate that this protective protein antigen plays no detectable role in the expression of virulence by Hib, as assessed in an animal model system.

Haemophilus influenzae type b (Hib) is the most important cause of meningitis in the United States (5). While new vaccines composed of purified Hib capsular polysaccharide covalently coupled to carrier proteins (e.g., tetanus and diphtheria toxoids) are currently being tested for their efficacy (44), there remains considerable interest in outer membrane proteins of this organism as potential vaccine candidates (11, 19). At least four different Hib outer membrane proteins have been shown to be targets for antibodies protective against experimental Hib disease. These include a surface protein with an apparent molecular weight of 98,000 (98K protein) (21), the P1 heat-modifiable major outer membrane protein (10, 11, 25), the P2 porin protein (32), and the P6 lipoprotein (12, 30). Of these proteins, only P2 has been assigned a physiologic function (42, 43).

Recent data suggest that the P1 protein may possess surface epitopes common to most or all strains of this pathogen, because immune polyclonal serum raised against purified P1 from one Hib strain provided immunoprotection against several different Hib strains in an animal model (25). However, no physiologic function has yet been ascribed to this protein and, similarly, it is not known whether this protein plays any essential role in the expression of virulence by Hib. Towards these ends, the Hib gene encoding this protein has been cloned and expressed in *Escherichia coli* by our laboratory (10) and by Munson and Grass (31). Using the cloned Hib gene encoding P1, we constructed both a mutant of Hib which lacks the ability to express detectable P1 protein and a Hib strain which expresses unusually large amounts of this protein. These two strains were used to establish that expression of the P1 protein is not required for growth of Hib in vitro and that, while this protein is a target for protective antibodies, it plays no detectable role in the expression of virulence by Hib in an animal model.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Hib strains DL41 and DL42, originally isolated from the cerebrospinal fluid of infants with Hib meningitis, have been described previously (14). The P1 proteins expressed by these two strains share reactivity with different P1-directed monoclonal antibodies (MAbs) (10) and exhibit the same apparent molecular weight in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). DB117, a recombination-deficient (*rec-1*) strain of *H. influenzae* Rd (37), was used as the host for plasmid pGJB103 and its derivatives. Competent cells of *E. coli* HB101 were obtained from a commercial source (Bethesda Research Laboratories, Gaithersburg, Md.). *H. influenzae* strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal base (BHIS) as described previously (17). Where appropriate, ampicillin or tetracycline was included at a concentration of 10 or 5 μg/ml, respectively. *E. coli* HB101 was grown in LB broth (27), with 100 μg of ampicillin per ml or 20 μg of tetracycline per ml as appropriate. Solid media included 1.5% (wt/vol) agar (Difco).

Plasmid pFRG100 is a pBR322 derivative bearing a 6.1-kilobase (kb) *Eco*RI fragment of Hib strain DL41 chromosomal DNA which contains the gene encoding the Hib P1 protein (10). Plasmid pGJB103 is a derivative of the shuttle vector pHVT1 (7) which can replicate in both *E. coli* and *H. influenzae* and encodes resistance to both ampicillin and tetracycline (18; G. J. Barcak, personal communication).

P1 protein-directed MAbs and polyclonal antisera. The P1-directed MAbs 6B1 and 7C8 have been described previously (10). The production of rat antiserum against whole

DL41 cells has also been described previously (14, 16). Antiserum was raised against purified P1 protein by immunizing adult Sprague-Dawley rats (Simonsen Labs, Gilroy, Calif.) with P1 protein obtained by preparative SDS-PAGE. The rats received three intraperitoneal injections of approximately 50 μg of protein in 0.5 ml of elution buffer (see below) homogenized with an equal volume of monophosphoryl lipid A and trehalose dimycolate adjuvant (Ribi Immunochem Research, Hamilton, Mont.) at 3- to 4-week intervals. Immune serum was prepared by standard methods from the blood of animals exsanguinated by cardiac puncture 10 days after the last booster immunization.

Normal human serum was obtained from blood drawn from four adult volunteers and was processed to preserve complement activity. Normal infant rat serum was prepared from the blood of infant rats exsanguinated at 8 days of age; this serum was also processed to preserve complement activity. Bactericidal assays using this normal infant rat serum together with serum-sensitive and serum-resistant strains of Hib described previously (22) confirmed that this serum was complement sufficient.

Colony blot-RIA. The colony blot-radioimmunoassay (RIA) was accomplished as described previously (14) by using MAbs 6B1 and 7C8 as primary antibodies.

SDS-PAGE and Western blotting (immunoblotting). Modifications to standard techniques for SDS-PAGE and Western blotting have been previously described (17, 21, 22). Whole-cell lysates of bacteria grown on solid media were prepared as described by Patrick et al. (35). Outer membrane vesicles were prepared by the lithium chloride-based procedure of McDade and Johnston (28) as modified by Gulig et al. (13). Lipooligosaccharides (LOS) were resolved by electrophoresis of proteinase K-treated cell lysates in SDS-polyacrylamide gradient gels (21) and were visualized by silver staining (41).

Preparative SDS-PAGE. Proteins present in outer membrane vesicles prepared from Hib strain DL42 were solubilized in digestion buffer (16, 17) and resolved by SDS-PAGE in 16-cm-long separating gels (22). The gels were soaked briefly in 0.25 M KCl (15), and the clear protein bands were visualized against the cloudy background of SDS precipitated in the gel. The P1 band was excised and rinsed briefly in distilled water, and the protein was electrophoretically eluted into elution buffer (25 mM Tris [pH 8.4] containing 192 mM glycine and 0.1% SDS) by using an Elutrap (Schleicher & Schuell, Inc., Keene, N.H.). Protein was quantitated by comparison to standards of known concentration by using SDS-PAGE.

In vitro protein synthesis. Plasmid pFRG100 and its hydroxylamine-treated derivatives were purified on CsCl gradients (27) and used as templates in a procaryotic DNA-directed translation kit (Amersham Corp., Arlington Heights, Ill.). [^{3}H]leucine was used to radiolabel proteins synthesized in vitro by following the instructions of the manufacturer. The radiolabeled proteins were resolved by SDS-PAGE and visualized by fluorography (16).

Mutagenesis and analysis of plasmid DNA. Purified pFRG100 was mutagenized by treatment with hydroxylamine by a method (18) modified from that of Eichenlaub (9). Competent *E. coli* HB101 cells were transformed with the mutagenized plasmid DNA as recommended by the supplier (Bethesda Research Laboratories). *E. coli* transformants lacking reactivity with the P1-directed MAb 7C8 were identified in a colony blot-RIA. Plasmid DNA was prepared from these P1-negative transformants by using a rapid cleared-lysate method recommended by the supplier (Bethesda Research Laboratories, Focus **6(4)**:8, 1984). This plasmid DNA was digested to completion with *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and compared with *Eco*RI digests of nonmutagenized pFRG100 in agarose gel electrophoresis. Restriction digests were electrophoresed in 0.8% or 1.0% SeaKem HGT (FMC Bioproducts, Rockland, Maine) agarose gels under standard conditions (27).

Subcloning procedures. Complete *Eco*RI digests of pFRG100 plasmid DNA were resolved by agarose gel electrophoresis by using SeaKem GTG agarose (FMC Bioproducts), and the 6.1-kb band representing the Hib DNA insert was excised. DNA was electrophoretically eluted from this gel band by using the Elutrap as recommended by the manufacturer (Schleicher & Schuell). Plasmid pGJB103 was similarly digested with *Eco*RI, which cuts this plasmid in the Tet^r gene, and then was treated with calf intestine alkaline phosphatase (Promega Biotec, Madison, Wis.) as recommended by the supplier, phenol extracted, and ethanol precipitated. The vector and insert DNAs were ligated by standard techniques (27) by using T4 DNA ligase (Promega Biotec). *E. coli* HB101 was transformed with a portion of the ligation reaction, and Amp^r Tet^s transformants were screened for production of P1 by Western blot analysis using MAb 7C8 as the probe for P1.

Transformation of *H. influenzae*. Plasmid DNA from pFRG100 mutants deficient in P1 expression was digested to completion with *Eco*RI, and the linear molecules were used to transform DL42 cells made competent by the method of Herriott et al. (20). P1-deficient transformants were identified by screening with MAb 7C8 in the colony blot-RIA as described above.

H. influenzae DB117 was used as a host for propagation of pGJB103-derived plasmids intended for subsequent transformation into Hib strain DL42. The pGJB103 plasmid can be transformed into *H. influenzae* much more readily when it is obtained from *H. influenzae* than when it is obtained from *E. coli* (E. J. Hansen, unpublished observations). Plasmid pGJB103 and its derivatives were used to transform DB117 by the method of Stuy and Walter (38) as modified by Barcak (18; G. J. Barcak, personal communication). Plasmid DNA was isolated from log-phase cultures of DB117 by the method of Birnboim and Doly (3). This DNA was used to transform Hib strain DL42 (20). Plasmid DNA preparations from Amp^r Tet^s transformants of DL42 were subjected to restriction enzyme analysis to confirm the presence of the P1-encoding DNA insert. The P1 protein content of these transformants was analyzed by SDS-PAGE followed by Coomassie blue staining and Western blotting with MAb 7C8.

Indirect antibody accessibility RIA. The indirect antibody accessibility RIA was used to compare the relative number of P1 surface epitopes recognized by MAb 7C8 among Hib strains. This method was performed as described previously (22).

Infant rat challenge experiments. The virulence of the wild-type parent strain DL42, the P1-negative mutant, and plasmid-bearing derivatives of DL42 altered in P1 expression was assessed in the infant rat model system as described previously (23, 45).

Serum bactericidal assays. Determination of the susceptibility of the wild-type, mutant, and plasmid-containing strains to killing by bactericidal antibody was accomplished by using a standard serum bactericidal assay (22).

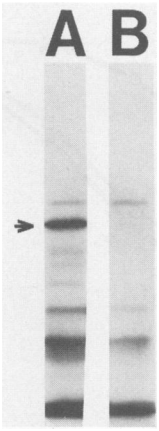

FIG. 1. Fluorograph of proteins synthesized in an in vitro DNA-directed translation system. [^{3}H]leucine was used to radiolabel proteins encoded by the recombinant plasmid pFRG100 (lane A) and by the mutant plasmid obtained from the *E. coli* transformant that was unreactive with MAb 7C8 (lane B). The arrow indicates the position of the P1 protein encoded by pFRG100. Both samples were heated at 100°C for 3 min prior to SDS-PAGE.

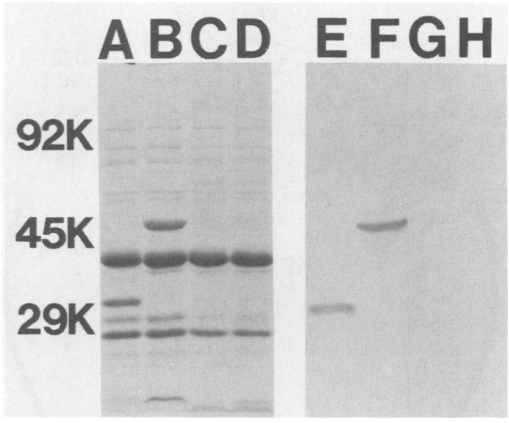

FIG. 2. SDS-PAGE and Western blot analysis of outer membrane proteins of the wild-type parent strain DL42 and its P1-negative mutant. Proteins in outer membrane vesicles from these strains were solubilized at two different temperatures, resolved by SDS-PAGE, and stained with Coomassie blue (lanes A to D) or transferred to nitrocellulose and probed in Western blot analysis with MAb 7C8 (lanes E to H). Lanes A and E, DL42 vesicles solubilized at 37°C; lanes B and F, DL42 vesicles solubilized at 100°C; lanes C and G, vesicles from the MAb 7C8-unreactive Hib transformant solubilized at 37°C; lanes D and H, vesicles from the MAb 7C8-unreactive Hib transformant solubilized at 100°C. Numbers on the left indicate apparent molecular weights.

Capsular polysaccharide quantitation. Cell-associated capsular polysaccharide was quantitated by rocket immunoelectrophoresis as described by Sutton et al. (39).

RESULTS

Construction of a P1-negative mutant of Hib. The recombinant plasmid pFRG100, carrying a 6.1-kb *Eco*RI fragment of Hib strain DL41 chromosomal DNA containing the gene encoding P1 (10), was mutagenized by treatment with hydroxylamine and transformed into *E. coli*. P1-negative transformants were identified in the colony blot-RIA by using the P1-directed MAb 7C8. Plasmid DNA was prepared from a selected P1-negative transformant. The restriction fragments derived from *Eco*RI digestion of this plasmid and the original pFRG100 plasmid were identical. The plasmid from this P1-negative *E. coli* transformant was then compared with pFRG100 in a DNA-directed in vitro translation system to confirm that this mutant plasmid could not express any P1 protein. The use of the recombinant plasmid pFRG100 in this translation system yielded a readily detectable P1 protein band which exhibited an apparent molecular weight of 49,000 after heating at 100°C prior to SDS-PAGE (Fig. 1, lane A). In contrast, the use of the mutant plasmid in the same translation system indicated that the 6.1-kb Hib DNA insert in this particular plasmid no longer expressed any detectable P1 protein (Fig. 1, lane B). In addition, P1 protein was not detectable in Western blots of whole-cell lysates of this *E. coli* transformant when probed with MAb 7C8 or with rat anti-DL41 immune serum (data not shown).

The Hib DNA insert in this mutant plasmid was excised by digestion with *Eco*RI. This linearized DNA preparation then was used to transform competent cells of Hib strain DL42. Transformants lacking reactivity with the P1-directed MAb 7C8 were identified in a colony blot-RIA. The proteins in outer membrane vesicles obtained from the wild-type parent strain (DL42) and one of these 7C8-unreactive transformants were analyzed by SDS-PAGE followed by Coomassie blue staining to visualize the proteins (Fig. 2). The outer membrane vesicles of DL42 solubilized at 37°C contained a major protein with an apparent molecular weight of approximately 34,000 in SDS-PAGE, characteristic of the P1 protein solubilized at this temperature (Fig. 2, lane A). When outer membrane proteins of DL42 were solubilized at 100°C, the P1 protein exhibited its heat-modified form, which migrated in SDS-PAGE with an apparent molecular weight of approximately 49,000 (Fig. 2, lane B). Outer membrane vesicles from the 7C8-unreactive transformant similarly treated at 37°C (Fig. 2, lane C) or 100°C (Fig. 2, lane D) did not yield any heat-modifiable protein with an M_r characteristic of P1. The use of MAb 7C8 in Western blot analysis of an identical set of four gel lanes transferred to nitrocellulose (Fig. 2, lanes E to H) confirmed the identity of the P1 protein in lanes A and B (Fig. 2, lanes E and F) and also established that the MAb 7C8-unreactive transformant did not express any immunologically detectable P1 protein (Fig. 2, lanes G and H). In addition, the use in Western blot analysis of polyclonal antiserum raised against purified P1 protein confirmed that no P1 protein was expressed by the MAb 7C8-unreactive transformant (data not shown). Therefore, the MAb 7C8-unreactive transformant is an isogenic mutant of Hib strain DL42 that no longer expresses detectable P1 protein.

Construction of a Hib strain that overexpresses P1. The availability of the shuttle vector pGJB103 made feasible the construction of a Hib strain that would express larger than normal quantities of P1. The new recombinant plasmid pFRG1100 (Fig. 3), consisting of pGJB103 containing the 6.1-kb Hib DNA insert of pFRG100, was obtained from a MAb 7C8-reactive *E. coli* transformant as described in Materials and Methods and was used to transform Hib strain DL42. An Amp^r Tet^s transformant of Hib DL42 was chosen for further analysis and was shown to contain pFRG1100. SDS-PAGE analysis of the protein content of outer membrane vesicles from this transformant revealed that it expressed more P1 protein (Fig. 4, lanes E and F) than did either the wild-type parent strain DL42 (Fig. 4, lanes A and B) or a transformant of DL42 carrying the shuttle vector pGJB103 (Fig. 4, lanes C and D). Western blot analysis

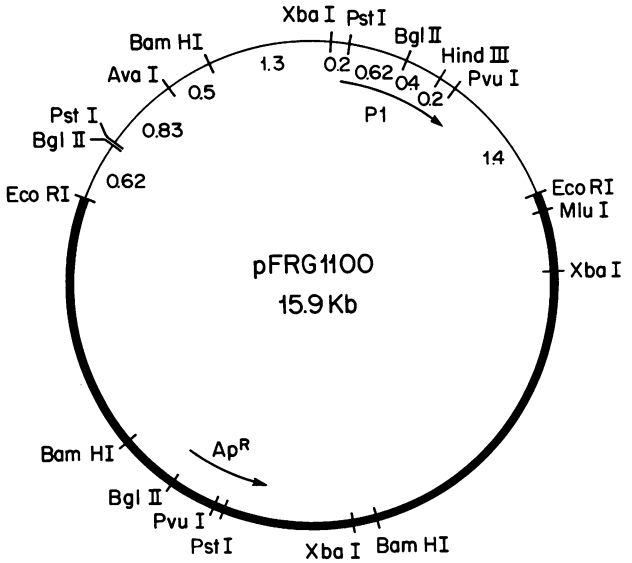

FIG. 3. Partial restriction map of the recombinant plasmid pFRG1100. Approximate distances between restriction sites are given in kilobases. The heavy line represents pGJB103 vector DNA. The indicated position of the structural gene encoding the P1 protein was described by Munson and Grass (31).

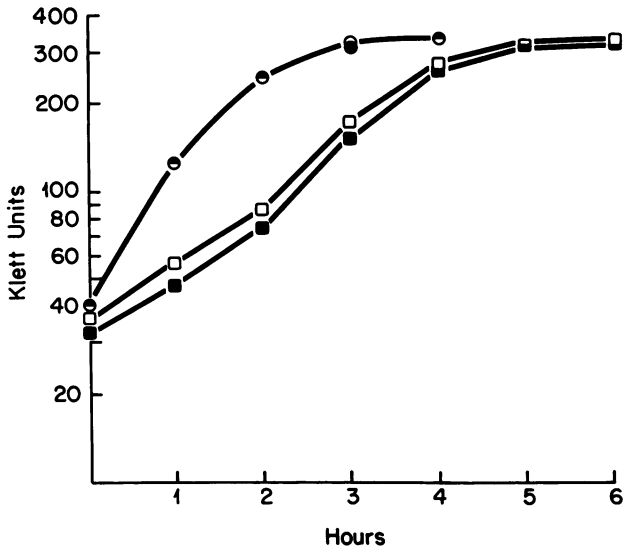

FIG. 5. Comparison of the growth characteristics of Hib strains in broth culture. Cells in the logarithmic phase of growth were inoculated into BHIS broth, and changes in the turbidity of the bacterial cultures with time were measured in a Klett-Summerson colorimeter (no. 66 filter). ●, Wild-type strain DL42; ○, P1-negative mutant; □, DL42(pGJB103); ■, DL42(pFRG1100). The latter two plasmid-containing strains were grown in BHIS containing 10 μg of ampicillin per ml.

involving MAb 7C8 confirmed the identity of the overexpressed protein as P1 (data not shown). This strain, which expresses larger than normal quantities of P1, was designated DL42(pFRG1100). Western blot analysis of whole-cell lysates of strains DL42, DL42(pGJB103), and DL42(pFRG1100) gave results similar to those described above.

The indirect antibody accessibility RIA was used to determine whether the increased quantity of P1 in DL42(pFRG1100) was surface exposed and accessible to antibodies in the same manner as P1 is in the wild-type parent strain. When compared on the basis of CFU, strain DL42(pFRG1100) bound more MAb 7C8 than did either the wild-type parent strain DL42 or the strain containing only the vector pGJB103. Radioiodinated goat anti-mouse immunoglobulin bound to MAbs on the surface of Hib cells previously incubated with MAb 7C8 in the following amounts (counts per minute): DL42 (wild type), 6,110; P1-negative mutant of DL42, 610; DL42(pGJB103), 2,240; DL42(pFRG1100), 15,860; and DL42 (negative control; wild-type DL42 incubated with an irrelevant MAb), 530. These data indicate that the increased quantity of P1 in DL42(pFRG1100) was translocated to the outer membrane and is, at least in part, exposed on the cell surface.

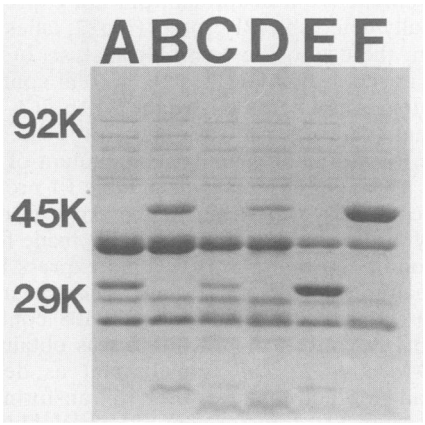

FIG. 4. SDS-PAGE analysis of the outer membrane proteins of the wild-type parent strain and two plasmid-containing derivatives. Proteins in outer membrane vesicles were solubilized at two different temperatures, resolved by SDS-PAGE, and stained with Coomassie blue. Lane A, DL42, 37°C; lane B, DL42, 100°C; lane C, DL42(pGJB103), 37°C; lane D, DL42(pGJB103), 100°C; lane E, DL42(pFRG1100), 37°C; lane F, DL42(pFRG1100), 100°C. Numbers on the left indicate apparent molecular weights.

Characterization of Hib strains with altered P1 expression: in vitro systems. The growth characteristics in broth culture of the mutant lacking P1 and the P1-overexpressing strain were compared with those of the wild-type parent strain DL42. The P1-negative mutant grew to the same cell density and had a generation time identical to that of the wild-type parent strain (Fig. 5). The P1-overexpressing strain DL42(pFRG1100) grew more slowly than did the wild-type parent strain DL42, but this difference was probably due to the presence of ampicillin (10 μg/ml) in the growth medium used with this plasmid-containing strain. Evidence for this conclusion is provided by the fact that when DL42(pGJB103) was grown in ampicillin-containing medium, both its rate of growth and its extent of growth were nearly identical to those of DL42(pFRG1100).

The susceptibility of these strains with altered P1 expression to killing by bactericidal antibody was assessed by using pooled normal human serum. This serum was bactericidal for the wild-type parent strain at a concentration of 20% (vol/vol) and contained antibodies directed against the type b polysaccharide capsule as well as antibodies to noncapsular somatic antigens (data not shown). Both the P1-negative mutant and the P1-overexpressing strain were killed as readily by this serum as was the wild-type parent strain (Fig.

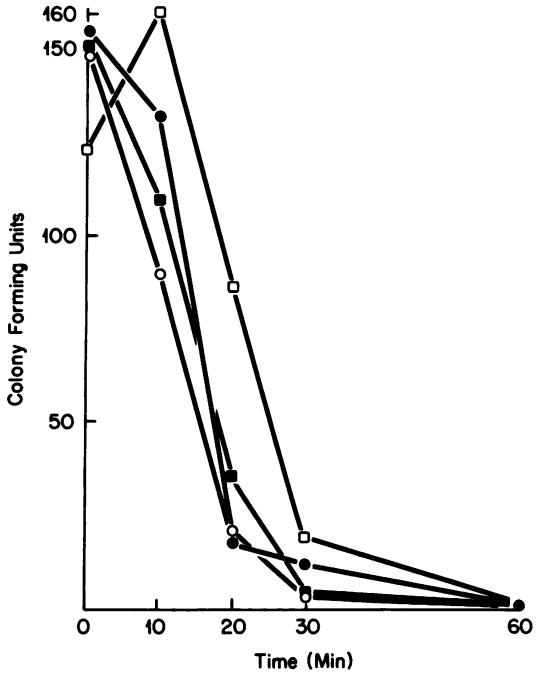

FIG. 6. Susceptibility of Hib strains to killing by pooled normal human serum. The serum bactericidal assay using pooled normal human serum was performed as described in Materials and Methods. The final concentration of serum was 20% (vol/vol). ●, Wild-type strain DL42; ○, P1-negative mutant; □, DL42(pGJB103); ■, DL42(pFRG1100).

6). Similarly, the presence of the shuttle vector pGJB103 in DL42 did not affect serum killing of this strain (Fig. 6). In addition, neither the wild-type parent strain nor the P1-negative mutant was susceptible to the bactericidal activity of normal infant rat serum (Table 1).

Quantification of the amount of cell-associated capsular polysaccharide expressed by the P1-negative mutant showed that this mutant synthesized a quantity of capsular polysaccharide identical to that associated with the wild-type parent strain DL42 (30 μg/10^9 CFU). Similarly, the P1-overexpressing strain DL42(pFRG1100) and the strain carrying only the shuttle vector [DL42(pGJB103)] each made identical quantities of cell-associated capsular polysaccharide (33 μg/10^9 CFU).

Characterization of the LOS synthesized by the Hib strains with altered P1 expression involved both antigenic

TABLE 1. Sensitivity of the wild-type strain and the P1-negative mutant to killing by normal infant rat serum

Hib strain and serum[a]	CFU/10 μl (mean ± SEM) at the following times (min):	
	0	60
DL42 (wild type)		
Normal serum	190 ± 16	697 ± 41
Heat-inactivated serum	211 ± 3	494 ± 42
P1-negative mutant of DL42		
Normal serum	251 ± 30	728 ± 45
Heat-inactivated serum	221 ± 13	588 ± 3

[a] Serum sensitivity was assessed by using infant rat serum at a final concentration of 50% (vol/vol) in the standard serum bactericidal assay, as described in Materials and Methods. Negative control was with heat-inactivated serum; normal infant rat serum was incubated at 56°C for 30 min to inactivate complement.

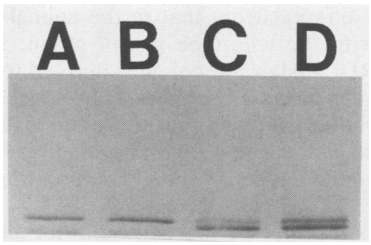

FIG. 7. Analysis of LOS by SDS-polyacrylamide gradient gel electrophoresis. Proteinase K-treated samples of detergent-solubilized cells were subjected to gradient gel electrophoresis and stained with silver by the method of Tsai and Frasch (41). Lane A, DL42; lane B, P1-negative mutant; lane C, DL42(pGJB103); lane D, DL42(pFRG1100).

and SDS-polyacrylamide gradient gel electrophoretic analyses of the LOS molecules. Colony blot-RIA using MAbs 4C4 and 5G8, which are directed against two different epitopes in the oligosaccharide region of DL42 LOS (14), showed that both the P1-negative mutant and the P1-overexpressing strain bound these two antibodies (data not shown). In addition, SDS-polyacrylamide gradient gel electrophoresis was used to resolve proteinase K-treated whole-cell lysates of the wild-type parent strain, the P1-negative mutant, strain DL42(pGJB103), and strain DL42(pFRG1100) which were then stained with silver to reveal the LOS bands. The absence of P1 in the P1-negative mutant did not affect expression of LOS by this strain (Fig. 7, lane B) relative to that observed with the wild-type parent strain (Fig. 7, lane A). Both DL42 containing the shuttle vector pGJB103 (Fig. 7, lane C) and DL42 containing the recombinant plasmid pFRG1100 (Fig. 7, lane D) had the same LOS profile by silver stain analysis.

Virulence of Hib strains with altered P1 expression. The wild-type parent strain and the P1-negative mutant were compared for their abilities to produce bacteremia in infant rats after intranasal challenge. In two different experiments, the wild-type strain DL42 produced bacteremia in a majority of animals challenged with this fully virulent Hib strain (Table 2). Similarly, the P1-negative mutant, at inoculum levels equivalent to those used with DL42, produced bacteremia in most of the challenged animals (Table 2). In fact, the number of animals rendered bacteremic by challenge with the P1-negative mutant was identical to that of animals in which bacteremia was caused by the wild-type parent strain in each experiment. In addition, the magnitude of bacteremia in animals infected with the P1-negative mutant was not

TABLE 2. Relative virulence of Hib strain DL42 and its P1-negative mutant[a]

Bacterial strain	Inoculum size (CFU)	No. of bacteremic animals/no. of animals challenged	CFU/10 μl of blood (mean ± SD)
Expt 1			
DL42 (wild type)	1.9 ± 10^8	9/10	79 ± 109
P1-negative mutant	3.5 ± 10^8	9/10	163 ± 246
Expt 2			
DL42 (wild type)	1.6 ± 10^8	8/10	144 ± 159
P1-negative mutant	1.8 ± 10^8	8/10	103 ± 152

[a] Virulence was determined in 5- to 6-day old infant rats that were challenged by the intranasal route, as described previously (23, 45).

substantially different from that in the animals which were bacteremic with the wild-type parent strain. Furthermore, colony blot-RIA analysis of colonies derived from the blood of animals which became bacteremic after challenge with the P1-negative mutant showed that these bloodstream-derived organisms did not express P1. This finding indicates that growth in vivo did not select for a P1-expressing revertant of this mutant.

Overexpression of P1 apparently did not affect the virulence of Hib, because intranasal challenge of infant rats with DL42(pGJB103) and DL42(pFRG1100) produced similar numbers of bacteremic animals (7 of 9 animals challenged and 8 of 10 animals challenged, respectively), and the magnitudes of bacteremia in the two sets of infected animals were also similar (data not shown). However, analysis of the antibiotic resistance characteristics of the Hib colonies derived from the blood of bacteremic animals revealed that all of the colonies obtained from the DL42(pGJB103)-infected animals contained the vector while the majority (64%) of the colonies obtained from the DL42(pFRG1100)-infected animals no longer contained the recombinant plasmid.

DISCUSSION

Heat-modifiable outer membrane proteins have been identified in a number of different bacteria. The OmpA protein of *E. coli* (34) and the PII protein of *Neisseria gonorrhoeae* (6, 40) have been studied extensively. More recently, another heat-modifiable outer membrane protein, the *fadL* gene product involved in fatty acid transport, was identified in *E. coli* (4), and a heat-modifiable outer membrane protein has been identified in *Francisella tularensis* (33). The OmpA protein of *E. coli* is thought to play primarily a structural role in the outer membrane of this organism (34), while the PII protein of *N. gonorrhoeae* is responsible for the colony opacity phenomenon (40) and may be involved in adherence functions (24).

The heat-modifiable P1 protein is one of the major protein components of the Hib outer membrane and has received considerable attention for two reasons. First, in the outer membrane protein subtyping scheme devised by Barenkamp et al. (2) and used in numerous studies of the epidemiology of Hib disease (1, 2), this protein was crucial in the classification of Hib strains. After heating in SDS at 100°C for 3 min, this protein usually migrates in SDS-PAGE with an apparent molecular weight of either 50,000 (designated H) or 49,000 (designated L) (1). Second, antibody to this protein has been shown to be protective against experimental Hib disease. Polyclonal antiserum raised against purified P1 was shown to be protective against Hib bacteremia in infant rats (11, 25), and a MAb against a surface epitope of P1 had a similar protective effect against systemic Hib disease (10).

The function of P1 in Hib has not yet been determined. The organism apparently does not require expression of this protein for growth in vitro, at least in a nutrient-rich broth medium. The fact that the growth curve of the wild-type parent strain and that of the P1-negative mutant were superimposable (Fig. 5) also indicates that not only is this protein unnecessary for growth of Hib in this broth medium but that the absence of P1 has no detectable effect on growth rate under these conditions. Neither an absence nor an overabundance of P1 in the outer membrane affected the susceptibility of Hib to killing by pooled normal human serum (Fig. 6). This serum, which contains antibodies to both the type b capsular polysaccharide and noncapsular somatic antigens (L. D. Cope and E. J. Hansen, unpublished data), was bactericidal for the wild-type parent strain, and it is conceivable that the multitude of bactericidal antibodies in this serum could have obscured possible minor differences in serum susceptibility between the P1-negative mutant and the wild-type parent strain. Nonetheless, the use in bactericidal assays of normal infant rat serum, which can distinguish serum-sensitive from serum-resistant strains of Hib (22, 23), also did not detect any differences between the P1-negative mutant and the wild-type parent strain (Table 1). In addition, lack of P1 expression did not affect the genetic competence of Hib, because the P1-negative mutant could be transformed to streptomycin resistance as readily as could the wild-type parent (data not shown).

It might be argued that over- or underexpression of other Hib virulence factors might somehow compensate for alterations in expression of P1, if this protein did indeed play a role in virulence. It was found that lack of expression of P1 by Hib did not affect expression of either of the other two Hib antigens associated with the expression of virulence by this pathogen. The type b polysaccharide capsule is known to be the primary virulence factor of this organism (29, 36); the P1-negative mutant possessed as much cell-associated capsular polysaccharide as did the wild-type parent strain. Similarly, the absence of P1 in the outer membrane did not affect the expression of LOS by Hib; changes in LOS phenotype have been associated with changes in virulence of Hib (22, 23, 46). While the LOS molecules synthesized by DL42(pGJB103) and by DL42(pFRG1100) were identical to each other, they were different from that synthesized by the wild-type parent strain (Fig. 7). Whether this change in LOS expression was the result of the presence of plasmids in these two strains or was caused by growth in an antibiotic-containing medium cannot be determined from the available data, but it can be assumed that overexpression of P1 in DL42(pFRG1100) was not responsible for this change in this recombinant strain.

Challenge with the vector-containing strain DL42 (pGJB103) and the recombinant strain DL42(pFRG1100) produced similar numbers of bacteremic animals, but quantitative analysis of the resultant levels of bacteremia was confounded by the apparent loss of the recombinant plasmid during growth in vivo. Loss of plasmids in the absence of antibiotic selection has been previously reported to occur in Hib strains grown in vivo (8). However, it is clear from the available data that the ability of DL42(pFRG1100) to overexpress P1 did not confer a selective in vivo growth advantage on this strain, relative to DL42(pGJB103).

Investigation of the possible involvement of P1 in the expression of virulence by Hib in the infant rat model established that expression of this protein by Hib was not required for production of systemic disease, because the P1-negative mutant was as virulent as the wild-type parent strain in this model. These findings strongly suggest that P1 plays no role in the ability of Hib to penetrate from the nasopharynx into the systemic circulation and to survive and produce a sustained bacteremia in this animal model. Whether P1 might play some specialized role in the pathogenesis of Hib disease in humans cannot be determined from the available data. It should be noted that all strains of Hib isolated to date appear to contain P1 (1, 2), indicating that this protein has been highly conserved among Hib strains, and recent genetic evidence suggests that only minor amino acid sequence differences exist between the H and L forms of this protein (R. S. Munson, Jr., M. Einhorn, S. Grass, and C. Newell, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1124, 1988). It cannot be

ruled out that P1 may play some role in facilitating colonization of the human nasopharynx by Hib, independent of the ability of this organism to produce invasive disease.

A previous study involving a mutant of Hib lacking the ability to synthesize the 98K surface protein of Hib demonstrated that, at least in Hib, a surface antigen that is a target for protective antibodies does not necessarily have to be a virulence factor (45). While this earlier study was subject to the same experimental limitations as the present study with regard to the infant rat model (45), it would now appear that the P1 protein of Hib is the second example of a protective antigen which apparently is not involved in the expression of virulence by Hib, as measured in an animal model system.

The demonstrated ability of purified P1 protein to induce the synthesis of antibodies protective against experimental Hib disease indicates that this surface protein can function as a protective antigen (11, 25). These data were obtained by using a passive immunization system and thus pertain specifically to bloodstream clearance of Hib. It is not known whether parenteral immunization with P1 could induce the synthesis in the upper respiratory tract of antibodies that would hinder or prevent colonization of the nasopharynx by Hib. In this regard, experience with the pneumococcal capsular polysaccharide vaccine has shown that systemic immunization against the pneumococcus will not eliminate nasopharyngeal colonization with this organism but will result in a decreased incidence of acquisition of vaccine-type strains in the nasopharynx (26). If immunization with P1 protein adversely affects nasopharyngeal colonization by Hib, and if P1 is not involved in this colonization process, then this situation might provide the opportunity for selection of P1-negative mutants in the nasopharynx. Reassessment of the appropriateness of P1 as a vaccine candidate may be indicated if P1 is truly not essential for expression of virulence by Hib. Conversely, if P1 is essential for nasopharyngeal colonization by Hib or for some other process in the pathogenesis of systemic Hib disease in humans, then systemic immunization with P1 would have the potential to be efficacious against systemic Hib disease. In this case, the P1-overexpressing strain DL42(pFRG1100) might be an excellent source of P1 for purification purposes.

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