INFECTION AND IMMUNITY, Oct. 1988, p. 2709–2716
0019-9567/88/102709-08$02.00/0

Vol. 56, No. 10

Cloning of the Gene Encoding the Major Outer Membrane Protein of *Haemophilus influenzae* Type b

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Received 24 March 1988/Accepted 5 July 1988

The major outer membrane protein (P2) of *Haemophilus influenzae* type b (Hib) with an apparent molecular weight of 37,000 to 40,000 has been previously shown to function as a porin and also as a target for antibodies protective against experimental Hib disease. The gene encoding the Hib P2 protein was cloned by using a shuttle vector capable of replication in both *Escherichia coli* and *H. influenzae*. The amino acid sequence of the amino terminus of the Hib P2 protein was determined and used to design an oligonucleotide probe corresponding to the first 20 amino acids of this protein. This oligonucleotide probe was used to identify Hib chromosomal DNA fragments containing the Hib P2 gene. These DNA fragments were ligated into the plasmid vector pGJB103 and then used to transform a *rec-1* mutant of *H. influenzae* Rd. Recombinant clones expressing the Hib P2 protein were identified in a colony blot-radioimmunoassay by using a monoclonal antibody specific for a surface epitope of the Hib P2 protein. The gene encoding this Hib protein was present on a 10-kilobase Hib DNA insert in the recombinant plasmid. Transformation experiments involving the recombinant plasmid suggested that unregulated synthesis of Hib P2 is a lethal event in *E. coli*. The recombinant Hib P2 protein was exposed on the surface of the recombinant *H. influenzae* strain. This recombinant strain was used to develop a system for detecting polyclonal serum antibodies directed against surface determinants of the Hib P2 protein. The availability of the gene encoding the Hib P2 protein should facilitate investigation of both the immunogenicity and the structure-function relationship(s) of this major outer membrane protein.

The most important cause of meningitis in the United States is *Haemophilus influenzae* type b (Hib) (5). Most efforts to develop an efficacious Hib vaccine have been focused on the Hib capsular polysaccharide, which is known to be both the primary virulence factor of this organism as well as a target for antibodies protective against systemic Hib disease (5, 13, 43). More recently, however, surface-exposed outer membrane proteins of this organism have received attention as possible vaccine candidates (13, 20). Monoclonal and polyclonal antibodies to several different Hib outer membrane proteins have been shown to have a protective effect against experimental Hib disease (13, 14, 25, 28, 36, 38, 44).

The first Hib outer membrane protein shown to induce the synthesis of such protective antibodies was the major outer membrane protein of this organism which exhibits an apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 37,000 to 40,000 (38). This protein is quantitatively the most abundant protein species in the Hib outer membrane (6, 15, 29, 32, 50). This outer membrane protein, referred to as the 39K protein (15, 16, 18), the P2 protein (38), and protein b/c (30, 51) by different laboratories, also has been shown to be a porin, forming transmembrane permeability channels in planar lipid bilayer membranes (48, 49). This protein also binds avidly to Hib lipooligosaccharide (LOS) (15). Recent data suggest that nontypeable *H. influenzae* possess a protein with a similar apparent molecular mass (ca. 40 kilodaltons) which functions as a porin (3).

The potential importance of the P2 protein as a vaccine candidate and its apparent functional role in the physiology of Hib warrant detailed investigation of the antigenic, biochemical, and biophysical characteristics of this protein. The availability of the Hib gene encoding this protein would facilitate such investigations and would also permit genetic analysis of this protein among Hib strains. We cloned the gene for the Hib P2 protein by using a plasmid shuttle vector capable of replication in both *H. influenzae* and *Escherichia coli* (7). In addition, the recombinant strain of *H. influenzae* expressing the Hib P2 protein was used to develop a system for detecting polyclonal serum antibodies directed against surface determinants of this protein.

MATERIALS AND METHODS

Bacterial strains and DNA cloning vectors. The Hib strain used in these experiments was DL42 and has been described previously (17). The recombination-deficient (*rec-1*) strain of *H. influenzae* Rd utilized for cloning purposes was DB117 (45). Competent cells of *E. coli* HB101 and DH5α were obtained from a commercial source (Bethesda Research Laboratories, Gaithersburg, Md.). All *H. influenzae* strains and *E. coli* strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal base (BHIS) as described previously (1, 19).

The plasmid vector pBR322 and the bacteriophage expression vector λgt11 were employed in shotgun cloning experiments as described previously (23, 33). Plasmid pHVT1 is a shuttle vector constructed by Danner and Pifer (7) that will replicate in both *H. influenzae* and *E. coli*. It contains the ampicillin resistance gene and the replication origin from the *H. influenzae* plasmid pRSF0885. This plasmid also contains the replication origin from pBR322 and the tetracycline resistance gene from the transposon Tn*10* (7). Plasmid pGJB103 is a derivative of pHVT1 with a deletion from the *Ava*I site to the *Sph*I site and also contains a *Bgl*II linker

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inserted at the *Ssp*I site of the β-lactamase gene located in the untranslated leader region (G. J. Barcak, personal communication).

Preparation of the oligonucleotide probe. P2 was purified from Hib DL42 by the method of Munson et al. (38). The amino acid sequence at the amino-terminal end of this protein was determined by the use of a gas-phase sequencer (model 470; Applied Biosystems, Foster City, Calif.) with an on-line high-pressure liquid chromatography phenylthiohydantoin amino acid identification system (model 120; Applied Biosystems). Standard *E. coli* codon usage assignments were used to design an oligonucleotide probe comprising 60 bases encoding the first 20 amino acids of the P2 protein (12) (see Fig. 1). This oligonucleotide was synthesized by using phosphoramidite chemistry in a DNA synthesizer (model 380; Applied Biosystems) and purified by PAGE.

P2-specific monoclonal antibodies. Monoclonal antibodies (MAbs) 9F5 and 2F4 are directed against the P2 protein of Hib DL42 and also react with every Hib strain tested to date (E. J. Hansen, S. E. Pelzel, K. Orth, and C. Slaughter, manuscript in preparation). MAb 9F5 is specific for a determinant of P2 that is not expressed on the Hib cell surface; this antibody is functional in both the colony blot-radioimmunoassay (RIA) system and in a Western blot (immunoblot) system. MAb 2F4 is specific for a surface-exposed epitope of P2; this antibody is functional in the colony blot-RIA but does not bind to Hib P2 in Western blot analysis (Hansen et al., in preparation). MAb 9F5 reacts with the P2 proteins of both Hib DL42 and *H. influenzae* Rd DB117, while MAb 2F4 reacts with the P2 protein of Hib DL42 but not with the P2 protein of strain DB117.

Construction of recombinant plasmids. Chromosomal DNA was purified from Hib DL42 as described by Bricker et al. (2). Plasmids were extracted and purified by cesium chloride density centrifugation as described previously (33). Complete enzymatic digestion of Hib chromosomal DNA was achieved by incubating 8.8-μg portions of DL42 DNA with 22 U of *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 20 U of *Pst*I (Boehringer Mannheim), 25 U of *Pvu*II (Boehringer Mannheim), and 40 U of *Bam*HI (Promega Biotech, Madison, Wis.) in 80-μl reaction volumes under the conditions recommended by the manufacturers. Southern blot analysis of complete restriction enzyme digests of Hib DL42 chromosomal DNA (2 μg) or plasmid DNA (1 μg) resolved by agarose gel electrophoresis was performed as described by Maniatis et al. (33) by using the P2-derived oligonucleotide probe. This oligonucleotide probe was end labeled with polynucleotide kinase (Promega Biotech) and adenosine 5′-[γ-^{32}P]triphosphate (Amersham Corp., Arlington Heights, Ill.) (33). Molecular mass markers obtained from Bethesda Research Laboratories were used in every agarose gel.

Cloning of the 10-kilobase (kb) *Pst*I gene fragment which hybridized to the oligonucleotide probe was accomplished as follows. A complete *Pst*I digest of Hib DL42 DNA was prepared by digesting 375 μg of DNA with 1,000 U of *Pst*I for 2 h at 37°C. The digested DNA was then subjected to electrophoresis in a 1% (wt/vol) agarose gel. The region of the gel containing DNA fragments between 8 and 15 kb in size was excised, and the DNA was eluted into dilute Tris-EDTA buffer at 55°C. The eluted DNA was purified by passage over a NACS column (Bethesda Research Laboratories). Ligation of this DNA into the vector was accomplished by incubating 1.5 μg of the sized DNA fragments with 0.43 μg of *Pst*I-digested and alkaline phosphatase-treated pGJB103 plasmid together with ATP and T4 DNA ligase (33). After ligation, the DNA was concentrated by ethanol precipitation and solubilized in 100 μl of sterile phosphate-buffered saline (pH 7.3).

The entire ligation mixture was used to transform *H. influenzae* Rd DB117 by a method devised by Stuy and Walter (46) as modified by Barcak (G. J. Barcak, personal communication). Briefly, a 10-ml portion of DB117 cells (1.1×10^9 CFU/ml) in the logarithmic phase of aerobic growth was transferred into a 17-by-100-mm polypropylene tube and incubated for an additional 75 min at 37°C without shaking. A 0.2-ml portion of these cells was then transferred to a new 17-by-100-mm tube, 1.8 ml of phosphate-buffered saline containing 900 μg of lactate per ml (pH 7.2) was added, and the mixture was incubated for an additional 65 min at 30°C without shaking. The entire DNA ligation mixture was then added to this tube with gentle but thorough mixing, and this tube was incubated for 30 min at 30°C without shaking. A 1.35-ml volume of warmed (37°C) 80% (vol/vol) glycerol was then added with thorough mixing, and the tube was allowed to stand for 10 min at room temperature. The entire mixture was then transferred into a 125-ml flask containing 10 ml of BHIS (prewarmed to 37°C), and the flask was shaken at 37°C for 3 h. The entire culture was then transferred into a sterile centrifuge tube and spun for 10 min at 6,700 × *g* at 4°C. The cell pellet was suspended in 1 ml of BHIS, and 0.1-ml portions of this were spread on 10 BHIS agar plates containing 5 μg of tetracycline hydrochloride per ml to select plasmid-containing transformants. These plates were incubated at 37°C in a candle extinction jar for 18 to 20 h. The resultant colonies were probed with MAb 2F4 in the colony blot-RIA.

Colony blot-RIA. The colony blot-RIA was accomplished as described by Gulig et al. (17) with MAb 2F4 as the primary antibody.

SDS-PAGE and Western blot analysis. Whole-cell lysates of *H. influenzae* strains were prepared as described by Patrick et al. (42) except that organisms were grown on BHIS agar plates. Outer membrane vesicles were prepared by the lithium chloride-based method of McDade and Johnston (34) as modified by Gulig et al. (16). Portions of these whole-cell lysates or outer membrane vesicle preparations were subjected to SDS-PAGE as described previously (19) and then stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis (25).

Indirect antibody accessibility RIA. The indirect antibody accessibility RIA was used to investigate the surface exposure of P2 and was performed as described by Kimura et al. (25, 26). MAb 4C4, directed against a surface-exposed epitope in the oligosaccharide region of Hib DL42 LOS, was used as a control for nonspecific antigen binding in these experiments (17).

In vitro protein synthesis. Purified plasmids pGJB103 and pEJH39-1 were used as templates in a procaryotic DNA-directed translation kit (Amersham). [^{3}H]leucine was used to radiolabel proteins synthesized in vitro, according to the instructions of the manufacturer. After termination of protein synthesis by chilling the reaction mixture in ice water, the contents of each reaction tube were used in the presolubilized radioimmunoprecipitation (RIP) system together with MAb 2F4 as described previously (18). Immune precipitates were resolved by SDS-PAGE, and the radiolabeled proteins present in these gels were visualized by fluorography (18).

Mutagenesis of plasmid DNA. Purified pEJH39-1 was mutagenized by treatment with hydroxylamine as described by Eichenlaub (9) with minor modifications. Briefly, 1 μg of

Ala	Val	Val	Tyr	Asn	Asn	Glu
GCT	GTT	GTT	TAT	AAC	AAC	GAA

Gly	Thr	Asn	Val	Glu	Leu	Gly
GGT	ACC	AAC	GTT	GAA	CTG	GGT

Gly	Arg	Leu	Ser	Ile	Ile
GGT	CGT	CTG	TCT	ATC	ATC

FIG. 1. Amino acid sequence of residues 1 to 20 of the P2 protein of Hib DL42 and the corresponding oligonucleotide (5′ to 3′) synthesized from this sequence.

pEJH39-1 was incubated with 40 μl of 1.0 M hydroxylamine (pH 6.0) in 0.1 M sodium phosphate buffer (pH 6.0) in a final volume of 100 μl for 41 h at 37°C. This preparation was then dialyzed overnight at 4°C against 100 mM Tris hydrochloride (pH 7.5) containing 50 mM sodium chloride and 5 mM EDTA with two changes of buffer (500 ml each). The plasmid DNA was then ethanol precipitated and suspended in 10 μl of 100 mM Tris containing 50 mM sodium chloride and 5 mM EDTA.

Detection of P2-directed polyclonal antibodies. Immune rat serum was prepared by injecting 10^8 CFU of Hib DL42 intraperitoneally into adult rats three times over an 8-week period. Immune serum (1.0 ml) prepared from the blood of these animals was adsorbed exhaustively with whole cells of the mutant strain DB117(pEJH39-1)2F4$^-$ as described previously (21). Individual portions (400 μl) of this adsorbed immune serum, MAb 2F4, and control (nonimmune) rat serum were used in the whole-cell RIP system together with radioiodinated whole cells of strains DB117(pEJH39-1) and DB117(pEJH39-1)2F4$^-$. The resultant immune precipitates were subjected to SDS-PAGE and analyzed by autoradiography as described previously (18).

RESULTS

Preliminary attempts to shotgun clone the gene encoding the Hib P2 protein into *E. coli* by using either λgt11 or pBR322 together with immunoscreening by means of a MAb reactive with the P2 protein were unsuccessful. We therefore proceeded to use a more direct approach to identify the gene encoding the P2 protein of Hib. The amino-terminal amino acid sequence of the P2 protein of Hib DL42 was determined, and an oligonucleotide probe theoretically corresponding to the first 20 amino acids in P2 was constructed by using standard *E. coli* codon usage assignments (12) (Fig. 1). This oligonucleotide was used to probe complete restriction enzyme digests of Hib DL42 chromosomal DNA in Southern blot analysis (Fig. 2). The oligonucleotide probe hybridized to a 10-kb band from the *Pst*I digest (Fig. 2, lane A), to a 1- to 2-kb band from the *Eco*RI digest (Fig. 2, lane B), to a 48-kb band from the *Bam*HI digest (Fig. 2, lane C), and to a 1- to 2-kb band from the *Pvu*II digest (Fig. 2, lane D).

The *Pst*I restriction fragments ranging in size from approximately 8 to 15 kb were ligated into pBR322 and used to transform *E. coli* HB101 and DH5α. The resultant transformants were screened in a colony hybridization assay with the Hib P2-specific oligonucleotide probe. No recombinant clones reactive with this oligonucleotide were identified in several different experiments involving over 8,000 transformants.

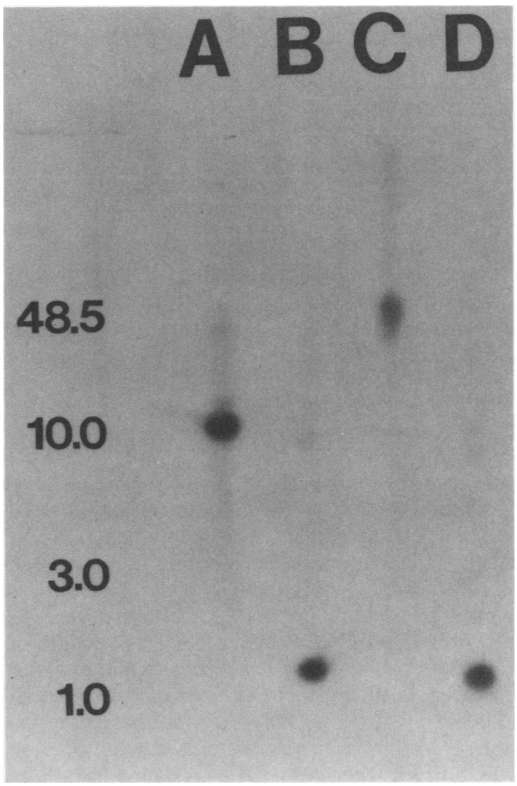

FIG. 2. Southern blot analysis of complete restriction enzyme digests of Hib DL42 chromosomal DNA hybridized with the P2-derived oligonucleotide probe. The restriction endonucleases used in this experiment were *Pst*I (lane A), *Eco*RI (lane B), *Bam*HI (lane C), and *Pvu*II (lane D). Numbers on the left side of the figure indicate molecular size in kilobases.

The failure to identify a recombinant *E. coli* clone containing the P2 gene suggested that expression of P2 in *E. coli* might be a lethal event. We chose to circumvent this possible toxicity problem by using an approach which involved the cloning and expression of the Hib P2 gene in a more native environment (i.e., within *H. influenzae*). The availability of a recombination-deficient Rd strain of *H. influenzae* (DB117) permitted the efficient use of plasmid pGJB103 as a cloning vector (7, 45). Additionally, we screened a number of Hib P2-directed MAbs for their reactivity with Hib DL42 and with the Rd strain DB117 (*rec-1*) in order to determine whether immunoscreening methods could be employed to identify *H. influenzae* Rd recombinants expressing the Hib P2 protein. One Hib P2-specific MAb (2F4) was identified which was reactive with Hib DL42 but not with the Rd strain (Fig. 3).

The size-selected (8- to 15-kb) *Pst*I DNA fragments were ligated into *Pst*I-cleaved pGJB103, and the entire ligation mixture was used to transform Rd strain DB117. The resultant transformants were selected on BHIS agar containing tetracycline and were analyzed in the colony blot-RIA system with the Hib P2-specific MAb 2F4. Of 364 transformants, 10 were found to be reactive with this MAb. These strains were purified by single-colony passage and retested in the colony blot-RIA; all 10 strains were positive. The 10 strains also were sensitive to ampicillin, a finding consistent with insertional inactivation of the β-lactamase gene containing the *Pst*I recognition site in pGJB103.

Characterization of the antibody-reactive transformant.

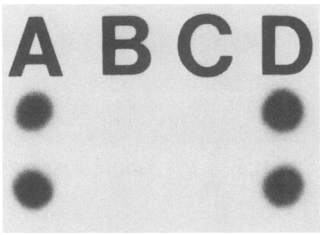

FIG. 3. Colony blot-RIA of Hib DL42 and *H. influenzae* Rd strains probed with MAb 2F4. Strains probed with this MAb were Hib DL42 (lane A), *H. influenzae* Rd DB117 (lane B), *H. influenzae* Rd DB117(pGJB103) (lane C), and Mab 2F4-reactive transformant (recombinant strain) (lane D). Each strain was spotted in duplicate on the filter pad.

Whole-cell lysates of Hib DL42, the Rd host strain DB117, DB117 containing the vector pGJB103, and a MAb 2F4-reactive DB117 transformant were subjected to SDS-PAGE followed by Coomassie blue staining and Western blot analysis of total cell proteins. Coomassie blue staining of these whole-cell lysates revealed a readily detectable P2 protein in Hib DL42 (Fig. 4A, lane A). The lysate of strain DB117 exhibited two major protein bands in the same general region with apparent molecular weights of 39,000 and 36,000 (Fig. 4A, lane B). Introduction of the pGJB103 vector into DB117 resulted in reduced expression of proteins in this region of the gel (Fig. 4A, lane C). The MAb 2F4-reactive transformant strain (Fig. 4A, lane D) expressed a protein with a molecular weight apparently identical to that of the P2 protein of DL42; this protein was not present in the host strain containing only the vector (Fig. 4A, lane C).

Western blot analysis of the same whole-cell lysates was performed with a second MAb (9F5) which is reactive with the P2 protein of Hib DL42 in Western blot analysis. This antibody bound to the P2 protein of Hib DL42 (Fig. 4B, lane A). This antibody bound to a protein with an apparent

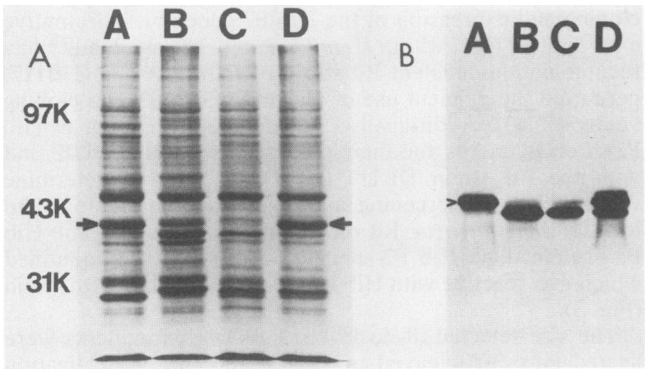

FIG. 4. (A) Total cell proteins of Hib and *H. influenzae* Rd strains. Whole-cell lysates of Hib DL42 (lane A), *H. influenzae* Rd DB117 (lane B), *H. influenzae* Rd DB117(pGJB103) (lane C), and MAb 2F4-reactive transformant (lane D) were resolved by SDS-PAGE and stained with Coomassie blue. The arrows indicate the position of the P2 major outer membrane protein in Hib DL42. Numbers indicate apparent molecular weights. (B) Western blot analysis of whole-cell lysates probed with MAb 9F5. Whole-cell lysates of each strain were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis with the Hib P2-directed MAb 9F5. Lanes are as in panel A. The arrowhead indicates the position of the Hib P2 protein in Hib DL42.

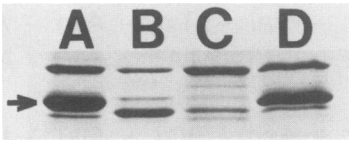

FIG. 5. Presence of P2 protein in outer membrane vesicles of Hib DL42 (lane A), *H. influenzae* Rd DB117 (lane B), *H. influenzae* Rd DB117(pGJB103) (lane C), and MAb 2F4-reactive transformant (lane D). Outer membrane vesicles were resolved by SDS-PAGE and stained with Coomassie blue. Only the relevant midsection of the gel is shown. The arrow indicates the position of the P2 major outer membrane protein in Hib DL42.

molecular weight of 36,000 which was present in both DB117 and DB117(pGJB103) (Fig. 4B, lanes B and C); this protein is presumably the P2 protein of DB117. The MAb 2F4-reactive transformant (Fig. 4B, lane D) expressed two proteins reactive with MAb 9F5, the 36K protein and a protein with an apparent molecular weight identical to that of the P2 protein of Hib DL42 (Fig. 4B, lane A).

Outer membrane vesicles were prepared from these four strains. Comparison of the proteins present in outer membrane vesicles of Hib DL42 (Fig. 5, lane A) with those in the MAb 2F4-reactive transformant (Fig. 5, lane D) showed that the 39,000-molecular-weight proteins detected in whole-cell lysates of these two strains (Fig. 4A, lanes A and D) are present in the outer membranes of these organisms. Western blot analysis of these vesicles confirmed that the 39,000-molecular-weight proteins were both Hib P2.

Characterization of the recombinant plasmid. Agarose gel electrophoresis of complete restriction enzyme digests of the vector pGJB103 and the plasmid extracted from the MAb 2F4-reactive transformant revealed that the recombinant plasmid contained a DNA insert approximately 10 kb in size. Southern blot analysis involving the use of the Hib P2-derived oligonucleotide probe confirmed that this insert was comprised of Hib chromosomal DNA. This recombinant plasmid was designated pEJH39-1.

A partial restriction enzyme map of the recombinant plasmid pEJH39-1 is illustrated in Fig. 6. The Hib DNA insert in this plasmid contained several recognition sites for *Pvu*II and *Eco*RI and a single recognition site for *Mlu*I. This insert has no *Bam*HI site.

An in vitro DNA-directed translation system also was used to characterize the Hib insert in the recombinant plasmid. By using the vector pGJB103 and the recombinant plasmid pEJH39-1 as templates, translation products were radiolabeled with [^{3}H]leucine and then immunoprecipitated with MAb 2F4. The resultant immune precipitates were subjected to SDS-PAGE and analyzed by fluorography. Positive control experiments included the use of MAb 2F4 to immunoprecipitate radiolabeled P2 protein from Hib DL42 cells intrinsically radiolabeled by growth in the presence of [^{3}H]leucine. This control experiment yielded a single radiolabeled band with an apparent molecular weight of 39,000 (Fig. 7, lane C). The immune precipitate derived from proteins encoded in pEJH39-1 contained a major band slightly larger than that of the native P2 protein and a smaller amount of a protein that migrated identically to the native P2 protein (Fig. 7, lane B). The slightly larger (upper) band presumably represents P2 protein from which the signal peptide has not been removed (27). The immune precipitate derived from the proteins encoded in the vector pGJB103 contained only very faint bands (Fig. 7, lane A).

Transformation of *E. coli* with the recombinant plasmid pEJH39-1. Both the vector plasmid pGJB103 and the recom-

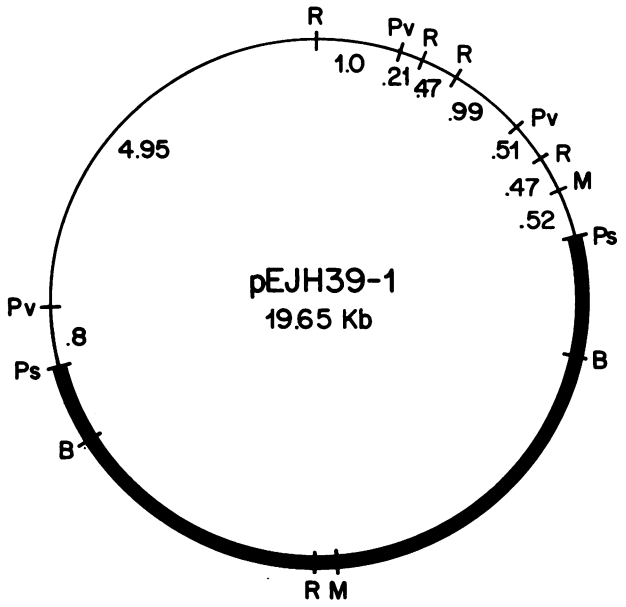

FIG. 6. Partial restriction map of the recombinant plasmid pEJH39-1. Approximate distances between restriction sites are given in kilobases. The heavy line represents vector DNA. Abbreviations for restriction enzyme sites: B, *Bam*HI; Ps, *Pst*I; R, *Eco*RI; Pv, *Pvu*II; M, *Mlu*I.

binant plasmid pEJH39-1 were used to transform *E. coli* HB101 and DH5α. Transformation of *E. coli* HB101 with pGJB103 in two separate experiments yielded a total of approximately 12,000 tetracycline-resistant transformants, while transformation of this strain with pEJH39-1 yielded no tetracycline-resistant transformants. Transformation of *E. coli* DH5α with pGJB103 in three separate experiments yielded a total of 22,000 transformants, but only 3 tetracycline-resistant transformants were obtained when pEJH39-1 was used to transform *E. coli* DH5α. Colony blot-RIA analysis revealed that these latter three transformants did not express an antigen reactive with the Hib P2-specific MAbs (data not shown). Analysis of plasmid DNA from

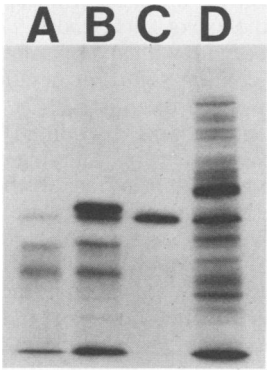

FIG. 7. Immunoprecipitation of proteins synthesized in an in vitro DNA-directed translation system. MAb 2F4 was used in the presolubilized RIP system to immunoprecipitate [^{3}H]leucine-labeled proteins synthesized in vitro by using plasmids pGJB103 (lane A) and pEJH39-1 (lane B) as templates. MAb 2F4 also was used in this RIP system to immunoprecipitate P2 from Hib DL42 cells grown in the presence of [^{3}H]leucine. Lane C, P2 immunoprecipitated from Hib DL42 cells; lane D, solubilized ^{3}H-labeled DL42 cells.

TABLE 1. Antibody accessibility of P2 epitopes on the surface of *H. influenzae* strains

Strain	Binding[a] of MAb:	
	4C4[b]	2F4[c]
DB117	160	127
DB117(pGJB103)	128	72
DB117(pEJH39-1)	191	1,405
DL42	6,286	2,208

[a] Counts per minute of ^{125}I-labeled goat anti-mouse immunoglobulin bound to MAbs attached to the bacterial cell surface as determined in the indirect antibody accessibility RIA. These data represent the average of values obtained in two separate experiments.
[b] MAb 4C4 is an immunoglobulin G3 antibody specific for a surface epitope of LOS in DL42; it is included here as a control for nonspecific binding.
[c] MAb 2F4 is an immunoglobulin G3 antibody specific for a surface epitope of the Hib P2 protein.

these three strains revealed that all three plasmids were smaller than pEJH39-1 and lacked the Hib DNA insert present in pEJH39-1 (data not shown).

Localization of the Hib P2 protein in DB117(pEJH39-1). The specificity of MAb 2F4 for a surface epitope of the Hib P2 protein permitted investigation of the possible surface exposure of Hib P2 in the recombinant strain DB117 (pEJH39-1). Both this immunoglobulin G3 antibody and another immunoglobulin G3 MAb specific for a surface epitope of Hib DL42 LOS were used in the indirect antibody accessibility RIA together with whole cells of DB117, DB117(pGJB103), DB117(pEJH39-1), and Hib DL42 (Table 1). The control MAb (4C4) exhibited low levels of binding to DB117 and to the DB117-derived strains but bound to Hib DL42 at a much higher level. MAb 2F4 bound at only very low levels to DB117 and to DB117(pGJB103) and bound at much higher levels to both the recombinant strain and Hib DL42, indicating that at least one epitope of the Hib P2 protein is expressed on the surface of this recombinant strain.

Detection of Hib P2-directed polyclonal antibodies. The availability of a recombinant strain of *H. influenzae* expressing Hib P2 on its cell surface presented the opportunity for the development of an immunoassay system to detect polyclonal serum antibodies specific for surface epitopes of Hib P2. The first step in this process involved the construction of a strain which could be used as an immunoadsorbent to remove serum antibodies to all surface antigens of DB117(pEJH39-1) except those antibodies specific for surface epitopes of the Hib P2 protein. Hydroxylamine mutagenesis of plasmid pEJH39-1 and subsequent transformation of the mutagenized plasmids into strain DB117 yielded a strain which failed to bind MAb 2F4 in the colony blot-RIA. This transformant [DB117(pEJH39-1)2F4$^-$] expressed no detectable Hib P2 protein as determined by Coomassie blue staining and Western blot analysis of SDS-PAGE-resolved outer membrane vesicles (data not shown). Similarly, the LOS profiles of strains DB117(pEJH39-1) and DB117 (pEJH39-1)2F4$^-$ were identical as determined by SDS-polyacrylamide gradient gel analysis and silver staining (data not shown). The plasmid present in this latter strain was the same size as pEJH39-1, indicating that the Hib DNA insert was still present in the plasmid of this strain.

Immune rat serum raised against Hib DL42 was adsorbed exhaustively with whole cells of strain DB117(pEJH39-1)2F4$^-$ and was then used in the whole-cell RIP system together with radioiodinated whole cells of strains DB117(pEJH39-1) and DB117(pEJH39-1)2F4$^-$. Control an-

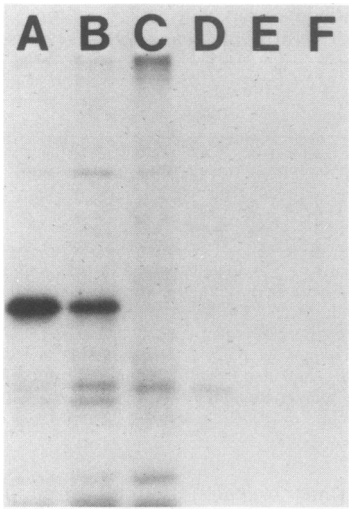

FIG. 8. Detection of antibodies directed against surface determinants of Hib P2. Radioiodinated whole cells of DB117(pEJH39-1) (lanes A to C) and DB117(pEJH39-1)2F4$^-$ (lanes D to F) were used in the whole-cell RIP system with Hib DL42 immune serum adsorbed with whole cells of DB117(pEJH39-1)2F4$^-$ (lanes A and D), MAb 2F4 (lanes B and E), or control rat serum from normal (nonimmune) animals (lanes C and F).

tibodies used in this whole-cell RIP system included MAb 2F4 (positive control) and serum from normal, nonimmune rats (negative control). Antibodies in the preadsorbed immune serum immunoprecipitated the Hib P2 protein present in DB117(pEJH39-1) (Fig. 8, lane A) and did not immunoprecipitate any proteins from DB117(pEJH39-1)2F4$^-$ (Fig. 8, lane D). MAb 2F4 similarly immunoprecipitated its homologous antigen (Hib P2) from DB117(pEJH39-1) (Fig. 8, lane B) and no proteins from DB117(pEJH39-1)2F4$^-$ (Fig. 8, lane E). Control serum did not immunoprecipitate radioiodinated P2 protein from either type of cell (Fig. 8, lanes C and F). These data indicate that these two strains, differing only in the expression of the Hib P2 protein, can be used in conjunction with a RIP system to detect serum antibodies directed against surface-exposed epitopes of Hib P2.

DISCUSSION

The genes encoding two Hib surface proteins that bind protective antibodies have been cloned previously in *E. coli* (8, 10, 37). Purified preparations of the heat-modifiable major outer membrane protein of Hib, designated P1 (13) or protein a (28), have been shown to be able to induce the synthesis of polyclonal antibodies protective against experimental Hib disease (13, 28), and a Mab to P1 also has been shown to have a protective effect (10). When expressed in recombinant *E. coli*, this protein is exposed on the cell surface (10). The P6 protein is another surface-exposed macromolecule which is a target for protective antibodies and which appears to be highly conserved among both Hib strains and strains of nontypeable *H. influenzae* (14, 36, 39, 40). The gene encoding the P6 protein of Hib has been cloned, as has the gene encoding the P6 protein of a nontypeable strain of *H. influenzae*, and the amino acid sequences deduced from the DNA sequences of these two genes have been found to be identical (9, 41).

Our initial efforts involving the use of both monoclonal and polyclonal antibody probes to detect expression of the Hib P2 protein in recombinant strains of *E. coli* derived from shotgun cloning experiments were uniformly unsuccessful, leading us to suspect that unregulated expression of this Hib protein in *E. coli* might be lethal. The fact that *H. influenzae* promoters apparently function effectively in *E. coli* (8, 10, 22, 41) reinforced the validity of this concern. Therefore, we chose to circumvent this potential problem of lethal gene expression by using *H. influenzae* as the host for cloning the Hib P2 gene. The use of an oligonucleotide probe derived from the amino-terminal amino acid sequence of P2 together with the pGJB103 shuttle vector permitted the cloning of the gene encoding the Hib P2 protein in a strain of *H. influenzae* Rd. This gene product was expressed on the surface of the recombinant *H. influenzae* strain, and it was also present in the outer membrane of this same strain. It is of interest to note that the introduction of the pGJB103 plasmid vector into *H. influenzae* Rd DB117 reduced the amount of the homologous P2 protein expressed by this strain (Fig. 4A, compare lanes B and C). The basis for this apparent suppression of P2 expression is not known.

Transformation experiments involving the use of recombinant plasmid pEJH39-1 encoding the Hib P2 protein together with two different *E. coli* strains again strongly suggested that unregulated expression of this P2 protein in *E. coli* is lethal. The only transformants obtained in these experiments no longer expressed an antigen reactive with the Hib P2-specific MAb 2F4. The basis for the apparent lethality of Hib P2 for *E. coli* is not known but could involve interference with *E. coli* porin function, and it has been suggested that the physical arrangement of the pore formed by Hib P2 porins may be very different from that of the pores normally found in *E. coli* (48). The fact that *E. coli* regulates the synthesis of its own porins (35) also indicates that unregulated production of Hib P2 could disturb the integrity of the outer membrane, especially in view of the fact that other *H. influenzae* proteins are known to be translocated to the surface of *E. coli* when expressed from cloned genes (10, 22, 41). The apparently lethal expression of Hib P2 in *E. coli* is similar to that recently reported for the gonococcal porin protein (4, 11). Cloning of the gonococcal P.1 gene in *E. coli* only became possible when the −35 region of the gonococcal promoter was removed and the remaining structural gene was placed under the control of a foreign inducible promoter (4). It is probable that the Hib P2 gene could be cloned and expressed in *E. coli* by using such an approach. A recent report describing the use of λgt11 and *E. coli* to clone a gene encoding a protein of Hib with an apparent molecular weight of 39,500 did not contain sufficient detailed information to determine whether this protein is P2 (47).

The availability of the gene encoding the Hib P2 protein opens up new possibilities for studying both the immune response to this protein and its role in the pathogenesis of Hib disease. If the gene encoding this protein can be cloned and expressed at high levels in *E. coli*, then this protein can be purified free of contamination with Hib LOS for use in immunogenicity studies. The fact that the P2 protein avidly binds Hib LOS has precluded the use of Hib LOS-free P2 in immunogenicity experiments (15, 38), and the fact that lipopolysaccharide is known to affect antibody response to other outer membrane proteins suggests that this interaction requires evaluation with regard to the immunogenicity of Hib P2 (24). Similarly, not much is known about antibody response to surface determinants of Hib P2. Passive protection experiments involving polyclonal rabbit antisera raised against purified Hib P2 proteins suggested that the surface-exposed antigenic determinants of this protein may vary

among strains of this pathogen (38). Experiments designed to examine antibody response to P2 as a result of either invasive Hib disease in humans or immunization of experimental animals with viable Hib cells have been hampered by two different problems. The interaction of Hib P2 with Hib LOS has made accurate interpretation of earlier results obtained with certain RIP systems very difficult (15). Similarly, the apparent denaturation or destruction of P2 epitopes in gel-immunoradioassay methods yielded negative results when sera from infants convalescing from Hib meningitis were assayed for P2-directed antibodies (30, 51). Accordingly, it should be noted that the Mab (2F4) used in this study and another MAb, which are specific for two different surface epitopes of the Hib P2 protein, do not function in Western blot systems, again suggesting that the relevant epitopes are denatured by these techniques (Hansen et al., in preparation). More recently, a study using adsorption of serum antibodies on whole Hib cells prior to use of the serum in Western blot assays was confounded by the apparent association of Hib LOS with P2 in the polyacrylamide gel and subsequent Western blot (31).

We used the recombinant strain of *H. influenzae* expressing the Hib P2 protein to produce a strain of *H. influenzae* which could be used to adsorb irrelevant antibodies away from P2-specific antibodies in polyclonal sera. Chemical mutagenesis of the recombinant plasmid pEJH39-1 permitted construction of a mutant strain which no longer expressed the Hib P2 protein but which had an outer membrane protein profile and LOS characteristics identical to those of the recombinant strain DB117(pEJH39-1). This mutant strain [DB117(pEJH39-1)2F4$^-$] was used to adsorb immune serum raised against Hib DL42 in order to remove any antibodies to surface antigens of strain DB117(pEJH39-1) except antibodies to surface determinants of the recombinant Hib P2 protein. This adsorbed immune serum immunoprecipitated the Hib P2 protein of DB117(pEJH39-1) but did not precipitate any proteins from the mutant strain DB117(pEJH39-1)2F4$^-$ (Fig. 8). If there had been any antibodies to surface determinants of DB117 LOS in this adsorbed immune serum, then the DB117 P2 (with an apparent molecular weight of 36,000) would have been immunoprecipitated by these antibodies in a complex with DB117 LOS (15). The possibility exists that the epitopes of Hib P2 expressed on the surface of DB117(pEJH39-1) are only a subset of those found on native Hib cells, but at the very least this system would allow, for the first time, measurement of the humoral immune response to epitopes of the Hib P2 protein which are potentially involved in the host-parasite interaction.

Finally, the availability of this gene encoding the Hib P2 protein will permit detailed studies on structure-function relationships of this protein through DNA sequence analysis and the use of site-directed mutagenesis. Additionally, it may be possible to construct mutant strains expressing little or no P2 protein; such mutants could be used to determine whether this protein is involved in the expression of virulence by Hib.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17621 from the National Institute of Allergy and Infectious Diseases to E.J.H.

We thank Gerard J. Barcak, Hanna Tomb, and Hamilton O. Smith of Johns Hopkins University for generously providing plasmid pGJB103 to us prior to publication of their own work with this plasmid and for many helpful discussions concerning the genetics of *H. influenzae*. We also thank Leon Eidels, Robert Munford, and Justin Radolf for their comments concerning this manuscript, which was expertly typed by Cindy Baselski.

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