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Mapping of B-Cell Epitopes on the Outer Membrane P2 Porin Protein of *Haemophilus influenzae* by Using Recombinant Proteins and Synthetic Peptides

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The P2 protein of *Haemophilus influenzae* type b has a porin activity and is the most abundant protein in the outer membrane. We have employed fusion protein constructs and synthetic peptides along with monoclonal antibodies to map B-cell epitopes in this protein. A linear, surface-exposed epitope was identified between residues 158 and 174. A second surface-exposed epitope was identified near the carboxy-terminal end of the protein (residues 319 to 341). Two additional B-cell epitopes were identified. One was localized between residues 28 and 55, whereas the other was located between residues 148 and 174. These epitopes were not present on the surface of intact *H. influenzae* cells. Thus, four distinct immunogenic and antigenic regions on the P2 protein have been identified.

The great majority of invasive *Haemophilus influenzae* disease in North America, Europe, and other regions of the world is caused by strains expressing the type b capsular polysaccharide (33, 37). The capsular polysaccharide, polyribosyl-ribitol phosphate, has been shown to be one of the primary virulence factors for this microorganism (24, 41). Recently, a conjugate vaccine composed of the type b polysaccharide covalently coupled to a mutant diphtheria toxin protein has been licensed for use in 2-month-old infants in the United States. This vaccine and other conjugate vaccines which have been licensed for use in older children all employ irrelevant protein carriers. It is likely that future vaccines will contain capsular polysaccharides coupled to *Haemophilus* proteins or peptides derived from *Haemophilus* proteins. Several *Haemophilus* outer membrane proteins (OMPs) are being investigated as potential vaccine candidates (7). Antibodies directed against five *Haemophilus* OMPs have been shown to have protective or bactericidal activity (4, 7–9, 15, 19, 27, 28). One of these surface molecules, OMP P2 (also designated b/c [20]), has porin activity and is the most abundant protein in the *Haemophilus* outer membrane (20, 28, 38, 39). A monoclonal antibody (MAb) directed against the P2 protein and a human polyclonal anti-P2 serum were shown to have bactericidal activity against nontypeable *Haemophilus* isolates (29, 30). Furthermore, Munson et al. (28) have demonstrated that antibodies directed against the *H. influenzae* serotype b P2 protein are protective in an infant rat model of bacteremia. This study also indicated that some antigenic heterogeneity exists among type b strains. Polyclonal antibody directed against OMP P2 isolated from an OMP subtype 1H strain had protective activity in the infant rat model of bacteremia when the animals were challenged with the same strain used to produce the immunogen but not when they were challenged with another strain with the OMP subtype 2L.

Common surface-exposed epitopes must be identified on P2 if that protein is to be considered for inclusion in a new generation of *H. influenzae* vaccine. Hamel et al. (10) and Hansen et al. (12) have generated porin-specific MAbs that recognized 99 and 100%, respectively, of the type b strains tested. These data indicate that some antigenic epitopes which are highly conserved among *Haemophilus* isolates exist on P2. The DNA sequence of several P2 genes from prototype strains has been determined (11, 25, 26). These genes are highly conserved, which is consistent with the isolation of these broadly cross-reactive MAbs. Martin et al. (21) recently reported the generation of a panel of MAbs directed against P2. This panel of MAbs was characterized with respect to reactivity with representatives of the major clonal groups (A1, A2, and B1) of type b *Haemophilus* isolates as well as with serotype a and d strains (clonal groups B2 and B1, respectively). The surface accessibility of the epitopes recognized by this panel of MAbs was also determined (21). In the present study, gene fusions expressing portions of P2 as well as synthetic peptides were employed to localize the epitopes recognized by these anti-P2 MAbs. Identification of surface-exposed epitopes is important for the development of future vaccines and also provides preliminary information on the folding of the P2 protein in the outer membrane of *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *H. influenzae* type b strain 3175, a strain with the OMP subtype 3L, has been described previously (25). The P2 gene from this strain is identical to the gene from strain MinnA. *Escherichia coli* BL21(DE3)/pLysS was the gift of F. William Studier (35). This strain contains a single copy of T7 RNA polymerase downstream from the *lac* control region. The pLysS plasmid

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contains the T7 lysozyme gene. T7 lysozyme binds to T7 RNA polymerase in vitro, and pLysS stabilizes many T7 expression constructs, presumably by binding and inactivating the small quantities of T7 RNA polymerase produced by BL21(DE3) in the absence of isopropylthio-β-galactoside (22). The bacteriophage T7 expression vector pT7-7 and M13 phage mGP1-2 (containing the bacteriophage T7 RNA polymerase gene) were the gifts of Stan Tabor (36). pGEMEX-1 was obtained from Promega (Madison, Wis.). M13mp18, M13mp19, and *E. coli* JM101 were obtained from New England BioLabs (Beverly, Mass.).

H. influenzae was grown in supplemented brain heart infusion medium as described elsewhere (1). *E. coli* strains were grown in L medium supplemented with ampicillin and/or chloramphenicol where appropriate.

Molecular cloning. The cloning and sequence of the P2 gene from strain 3175 (OMP subtype 3L) was reported previously (25). The gene was cloned into M13mp18 after amplification of the gene from chromosomal DNA with the polymerase chain reaction. The oligonucleotide primer for the polymerase chain reaction, 5′ to the coding sequences for the mature P2 gene, contained a *Bam*HI site. The complementary nucleotide 3′ to the gene contained a *Hin*dIII site. Thus, the polymerase chain reaction product had been cloned into M13 as a *Bam*HI-to-*Hin*dIII fragment. The *Bam*HI-to-*Hin*dIII fragment from the M13mp18 clone was cloned into pGEMEX-1, which has a multiple cloning site near the 3′ end of the bacteriophage T7 capsid gene. Expression of the T7 capsid gene as well as fusion proteins generated in this plasmid are controlled by the T7 promoter. A plasmid with the correct restriction map was designated pRSM741 and saved for further analysis. In this construct, a fusion protein between the T7 capsid gene and P2 was created. In strain JM101/pRSM741, the capsid-P2 fusion protein accumulated after infection with mGP1-2, an M13 phage containing the T7 RNA polymerase gene, or after induction of T7 RNA polymerase in strain BL21(DE3)/pLysS/pRSM741 by the addition of isopropylthio-β-galactoside.

A series of nested deletions were constructed by employing ExoIII. pRSM741 was digested with *Sac*I and *Bam*HI, digested for various periods with ExoIII, blunt ended, religated, and transformed into *E. coli* BL21(DE3)/pLysS with an Erase-a-Base kit (Promega) according to the manufacturer's instructions. Recombinants were screened immunologically, and the junction between the truncated 5′ portion of the P2 gene and the T7 capsid gene in the individual constructs was determined by sequence analysis. Inefficient expression of some fusion proteins was observed in the BL21(DE3)/pLysS background; therefore, plasmids of interest were transformed into *E. coli* JM101 for further analysis.

DNA sequence analysis. Plasmids were prepared by an alkaline-sodium dodecyl sulfate (SDS) protocol (Promega) and denatured in 0.2 M NaOH–0.2 mM EDTA 37°C for 30 min. Dideoxy sequencing was performed with Sequenase (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's directions. [α-thio-^{35}S]ATP was purchased from New England Nuclear Corp. The oligonucleotide primer 5′TGGTACCGGATCGAATTGGC hybridizes upstream of the multiple cloning site in pGEMEX-1 and was employed for sequencing across the gene 10-P2 gene fusion. Data were analyzed by Compugene software (2) on a Digital VAX 8530 computer.

MAbs. MAbs reactive with the P2 protein of *Haemophilus* isolates have been previously characterized by their reactivity with 119 type a, b, and d isolates as determined by immunoblots and immunodots (21). Each member of the MAb groups designated A through D contained antibodies of the identical specificity when the panel of isolates was examined. The reactivity of these antibodies with serotype b strains of the major clonal groups A1, A2, and B1 and the surface accessibility of the epitopes recognized by these MAbs as determined by binding to live bacteria in suspension are summarized in Table 1.

TABLE 1. Reactivities of the P2-specific MAbs[a]

MAb group[b]	MAb	Class and subclass	Binding activity (cpm)[c]	% Reactivity with *H. influenzae* type b[d]
None	P2-2	IgG3(κ)	9,650	91
A	P2-3	IgG3(κ)	10,397	92
	P2-8	IgG3(κ)	12,224	92
	P2-11	IgG3(κ)	12,409	92
B	P2-4	IgG2a(κ)	58,257	100
	P2-6	IgG2b(κ)	12,454	100
C	P2-5	IgG1(κ)	19,085	78
	P2-7	IgG1(κ)	13,756	78
	P2-9	IgG1(κ)	18,161	78
	P2-12	IgG1(κ)	15,203	78
D	P2-10	IgG2a(κ)	33,458	99
	P2-13	IgG2a(κ)	15,657	99
None	P2-14	IgG3(κ)	4,922	92
	P2-16[e]	IgG2a(κ)	2,621	100
	P2-17[e]	IgG1(κ)	2,720	82

[a] Part of the data is summarized from the report of Martin and coworkers (21).

[b] MAb groups were defined on the basis of reactivity with 119 isolates of serotype a, b, and d organisms from clonal groups A1, A2, B1, B2, H1, I1, and J1. Each group of MAbs was distinguished by their ability to recognize the same set of strains.

[c] Bacterial cells were incubated sequentially with hybridoma culture supernatants containing MAbs and ^{125}I-labeled sheep anti-mouse Ig, and the amount of cell-bound ^{125}I was determined. *H. influenzae*-unrelated control MAbs were used as a control and gave counts lower than 1,800 cpm.

[d] Eighty-nine isolates representative of the three major clonal families (A1, A2, and B1) of serotype b isolates were tested by dot immunoassay for reactivity with the MAbs.

[e] Cells were boiled 5 min before application to nitrocellulose for dot blot reactivity assays.

Peptide synthesis. Nineteen peptides of 12 to 30 amino acid residues were selected to cover the entire P2 protein sequence. The peptide sequences and their locations in the protein are summarized in Fig. 1. All peptides were synthesized with the Applied Biosystems (Foster City, Calif.) model 430A automated peptide synthesizer. All peptides, except Pep-25 and Pep-26, were synthesized with an additional cysteine residue at the end distal to the predicted β-turn sequence, so it would be possible to link them to a carrier protein for immunization. The following peptides have the added cysteine at their carboxyl end: Pep-P1 (1 to 14), Pep-1 (28 to 55), Pep-2 (53 to 81), Pep-4 (101 to 129), Pep-5 (125 to 150), Pep-7 (171 to 196), Pep-8 (193 to 219), Pep-10 (241 to 265), Pep-12 (285 to 306), and Pep-13 (302 to 319). The additional cysteine was added at the N terminus of the following peptides: Pep-3 (79 to 106), Pep-6 (148 to 174), Pep-9 (219 to 244), Pep-11 (263 to 289), and Pep-C (314 to 341). Synthetic peptides were cleaved from resin by using hydrofluoric acid and purified by reverse-phase high-pressure liquid chromatography using a Vydac C4 column (The

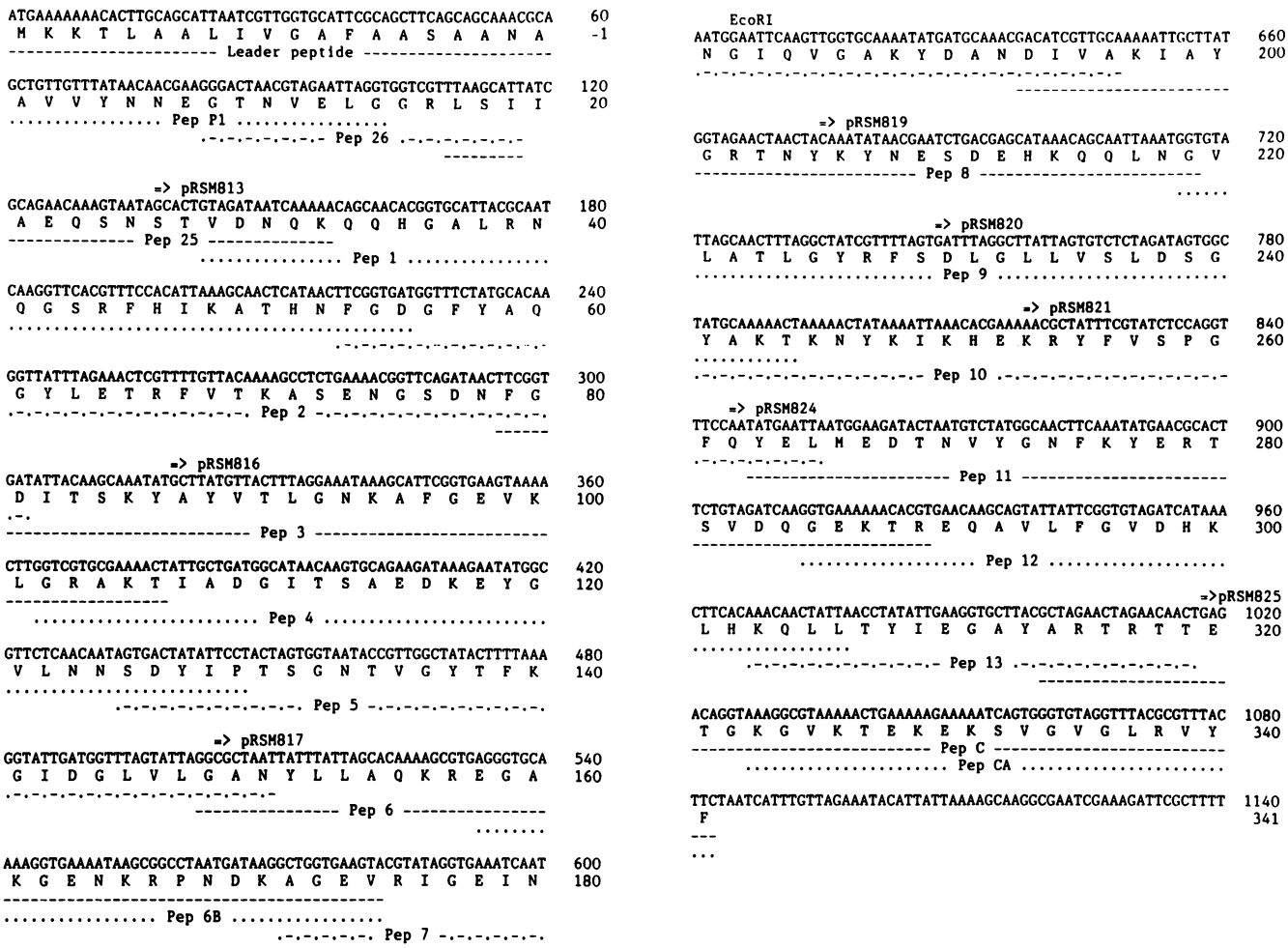

FIG. 1. Nucleotide and derived amino acid sequences of the *H. influenzae* MinnA P2 protein, detailing endpoints of fusion constructs and synthetic peptides.

Separations Group, Hesperia, Calif.). The amino acid analyses of the peptide hydrolysates were in good agreement with their theoretical compositions. Peptides Pep-25 (17 to 32), Pep-26 (8 to 19), Pep-2 (53 to 81), Pep-3 (79 to 106), Pep-7 (171 to 196), Pep-9 (219 to 244), and Pep-12 (285 to 306) were solubilized in a small volume of either dimethylformamide (Sigma Chemical Co., St. Louis, Mo.) or 6 M guanidine-HCl (Sigma). These peptides were then adjusted to 200 μg/ml with distilled water. All of the other peptides were freely soluble in distilled water.

Immunoassays. Fusion protein clones in the BL21(DE3)/pLysS background were induced for the expression of fusion proteins downstream of the T7Φ10 promoter by the addition of 2 mM isopropylthio-β-galactoside. Fusion protein clones in the JM101 background were induced by infection with mGP1-2 in the presence of isopropylthio-β-galactoside as described previously (26). After 2 h of continued incubation at 37°C, the cells were harvested, suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4), and sonicated. For dot blot immunoassay, 2.5 μg of crude sonicate or microcentrifuge-pelleted protein was applied to nitrocellulose (Bio-Rad, Richmond, Calif.). Nonspecific protein-binding sites were blocked with gelatin. The blot was sequentially incubated with undiluted culture supernatants containing the MAb, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) and IgM, and developed as described elsewhere (26).

Peptide enzyme-linked immunosorbent assays (ELISAs) were performed by coating synthetic peptides onto microtitration plates (Maxisorp immunoplate; Nunc, Naperville, Ill.) at concentrations ranging from 0.03 to 50 μg/ml in 50 mM carbonate buffer, pH 9.6. After overnight incubation at room temperature, the plates were washed with phosphate-buffered saline (PBS) containing 0.05% (wt/vol) Tween 20 (Sigma) and blocked with PBS plus 0.5% (wt/vol) bovine serum albumin (Sigma). One hundred microliters of culture supernatants containing the P2-specific MAb per well was incubated for 1 h at 37°C. The plates were washed three times, and 100 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Bethesda Research Laboratories, Gaithersburg, Md.) diluted 1 in 1,500 in PBS containing 3% (wt/vol) bovine serum albumin was added. After incubation for 1 h at 37°C, the plates were washed and 100 μl of diethanolamine (10% [vol/vol], pH 9.8) containing *p*-nitrophenylphosphate (Sigma) at 1 mg/ml was added. After 60 min, the A_{410} was determined spectrophotometrically with a microplate reader (MR 600; Dynatech Industries, Inc., Mclean, Va.).

For the inhibition assay, microtitration plates were coated with 100 μl of OMP preparations (3) adjusted to 7.5 μg/ml in carbonate buffer. After overnight incubation at room temperature, the plates were washed with PBS-Tween 20 and blocked with PBS plus 0.5% (wt/vol) bovine serum albumin. Diluted hybridoma culture supernatants containing the P2-specific MAb were incubated overnight at 4°C with several concentrations (0.05 to 50 μg/ml) of peptide. Peptide-treated and control culture supernatants (100 μl) were then transferred to the plates and incubated at 37°C for 15 min. The remaining steps of the inhibition assay were performed as described above for the ELISA.

Other techniques. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described elsewhere (26). Protein concentrations were determined by the bicinchoninic acid method (BCA protein assay kit; Pierce Chemical Co., Rockford, Ill.). Oligonucleotides were synthesized on an Applied Biosystems DNA synthetizer, model 380B.

RESULTS

The MAbs react with P2 isolated from *H. influenzae* MinnA on Western immunoblots as well as with recombinant P2 produced in JM101/pRSM478 (26). The gene from strain 3175 has the identical sequence and was cloned such that a *Bam*HI site was inserted 5′ to the coding sequences for the mature protein. This modified gene was convenient for the present study, as an in-frame fusion between the bacteriophage T7 protein 10 gene and the *ompP2* gene was generated by cloning it into the *Bam*HI site of the T7 expression vector pGEMEX-1. A construct with the appropriate restriction map was designated pRSM741. After induction of T7 RNA polymerase, an immunologically reactive fusion protein accumulated in *E. coli* strains containing pRSM741. The protein apparently precipitates in the cytoplasm, and an enriched fraction was obtained by centrifugation in a microcentrifuge for 5 min. This protein could be readily detected in Coomassie blue-stained SDS-PAGE gels (Fig. 2).

In order to determine the portion of the P2 protein which was reactive with the members of the panel of MAbs, a series of nested deletions was constructed from pRSM741. A subset of the recombinant strains producing immunologically reactive fusion proteins were further characterized to determine the size of the fusion protein (Fig. 2). The 5′ end of the truncated P2 gene in the constructs was determined by sequencing (Fig. 1 and 3). These plasmids were designated pRSM813 through pRSM825. Plasmids expressing the portion of the P2 gene 5′ to the *Eco*RI site and the portion of P2 gene from *Eco*RI to the 3′ end of the gene were expressed independently in pT7-7. The portion of the P2 gene encoded by these constructs, pRSM705 and pRSM707, respectively, is also shown in Fig. 3.

Dot blot immunoassay was employed to determine the reactivity of fusion protein constructs with the panel of MAbs (Fig. 4). Group A and C MAbs and MAbs P2-2, P2-14, and P2-16 reacted with the fusion protein expressed by JM101/pRSM817 but not with the protein expressed by JM101/pRSM819. The same MAbs (P2-16 not tested) reacted with extracts of strains containing pRSM705. Thus, the epitopes recognized by these groups of MAbs are localized between amino acid residues 149 and 182 of the P2 protein. MAbs P2-4 and P2-6 (group B), as well as P2-10 and P2-13 (group D), reacted with JM101/pRSM825, which expresses the carboxy-terminal portion of the P2 protein (residues 319 to 341). The construct localized the epitopes recognized by group B and D MAbs to the carboxy-terminal portion of the P2 protein. MAb P2-17 reacted with the fusion protein expressed by JM101/pRSM813 but not with the fusion protein produced by JM101/pRSM816. These data localize the epitope recognized by MAb P2-17 to between residues 27 and 87 of the P2 protein.

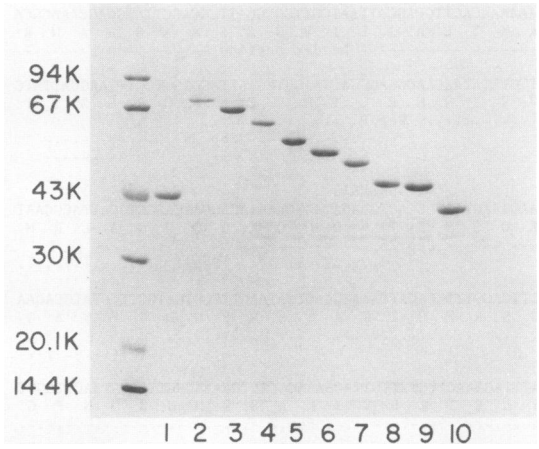

FIG. 2. Coomassie blue-stained 11% SDS-PAGE gel of partially purified T7 gene 10-P2 fusion proteins expressed in *E. coli*. Plasmid pRSM740 contains the T7 gene 10 (lane 1), and plasmid pRSM741 contains the gene 10-P2 fusion gene (lane 2). Lanes 3 through 10, Extracts of *E. coli* strains containing a series of plasmids with P2 genes which are truncated at the 5′ end: pRSM813, pRSM816, pRSM817, pRSM819, pRSM820, pRSM821, pRSM824, and pRSM 825, respectively. Each lane contains 10 μg of protein. The samples were solubilized in buffer containing 5% β-mercaptoethanol at 100°C for 5 min. Molecular weight standards are indicated on the left.

To confirm the fusion protein data and further localize the epitopes recognized by the panel of MAbs, 19 overlapping peptides spanning the P2 amino acid sequence were generated and analyzed for activity by ELISA (Fig. 1). Since the conjugated anti-mouse antiserum nonspecifically reacted with wells coated with peptide Pep-10 (241 to 265), the reactivity of the MAbs with this peptide was evaluated only by inhibition ELISA. The reactivities of four representative MAbs with the synthetic peptides in ELISA are shown in Fig. 5. The MAbs reacted strongly with one or two of the following four peptides: Pep-1 (28 to 55), Pep-6 (148 to 174), Pep-6B (158 to 174), and Pep-C (314 to 341). Group A MAbs, group C MAbs, P2-2, P2-14, and P2-16 reacted with peptide Pep-6 (148 to 174) by ELISA. MAb P2-16 did not react with Pep-6B (158 to 174), while the other MAbs retained reactivity with the smaller peptide. The group B and D MAbs recognized peptide Pep-C (314 to 341). MAb P2-17 was reactive with peptide Pep-1 (28 to 55).

An inhibition assay was used to confirm the results obtained by ELISA (Table 2). This test also gave valuable information about the relationship between the conformation adopted by the soluble peptide and its antigenicity. In this assay, the MAbs were incubated overnight at 4°C with decreasing amounts of specific and control peptides prior to incubation with OMP preparations. The ELISA plates were coated with OMP preparations in order to evaluate the ability of a peptide to inhibit the binding of the MAbs to their specific epitopes on intact P2 protein integrated in the OMP preparations. The smallest concentration of peptide inhibiting 50% of the binding of the MAb to OMP-coated wells was then determined. The binding of group A and C MAbs, P2-2,

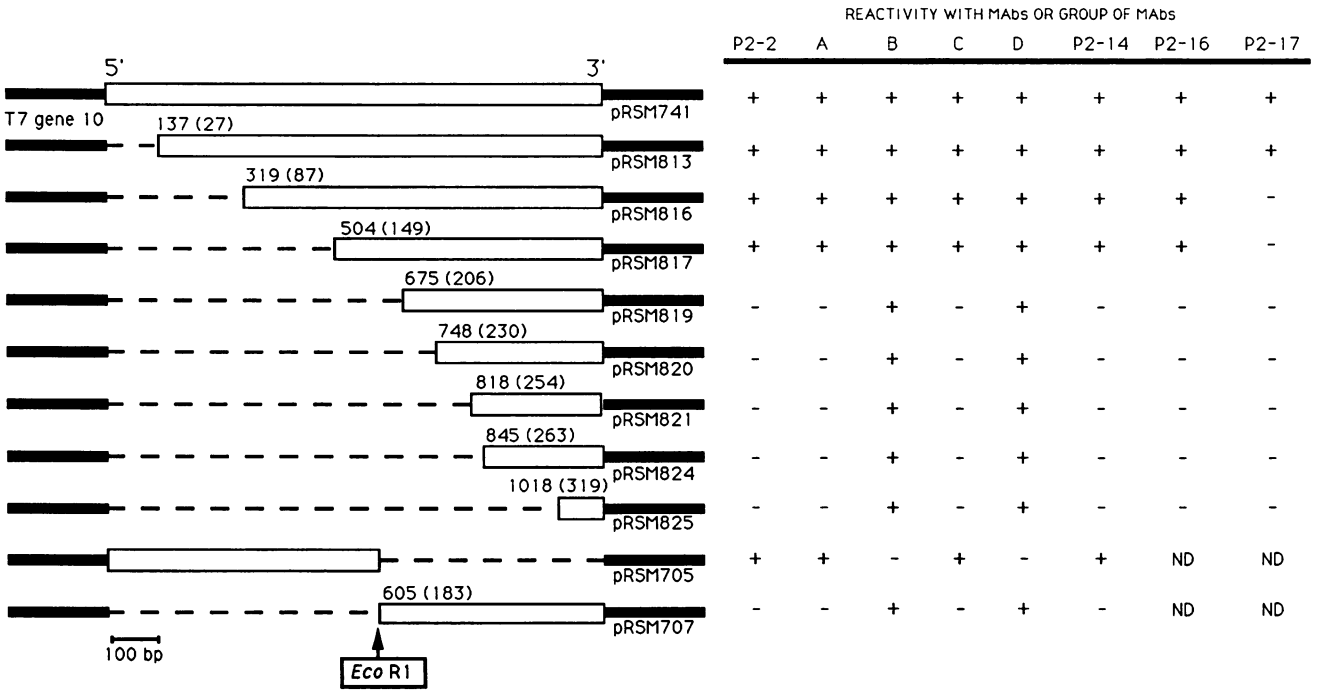

FIG. 3. Structures of recombinant plasmids with deletions in *ompP2* and reactivities of the P2-specific MAbs with the fusion proteins expressed by these plasmids in *E. coli*. The numbers and the numbers in parentheses correspond to the first nucleotide and amino acid residue of P2 at the junction of the gene 10-P2 fusion. The reactivity of the MAbs with the partially purified fusion proteins was determined by dot blot immunoassay. Each MAb in a group had the same reactivity pattern with the panel of Hi strains (Table 1): group A, P2-3, P2-8, and P2-11; group B, P2-4 and P2-6; group C, P2-5, P2-7, P2-9, and P2-12; group D, P2-10 and P2-13.

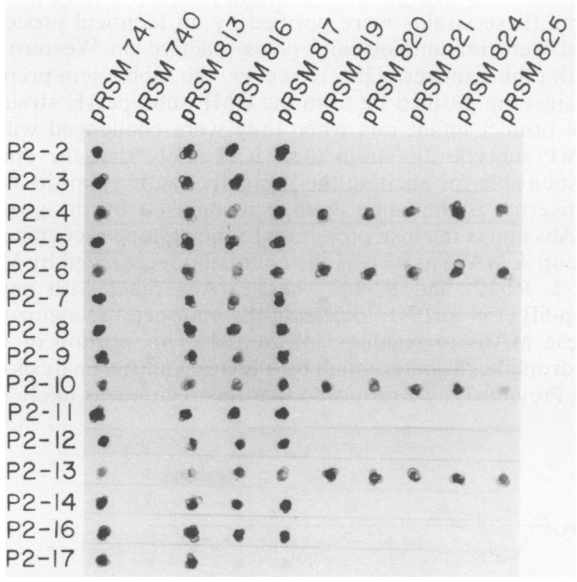

FIG. 4. Dot blot immunoassay demonstrating the reactivity of the gene 10-P2 fusion proteins with the panel of P2 MAbs. Partially purified fusion protein (2.5 μg) was spotted on the nitrocellulose, incubated sequentially with undiluted culture supernatants containing the MAb and alkaline phosphatase-conjugated second antibody, and developed. The fusion protein constructs are the same as those in Fig. 1 to 3.

and P2-14 to OMP preparations was inhibited by concentrations of less than 0.20 μg of peptide Pep-6 (148 to 174) per ml. Similarily, the binding of these MAbs was inhibited by concentrations of less than 0.40 μg of peptide Pep-6B (158 to 174) per ml. The binding of MAb P2-16 was inhibited by 3.25 μg of peptide Pep-6 (148 to 174) per ml but not by 50 μg of Pep-6B (158 to 174) per ml. The binding of the group B MAbs, P2-4 and P2-6, was inhibited by 1.55 and 50 μg of peptide Pep-C (314 to 341) per ml, respectively. The binding of group D MAbs P2-10 and P2-13 was inhibited by 1.55 and 12.50 μg of peptide Pep-C (314 to 341) per ml, respectively. An additional peptide, Pep-CA (323 to 341), which lacks the first 9 residues of Pep-C (314 to 341), was also tested for inhibition activity with group B and D MAbs. No inhibition was observed at a concentration of 50 μg/ml, indicating that the epitope recognized by these antibodies is near the amino-terminal portion of peptide Pep-C (314 to 341). Binding of MAb P2-17 was inhibited by 1.55 μg of peptide Pep-1 (28 to 55) per ml. Both ELISA and inhibition ELISA data are consistent with the epitope assignments made by analysis of the recombinant protein data.

DISCUSSION

In this study, we have used a panel of MAbs to investigate the antigenic structure of *Haemophilus* P2 porin protein. These P2-specific MAbs were described in an earlier report (21) and were demonstrated to react with B-cell epitopes that are widely distributed among *Haemophilus* strains. The strains used in that study have been clustered into genetically related groups on the basis of alloenzyme electrophoretic typing (31). Group A MAbs and MAb P2-14 were reactive with 100% of type b isolates from clusters A1 and

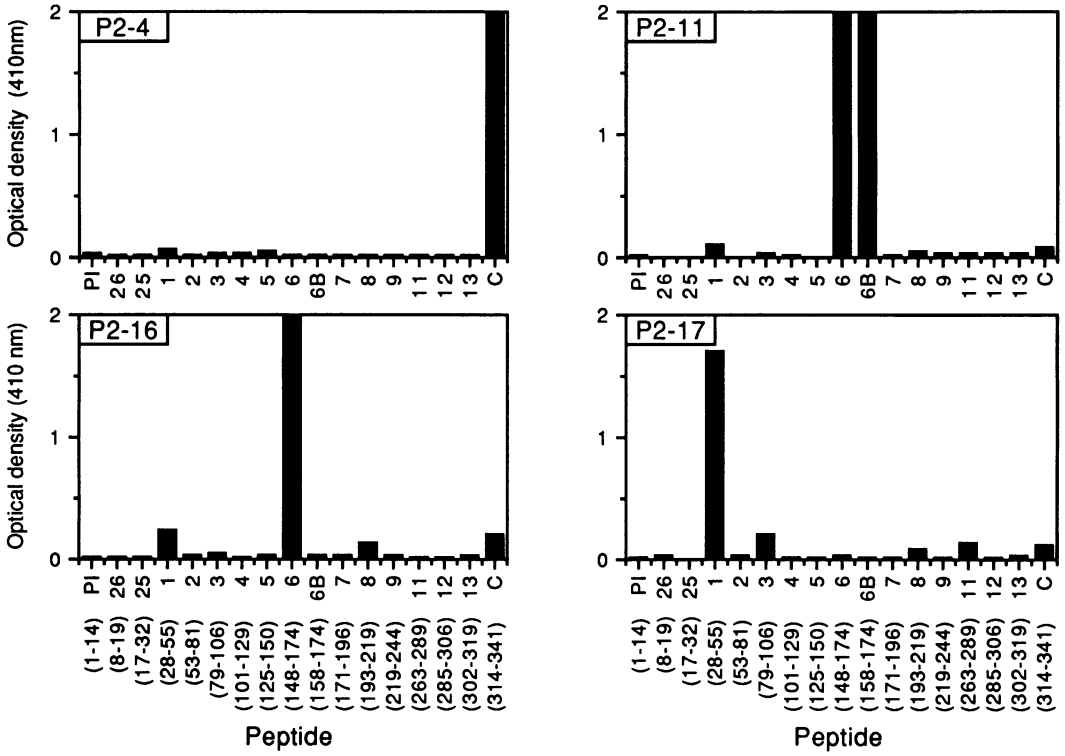

FIG. 5. Reactivities of four representative MAbs with 17 overlapping synthetic peptides spanning the P2 protein as measured by ELISA. Peptides were coated on the microtitration plates at a concentration of 5 μg per well. Reactions were read after 60 min. The numbers in parentheses are the P2 residues in each peptide.

A2. MAb P2-2 recognized all isolates tested in cluster A1 and 56 of 57 isolates tested in cluster A2. Interestingly, none of these MAbs reacted with isolates from serotype b isolates in cluster B1. These MAbs reacted with an epitope(s) located between amino acid residues 158 and 174 of P2 from *H. influenzae* MinnA. Munson et al. (25) have previously demonstrated that the P2 genes from a representative serotype b, cluster B1 isolate and a serotype b, cluster A1 isolate diverge at 13 nucleotides; six of these differences result in four amino acid changes in the same region recognized by these MAbs. These changes are sufficient to prevent the binding of these MAbs to the P2 protein expressed by the serotype b, cluster B1 group of strains.

Group C MAbs are distinct from group A MAbs in their lack of reactivity with a subset of the type b isolates in clusters A1 and A2. Type b *Haemophilus* isolates have also been subgrouped by their OMP profiles on SDS-PAGE (1). The isolates in clusters A1 and A2 that were not recognized by the group C MAbs had the OMP subtype 2L (unpublished data). The P2 protein of a prototype strain with the OMP subtype 2L differs from the P2 of prototype OMP subtype 3L and 1H strains by a single amino acid change from a glutamine to an arginine at position 166 (25). The P2 proteins from these strains were purified by an identical procedure and were immunologically cross-reactive on Western blot with rabbit antisera (28). However, the rabbit sera prepared against the purified P2 from the OMP subtype 1H strain did not protect infant rats when they were challenged with an OMP subtype 2L strain (28). It is likely that the epitope responsible for eliciting the protective activity in the rabbit antiserum is the same epitope recognized by the group C MAbs and is in close proximity to the epitope recognized by group A MAbs as well as the epitope(s) recognized by MAbs P2-2, P2-12, and P2-14. These MAbs react with peptide Pep-6B (158 to 174), localizing the epitope(s) recognized by these MAbs to residues 158 to 174. This portion of P2 is hydrophilic, as determined by a Kyte-Doolittle analysis (Fig. 6). Previously, we proposed that this region was likely to be

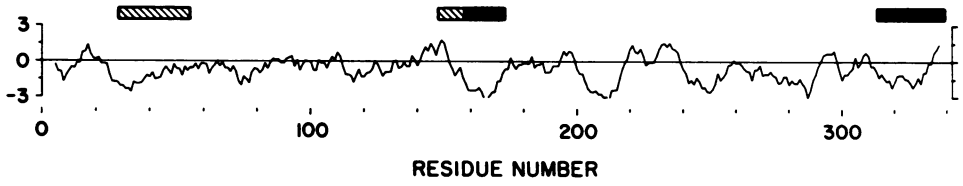

FIG. 6. Schematic representation of the *Haemophilus* porin with the four antigenic sites identified by the MAbs superimposed on a Kyte-Doolittle hydrophobicity plot of the MinnA P2 protein (25). The hatched boxes represent the location on the porin of the antigenic sites which are not exposed or accessible at the surface of intact bacteria. The filled boxes represent the locations on the porin of the antigenic sites which are exposed and accessible at the surface of intact bacteria.

TABLE 2. Reactivity of the P2-specific MAbs with the synthetic peptides as determined by ELISA and inhibition ELISA

MAb group[a]	MAb	Peptides (amino acids) recognized by MAb by ELISA	Amt (μg/ml) of peptide in inhibition ELISA[b]
None	P2-2	Pep-6 (148–174)	0.20
		Pep-6B (158–174)	0.10
A	P2-3	Pep-6 (148–174)	0.05
		Pep-6B (158–174)	0.20
	P2-8	Pep-6 (148–174)	0.05
		Pep-6B (158–174)	0.10
	P2-11	Pep-6 (148–174)	0.10
		Pep-6B (158–174)	0.40
B	P2-4	Pep-C (314–341)	1.55
	P2-6	Pep-C (314–341)	50.00
C	P2-5	Pep-6 (148–174)	0.10
		Pep-6B (158–174)	0.05
	P2-7	Pep-6 (148–174)	0.20
		Pep-6B (158–174)	0.05
	P2-9	Pep-6 (148–174)	0.10
		Pep-6B (158–174)	0.05
	P2-12	Pep-6 (148–174)	0.20
		Pep-6B (158–174)	0.20
D	P2-10	Pep-C (314–341)	1.55
	P2-13	Pep-C (314–341)	12.50
None	P2-14	Pep-6 (148–174)	0.20
		Pep-6B (158–174)	0.20
	P2-16	Pep-6 (148–174)	3.25
	P2-17	Pep-1 (28–55)	1.55

[a] Each MAb in a group had the same reactivity pattern with the panel of Hi strains.

[b] Amount of peptide required for 50% inhibition of the binding of the MAb to OMP-coated plates.

surface exposed (25).

A distinct B-cell epitope was identified in this same region by the peptide inhibition assay (Table 2). This B-cell epitope was recognized only by MAb P2-16, which reacted with peptide Pep-6 (148 to 174) but not with Pep-6B (158 to 174), localizing this epitope to the amino-terminal portion of peptide Pep-6 (148 to 174). This epitope is not in a hydrophilic portion of P2 (Fig. 6) and is not surface exposed.

The reactivity of group B and D MAbs with JM101/pRSM825 expressing residues 319 to 341 of P2 indicates that a third important antigenic region is located at the carboxy-terminal portion of the P2 protein (Fig. 3). Inhibition assays using peptides Pep-C (314 to 341) and Pep-CA (322 to 341) clearly establish that the epitope(s) is near the amino terminus of peptide Pep-C (314 to 341) (Table 2). However, the fusion protein data indicate that residues 314 to 318 are not in the epitope(s). The epitope(s) recognized by group B MAbs was expressed in all serotype b isolates tested in clusters A1, A2, and B1. The epitope recognized by group D MAbs was present in 100% of the serotype b isolates tested in clusters A1 and A2 and in six of seven serotype b isolates tested in cluster B1. Reactivity of the group D MAbs with the six cluster B1 isolates was weak. As noted above, sequence data are available for the P2 protein of prototype isolates in the A1 and B1 clusters. In this region, the derived amino acid sequences of these two proteins differ by a single amino acid, glutamic acid to glycine at position 320 (25), which is likely a part of the epitope recognized by the group D MAbs. The epitope(s) recognized by the group B and group D MAbs is localized to a hydrophilic portion of the P2 protein (Fig. 6).

A fourth antigenic region (residues 28 to 55) near the N-terminal region on the P2 protein was identified with MAb P2-17 (Fig. 5 and Table 2). In contrast to the epitopes defined by the group A to D MAbs, this epitope is not exposed on the surface of intact cells.

The structures of the porin proteins from *E. coli* have been intensively investigated. Models have been generated for the LamB protein, the PhoE protein, and the OmpF protein (16, 23, 40). These proteins lack transmembrane α helices and are rich in β-sheet structure. The β-sheet strands are thought to traverse the membrane numerous times. Electron crystallographic analyses of these proteins are consistent with this interpretation (14, 18, 32). In contrast, little structural information on the *Haemophilus* porin is available. Cross-linking studies indicate the porin exists as a trimer (17), although unlike the *E. coli* porins, P2 exists as a monomer when solubilized in SDS at room temperature.

Mutations in *E. coli* porins which render the *E. coli* cell expressing these mutant proteins resistant to bacteriophages or unreactive with MAbs have been important in identifying surface-exposed residues (5, 6, 16, 34, 40). Although the majority of the antiporin MAbs recognize conformational epitopes, several MAbs which recognize surface-exposed linear epitopes on *E. coli* and *Neisseria* porins have been recently identified (13, 16, 23). Furthermore, antipeptide sera which react with porin epitopes exposed on the surfaces of *E. coli* and *Neisseria* cells have been generated (13, 23). We have selected MAbs which recognize linear epitopes on the *Haemophilus* porin and have identified four epitopes, two of which are exposed on the surface of intact bacterial cells.

Further experiments are required to identify all of the surface-exposed regions and to understand how they are associated in the trimeric form of the porin channel in the *Haemophilus* outer membrane. Furthermore, the antigenic and protective potentials of some of the surface-exposed regions identified on the P2 protein will have to be evaluated in order to gain evidence about their usefulness in future vaccines.

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