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Diversity of the P2 Protein among Nontypeable *Haemophilus influenzae* Isolates

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The genes for outer membrane protein P2 of four nontypeable *Haemophilus influenzae* strains were cloned and sequenced. The derived amino acid sequences were compared with the outer membrane protein P2 sequence from *H. influenzae* type b MinnA and the sequences of P2 from three additional nontypeable *H. influenzae* strains. The sequences were 76 to 94% identical. The sequences had regions with considerable variability separated by regions which were highly conserved. The variable regions mapped to putative surface-exposed loops of the protein.

Nontypeable *Haemophilus influenzae* (NTHI) is responsible for a number of human diseases. In the developed world this organism is responsible for recurrent respiratory tract infections in patients with chronic obstructive pulmonary disease (10, 20, 30) and causes sinusitis and otitis media in children (9, 20, 30, 32). In the developing world NTHI is an important cause of invasive disease of the lower respiratory tract, resulting in severe morbidity and mortality (17, 33).

The predominant outer membrane protein (OMP) of *H. influenzae* has porin activity (29) and was designated P2 (18). The P2 gene from several prototype serotype b strains has been cloned and sequenced; it is highly conserved. The P2 proteins from the most genetically diverse type b strains characterized differ by only 10 amino acid residues (16). In contrast to the highly conserved structure of the type b P2 proteins, the P2 proteins from NTHI isolates show considerable variation in apparent molecular size when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2, 8, 10, 11, 22). Western blot (immunoblot) analysis with polyclonal antisera indicates that the P2 proteins of NTHI share epitopes, but analysis of the surface-exposed epitopes with polyclonal and monoclonal reagents indicates that these proteins are antigenically different (7, 10, 11, 21, 31). Sikkema and Murphy have characterized the P2 proteins from three NTHI strains and suggested that these antigenic differences reside in four hypervariable regions of the porin protein (25). Two of these hypervariable regions correspond to regions in which surface-exposed epitopes were identified in the type b porin. We have extended these studies by characterizing the P2 protein from four additional NTHI strains and by comparing their sequences with the sequences of the previously published P2 proteins.

Bacterial strains and bacteriophages. NTHI strains 12049 and 12085 were isolated from the blood of children in Pakistan (33), and strains 12 and 3232 were recovered from middle ear aspirates of children with otitis media (1, 23). Bacteriophages M13mp18 and M13mp19 as well as *Escherichia coli* JM101 were obtained from New England Biolabs, Inc. (Beverly, Mass.). NTHI strains were grown on chocolate agar and in supplemented brain heart infusion medium as described previously (18).

Molecular cloning and DNA sequence analysis. We previously reported the cloning of the P2 gene from several serotype b strains employing the PCR (16). The 5′ oligonucleotide primer recognized sequences in the leader peptide, and the 3′ primer recognized sequences 3′ to the gene. The 5′ primer contained a *Bam*HI site and the 3′ primer contained a *Hin*dIII site to facilitate cloning. The sequences of the primers have been reported (16). Template DNA was prepared by a modification of the method of Marmur (14) (strains 12 and 12085) or by the method of Joshi et al. (13) (strains 3232 and 12049). Twenty-five cycles of PCR were performed to amplify the P2 genes. In the first cycle, the template was denatured at 94°C for 1 min, annealed at 37°C for 2 min, and elongated at 50°C for 6 min. In subsequent cycles, the template was denatured at 93°C, annealed at 65°C, and extended at 75°C, each step for 2 min. PCR products were purified from unincorporated primers by using a Magic PCR Prep kit (Promega, Madison, Wis.). The PCR products were then digested with *Bam*HI and *Hin*dIII, purified from 0.7% agarose gels, and ligated as a *Bam*HI-*Hin*dIII fragment into M13mp18 and M13mp19 which had been digested with the same enzymes. Two independent PCR reactions were performed with each chromosomal DNA preparation. One PCR product was cloned into M13mp18, and the other product was cloned into M13mp19. Thus, each sequence was determined in both directions from clones derived from independent PCR reactions. Enzymes were purchased from GIBCO/BRL (Gaithersburg, Md.), Promega and Perkin Elmer Cetus (Norwalk, Conn.). Enzymes and recombinant DNA

TABLE 1. Percent identity of the P2 proteins of *H. influenzae* strains

Protein from strain:	% Identity with protein from strain:						
	12085	3232	2019	12	5657	MinnA	12049
3232	94						
2019	92	93					
12	91	89	89				
5657	87	87	85	87			
MinnA	83	82	82	84	83		
12049	82	84	81	83	82	81	
1479	79	78	80	79	81	79	76

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12085      AVVYNNEGTN VELGGRLSII AEQSNSTIKD QKQQHGALRN QGSRFHIKAT  50
3232       AVVYNNEGTN VELGGRLSII AEQSNSTIKD QKQQHGALRN QSSRFHIKAT
2019       AVVYNNEGTN VELGGRLSII AEQSNSTIKD QKQQHGALRN QSSRFHIKAT
12         AVVYNNEGTK VELGGRLSII AEQSNSTVND QKQQHGALRN QGSRFHIKAT
5657       AVVYNNEGTK VELGGRLTII AEQSSNTLDD QKQQHGALRN QGSRFHIKAT
Minna      AVVYNNEGTN VELGGRLSII AEQSNSTVDN QKQQHGALRN QGSRFHIKAT
12049      AVVYNNEGTK VELGGRLSVI AEQSNTTVDD QKQQHGALRN QGSRFHIKAT
1479       AVVYNNEGTK VELGGRLSVI AEQSSSTEDN QEQQHGALRN QGSRFHIKAT
Consensus  AVVYNNEGT- VELGGRL--I AEQS--T--- Q-QQHGALRN Q-SRFHIKAT
                     β1                L1                β2

12085      HNFGDGFYAQ GYLETRLVSA QSGTESDNFG HIITKYAYVT LGNKALGEVK 100
3232       HNFGDGFYAQ GYLETRLVSA QSGTESDNFG HIITKYAYVT LGNKAFGEVK
2019       HNFGDGFYAQ GYLETRLVSA QSGTESDNFG HIITKYAYVT LGNFAKGEVK
12         HNFGDGFYAQ GYLETRFVAA QSGTKSDDFG HIITKYAYVT LGNKAFGEVK
5657       HNFGDGFYAQ GYLETRFVSK YKD.NADHFD SITTKYAYVT LGNKALGEVK
Minna      HNFGDGFYAQ GYLETRFVTK ASENGSDNFG DITSKYAYVT LGNKAFGEVK
12049      HNFSDGFYAQ GYLETRLISS YDGENTDGFG GIVTRYAYVT LGNKAFGEVK
1479       HNFGDGFYAQ GYLETRFVSK ASKEKADQFA DIVNKYAYLT LGNNTFGEVK
Consensus  HNF-DGFYAQ GYLETR---- ------D-F- -I---YAY-T LGN---GEVK
                    β3               L2            β4         β5

12085      LGRAKTIADG ITSAEDKEYG VLNNSKYIPT DGNTVGYTFK GIDGLVLGAN 150
3232       LGRAKTISDG ITSAEDKEYG VLNNSKYIPT NGNTVGYTFK GIDGLVLGAN
2019       LGRAKTIADG IISAEDKEYG VLNNSKYIPT NGNTVGYTFK GIDGLVLGAN
12         LGRAKTIADG ITSAEDKEYG VLNNSKYIPT DGNTVGYTFK GIDGLVLGAN
5657       LGRAKTIADG ITSAEDKEYG VLNNSKYIPT NGNTVGYTFE GIDGLVLGAN
Minna      LGRAKTIADG ITSAEDKEYG VLNNSDYIPT SGNTVGYTFK GIDGLVLGAN
12049      LGRAKTIADG ITSAEDKEYG VLNNSKYIPT NGNTAGYTFK GIDGLVLGAN
1479       LGRAKTIADE ITTAEDKEYG LLNSKKYIPT NGNTVGYTFN GIDGLVLGAN
Consensus  LGRAKTI-D- I--AEDKEYG -LN---YIPT -GNT-GYTF- GIDGLVLGAN
                          L3                  β6            β7

12085      YLLAQERHKY T......GA. .GAGAVAGEV YQQKISNGVQ VGAKYDANNI 192
3232       YLLAQERYKY G......GAA GGAGAVAGEV YPQKISNGVQ VGAKYDANNI
2019       YLLAQQRHKY TAAAAAAAAA GGARAVAGEV YPQKISNGVQ VGAKYDANNI
12         YLLAQDRSKY T......... .ASGSVAGEV TPQSISNGVQ VGAKYDANNI
5657       YLLAQQRN.. .......... .AHGSTAGEV VAQVISNGVQ VGAKYDANNI
Minna      YLLAQKRE.. ....GAKGEN KRPNDKAGEV RIGEINNGIQ VGAKYDANDI
12049      YLLAQK.... .......... YDTAGVAGEV QRQSISNGVQ VGAKYDANNI
1479       YLLAQERDLR TL......DS RTNLSKSGEV TVGEVSNGIQ VGAKYDANNI
Consensus  YLLAQ----- ---------- -------GEV ------NG-Q VGAKYDAN-I
                                L4                  β8
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FIG. 1. Alignment of the P2 sequences from the serotype b strain MinnA and seven nontypeable strains. Putative β strands are indicated by the solid blocks and numbered β1 through β16 starting from the amino terminus of the proteins. The putative loops are labeled L1 through L8.

techniques were performed according to standard methods or according to the manufacturers' instructions. Clones were generally identified by sequencing; however, when clones containing the P2 gene represented only a fraction of the clones with inserts, clones containing the P2 gene were identified by hybridization to pRSM478 (19), a plasmid containing the P2 gene from the serotype b strain MinnA.

DNA sequence analysis was performed by the dideoxy method, using the U.S. Biochemicals (Cleveland, Ohio) Sequenase kit as suggested by the manufacturer. M13 clones were sequenced by using a combination of the M13 universal primer and 20-mer primers synthesized on the basis of previously determined sequences. In some instances, nested deletions of M13 clones were constructed by using the Erase-a-Base kit (Promega). M13mp18 clones containing the P2 gene from strain 3232 were not stable. In this instance, the sequence was determined by direct sequencing of the PCR product, using the fmol DNA sequencing system (Promega). Data were analyzed with the Compugene software (3) and by the GCG software (6) on a Digital VAX computer.

Molecular characteristics of NTHI OMP P2. The OMP P2 genes were cloned and sequenced from four NTHI strains. The nucleotide sequences encoding the mature P2 protein range in identity between 87 and 96%. The P2 proteins in this study range in length between 341 and 345 amino acids, with M_rs ranging between 37,691 and 37,948.

Alignment of NTHI porin protein sequences. The derived amino acid sequences of the four P2 proteins were aligned together with the derived amino acid sequences of three additional NTHI P2 genes which were sequenced by Sikkema and Murphy (25) and the P2 sequence from the serotype b strain MinnA (19). The GAP program in the GCG package was used for sequence comparisons. The sequences of the P2 proteins from the eight strains analyzed range in amino acid identity between 76 and 94%. The P2 proteins from the NTHI strains were 79 to 84% identical to the P2 protein from *H. influenzae* type b strain MinnA (Table 1). P2 sequences from strains 12085 and 3232 are the most closely related, whereas the P2 sequences from strains 12049 and 1479 have the least identity (Table 1). When a multiple alignment was performed with the Pileup program of the GCG software package, it was apparent that the sequences contained alternating regions of

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12085      IAGIAYGRTN YRE....... .......... D..IIAGSDS .GKKQQVNGA 222
3232       IAGIAYGRTN YRE....... .......... S...IHEKDL .GKKQQVNGA
2019       IAGIAYGRTN YRE....... .......... DITITPADKL .GKKQQVNGA
12         IIGIAYGRTN YRE....... .......... DIISKQLNNL VGTKQQVNGA
5657       IAGIAYGRTN YRE....... .......... DLAAQGDSD. ..KKQQVNGA
Minna      VAKIAYGRTN YK........ .......... ......YNES DEHKQQLNGV
12049      VAAIAFGRTN YKE....... .......... SSVIA..... LGRKEQLKGV
1479       IVAIAYGRTN YKDSNHSYTQ KIPKANAADA DTDTTIIYPH HGKKQEVNGA
Consensus  ---IA-GRTN Y--------- ---------- ---------- ---K----G-
              β9                      L5

12085      LSTLGYRFSD LGLLVSLDSG YAKTKNYKAK H......... .EKSYFVSPG 262
3232       LSTLGYRFSD LGLLVSLDSG YAKTKNYKDK H......... .EKSYFVSPG
2019       LSTLGYRFSD LGLLVSLDSG YAKTKNYKAK H......... .EKSYFVSPG
12         LSTLGYRFSD LGLLVSLDSG YAKTKNYKAK H......... .EKSYFVSPG
5657       LSTLGYRFSD LGLLVSLDSG YAKTKNYKDK H......... .EKRYFVSPG
Minna      LATLGYRFSD LGLLVSLDSG YAKTKNYKIK H......... .EKRYFVSPG
12049      LSTLGYRFSD LGLLVSLDSG YAKTKNHKEL NKPTGAKPAY DEKRYFVSPG
1479       LASLGYRFSD LGLLVSLDSG YAKTKNYKAK H......... .EKSYFVSPG
Consensus  L--LGYRFSD LGLLVSLDSG YAKTKN-K-- ---------- -EK-YFVSPG
           β10              β11                 L6

12085      FQYELMEDTN FYGNFKYERN SVDQGKKERE HAVLFGVDHK LHKQVLTYIE 312
3232       FQYELMEDTN FYGNFKYERN SVDQGKKERE QAVLFGIDHK LHKQVLTYIE
2019       FQYELMEDTN VYGNFKYERN SVDQGEKERE QAVLFGIDHK LHKQVLTYIE
12         FQYELMEDTN VYGNFKYERD SVDQGKKARE HAVLFGVDHK LHKQVLTYIE
5657       FQYELMEDTN VYGNFKYERN SVDQGKKARE HAVLFGVDHK LHKQVLTYIE
Minna      FQYELMEDTN VYGNFKYERT SVDQGEKTRE QAVLFGVDHK LHKQLLTYIE
12049      FQYELMEDTN VYGNFKYERT SSDEGKKTHE QAVLFGVDHK LHKQVLTYIE
1479       FQYELMEDTN VYGNFKYERN SVDQGEKERE QALLFGIDHK LHKQVLTYIE
Consensus  FQYELMEDTN -YGNFKYER- S-D-G-K--E -A-LFG-DHK LHKQ-LTYIE
           β12          β13        L7          β14           β15

12085      GAYARTRTT. ...QATGTKV KTEKEKSVGV GLRVYF                 344
3232       GAYARTRTN. ...DKSKAE. KTEKEKSVGV GLRVYF
2019       GAYSRTRTTT VGSKTNASKV KTEKEKSVGV GLRVYF
12         GAYARTRTT. ....ESKKGV KTEKEKSVGV GLRVYF
5657       GAYARTRTN. ....DKGKTE KTEKEKSVGV GLRVYF
Minna      GAYARTRTT. ....ETGKGV KTEKEKSVGV GLRVYF
12049      GAYARTKTN. ....DKNKPE KTGKEKSVGV GLRVYF
1479       GAYSRTRTTS VGDKQVASKV KTEKEKSVGV GLRVYF
Consensus  GAY-RT-T-- ---------- KT-KEKSVGV GLRVYF
                          L8          β16
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FIG. 1—*Continued.*

variable sequences separated by well-conserved regions of the sequences (Fig. 1).

The structure of the *Haemophilus* porin family was predicted as described by Jeanteur and coworkers (12). The structural prediction is based on a number of parameters, including hydrophobicity and hydrophobic moment. The protein is predicted to contain 16 β strands which traverse the membrane. At the external face of the cell, these β strands are linked by eight loops of variable size. Beginning at the amino terminus, we have designated the β strands β1 through β16 and the loops L1 through L8 (Fig. 1). The regions proposed to contain the 16 β strands are highly conserved among all of the P2 proteins (Fig. 1). Thus, amino acid changes among the P2 proteins are localized mainly to the eight proposed loop regions. Variation is particularly notable in loops L2, L4, L5, and L8. Loops L4, L5, and L6 are also notable for variability in the length of the loop. For example, loop L5 of the porin from strain MinnA is 24 residues shorter than the loop from the porin of strain 1479. Relatively conserved regions are also present in the proposed loop regions. For example, the conserved portion of loop 6 is 82% identical among the proteins studied. Relatively conserved regions are also apparent in L1, L3, and L7.

Despite a lack of sequence similarity between the porins of *E. coli* and *Rhodobacter capsulatus*, these porins share a common channel-forming motif (5, 24, 34). Jeanteur et al. (12) have used sequence alignments and computer modeling to predict the topographical organization of porins in a bacterial superfamily consisting of enteric proteins and porins from *Neisseria* spp. Their data indicate that the 16 transmembrane β-strand structures are conserved among these additional porins. We have now employed this methodology to predict the structure of the *Haemophilus* porins. Our model is similar to the model for the type b P2 protein proposed by Srikumar and coworkers (26) in that the porin contains 16 transmembrane β strands and eight loops of variable length facing the exterior side of the outer membrane, although there are differences in the boundaries of several of the β strands.

We examined the sequences of the P2 proteins produced by seven NTHI isolates, four of which were determined in this study and three of which were recently reported by Sikkema and Murphy (25). Our data extend the observations of Sikkema and Murphy in that the putative β-strand regions are highly conserved and four of the eight putative loop regions are highly variable in sequence.

We (15) and others (26) have identified surface-exposed epitopes in L4 and L8 of the P2 protein of *H. influenzae* type b.

These amino acid changes resulted in altered reactivity of murine monoclonal antibodies directed against a surface-exposed epitope in the L4 loop of P2. A second surface-exposed epitope was mapped to a region including loop L8 of P2 (16). Srikumar et al. (26) have also reported that surface-exposed epitopes are present in loops L4 and L8. Additionally, they identified an epitope which is localized near, or in, L3 which was not surface exposed. The L3 loop of the *E. coli* OmpF and PhoE porins folds into the barrel structure and plays a role in constriction of the pore (5, 28). If the P2 protein is folded in a similar manner and the epitope is localized in the L3 loop, then the folding of the L3 loop into the β barrel would explain the inaccessibility of this epitope to antibody added to intact cells. Recently, Duim et al. (7) reported antigenic variation in the P2 protein from serial isolates of the same nontypeable strains obtained from patients with chronic obstructive pulmonary disease. The sequence changes which result in these antigenic changes were localized to L6, suggesting that a portion of L6 is also exposed at the cell surface.

Thus, it is apparent that *H. influenzae* has the ability to produce porin proteins which differ significantly in the structure of the loop regions. These data and the recent recognition that the protective epitopes of P2 are conformational in nature diminish the prospects for rapid development of a P2-based universal *Haemophilus* vaccine (4, 27).

Nucleotide sequence accession number. The nucleotide sequences of the P2 genes from NTHI strains 12, 3232, 12049, and 12085 have been assigned GenBank accession numbers UO8206, UO8205, UO8207, and UO8208, respectively.

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