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Molecular Analysis of the P2 Porin Protein of Nontypeable *Haemophilus influenzae*

DANIEL J. SIKKEMA* AND TIMOTHY F. MURPHY

Division of Infectious Diseases, Department of Medicine, and Department of Microbiology, State University of New York at Buffalo, and Veterans Affairs Medical Center, Medical Research 151, 3495 Bailey Avenue, Buffalo, New York 14215

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The P2 porin protein is the most abundant outer membrane protein (OMP) of nontypeable *Haemophilus influenzae* (NTHI) and shows extensive antigenic heterogeneity among strains. To study the molecular basis of this heterogeneity, the DNA sequences of the genes encoding the P2 proteins of three unrelated strains of NTHI were determined, and restriction fragment length polymorphisms around the P2 genes of 35 strains were analyzed. The deduced amino acid sequences of the P2 genes from the three strains of NTHI revealed four major (12 to 35 amino acids long) and several smaller (2 to 7 amino acids) hypervariable regions in each protein. The major variations occurred in identical portions of the genes, and these regions showed a high antigenic index and surface exposure probability in computer modeling analysis. Differences in the molecular mass of the P2 protein correlate with differences in the size of the variable region in each strain. Oligonucleotide primers suitable for amplification of the P2 genes by polymerase chain reaction were developed. Restriction fragment length polymorphism analysis showed marked heterogeneity in and around the *ompP2* locus of 35 NTHI strains. These results contrast with the high degree of conservation of the P2 genes in *H. influenzae* type b strains. We conclude that the molecular mass and antigenic heterogeneity of the P2 molecule of NTHI is due to variations in gene sequence that are clustered primarily in four large hypervariable regions of the gene.

Nontypeable *Haemophilus influenzae* (NTHI) strains are an important cause of infection in patients with chronic obstructive pulmonary disease and are a common cause of acute otitis media in children (3, 4, 30). Recurrent infections are usually caused by different strains of NTHI in which the major outer membrane protein (OMP) profile is altered in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (2, 16, 34). Differences in immunoreactivity of certain OMPs to polyclonal and monoclonal antibodies are observed in these new isolates as well (16, 17, 31, 49). When the same strain persists in a patient with chronic obstructive pulmonary disease, immunochemical changes in the surface epitopes of OMP P2 are seen (49).

OMP P2, the most predominant OMP, functions as a porin (45, 47, 48) and has a molecular mass that varies among strains from 36,000 to 42,000 Da (1, 22, 35). This variation in molecular mass among P2 molecules provides the basis for a subtyping system for NTHI (34, 35). The P2 molecule of nontypeable strains expresses highly strain-specific, immunodominant epitopes on the bacterial surface (17). P2 is also a target for human serum bactericidal antibody (32). The antigenic differences in the P2 molecules among strains of NTHI may induce a strain-specific host immune response and allow other strains to cause recurrent infection.

The goal of this study is to determine the molecular basis of the antigenic heterogeneity of P2 molecules among strains. The sequences of the P2 gene of three strains of NTHI were determined. Restriction fragment length polymorphism analysis of the *ompP2* locus of 35 strains was used to chart relationships among a diverse collection of strains.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. A total of 35 strains of NTHI was used in this study. Strains were chosen so that a diverse collection of isolates could be studied. The strains represent a variety of OMP subtypes (34, 35), OMP serotypes (29), and lipooligosaccharide serotypes (6). The isolates were recovered from a variety of clinical samples and from several different cities in the United States (Table 1). One strain of *H. influenzae* biogroup aegyptius was recovered from a patient with Brazilian purpuric fever (5). *Escherichia coli* DH5αF′ and bacteriophages M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Plasmid pGEM7ZF− was obtained from Promega Corp. (Madison, Wis.).

Molecular cloning and sequencing. To clone the P2 gene from NTHI strain 5657, a family of 16 oligonucleotide probes corresponding to the reverse-translated N-terminal amino acid sequence of the mature protein was synthesized (39). A λgt11 genomic library of this strain containing inserts of 2 to 8 kb was screened by hybridization with oligonucleotides end labeled with [γ-^{32}P]ATP (New England Nuclear) by using T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) (42). Hybridization was carried out overnight at 37°C, and washes were performed at 52°C in the presence of tetramethylammonium chloride (51). One clone (no. 18A), determined by restriction endonuclease analysis to contain the entire P2 gene, was subcloned into bacteriophage M13 for sequence determination (18).

Amplification of the P2 gene by PCR. Polymerase chain reaction (PCR) was used to isolate the P2 genes from chromosomal DNA of NTHI strains 1479 and 2019. Chromosomal template DNA was purified by the method of Silhavy et al. (44); 1 μg was incubated with 100 ng of oligonucleotides, and the P2 gene was amplified in overlapping halves by 30 rounds of denaturation and polymerization. PCRs were performed with a 1-min annealing time at

TABLE 1. Sources of NTHI strains

Geographic source	Clinical source					
	Sputum	Eye	Middle ear fluid/otitis[a]	Blood	Cerebrospinal fluid	Nasopharynx
Boston	2	1				
Brazil				1		
Buffalo	10		4	1	1	1
Dallas			3			
Houston	6			1		
St. Louis				3	1	

[a] Middle ear fluid obtained by tympanocentesis.

50°C, an extension time of 2 min at 72°C, and a denaturation time of 1 min at 95°C. PCR fragments of approximately 600 bp each were purified from 1.2% agarose gels with GeneClean (Bio 101, La Jolla, Calif.) and cloned by using the TA Cloning Kit (Invitrogen Corp., San Diego, Calif.) as described by the manufacturer. *Taq* DNA polymerase and other PCR reagents were purchased from Cetus (Norwalk, Conn.). Other enzymes were purchased from Bethesda Research Laboratories, Inc., or Promega Corp. and used in accordance with the manufacturers' instructions.

Determination of DNA sequence. Dideoxy sequencing was performed on M13 and pCr2000 clones with Sequenase (US Biochemicals, Cleveland, Ohio) according to the manufacturer's instructions. Two independent clones were sequenced from each PCR-derived ligation. [^{35}S]deoxyadenosine 5′-(α-thio)-triphosphate was purchased from New England Nuclear Corp.

DNA sequence and peptide structural analysis. DNA and amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software package (8). Amino acid sequences were analyzed for antigenic index, surface exposure probability, and hydrophilicity. Antigenic index is measured by summing several weighted measures of secondary structure as described by Modrow and Wolf (24). Probability of surface exposure is calculated by the formula of Emini et al. (10), and hydrophilicity was calculated by the algorithm of Hopp and Woods (21).

Restriction fragment length polymorphism analysis. Chromosomal DNA from 35 strains of NTHI was isolated as described by Silhavy et al. (44). Genomic DNA (5 μg) was digested to completion with *Eco*RI or *Pvu*II. Fragments were electrophoresed on 0.8% agarose gels and transferred to Nytran (Schleicher & Schuell, Inc., Keene, N.H.) in accordance with the manufacturer's recommendations.

Oligonucleotides were purchased from Biosynthesis, Inc. (Denton, Tex.) and end labeled with [^{32}P]ATP (New England Nuclear Corp.) by using T4 polynucleotide kinase. Hybridization and washes were performed at 5 to 7°C below the temperature of dissociation for the probes (51), which was 48 to 52°C for all probes. Autoradiography was performed overnight at −70°C.

Nucleotide sequence accession numbers. The P2 gene sequences of strains 1479, 2019, and 5657 have been assigned GenBank accession numbers M93268, M93269, and M93270, respectively.

RESULTS

Molecular cloning and sequencing. The λgt11 genomic library of NTHI strain 5657 was immunoscreened with monoclonal antibody 2E6, which recognizes a surface epitope in the P2 protein (17, 31). Screening of 20,000 plaques failed to yield any reactive plaques. Therefore, the library was screened by hybridization with a family of end-labeled oligonucleotides corresponding to reverse translation of the N-terminal amino acid sequence of purified P2 from strain 5657 (39, 42). A mixture of 16 oligonucleotides was used (Fig. 1) to accommodate codon degeneracy. Seven plaques hybridized with the oligonucleotides. These clones were plaque purified and tested for reactivity to monoclonal antibody 2E6 (17, 31). Clone 18A was very weakly reactive with 2E6 in the immunoblot assay. Since the band corresponded to the molecular mass of native P2, clone 18A was assumed to contain the entire P2 gene. The 1.1-kb insert was excised from λgt11 with *Eco*RI but could not be subcloned into M13mp18 or M13mp19 for sequence determination until the portion of the gene encoding the leader peptide was removed by digestion with PvuII. The sequence of both strands of the entire insert was determined by using newly synthesized primers as required.

Analysis of the sequence revealed that portions of the gene had homology with the sequence for P2 from *H. influenzae* type b strains (19, 25, 27). In addition, several large regions differed from the sequence of type b strains (Fig. 2).

Having cloned and determined the sequence of the P2 gene of strain 5657, we set out to clone the P2 gene from strain 1479. A λgt11 genomic library of strain 1479 was screened with oligonucleotides corresponding to apparently conserved regions of the P2 gene. Several screenings yielded only small fragments of the gene, as determined by sequence analysis of M13 subclones. The first clone obtained was 600 bp in length, with only 144 bases contained within the P2 gene and the remainder lying upstream. Another clone of 4.2 kb gave only the N-terminal 486 bp of the gene; the rest was apparently rearranged as a result of toxicity associated with unregulated expression of porin molecules in *E. coli* (7, 14, 28). To clone the remainder of the P2 gene from strain 1479, oligonucleotide primers suitable for amplification of overlapping fragments of the gene were constructed, based on the sequence of strain 5657 (Table 2). PCR-generated fragments from the genomic DNA of strain 1479 were cloned into the TA cloning vector pCR2000 by standard ligation techniques and transformed into *E. coli* DH5αF′ (18).

Sequence determination of the P2 gene from strain 1479 showed regions of homology with the P2 gene of strain 5657 and type b strains (25, 27) (Fig. 2). There were also several regions which showed significant heterogeneity. The gene from strain 1479 encoded 33 additional amino acids, accounting for the molecular mass variations observed between the P2 proteins of the two strains of NTHI (Table 3, Fig. 3).

The P2 gene from a third strain (2019) was cloned, and the sequence was determined in overlapping halves by PCR.

Asn	Asn	Glu	Gly	Thr	Lys	Val
AAC	AAC	GAA	GGI	ACI	AAA	GT.
T	T	G			G	

FIG. 1. Family of 16 oligonucleotides corresponding to the reverse translation of the N terminus region of purified OMP P2 of strain 5657. The top line represents the amino acid sequence beginning with amino acid 25, with position 1 defined as the methionine. The bottom line represents the sites at which a mixture of two nucleotides was used in the synthesis of the oligonucleotides.

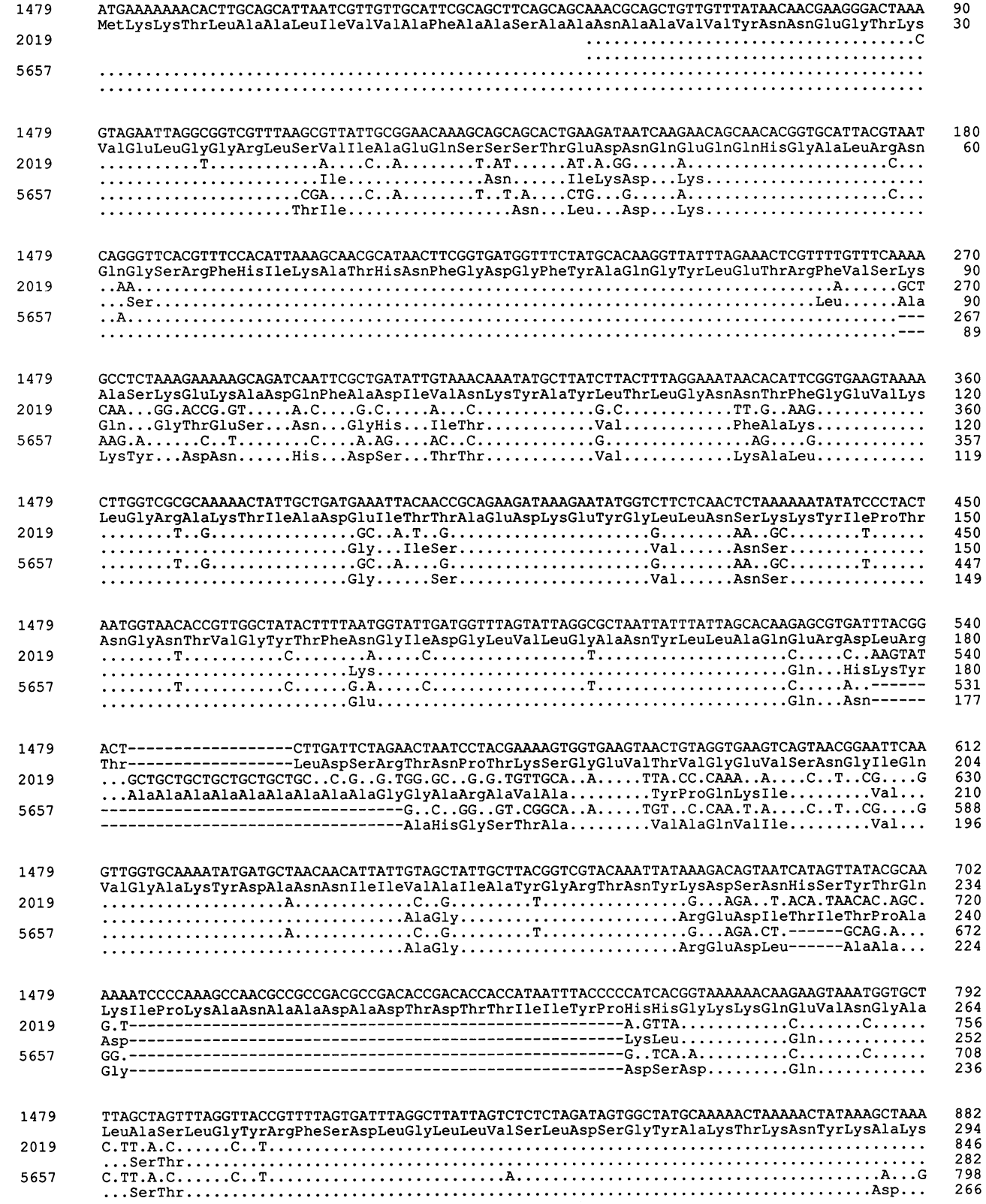

FIG. 2. Complete nucleotide and derived amino acid sequence of the P2 genes from NTHI strains 1479, 2019, and 5657. Regions of identity are denoted as periods, and gaps are depicted as dashes. The amino acid and nucleotide position are displayed on the right-hand side of each row, while the strain is listed on the left-hand side.

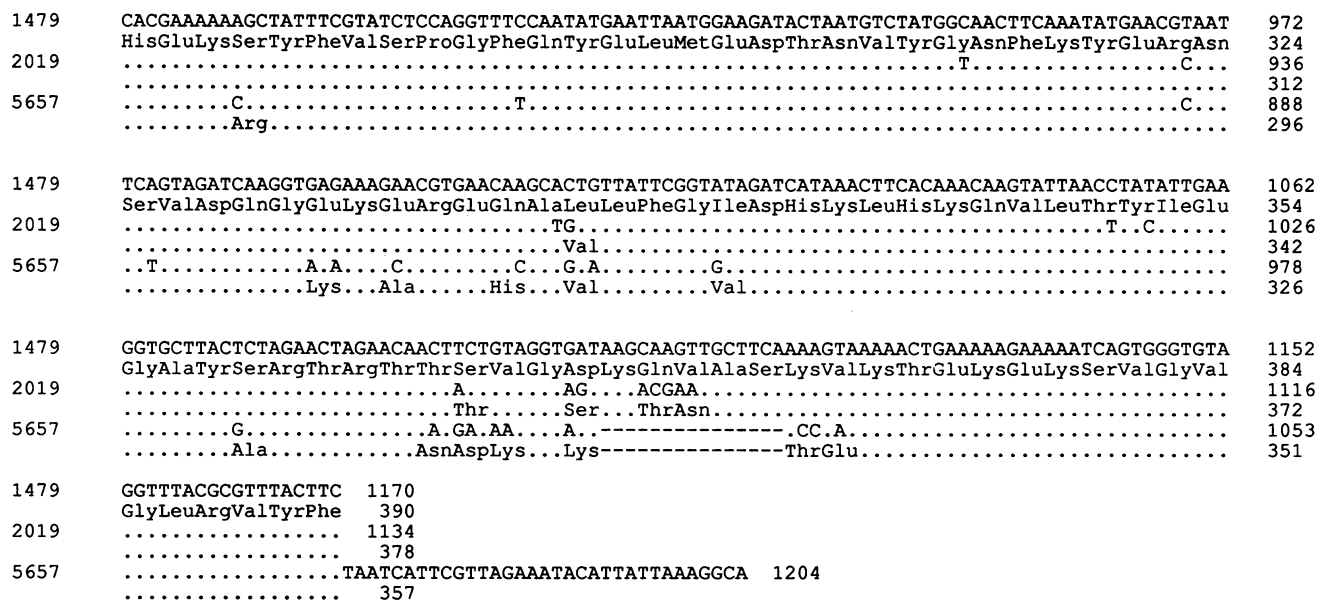

FIG. 2—*Continued*

Sequence data again showed marked heterogeneity among strains; however, as before, long stretches of conserved sequences occurred between the variable portions. Amino acid identity among strains ranged from 79 to 85% with the percent similarity being slightly higher (Table 3).

The sequence of the region corresponding to the carboxy-terminal primer used for amplification of the P2 genes was determined as follows. The carboxy-terminal portion of the P2 gene of strains 1479 and 2019 was amplified by PCR with a primer downstream of the P2 gene and a primer in the coding region of the gene (Table 2). The resulting fragment of strain 1479 was sequenced directly following gel purification of the ~600-bp EcoRI-digested PCR product. The analogous fragment from strain 2019 was similarly sequenced following digestion with *Xba*I. The sequences of these regions of the P2 gene were identical for all three strains.

Analysis of sequences of the P2 genes. Variations in the molecular mass of P2 among strains of NTHI form the basis of a subtyping system (34, 35). The gene sequences presented here encode proteins with calculated molecular masses of 40,915 Da (strain 1479), 39,072 Da (strain 2019), and 37,271 Da (strain 5657). These data correlate well with estimates from SDS-PAGE (Fig. 3, Table 3).

Regions of dissimilarity could be broken down into four major hypervariable regions: V1, amino acids 85 to 115; V2, amino acids 175 to 209; V3, amino acids 226 to 255; and V4, amino acids 363 to 374. These numbers vary among strains due to differences in the lengths of the variable regions among strains, yet all the differences occur in the same relative region of the protein molecule (Fig. 4).

The variable regions of each gene were analyzed for surface exposure and antigenic index (10, 24). All of these variable regions contain potentially surface-exposed regions which vary in their surface exposure probability and hydrophilicity (10, 21, 24). These data were incorporated into the new model for the porin molecule of *H. influenzae* type b developed by Srikumar et al. (46) (Fig. 5).

With regard to previously reported P2 sequences in *H. influenzae* type b, amino acids 181 to 188 in OMP subtypes 2L and 6U varied from those in the P2 of strain Minn A (OMP subtype 1H) (25). These differences coincide with V2 in NTHI (Fig. 4 and 5) or hydrophilic loop number 4 in the type b topology model (46). An additional amino acid variation at position 340 in OMP subtype 6U maps to V4 of NTHI and hydrophilic loop number 8 of the type b model (46). All other amino acid variations in P2 molecules of OMP subtype 6U isolates map to proposed transmembrane beta regions in the type b model (25, 46).

Restriction fragment length polymorphism. To further characterize the *ompP2* locus at the nucleotide level, ge-

TABLE 2. Oligonucleotides used in PCR and restriction fragment length polymorphism analysis

Oligonucleotide (5′→3′)	Strand	Nucleotides[a]
TTCGCAGCTTCAGCAGC[b]	Gene	37–53
GCATCATATTTTGCACC	Opposite	591–607
TATTAGCACAACAGCGT	Gene	512–528
GAAGTAAACGCGTAACC[b]	Opposite	1054–1071
TAATAATGTATTTGTAAC	Opposite	1081–1099

[a] Relates to the nucleotide positions in Fig. 2 for strain 5657.
[b] Probes used in restriction fragment length polymorphism analysis (Fig. 6 and 7).

TABLE 3. Properties of the P2 gene products of three NTHI strains and one type b strain

Strain	Molecular mass[a] (Da)	No. of amino acids[b]	% Identity with strain 1479 sequence
NTHI			
1479	40,915	370	
2019	39,072	358	80.0
5657	37,271	337	80.7
H. influenzae type b Minn A[c]	37,782	341	78.8

[a] Calculated from predicted amino acid sequence.
[b] Amino acid chain length of the mature P2 peptide.
[c] From reference 27.

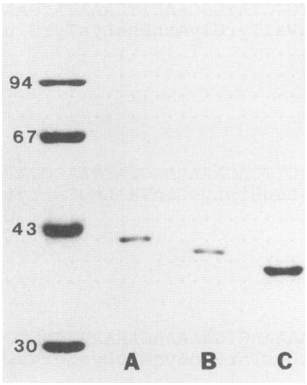

FIG. 3. SDS-polyacrylamide gel stained with Coomassie blue. Molecular mass standards are shown on the left (in kilodaltons). Purified P2 was obtained from the following NTHI strains: A, 1479; B, 2019; C, 5657.

nomic DNA from 35 strains of NTHI was digested with *Eco*RI or *Pvu*II and subjected to restriction fragment length polymorphism analysis. Digested genomic DNA was subjected to agarose gel electrophoresis and transferred to a nylon membrane. The resulting Southern blot was incubated with two end-labeled oligonucleotide probes corresponding to regions of the genes where DNA sequences are the same among strains 1479, 2019, and 5657 (Fig. 6 and 7). One probe corresponded to sequence near the amino terminus, and the other corresponded to the carboxy terminus (Table 2). The probes hybridized to one or two bands in each of the 35 strains, depending on the presence of restriction endonuclease sites within the gene (Fig. 6 and 7).

While several strains showed similar banding patterns (Fig. 6 and 7), a great deal of heterogeneity in restriction fragment size was observed with both *Eco*RI- and *Pvu*II-restricted DNA. Fourteen of 35 strains contained a central *Eco*RI site, contrasting to 100% of type b strains examined (25, 27). The P2 gene of 13 of 35 strains contained PvuII sites, again distinguishing the nontypeable strains from the type b isolates, the P2 genes of which all contain a single *Pvu*II site at the N terminus of the mature protein (25, 27). No correlation between restriction fragment length patterns and the geographic origin or body site of isolation of the strains was noted.

Together, these data indicate that antigenic differences in the P2 protein are due to heterogeneity in the P2 genes of NTHI strains and that most of the heterogeneity is present in four hypervariable regions of the P2 gene.

DISCUSSION

The data reported in this study indicate that the antigenic heterogeneity of P2 molecules among NTHI strains is due to gene sequence variation in defined regions of the P2 gene. Molecular mass variation among P2 proteins is due to differences in the size of these variable regions. Four major hypervariable regions (affecting 12 to 35 amino acids) were identified in each of the three P2 genes sequenced; all occurred in identical regions of the gene. Several smaller variable regions were also noted (affecting two to seven amino acids).

Restriction fragment length polymorphism analysis of DNA from 35 NTHI strains demonstrated significant heterogeneity in and around the *ompP2* locus. While some strains showed similar-sized fragments on Southern blot analysis, no correlation was noted between restriction fragment length patterns and biotype, clinical source, or geographic origin.

The P2 proteins of nontypeable strains of *H. influenzae* differ from the P2 proteins of type b strains in molecular mass in SDS-PAGE and antigenic heterogeneity (28, 29, 35). Studies by Munson et al. (25) have shown a high degree of conservation of the P2 gene among type b strains. Furthermore, the DNA sequence of the P2 genes of two epidemiologically unrelated type b strains showed 100% homology (19, 27). The sequence of P2 genes from NTHI has not been reported previously. The P2 proteins of nontypeable strains are quite different from each other, while the P2's of type b strains are conserved (17, 19, 20, 25). The present study establishes that the differences among P2 proteins of nontypeable strains are due to marked variability at specific locations within the P2 gene. These observations are consistent with alloenzyme electrophoretic typing studies showing that type b strains are basically clonal, whereas nontypeable strains are genetically diverse (36, 37, 40, 41).

Previous studies have shown that strain-specific epitopes on the P2 proteins of nontypeable strains appear to be immunodominant epitopes. Groeneveld et al. (16) showed that immunization of rabbits with intact bacteria resulted in the production of anti-P2 antibodies that were protective only against the immunizing strain. Studies in our laboratory identified the presence of a strain-specific immunodominant epitope by analysis with monoclonal antibodies to P2 (17). Sequence data obtained in this study reveal several potentially heterogeneous epitopes. Computer modeling indicates that these variable regions are likely surface exposed and possess a high antigenic index. Epitope mapping experiments are under way to identify which variable portion(s) of the P2 gene encodes the major immunodominant surface-exposed epitope(s) in these bacteria.

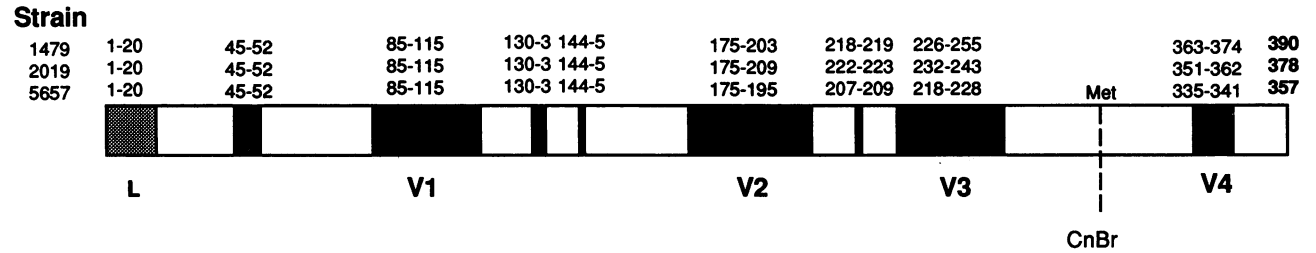

FIG. 4. Diagrammatic representation of the P2 genes of NTHI strains 1479, 2019, and 5657. Regions of sequence variability are shown as solid rectangles. Amino acid positions affected by the variable regions of each gene are shown. The total number of amino acids present in each P2 is shown in bold at the far right. L, leader peptide; V1 through V4, four major variable regions of the P2 gene; CnBr, cyanogen bromide.

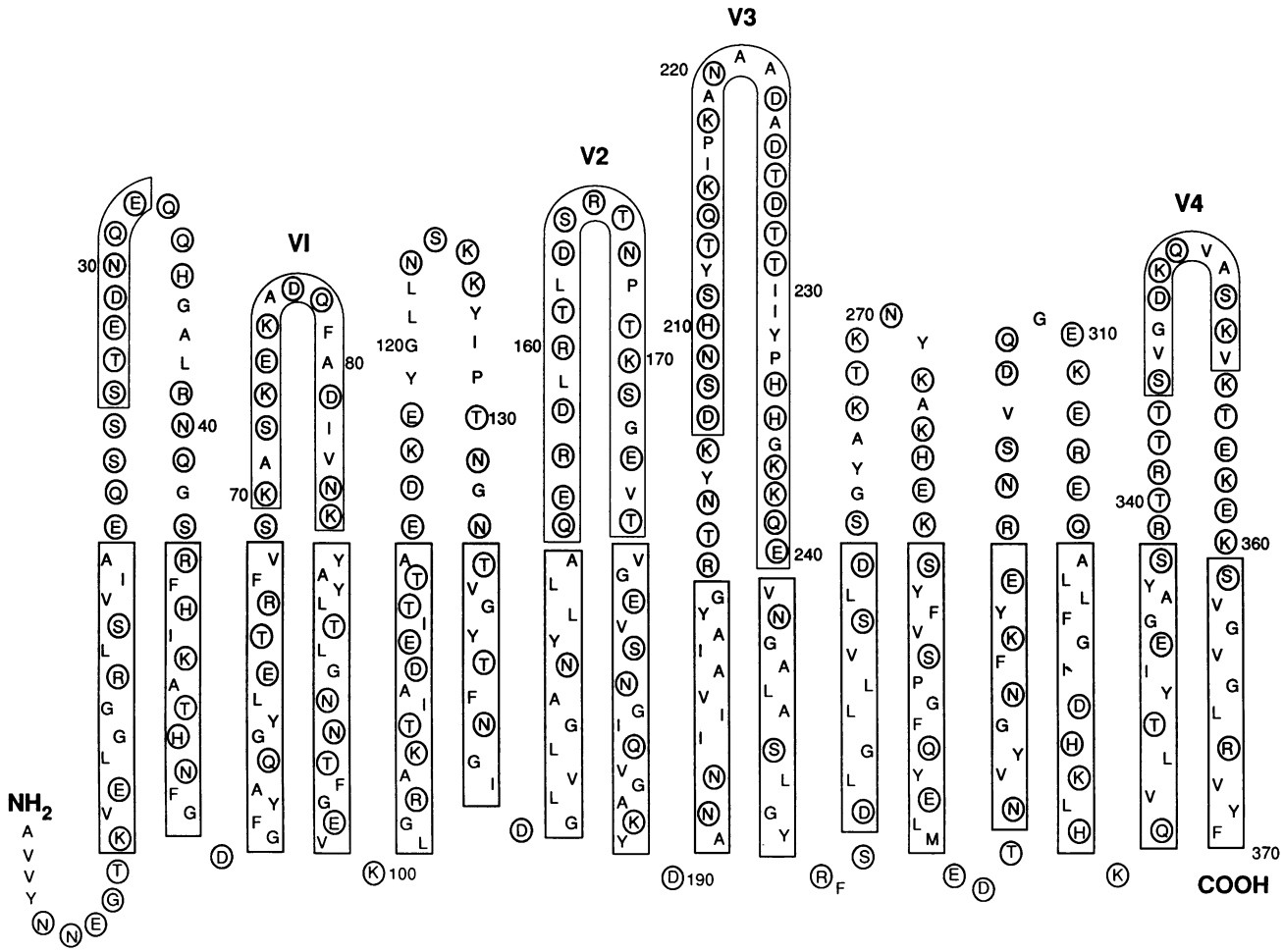

FIG. 5. Predicted topological structure of NTHI porin (strain 1479) as it conforms to the model developed by Srikumar et al. (46). Sixteen beta strands nearly identical to those of *H. influenzae* type b are shown as rectangles. For each beta strand, the right side shows residues that are proposed to face the water-filled lining of the pore, and the left side shows residues that are hypothesized to interact with the outer membrane bilayer. Some amino acid positions of the mature peptide are shown numerically throughout the diagram. Residues of significant hydrophilicity are circled; variable portions of the P2 molecule outside the beta regions are enclosed. V1, V2, V3, and V4 correspond to the regions depicted in Fig. 4.

High levels of homology among the P2 proteins suggest a common ancestry, structure, and function for these molecules. The four variable regions, differing in length among strains, do not appear likely to contribute to the overall tertiary structure of the molecule but may form extramembranous loops outside the cell surface, where they interact with the host. Analysis of the amino acid sequence of the P2 proteins reveals 16 beta-pleated sheet domains within the various conserved regions of each gene (Fig. 5). This is similar to the porin proteins of *E. coli*. These OMPs represent a "beta barrel" conformation (11, 50). A similar scenario has been hypothesized for the meningococcal class 1 OMPs and *H. influenzae* type b (23, 46). The size and spacing of the conserved regions in the P2 protein of NTHI in conjunction with their beta-pleated sheet domains fit well with the hypothesis that P2 shows a similar beta barrel conformation, which exposes variable regions on the surface of the bacterium.

Expression of a strain-specific and highly immunodominant antigenic epitope on the surface of the bacterium may represent a mechanism of host evasion by NTHI, permitting

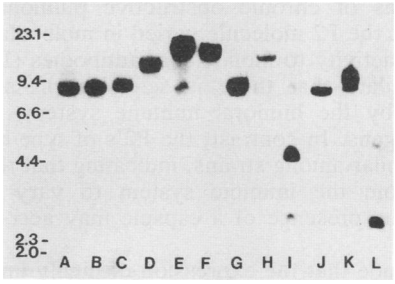

FIG. 6. Southern blot assay showing restriction fragment length polymorphisms of the *ompP2* locus from 12 isolates of NTHI. Five micrograms of chromosomal DNA was digested to completion with *Eco*RI. The fragments were separated on a 0.8% agarose gel, transferred to Nytran, and probed with end-labeled oligonucleotides corresponding to conserved regions of the P2 genes. Lanes contained genomic DNA from *H. influenzae* strains (A) biogroup aegyptius 16, (B) SL820, (C) 7891, (D) 1749, (E) C83, (F) SL1484, (G) 4971, (H) 39, (I) 7172, (J) 5657, (K) 2019, and (L) 1479. Molecular size standards are noted on the left (in kilobases).

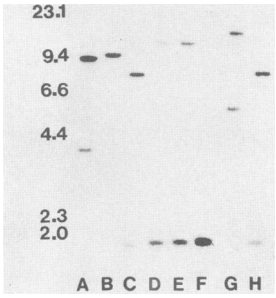

FIG. 7. Southern blot assay showing restriction fragment length polymorphisms of the *ompP2* locus from eight isolates of NTHI. All conditions were the same as described in the legend to Fig. 6 except that DNA was digested with *Pvu*II. Lanes contain genomic DNA from *H. influenzae* strains (A) 5657, (B) biogroup aegyptius 16, (C) C304, (D) C83, (E) DL205, (F) 2627, (G) 2019, and (H) 1479. Molecular size standards are noted on the left (in kilobases).

the organism to cause recurrent infections. Recurrent otitis media continues to be a problem despite the presence of antigenically conserved structures, such as the P1 and P6 proteins, on the surface of the bacterium (15, 26, 33, 38). However, infants and children mount a strain-specific bactericidal antibody response following otitis media due to NTHI (12). We hypothesize that the presence of a strain-specific immunodominant epitope on the bacterial surface induces the host to make antibody to this epitope and hides the antigenically conserved epitopes. Analysis of the specific epitopes to which children make antibody following otitis media will allow direct testing of this hypothesis.

Since none of the three strains whose P2 gene was sequenced was isolated from the same patient, it is unknown at this time whether sequence variation among strains is the result of large changes in variable regions or of single point mutations accumulated over long periods of time. Recently, van Alphen et al. showed that the mutation rate among spontaneously emerging mutants was very low (10^{-9} to 10^{-11}) (49). Despite the low frequency of mutation, estimates of the number of bacteria in the sputum of chronic obstructive pulmonary disease patients were on the order of 10^9/ml. A typical chromosomal mutation rate in this setting would be sufficient to allow the spontaneous emergence of strains which varied in a particular surface epitope. The sequential isolation of strains of NTHI from patients with exacerbations of chronic obstructive pulmonary disease showed that the P2 molecule varied in molecular mass and antigenic reactivity to monoclonal antibodies (16, 49). One might speculate that there is selective pressure on the bacterium, by the humoral immune system, to vary its surface antigens. In contrast, the P2's of type b organisms are very similar among strains, indicating that there is little pressure from the immune system to vary its surface epitopes. The presence of a capsule may account for this difference.

We conclude that the expression of highly immunodominant and strain-specific epitopes on the surface of NTHI is the result of substantial heterogeneity within the *ompP2* locus among strains. A series of spontaneous mutations within the surface-exposed epitopes may have emerged because of immunologic pressure from patients with recurrent infections. Alternatively, horizontal transformation of genetic material from a mixture of strains present in the human host may be a second mechanism to account for this observation (9, 13, 23, 43).

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