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Molecular Variation in the Major Outer Membrane Protein P5 Gene of Nonencapsulated *Haemophilus influenzae* during Chronic Infections

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During the course of persistent infections by nonencapsulated *Haemophilus influenzae* in patients with chronic bronchitis, the major outer membrane protein (MOMP) P5 varies in molecular weight. The nature of this variability was determined by DNA sequence analysis of the P5 gene from five different *H. influenzae* strains and their seven MOMP P5 variants which were isolated from patients with chronic infections of the lower respiratory tract. Analysis of the P5 sequence data from the different strains revealed four well-defined, heterogeneous regions. These regions of variable sequence appeared to correspond to the regions of the gene encoding the putative surface-exposed loops of MOMP P5. The MOMP P5 variants with alterations in MOMP P5 were shown to result from DNA point mutations and codon deletions. In addition, in three variants derived sequentially from one *H. influenzae* strain, a frameshift mutation resulted in the formation of a stop codon in the region encoding the signal sequence of the MOMP P5 gene. Strikingly, all nucleotide substitutions in the MOMP P5 loop regions of variants were nonsynonymous, suggesting that variants with alterated amino acid compositions of the surface-exposed parts of MOMP P5 obtained a selective advantage during persistence of the infection by nonencapsulated *H. influenzae* in chronic bronchitis patients.

Nonencapsulated *Haemophilus influenzae* causes persistent infections in the respiratory tract of patients with chronic bronchitis (24, 26, 38). During these persistent infections by *H. influenzae*, variants with alterations in the molecular mass of the major outer membrane protein (MOMP) P2 (39 to 42 kDa) and/or P5 (37 to 39 kDa) have been isolated (11, 12). The heterogeneous MOMP P2 is an immunodominant protein of *H. influenzae* and is an important target for bactericidal and opsonic antibodies (25, 37, 39). Variation in MOMP P2 appeared to result from DNA point mutations all causing amino acid changes (nonsynonymous substitutions) in immunodominant surface-exposed loops of MOMP P2. These changes resulted in antigenic drift of *H. influenzae* (6, 13). Since bactericidal antibodies elicited against MOMP P2 were variant specific, immune selection of MOMP P2 variants in the host has been proposed (6).

MOMP P5 is a heat-modifiable outer membrane protein of *H. influenzae*. Antibodies elicited against purified MOMP P5 cross-reacted with the OmpA protein of *Escherichia coli* (22, 41). The gene encoding MOMP P5 was approximately 50% homologous to the *ompA* gene (23). OmpA was shown to have a weak pore-forming activity (36). It has been proposed that the N-terminal domain of this protein transverses the outer membrane eight times, resulting in the formation of four short loops extending on the bacterial surface (16).

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As for MOMP P2, molecular weight variation of MOMP P5 was observed during chronic infections by *H. influenzae* (11), but the host immune response to MOMP P5 in humans has not been extensively studied. Antibodies specific for MOMP P5 elicited in rabbits had no protective activity (22), although recently it was shown that antibodies elicited against purified MOMP P5 in mice were bactericidal for the homologous and a few heterologous nonencapsulated *H. influenzae* strains (31). Since these data suggest that MOMP P5 may also be under immunological pressure, in this study the nature of the observed variability of the MOMP P5 gene of *H. influenzae* isolates obtained from persistently infected chronic bronchitis patients was determined. We discuss whether changes observed in the MOMP P5 gene during persistent infections in patients with chronic bronchitis may indicate selective pressure encountered by the host immune system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Nonencapsulated *H. influenzae* strains were isolated from sputum samples of patients with chronic bronchitis. These included different *H. influenzae* strains isolated independently and derived MOMP variants. MOMP variants are genotypically identical *H. influenzae* strains showing differences in mobilities of their MOMP P2 and/or P5 proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Genotyping was performed by analysis of DNA fingerprints after digestion with two restriction enzymes (11) or by random amplified polymorphic DNA analysis (42). In this study, all of the MOMP P5 variants obtained during a 2-year longitudinal study of *H. influenzae* isolates from chronic bronchitis patients were analyzed (11, 12). Strain characteristics and intervals between isolation of variants are summarized in Table 1. *H. influenzae* strains were cultured on chocolate agar plates or in brain heart infusion broth (Difco), both supplemented with hemin (10 μg/ml) and NAD (10 μg/ml), at 37°C in a humid atmosphere enriched with 5% CO_2.

Analysis of outer membrane proteins and immunoblotting. Cell envelopes were isolated and analyzed by SDS-PAGE as described by van Alphen et al. (41). MOMP P5 was identified with immunoblotting using the cross-reactive monoclonal antibody (MAb) 4BF8. This MAb had the same specificity as MAb 1CG7, described previously (40).

TABLE 1. Characteristics of isolated MOMP P5 variants of nonencapsulated *H. influenzae*

Patient	*H. influenzae* strains and variants	Mol wt variation		Interval between isolation of strains and variants (mo)
		MOMP P5	MOMP P2	
1	d1, d2, d3, d4[a]	–[b]	+	0, 6, 6
2	A850053, A860509	+	–	9
3	A850047, A860501	+	+	11
4	A850048, A860503	+	+	8
5	A850079, A850080	+	+	3

[a] *H. influenzae* d1 and d2 were isolated from one sputum sample; *H. influenzae* d3 and d4 were obtained from one sputum sample obtained 6 months later.
[b] MOMP P5 deletion.

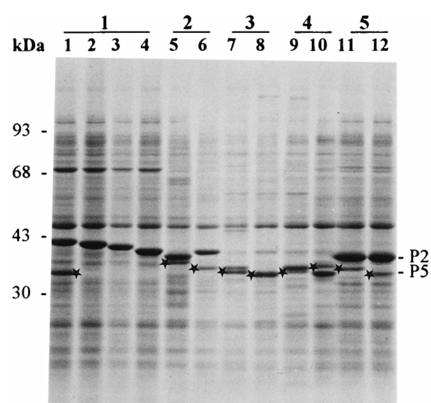

FIG. 1. Outer membrane protein patterns of nonencapsulated *H. influenzae* as analyzed with SDS-PAGE. Five *H. influenzae* strains and their seven MOMP P5 variants isolated from five patients with chronic bronchitis are shown. Lanes 1 to 4, d1, d2, d3, and d4; lanes 5 and 6, A850053 and A860509; lanes 7 and 8, A850047 and A860501; lanes 9 and 10, A850048 and A860503; lanes 11 and 12, A850079 and A850080. Variants of the same strain are connected with lines. Stars indicate MOMP P5. In lane 8, MOMP P5 comigrated with MOMP P2.

Oligonucleotide primers. Oligonucleotides used for amplification and sequencing of the P5 gene of nonencapsulated *H. influenzae* were synthesized on an Applied Biosystems model 381A oligonucleotide synthesizer. Primer sequences and the locations of the sequence in the P5 gene are indicated in Table 2. Oligonucleotide primers P5.1 and P5.2 were extended at their 5′ ends with a −21M13 priming sequence, and primers P5.1r and P5.24 were extended with the M13 reverse priming sequence. For determination of the DNA sequence upstream of the P5 gene, primers P5A and P5B (sequences corresponding to the 5′ coding end of the P5 gene) and random primer Pho, derived from the *phoE* gene sequence of *E. coli*, were used (30).

Amplification of the P5 gene. Overlapping templates of the P5 gene were obtained by PCR amplification using the primers described above. Cell lysates of *H. influenzae* isolates were used as the source of chromosomal DNA (4). PCR was carried out in a total volume of 100 μl containing 10 μl of chromosomal DNA preparation, 100 pmol of each primer, 200 μM each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 0.01% gelatin, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus). Following initial denaturation at 95°C for 5 min, 35 amplification cycles were performed in a Trio Thermoblock (Biometra). Each cycle comprised 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The PCR was finished with a final incubation at 72°C for 5 min. PCR products were analyzed on a 1% agarose gel, using a Tris-acetate-EDTA buffer system (19). Relevant products were excised from the gel and purified by using Qiaex as instructed by the manufacturer (Qiagen Inc., Chatsworth, Calif.). The DNA concentration was adjusted to approximately 1 ng/μl in distilled water for sequencing. To obtain the upstream sequence of the P5 gene, a random PCR was performed with primer P5A and the random primer Pho. The PCR conditions were the same as described above except that an annealing temperature of 37°C was used.

DNA sequence analysis. Both strands of the PCR products were sequenced by using the Taq Dye-Primer cycle sequencing system with fluorescent dye-labeled −21M13 and M13 reverse sequence primers (Applied Biosystems). The PCR products obtained from the DNA upstream the P5 gene were sequenced with a Taq Dye-Terminator sequencing kit (Applied Biosystems), using fluorescent dye-labeled dideoxy nucleotides and the PCR primers P5A and Pho. DNA analysis was performed with an automated fluorescence DNA sequencer (model 370A; Applied Biosystems). The derived sequences were analyzed by using computer programs included in the program package PCgene (Intelligenetics, Inc.). The DNA sequences were aligned with the program Clustal, using the method developed by Higgins and Sharp (15). Secondary protein structure predictions were made by the method of Garnier et al. (10). For calculation of numbers of variable sites present in different P5 genes, the method of Nei and Gojobori (28), as implemented in the computer program MEGA (version 1.01; Institute for Molecular Evolutionary Genetics, Pennsylvania State University) was used.

Nucleotide sequences. The nucleotide sequences of the P5 genes will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession numbers X90505, X90506, X90507, X90508, X90494, X90504, X90490, X90500, X90492, X90502, X90496, and X90498.

RESULTS

Identification of MOMP P5 variants of nonencapsulated *H. influenzae*. Outer membrane protein analysis by SDS-PAGE of five *H. influenzae* strains and the MOMP P5 variants derived from these strains, obtained from five patients with chronic bronchitis, showed molecular masses of MOMP P5 ranging between 37 and 39 kDa (Fig. 1, lane 5 to 12). By immunoblotting using the cross-reactive MOMP P5-specific MAb 4BF8 (40), the presence of MOMP P5 was shown in these strains and variants, except for *H. influenzae* variants d2, d3, and d4 of strain d1 (Fig. 1, lanes 1 to 4).

Diversity in the P5 genes of different nonencapsulated *H. influenzae* strains. The MOMP P5 gene sequences of five different independently obtained nonencapsulated *H. influenzae* strains, d1, A850053, A850047, A850048, and A850079, were

TABLE 2. Oligonucleotide primers used for amplification of the MOMP P5 gene by PCR

Name	Position in gene[a]	Sequence (5′-3′)[b]
P5 gene		
P5.1	19–36	*TGT AAA ACG ACG GCC AGT* GCA TTA GTA GTT GCT GGC
P5.2	427–444	*TGT AAA ACG ACG GCC AGT* GGT ACT CGT GAC CAC AAG
p5.1r	1027–1044	*CAG GAA ACA GCT ATG ACC* TGC GAT TTC TAC ACG ACG
P5.2r[c]	667–684	*CAG GAA ACA GCT ATG ACC* TAC GAT TTC AGG TGC TGC
P5A	94–114	AGA TCC TTG ACC AGC TTT AAC
P5B	55–72	TTC TTG TGG AGC TGC TTG AGC
Pho[d]		GGC GCA TGC ACG AGC CGG TGA AGT TAA CGG

[a] Numbers are based on the published sequence of the MOMP P5 gene of *H. influenzae* type b strain 1613 (23).
[b] The −21M13 sequence (TGT AAA ACG ACG GCC AGT) and the M13 reverse sequence (CAG GAA ACA GCT ATG ACC), added at the 5′ end for sequencing of PCR products, are indicated in italics.
[c] Based on the P5 sequences obtained in this study.
[d] Used as a random primer; derived from the *E. coli phoE* sequence (30).

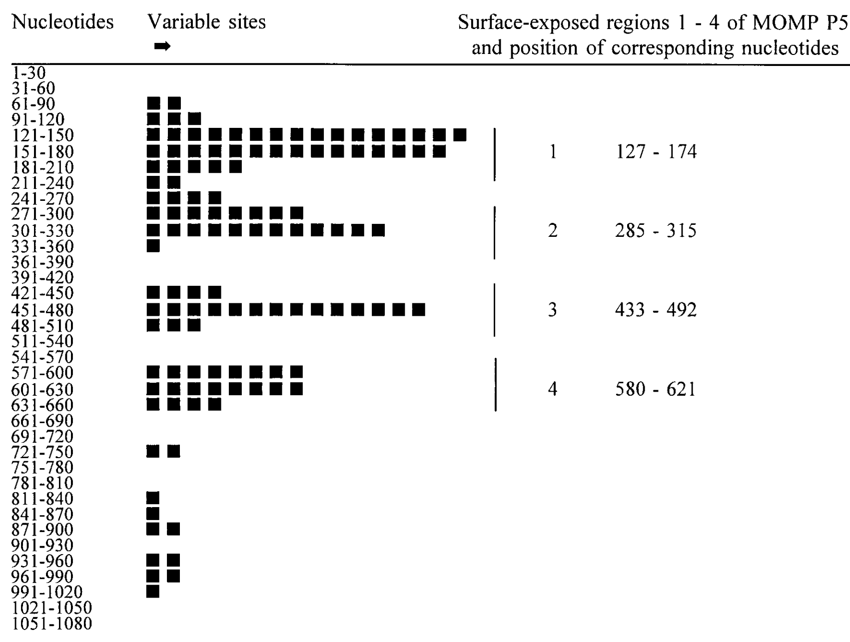

FIG. 2. Distribution of sequence diversity along the MOMP P5 gene. The MOMP P5 genes of five *H. influenzae* strains, A850053, A850047, A850048, A850079, and d1, were aligned, and the numbers of sites containing variable sequences (in nonoverlapping windows with a size of 30) were calculated with MEGA (28). Each box represents a site with variable sequence. Variable regions 1 to 4 encode putative surface-exposed parts of MOMP P5, based on comparison with the topology model described for OmpA of *E. coli* (16). Numbering is based on the MOMP P5 gene sequence of strain d1.

compared. With primers based on conserved sequences of the published sequence of the P5 gene of *H. influenzae* type b strain 1613 (23), the MOMP P5 genes of the isolated *H. influenzae* strains yielded a relevant PCR product. The resulting PCR products represented approximately 97% of the coding region for the mature MOMP P5. Alignment of the MOMP P5 sequences of these five strains revealed DNA mutations throughout the whole gene that did not all result in amino acid substitutions. The greatest sequence diversity appeared to be concentrated in four areas, located between nucleotides 127 and 171, 285 and 315, 430 and 492, and 577 and 618, when the P5 gene of strain d1 was used as the reference sequence (Fig. 2). Using the topology model for OmpA of *E. coli* (21) and sequence comparison with the *ompA* gene, the regions showing diversity in the MOMP P5 gene corresponded largely to the probable surface-exposed parts of the MOMP P5 protein. A secondary structure prediction of the MOMP P5 protein determined by the method of Garnier et al. (10) also indicated that the regions showing the highest diversity were likely to be surface exposed.

Variation in the MOMP P5 gene of *H. influenzae* during chronic infection. Sequence analysis after PCR amplification of the P5 genes of four MOMP P5 variants appearing during chronic infection revealed accumulation of nucleotide substitutions, deletions, and insertions in the P5 genes of these variants. All mutations were localized in three regions encoding the putative surface-exposed loops of MOMP P5. In Fig. 3, the changes in the DNA sequences and the derived amino acid sequences of the variable regions are shown. The remaining sequences of the P5 genes were identical and are not shown. All of the nucleotide changes resulted in amino acid substitutions, deletions, or insertions (nonsynonymous mutations).

Analysis of the MOMP P5 genes of *H. influenzae* variants no longer expressing the MOMP P5. In contrast to *H. influenzae* strain d1, its variants d2, d3, and d4 did not express MOMP P5 (Fig. 1). However, the presence of a MOMP P5-encoding gene in each of the MOMP P5 deletion mutants was confirmed by using PCR and hybridization of digested chromosomal DNA with the PCR-amplified P5 gene of strain d1 as a probe (not shown). To determine whether expression of MOMP P5 is regulated at the transcriptional level, the region upstream of the MOMP P5 gene of variants d2 to d4, not expressing MOMP P5, was analyzed. Using one specific primer for the P5 gene (P5A located at the 5′ end) and a random primer in a PCR, we obtained a fragment of approximately 800 bp that hybridized with the internal primer P5B (derived from nucleotide sequences within the signal sequence). With primer P5B and the random primer, a nested PCR was performed, and the obtained fragment was sequenced. The nucleotide sequences upstream of the P5 gene of *H. influenzae* variants d1 to d4 were found to be identical and homologous to the corresponding sequence of the P5 gene described by Sirakova et al. (35). The loss of expression of MOMP P5 appeared to be the result of a frameshift mutation caused by the deletion of an A nucleotide in a stretch of seven A nucleotides in the signal sequence of the variants d2, d3, and d4 compared to that of strain d1, since this frameshift resulted in the formation of two stop codons at position 22 (Fig. 4). Upstream of the initiation codon, a putative ribosome binding site, AGGA, similar to the consensus sequence of the *E. coli* ribosome binding site was found. In addition to the frameshift mutation, a nucleotide substitution and a codon deletion were observed between strain d1 and its variants d2 to d4 (Fig. 3).

DISCUSSION

During persistent infections by nonencapsulated *H. influenzae* in patients with chronic bronchitis, *H. influenzae* variants manifesting alterations in their outer membrane proteins MOMP P2 and P5 appear (11, 12). The variability of MOMP

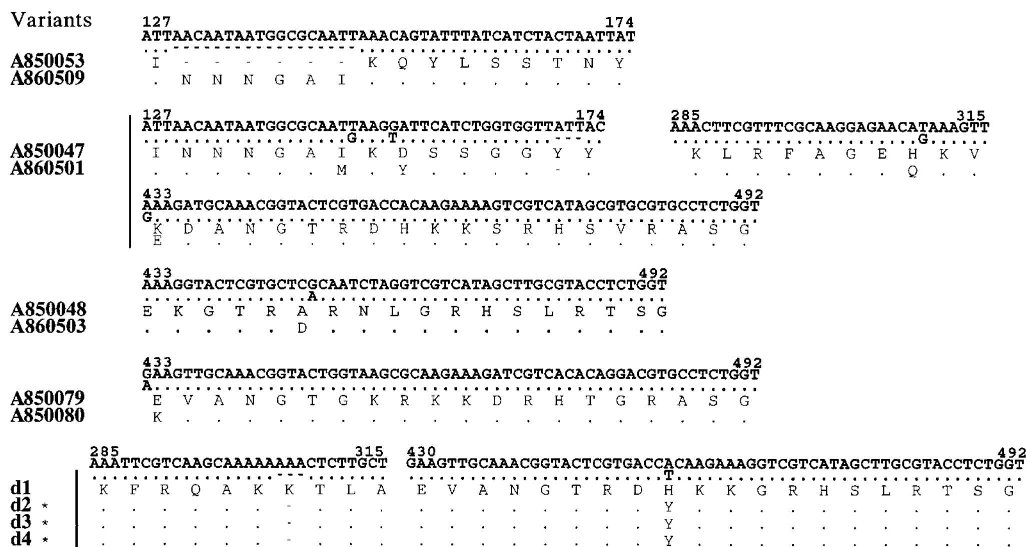

FIG. 3. Sequence variation in MOMP P5 of nonencapsulated *H. influenzae* variants isolated during persistence in patients with chronic bronchitis. Nonsynonymous mutations were detected exclusively in the variable regions of MOMP P5 that are putative surface-exposed loops. Numbering is based on the MOMP P5 gene of strain d1. The DNA sequence and the deduced amino acid sequence of the variable regions of *H. influenzae* MOMP P5 variants derived from five strains are shown. *, the nucleotide deletion that results in a stop codon at position 22 of the P5 gene. Dashes represent deletions, and dots indicate identical nucleotides and amino acids.

P2 has extensively been studied, both genetically and antigenically, in contrast to the variation of MOMP P5. In this study, alignment of the MOMP P5 genes from different *H. influenzae* strains revealed conservation of the sequence except for four regions showing high levels of diversity in the gene encoding MOMP P5 (Fig. 2). According to the topology model for the *E. coli* OmpA protein, which has 50 to 60% amino acid sequence identity with MOMP P5 (21), the sequence conservation of 77% homology occurs in the membrane-spanning regions and the C-terminal part of the protein. The sequence conservation in the membrane-spanning regions of MOMP P5 probably results from the structural constraints imposed on transmembrane proteins (33). It has been suggested that the C-terminal sequences of OmpA and related proteins, which are highly conserved and predicted to be periplasmic (21), are peptidoglycan associated (3, 17). The C-terminal sequences of OmpA and MOMP P5 also show some sequence homology with a probable surface-exposed domain of the *Pseudomonas* outer membrane protein OprF (7). Both the deduced hydropathy profile of MOMP P5 and a comparison with the predicted topology of the *E. coli* OmpA suggest that the highly diverse regions are located at the bacterial cell surface, similar to what has been observed for the variable regions of MOMP P2 (4, 34).

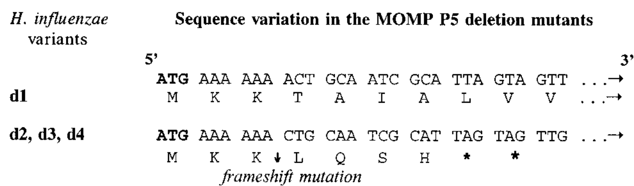

FIG. 4. Part of the MOMP P5 gene sequence along with the N-terminal amino acid sequences of the MOMP P5-positive strain d1 and variants d2, d3, and d4, lacking MOMP P5. The initiation codon is shown in boldface, and part of the signal sequence of the P5 gene is shown. Variants d2, d3, and d4 of *H. influenzae* strain d1, not expressing MOMP P5, contain a base pair deletion at position 10 of the signal sequence of the coding P5 sequence generating two stop codons in the translated reading frame. The stop codons are indicated with asterisks.

H. influenzae variants with changes in the molecular mass of MOMP P5 were isolated from sputum samples obtained at 3- to 11-month intervals from different patients suffering from chronic infections. Differences in MOMP P5 between variants were found to result exclusively from nonsynonymous nucleotide substitutions, resulting in amino acid changes in the four putative surface-exposed regions. The accumulation of amino acid-altering changes in the surface-exposed regions of MOMP P5 of variants may have occurred by chance. However, it is also possible that as for the MOMP P2 gene, the amino acid-altering changes have accumulated as the result of a specific selection process (6). Suggestive of specific selection is the occurrence of only nonsynonymous substitutions in the MOMP P5 gene during chronic infection. This is striking, since for coding sequences in bacteria, the average ratio of synonymous to nonsynonymous substitutions per respective site is 20 (29). A selective pressure may be exerted by antibody-mediated defenses of the host (6, 27). Since antibodies elicited against purified MOMP P5 in mice have been shown to be bactericidal in the presence of complement (31), these antibodies may contribute to selection of MOMP P5 variants. However, the data obtained in this study are insufficient to demonstrate significant selection of nonsynonymous changes in the variable loop regions.

Since the MOMP P5 variants were often isolated as pure cultures from sputum samples of patients with chronic bronchitis, the MOMP P5 variation was advantageous to nonencapsulated *H. influenzae* during the chronic infections in those patients. The appearance of MOMP P5 variants was not always accompanied by variation in MOMP P2 (Table 2), indicating that variation in MOMP P5, or deletion of it, itself is sufficient to be advantageous. However, we cannot exclude that the mutation coincides with another unknown advantageous mutation elsewhere in the genome.

Variation of *H. influenzae* proteins during chronic infections has now been demonstrated for MOMP P2 and P5, the immu-

noglobulin A1 protease (changes in cleavage specificity and antigenic properties) (18), and metabolic enzymes determining the biotype of *H. influenzae* (20). In contrast, the sequences of other *H. influenzae* proteins, such as protein D and lipoprotein P6, appeared to be highly conserved (2, 5), but this is most likely due to some structural or functional constraints (33).

Since the mutations in the MOMP P2 and P5 gene are base substitutions, DNA polymerase errors possibly as a result of slipped-strand mispairing during DNA replication may be responsible for spontaneous mutations (1, 32). The specific accumulation of mutations during starvation (so-called adaptive mutations) has been proposed (8, 9, 14). During persistence in patients with chronic bronchitis, growth restriction due to nutrient limitation of *H. influenzae* is conceivable, especially in periods in which the persisting bacteria cannot be cultured from sputum samples (11). Generating variant phenotypes may enable the bacteria to adapt to adverse life conditions during the course of a chronic infection. Besides a possible increase in genetic diversity, immunological selection for variation in surface-exposed parts of MOMP P5 may contribute to the appearance of the spontaneous mutant phenotype.

In conclusion, four well-defined, probably surface-exposed regions of MOMP P5 of nonencapsulated *H. influenzae* have been shown to vary in sequence. The variation observed during the course of persistent infections in chronic bronchitis patients resulted from nonsynonymous substitutions or in one case from a frameshift mutation as the consequence of a single nucleotide deletion in the MOMP P5 gene. Since these mutations became fixed in the population, some selective advantage for these mutants must exist. One possibility is immune selection similarly responsible for the antigenic drift of MOMP P2.

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