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Antigenic Heterogeneity and Molecular Analysis of CopB of *Moraxella* (*Branhamella*) *catarrhalis*

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Outer membrane protein (OMP) CopB, an iron-repressible 81-kDa major OMP of *Moraxella* (*Branhamella*) *catarrhalis* has been a major focus of investigation. To assess CopB as a potential vaccine antigen, we elucidated the degree of antigenic and sequence heterogeneity in this protein among strains of *M. catarrhalis*. Two monoclonal antibodies, 1F5 and 2.9F, which bind to surface-exposed epitopes on CopB recognized 60 and 70% of the strains, respectively. The degree of sequence heterogeneity in CopB was assessed by cloning and sequencing the CopB gene from two different strains of *M. catarrhalis* and comparing with the published sequence. There was 92 to 96% homology between the sequences at the nucleotide level and 90 to 95% homology at the amino acid level. The variability in the protein sequence is confined mainly to three moderately variable regions. Restriction fragment length polymorphism (RFLP) analysis of the CopB genes obtained from 20 diverse strains by PCR was performed. Ninety percent of the potential restriction sites in the constant regions and 47% of the potential restriction sites in the variable regions were present in the 20 strains, indicating that the pattern of variable and constant areas in the CopB gene is a general pattern among strains of *M. catarrhalis*. We conclude that the CopB gene is largely conserved among strains of *M. catarrhalis* and contains discrete regions which show moderate heterogeneity among strains.

Moraxella (*Branhamella*) *catarrhalis* has emerged as an important human pathogen over the last two decades. In recent studies, it is the third most common bacterial pathogen isolated from the middle ear fluid of children with otitis media (2, 7). There has been a steady increase in the rate of isolation of *M. catarrhalis* from the sputum of adults with acute exacerbations of chronic obstructive pulmonary disease (9, 15, 18, 19).

The basic characteristics of the outer membrane proteins (OMPs) of *M. catarrhalis* have been well described. The major OMPs of *M. catarrhalis* are 98 to 20 kDa in size and have been named A to H (3, 16). In addition, a high-molecular-weight OMP of 300 to 700 kDa has been identified (12, 14).

CopB, an 81-kDa major OMP, has been a major focus of investigation (1, 10, 11). This protein is identical to OMP B2 (16, 21). The gene encoding this OMP has been cloned and sequenced from one strain (10). Though its precise function is not known, CopB is iron repressible and is involved in iron uptake by *M. catarrhalis* from lactoferrin and transferrin (1, 5).

Several lines of evidence suggest that CopB is a potential vaccine antigen. In a murine model, a monoclonal antibody directed towards this OMP enhances clearance of *M. catarrhalis* from the lung (10). An isogenic mutant which does not express CopB has diminished ability to survive in the same murine pulmonary clearance model (11). This isogenic mutant also has diminished serum resistance to normal human sera in comparison to the wild type strain (11). To further assess CopB as a potential vaccine antigen, it will be important to know the degree of antigenic and sequence heterogeneity in this protein among strains of *M. catarrhalis.*

In this study, the development of two monoclonal antibodies directed at CopB is described. Flow cytometry was used to determine the surface exposure of the epitopes recognized by these antibodies. Antigenic variability among strains in the CopB protein for these epitopes has been elucidated by immunodot assays. To assess the degree of sequence heterogeneity in CopB, we have cloned and sequenced the gene encoding CopB from two different strains of *M. catarrhalis* and have compared the sequences with the published sequence. To extend these observations to multiple strains, PCR-restriction fragment length polymorphism (RFLP) analysis of the CopB genes from twenty diverse strains is also described.

MATERIALS AND METHODS

Bacterial strains. *M. catarrhalis* 25240 is from the American Type Culture Collection. *M. catarrhalis* 8184 was recovered by tympanocentesis from the middle ear of an infant with otitis media in Buffalo, N.Y. *M. catarrhalis* 56 is a clinical isolate from the sputum of an adult patient from Johnson City, Tenn. Strains of *M. catarrhalis* isolated from various body sites and from diverse geographic sources were used for immunodot assays and PCR-RFLP analysis (Table 1). *Escherichia coli* JM 109 was the host strain for pGEM 7zf- and pGEX 4T3.

Purification of outer membrane fractions. The outer membrane of *M. catarrhalis* was purified by collecting vesicles induced by a combination of heat and EDTA (17) or by the zwittergent extraction method (16). CopB of strain 25240 was partially purified to immunize mice for the development of monoclonal antibodies as follows. OMPs purified by the zwittergent extraction method were precipitated by the addition of ethanol to a final concentration of 80%. Following centrifugation at 16,000 × *g* for 10 min at 4°C, the pellets were suspended in 60 ml of a buffer containing 3% ampholytes (Bio-Rad), 2% zwittergent, and 10% glycerol. This sample was then subjected to isoelectric focusing with a Bio-Rad Rotofor apparatus according to the instructions of the manufacturer. To establish a pH gradient from 3 to 11, the electrode buffers were 0.1 M sodium hydroxide and 0.1 M phosphoric acid. Isoelectric focusing was performed at 12 W for 5 h. The resulting 20 fractions were collected, and aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing CopB (pH 8.3 to 9.8) were pooled, precipitated with ethanol, and used to immunize mice.

Development of monoclonal antibodies. Antibody 1F5 was developed from BALB/c mice immunized with outer membranes purified from strain 8184 by the zwittergent extraction method. Twenty-five micrograms of outer membrane was administered to each mouse at each injection. The sample was emulsified with an equal volume of adjuvant. The following schedule was followed: day 0, 25 μg of OMPs with complete Freund's adjuvant subcutaneously; days 7 and 14, 25 μg of OMPs with incomplete Freund's adjuvant subcutaneously; day 28, 25 μg of

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OMPs intraperitoneally (no adjuvant). On day 32 following the initial immunization a fusion was performed.

Antibody 2.9F was developed from mice immunized with partially purified CopB of strain 25240. Based on SDS-PAGE, we estimate that CopB made up approximately 50% of the protein content of the mixture used to immunize the mice. Proteins were precipitated with ethanol and resuspended in phosphate-buffered saline (PBS). The following schedule was used: day 0, 150 μg of total protein emulsified with an equal volume of incomplete Freund's adjuvant administered intraperitoneally; day 25, 55 μg of protein in PBS intraperitoneally; day 29, 100 μg of protein in PBS intraperitoneally. The fusion was performed on day 32 following the initial immunization.

To perform the fusions, mouse splenocytes were fused with SP2/O-Ag14 plasmacytoma cells to obtain antibody-producing hybridomas by previously described methods (13). Clones producing antibodies were identified initially by screening hybridoma supernatants in immunodot assays or by enzyme-linked immunosorbent assays using whole-cell lysates of the homologous strain. The specificity of the antibody reactivity was determined by immunoblot assays. Hybridomas were cloned by limiting dilution and were inoculated intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid. Antibodies from ascites fluid were affinity purified by elution from protein G.

Flow cytometry. Flow cytometry was used to determine whether monoclonal antibodies recognize epitopes which are exposed on the surface of the intact bacterium. Bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.2 in brain heart infusion broth. Two hundred microliters of the broth culture was diluted with 800 μl of PBS containing 25 μg of DNase (PBS-DNase) (Amersham) per ml to reduce clumping, and the cells were harvested by centrifugation at 14,000 × *g* for 2 min in a microfuge (6). Cells were then incubated with 200 μl of monoclonal antibody (undiluted tissue culture supernatant) containing 25 μg of DNase per ml. After incubation at 37°C for 1 h, the cells were centrifuged and resuspended in 200 μl of fluorescein-labeled goat anti-mouse immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories [KPL]) diluted 1:10 in PBS-DNase and incubated for 30 min. Then, 1.8 ml of PBS-DNase was added, and the sample was subjected to flow cytometry with a FACScan (Becton-Dickinson). A total of 20,000 cells were counted in a gated region corresponding to unclumped cells. Negative controls were performed with each experiment and included incubation of cells with (i) an aliquot of tissue culture supernatant from SP2 or purified ascites fluid prepared exactly as the antibodies were prepared and (ii) an irrelevant monoclonal antibody.

Strain specificity of the monoclonal antibodies. Whole-cell lysates of the bacterial strains were prepared by centrifuging bacteria grown in brain heart infusion broth and resuspending the bacterial pellet in PBS to an OD_{600} of 0.2, followed by sonication. A volume of 3 μl of these lysates was dotted on nitrocellulose and air dried. The nitrocellulose was then blocked with 1% nonfat milk and incubated with 1F5 (undiluted tissue culture supernatant) or 2.9F (1:1,000-diluted ascites fluid) overnight at room temperature. After being washed with PBS, the dot blot was incubated with 1:1,000-diluted peroxidase-labeled goat anti-mouse IgG-IgM antibody (KPL) and then developed with horseradish peroxidase developing reagent (Bio-Rad).

Cloning and sequencing of the CopB gene. The published sequence of the CopB gene of *M. catarrhalis* O35E (10) was used to design primers corresponding to the amino terminus (B1; 5′ GCTGTTAGCCAGCCTAAG 3′) and carboxy terminus (B2; 5′ GTAAGTGAAGTTCACGCC 3′) of the mature protein and to upstream (U2; 5′ TCAAGCCTCATAATCGGAG 3′) and downstream (D; 5′ AGAACACCCAAGCGTGCT 3′) flanking sequences. *Bam*HI recognition sites were incorporated in these primers. These primers were used in PCR to amplify the CopB gene from genomic DNA of *M. catarrhalis* 25240 and 56 with Vent DNA polymerase (Boehringer). One microgram of template DNA was incubated with 300 ng of oligonucleotides, and the CopB gene was amplified by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min. DNA fragments of 2.2 kb were precipitated with 2 M ammonium acetate and cloned into the *Bam*HI sites of pGEM 7Zf-plasmid vectors by standard methods. The nucleotide sequences of both strands of the inserts in the resultant plasmids pGM4 (strain 25240) and p561 (strain 56) were determined. Both dideoxy sequencing with Sequenase (U.S. Biochemicals) and automated sequencing at the University of Buffalo microsequencing facility were utilized.

PCR-RFLP analysis of the CopB gene. Genomic DNA was isolated from overnight broth cultures of twenty strains of *M. catarrhalis* by standard methods. Primers B1 and B2 were used in PCR to amplify the CopB gene from the genomic DNA of the 20 strains as described above except that the enzyme used was *Taq* polymerase (Promega).

Sequences of the published sequence of strain O35E and of strain 25240 were used to select the appropriate enzymes for RFLP analysis (Fig. 4). Restriction enzyme digestions were performed according to the manufacturer's instructions. Restriction digests were analyzed by agarose or polyacrylamide gel electrophoresis depending on the size of the expected DNA fragments.

Expression of recombinant CopB. The methodology used has been described previously (21). Briefly, the CopB gene was amplified from the genomic DNA of *M. catarrhalis* 25240 and 8184 by PCR with B1 and B2 primers as described above. DNA fragments of 2.2 kb were cloned into plasmid vector pGEX 4T3. The cloned gene was expressed in *E. coli* JM 109 as follows. A single colony of each clone was inoculated into 2 ml of Luria-Bertani (LB) broth–ampicillin (100 μg/ml) and grown for 3 to 5 h at 37°C until visually turbid. Isopropylthiogalactopyranoside (IPTG; 100 mM) was added to a final concentration of 0.1 mM, and incubation continued for an additional 2 h. The liquid culture was transferred to microcentrifuge tubes and centrifuged at 14,000 × *g* for 2 min. The supernatant was discarded, and the bacterial pellet was resuspended in 100 μl of 2× SDS-PAGE sample buffer (0.12 M Tris, 2.4% SDS, 2% mercaptoethanol, 23.8% glycerol, 0.006% bromophenol blue) and boiled for 10 min (whole-cell lysate). A control whole-cell lysate was prepared with *E. coli* JM 109 transformed with pGEX 4T3 with an unrelated DNA insert.

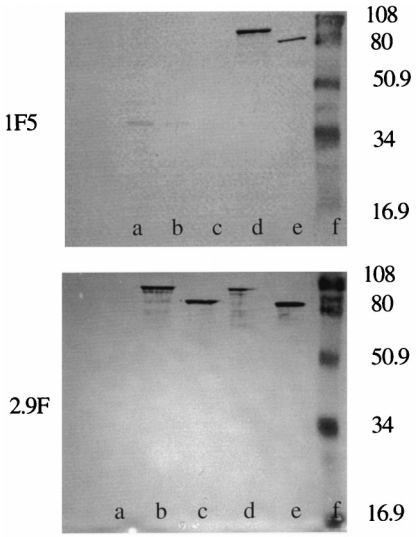

FIG. 1. Immunoblot of 1F5 (top) and 2.9F (bottom) with whole-cell lysates of *E. coli* JM 109 transformed with control pGEX 4T3 (a), *E. coli* JM 109 expressing recombinant CopB of *M. catarrhalis* 25240 as a fusion protein with glutathione *S*-transferase (GST) (b), *M. catarrhalis* 25240 (c), *E. coli* JM 109 expressing recombinant CopB of *M. catarrhalis* 8184 as a fusion protein with GST (d), and *M. catarrhalis* 8184 (e). The conjugate antibody in all lanes is peroxidase-labeled goat anti-mouse IgG. Molecular mass standards are noted on the right in kilodaltons.

Antigenic specificity of antibodies 1F5 and 2.9F. Whole-cell lysates of *E. coli* JM 109 transformed with control pGEX 4T3, *E. coli* JM 109 expressing recombinant CopB of *M. catarrhalis* 25240, *E. coli* JM 109 expressing recombinant CopB of *M. catarrhalis* 8184, and whole-cell lysates of *M. catarrhalis* 25240 and 8184 were tested in an immunoblot assay with monoclonal antibodies 2.9F and 1F5 as follows. The whole-cell lysates were separated in an SDS–11% PAGE gel and transferred to nitrocellulose. The nitrocellulose was blocked with 1% nonfat dried milk in PBS and incubated with 2.9F mouse ascites fluid diluted 1:10,000 and undiluted 1F5 tissue culture supernatant overnight at 4°C. After being washed with PBS, the immunoblot was incubated with peroxidase-conjugated goat anti-human IgG-IgM at a 1:2,000 dilution (KPL) for 1 h. The immunoblots were washed and developed with horseradish peroxidase developing reagant (Bio-Rad).

RESULTS

Antigenic specificities of antibodies 1F5 and 2.9F. To analyze antigenic determinants on CopB, monoclonal antibodies were developed by immunizing mice with purified outer membrane of strain 8184. Immunoblot assays identified antibody 1F5 which is an IgG1 and which recognized a band of ~80 kDa in whole-organism lysates and purified outer membrane. This antibody recognizes recombinant CopB of strain 8184 (Fig. 1) when it is expressed as a fusion protein with glutuathione *S*-transferase in *E. coli* JM 109.

Subsequent fusions were performed with splenocytes from mice immunized with purified outer membrane of different strains. None of these fusions yielded monoclonal antibodies to CopB. Therefore, CopB was partially purified by subjecting zwittergent-extracted outer membrane of strain 25240 to preparative isoelectric focusing as described above. Immunoblot assays with antibody 2.9F, an IgG1, revealed a band of ~80 kDa in whole-organism lysates and purified outer membrane.

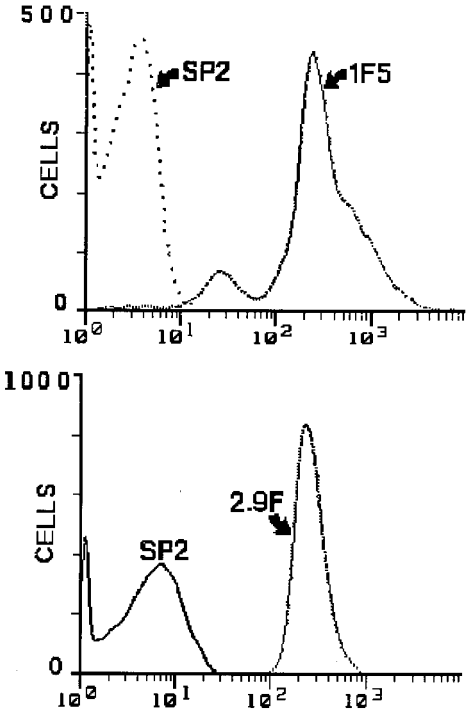

FIG. 2. Fluorescence histograms to determine surface exposure of 1F5 and 2.9F epitopes. The number of cells is depicted on the *y* axis and the fluorescence (FL-1) is shown on the *x* axis. SP-2 is protein G affinity-purified SP2 ascites fluid prepared exactly as the antibodies were prepared and tested simultaneously.

This antibody recognizes recombinant CopB of strains 25240 and 8184 (Fig. 1) when they are expressed as fusion proteins with glutuathione *S*-transferase in *E. coli* JM 109.

Surface exposure of epitopes on CopB. Whether antibodies 1F5 and 2.9F recognize epitopes which are exposed on the surface of the intact bacterium was assessed by flow cytometry as described above. Both antibodies recognized epitopes which are present on the bacterial surface (Fig. 2). Three negative controls were performed to exclude the possibility that nonspecific binding of antibodies was occurring. (i) Protein G affinity-purified SP2 ascites fluid (prepared exactly as the antibodies were prepared) used in place of antibodies in the same experiment gave a negative result. (ii) An irrelevant IgG1 monoclonal antibody which recognizes the P2 porin protein of nontypeable *Haemophilus influenzae* yielded a negative result (data not shown). (iii) Strains of *M. catarrhalis* which lacked the epitopes according to the immunodot assay gave negative results in flow cytometry as expected (data not shown).

Strain specificities of epitopes recognized by antibodies 1F5 and 2.9F. Fifty strains were tested in an immunodot assay with these monoclonal antibodies. The strains were isolated from diverse clinical sites, including sputum, blood, sinus aspirate, and middle ear fluid. Six of these strains had been isolated in the United Kingdom (provided by Susan Hill, Birmingham General Hospital), while the rest were from four different geographic locations in the United States (Buffalo, N.Y.; Johnson City, Tenn.; Houston, Tex.; and Philadelphia, Pa.). 1F5 recognized 30 (60%) of the strains tested, while 2.9F recognized 35 (70%) of the strains tested. Twenty-four (48%) of the strains were recognized by both antibodies, 6 (12%) were recognized by 1F5 alone, and 11 (22%) were recognized by 2.9F alone. Nine (18%) strains did not express either of the two epitopes recognized by these monoclonal antibodies. The pattern of strain recognition of 1F5 was different from that of

TABLE 1. Characteristics of strains of *M. catarrhalis*

Strain		Source	Geographic source	Recognition pattern of monoclonal antibody[a]:		Variable-region restriction sites for enzyme[b]:			Constant-region restriction pattern for enzyme[c]:			
No.	Designation			1F5	2.9F	*Xmn*I	*Bsp*1286I	*Bsi*EI	*Bsm*AI	*Hinc*II	*Hga*I	*Bsr*I
1	451	Sputum	Birmingham, U.K.[d]	+	+	+	+	−	2/2	3/4	3/3	4/4
2	1084	Sputum	Birmingham, U.K.	+	+	+	−	−	2/2	4/4	3/3	4/4
3	640	Sputum	Birmingham, U.K.	−	−	−	−	−	2/2	4/4	3/3	3/4
4	857	Sputum	Birmingham, U.K.	+	+	−	−	−	2/2	4/4	3/3	3/4
5	342	Sputum	Birmingham, U.K.	−	+	−	+	+	2/2	4/4	3/3	3/4
6	1592	Sputum	Birmingham, U.K.	−	−	−	−	−	2/2	3/4	3/3	3/4
7	25240		ATCC[e]	−	+	−	+	+	2/2	4/4	3/3	4/4
8	3	Sputum	Johnson City, Tenn.	+	−	−	−	−	2/2	4/4	3/3	4/4
9	56	Sputum	Johnson City, Tenn.	+	−	−	+	−	2/2	4/4	2/3	3/4
10	M10	Sputum	Houston, Tex.	+	+	+	−	−	2/2	3/4	3/3	3/4
11	585	Blood	Buffalo, N.Y.	+	+	+	+	−	2/2	4/4	3/3	3/4
12	8184	Middle ear	Buffalo, N.Y.	+	+	+	−	−	2/2	4/4	3/3	3/4
13	5192	Nasopharynx	Buffalo, N.Y.	−	+	−	+	+	2/2	4/4	3/3	3/4
14	5193	Middle ear	Buffalo, N.Y.	−	+	−	+	−	2/2	4/4	3/3	4/4
15	6349	Middle ear	Buffalo, N.Y.	+	+	+	+	−	2/2	4/4	3/3	3/4
16	M4	Sputum	Houston, Tex.	+	−	−	−	−	2/2	4/4	3/3	3/4
17	20	Sputum	Johnson City, Tenn.	−	−	−	+	−	2/2	4/4	2/3	3/4
18	34053 A	Blood	Buffalo, N.Y.	+	+	+	−	−	2/2	4/4	3/3	3/4
19	9790	Sputum	Buffalo, N.Y.	−	+	−	+	+	2/2	4/4	3/3	3/4
20	Tal1	Sinus	Philadelphia, Pa.	+	+	+	+	+	2/2	3/4	3/3	3/4

[a] Pattern of recognition of these strains by the two monoclonal antibodies to CopB. +, epitope present; −, epitope absent.
[b] Presence or absence of restriction sites in the variable regions of the CopB gene. +, restriction site present; −, restriction site absent.
[c] Restriction pattern of the CopB gene with the constant-region enzymes (number of restriction sites present/number of possible restriction sites).
[d] U.K., United Kingdom.
[e] ATCC, American Type Culture Collection.

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      1                                                                                                  100
035E  MNKFQLLPLTLAVSAAFTTTAFAAVSQPKVVLAGDTVVSDRQGAKIKTNVVTLREKDESTATDLRGLLQDEPAIGFGGGNGTSQFISIRGMGHNAIDLKI
25240 ....................................................................................................
56    ...................................................................................V................

      101                                                                                                200
035E  DNAYQDGQLHYHQGRFMLDPQMVKVVSVQKGAGFASAGIGATNGAIVTKTLDADELLRNSDKDYGFKVGAGLSTNKGHSYHGSAFGKAQTGFGQVDALVS
25240 ....................................................................................................
56    ....................................................................................................

      201                                                                                                300
035E  YNQVNDSDYKGGKGYTNLLGNDVVTRSALDKSSYLVKAGLTAGDHRFVVSHLNEVHKGIRGVREEFDFANRALTLDIEKDKKKRTDEQLQAELDNKYAGK
25240 ..........................................................................K..D.TL.ETE............
56    ..........................................................................I....KG.I..EAE.L...NKD.P.Q

      301                                                                                                400
035E  GYKLGSKTPDGKK----YNVVDANGKLVADLDRNNPTQRETYQKLTNLEWTGKNLGFANEVTANVYKLEHGRNSSSDKGNSYILRDVPNTINDNGDSPSN
25240 ............----..........................................................Q..T..TK...KE.I..V.T...
56    .....K.IN.KDNKVVG.H......RQ.PN.N.....................A....L.....................Q..T..TK...KE.I..V.T...

      401                                                                                                500
035E  MHVVATGANINFDKEFNHGLLKGFGVDHTLLKYGINYRHQEAVPPRGIRPGFQNQEKTDAGIYLEAVNQINDFTINTGVRVDRFDFKAMDGKKVGKTDIN
25240 ..................SP.....................K.....SLK..VVH.....................................................
56    ..................SP.....................T.....SLK..VVH.....................................................

      501                                                                                                600
035E  PSFGVIYDVNPNLSVSGNLIYATRSPRFADAILSRGFRDGVVSIADNAKAEKARNTEIGFNYNNGPYTAFGSYFWQRVDNARAT-ADAVQHPTVTTA---
25240 ..................................Y.G..I..D.....................................-..ITR.G.TDANGKP
56    ............................V.......Y.G..I..D..............I.................V.....VT.ISR.G.TDANGKS

      601                                                                                                700
035E  -KITYLGNQGHQTNHGYELGVGYTEGAWRARAGVAHSKPTMHNVKFKANPEYAVRTGRTWTADVAYRLPNPSVELGVRHTLVEGVDAKDTSILSGKFDDK
25240 I.VPT.........Q.................................SG.....................................................I..-----
56    I.VPA..T......Q.....................Y.T.S....TLDG.....A.....................................................

      701                                                                                                768
035E  DGAILNREGYNVSDIYANWKPYGNDKVNVNFAVNNVFNKNYRPHTQRASIDTLPGAGRDFRVGVNFTY
25240 EVSK.....................................................................
56    .....................................................................
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FIG. 3. Alignment of the amino acid sequences of CopB of *M. catarrhalis* O35E, 25240, and 56. Identical amino acids are shown as dots, and gaps are shown by dashes. Amino acid sequences were deduced from the nucleotide sequences. Strain O35E, GenBank accession no. L12346 (Helminen et al. [10]); strain 25240, GenBank accession no. U83900; strain 56, GenBank accession no. U83901.

2.9F, indicating that the two antibodies see different epitopes on CopB. The pattern of strain recognition by the monoclonal antibodies did not follow any geographic pattern and did not correspond to the presence of any of the variable restriction sites.

Sequence comparison of CopB gene. The three strains, O35E, 25240, and 56, are from different geographic areas and have different patterns of reacting to monoclonal antibodies to CopB (Table 1). The deduced CopB amino acid sequences are shown in Fig. 3. There was 92 to 96% homology among the sequences at the nucleotide level. The GenBank accession number for the strain 25240 sequence is U83900, and that for the strain 56 sequence is U83901. At the amino acid level, there was 90 to 95% homology. The variability in the protein sequence is confined mainly to three moderately variable regions (V1, V2, and V3). Four conserved regions, the N- and C-terminal regions, and two internal regions can be delineated (Fig. 4). Three more short variable regions can also be delineated (amino acids 447 to 454, 636 to 649, and 696 to 704). All variable regions are composed predominantly of hydrophilic amino acids.

FrpB is an OMP of pathogenic *Neisseria* spp. with 49 to 52% identity to CopB (4, 20). Sequence variability in the FrpB protein of *Neisseria* has been described mainly in two well-defined regions, the putative loop 5 and loop 7 regions (22). When the FrpB sequences were aligned with the CopB sequences, there was found to be partial overlap between the loop 5 variable region of FrpB and the first variable region (V1) in CopB. There was also partial overlap between the loop 7 variable area of FrpB and the first minor variable region (m1) of CopB (data not shown).

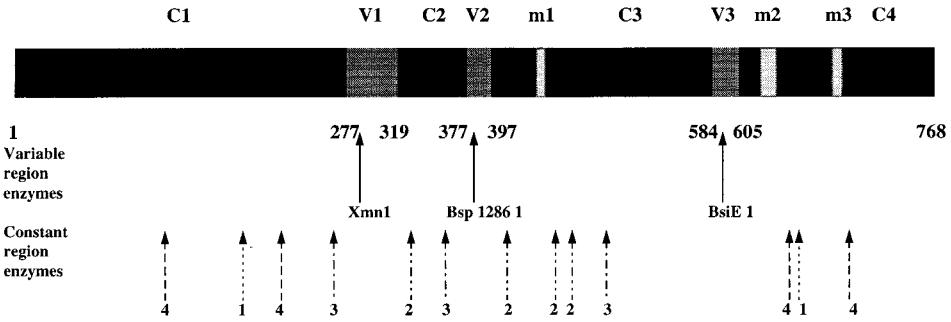

FIG. 4. Diagrammatic representation of the constant and major variable regions in the CopB protein deduced from the DNA sequence. C1 to C4 are the constant regions, and V1 to V3 are the major variable regions. m1 to m3 are the minor variable regions; m1 (amino acid [aa] 447 to 454) lies within C3, and m2 (aa 636 to 649) and m3 (aa 696 to 704) lie within C4. Numbers denote the amino acid residues of the CopB protein. Arrows denote the restriction sites of enzymes used for PCR-RFLP analysis. Numbered constant-region enzymes are as follows: 1, *Bsm*AI; 2, *Hin*cII; 3, *Hga*I; 4, *Bsr*I.

PCR-RFLP analysis. Sequence analysis suggested a pattern of constant and variable regions in the CopB gene. To generalize this observation to other strains of *M. catarrhalis*, we performed PCR-RFLP analysis of 20 strains with seven restriction enzymes (Table 1 and Fig. 4). Restriction sites in the constant regions were highly conserved among the 20 strains, with 239 of the 260 (90%) potential sites present (Table 1). Restriction sites in the variable regions were much less conserved, with 28 of the 60 (47%) potential sites found (Table 1). These observations indicate that the pattern of variable and constant areas in the CopB gene is a general pattern among strains of *M. catarrhalis.*

DISCUSSION

We have shown in our previous studies that the OMP patterns are very similar among strains of *M. catarrhalis* (3). CopB, an 80-kDa major OMP has been consistently present in all strains studied with minor variations in apparent molecular weight. As discussed above, this OMP is a potential vaccine antigen. The antigenic and genetic heterogeneity in this OMP among strains of *M. catarrhalis* is therefore of considerable importance. Antigenic heterogeneity in CopB was elucidated in this study with two IgG monoclonal antibodies. Both antibodies recognize surface-exposed epitopes on this OMP. Immunodot assays show that these epitopes are present in 60 to 70% of strains tested. Therefore, CopB has surface-exposed antigenic determinants which are moderately heterogeneous among strains.

We then elucidated the degree of sequence heterogeneity in CopB. Sequence comparison and PCR-RFLP analysis results indicate that the CopB gene is mostly conserved among strains except for three major well-defined areas of sequence heterogeneity which translate to three variable regions (V1, V2, and V3). These variable regions are composed of predominantly hydrophilic amino acids and are likely to be exposed on the surface of the intact bacterial cell. In view of the strain specificities of monoclonal antibodies 1F5 and 2.9F, it is likely that these antibodies bind to epitopes within these variable regions. However, there is no correlation between the restriction pattern in these variable regions and strain recognition of a monoclonal antibody. This observation does not indicate that the epitopes of these monoclonal antibodies lie outside these regions, as the restriction enzyme site is only a small portion of each variable region. The three minor areas of sequence heterogeneity also present in the CopB gene could represent the binding sites of these antibodies. Further investigation is required to precisely map the binding sites of these monoclonal antibodies.

The precise function of CopB is not known, but it is involved in iron uptake from mucosal surfaces. Campagnari et al. have shown that the expression of CopB increases substantially when the bacterium is grown in iron-deficient media (5). An isogenic CopB mutant shows severe impairment of utilization of iron from transferrin and lactoferrin (1). FrpB is a 76-kDa major OMP protein of *Neisseria gonorrhoeae* which is 52% identical to CopB (4). FrpB is also iron regulated and is speculated to be a TonB-dependent receptor, but its precise physiological role has not been determined. The FrpB protein of *Neisseria meningitidis* shows 49% identity to CopB, but mutation in FrpB does not affect iron uptake by this pathogen (20).

Is CopB an important antigen for the human immune response? It plays a role in mediating resistance to the bactericidal activity of normal human serum (11). The predominant antibody response to chronic lower respiratory tract infection with *M. catarrhalis* in patients with bronchiectasis is to OMP B1, a 84-kDa minor OMP. Serum IgG antibodies to CopB were seen in lower titer in these patients (21). Goldblatt et al. have shown that an approximately 82-kDa OMP is an important antigenic target for IgG antibodies to *M. catarrhalis* in children. Whether this OMP is OMP B1 or CopB is not known (8).

Further studies of CopB should address several important issues. The role of this OMP as an antigen in diverse populations after acute infections with *M. catarrhalis*, i.e., children with otitis media and adults with chronic obstructive pulmonary disease, needs to be further defined. The moderate degree of heterogeneity in this protein could be exploited to develop a typing system for *M. catarrhalis* based on a panel of monoclonal antibodies directed at this OMP. A map of the epitopes on this OMP recognized by an array of monoclonal antibodies will allow us to understand the three-dimensional structure of this protein. The exact role of this OMP in iron uptake also needs to be defined.

We conclude that the CopB is largely conserved among strains of *M. catarrhalis* and contains discrete regions which show moderate heterogeneity among strains. Whether this moderate degree of heterogeneity in this OMP precludes its use as a vaccine antigen needs further study. Further investigation of this OMP may identify epitopes in the conserved regions which are surface exposed and are targets of protective human antibody and which therefore are good vaccine candidates.

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