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Molecular Characterization of the BRO β-Lactamase of *Moraxella* (*Branhamella*) *catarrhalis*

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A rapid increase in the prevalence of β-lactamase-producing *Moraxella* (*Branhamella*) *catarrhalis* strains has been noticed during the last decades. Today, more than 80% of strains isolated worldwide produce β-lactamase. To investigate β-lactamase(s) of *M. catarrhalis* at the molecular level, the BRO-1 β-lactamase gene (*bla*) was isolated as part of a 4,223-bp *Hin*dIII fragment. Sequence analysis indicated that *bla* encodes a polypeptide of 314 amino acid residues. Insertional inactivation of *bla* in *M. catarrhalis* resulted in complete abrogation of β-lactamase production and ampicillin resistance, demonstrating that *bla* is solely responsible for β-lactam resistance. Comparison with other β-lactamases suggested that *M. catarrhalis* β-lactamase is a unique enzyme with conserved residues at the active sites. The presence of a signal sequence for lipoproteins suggested that it is lipid modified at its N terminus. In keeping with this assumption was the observation that 10% of β-lactamase activity was found in the membrane compartment of *M. catarrhalis*. *M. catarrhalis* strains produce two types of β-lactamase, BRO-1 and BRO-2, which differ in their isoelectric points. The BRO-1 and BRO-2 genes from two ATCC strains of *M. catarrhalis* were sequenced, and only one amino acid difference was found between the predicted products. However, there was a 21-bp deletion in the promoter region of the BRO-2 gene, possibly explaining the lower level of production of BRO-2. The G+C content of *bla* (31%) was significantly lower than those of the flanking genes (47 and 50%), and the overall G+C content of the *M. catarrhalis* genome (41%). These results indicate that *bla* was acquired by horizontal gene transfer from another, still unknown species.

Moraxella (*Branhamella*) *catarrhalis* is a gram-negative diplococcus previously considered to be a harmless commensal. However, in recent years it has become evident that this bacterium can indeed be pathogenic. It is the third most common cause of upper respiratory tract infections in children and, particularly in adults with chronic obstructive pulmonary disease, of lower respiratory tract disease (7, 10, 18, 30).

An important feature of *M. catarrhalis* is the production of β-lactamase, which was first reported in 1977 (27, 33). Today, more than 90% of the strains isolated worldwide produce β-lactamase (12, 16, 31, 34). Apart from protecting the producing bacteria against β-lactam antibiotics, the β-lactamase of *M. catarrhalis* can also have indirect pathogenic effects by blocking antibiotic therapy of concomitant infections with more dangerous respiratory pathogens such as pneumococci, as suggested by Wardle (41) and as experimentally confirmed by Hol et al. (21).

The β-lactamases of *M. catarrhalis* can be divided into two closely related types based on isoelectric focusing patterns (40). The commoner pattern has been designated BRO-1 (previously Ravasio type [15]) and can be found in approximately 90% of the β-lactamase-producing strains in both Europe and the United States (16, 31, 34). The remaining 10% has a different isoelectric focusing pattern, referred to as BRO-2. A possible third type (BRO-3), only detectable on isoelectric focusing gels after mild papain treatment of cell extracts, has been described by Christensen et al. (11). However, Steingrube et al. (37) considered this to be a membrane-bound precursor molecule rather than a different enzyme. Isolates producing the BRO-1 enzyme have been shown to be consistently more resistant to ampicillin than strains of the BRO-2 type (16, 26). This difference in MICs correlates well with the observation that BRO-1 is produced at a level two to three times higher than that of BRO-2 (39, 40).

While much is known about the biochemical properties of the BRO enzymes, not much data concerning molecular aspects of the BRO genes are available. Although initial reports suggested the presence of plasmid-encoded BRO-1 (13, 23), it is currently believed that the genes encoding BRO-1 and BRO-2 are located on the chromosome (14, 40). At present, it is unclear whether BRO-1 and BRO-2 are the product of one gene or are encoded by separate genes. We report here the cloning, analysis, and insertional inactivation of the β-lactamase gene from an *M. catarrhalis* patient isolate. Furthermore, the BRO-1 and BRO-2 genes from ATCC strains 43618 and 43617 were sequenced, and differences observed in coding and regulatory regions are discussed. Finally, our results strongly suggest that the BRO genes were acquired by interspecies gene transfer.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *M. catarrhalis* strains used were 78301 (patient isolate, β-lactamase positive), 3.21S (carrier isolate, β-lactamase negative) (22), and ATCC strains 43617 (BRO-2) and 43618 (BRO-1). *Escherichia coli* DH5α was used as the host strain for cloning and further analysis of the β-lactamase gene in plasmid pUK21 (kanamycin resistant [38]). Plasmid pAlter-1 (tetracycline resistant; Promega Corporation, Madison, Wis.) was used for the mutagenesis of the β-lactamase gene.

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Culture conditions. *M. catarrhalis* strains were grown by overnight incubation at 37°C on blood agar plates or in brain heart infusion medium (Difco Laboratories, Detroit, Mich.). *E. coli* strains were cultured in NZ broth or on NZ agar (Bio 101 Inc., La Jolla, Calif.). Antibiotics were added as required at the following concentrations: ampicillin, 10 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml.

DNA techniques. Unless otherwise mentioned, DNA techniques were performed according to the protocols described by Ausubel et al. (3). Electrocompetent *M. catarrhalis* cells were prepared as described by Helminen et al. (20), except that cells were stored at −70°C in 10% glycerol until use. Electroporation was performed with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) set at a field strength of 12.5 kV/cm, with a pulse time of 5 ms (i.e., a resistance value of 200 Ω). The capacity was held constant at 25 μF.

Cloning, mapping, and sequencing of the β-lactamase gene. Chromosomal DNA from *M. catarrhalis* 78301 was digested with *Hin*dIII and was ligated to *Hin*dIII-digested pUK21. The ligation products were transformed to *E. coli*, and clones harboring the *bla* gene were selected for on plates containing both kanamycin and ampicillin. Five recombinants were obtained, all with the same 4.2-kb insert as confirmed by restriction analysis and Southern blotting (not shown). One clone, pMC100, was used for further characterization. To localize the *bla* gene within the *Hin*dIII fragment, subclones with deletions on either side of the *Hin*dIII fragment were generated with the Erase-a-base kit (Promega). DNA sequencing was performed with an ABI 373A DNA sequencer according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, Calif.). Both strands were sequenced with either the M13-sequencing primers or primers deduced from the newly sequenced DNA.

Sequence analysis. For initial similarity searches, DNA and protein sequences were compared with sequences in the GenBank and EMBL libraries by using the BLAST algorithm (1). Further analysis of sequence data was performed with the PC/Gene package (IntelliGenetics Inc., Mountain View, Calif.).

Insertional mutagenesis of the β-lactamase gene. A *Hin*dIII-*Eco*RV fragment containing the *bla* gene (see Fig. 1) was subcloned into the pAlter-1 vector digested with *Hin*dIII and *Sma*I, resulting in plasmid pMC200. Subsequently, a kanamycin cassette was inserted into the unique restriction site *Bst*XI located within the *bla* gene as follows. After digestion of pMC200 with *Bst*XI, the DNA was treated with T4 polymerase to create blunt ends. The kanamycin cassette (1.4 kb) was excised from plasmid pKan (5) by cleavage with *Hin*dII and was subsequently ligated to the linearized pMC200. Recombinants were selected for on tetracycline- and kanamycin-containing plates. One clone, designated pMC200K, was used for further experiments. Proper insertion of the kanamycin cassette into the *Bst*XI site was verified by sequence analysis. The *bla* gene in *M. catarrhalis* 78301 was inactivated as follows. Plasmid pMC200K was linearized by digestion with *Eco*RV, which cuts once in the vector but not in the insert (the *Eco*RV site used to clone the insert containing the *bla* gene was changed into a hybrid *Eco*RV-*Sma*I site and was therefore lost). A 20-μl portion of electrocompetent *M. catarrhalis* cells was mixed with 0.5 to 1.0 μg of linear DNA in 5 μl of distilled water in an electroporation cuvette (Bio-Rad Laboratories). Immediately after electroporation, the cells were added to 1.5 ml of brain heart infusion broth and incubated at 37°C for 6 h. The suspension was then plated on brain heart infusion agar containing kanamycin. Several kanamycin-resistant *M. catarrhalis* clones were obtained, six of which were analyzed by Southern blotting. All *M. catarrhalis* clones were found to be sensitive to tetracycline and did not contain plasmid DNA, indicating that the kanamycin resistance gene was integrated into the chromosome.

Southern blot analysis. Chromosomal DNA was digested with *Hin*dIII, separated on a 1% agarose gel, and transferred to a Hybond N^+ membrane (Amersham, 's-Hertogenbosch, The Netherlands). The probes were purified by gel electrophoresis and were labelled with horseradish peroxidase with the enhanced chemiluminescence (ECL) system (Amersham). Hybridization and detection of hybridizing bands were performed under conditions indicated by the manufacturer of the ECL system (Amersham). A 1-kb DNA ladder (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) was used as a DNA size reference.

Detection of β-lactamase activity. To detect β-lactamase activity, a commercially available chromogenic cephalosporin disk β-lactamase assay was used, containing nitrocefin as substrate (Cefinase disks; BBL Microbiology Systems, Cockeysville, Md.). The disks were examined for a yellow-to-red change as evidence of a positive reaction. The amount of β-lactamase produced was quantitated according to the spectrophotometric method, by measuring the degradation of nitrocefin (25 μM; Oxoid Limited, Basingstoke, Hampshire, United Kingdom) at 486 nm essentially as described by O'Callaghan et al. (32). All measurements were performed at room temperature in phosphate (10 mM)-buffered saline (PBS; pH 7.3) and repeated at least three times. β-Lactamase activities were expressed as changes in absorbance (ΔA) per minute per milligram of total protein present in cell extract (CFE), measured in the linear portion of the hydrolysis curve.

Enzyme preparation and subcellular localization of β-lactamase. Overnight cultures of bacteria were washed once with PBS and resuspended in the same buffer. The suspension was subjected to sonication for fives times each for 1 min at 4°C in a Sonifier B-12 (Branson Sonic Power Co, Danbury, Conn.) sonicator, after which it was centrifuged three times for 15 min at 3,000 × *g* to remove unbroken cells. The total protein content of the CFE obtained in this way was measured by the Bio-Rad assay with bovine serum albumin as a reference and was subsequently adjusted to 50 μg/ml. β-Lactamase activity was determined as described above with 1 ml of CFE at this concentration. For fractionation experiments, the CFE was centrifuged for 60 min at 150,000 × *g* to separate membranes from soluble proteins. The pelleted membrane fraction was washed once with PBS–10% (vol/vol) glycerol before reconstitution to its original volume in PBS. β-Lactamase activities for all fractions were determined as described above.

PCR amplification and sequencing of BRO-1 and BRO-2. Chromosomal DNA (10 ng/μl) of *M. catarrhalis* ATCC 43617 and ATCC 43618 was used for PCR amplification of the BRO-2 and BRO-1 genes, respectively. Amplification was performed in 25-μl reaction volumes containing 5 pmol of each primer, 0.1 U of SuperTth DNA polymerase (HT Biotechnology Ltd, Cambridge, United Kingdom), and standard amounts of amplification reagents (200 μM each deoxynucleoside triphosphate, 50 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100). Primers at positions 1494 (3F; 5′-CTTGGCGATGTCTACACC-3′) and 2958 (3R; 5′-AAGTTTGGCATTGAC ACG-3′) in the *Hin*dIII fragment were used for 25 cycles of amplification (30 s at 95°C, 1 min at 55°C, and 1 min at 72°C) with a ThermoCycler (Perkin-Elmer Cetus, Gouda, The Netherlands). Sequencing of both strands was performed with the same primers as well as with primers located within the amplified product.

Nucleotide sequence accession numbers. The sequences reported in this study are available under EMBL accession numbers Z54180 (BRO-1) and Z54181 (BRO-2).

RESULTS

Isolation and mapping of the *M. catarrhalis* β-lactamase gene. Although the *bla* gene conferred only low levels of ampicillin resistance (MIC for strain 78301, 4 μg/ml) to *M. catarrhalis*, we anticipated that insertion of the gene into a multicopy plasmid would result in significantly higher levels of ampicillin resistance. Indeed, when a gene bank of *M. catarrhalis* 78301, consisting of *Hin*dIII fragments in the multicopy plasmid pUK21, was transformed to *E. coli*, five clones with high levels of ampicillin resistance were obtained (MIC, >50 μg/ml). All clones analyzed contained vector DNA with an apparently identical 4.2-kb *Hin*dIII insert. One of the recombinants, designated pMC100, was used for further analysis. The insert consisting of 4,223 bp was sequenced on both strands. The DNA sequence revealed the presence of three open reading frames (ORFs), all in the same orientation (Fig. 1). To map the β-lactamase gene, deletion derivatives of plasmid pMC100 were generated by exonuclease III treatment and were tested for their ability to confer ampicillin resistance and nitrocefin conversion. Analysis of the deletion derivatives of pMC100 (i.e. pMC097, pMC096, pMC105, and pMC106 [Fig. 1]) indicated that ORF2 constituted the gene coding for the *M. catarrhalis* β-lactamase (designated *bla*). Sequence comparison of the translated ORF2 to other β-lactamases confirmed this assumption. Similarity searches indicated that ORF1 (493 amino acids) was homologous to *Rhodococcus* enantiomer-selective amidase (28) (34.4% identical amino acid residues), whereas ORF3 showed surprising similarity to PET112, a *Saccharomyces cerevisiae* nuclear gene product (29), i.e., 29% identical amino acid residues. It has been suggested that the PET112 protein is involved in the expression of mitochondrial genes at the level of translation. Furthermore, ORF3 showed significant similarity to hypothetical proteins found in *Chlamydia trachomatis* (24) and *Mycoplasma genitalium* (accession number U01799).

Structure of the *bla* gene and its product. The nucleotide sequence of the β-lactamase gene and its deduced amino acid sequence are shown in Fig. 2. The *bla* gene encodes a polypeptide of 314 amino acid residues. A putative ribosome-binding site (AAGGAG) is located 9 bp upstream of the first initiation codon. Putative promoter sequences were found 29 bp upstream of the initiation codon. The potential −10 sequence (AATAAT) begins at position 1732, with a possible −35 sequence (ATGATA) located 17 bp upstream of the −10 sequence, which is a spacing consistent with that described for

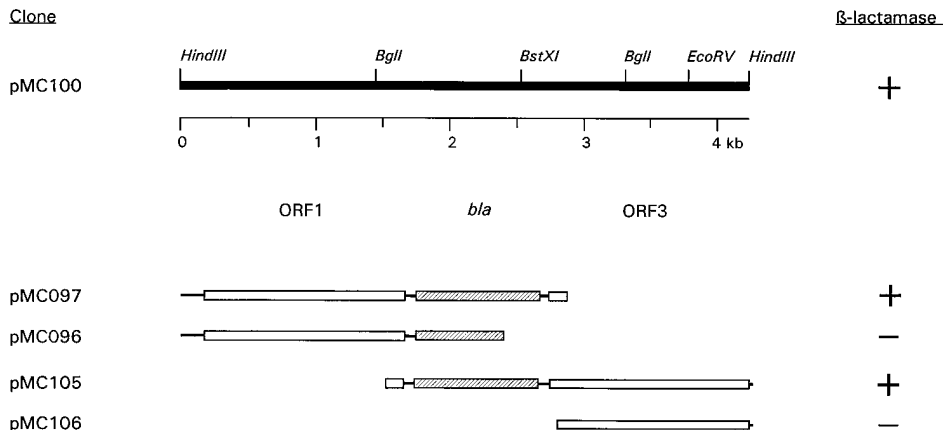

FIG. 1. Physical and genetic map of the *M. catarrhalis Hin*dIII fragment contained in pUK21. Boxes represent ORFs; the β-lactamase gene (*bla*) is indicated by the hatched boxes. The production of β-lactamase by pMC100 and its deletion derivatives (pMC097, pMC096, pMC105, and pMC106) is indicated on the right. Only relevant restriction sites are shown.

E. coli promoters. The overall G+C content of the *bla* gene was 31%, which is significantly lower than those of the flanking ORFs (ORF1, 50.4%; ORF3, 46.8%) and of the reported G+C content (41%) for the entire *M. catarrhalis* genome (10).

The amino acid sequence at positions 24 to 27 (LTGC) resembles the consensus sequence of a prokaryotic membrane lipoprotein lipid attachment site (19), suggesting that the *M. catarrhalis* β-lactamase is a lipoprotein and is membrane bound rather than periplasmic. Taking into account this potential signal peptidase II cleavage site, the proposed mature β-lactamase would have an estimated relative molecular mass (M_r) of 32,504.

The motifs involved in the formation of the active site of serine β-lactamases (17) could all be traced in the predicted amino acid sequence of *bla*. In the ABL numbering scheme for class A β-lactamases (2), the S of the S/XXK motif is at position 70, the S of the (S/Y)DN motif is at position 130, the E of the E/D motif is at position 166, and the K of the (K/H)(T/S)G motif is at position 234. Taking into account the potential signal sequence of 26 amino acids, for *bla* the S of the STIK sequence is at position 65, the S of the SDN motif is at position 127, the E of the E/D motif is at position 162, and the K of the KTG sequence is at position 230 (Fig. 2), which is in good agreement with the ABL numbering mentioned above.

Comparison of the *M. catarrhalis* β-lactamase to other β-lactamases. The nucleotide sequence of the *bla* gene did not show significant sequence similarity to those of any known β-lactamase genes. However, amino acid sequence comparison did reveal a similarity of the *bla* product to other β-lactamases, especially class A β-lactamases. Sequence alignment of *bla* to five other β-lactamases (Fig. 3) revealed the presence of the highly conserved serine active-site motifs at a similar location within the amino acid sequence. The closest similarity of *M. catarrhalis* β-lactamase was found with β-lactamase from *Lysobacter enzymogenes*, a gram-negative, nonpathogenic soil bacterium (6), i.e., 32% identity and 45% overall similarity. The percentages of identical amino acid residues with the other β-lactamases varied between 26 and 29%. No clear indication as to the possible origin of the *M. catarrhalis* β-lactamase could be obtained from these comparisons.

Generation of isogenic *bla* disruption mutants. To confirm that the cloned gene is the only determinant of β-lactamase and ampicillin resistance in *M. catarrhalis*, isogenic mutants were generated by insertional inactivation of the *bla* gene. β-Lactamase-negative mutants were constructed by allelic exchange with a cloned copy of the *bla* gene which was inactivated by the insertion of a kanamycin cassette into the *Bst*XI site (Fig. 1). The inactivated β-lactamase gene was introduced into *M. catarrhalis* 78301 by electroporation. To confirm that

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                                                  -35                -10
1681  TGGTGAAGTGATTTTTGGATTGGGGTGAATGATAAACTTATCCCAAACAATAATAAATTT
        l  v  k  *
                                RBS
1741  ATCCCAAACAATAAAAGGAGATAATGATGCAACGCCGTCATTTTTTACAAAAAACCTTAT
                                          M  M  Q  R  R  H  F  L  Q  K  T  L    12

1801  TGGCACTACCTATTATTTTTTCTGGCAATTTATTAACTGGATGTAAAACGAATTTATCTG
        L  A  L  P  I  I  F  S  G  N  L  L  T  G  C  K  T  N  L  S    32

1861  ATGATTATTTGCCCGATGATAAGATAACAAACAATCCAAATTTATTACAAAATAAATTAA
        D  D  Y  L  P  D  D  K  I  T  N  N  P  N  L  L  Q  N  K  L    52

1921  AAGAGATATTGCCAATTTGGGAAAATAAATTTAATGCCAAAATTGGTATGACGATTATTG
        K  E  I  L  P  I  W  E  N  K  F  N  A  K  I  G  M  T  I  I    72

1981  CTGACAATGGTGAACTATCCAGTCATCGTGGTAATGAATATTTTCCTGTTAATAGTACCA
        A  D  N  G  E  L  S  S  H  R  G  N  E  Y  F  P  V  N  S  T    92

2041  TTAAAGCCTTTATTGCAAGTCATATATTATTACTTGTAGATAAAGAAAAATTGGATTTAA
        I  K  A  F  I  A  S  H  I  L  L  L  V  D  K  E  K  L  D  L   112

2101  ACGAAAAAATCATCATTAAAGAAAGCGATTTGATAGAATATTCTCCTGTCTGTAAAAAAT
        N  E  K  I  I  I  K  E  S  D  L  I  E  Y  S  P  V  C  K  K   132

2161  ACTTTGATGAGAATAAACCAATTTCTATTAGTGAATTGTGCGAAGCTACCATAACACTGA
        Y  F  D  E  N  K  P  I  S  I  S  E  L  C  E  A  T  I  T  L   152

2221  GTGATAATGGTTCTGCTAATATCTTGTTGGATAAAATTGGGGGTTTGACTGCATTCAATC
        S  D  N  G  S  A  N  I  L  L  D  K  I  G  G  L  T  A  F  N   172

2281  AATTTTTGAAAGAGATTGGGGCGGATATGGCGCTGGCAAATAATGAGCCTTTATTAAATC
        Q  F  L  K  E  I  G  A  D  M  A  L  A  N  N  E  P  L  L  N   192

2341  GCTCACATTATGGTGAAACCAGTGATACCGCAAAACCAATTCCTTACACAAAAAGCCTAA
        R  S  H  Y  G  E  T  S  D  T  A  K  P  I  P  Y  T  K  S  L   212

2401  AAGCACTGATTGTAGGCAATATCCTATCCAATCAAAGCAAAGAACAGTTGATAACTTGGC
        K  A  L  I  V  G  N  I  L  S  N  Q  S  K  E  Q  L  I  T  W   232

2461  TTATCAATGATAAAGTTGCTGATAATTTATTGAGAAAAATATTTACCAAAAAATTGGCGAA
        L  I  N  D  K  V  A  D  N  L  L  R  K  Y  L  P  K  N  W  R   252

2521  TTGGCGACAAAACAGGCACAGGTAGTGAATCAAAAAATATCATTGCTGTGATTTGGAATG
        I  G  D  K  T  G  T  G  S  E  S  K  N  I  I  A  V  I  W  N   272

2581  AAAATAATAAACCTTATTTTATCAGCCTATTTATCACCCAGCCCCATGATGGTAAATCCC
        E  N  N  K  P  Y  F  I  S  L  F  I  T  Q  P  H  D  G  K  S   292

2641  TTGATTTTAAAAATCAAAAAGATGAAATAATGGCACAAATTGGTAAAGAAATTTATCCAT
        L  D  F  K  N  Q  K  D  E  I  M  A  Q  I  G  K  E  I  Y  P   312

2701  TTTTATAATCCATTAAATTTTTATAGTGAATAGAAAAATTTATGACCCAACAAGCCCCCC
        F  L  *                                          m  t  q  q  a  p   314
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FIG. 2. DNA sequence of the *M. catarrhalis* β-lactamase gene and the predicted amino acid sequence of its product. Nucleotide numbering is according to the position in the 4,223-bp *Hin*dIII insert and is indicated on the left. Amino acid numbering is presented on the right. The putative ribosome binding site (RBS) and promoter sequences (−10 and −35) are underlined. The stop codon is marked by an asterisk. The potential lipid attachment site (amino acids 24 to 27) is indicated by boldface letters, and the signal peptidase II cleavage site is indicated by an arrow. The serine active-site motifs SXXK, (S/Y)XN, E/D, and (K/H)(T/S)G as defined by Ghuysen (17) are shown in boldface type and are underlined. The amino acid residues constituting the end of ORF1 and the start of ORF3, respectively, depicted at the beginning and end of the sequence, are indicated by small letters.

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Mcbla    MMQRRHFLQKTLLALPIIFSGNLLTGCKTNLSDDYLPDDKITNNPNLLQN    50
Lebla     ....A...C.GSL.LAG-GAVA------SFGAAA.SPKP---AASADAA    39
Rcbla     .R---.---.ATV.SRV-ATG.------A.GLSMATASL---AETPVEA    33
CARB-3    .LYK---MCDNQNYGVTYMKF.-------.AFSL.IPSVVFASSSKF.Q    39
BlaP      .NV.---.HKASFFSVV-ITF.------C.TLSL--------.A.ATDS    31
SHV-1                                                 SPQ      3
           *

Mcbla    KLKEILPIWENKFNAKIGMTIIADN-GE-LSSHRGNEYFPVNSTIKAFIA    98
Lebla    LRARLAEL-.KRSGGRL.VSALDTAD.RRFDV-..D.R.AMC..F.FLL.    87
Rcbla    LSETVAR.-.EQLG.RV.LSLMETGT.WSW.H-.ED.L.LM...V.VPVC    81
CARB-3   VEQDVKA.-.VSLS.R..VSVLDTQN..YWD-YN..QR..LT..F.TIAC    87
BlaP     V.EAVTNA-.TELG.R..LAAHDLET.KRWE-.KS..R..LS..F.TLAC    79
SHV-1    P.EQ.KLS-.SQLSGRV..IEMDLAS.RT.TAWRAD.R..MM..F.VVLC    52
                  *       *       *       *   ** *

Mcbla    SHILLLVDKEKLDLNEKIIIKESDLIEYSPVCKKYFDENK-PISISELCE   147
Lebla    AAV.RKA.LGE..MQQRLAVPK.A..SH..ITT-PIA.AGGSA.LAQL..   136
Rcbla    GA..ARW.AGR.S.SDALPVRKA..VP.A..TE---TRVGGNMTLDEL.L   128
CARB-3   AKL.YDAEQG.VNP.STVE..KA..VT....IE.QVGQ-A--.TLDDA.F   134
BlaP     ANV.QR..LG.ERIDRVVRFS..N.VT....TE.HVGKKG--M.LA...Q   127
SHV-1    GAV.AR..AGDEQ.ER..HYRQQD.VD....SE.HLAD-G--MTVG...A    99
            *                   *    *                  *

Mcbla    ATITLSDNGSANILLDKIGGLTAFNQFLKEIGADMA-LANNEPLLNRSHY   196
Lebla    ..M.I...AA..L..PLV.NPAGLTR.LRG..DKVTR.DR...E..TALP   186
Rcbla    .A.DM...VA....IGHL..PE.VT...RSV.DPTSR.DRI..K..DFAS   178
CARB-3   ..M.T...TA...I.SAV..PKGVTD..RQ..DKETR.DRI..D..EGKL   184
BlaP     ..LST...SA..FI.QA...PK.LTK..RS..D.TTR.DRW..E..EAVP   177
SHV-1    .A..M...SA..L..TAV..PAGLTA..RQ..DNVTR.DRW.TE..EALP   149
         *    ***  **       *       *     *    *  **

Mcbla    GETSDTAKPIPYTKSLKALIVGNILSNQSKEQLITWLINDKVADNLLRKY   246
Lebla    .DPR..TS.NAMLA.MRT.LL.DA..AA.RR..TD...ANQTG.KR..AG   236
Rcbla    .DER..TS.AAMSET.R..LL.DV..PEARGK.AE.MRHGG.TGA...AE   228
CARB-3   .DLR..TT.KAIAST.NK.LF.SA..EMNQKK.ES.MV.NQ.TG....SV   234
BlaP     .DKR..TT..AMVTT.EK.LIDET..IK.RQ..ES..KGNE.G.A.F..G   227
SHV-1    .DAR..TT.ASMAAT.RK.LTSQR..AR.QR..LQ.MVD.R..GP.I.SV   199
         *   **  *         *     **      *  *         *

Mcbla    LPKNWRIGDKTGTGS-ESKNIIAVIWNENNKPYFISLFITQPHDGKSLDF   295
Lebla    ...S........S.N-GTT.D..I..PQGR..V..TTYL..AQGEDD---   282
Rcbla    AEDA.L.L..S.S..-HTR.LV...QP.GGA.WIATM..SDTDAEFE---   274
CARB-3   ..AG.N.A.RS.A.GFGARS.T..V.S.HQA.IIV.IYLA.TQASMA---   281
BlaP     V.SD.IVA.R..A.GYG.RA.T..M.PP.R..IVAA.Y..ETDASFE---   274
SHV-1    ..AG.F.A....A.ERGARG.V.LLGPN.KAERIVVIYLRDTPASMA---   246
            *   *  * *       *

Mcbla    KNQKDEIMAQIGKEIYPFL          314
Lebla    --ARSA.L.DAARALAQHWIGAA      303
Rcbla    --VRN.ALKDL.RAVVA--VVRE      293
CARB-3   --ERNDAIVK..HS.FD-VYTSQSR    303
BlaP     --ERNAVI.K..EQIAKTVLMENSRN   298
SHV-1    --ERNQQI.G..AA-----LIEHWQR   265
```

FIG. 3. Alignment of the *M. catarrhalis* β-lactamase with other β-lactamases. Amino acid residues conserved in all sequences are indicated by asterisks. Sequence identity with *M. catarrhalis* β-lactamase is represented by dots. Dashes indicate gaps introduced to maximize alignment. The serine active-site motifs are shown in boldface type and are underlined in the *bla* sequence. Mcbla, *M. catarrhalis* β-lactamase; Lebla, *L. enzymogenes* β-lactamase (6); Rcbla, *Rhodobacter capsulatus* β-lactamase (9); CARB-3, *Pseudomonas aeruginosa* β-lactamase (25); BlaP, *Proteus mirabilis* β-lactamase (35); SHV-1, *Klebsiella pneumoniae* β-lactamase (4).

the proper allelic exchange had occurred, Southern blot analysis of the resulting kanamycin-resistant *M. catarrhalis* clones was performed. Chromosomal DNAs from the wild-type *M. catarrhalis* strain 78301 and from two mutant derivatives were digested with *Hin*dIII and were probed with the kanamycin cassette. Since the *bla* gene is located on a 4,223-bp *Hin*dIII fragment and the kanamycin cassette (1,400 bp) contains an additional *Hin*dIII site, two DNA fragments (2,200 and 3,400 bp) were expected to hybridize with the kanamycin probe. Indeed, these bands were observed in the mutant strains, whereas no hybridizing bands were detected in the parental strain (Fig. 4, left panel). When these same *Hin*dIII digests were probed with a 1,900-bp *Bgl*I fragment containing the *bla* gene (Fig. 1), the same bands hybridized in the mutant strains and the expected 4.2-kb *Hin*dIII band reacted in the wild-type strain (Fig. 4, right panel). These results suggest that an allelic exchange has occurred in the mutant strains; the mutated *bla* gene containing the 1.4-kb kanamycin cassette replaces the wild-type allele.

β-Lactamase production by the wild-type and *bla* mutant strains. To determine whether the wild-type strain 78301 was able to produce more than one β-lactamase, β-lactamase production by the mutant derivatives was measured with a quantitative spectrophotometric assay. CFEs of wild-type strain 78301, two mutant strains, and one strain which did not contain

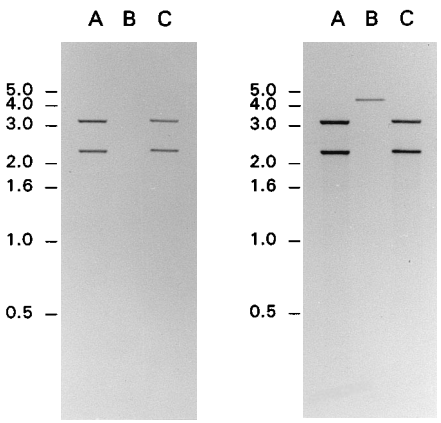

FIG. 4. Southern blot analysis of wild-type and mutant strains. *Hin*dIII-digested chromosomal DNAs from *M. catarrhalis* wild-type strain 78301 (lane B) and two isogenic mutants (lanes A and C) were probed with either the kanamycin cassette (left panel) or a 1.9-kb *Bgl*I-fragment containing the *bla* gene (right panel). Molecular size markers in kilobases are indicated to the left.

the *bla* gene (3.21S) were prepared by sonication. The β-lactamase activities observed in these extracts are listed in Table 1, clearly showing the absence of any β-lactamase production in the mutant strains and the isolate (3.21S) lacking the *bla* gene. Even when a 20× excess of CFE was used, no β-lactamase activity could be observed in these strains. In contrast, the wild-type strain 78301 showed a high level of β-lactamase activity. Therefore, it appears that *M. catarrhalis* 78301 contains only β-lactamase determinant.

Subcellular localization of β-lactamase activity. As mentioned before, a putative prokaryotic lipoprotein lipid attachment site was observed in the amino acid sequence of *M. catarrhalis* β-lactamase. Such sequences are lipid modified and recognized by a distinct signal peptidase (i.e., type II). Furthermore, lipoproteins are membrane associated through their lipid moiety. To determine the subcellular location of *M. catarrhalis* β-lactamase, we purified the membrane fraction from the CFEs of three strains. Apart from *M. catarrhalis* 78301, two *E. coli* strains were used: one harboring the *M. catarrhalis bla* gene on plasmid pMC100 and one with the gene for the periplasmic *E. coli* TEM enzyme on plasmid pUC18. In *E. coli*, 44% of *M. catarrhalis* β-lactamase appeared to be membrane bound (Fig. 5, upper panel; Table 2), in contrast to the periplasmic TEM enzyme, of which only a negligible amount (0.2%) was detected in the membrane fraction (Fig. 5, middle panel; Table 2). In *M. catarrhalis* (Fig. 5, lower panel; Table 2), 10% of β-lactamase was found to be associated with the mem-

TABLE 1. β-Lactamase production by *M. catarrhalis* strains and knockout mutants

Strain	β-Lactamase type	β-Lactamase activity[a]	% of BRO-1 activity[b]
78301	BRO-1	0.83 ± 0.06	93
bla mutant 1	Knock out	Not detectable	Not detectable[c]
bla mutant 6	Knock out	Not detectable	Not detectable[c]
3.21S	None	Not detectable	Not detectable[c]
ATCC 43618	BRO-1	0.89 ± 0.22	100
ATCC 43617	BRO-2	0.27 ± 0.04	30

[a] Expressed as ΔA per minute per milligram of protein ± standard errors of the means.
[b] Relative to strain ATCC 43618.
[c] <0.1%.

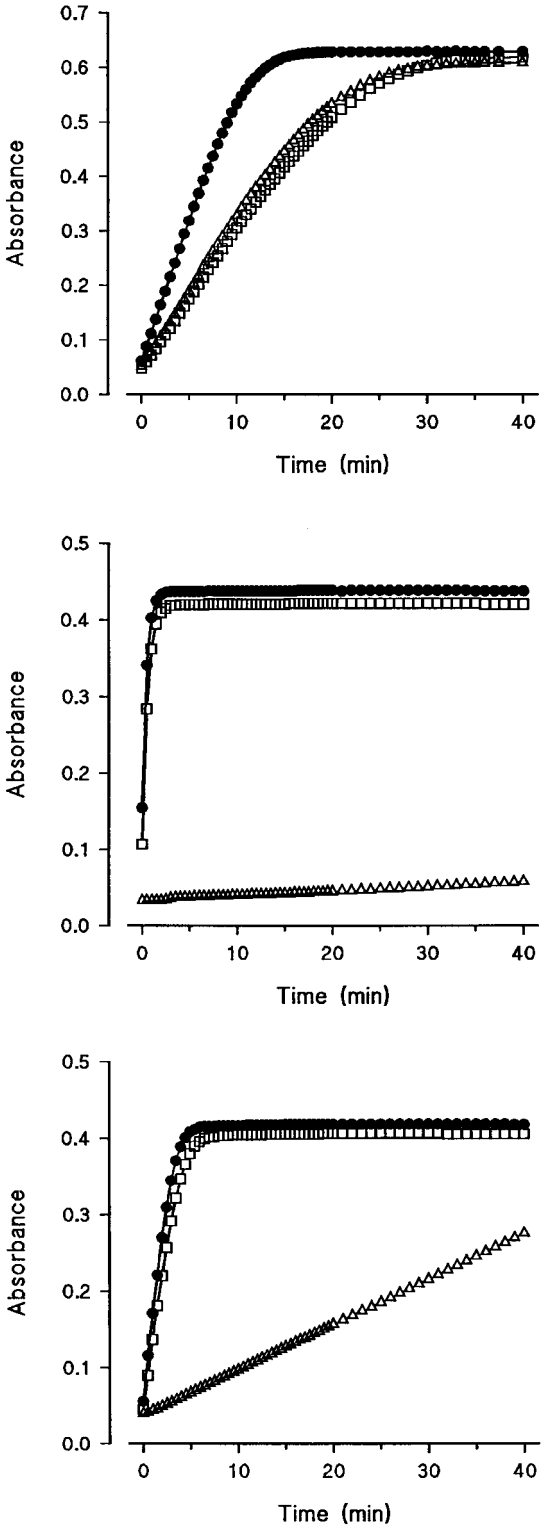

FIG. 5. β-Lactamase activity in subcellular fractions. CFEs (closed circles) from *E. coli* harboring either pMC100 (upper panel) or pUC18 (middle panel) and from *M. catarrhalis* 78301 (lower panel) were fractionated into membranes (open triangles) and soluble proteins (open squares) by ultracentrifugation. Nitrocefin conversion was measured for 40 min at 486 nm.

TABLE 2. β-Lactamase activities in subcellular fractions

Strain	Fraction	β-Lactamase activity[a]	% activity of CFE
M. catarrhalis 78301	Cell extract	0.83 ± 0.06	100
	Membranes	0.08 ± 0.02	10
	Soluble proteins	0.60 ± 0.09	73
E. coli pMC100	Cell extract	3.1 ± 0.4	100
	Membranes	1.4 ± 0.1	44
	Soluble proteins	1.8 ± 0.4	60
E. coli pUC18	Cell extract	31 ± 1.9	100
	Membranes	0.07 ± 0.02	0.21
	Soluble proteins	31 ± 2.6	97

[a] Expressed as ΔA per minute per milligram of protein ± standard error of the means.

brane fraction. Therefore, it appears that the mature *M. catarrhalis* β-lactamase is, at least partly, produced as a membrane-bound protein, suggesting that it is lipid modified.

Comparison of the BRO-1 and BRO-2 β-lactamase genes. Two types of β-lactamase, designated BRO-1 and BRO-2, have been described for *M. catarrhalis*. They differ in their isoelectric focusing patterns (40). In addition, the BRO-1 enzyme is produced at levels higher than those for the BRO-2 enzyme (39, 40). To determine the molecular basis of these differences, we sequenced the *bla* gene derived from two ATCC strains (43618 and 43617, respectively) producing either BRO-1 or BRO-2 β-lactamase. PCR amplification of the BRO-1 and BRO-2 genes, with primers flanking the *bla* gene derived from strain 78301, yielded a product of the expected size (1.4 kb) from both ATCC strains. Both strands of the two products were sequenced and compared with each other and to the gene cloned from strain 78301. The sequence of the BRO-1 gene (strain 43618) appeared to be the same as that of the cloned β-lactamase gene (Fig. 2). When the coding sequence of BRO-1 and BRO-2 were compared, five base differences were observed (not shown). However, only one (an A→G substitution at position 2644 in Fig. 2) resulted in a difference in amino acid composition between BRO-1 and BRO-2: a glycine instead of an aspartic acid residue at position 294. Alignment of the upstream regions of both BRO genes, however, did show some major differences (Fig. 6). First, a base change in the putative ribosome-binding site was observed. The most prominent differences were the two substitutions of five and four consecutive bases and the 21-bp deletion ob-

```
BRO-1   (1551)   CCTGTGGGACAAGCAAACGGCTTACCTGTGGGCTTACAGC
BRO-2            ..............G.........G.........A..G...T

BRO-1   (1591)   TCATCGGCAAACATTGGGCGGAAAGCGAGCTGTTAAAAAC
BRO-2            .G..T.....G.....................A.........

BRO-1   (1631)   CGCTCACATTTACCAAAGCCATACCGATTTTCATCAGGCA
BRO-2            ......T.....T..GGCGA.......G.........A.AG

BRO-1   (1671)   AAGGCGGATTTGGTGAAGTGATTTTTGGATTGGGGTGAAT
BRO-2            .T.........A..............GAAC.....A.A...
                                          -35

                 -35                    -10
BRO-1   (1711)   GATAAACTTATCCCAAACAATAATAAATTTATCCCAAACA
BRO-2            .----------------------.....------.......
                                                -10
                    RBS
BRO-1   (1751)   ATAAAAGGAGATAATGATGCAACGCCGTCATTTTTTACAA
BRO-2            ........G.....................................
                              M  M  Q  R  R  H  F  L  Q
```

FIG. 6. Comparison of the putative promoter regions of the BRO-1 and BRO-2 genes. The upstream region of the two BRO genes as well as the sequence coding for the first nine amino acids, is aligned. The dashed line indicates the 21-bp deletion in the upstream region of the BRO-2 gene. The putative ribosome-binding site (RBS) and promoter sequences (−10 and −35) of BRO-1 are shown in boldface type. Potential promoter sequences for BRO-2 are underlined. The numbering of the BRO-1 sequence corresponds to that in Fig. 2.

served 31 bp upstream of the BRO-2 gene. This deletion strongly affects the putative promoter sequences (−10 and −35) indicated for BRO-1. However, within the upstream sequence of the BRO-2 gene, other possible promoter sequences could be identified (Fig. 6). All of these variations might contribute to the lower level of production of the BRO-2 enzyme. To confirm that the BRO-1 and BRO-2 enzymes were produced at different levels, CFEs of ATCC strains 43617 and 43618 were prepared and β-lactamase activities were measured. The results (Table 1) clearly demonstrated that BRO-1 was produced in much higher quantities than BRO-2.

DISCUSSION

The β-lactamase (*bla*) gene of *M. catarrhalis* was cloned and found to be localized on a 4.2-kb *Hin*dIII fragment, flanked by genes sharing sequence similarity with *Rhodococcus* enantiomer-selective amidase (ORF1) and, surprisingly, with *S. cerevisiae* nuclear gene product PET112 (ORF3), which is thought to be involved in the expression of mitochondrial genes. The *bla* gene consists of 945 bp, potentially encoding a polypeptide of 314 amino acids. This gene seems to be controlled by its own promoter in *E. coli*, since β-lactamase production was detected from clones with inserts in either orientation (not shown). Promoter sequences consistent with consensus sequences of *E. coli* promoters were found in the upstream region of the *bla* gene. The product of the *bla* gene contains the consensus sequence of bacterial lipoproteins which is recognized by signal peptidase II and is modified by the attachment of a lipid group to the cysteine residue. The unmodified, mature protein of 288 amino acids has a predicted molecular mass of 32,504. Yokota et al. (42) reported an M_r of 33,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for *M. catarrhalis* β-lactamase purified from crude cell extract, while Eliasson et al. (14) reported M_rs of 28,000 for papain-solubilized BRO-1 and BRO-2, which were also determined by SDS-PAGE. Moreover, values ranging from 36,500 to 41,000 based on gel filtration of bands isolated from isoelectric focusing gels have been described elsewhere (36). This heterogeneity in molecular mass might be explained by the occurrence of unmodified and lipid-modified forms of the β-lactamase.

The presence of a consensus sequence characteristic for gram-negative lipoproteins suggests that *M. catarrhalis* β-lactamase is produced as a membrane-bound enzyme. Fractionation of the β-lactamase activity (Fig. 5; Table 2) showed that the enzyme is at least partly (10%) associated with membranes in *M. catarrhalis*. When expressed in *E. coli*(pMC100), the association of *M. catarrhalis* β-lactamase to membranes is even more prominent; about half of the enzyme activity was found in the membrane fraction. The observation that not all of the β-lactamase activity is associated with the membrane fraction may be due to proteolytic removal of the lipid group. This processing step may be more prominent in *M. catarrhalis* than in *E. coli*, explaining why a smaller fraction of enzyme activity is associated with membranes in the former. Definite proof that *M. catarrhalis* β-lactamase is a lipoprotein can be obtained only from experiments showing that it actually contains lipids. To our knowledge, lipid-modified β-lactamases have been described only for gram-positive organisms.

The G+C content (31%) of the *bla* gene is unusually low compared with the overall G+C content (41%) for *M. catarrhalis*, and even more so compared with those of the two ORFs flanking the *bla* gene (50.4 and 46.8%). This could mean that *M. catarrhalis* has acquired its β-lactamase gene by horizontal transfer from another species. Comparison of the nucleotide sequence to sequences present in the data base, however, did not reveal significant similarity with any known β-lactamase genes. For serine active-site β-lactamases of class A, four peptide motifs have been defined that are involved in the formation of the active site (17). The predicted protein sequence of *M. catarrhalis* β-lactamase conforms in every respect to these consensus motifs and their distribution in penicillin-interacting proteins (Fig. 3). Comparison of the amino acid sequence of *M. catarrhalis* β-lactamase did reveal similarity with other β-lactamases, especially with those of molecular class A. The highest degree of identity obtained was with *L. enzymogenes* β-lactamase (6), i.e., 32.3% identical amino acid residues. Remarkably, the genes encoding these β-lactamases must be quite different as judged by their G+C contents: 31% for *bla* compared with 71.5% for the *L. enzymogenes* β-lactamase. In a recent classification scheme based on functional characteristics described by Bush et al. (8), *M. catarrhalis* β-lactamases were assigned to group 2c, which are carbenicillin-hydrolyzing β-lactamases inhibited by clavulanic acid. However, not all of the β-lactamases presented in Fig. 3 were placed in this group. Therefore, although the enzymes with highest similarity to *bla* all belong to the same molecular class, the amino acid comparison does not seem to correlate with the functional classification.

The insertion of a kanamycin cassette into the *bla* gene of *M. catarrhalis* 78301 resulted in a complete abrogation of β-lactamase production (Table 1). In fact, the level of nitrocefin degradation and ampicillin resistance (MIC, <0.5 μg/ml) of the mutant strains was similar to that of a strain which did not contain the *bla* gene. Therefore, the gene described in this paper does indeed encode the only detectable *M. catarrhalis* β-lactamase.

Two types of β-lactamase, BRO-1 and BRO-2, have been described for *M. catarrhalis* (40). They can be distinguished by their isoelectric focusing patterns and by their levels of production. To study the molecular basis for these differences, we sequenced the β-lactamase genes from two ATCC strains known to produce either BRO-1 or BRO-2. The genes coding for BRO-1 and BRO-2 displayed only minor differences in the coding sequence. Most of the nucleotide replacements were silent, with only one mutation (A→G) resulting in an amino acid substitution; BRO-2 contains a Gly residue instead of Asp at position 294 (Fig. 2). Eliasson et al. (14) have described distinctive isoelectric focusing patterns for papain-solubilized enzymes with main bands of pI 6.5 for BRO-1 and pI 6.9 for BRO-2. These differences could be explained by the amino acid substitution observed: replacement of an acidic amino acid residue (aspartic acid for BRO-1) by a neutral one (glycine for BRO-2). More pronounced sequence diversity was observed in the upstream region of the two genes (Fig. 6). The 21-bp deletion in the BRO-2 promoter region is the one most likely to have a notable effect on promoter activity, affecting both the potential −10 and the −35 sequence of BRO-1. However, other putative promoter sequences could be recognized in the BRO-2 upstream region. As suggested by Wallace et al. (39, 40) and experimentally confirmed by us (Table 1), BRO-2 is produced in amounts lower than those of BRO-1. On the basis of the results presented in this paper, it seems that the differences in MICs and production levels observed for BRO-1 and BRO-2 must be explained by a variation in promoter sequence, while the enzymes themselves are more or less similar in composition and activity.

One intriguing question as to the origin of the β-lactamase of *M. catarrhalis* remains, especially since the G+C content of the gene is not typical for this species. Only since the late 1970s have β-lactamase-producing strains been reported, while today the majority of the *M. catarrhalis* strains isolated are β-lacta-

mase positive. We found no evidence for a transposon-encoded β-lactamase, suggesting that *bla* was acquired through natural transformation. The rapid rise in β-lactamase-positive strains might be explained by the acquisition of the *bla* gene by horizontal gene transfer by a single progenitor strain, which is followed by clonal expansion of this strain, having an advantage over ampicillin-susceptible strains. Alternatively, many different strains may have acquired the *bla* gene more or less simultaneously. We are currently investigating these two hypotheses.

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