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Molecular and Biochemical Heterogeneity of Class B Carbapenem-Hydrolyzing β-Lactamases in *Chryseobacterium meningosepticum*

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Although the carbapenem-hydrolyzing β-lactamase (CHβL) BlaB-1 is known to be in *Chryseobacterium meningosepticum* NCTC 10585, a second CHβL gene, bla_{GOB-1}, was cloned from another *C. meningosepticum* clinical isolate (PINT). The G+C content of bla_{GOB-1} (36%) indicated the likely chromosomal origin of this gene. Its expression in *Escherichia coli* DH10B yields a mature CHβL with a pI of 8.7 and a relative molecular mass of 28.2 kDa. In *E. coli*, GOB-1 conferred resistance to narrow-spectrum cephalosporins and reduced susceptibility to ureidopenicillins, broad-spectrum cephalosporins, and carbapenems. GOB-1 had a broad-spectrum hydrolysis profile including penicillins and cephalosporins (but not aztreonam). The catalytic efficiency for meropenem was higher than for imipenem. GOB-1 had low amino acid identity with the class B CHβLs, sharing 18% with the closest, L-1 from *Stenotrophomonas maltophilia*, and only 11% with BlaB-1. Most of the conserved amino acids that may be involved in the active site of CHβLs (His-101, Asp-103, His-162, and His-225) were identified in GOB-1. Sequence heterogeneity was found for GOB-1-like and BlaB-1-like β-lactamases, having 90 to 100% and 86 to 100% amino acid identity, respectively, among 10 unrelated *C. meningosepticum* isolates. Each isolate had a GOB-1-like and a BlaB-1-like gene. The same combination of GOB-1-like and BlaB-1-like β-lactamases was not found in two different isolates. *C. meningosepticum* is a bacterial species with two types of unrelated chromosome-borne class B CHβLs that can be expressed in *E. coli* and, thus, may represent a clinical threat if spread in gram-negative aerobes.

Chryseobacterium meningosepticum is the most clinically important human pathogen among the *Chryseobacterium* and *Flavobacterium* genera. It is responsible for neonatal meningitis, with a mortality of up to 50% (17). *C. meningosepticum* is also found in pneumonia (J. Fujita, Y. Hata, and S. Irino, Letter, Lancet **335:**544, 1990) and endocarditis (7, 52) in immunocompromised patients.

C. meningosepticum (formerly known as *Flavobacterium meningosepticum*) belonged to the *Flavobacterium* genus until 1994. Since then, it has been reclassified and belongs now to the *Chryseobacterium* genus, like *Chryseobacterium indologenes* and *Chryseobacterium gleum* (56).

C. meningosepticum is naturally resistant to most β-lactams, including carbapenems (16). A carbapenem-hydrolyzing β-lactamase, (CHβL) BlaB (BlaB-1), from *C. meningosepticum* NCTC 10585 (CIP 6058) has been described (46). This enzyme belongs to the Ambler class B metallo-β-lactamase group (2), with a broad substrate profile, a relative molecular mass of 26 kDa, and a pI value of 8.5 (46). Recently, in the same species, Ambler class A extended-spectrum β-lactamases have also been characterized (6, 45). These extended-spectrum β-lactamases are inhibited by clavulanic acid, cefoxitin, moxalactam, and imipenem, and their substrate profile does not include carbapenems.

Metalloenzymes usually have a broad spectrum of hydrolysis, except for CphA-1 from *Aeromonas hydrophila* (31, 49), and are resistant to clinically available β-lactamase inhibitors (8). Within the last few years, metallo-β-lactamases IMP-1, VIM-1, and VIM-2 have been identified as chromosome, plasmid, and/or integron located in several pathogens, such as *Acinetobacter baumannii* (14), *Alcaligenes xylosoxydans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (3, 21, 22, 26, 30, 36, 42). IMP-1 is widespread in Japan (50, 51). The origin of these CHβLs remains, however, unknown.

Our preliminary experiment using isoelectric focusing (IEF) electrophoresis revealed a heterogeneity of pI values in *C. meningosepticum* isolates. Thus, characterization of the β-lactamase content of *C. meningosepticum* initiated with the class A ESBLs was continued (6). We report the molecular and biochemical characterization of the CHβL GOB-1 that was weakly related to any class B CHβLs, including BlaB-1. Additionally, sequence analysis of the CHβL genes of 10 *C. meningosepticum* isolates revealed that each isolate possessed a combination of both types of CHβLs. A combination of two naturally occurring CHβL genes in the same bacterial species had not been reported previously.

MATERIALS AND METHODS

Bacterial strains. *C. meningosepticum* PINT was isolated at the Raymond Poincaré hospital (Garches, France). *C. meningosepticum* AMA and GEO were isolated at the Bicêtre hospital (Le Kremlin-Bicêtre, France), both from tracheoalveolar aspirations. *C. meningosepticum* AB1572 and H01J100 were from Brita Bruun (11), and reference strains *C. meningosepticum* CIP 6057 (NCTC 10016), CIP 6058 (NCTC 10585), CIP 6059 (NCTC 10586), CIP 7830 (NCTC 11305), and CIP 79.5 (NCTC 11306) were from the Pasteur Institute (Paris, France). The *C. meningosepticum* isolates and reference strains were epidemiologically unrelated (data not shown).

Escherichia coli DH10B and rifampin-resistant *E. coli* JM109 were used for cloning and conjugation assays, respectively, and have been described previously (40, 41). *C. meningosepticum* isolates were identified as previously described (6, 39, 56). All strains were stored at −70°C in Trypticase soy (TS) broth supplemented with 15% glycerol until testing.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant genotype or phenotype	Source or reference
pBK-CMV phagemid	Neomycin and kanamycin resistant	Stratagene
pPCRScript Cam SK	Chloramphenicol resistant	Stratagene
pBS2	A 2.4-kb DNA fragment from *C. meningosepticum* PINT that contained bla_{GOB-1} in the *Bam*HI site of pBK-CMV	This study
pBS3	Entire bla_{GOB-1} gene in the *Srf*I site of pPCRScript Cam SK	This study
pBS4	Entire bla_{BlaB-1} in the *Srf*I site of pPCRScript Cam SK	This study

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study have been described (41). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur) with an inoculum of 10^4 CFU per spot (34). The plates were incubated at 35°C for 18 h before MIC determinations were performed as previously described (34).

Cloning experiments, PCR amplifications, and recombinant plasmids. Genomic DNAs were extracted as described previously (35). Fragments from *Sau*3AI partially digested genomic DNA from *C. meningosepticum* PINT were cloned in pBK-CMV phagemid (Stratagene, Ozyme, Amsterdam, The Netherlands) (Table 1) and expressed in *E. coli* DH10B as previously described (35). Antibiotic-resistant colonies were selected onto amoxicillin (30 μg/ml) and kanamycin (30 μg/ml) containing TS agar plates.

Recombinant plasmid DNA was obtained from 100-ml TS broth cultures grown overnight in the presence of amoxicillin (30 μg/ml) at 37°C. Plasmid DNAs were recovered by using Qiagen columns (Qiagen, Courtaboeuf, France) before restriction digest analyses.

16S rDNA fragments were amplified by PCR using the universal 16S RNA primers 5′-AGAGTTTGATCHTGGYTYAGA-3′ and 5′-ACGGYTACCTTG TTACGACTTC-3′, where Y is C or T and H is A, C, or T (4), and genomic DNAs of *C. meningosepticum* isolates as the template. Primers used to amplify bla_{GOB-1}-like genes were primer 1 (5′-GCTATGAGAAATTTTGCTACACTG-3′) or primer 3 (5′-GGAGTGGTAAAAGATGAAATGTGC-3′) and primer 2 (5′-TCATACTTATTTATCTTGGG-3′) (Fig. 1).

In order to establish a comparison of MICs of β-lactams for *E. coli* DH10B harboring either bla_{GOB-1} or bla_{BlaB-1}, PCR products of bla_{GOB-1} from *C. meningosepticum* PINT were obtained using primers 2 and 3, and those for bla_{BlaB-1} from *C. meningosepticum* CIP 6058 were obtained using primers 4 (5′-GTGAA TGTAGCAGAGTGTTAATG-3′) and primer 5 (5′-GTTGTCTGGTTAAGCG TTCG-3′) located at the 5′ and the 3′ end of bla_{BlaB-1} (Table 1) (46). Each PCR fragment was cloned into the same pPCR-Script CamSK vector (Stratagene) and electrotransformed into *E. coli* DH10B.

Conjugation assays, plasmid content, and Southern hybridization. Plasmid DNA extractions of *C. meningosepticum* isolates were attempted according to two different methods (18, 24). Direct transfer of resistance genes into in vitro-obtained rifampin-resistant *E. coli* JM109 was attempted by liquid and solid conjugation assays and by electroporation of the putative plasmid DNA suspension into *E. coli* DH10B (41). Transconjugants and electroporants were selected on TS agar plates containing either rifampin (200 μg/ml) and amoxicillin (30 μg/ml) or amoxicillin, respectively. Southern hybridizations were performed using a 0.8% electrophoresis gel containing unrestricted genomic DNAs of *C. meningosepticum* isolates and a PCR-prepared internal probe for bla_{GOB-1} (see below). Visualization was made using the ECL nonradioactive hybridization kit as described by the manufacturer (Amersham Pharmacia Biotech, Orsay, France).

DNA sequencing and protein analysis. Sequencing of the 2.4-kb cloned DNA fragment of recombinant plasmid pBS2, of 16S rDNA fragments, and of PCR products that contained bla_{BlaB-1}-like and bla_{GOB-1}-like genes was performed using an ABI 373 sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide and deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov.) and at Pedro's BioMolecular Research Tools website (http://www.fmi.ch/biology/research_tools.html.), and hydrophobicity analysis of the N-terminal region of the open reading frame (ORF) was performed as described (http://genome.cbs.dtu.dk./services/SignalP/ [25]). Multiple nucleotide or protein sequence alignments were carried out using the program ClustalW (http://www2.ebi.ac.uk/clustalw). A dendrogram of GOB-1 β-lactamase was derived from the multiple sequence alignment by a parsimony method using the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony) version 3.0 (53).

β-Lactamase extraction. A culture of *E. coli* DH10B (pBS2) was grown overnight at 37°C in 4 liters of TS broth containing kanamycin (30 μg/ml) and amoxicillin (30 μg/ml). Bacterial suspensions were pelleted, resuspended in 40 ml of 20 mM Tris-HCl buffer (pH 8), disrupted by sonification (three times at 50 W for 30 s using a Vibra Cell 75022 Phospholyser [Bioblock, Illkirch, France]), and centrifuged for 1 h at 48,000 × *g* at 4°C. Nucleic acids were precipitated by addition of 0.2 M spermine (7% [vol/vol]) (Sigma, Saint-Quentin Fallavier, France) overnight at 4°C. This suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant contained the β-lactamase extract.

β-Lactamase purification. The β-lactamase extract from *E. coli* DH10B (pBS2) was filtered through a 0.45-μm-pore-size filter (Millipore, Saint-Quentin-en-Yvelines, France) prior its loading onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The enzyme which was recovered in the flowthrough was then dialyzed overnight at 4°C against 50 mM phosphate buffer, pH 7. The enzymatic fraction was then loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted by a linear NaCl gradient (0 to 1 M) in phosphate buffer (pH 7). The β-lactamase was eluted at a concentration of 170 mM NaCl. The fraction containing the β-lactamase activity was dialyzed overnight against 30 mM cacodylate buffer, pH 6.5, containing 50 μM $ZnCl_2$. The specific activities of the β-lactamase extract and of the purified β-lactamase from *E. coli* DH10B (pBS2) were compared using 100 μM of imipenem as substrate as previously described (40).

N-terminal sequencing and isoelectric focusing. In order to determine the site for cleavage of the mature protein of GOB-1 β-lactamase, the purified enzyme was submitted to an Edman analysis (19) at the laboratory for protein microsequencing at the Pasteur Institute, Paris, France. Purified enzyme and marker proteins were subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (20 mA, 5 h, room temperature). Proteins were then electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by using the Mini Protean II transfer cell (8 by 7.3 cm) (Bio-Rad) in 50 mM Tris–50 mM borate buffer (pH 8.7) at room temperature (3.5 V/cm, overnight). The membrane was then rinsed in distilled water and stained with a solution made of 0.05% Coomassie brilliant blue R-250 in methanol and water (50:50 [vol/vol]) for 5 min. The membrane was then destained in methanol and water (50:40 [vol/vol]) and acetate and water (10:40 [vol/vol]). The protein band was then excised with a razor blade and allowed to air dry. The amino-terminal sequence of the β-lactamase was determined with an automated Edman sequencer on a model 473A gas phase sequencer (Applied Biosystems).

The purified enzyme from a culture of *E. coli* DH10B (pBS2) and β-lactamase extracts from cultures of 10 *C. meningosepticum* isolates were subjected to analytical IEF on an ampholine polyacrylamide gel with a pH of 3.5 to 9.5 (Ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Paris, France) or with an iodine-starch agar gel containing 0.5% (wt/vol) of imipenem (31) in 100 mM phosphate buffer (pH 7.0). These gels were preincubated with or without 100 mM EDTA (ethylenediaminetetraacetic acid) and with or without 5 mM clavulanic acid (38). The pI values were determined and compared to those of known β-lactamases.

Kinetic measurements and relative molecular mass determination. Purified β-lactamase was used for kinetic measurements performed at 30°C in 30 mM cacodylate (pH 6.5) supplemented with 50 μM $ZnCl_2$ as described previously (46). The rates of hydrolysis were determined with a Pharmacia ULTROSPEC 2000 spectrophotometer and were computer analyzed using the SWIFT II software (Amersham Pharmacia Biotech).

K_m and k_{cat} values were determined by analyzing the β-lactam hydrolysis under initial rate conditions by using the Eadie-Hoffstee linearization of the Michaelis-Menten equation as previously described (13, 41).

Various concentrations of EDTA or clavulanic acid were preincubated with the enzyme for 10 min at 30°C before testing the rate of imipenem hydrolysis. The 50% inhibitory concentration (IC_{50}) of these inhibitors was then determined.

The relative molecular mass of the purified β-lactamase was determined by gel filtration using a 1.6- by 47-cm column packed with Superdex 75 (Amersham Pharmacia Biotech) equilibrated and eluted with phosphate buffer (pH 7) containing 150 mM NaCl. Each elution peak was tested for β-lactamase activity by using nitrocefin as substrate. The peak that showed the highest β-lactamase activity was linearly plotted against the logarithm of the molecular masses of the standard proteins (Amersham Pharmacia Biotech) to determine the relative molecular mass of the purified β-lactamase.

Nucleotide sequence accession numbers. The nucleotide and deduced β-lactamase amino acid sequences reported in this work have been assigned to the GenBank and EMBL databases under the accession no. AF189290 to AF189305 and AF090141. The nucleotide sequences of the 16S rDNAs have been assigned to the accession no. AF207070 to AF207079.

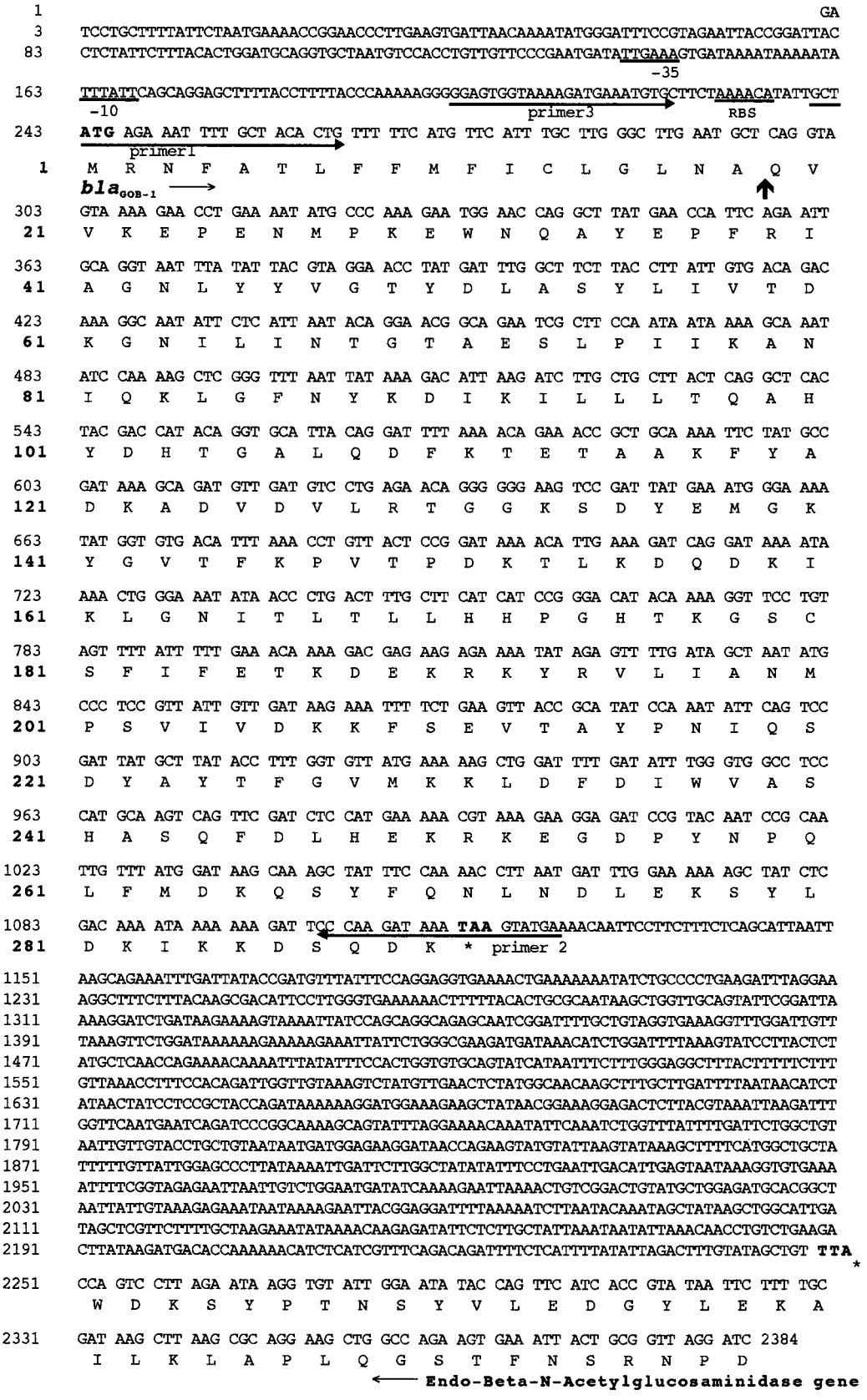

FIG. 1. Nucleotide sequence of a 2,384-bp DNA fragment of recombinant plasmid pBS2 carrying bla_{GOB-1} and the 117 bp of the 3′ end of the endo-beta-*N*-acetylglucosaminidase gene of *C. meningosepticum* PINT. The deduced amino acid sequences are given in a single-letter code. The start and stop codons of the bla_{GOB-1} gene and the stop codon of the endo-beta-*N*-acetylglucosaminidase gene are in bold. The vertical arrow indicates the peptide leader cleavage site in *E. coli* as determined by N-terminal sequencing. The putative −35 and −10 sequences of the putative promoter and ribosome binding site (RBS) for bla_{GOB-1} are underlined. Primers 1, 2, and 3 used to PCR amplify bla_{GOB-1}-like genes from other *C. meningosepticum* isolates are indicated by an arrow.

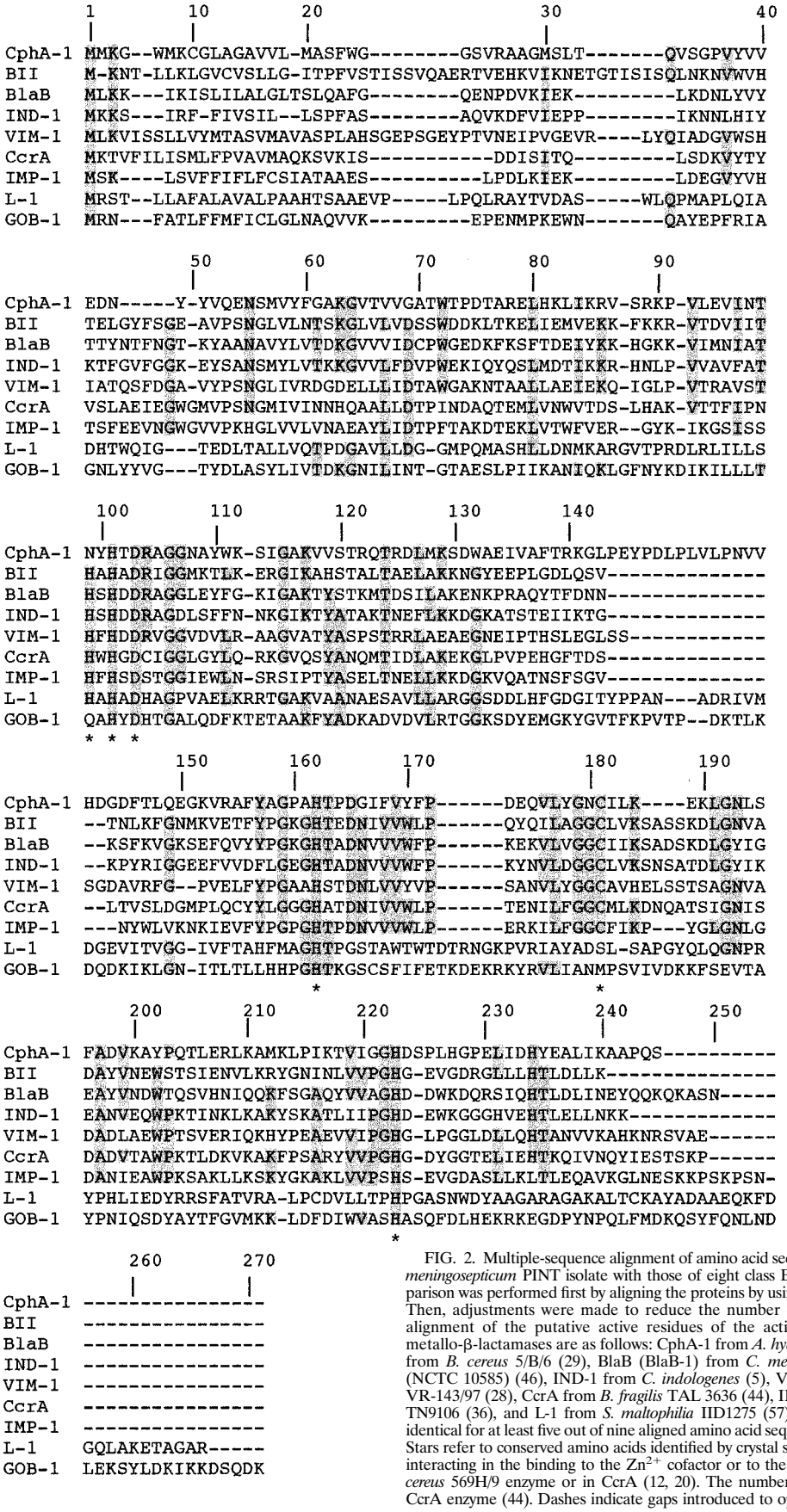

FIG. 2. Multiple-sequence alignment of amino acid sequence of GOB-1 from *C. meningosepticum* PINT isolate with those of eight class B CHβLs. Sequence comparison was performed first by aligning the proteins by using the ClustalW program. Then, adjustments were made to reduce the number of gaps and to maintain alignment of the putative active residues of the active sites. The origins of metallo-β-lactamases are as follows: CphA-1 from *A. hydrophila* AE036 (31), BII from *B. cereus* 5/B/6 (29), BlaB (BlaB-1) from *C. meningosepticum* CIP 6058 (NCTC 10585) (46), IND-1 from *C. indologenes* (5), VIM-1 from *P. aeruginosa* VR-143/97 (28), CcrA from *B. fragilis* TAL 3636 (44), IMP-1 from *S. marcescens* TN9106 (36), and L-1 from *S. maltophilia* IID1275 (57). Amino acids that were identical for at least five out of nine aligned amino acid sequences are shaded in grey. Stars refer to conserved amino acids identified by crystal structure determination as interacting in the binding to the Zn^{2+} cofactor or to the water molecule in the *B. cereus* 569H/9 enzyme or in CcrA (12, 20). The numbering scheme refers to the CcrA enzyme (44). Dashes indicate gaps introduced to optimize the alignment.

RESULTS

Cloning and sequence analysis of bla_{GOB-1}. Partially *Sau*3AI-digested genomic DNA from *C. meningosepticum* PINT was cloned into the *Bam*HI site of pBK-CMV. Three recombinant *E. coli* DH10B clones were obtained. One of them, harboring pBS2 (the smallest insert [2.4 kb]), was selected for further studies.

DNA sequence analysis of the 2,384-bp insert of pBS2 revealed an ORF of 873 bp, encoding a 290-amino-acid preprotein (Fig. 1). Putative −35 (TTGAAA) and −10 (TTTATT) promoter regions and a ribosome binding site (AAAACA) were found along with a putative ATG initiation codon at position 243 (Fig. 1).

The G+C content of this ORF was 36%, which lies close to the G+C ratio found for other *C. meningosepticum* genes recorded in the EMBL and GenBank sequence database (36.1 to 41.6%). The codon usage of this ORF was also similar to those calculated for the set of these *C. meningosepticum* genes (data not shown). From the sequencing data, one would expect the first 18 amino acids of this ORF, which contains numerous hydrophobic residues found by hydrophobicity analysis, to be the leader peptide (Fig. 1). This was indeed the case, since Edman analysis (nine cycles) determined the N-terminal sequence of the purified protein from a culture of *E. coli* DH10B (pBS2) cells as being QVVK. The cleavage site of the leader peptide was therefore deduced to be just after the alanine residue at position 18 (Fig. 1).

Further DNA sequence analysis of the downstream region of this ORF identified the 3′ end terminal sequence of an endo-beta-*N*-acetylglucosaminidase F1 gene (Fig. 1) (54).

The mature protein (named GOB-1 for class B β-lactamase of *C. meningosepticum*) expressed in *E. coli* DH10B cells had a relative molecular mass determined by gel filtration to be 28.2 kDa. His-101, Asp-103, His-162, and His-225 identified by biochemical analysis or by crystal structure analysis as interacting with a Zn^{2+} cofactor in *Bacillus cereus* 569H/9 enzyme or in CcrA were found in GOB-1 (Fig. 2) (10, 12). However, the histidine residue at position 99 found in most class B CHβLs was changed for a glutamine residue in GOB-1 (Fig. 2). The comparison of GOB-1 with other class B β-lactamases revealed only weak identity (Fig. 3). The highest percentage of identity was with L-1 from *Stenotrophomonas maltophilia* (18%) and only 11% with BlaB-1 from *C. meningosepticum*.

β-Lactam resistance phenotype and plasmid analysis. The MICs of β-lactams for *C. meningosepticum* PINT showed that it was resistant to all tested β-lactams except piperacillin, as previously reported (Table 2) (6, 16). Similar MICs (within a two-dilution range) were obtained for the *C. meningosepticum* isolates except for *C. meningosepticum* H01J100, for which MICs of all β-lactams were lower (data not shown).

Recombinant plasmids pBS3 and pBS4 were constructed by cloning the ORF of bla_{GOB-1} and bla_{BlaB-1} in plasmid pPCR-Script Cam SK, respectively, without the putative promoter regions of these β-lactamase genes (Table 1). *E. coli* DH10B (pBS3) showed a decreased susceptibility to all β-lactams except to aztreonam (Table 2), thus indicating that bla_{GOB-1} was involved at least partially in the resistance to carbapenems of *C. meningosepticum* PINT. MICs of penicillins were higher against *E. coli* DH10B (pBS4) than those against *E. coli* DH10B (pBS3), while the opposite was found for cephalosporins (Table 2). Both recombinant *E. coli* strains remained fully susceptible to aztreonam. MICs of carbapenems were similar against *E. coli* DH10B (pBS3) and *E. coli* DH10B (pBS4), although the amino acid identity of GOB-1 and BlaB-1 was low.

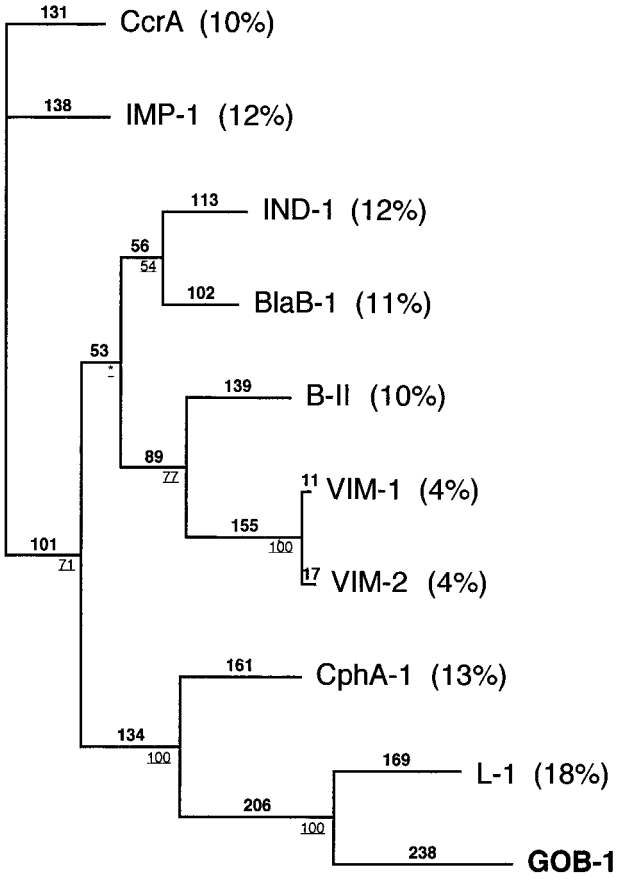

FIG. 3. Dendrogram obtained for nine representative CHβLs calculated with ClustalW followed by adjustments to reduce the number of gaps and to maintain alignment of the residues identified in the active sites of some CHβLs. Branch lengths are to scale and proportional to the number of amino acid changes. The percentages at the branching point (bold and underlined) refer to the number of times a particular node was found in 100 bootstrap replications (the stars indicate uncertainty of nodes with bootstrap values of less than 50%). The distance along the vertical axis has no significance. BlaB-1 (BlaB) and GOB-1 were from *C. meningosepticum*, IND-1 was from *C. indologenes*, CphA-1 was from *A. hydrophila*, L-1 was from *S. maltophilia*, BII was from *B. cereus*, VIM-1 and VIM-2 were from *P. aeruginosa*, CcrA was from *B. fragilis*, and IMP-1 was from *S. marcescens*. Percent amino acid identities to GOB-1 are indicated in parentheses.

Plasmid analysis and attempts to transfer the β-lactam resistance markers from *C. meningosepticum* to *E. coli* failed, thus suggesting the likely chromosomal origin of bla_{GOB-1}.

Biochemical properties of GOB-1. IEF analysis revealed that *E. coli* DH10B (pBS2) produced only one β-lactamase activity with a pI value of 8.7. This pI value did not correspond to the pI value of 8.3 found for the carbapenem-hydrolyzing activity identified in *C. meningosepticum* PINT.

Specific activity prior to and after purification enabled us to determine the 400-fold purification factor for GOB-1 from *E. coli* DH10B (pBS2). The specific activity of the purified enzyme was 73.2 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$.

Kinetic parameters of GOB-1 revealed a broad spectrum of hydrolysis with a strong activity against meropenem, compared to that against imipenem (Table 3). GOB-1 β-lactamase has a strong activity against amoxicillin, benzylpenicillin, piperacillin, and extended-spectrum cephalosporins. Hydrolysis of aztreonam was not detectable. The hydrolytic activity of GOB-1 β-lactamase was inhibited by EDTA (IC_{50}, 25 μM) but not by class A β-lactamase inhibitors, such as clavulanic acid (IC_{50},

TABLE 3. Kinetic parameters of β-lactam antibiotics for the purified carbapenem-hydrolyzing β-lactamase GOB-1

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)[a]
Benzylpenicillin	109	204	1.87
Amoxicillin	1,024	357	0.35
Ticarcillin	147	76.3	0.52
Piperacillin	169	279	1.66
Cephalothin	24	16.1	0.67
Cefepime	52.6	10.7	0.20
Cefoxitin	46.8	11.9	0.25
Cefuroxime	26.9	26.3	0.98
Ceftazidime	71.4	54.5	0.76
Cefotaxime	51.1	43.7	0.85
Aztreonam	ND[b]	<0.5	—[c]
Moxalactam	78.8	10.4	0.13
Imipenem	60	39.4	0.66
Meropenem	5.4	29.2	5.34

[a] Standard deviations were within 10%.
[b] ND, not determinable.
[c] —, the hydrolysis parameter could not be calculated.

>10 mM). GOB-1 was therefore classified the functional CHβL group 3a according to the Bush classification (9, 43).

Distribution of GOB-1-like and BlaB-like β-lactamases and 16S rDNA sequencing. EDTA-inhibited activities obtained by comparison of pI values with or without EDTA were heterogeneous for the 10 *C. meningosepticum* isolates (Table 4). Only one EDTA-inhibited hydrolysis activity was detected for *C. meningosepticum* PINT, AMA, 7830, 79.5, CIP 6057, AB1572, and H01J100 isolates. Three isolates produced two EDTA-inhibited activities (Table 4). Additionally, clavulanic acid-inhibited β-lactamase activities varied from one isolate to the other (Table 4). Southern hybridization experiments using nonrestricted genomic DNA of *C. meningosepticum* isolates and a PCR-amplified 731-bp fragment internal to bla_{GOB-1} as a probe yielded a hybridization signal that corresponded to the chromosomal band (data not shown), showing that each *C. meningosepticum* isolate possessed a chromosomally located bla_{GOB-1}-like gene. PCR fragments of GOB-1- and BlaB-1-like genes of 10 *C. meningosepticum* isolates (except for a bla_{GOB-1}-like gene from *C. meningosepticum* CIP 7830 that failed to yield a PCR-positive result) were sequenced on both strands. Sequences for entire bla_{BlaB-1}-like genes and a partial portion of the bla_{GOB-1}-like genes yielding 252 out of 290 amino acids were obtained using the designed PCR primers. The deduced amino acid sequences revealing heterogeneity among GOB-1-like β-lactamases having 90 to 100% amino acid identity (Fig. 4).

Alignment of the BlaB-1-like sequences of 10 *C. meningosepticum* isolates also revealed heterogeneity, with 86 to 100% amino acid identity (Fig. 5). The same GOB-1-like or BlaB-1-like sequences were found in several isolates, for example, GOB-1 in *C. meningosepticum* PINT, AB1572, and CIP 6057 and BlaB-1 in *C. meningosepticum* PINT and CIP 6058 (Table 4). However, within two given *C. meningosepticum* isolates, the same combination of GOB-1-like and BlaB-1-like β-lactamases was not found (Table 4). 16S rDNA sequencing identified homogeneous sequences (from 96 to 99% identity) among the studied *C. meningosepticum* isolates (data not shown, accession numbers available).

DISCUSSION

GOB-1 is a broad-spectrum class B β-lactamase like the previously identified BlaB-1 (BlaB) in *C. meningosepticum* NCTC 10585 (CIP 6058). Comparison of their kinetic constants revealed that BlaB-1 hydrolyzed benzylpenicillin better than GOB-1. Additionally, a comparison of MICs of β-lactams for *E. coli* expressing either GOB-1 or BlaB-1 revealed that GOB-1 hydrolyzed ceftazidime and cefoxitin more significantly than BlaB-1 does (hydrolysis constants of BlaB-1 for ceftazidime are not available [46]). GOB-1 β-lactamase hydrolysis of meropenem was greater than that of imipenem. Imipenem is usually hydrolyzed better than meropenem by class B CHβLs with two exceptions, the group 3a *B. cereus* II enzyme and the group 3b AsbM1 enzyme from *A. hydrophila* (43). GOB-1, like BlaB-1, conferred only a slight increase in the MICs of carbapenems once its gene was cloned on a multicopy plasmid and expressed in *E. coli*. Similar results have been found for the CHβLs IMP-1, VIM-1, and VIM-2 (28, 36, 42). These results, together with data for kinetic constants of carbapenems, may indicate that an additional decrease of outer membrane permeability for carbapenems may explain the resistance to carbapenems observed for *C. meningosepticum* (32). In this regard, the pI value of 8.3 for the EDTA-inhibited β-lactamase identified in *C. meningosepticum* PINT did not correspond to the pI value of 8.7 for GOB-1 expressed in *E. coli* DH10B. This result may be explained either by a pI value of 8.3 corresponding to BlaB-1 also found in *C. meningosepticum* PINT, by a weak or lack of expression of GOB-1, or to differences in leader peptide cleavage in *E. coli* and in *C. meningosepticum*. Such a difference in the N-terminal end of the mature protein of GOB-1 may lead to its low concentration in the periplasmic space in *E. coli*. Whatever the reason is, the low level of resistance to carbapenems conferred by GOB-1 in *E. coli* may explain its difficulty in being detected once expressed in enterobacterial clinical isolates. Studies of the pI values of the *C. meningosepticum* isolates revealed that GOB-1-like and BlaB-1-like β-lactamases may not always be expressed since for some *C. meningosepticum* isolates, only one EDTA-inhibited β-lactamase was evidenced by IEF gel electrophoresis although two CHβL genes had been identified (Table 4). However, since the pI values of GOB-1 and BlaB-1 were very close, one cannot exclude that they cannot be distinguished on the IEF gel.

Several variants of CHβLs have been found in *S. maltophilia*, *A. hydrophila*, and *Bacteroides fragilis*. However, in these species, it was determined that variants from reference CHβLs had 88 to 95% identity (31, 38, 43, 47, 48). To the best of our knowledge, it is the first time that two CHβLs with only 11%

TABLE 2. MICs of β-lactams for *C. meningosepticum* PINT, *E. coli* DH10B (pBS3), *E. coli* DH10B (pBS4), and the *E. coli* DH10B reference strain

β-Lactam	MIC (μg/ml)			
	C. meningosepticum PINT	*E. coli* DH10B (pBS3)[a]	*E. coli* DH10B (pBS4)[b]	*E. coli* DH10B
Amoxicillin	256	64	128	4
Ticarcillin	256	64	256	4
Piperacillin	32	2	4	1
Cephalothin	512	32	16	2
Cefepime	32	0.06	0.03	0.03
Cefoxitin	32	16	2	1
Cefpirome	32	0.5	0.06	0.06
Ceftazidime	256	16	0.5	0.5
Cefotaxime	64	0.25	0.12	0.12
Aztreonam	>512	0.25	0.25	0.25
Moxalactam	64	1	0.12	0.12
Imipenem	32	0.5	0.5	0.12
Meropenem	16	0.12	0.12	0.06

[a] *E. coli* DH10B (pBS3) expressed GOB-1.
[b] *E. coli* DH10B (pBS4) expressed BlaB-1.

TABLE 4. pI values of β-lactamase activity detected in *C. meningosepticum* isolates and in *E. coli* DH10B harboring pBS2 (GOB-1) or pBS4 (BlaB-1) and the corresponding GOB-1-like and BlaB-1-like sequences

Strains	pI values		Shared amino acid sequence	
	Clavulanic acid-inhibited β-lactamase activity	EDTA-inhibited β-lactamase activity	GOB-1 like	BlaB-1 like
C. meningosepticum PINT	7.6	8.3	GOB-1	BlaB-1
C. meningosepticum CIP 6058	8.5	7.8, 8.7	GOB-5	BlaB-1
C. meningosepticum AMA	8.5	8.3	GOB-6	BlaB-2
C. meningosepticum GEO	7.0	7.5, 8.5	GOB-4	BlaB-3
C. meningosepticum CIP 7830	7.5	8	+[a]	BlaB-6
C. meningosepticum CIP 6059	8.4	7.5, 8.5	GOB-3	BlaB-3
C. meningosepticum CIP 79.5	7.6	8.4	GOB-7	BlaB-5
C. meningosepticum AB1572	7.6	8.3	GOB-1	BlaB-7
C. meningosepticum H01J100	8.1	8.3	GOB-2	BlaB-8
C. meningosepticum CIP 6057	7.6	8.6	GOB-1	BlaB-4
E. coli DH10B (pBS2)	—[b]	8.7	GOB-1	—
E. coli DH10B (pBS4)	—	8.5	—	BlaB-1

[a] +, GOB-1-like positive results by hybridization but negative by PCR amplification.
[b] —, not detectable.

amino acid identity were identified in the same bacterial species. The significance of this result remains to be determined. The regulation of these CHβLs, if any, would be of interest as described for the *A. hydrophila* CHβL (1). It may be hypothesized that CHβLs in *C. meningosepticum* may counteract the effect of antibiotics produced by this *Chryseobacterium* species (33, 37). Additionally, the presence of two CHβL genes in *C. meningosepticum* may be used as a tool for a PCR-based identification of this species.

The amino acid sequence of GOB-1 allowed its classification in the sequence-based subclass B3 of metallo-CHβLs (43) along with L-1, the only other member of this subgroup, whereas BlaB-1 is a member of the subclass B1.

The primary structure of GOB-1 β-lactamase keeps most of the conserved amino acid residues of class B β-lactamases that act in the interaction with the Zn^{2+} cofactor or with the water molecule located in the active site, as shown for CcrA (58) or for L-1 (55): His-101, Asp-103, His-162, and His-225 (43). However, like CphA-1, GOB-1 lacks the His-99 residue, which is also involved in Zn^{2+} binding, but possesses instead a glutamine residue (asparagine in CphA) (31). Therefore, the absence of His-99 does not seem to be involved in narrowing the spectrum of GOB-1 (Fig. 2).

GOB-1 β-lactamase, like L-1, lacks a cysteine residue at position 181 that is involved in the interaction with a Zn^{2+} ion. In L-1, Cys-181 function is replaced by a histidine residue located at position 104 (55). It could be the same for GOB-1, which possesses also a histidine residue at this same position.

While this work was in progress, two BlaB variants were reported in GenBank, BlaC from *C. meningosepticum* NCTC

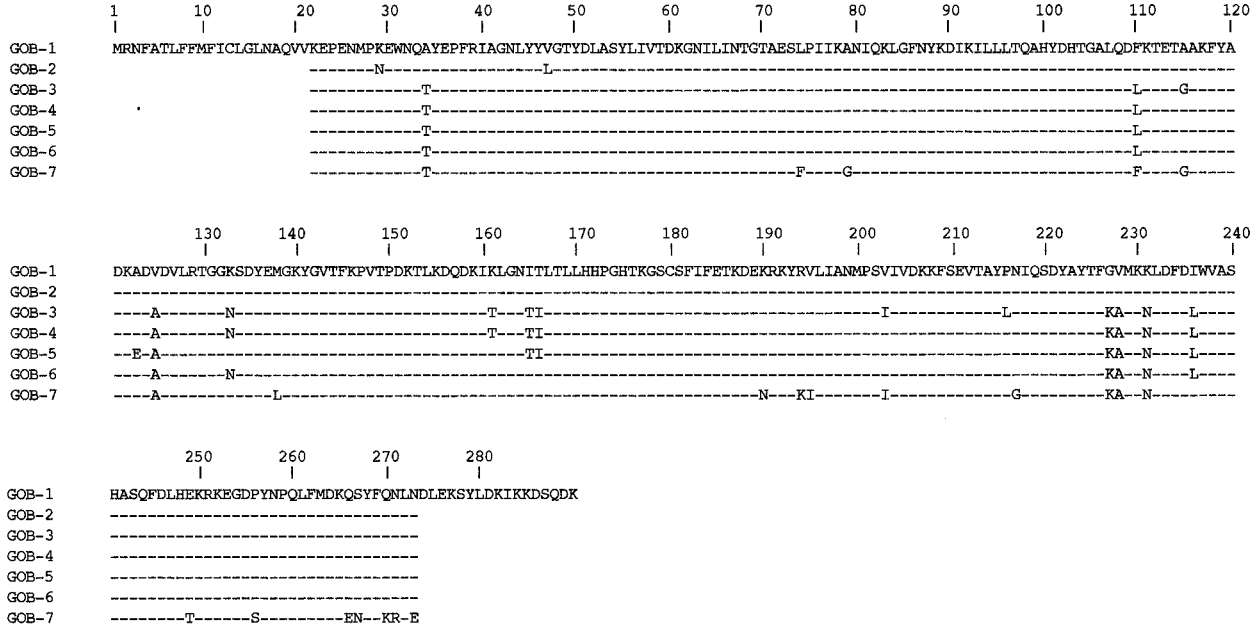

FIG. 4. Amino acid comparison of the GOB-1-like β-lactamases from nine *C. meningosepticum* isolates. Dashes indicate identical amino acids, and dots indicate undetermined sequences. GOB-1 was from *C. meningosepticum* PINT, CIP 6057, and AB1572, GOB-2 was from *C. meningosepticum* HO1J100, GOB-3 was from *C. meningosepticum* CIP 6059, GOB-4 was from *C. meningosepticum* GEO, GOB-5 was from *C. meningosepticum* CIP 6058, GOB-6 was from *C. meningosepticum* AMA, and GOB-7 was from *C. meningosepticum* CIP 79.5. Numbering is according to the GOB-1 sequence.

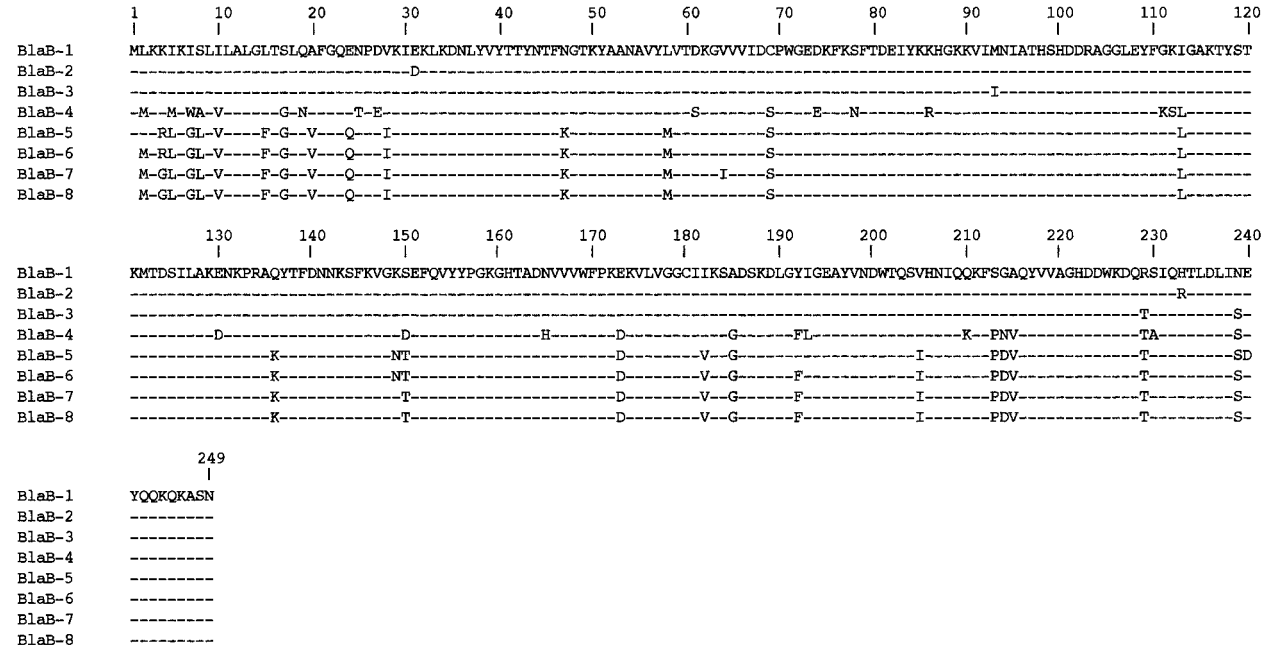

FIG. 5. Amino acid comparison of the BlaB-1-like β-lactamases from 10 *C. meningosepticum* isolates. Dashes indicate identical amino acids. BlaB-1 was from *C. meningosepticum* CIP 6058 and PINT, BlaB-2 was from *C. meningosepticum* AMA, BlaB-3 was from *C. meningosepticum* GEO and CIP 6059, BlaB-4 was from *C. meningosepticum* CIP 6057, BlaB-5 was from *C. meningosepticum* CIP 79.5, BlaB-6 was from *C. meningosepticum* CIP 7830, BlaB-7 was from *C. meningosepticum* AB1572, and BlaB-8 was from *C. meningosepticum* H01J100. Numbering is according to the BlaB-1 sequence.

10016 and BlaB-2 from *C. meningosepticum* 97/P/5443. We have also identified BlaC (BlaB-4) from the same *C. meningosepticum* NCTC 10016 isolate and BlaB-2 from another *C. meningosepticum* isolate (*C. meningosepticum* AMA).

Although some genetic variation was identified among BlaB-1-like and GOB-1-like sequences, none of the studied *C. meningosepticum* isolates could be assigned to a special *C. meningosepticum* subgroup. Indeed, the *C. meningosepticum* isolates had 96 to 99% identity, according to the results of 16S rDNA sequencing.

Time will tell if gram-negative aerobes, such as *C. meningosepticum*, may be a reservoir for diffusion of CHβL genes to opportunistic pathogens. *P. aeruginosa* and *Acinetobacter* spp. that share low natural permeability towards most β-lactams are good candidates for expressing these carbapenem resistance genes. Finally, since *C. meningosepticum* CHβLs provide only a low level of resistance to carbapenems once they are expressed in *E. coli*, their routine detection in gram-negative clinical pathogens shall be performed at best with PCR-based methods previously described for $bla_{\text{IMP-1}}$ detection among American and Japanese isolates (23, 52).

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