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Genetic-Biochemical Analysis and Distribution of the Ambler Class A β-Lactamase CME-2, Responsible for Extended-Spectrum Cephalosporin Resistance in *Chryseobacterium* (*Flavobacterium*) *meningosepticum*

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In vitro synergy between extended-spectrum cephalosporins and either clavulanic acid or cefoxitin was found for *Chryseobacterium meningosepticum* isolates during a double-disk assay on an agar plate. An extended-spectrum β-lactamase (ESBL) gene from a *C. meningosepticum* clinical isolate was cloned and expressed in *Escherichia coli* DH10B. Its protein conferred resistance to most β-lactams including extended-spectrum cephalosporins but not to cephamycins or to imipenem. Its activity was strongly inhibited by clavulanic acid, sulbactam, and tazobactam, as well as by cephamycins and imipenem. Sequence analysis of the cloned DNA fragment revealed an open reading frame (ORF) of 891 bp with a G+C content of 33.9%, which lies close to the expected range of G+C contents of members of the *Chryseobacterium* genus. The ORF encoded a precursor protein of 297 amino acids, giving a mature protein with a molecular mass of 31 kDa and a pI value of 9.2 in *E. coli*. This gene was very likely chromosomally located. Amino acid sequence comparison showed that this β-lactamase, named CME-2 (*C. meningosepticum* ESBL), is a novel ESBL of the Ambler class A group (Bush functional group 2be), being weakly related to other class A β-lactamases. It shares only 39 and 35% identities with the ESBLs VEB-1 from *E. coli* MG-1 and CBL-A from *Bacteroides uniformis*, respectively. The distribution of bla_{CME-2} among unrelated *C. meningosepticum* species isolates showed that bla_{CME-2}-like genes were found in the *C. meningosepticum* strains studied but were absent from strains of other *C. meningosepticum*-related species. Each *C. meningosepticum* strain produced at least two β-lactamases, with one of them being a noninducible serine ESBL with variable pIs ranging from 7.0 to 8.5.

Chryseobacterium meningosepticum (formerly classified as *Flavobacterium meningosepticum* [45]) is a waterborne saprophytic bacterium. Among *Chryseobacterium* species, *C. meningosepticum* is most commonly associated with infections in humans. It may cause meningitis in newborns and pneumonia and sepsis in immunocompromised patients, especially those hospitalized in intensive care units (3, 39). *C. meningosepticum* is naturally resistant to most β-lactams, including extended-spectrum cephalosporins and carbapenems, with only some isolates remaining susceptible to ureidopenicillins (6).

Phenotype analysis of the β-lactam resistance pattern of a *C. meningosepticum* PINT clinical isolate revealed the presence of a putative extended-spectrum β-lactamase (ESBL) according to the synergy found between clavulanic acid and most extended-spectrum cephalosporins when a double-disk assay was performed on an agar plate (20). Uncommonly, a similar synergy was also found between cephamycins such as cefoxitin or moxalactam and extended-spectrum cephalosporins. Recently, an Ambler class B carbapenem-hydrolyzing β-lactamase has been reported from *C. meningosepticum* CIP 6058 (36). Although the hydrolysis spectrum of this β-lactamase is broad, its presence cannot be responsible for the extended-spectrum cephalosporin resistance profile observed in *C. meningosepticum*.

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The aim of this work was to analyze both biochemically and genetically the β-lactamase responsible for the observed phenotype and to determine its distribution among nonepidemiologically related *C. meningosepticum* isolates and other *Chryseobacterium* species strains.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. *C. meningosepticum* PINT was isolated at the Raymond Poincaré Hospital in Garches, France, a suburb of Paris. *C. meningosepticum* AMA and GEO were isolated at the Bicêtre Hospital (Le Kremlin-Bicêtre, France), and both were from tracheoalveolar aspirates. Reference strains were from the Pasteur Institute (Paris, France) and Denmark (7). The *C. meningosepticum* isolates and reference strains were epidemiologically unrelated (data not shown).

Escherichia coli DH10B and nalidixic acid- and rifampin-resistant *E. coli* JM109 were used for cloning and conjugation assays, respectively (Table 1). The *Chryseobacterium* sp. strains were identified by standard techniques as described previously (30, 39), and their identities were confirmed with the API 32GN system (bioMérieux, Marcy l'Etoile, France). All strains were stored at −70°C in Trypticase soy (TS) broth (Becton Dickinson, Le Pont de Claix, France) supplemented with 15% glycerol until testing.

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study were obtained in the form of standard laboratory powders and were used immediately after their solubilization. The agents and their sources have been described elsewhere (32). Antibiotic disks were used for routine antibiograms (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France).

MICs were determined by an agar dilution technique on Mueller-Hinton (MH) agar (Sanofi-Diagnostics Pasteur) with an inoculum of 10^4 CFU per spot (8, 23). All drugs were incorporated into MH agar at serial twofold concentrations, and the antimicrobial susceptibilities of all isolates were determined concomitantly. The plates were incubated at 35°C for 18 h. The MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml), tazobactam (4 μg/ml), cefoxitin (0.1 μg/ml), or moxalactam or imipenem (0.05 μg/ml each).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli DH10B	F′ *mcrA* Δ(*mrr-hsdRMS-mrcBC*) Φ80Δ*lacZDM15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK1 rpsL nupG*	Gibco BRL
E. coli JM109	*endA1 hsdR17 gyrA96* Δ(*lac proA*) *recA1 relA supE44 thi* F′ (*lacI*^{q} *lacZ*Δ*M15 proAB*$^+$ *traD36*)	48
Rifampin-resistant *E. coli* JM109 obtained in vitro	Rifampin-resistant	This study
C. meningosepticum PINT	Extended-spectrum cephalosporin and carbapenem resistant	This study
C. meningosepticum AMA	Extended-spectrum cephalosporin and carbapenem resistant	This study
C. meningosepticum GEO	Extended-spectrum cephalosporin and carbapenem resistant	This study
C. meningosepticum CIP 6058	Extended-spectrum cephalosporin and carbapenem resistant	IP[a]
C. meningosepticum CIP 6059	Extended-spectrum cephalosporin and carbapenem resistant	IP
C. meningosepticum CIP 7830	Extended-spectrum cephalosporin and carbapenem resistant	IP
C. meningosepticum CIP 7905	Extended-spectrum cephalosporin and carbapenem resistant	IP
C. meningosepticum AB1572	Extended-spectrum cephalosporin and carbapenem resistant	7
C. meningosepticum H01, J100	Extended-spectrum cephalosporin and carbapenem resistant	7
C. indologenes CIP 101026	Carbapenem resistant	IP
C. indologenes PIT	Carbapenem resistant	This study
S. multivorum CIP 103686	Carbapenem resistant	IP
M. odoratus CIP 103105	Carbapenem resistant	IP
Plasmids		
pBK-CMV phagemid	Neomycin and kanamycin resistant	Stratagene
pBR322	Recombinant plasmid containing the 560-bp *Ssp*I-*Pst*I internal fragment of $bla_{\text{TEM-1}}$	42
pHUC37	Recombinant plasmid containing the 435-bp *Pst*I-*Not*I internal fragment of $bla_{\text{SHV-3}}$	24
pPZ1	Recombinant plasmid containing the 1.1-kb *Sna*BI fragment of $bla_{\text{PER-1}}$	25
pRLT1	Recombinant plasmid containing the 329-bp *Dra*I-*Xmn*I internal fragment of $bla_{\text{VEB-1}}$	32
pPL1	Recombinant plasmid containing the 396-bp *Eco*RI-*Pvu*I internal fragment of $bla_{\text{OXA-18}}$	29
pBS1	1.9-kb *Sau*3AI fragment from *C. meningosepticum* PINT cloned into pBK-CMV	This study

[a] IP, Institut Pasteur, Paris, France.

Cloning experiments and analysis of recombinant plasmids. Genomics DNAs from *C. meningosepticum* PINT and from other strains were extracted as described previously (25). The *Xmn*I restriction endonuclease was from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France), while all the other enzymes used in the cloning experiments were from Amersham Pharmacia Biotech (Orsay, France). Fragments from genomic DNA partially digested with *Sau*3AI were ligated into *Bam*HI-restricted phagemid pBK-CMV from Stratagene (Ozyme) (37). Ligation was performed at a 1:2 vector-insert ratio with 200 ng of restricted genomic DNA in a ligation mixture containing 1 U of T4 DNA ligase at 4°C for 18 h. Recombinant plasmids were transformed by electroporation (Bio-Rad Gene Pulser II) into *E. coli* DH10B electrocompetent cells (Gibco BRL, Life Technologies, Cergy Pontoise, France). Antibiotic-resistant colonies were selected on TS agar plates containing amoxicillin (50 μg/ml) and kanamycin (30 μg/ml).

Recombinant plasmid DNA was obtained from 100-ml TS broth cultures grown overnight in the presence of amoxicillin (100 μg/ml) at 37°C. Plasmid DNAs were recovered by using Qiagen columns (Qiagen, Courtaboeuf, France). Plasmid mapping was performed after double restriction analysis. Fragment sizes were estimated by comparison with the fragment sizes on a 1-kb DNA ladder (Amersham Pharmacia Biotech).

Conjugation assays and plasmid content. Direct transfer of resistance genes into rifampin-resistant *E. coli* JM109 obtained in vitro was attempted by liquid and solid conjugation assays at 30 and 37°C. Transconjugants were selected on TS agar plates containing rifampin (200 μg/ml) and amoxicillin (50 μg/ml). Extraction of plasmid DNA from *C. meningosepticum* PINT was attempted by two different methods (10, 15).

DNA sequencing and protein analysis. Both strands of the 1.9-kb cloned DNA fragment of recombinant plasmid pBS1 were sequenced with an Applied Biosystems sequencer (ABI 373). The nucleotide sequence and the deduced protein sequence were analyzed with software available over the Internet at the National Center of 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). Multiple protein sequence alignments were carried out with the program Clustal W, available over the Internet at the University of Cambridge. A dendrogram was derived from the multiple sequence alignment by a parsimony method with the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony), version 3.0 (44). Among the Ambler class A β-lactamases, 11 were compared to CME-2: PER-1 from *Pseudomonas aeruginosa* RNL-1 (25), VEB-1 from *E. coli* MG-1 (32), CEP-A from *Bacteroides fragilis* CS30 (35), CFX-A from *Bacteroides vulgatus* CLA341 (26), CBL-A from *Bacteroides uniformis* WAL-7088 (40), TEM-3 from *Klebsiella pneumoniae* CFF104 (41), SHV-2 from *Klebsiella ozaenae* (11), MEN-1 from *E. coli* MEN (2), L-2 from *Stenotrophomonas maltophilia* 1275 IID (47), TOHO-1 from *E. coli* TUH12191 (13), and NMC-A from *Enterobacter cloacae* NOR-1 (22) as a representative of the serine carbapenem-hydrolyzing β-lactamase group (Bush group 2f) (5), which possesses an extended hydrolysis spectrum toward aztreonam.

β-Lactamase preparations. Cultures of *C. meningosepticum* clinical isolates and *E. coli* DH10B harboring recombinant plasmid pBS1 were grown overnight at 37°C in 100 ml of TS broth containing amoxicillin (100 μg/ml) and 4 liters of TS broth, respectively. Bacterial suspensions were pelleted, resuspended in 40 ml of Tris-HCl (50 mM) buffer (pH 8), disrupted by sonification (three times at 50 W for 30 s each time with a Vibra Cell 75022 Phospholyser [Bioblock, Illkirch, France]), and centrifuged at 48,000 × *g* for 1 h at 4°C. Nucleic acids were precipitated by the addition of spermin (0.2 M; 7% [vol/vol]; Sigma, Saint-Quentin Fallavier, France) for 1 h on ice. This suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4°C.

Then, the β-lactamase extract from *E. coli* DH10B(pBS1) was loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The β-lactamase was recovered in the flowthrough and was subsequently dialyzed overnight against 100 mM phosphate buffer (pH 7.0). The β-lactamase was loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted with a linear NaCl gradient (0 to 1 M) in phosphate buffer (pH 7). The β-lactamase was eluted with NaCl at a concentration of 320 to 350 mM. The fraction containing the β-lactamase activity was dialyzed overnight against 100 mM phosphate buffer (pH 7.0) prior to a 10-fold concentration with a Centrisart-C30 microcentrifuge filter (Sartorius, Goettingen, Germany).

Isoelectric focusing. Enzyme preparations from cultures of *C. meningosepticum* clinical isolates and *E. coli* DH10B(pBS1) were subjected to analytical isoelectric focusing on a pH 3.5 to 9.5 Ampholine polyacrylamide gel (Ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamase was detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Paris, France) in 100 mM phosphate buffer (pH 7.0). Since a metalloenzyme has previously been described in *C. meningosepticum* (36), detection of the pIs of the serine β-lactamases were additionally performed by incubating the enzyme extracts with 100 μM clavulanic acid prior to their loading and focusing on an isoelectric focusing gel, followed by nitrocefin detection (28). The pI values were determined and compared to those of known β-lactamases run on the same gels.

Kinetic measurements. Purified β-lactamase was used for kinetic measurements (k_{cat}, K_m), which were made at 30°C in 100 mM sodium phosphate (pH 7.0) with a Pharmacia ULTROSPEC 2000 spectrophotometer as described previously (17). Various concentrations of clavulanic acid, sulbactam, tazobactam, cefoxitin, imipenem, or moxalactam were preincubated with the enzyme for 3 min at 30°C before testing the rate of benzylpenicillin (100 μM) hydrolysis. The 50% inhibitory concentrations (IC_{50}s) of these inhibitors were determined. Results were expressed in micromolar units.

Induction experiments. To test the inducibility of the serine β-lactamase in *C. meningosepticum* clinical isolates, induction experiments were performed as described previously (31) with cefoxitin (10 μg/ml) or imipenem (1 μg/ml) as the inducer. One unit of enzyme activity was defined as that which was required to hydrolyze 1 μmol of aztreonam per min (aztreonam is not hydrolyzed by metalloenzymes). The total protein content was measured with bovine albumin as the standard (Bio-Rad DC Protein assay kit).

Determination of the β-lactamase relative molecular mass. The relative molecular mass of the β-lactamase from *E. coli* DH10B harboring pBS1 was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis. Enzyme extracts and marker proteins were boiled for 10 min in a 1% SDS–3% β-mercaptoethanol solution and were then subjected to electrophoresis on a 12% polyacrylamide gel (25 mA, 4 h) (16). Renaturation of the β-lactamase activity after denaturing electrophoresis and visualization of the β-lactamase on a benzylpenicillin-containing agar gel were performed as described previously (18).

Hybridization experiments. Southern hybridizations were performed with an ECL nonradioactive hybridization kit as described by the manufacturer (Amersham Pharmacia Biotech). Genomic DNA from *C. meningosepticum* PINT was hybridized with the following DNA probes corresponding to some β-lactamase genes: the 1.1-kb *Sna*BI fragment from recombinant plasmid pPZ1 for bla_{PER-1}, the 450-bp *Pst*I-*Not*I fragment from recombinant plasmid pHUC37 for bla_{SHV-3}, the 560-bp *Ssp*I-*Pst*I fragment from plasmid pBR322 for bla_{TEM-1}, the 329-bp *Dra*I-*Xmn*I fragment from recombinant plasmid pRLT1 for bla_{VEB-1}, the 396-bp *Eco*RI-*Pvu*I from recombinant plasmid pPL1 for bla_{OXA-18}, or the 811-bp PCR-amplified fragment internal to bla_{CARB-2} (12). Two micrograms of denatured DNA was put onto a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech) and cross-linked with UV light for 2 min with a UV Stratalinker 2400 instrument (Stratagene). Membrane was incubated for 1 h at 60°C in a prehybridization buffer containing 100 μg of salmon sperm DNA per ml, 0.1% SDS, 5× SSC (0.75 M sodium chloride and 0.075 M sodium citrate), 5% dextran sulfate, and a 20-fold dilution of liquid block solution (supplied). Hybridizations were performed overnight at 60°C. Then, two washes were performed successively in the following solutions: 1× SSC–0.1% SDS for 15 min at 60°C and 0.5× SSC–0.1% SDS for 15 min at 60°C. The membrane was blocked and was then incubated with anti-fluorescein-horseradish peroxidase conjugate and finally washed. The signal was generated with a luminol solution and was detected with an autoradiographic film after 15 min of exposure.

Once the ESBL gene was identified, a set of primers was designed (primer 1, [5′-GTA GCT GTT TCT GTT TTG GGG-3′] and primer 2, [5′-CGA GAC CTG GGC AAT GAT TC-3′] at positions 438 to 458 and 1162 to 1181, respectively; (see Fig. 2) in order to attempt PCR amplification of this ESBL gene from the genomic DNAs of the *C. meningosepticum* isolates studied and from isolates of the related species studied (Table 1). In order to analyze the genetic organization of an ESBL-like gene in several *C. meningosepticum* strains, their corresponding genomic DNAs were digested with *Xmn*I and run on a 1% agarose gel. This gel was submitted to Southern hybridization as described above. Since *Xmn*I cut only once, in the middle of the ESBL gene (see below), *Dra*I-*Xmn*I and *Dra*I restriction digests gave three internal fragments for the ESBL gene used as probes (fragments S1, S2, and S3; see Fig. 1).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under the accession no. AF033200.

RESULTS

Preliminary hybridization experiments and cloning of the ESBL gene. Genomic DNA from *C. meningosepticum* PINT failed to hybridize with several probes corresponding to class A β-lactamase genes (bla_{SHV-3}, bla_{PER-1}, bla_{VEB-1}, bla_{TEM-1}, and bla_{CARB-2}) or with a probe for the gene of the clavulanic acid-inhibited extended-spectrum oxacillinase OXA-18. Genomic DNA from *C. meningosepticum* PINT that had been partially digested with *Sau*3AI was cloned into the *Bam*HI site of pBK-CMV. Sixteen recombinant *E. coli* DH10B clones harboring plasmids with inserts that varied in size (1.9 to 12 kb) were obtained after selection on amoxicillin- and kanamycin-containing TS agar plates. A schematic representation was generated for one of them, pBS1, which possesses the smallest insert (Fig. 1).

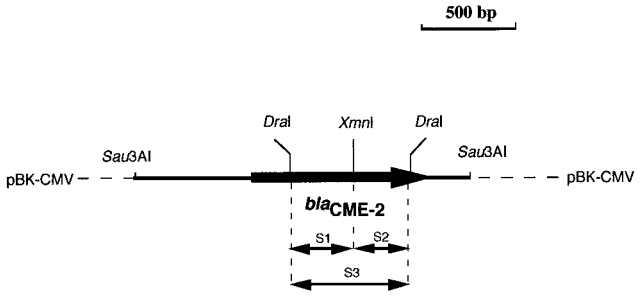

FIG. 1. Schematic representation of recombinant plasmid pBS1, which encodes the CME-2 β-lactamase from *C. meningosepticum* PINT. The thin line represents the cloned insert from *C. meningosepticum* PINT containing the CME-2 β-lactamase gene, with the arrow indicating its translational orientation (thick line), and the dotted lines indicate the vector pBK-CMV. The three internal probes (probes S1, S2, and S3) used for hybridization experiments are also represented by double-headed arrows.

β-Lactam resistance phenotype. Analysis of *C. meningosepticum* PINT by the conventional disk susceptibility assay suggested that its β-lactam resistance phenotype was partially due to the presence of an ESBL. A similar ESBL phenotype was found for *E. coli* DH10B harboring recombinant plasmid pBS1 (data not shown). This ESBL phenotype was peculiar since synergy was found not only between ceftazidime or cefotaxime and clavulanic acid or tazobactam but also with cefoxitin, moxalactam, or imipenem (data not shown). The MICs of β-lactams for *C. meningosepticum* PINT indicated that it was resistant to all tested β-lactams with the exception of ureidopenicillins such as piperacillin (Table 2). Similar MICs of β-lactams were obtained for all the *C. meningosepticum* isolates tested (data not shown). *E. coli* DH10B harboring recombinant plasmid pBS1 was resistant to the β-lactams tested but remained susceptible to cefepime, cephamycins, and carbapenems, therefore ruling out the role of the β-lactamase gene cloned into pBS1 in the carbapenem resistance of *C. meningosepticum* PINT. The MICs of the β-lactams were significantly lowered not only in the presence of clavulanic acid or tazobactam, as reported for a classical ESBL phenotype, but also in the presence of cefoxitin at concentrations as low as 0.1 μg/ml or of moxalactam and imipenem, both at 0.05 μg/ml.

Genetic and protein sequence analyses. Conjugation experiments failed to transfer any β-lactam resistance marker from *C. meningosepticum* PINT to rifampin-resistant *E. coli* JM109. However, no control was used in the assays for mating out from *C. meningosepticum* to *E. coli*. No plasmid was detected in *C. meningosepticum* PINT.

DNA sequence analysis of the 1,525-bp region downstream from the 1.9-kb insert of pBS1 revealed an open reading frame (ORF) of 891 bp encoding a 297-amino-acid preprotein (Fig. 2). No sequence typical of a class 1 integron was found within the insert. No ORF likely coding for a regulatory protein was found upstream of the β-lactamase gene.

The overall G+C content of the ORF was 33.9%, which lies close to the expected range of the G+C ratio of members of the *Chryseobacterium* (*Flavobacterium*) genus (37%) (34).

The mature protein (named CME-2 for *C. meningosepticum* ESBL) expressed in *E. coli* DH10B had a molecular mass of 31 kDa (data not shown). Within this protein sequence, seven boxes characteristic of β-lactamases possessing a serine-active site were found (14): box I, positions ABL 45 to 50; box II, positions ABL 70 to 73; box III, position ABL 105; box IV, position ABL 111; box V, position ABL 166; box VI, position ABL 210; and box VII, positions ABL 234 to 236 (Fig. 3). In

TABLE 2. MICs of β-lactams for *C. meningosepticum* PINT, *E. coli* DH10B harboring recombinant plasmid pBS1, and reference strain *E. coli* DH10B

β-Lactam[a]	MIC (μg/ml)		
	C. meningosepticum PINT	*E. coli* DH10B(pBS1)	*E. coli* DH10B
Amoxicillin	256	512	4
Amoxicillin + CLA	64	4	4
Amoxicillin + TZB	64	4	2
Amoxicillin + FOX	128	8	4
Amoxicillin + MOX	64	16	4
Amoxicillin + IPM	64	2	4
Ticarcillin	256	512	4
Ticarcillin + CLA	64	8	4
Ticarcillin + TZB	128	4	4
Ticarcillin + FOX	128	32	4
Ticarcillin + MOX	128	64	4
Ticarcillin + IPM	128	4	4
Piperacillin	32	8	1
Piperacillin + CLA	16	2	1
Piperacillin + TZB	16	2	1
Piperacillin + FOX	16	2	1
Piperacillin + MOX	32	2	1
Piperacillin + IPM	32	2	1
Cephalothin	512	256	2
Cefoxitin	32	4	1
Ceftazidime	256	32	0.5
Ceftazidime + CLA	16	0.5	0.5
Ceftazidime + TZB	16	0.25	0.5
Ceftazidime + FOX	16	1	0.5
Ceftazidime + MOX	64	2	0.5
Ceftazidime + IPM	64	0.5	0.5
Cefotaxime	64	4	0.12
Cefepime	32	0.5	0.03
Cefepime + CLA	4	0.03	0.03
Cefepime + TZB	4	0.03	0.03
Cefepime + FOX	8	0.06	0.03
Cefepime + MOX	16	0.06	0.03
Cefepime + IPM	16	0.06	0.03
Aztreonam	>512	16	0.25
Aztreonam + CLA	>512	0.12	0.25
Aztreonam + TZB	>512	0.25	0.25
Aztreonam + FOX	>512	0.25	0.25
Aztreonam + MOX	>512	0.5	0.25
Aztreonam + IPM	>512	0.25	0.25
Moxalactam	64	0.25	0.12
Imipenem	32	0.25	0.12

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml; FOX, cefoxitin at a fixed concentration of 0.1 μg/ml; IPM, imipenem at a fixed concentration of 0.5 μg/ml; MOX, moxalactam at a fixed concentration of 0.05 μg/ml.

addition, another class A conserved motif, serine-aspartic acid-asparagine (SDN), was found at positions 130 to 132 (Fig. 3).

The comparison of CME-2 with other class A β-lactamases revealed weak identity (Fig. 3). The highest degrees of homology were with VEB-1 from *E. coli* MG-1, CBL-A from *B. uniformis*, and PER-1 from *P. aeruginosa* RNL-1 (39, 35, and 34% identities, respectively). A dendrogram was constructed in order to relate CME-2 to representative Ambler class A ESBLs. CME-2 clustered with VEB-1, CFX-A, and CEP-A (Fig. 4).

Biochemical properties of CME-2 β-lactamase. Analytic isoelectric focusing revealed that *E. coli* DH10B harboring recombinant plasmid pBS1 produced only one β-lactamase with activity, and it had a pI of 9.2. In the original *C. meningosepticum* PINT isolate, β-lactamases with pI values of 7.6 and 8.3 were found. Only the β-lactamase with a pI of 7.6 corresponded to a serine β-lactamase.

After purification of the CME-2 β-lactamase from *E. coli* DH10B(pBS1), the specific activity of 22 $mU \cdot mg^{-1}$ of protein was determined with 100 μM benzylpenicillin as the substrate. The overall recovery of CME-2 was 41%, with a 120-fold β-lactamase purification. According to visual inspection of the SDS-polyacrylamide gel, the CME-2 β-lactamase gene product was weakly expressed from pBS1 in *E. coli*.

The kinetic parameters of the purified CME-2 β-lactamase revealed strong activity against cephalosporins, including extended-spectrum cephalosporins (Table 3), and its activity against aztreonam was also noticeable. CME-2 β-lactamase has no detectable activity against piperacillin, cephamycins, and imipenem (Table 3). IC_{50} results with benzylpenicillin as the substrate showed that CME-2 activity was strongly inhibited by clavulanic acid (0.05 μM), tazobactam (1.5 μM), and sulbactam (0.3 μM). In addition, unlike other class A ESBLs, CME-2 was inhibited more strongly by cefoxitin (0.004 μM), moxalactam (0.002 μM), and imipenem (0.015 μM).

Distribution of *bla*$_{CME-2}$ in *Chryseobacterium* sp. isolates. Using a set of primers designed to amplify a 743-bp internal fragment of *bla*$_{CME-2}$ by PCR, positive results were obtained for only five of nine *C. meningosepticum* strains (*C. meningosepticum* PINT, CIP 6058, AMA, GEO, and CIP 6059). Negative PCR results were obtained not only for four of nine *C. meningosepticum* isolates but also for all strains of the *C. meningosepticum*-related species (*Chryseobacterium indologenes*, *Sphingobacterium multivorum*, and *Myroides odoratus*) (data not shown). However, hybridization experiments with a *bla*$_{CME-2}$-specific internal probe gave positive results for the nine *C. meningosepticum* isolates and negative results for the strains of the *C. meningosepticum*-related species. In order to evaluate the distribution of a *bla*$_{CME-2}$-like gene within *C. meningosepticum* isolates, their DNAs were restricted with *Xmn*I and hybridized with three probes, probes S1 and S2, which are located upstream and downstream from the *Xmn*I site (*Xmn*I cuts in the middle of the *bla*$_{CME-2}$ gene), respectively, and S3, a *Dra*I internal fragment, giving a probe specific for the entire *bla*$_{CME-2}$ gene (Fig. 1). Six hybridization patterns were obtained for the nine *C. meningosepticum* isolates (Fig. 5). By using the S1 and S2 probes, *C. meningosepticum* PINT, CIP 6058, and AMA (pattern I) gave 3- and 1.8-kb fragments, respectively; *C. meningosepticum* GEO and CIP 6059 (pattern II) gave 2.7- and 1.8-kb fragments, respectively; *C. meningosepticum* CIP 7830 (pattern III) gave 3.5- and 1.9-kb fragments, respectively; *C. meningosepticum* CIP 7905 (pattern IV) gave 3.5- and 1.8-kb fragments, respectively; *C. meningosepticum* AB 1572 (pattern V) gave 3.7-kb fragments with both probes; and *C. meningosepticum* H01J100 (pattern VI) gave 3.8-kb fragments with both probes. S3 probe hybridizations gave patterns corresponding to the sum of the results obtained after the S1 and S2 probe hybridizations. Only patterns I and II gave positive results after *bla*$_{CME-2}$ PCR amplification.

Finally, induction studies with imipenem or cefoxitin as the inducer did not show that these ESBLs from *C. meningosepticum* would be inducible. As assessed by isoelectric focusing analysis, each isolate produced at least two β-lactamases. However, preincubation of the enzyme extracts with clavulanic acid showed that each *C. meningosepticum* isolate produced only one serine β-lactamase with variable pIs ranging from 7.0 to 8.5, with no correlation of the pIs with the hybridization patterns.

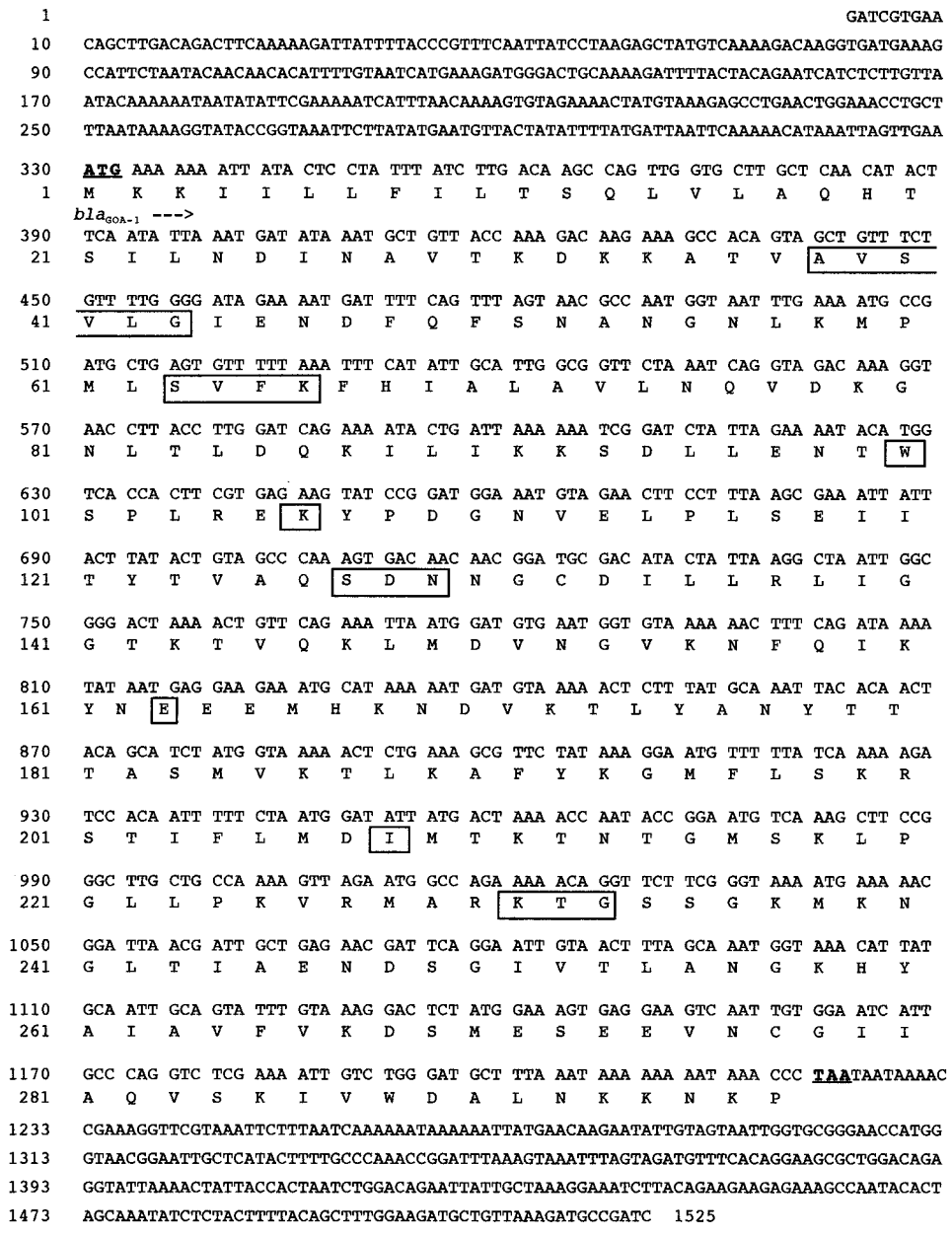

FIG. 2. Nucleotide sequence of the 1,525-bp fragment of pBS1 containing the 891-bp coding region for bla_{CME-2}. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. The seven conserved boxes described by Joris et al. (14) and the conserved SDN loop are boxed. The start and stop codons of the gene are in boldface type and are underlined.

DISCUSSION

The starting point of this study was the observation of a synergy between ceftazidime and cephamycins in a conventional antibiogram disk susceptibility assay performed with a *C. meningosepticum* clinical isolate. The overall β-lactam susceptibility pattern of this *C. meningosepticum* isolate corresponded to that previously described in these bacterial species characterized by resistance to all β-lactams tested, including extended-spectrum cephalosporins, monobactams, and carbapenems, and a moderated susceptibility to piperacillin (6, 20). This profile of resistance to broad-spectrum β-lactams may be explained for all β-lactams except cefepime and imipenem by the hydrolytic properties of the ESBL CME-2. For cefepime and imipenem the resistance is most likely mediated by the class B carbapenem-hydrolyzing β-lactamase recently identified in *C. meningosepticum* CIP 6058, a strain included in this study (36). The biochemical properties of CME-2 are similar to those of an unsequenced ESBL identified from another *C. meningosepticum* isolate (9). Both enzymes may be classified within Bush group 2be (5). This group includes several β-lactamases which are weakly related to CME-2, i.e., MEN-1, TOHO-1, and L-2. CME-2 activity not only was inhibited by clavulanic acid but was also strongly inhibited by cephamycins and imipenem. This inhibition profile is found in two other class A β-lactamases, VEB-1 from *E. coli* and CEP-A from *B. fragilis* (35). Comparison of their amino acid sequences did not provide evidence of

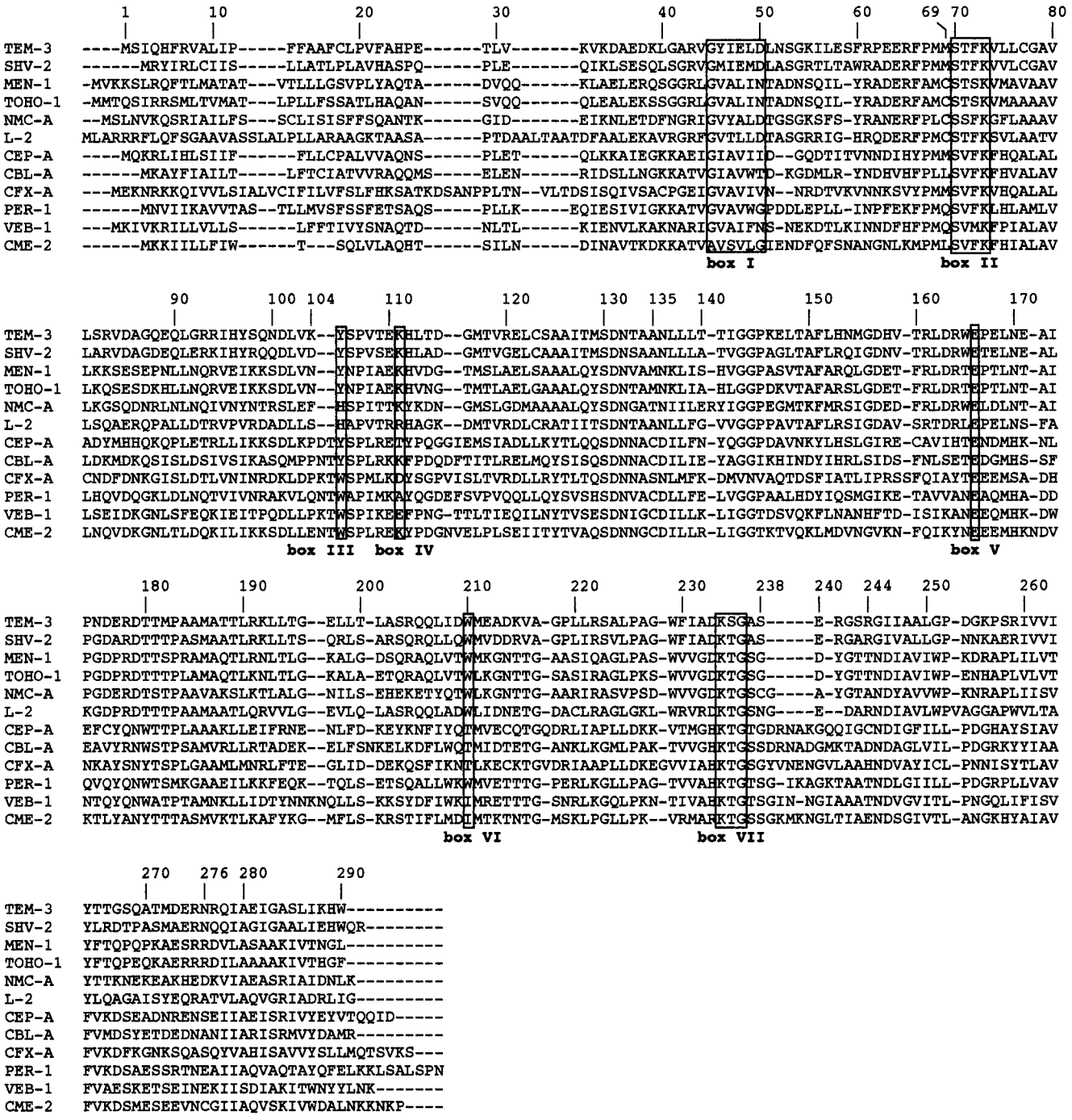

FIG. 3. Amino acid sequence alignment of CME-2 compared with those of selected class A β-lactamases. The numbering is according to Ambler (1). Roman numerals designate the boxes described by Joris et al. (14): box I, positions ABL 45 to 50; box II, positions ABL 70 to 73; box III, position ABL 105; box IV, position ABL 111; box V, position ABL 166; box VI, position ABL 210; box VII, positions ABL 234 to 236. The β-lactamases included in the alignment are TEM-3 from *K. pneumoniae* CFF104, SHV-2 from *K. ozaenae*, MEN-1 from *E. coli* MEN, TOHO-1 from *E. coli* TUH12191, NMC-A from *E. cloacae* NOR-1, L-2 from *S. maltophilia* 1275IID, CEP-A from *B. fragilis* CS30, CFX-A from *B. vulgatus* CLA341, VEB-1 from *E. coli* MG-1, CBL-A from *B. uniformis* WAL-7088, and PER-1 from *P. aeruginosa* RNL-1. Dashes indicate gaps within the alignment.

any particular amino acid identity which may explain this cephamycin inhibition property. This property may help to differentiate *C. meningosepticum* from other *Chryseobacterium* sp. isolates for which a synergy between cephamycins and ceftazidime is not observed.

CME-2 is only distantly related to other class A ESBLs. However, it belongs to a subgroup of class A β-lactamases, of which VEB-1 from *E. coli* MG-1 is the most closely related to CME-2 (39% amino acid identity).

Although isolated from *C. meningosepticum*, the CME-2 β-lactamase does not exhibit features common to class A β-lactamases isolated from some gram-negative species, such as

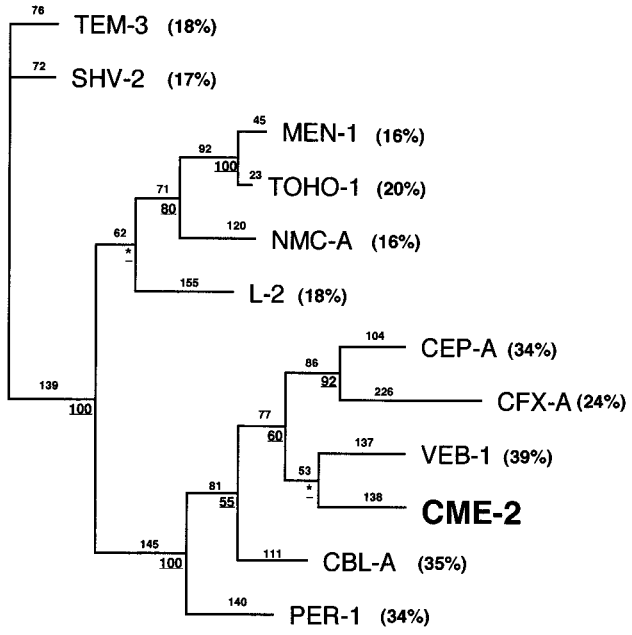

FIG. 4. Dendrograms obtained for 12 Ambler class A ESBLs by the parsimony method (44). Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The percentages at the branching points (underlined) refer to the number of times that a particular node was found in 100 bootstrap replications (the asterisks indicate uncertainty for nodes with bootstrap values of less than 50%). The distance along the vertical axis has no significance. Abbreviations for β-lactamases are given in the legend to Fig. 3. Percent amino acid identities to CME-2 are indicated in parentheses.

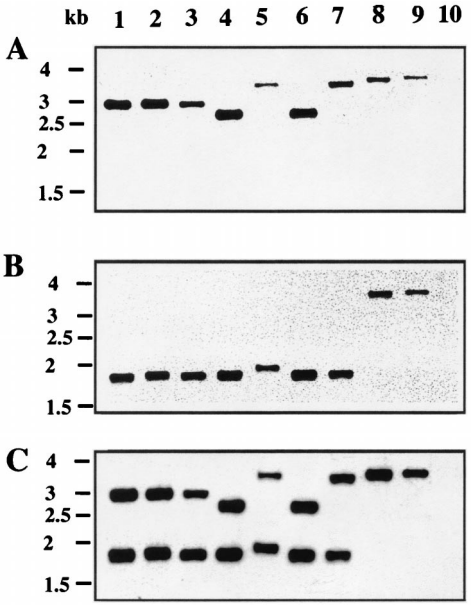

FIG. 5. Autoradiogram after Southern hybridizations of *C. meningosepticum* genomic DNAs. The DNAs were restricted with *Xmn*I, and the probe consisted of the 372-bp *Dra*I-*Xmn*I fragment (S1) of pBS1 (A), the 306-bp *Xmn*I-*Dra*I fragment (S2) of pBS1 (B), or the 667-bp *Dra*I fragment (S3) of pBS1 (C) (see Fig. 1 for the locations of S1, S2, and S3). Lanes: 1, *C. meningosepticum* PINT; 2, *C. meningosepticum* CIP 6058; 3, *C. meningosepticum* AMA; 4, *C. meningosepticum* GEO; 5, *C. meningosepticum* CIP 7830; 6, *C. meningosepticum* CIP 6059; 7, *C. meningosepticum* CIP 7905; 8, *C. meningosepticum* AB 1572; 9, *C. meningosepticum* H01J100; 10, *E. coli* DH10B (negative control). The sizes of the DNA fragments may be deduced from the scale (1.5 to 4 kb) shown on the left sides of the gels.

Cys77 and Cys123, which are thought to form a disulfide bridge (33, 38, 46). No cysteine residue is found at these positions in CME-2; instead, an alanine (as for VEB-1, PER-1, CFX-A, CBL-A, CEP-A, L-2, NMC-A, and TOHO-1) and an isoleucine (a leucine in VEB-1, PER-1, CFX-A, and CEP-A), respectively, are found (Fig. 3). Surprisingly, CME-2 possesses a cysteine residue at position 135, close to the SDN motif, as in VEB-1, PER-1, CBL-A, and CEP-A. It is interesting that within the non-TEM and non-SHV class A β-lactamases, cysteine residues are found close to either the SVFK or the SDN conserved motifs at a limited number of positions; position 69 (MEN-1, TOHO-1, NMC-A, and L-2), position 81 (CFX-A), position 123 (L-2), or position 135 (CEPA, CBL-A, PER-1, VEB-1, and CME-2). In CME-2, a disulfide bridge may be formed between cysteine 135 and cysteine 276, as evidenced for NMC-A between cysteine 69 and cysteine 238 (43).

TABLE 3. Kinetic parameters of the purified β-lactamase CME-2

Drug	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Cephaloridine	30.6	23.4	1.31
Benzylpenicilllin	12.3	7.4	1.66
Amoxicillin	6.7	26.5	0.26
Ticarcillin	1.8	5.8	0.31
Piperacillin	0.3	<1	—[a]
Cephalothin	60.6	41.5	1.46
Cefoxitin	<0.01	—	—
Cefuroxime	57.3	81.6	0.70
Cefoperazone	2.6	3.8	0.68
Cefotaxime	12.1	20.8	0.58
Ceftazidime	23.6	125	0.19
Aztreonam	3.2	10.5	0.31
Imipenem	<0.01	—	—

[a] —, not detectable.

The omega loop which goes from positions 169 to 179 is a structural element of class A enzymes. This loop is present in CME-2 but is totally different from those found in TEM or SHV derivatives. In this respect, CME-2 along with VEB-1, PER-1, CBL-A, and CEP-A possesses a histidine in place of an asparagine at position 170 (Fig. 3). This asparagine together with the highly conserved Glu166 and Ser70 is involved in the positioning of the active-site water molecule (19). It is tempting to speculate that this histidine may play a similar role in the catalytic properties of CME-2, but this needs to be confirmed by site-directed mutagenesis.

Many ESBLs are derived from the parental TEM-1, TEM-2, or SHV-1 enzymes by a few amino acid substitutions at position 104, 164, 238, or 240, leading to increased catalytic activities for cefotaxime, ceftazidime, and aztreonam. In the CME-2 β-lactamase, two additional amino acids are found at position 104 compared to those found in TEM-1, TEM-2, or SHV-1 derivatives. The Arg104Lys substitution in TEM-3 may correspond to either a glutamic acid, an asparagine, or a threonine in CME-2 (Fig. 3). In the PER-1 β-lactamase, additional amino acids (glutamine, asparagine, and threonine) are also found at position 104. PER-1 is the only non-TEM and non-SHV ESBL for which site-directed mutagenesis has so far been performed, but the roles of these amino acids at this position remain unclear (4).

At position 164, some TEM derivatives that possess ESBL properties have a serine or a histidine in place of an arginine. PER-1 possesses an alanine and CME-2 possesses a tyrosine.

Since the Ala164Arg substitution in PER-1 results in a mutant with no detectable activity (4), it would be interesting to study the effect of the Tyr164Arg substitution in CME-2. At positions 238 and 240, TEM-1, TEM-2, and SHV-1 possess glycine and glutamine residues, respectively. CME-2, like PER-1, possesses a serine at position 238, as found for some TEM and SHV ESBLs, and a glycine at position 240.

The G+C ratio of 33.9%, typical of *Chryseobacterium* species genes, together with negative conjugation and negative plasmid research results, indicated a likely chromosomal location of the bla_{CME-2} gene. No sequence typical of *E. coli* or *P. aeruginosa* promoters was identified upstream of the ATG initiation site. Interestingly, the pI value for CME-2 in *E. coli* DH10B (9.2) did not correspond to the pI value of the serine β-lactamase (7.6) found in *C. meningosepticum* PINT, thus indicating a putative difference in the cleavage site of the peptide leader. No regulatory protein gene was found immediately upstream of the bla_{CME-2} gene. Therefore, CME-2 expression may be either not regulated at all or not regulated like other chromosomally located class A ESBLs such as NMC-A and SME-1 (21, 22). In that respect, induction experiments failed to identify any inducible serine ESBL in *C. meningosepticum* isolates, whereas inducible L-2 enzymes are found in *S. maltophilia* (27). However, as found in *S. maltophilia* (28), several β-lactamases of Ambler class A and class B were identified in each *C. meningosepticum* isolate (data partially shown), and these may together account for the naturally occurring β-lactam resistance profiles of the *C. meningosepticum* isolates.

The results of PCR with nondegenerated primers for bla_{CME-2} detection showed that this technique may be too specific for the identification of bla_{CME-2}-like genes. However, hybridization experiments identified in each *C. meningosepticum* isolate a bla_{CME-2}-like gene that was present in only one copy. Moreover, they revealed that bla_{CME-2} gene variants are identified in all *C. meningosepticum* isolates tested, although their β-lactam resistance phenotypes were identical. The presence of a bla_{CME-2}-like gene in *C. meningosepticum* isolates makes it an identification marker for this bacterial species among *Chryseobacterium* species. Finally, the identification of CME-2 β-lactamase gives an additional clue that class A ESBLs may be a means for naturally occurring β-lactam resistance.

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ADDENDUM

After the work was submitted, we noticed a report from Rossolini et al. (36) showing a very similar enzyme, CME-1, from *C. meningosepticum* CCUG4310. CME-1 displays five amino acid changes with respect to CME-2: valine in CME-1 to isoleucine in CME-2 at position 157, isoleucine to methionine at position 278, and asparagine to lysine at position 295 and deletion of the last two amino acids, lysine and proline, of CME-1 in CME-2. This report explains why the name CME-2 was retained. None of the amino acid differences may be critical to the extended hydrolysis spectrum profile, which was found to be similar in both enzymes.

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