Characterization and sequence of the *Chryseobacterium* (*Flavobacterium*) *meningosepticum* carbapenemase: a new molecular class B β-lactamase showing a broad substrate profile

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The metallo-β-lactamase produced by *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum*, which is the flavobacterial species of greatest clinical relevance, was purified and characterized. The enzyme, named BlaB, contains a polypeptide with an apparent M_r of 26000, and has a pI of 8.5. It hydrolyses penicillins, cephalosporins (including cefoxitin), carbapenems and 6-β-iodopenicillanate, a mechanism-based inactivator of active-site serine β-lactamases. The enzyme was inhibited by EDTA, 1-10 phenanthroline and pyridine-2,6-dicarboxylic acid, with different inactivation parameters for each chelating agent. The *C. meningosepticum blaB* gene was cloned and sequenced. According to the G+C content and codon usage, the *blaB* gene appeared to be endogenous to the species. The BlaB enzyme showed significant sequence similarity to other class B β-lactamases, being overall more similar to members of subclass B1, which includes the metallo-enzymes of *Bacillus cereus* (Bc-II) and *Bacteroides fragilis* (CcrA) and the IMP-1 enzyme found in various microbial species, and more distantly related to the metallo-β-lactamases of *Aeromonas* spp. (CphA, CphA2 and ImiS) and of *Stenotrophomonas maltophilia* (L1).

INTRODUCTION

The clinical relevance of molecular class B β-lactamases [1], originally described in the non-pathogenic species *Bacillus cereus* [2], has significantly increased during the last few years. These metallo-enzymes have been found in several microbial species associated with infections in humans [3–5], and horizontal transfer of class B β-lactamase-encoding genes to clinically obnoxious species has also been demonstrated [4,6,7]. Their usually broad spectrum of activity, which includes carbapenems, together with their resistance to the potent mechanism-based inactivators of active-site serine β-lactamases [5] contribute to their threatening potential for a large proportion of our antimicrobial arsenal.

Enzymes belonging to this molecular class require a bivalent metal-ion cofactor (usually Zn^{2+}) for activity, and their primary structure and catalytic mechanism are completely different from those of the active-site serine β-lactamases [5,8,9].

A total of 12 genes encoding class B β-lactamases have been cloned and sequenced. These include: (i) three very similar alleles from *Bacil. cereus* 569/H, *Bacil. cereus* 5/B/6 and an alkalophilic *Bacillus* sp. respectively, encoding β-lactamase II variants [10–12]; (ii) four identical or very similar alleles from *Bacteroides fragilis* TAL3636, TAL3840, QMCN3 and QMCN4 respectively, encoding CcrA enzyme variants [5,13,14]; (iii) three very similar alleles from *Aeromonas hydrophila* AE036, *Ae. hydrophila* 19 and *Ae. veronii* bv. *sobria* 163a respectively, encoding CphA enzyme variants [5,15,16]; (iv) a gene from *Stenotrophomonas maltophilia* IID1275 encoding the L1 enzyme described in this species [17]; and (v) a gene encoding the IMP-1 β-lactamase originally found in *Serratia marcescens* clinical isolates [7,18] and subsequently detected also in isolates of other microbial species, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pseudomonas putida* and *Alcaligenes xylosoxidans* [4]. Although all class B enzymes share some conserved structural motifs, a considerable sequence divergence may occur among different members. According to the patterns of sequence similarity, the class B proteins can be grouped into three different subclasses: B1, which includes the *Bacillus* spp. and *Bact. fragilis* enzymes and the broad-host-range IMP-1 β-lactamase; B2, which includes the *Aeromonas* enzymes; and B3, which includes the *S. maltophilia* enzyme [5]. Structural differences parallel differences in enzymological properties, whereby members of subclasses B1 and B3 show very broad substrate profiles whereas the *Aeromonas* enzymes exhibit a much narrower substrate specificity [18–22].

Recently the crystal structures of two class B β-lactamases, Bc-II 569/H and CcrA, have been solved: both enzymes show a peculiar type of protein fold, including a $\beta\beta$ sandwich with helices on each external face [9,23].

Additional enzymes that probably belong to molecular class B have been reported in *Myroides odoratus* (formerly *Flavobacterium odoratum*) [24], *Chryseobacterium* (formerly *F.*) *meningosepticum* [25], *Legionella gormanii* [26] and *Burkholderia cepacia* [27]. However, a detailed molecular characterization of these enzymes has not been performed.

The objectives of this work were firstly to purify and characterize the metallo-β-lactamase produced by *C. meningosepticum* and secondly to clone and sequence the corresponding gene.

C. meningosepticum is currently considered as the ex-flavobacterial species of greatest clinical relevance, being responsible for cases of neonatal meningitis, adult septicaemia and nosocomial infections [28,29].

Abbreviations used: BHI, brain/heart infusion; LB, Luria–Bertani; ORF, open reading frame.

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Table 1 Purification of the metallo-β-lactamase of *C. meningosepticum* CCUG 4310

The crude lysate was obtained from 2 l of bacterial culture in BHI broth. These data are representative of three individual purifications performed. The specific carbapenemase activity of the crude enzyme extract and of the subsequent fractions was measured, for screening purposes, using 200 μM imipenem. This accounts for the apparent discrepancy between the k_{cat} for imipenem that could be calculated from this value and that reported in Table 2.

Step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification (fold)
Crude lysate	320	1.2	379	100	1
S-Sepharose FF	4.7	32	150	40	27
Mono Q HR-5/5	0.85	110	94	25	92

Table 2 Kinetic parameters of the purified metallo-β-lactamase

The K_m and k_{cat} values represent the means of three independent measurements, and are reported along with their S.D. values. Abbreviation: n.h., no hydrolysis detected. –, not done.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Imipenem	370 ± 15.5	350 ± 16.8	0.95
Penicillin G	32 ± 1.2	280 ± 10.3	8.75
Cephaloridine	29 ± 1.0	14 ± 0.6	0.48
Cefotaxime	180 ± 7.7	39 ± 1.7	0.22
Cefoxitin	24 ± 0.6	6 ± 0.2	0.25
Nitrocefin	66 ± 3.1	17 ± 0.8	0.26
Aztreonam	n.h.	n.h.	–
6-β-Iodopenicillanate	43 ± 2.1	300 ± 14.8	6.98

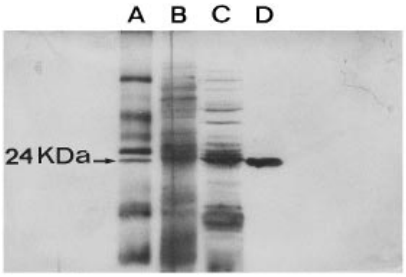

Figure 1 SDS/PAGE analysis of different purification steps of the *C. meningosepticum* metallo-β-lactamase

Lanes: A, mixture of standard proteins including (from top to bottom) BSA (M_r 66 000), chicken egg ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000), trypsin inhibitor (M_r 20 000), α-lactalbumin (M_r 14 000) and aprotinin (M_r 6500); B, crude protein extract; C, active peak from S-Sepharose chromatography; D, active peak from Mono Q chromatography. After electrophoresis, protein bands were detected by silver staining [33].

MATERIALS AND METHODS

Chemicals

Antibiotics

Nitrocefin was obtained from Unipath (Milan, Italy); penicillin G and cephaloridine were from Sigma (St. Louis, MO, U.S.A.); cefoxitin and imipenem were from Merck Sharp and Dohme (Rahway, NJ, U.S.A.); cefotaxime was from Hoechst (Frankfurt, Germany); aztreonam was from Squibb (Princeton, NJ, U.S.A.); and β-iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, U.K.). All antibiotic solutions were prepared immediately before use.

Other chemicals

S-Sepharose FF and the Mono Q HR-5/5 column were from Pharmacia (Uppsala, Sweden). The TSK-2000 column was from LKB (Uppsala, Sweden). Other chemicals were from Sigma unless otherwise specified.

Bacterial strains and culture conditions

C. meningosepticum CCUG 4310 (NCTC 10585) was used as the source of the original protein and of genomic DNA for construction of the library. *C. meningosepticum* was grown aerobically at 28 °C in brain/heart infusion (BHI) broth (Difco, Detroit, MI, U.S.A.). *Escherichia coli* DH5α [*supE*44 Δ*lac*U169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] (Gibco-BRL, Gaithersburg, MD, U.S.A.) was used as the host for recombinant DNA procedures. *E. coli* clones were grown aerobically at 37 °C in Luria–Bertani (LB) medium [30], supplemented with the appropriate antibiotics when necessary.

Assay for protein concentration

Protein concentrations were determined by the method of Bradford [31] with BSA as a standard.

Purification of the *C. meningosepticum* metallo-β-lactamase

Preparation of the crude enzyme extract

C. meningosepticum CCUG 4310 was grown overnight at 28 °C in 2 l of BHI broth. Thereafter, all purification steps were performed at 4 °C. Cells were harvested by centrifugation at 10 000 ***g*** for 30 min, washed twice with 50 mM sodium phosphate buffer, pH 6.5 (PB), resuspended in 100 ml of PB, and disrupted by sonication (5 × 30 s at 60 W). Cell debris were removed by centrifugation at 105 000 ***g*** for 1 h. The cleared supernatant constituted the crude enzyme extract. Carbapenemase activity was assayed in the crude enzyme extract and during the following steps, to monitor the purification process. These screening assays were performed by measuring the hydrolysis of 200 μM imipenem at 300 nm ($\Delta\epsilon = -9000\ M^{-1} \cdot cm^{-1}$) in 30 mM sodium cacodylate buffer, pH 6.5 (CB), at 30 °C. One unit of carbapenemase activity hydrolyses 1 μmol of substrate/min under these assay conditions.

S-Sepharose ion-exchange chromatography

After dialysis against PB, the crude enzyme extract was loaded on to a pre-equilibrated S-Sepharose FF column (column bed 2.5 cm × 25 cm) at a flow rate of 3 ml/min. The column was washed with PB until the A_{280} was below 0.05, and the enzyme was eluted by a linear NaCl gradient (0–1 M) in PB (gradient volume of 400 ml). The carbapenemase activity was eluted at 210–230 mM NaCl.

Mono Q ion-exchange chromatography

The fractions containing the carbapenemase activity were pooled, dialysed against 30 mM Tris/HCl buffer, pH 9, containing 50 μM $ZnCl_2$ and loaded on to a pre-equilibrated Mono Q HR

Table 3 Comparison of the k_{cat}/K_m values ($\mu M^{-1} \cdot s^{-1}$) of the *C. meningosepticum* metallo-β-lactamase with those of other class B β-lactamases

Bc-II, β-lactamase II of *Bacil. cereus* 5/B/6 [20]; CphA, CphA enzyme of *Ae. hydrophila* AE036 [20]; ImiS, ImiS enzyme of *Ae. veronii* bv. *sobria* 163a [22]; L1, L1 enzyme of *S. maltophilia* ULA-511 [20]; CcrA, CcrA enzyme of *Bact. fragilis* TAL3636 [19]. Abbreviations: Inact., inactivation; n.h., no hydrolysis detected; n.i., no interaction. –, not done.

	Enzyme					
Substrate	BlaB	Bc-II	CphA	ImiS	L1	CcrA
Imipenem	0.95	0.12	2.10	0.91	0.73	0.74
Penicillin G	8.75	0.45	0.0074	0.0021	22.00	4.80
Cephaloridine	0.48	0.019	0.0006	n.h.	0.093	7.00
Cefotaxime	0.22	0.67	0.0027	n.h.	2.60	3.60
Cefoxitin	0.25	0.00009	Inact.	n.h.	0.55	0.09
Nitrocefin	0.26	0.64	0.0003	0.004	2.90	12.50
Aztreonam	n.h.	n.i.	n.i.	–	n.i.	n.h.
β-Iodopenicillanate	6.98	0.06	0.0012	0.0028	1.00	–

5/5 column at a flow rate of 1 ml/min. The column was washed with the same buffer until the A_{280} was below 0.02, and the enzyme was eluted by using a linear NaCl gradient (0–0.7 M) in the same buffer (gradient volume of 25 ml). The fractions containing the carbapenemase activity, eluted at 210 mM NaCl, were pooled and immediately dialysed against CB containing 50 μM $ZnCl_2$. The enzyme preparation was stored at −80 °C.

SDS/PAGE analysis

SDS/PAGE of protein preparations was performed according to Laemmli [32], using a Mini Protean II apparatus (Bio-Rad, Richmond, CA, U.S.A.). The final acrylamide concentrations in the 1.0-mm-thick slab gel were 15% and 5% (w/v) for the separating and the stacking gels respectively. Before loading, all samples were heated for 2 min at 100 °C in Laemmli sample buffer [32]. Electrophoresis was carried out at a constant voltage of 140 V. The protein bands were stained either with Coomassie Brilliant Blue R-250 or with silver staining [33].

Isoelectric focusing

Analytical isoelectric focusing was performed with the help of a Multiphor II flat bed apparatus (Pharmacia) using pre-cast 6% acrylamide gels and an ampholine gradient in the pH range of 3.5–9.5 (Pharmacia). Proteins were focused at constant temperature (6 °C) for 3 h at 1 W/cm. After focusing, protein bands were stained with Coomassie Brilliant Blue R-250. Alternatively, the β-lactamase activity was revealed by the appearance of a purple-stained band after overlaying the gel with filter paper previously soaked in the chromogenic substrate nitrocefin at a 250 μM concentration in CB.

Gel-permeation chromatography

The M_r of the native protein was determined by means of gel-permeation chromatography using a TSK-2000 column (column bed 0.75 cm × 60 cm) equilibrated and eluted with PB, at a flow rate of 0.5 ml/min. The column was calibrated with standard proteins (Sigma) dissolved in the elution buffer. The peak fraction of each protein was determined by monitoring A_{280}. A linear plot of the partition coefficients against the logs of the molecular masses of the standard proteins was used to estimate the M_r of the β-lactamase.

N-terminus sequencing

The N-terminus sequence of the purified protein was determined using a gas-phase sequencer (Procise™-492; Applied Biosystems, Foster City, CA, U.S.A.), after resuspension of the protein (50 pmol) in a 0.1% (v/v) trifluoroacetic acid solution and loading the sample on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.).

Extraction of periplasmic proteins

Periplasmic proteins were extracted from bacterial cultures in the late-exponential phase of growth using the chloroform method [34]. Carbapenemase activity in periplasmic extracts was assayed as described for crude extracts.

Determination of kinetic parameters

Substrate hydrolysis was monitored by following the absorbance variation using a lambda 2 spectrophotometer (Perkin Elmer, Rahway, NJ, U.S.A.) equipped with thermostatically controlled cells and connected to an IBM-compatible personal computer via an RS232C serial interface. The wavelengths and absorbance variations were as previously described [35]. K_m and k_{cat} values were determined by analysing either the complete hydrolysis time courses [36] or under initial-rate conditions using the Hanes' linearization of the Michaelis–Menten equation. All the determinations were performed at 30 °C in CB supplemented with 50 μM $ZnCl_2$.

Inactivation by Zn^{2+} removal was studied at 30 °C using 200 μM imipenem as the reporter substrate in metal-free CB, in the presence of different concentrations of EDTA, 1-10 phenanthroline or pyridine-2,6-dicarboxylic acid.

The total reaction volume was 0.6 ml in all cases.

Genetic vectors

Plasmid pACYC184 [37] was used as the vector for construction of the *C. meningosepticum* library. Plasmid pBluescript SK (Stratagene, La Jolla, CA, U.S.A.) was used for some subcloning steps.

Recombinant DNA methodology

Basic recombinant DNA procedures were performed as described by Sambrook et al. [30]. Extraction of genomic DNA from *C. meningosepticum* was performed as previously described [38]. For construction of the *C. meningosepticum* genomic library, the DNA extracted from the reference CCUG 4310 strain was partially digested with the restriction endonuclease *Sau*3AI to obtain preferentially fragments in the 5–20 kb size range. Smaller fragments (< 1 kb) were removed by gel-permeation chromatography using a Cromaspin 1000 column (Clontech, Palo Alto, CA, U.S.A.) previously equilibrated with distilled water. Restriction fragments eluted from the column were ligated to a *Bam*HI-linearized and dephosphorylated pACYC184 vector, and the ligation mixture was used to transform *E. coli* DH5α by electroporation. Transformants were selected on LB agar plates containing 85 μg/ml chloramphenicol. The ratio of recombinant clones to clones carrying a re-ligated vector was > 25, as shown by replica plating of transformants on to plates containing both chloramphenicol and tetracycline (20 μg/ml). Southern-blot analysis was performed on nylon membranes (Hybond-N; Amersham, Little Chalfont, U.K.) according to the manufacturer's instructions, using random-primed ^{32}P-labelled probes. Restriction endonucleases and DNA modification enzymes were from Boehringer (Mannheim, Germany).

DNA sequencing and computer analysis of sequence data

DNA sequencing was performed by the dideoxy-chain termination method using a sequenase 2.0 DNA sequencing kit (Amersham) and custom sequencing primers. Sequences were completely determined on both strands, using denatured double-stranded DNA templates. Computer analyses of sequence data were performed using an updated version (8.0.1) of the UWGCG package [39]. Comparison of codon-usage tables was performed using the CORRESPOND program. Multiple sequence alignment was generated using the PILEUP program, and subsequently refined according to the structural information available for the *Bacil. cereus* Bc-II 569/H [9] and the *Bact. fragilis* CcrA [23] enzymes. Calculation of distance values between different amino acid sequences was performed using the DISTANCES program on the basis of the definitive sequence alignment. Phyletic analysis was performed using the GROWTREE program according to the neighbour-joining method [40], on the basis of the previously calculated distance matrix.

RESULTS

Purification of the metallo-β-lactamase produced by *C. meningosepticum* CCUG4310

C. meningosepticum isolates produce a metallo-β-lactamase active on carbapenem compounds ([25]; G. M. Rossolini, N. Franceschini, G. Lombardi and G. Amicosante, unpublished work).

The metallo-β-lactamase produced by the *C. meningosepticum* CCUG 4310 (NCTC 10585) reference strain, which was previously shown to be highly related to most *C. meningosepticum* clinical isolates [41], was purified by two sequential ion-exchange chromatography steps starting from the crude bacterial lysate as described in the Materials and methods section. Results of a typical purification experiment are shown in Table 1. The initial S-Sepharose chromatography step removed a high percentage of the contaminating proteins. After the Mono Q chromatography step, the protein was more than 90% pure, as evaluated by SDS/PAGE (Figure 1).

Molecular mass, pI and N-terminus sequence of the *C. meningosepticum* metallo-β-lactamase

The purified protein, analysed by SDS/PAGE, appeared as a single major band with an apparent M_r of approx. 26000 (Figure 1). Analysis of the purified protein by isoelectric focusing showed that the major protein component had an isoelectric pH of 8.5 and was endowed with β-lactamase activity (results not shown). The M_r of the β-lactamase, evaluated by gel-permeation chromatography, was approx. 25000, indicating that the native enzyme is monomeric. The N-terminus sequence of the protein was determined as NH_2-QENPD. The protein was named BlaB.

Kinetic parameters of the BlaB enzyme and sensitivity to inhibitors

The BlaB enzyme hydrolysed penicillins, cephalosporins (including cefoxitin), carbapenems and 6-β-iodopenicillanate, a mechanism-based inactivator of serine β-lactamases. No hydrolysis of aztreonam was detected. The catalytic efficiencies towards the studied substrates appeared to be generally high. 6-β-iodopenicillanate appeared to be one of the best substrates (Table 2).

A comparison of the catalytic efficiencies of the BlaB enzyme with those of other class B β-lactamases, considering various substrates, is reported in Table 3. The behaviour of BlaB was,

Table 4 Kinetic parameters for the inactivation of the purified metallo-β-lactamase by chelating agents

The K and k_{+2} values represent the means of three independent measurements and are reported along with their S.D. values.

Chelating agent	K (μM)	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} \cdot s^{-1}$)
EDTA	1540 ± 72.3	$1.24 \times 10^{-2} \pm 5.7 \times 10^{-4}$	8.1
1-10 Phenanthroline	213 ± 8.3	$0.96 \times 10^{-2} \pm 4.3 \times 10^{-4}$	45.1
Pyridine-2,6-dicarboxylic acid	155 ± 6.2	$4.06 \times 10^{-2} \pm 1.9 \times 10^{-3}$	261.9

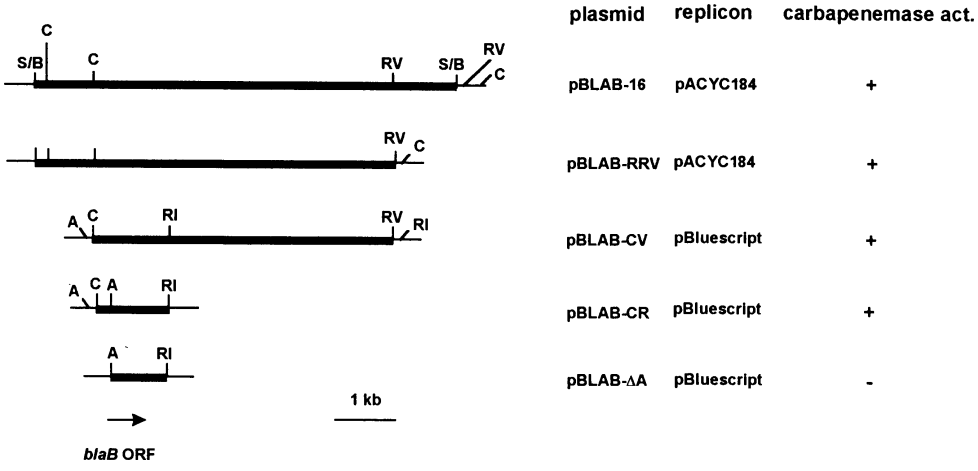

Figure 2 Restriction map of the DNA insert of plasmid pBLAB-16 and subcloning strategy

Insert sequences are represented by thick lines; vector sequences are represented by thin lines. The location and orientation of the *blaB* open reading frame (ORF) is indicated. Production of carbapenemase activity (act.) by *E. coli* clones carrying the recombinant plasmids was assayed in crude extracts as described in the Materials and methods section. Abbreviations: A, *Acc*I; C, *Cla*I; RI, *Eco*RI; RV, *Eco*RV; S/B, *Sau*3AI/*Bam*HI junction.

overall, similar to that of other broad-spectrum metallo-β-lactamases (Bc-II, CcrA and L1) and different from that of the narrow-spectrum *Aeromonas* enzymes. When compared with BC-II, CcrA and L1, BlaB appeared to be more active towards 6-β-iodopenicillanate and exhibited very similar catalytic efficiencies towards different cephalosporin substrates. It was also more active on cefoxitin than the two other monomeric enzymes of subclass B1 (the *Stenotrophomonas* L1 enzyme is a tetramer and belongs to subclass B3).

The activity of the BlaB enzyme was inhibited by various chelating agents, including EDTA, 1-10 phenanthroline and pyridine-2,6-dicarboxylic acid. The inactivation time courses followed pseudo-first-order kinetics. The inactivation rates increased with the chelating agent concentrations in a hyperbolic manner. This indicated that those compounds did not act by simply scavenging the free metal ions. In consequence, the data were analysed on the basis of the following model:

$$E \cdot Zn + C \overset{K}{\leftrightarrow} E \cdot Zn \cdot C \overset{k_{+2}}{\rightarrow} E + Zn \cdot C$$

where E is the enzyme, C is the chelating agent, K is the dissociation constant of the ternary $E \cdot Zn \cdot C$ complex and k_{+2} is a first-order rate constant. The individual values of K and k_{+2} (Table 4) were determined by fitting the value of k_i, the pseudo-first-order inactivation rate constant, to the following equation:

$$k_i = \frac{k_{+2}[C]}{K\frac{K_m^s + [S]}{K_m^s} + [C]}$$

where [S] and K_m^s are, respectively, the concentration and K_m value of the reporter substrate.

Under our experimental conditions, the second step appeared to be essentially irreversible.

Molecular cloning of the *blaB* gene

A genomic library of *C. meningosepticum* CCUG 4310 was constructed in the pACYC184 plasmid vector and transformed into the *E. coli* DH5α strain. Approx. 16000 transformants, grown on LB/chloramphenicol medium, were replica plated on to LB/chloramphenicol medium containing 50 μg/ml ampicillin. Eight clones were isolated that grew in the presence of ampicillin and produced a carbapenemase activity sensitive to inhibition by 1,10-phenanthroline. Restriction mapping and cross-hybridization experiments indicated that the eight clones carried overlapping DNA inserts (results not shown).

Clone pBLAB-16, which carried a 7 kb DNA insert, was selected for further characterization. Subclones of the DNA insert carried by pBLAB-16 were generated, and metallo-carbapenemase production was assayed for each subclone. This subcloning analysis indicated that the metallo-β-lactamase-encoding gene was apparently located within a 1.2 kb *Cla*I–*Eco*RI restriction fragment (Figure 2).

Southern-blot analysis performed on the genomic DNA of *C. meningosepticum* CCUG 4310 digested with *Cla*I, *Eco*RV and *Eco*RI, using the 1.2 kb *Cla*I–*Eco*RI DNA insert carried by pBLAB-CR as a probe, confirmed the origin of the cloned fragment from a single genomic region of the donor strain (results not shown).

Nucleotide sequence of the *blaB* gene and deduced amino acid sequence of the BlaB protein

The DNA insert of plasmid pBLAB-16CR was sequenced. A

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   ATCGATGTGGGAGACTGCTTTGGATATGAATACCAAGTATAAAGAAACTTCGGAAGGAGG
 1 ---------+---------+---------+---------+---------+---------+ 60

   GTTGGCTGTTGCAGTGAATGTAGCAGAGTGTTAATGATAAAATTGAATAAGAAATGTTGA
61 ---------+---------+---------+---------+---------+---------+ 120
                                                      M  L

   AAAAAATAAAAATAAGCTTGATTCTTGCTCTTGGGCTTACCAGTTTGCAGGCATTTGGAC
121 ---------+---------+---------+---------+---------+---------+ 180
   K  K  I  K  I  S  L  I  L  A  L  G  L  T  S  L  Q  A  F  G

   AGGAGAATCCTGATGTCAAAATTGAAAAGCTAAAAGATAATCTGTATGTATACACAACCT
181 ---------+---------+---------+---------+---------+---------+ 240
   Q  E  N  P  D  V  K  I  E  K  L  K  D  N  L  Y  V  Y  T  T

   ACAATACATTTAACGGGACTAAATATGCCGCAAATGCAGTATATCTGGTAACTGATAAGG
241 ---------+---------+---------+---------+---------+---------+ 300
   Y  N  T  F  N  G  T  K  Y  A  A  N  A  V  Y  L  V  T  D  K

   GTGTTGTGGTTATAGACTGTCCGTGGGGAGAAGACAAATTTAAAAGCTTTACGGACGAGA
301 ---------+---------+---------+---------+---------+---------+ 360
   G  V  V  V  I  D  C  P  W  G  E  D  K  F  K  S  F  T  D  E

   TTTATAAAAAACACGGAAAGAAAGTTATTATGAATATTGCAACACATTCTCATGATGATC
361 ---------+---------+---------+---------+---------+---------+ 420
   I  Y  K  K  H  G  K  K  V  I  M  N  I  A  T  H  S  H  D  D

   GTGCCGGAGGTCTTGAATATTTTGGTAAAATAGGTGCAAAAACTTATTCTACTAAAATGA
421 ---------+---------+---------+---------+---------+---------+ 480
   R  A  G  G  L  E  Y  F  G  K  I  G  A  K  T  Y  S  T  K  M

   CAGATTCTATTTTAGCAAAAGAGAATAAGCCAAGAGCACAATATACTTTTGACAATAATA
481 ---------+---------+---------+---------+---------+---------+ 540
   T  D  S  I  L  A  K  E  N  K  P  R  A  Q  Y  T  F  D  N  N

   AATCTTTCAAAGTAGGAAAATCCGAGTTTCAGGTTTACTATCCCGGAAAAGGGCACACAG
541 ---------+---------+---------+---------+---------+---------+ 600
   K  S  F  K  V  G  K  S  E  F  Q  V  Y  Y  P  G  K  G  H  T

   CAGATAATGTGGTGGTATGGTTTCCAAAAGAAAAAGTATTGGTTGGAGGTTGTATTATAA
601 ---------+---------+---------+---------+---------+---------+ 660
   A  D  N  V  V  V  W  F  P  K  E  K  V  L  V  G  G  C  I  I

   AAAGTGCTGATTCAAAGGACCTGGGGTATATTGGAGAAGCATATGTAAACGACTGGACGC
661 ---------+---------+---------+---------+---------+---------+ 720
   K  S  A  D  S  K  D  L  G  Y  I  G  E  A  Y  V  N  D  W  T

   AGTCTGTACACAATATTCAACAAAAGTTTTCCGGTGCTCAGTACGTTGTTGCAGGGCATG
721 ---------+---------+---------+---------+---------+---------+ 780
   Q  S  V  H  N  I  Q  Q  K  F  S  G  A  Q  Y  V  V  A  G  H

   ATGATTGGAAAGATCAAAGATCAATACAACATACACTAGACTTAATCAATGAATATCAAC
781 ---------+---------+---------+---------+---------+---------+ 840
   D  D  W  K  D  Q  R  S  I  Q  H  T  L  D  L  I  N  E  Y  Q

   AAAAACAAAAGGCTTCAAATTAATGAAGCCTTTTTCCTTAATTTAAACTGAACGCTTAAC
841 ---------+---------+---------+---------+---------+---------+ 900
   Q  K  Q  K  A  S  N  *
```

Figure 3 Nucleotide sequence of the *blaB* gene and flanking regions

Nucleotide number 1 corresponds to the first base of the *Cla*I restriction site located upstream of the gene. The deduced amino acid sequence of the BlaB protein is shown below the nucleotide sequence. The underlined region corresponds to the experimentally determined leader peptide for secretion. The two arrows indicate an 11 bp inverted repeat that could function as a transcriptional terminator.

747 nt open reading frame (ORF), which encoded a polypeptide containing a sequence identical with the N-terminal sequence of the purified BlaB proteins, was identified in the cloned insert (Figure 3). According to these findings, which were also consistent with results of subcloning and expression experiments (Figure 2), this ORF was identified as the *blaB* gene.

The N-terminus of the 247-residue polypeptide encoded by the *blaB* ORF exhibits a sequence pattern typical of signal peptides targeting proteins for secretion into the periplasmic space via the general secretory pathway [42]. Data from N-terminal sequencing of the purified protein confirmed that this region is actually removed from the mature protein and indicated that the signal peptidase cleavage site is located after the Gly^{22} residue (Figure 3). This would yield a mature protein of calculated molecular mass and pI value of 25803 Da and 9.00 respectively, which is in good agreement with the SDS/PAGE and isoelectric focusing data. The periplasmic location of the BlaB enzyme was confirmed by finding that the carbapenemase activity present in crude

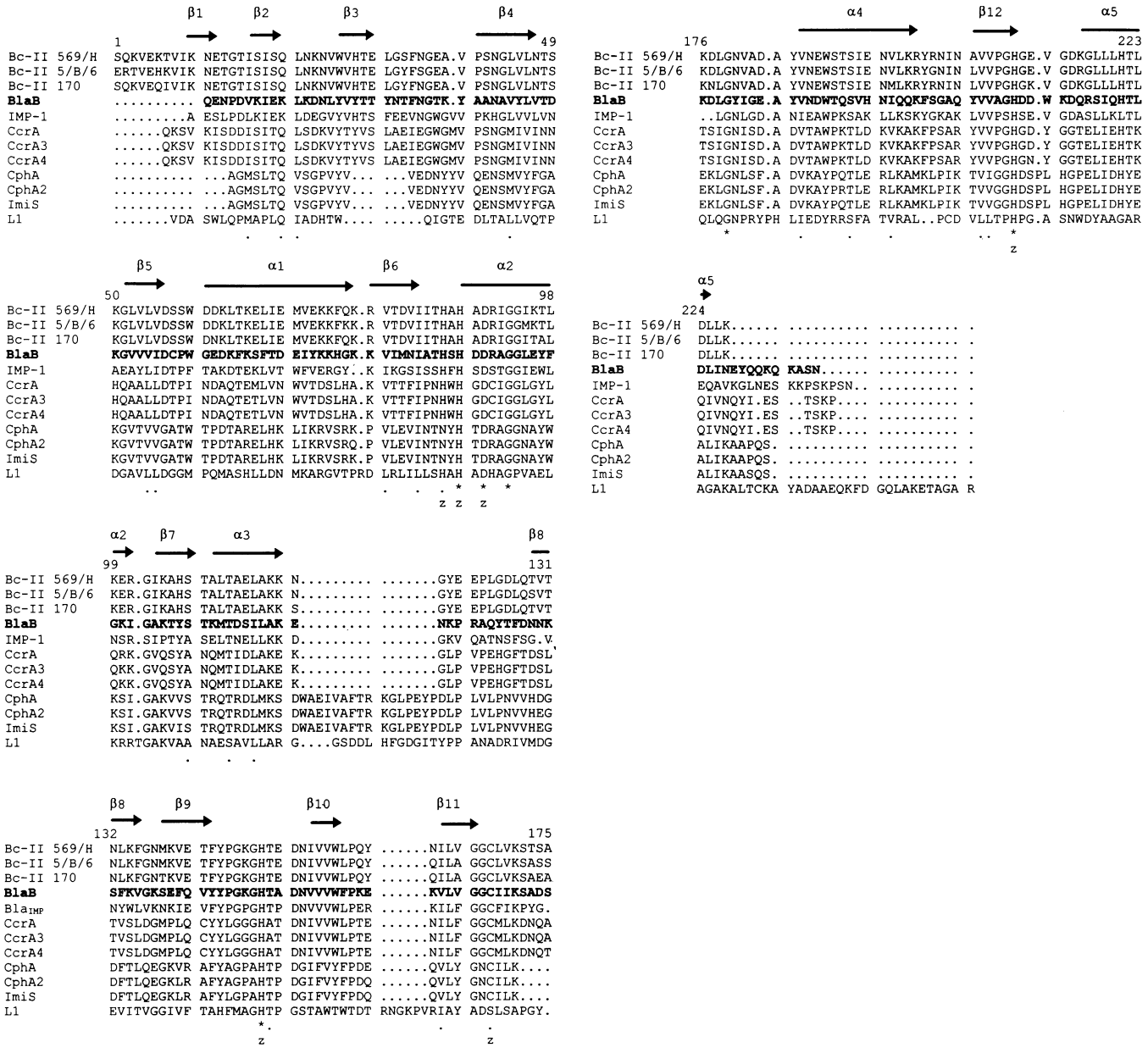

Figure 4 Amino acid sequence comparison of the mature metallo-β-lactamase of *C. meningosepticum* (BlaB) with the other sequenced class B metallo-β-lactamases

Bc-II 569/H, β-lactamase II of *Bacil. cereus* 569/H [10]; Bc-II 5/B/6, β-lactamase II of *Bacil. cereus* 5/B/6 [11]; Bc-II 170, β-lactamase II of alkalophilic *Bacil.* sp. strain 170 [12]; IMP-1, IMP-1 enzyme found in *S. marcescens* and also reported in *K. pneumoniae*, *P. aeruginosa*, *P. putida* and *Al. xylosoxidans* [4,7,18]; CcrA, CcrA/CfiA enzyme of *Bact. fragilis* strains TAL 3636/TAL 2840 [13,14]; CcrA3, CcrA3 enzyme of *Bact. fragilis* QMCN3 [5]; CcrA4, CcrA4 enzyme of *Bact. fragilis* QMCN4 [5]; CphA, CphA enzyme of *Ae. hydrophila* AE036 [15]; CphA2, CphA2 enzyme of *Ae. hydrophila* 19 [5]; ImiS enzyme of *Ae. veronii* bv. *sobria* 163a [16]; L1, L1 enzyme of *S. maltophilia* IID1275 [17]. The BlaB sequence is printed in bold. The numbering is that of the *Bacil. cereus* 569/H enzyme. Below the sequences, identical amino acid residues are indicated by an asterisk, conservative amino acid substitutions by a dot, and residues involved in zinc coordination by a z. Secondary-structure elements of Bc-II [9] are also indicated, above the sequences. Within the sequences, dots indicate gaps introduced to align the sequences.

extracts was quantitatively (> 80 %) recoverable in periplasmic preparations obtained from the same bacterial cultures.

At the 3′ end of the *blaB* ORF an 11 nt inverted repeat was found, which could function as a transcriptional terminator (Figure 3).

The G+C content of the *blaB* ORF (35.4 %) was similar to that of the other *C. meningosepticum* genes recorded in release 51 of the EMBL sequence database (36.1–41.6 %). The codon usage of the *blaB* ORF did not significantly differ (D-squared value = 1.35) from those calculated for the same set of *C. meningosepticum* genes.

Sequence comparison of the BlaB protein with other class B metallo-β-lactamases

The deduced amino acid sequence of the BlaB protein showed significant similarity to those of other sequenced molecular class B metallo-β-lactamases (Figure 4).

Table 5 Distances between the amino acid sequences of class N β-lactamases

Since values higher than 70 were obtained, no correction method was adopted for the calculation of the distance values. The names of the enzymes are as in Figure 4. –, not applicable.

Enzyme	Bc-II 569/H	Bc-II 5/B/6	Bc-II 170	BlaB	IMP-1	CcrA	CcrA3	CcrA4	CphA	CphA2	ImiS	L1
Bc-II 569/H	–	7.5	7.1	63.6	63.4	66.5	66.5	67.0	70.1	70.6	70.1	81.7
Bc-II 5/B/6		–	9.2	63.6	63.4	67.4	67.4	67.4	69.6	70.1	69.6	81.7
Bc-II 170			–	64.1	63.4	68.3	68.3	68.8	69.6	70.1	69.6	82.2
BlaB				–	68.9	71.4	71.0	71.4	71.6	72.6	71.6	88.8
IMP-1					–	63.2	63.2	63.2	77.2	76.7	76.7	84.7
CcrA						–	0.9	1.7	71.8	71.3	70.8	85.8
CcrA3							–	0.9	71.8	71.3	70.8	86.2
CcrA4								–	71.8	71.3	70.8	86.2
CphA									–	2.6	3.1	83.4
CphA2										–	2.2	83.9
ImiS											–	84.3
L1												–

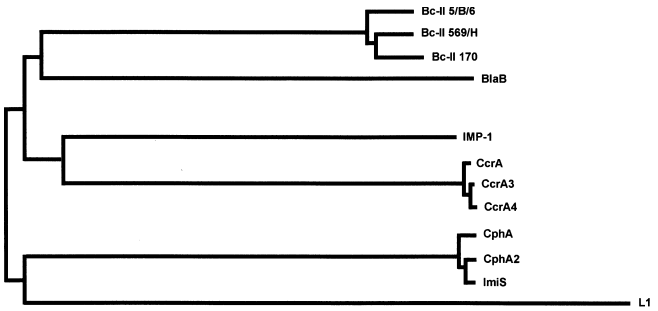

Figure 5 Unrooted phylogenetic tree of molecular class B β-lactamases

The names of the enzymes are as in Figure 4.

All the residues known to be involved either directly or indirectly in the co-ordination of Zn^{2+} ions (His^{86}, His^{88}, Asp^{90}, His^{149}, Cys^{168} and His^{210}, in the numbering of the Bc-II enzyme) [9,23] are conserved in the *C. meningosepticum* BlaB enzyme. Of the additional sixteen residues known to be in or close to the active site of the Bc-II enzyme [9], nine (Asn^{42}, Trp^{59}, Lys^{117}, Lys^{147}, Thr^{150}, Lys^{171}, Asp^{177}, Gly^{179} and Tyr^{185}) were found to be conserved in the BlaB protein, whereas four (Leu^{110}, Leu^{114}, Asp^{183} and Glu^{214}) were conservatively substituted. Only Asn^{118}, Glu^{151} and Asn^{180} were non-conservatively substituted, the latter two being replaced by hydrophobic residues (Figure 4).

According to the results of multiple sequence alignment (Figure 4) and to the analysis of the distance values among the various class B β-lactamases (Table 5), the *C. meningosepticum* BlaB protein appeared to be more closely related to members of subclass B1 (i.e. the *Bacil. cereus* and *Bact. fragilis* enzymes, and the broad-host-range IMP-1 enzyme).

Assuming a monophyletic origin for genes of this family, phylogenetic analysis suggested that the BlaB and Bc-II enzymes have evolved from a common ancestor within the lineage of subclass B1 enzymes (Figure 5).

DISCUSSION

Metallo-β-lactamases, although not as common as active-site serine β-lactamases, are becoming increasingly relevant for their contribution to β-lactam resistance in clinical isolates [4,5]. As a consequence, the study of these enzymes, initially considered a mere biochemical curiosity, has recently undergone a considerable increase in the eventual perspective of finding new β-lactam compounds resistant to the enzymes' hydrolytic activity and/or new molecules acting as therapeutically useful inhibitors.

Since production of metallo-β-lactamase activity has been reported in some members of the former *Flavobacterium* genus [24,25] but the enzymes have not been studied in detail and the corresponding genes have not been cloned, we initiated an investigation of the structural and functional properties of the metallo-β-lactamase produced by *C. meningosepticum*, which is the most clinically relevant flavobacterial species [28,29].

Kinetic analysis of the purified metallo-β-lactamase produced by *C. meningosepticum* showed that the enzyme has a broad substrate profile, including penicillins, cephalosporins, cephamycins and carbapenems. Its behaviour is thus generally similar to that of other class B β-lactamases, with the notable exception of the *Aeromonas* enzymes, which show an exceptionally narrow substrate profile [18–22]. Owing to its broad substrate profile and apparently constitutive production (G. M. Rossolini, N. Franceschini, G. Lombardi and G. Amicosante, unpublished work), it is likely that the BlaB enzyme provides a significant contribution to the high-level β-lactam resistance phenotype usually exhibited by *C. meningosepticum* clinical isolates [29,43]. The inactivation parameters calculated for various chelating agents were similar to those reported for the Bc-II and CphA metallo-β-lactamases [44] and indicated a mechanism where the formation of a ternary enzyme–metal–chelator complex precedes the removal of the metal from the enzyme active site.

The *blaB* gene was successfully isolated by means of a shotgun cloning technique based on the screening of a *C. meningosepticum* genomic library constructed in an *E. coli* plasmid vector for ampicillin-resistant clones able to produce 1-10 phenanthroline-sensitive carbapenemase activity. The *blaB* gene, therefore, can be expressed in *E. coli* resulting in the synthesis of a functional protein.

A comparative analysis showed that both G+C content and codon usage of the *blaB* gene were similar to those reported for other *C. meningosepticum* cloned genes. The *blaB* gene, therefore, appears to be endogenous to this microbial species, as are the genes encoding the Bc-II, CphA and L1 enzymes for their respective species. This is also consistent with the observation that metallo-β-lactamase production is a constant feature among *C. meningosepticum* clinical isolates ([25]; G. M. Rossolini, N.

Franceschini, G. Lombardi and G. Amicosante, unpublished work).

Sequence comparison with other molecular class B β-lactamases demonstrated that the BlaB enzyme is a member of this family. The overall degree of similarity of the BlaB enzyme with the Bc-II and CcrA enzymes, 35.2 % and 27.3 % of identical residues respectively, strongly suggests a similar protein fold for the *Chryseobacterium* enzyme. The similarity is even more striking when considering the residues in the immediate environment of the active site and those involved in the co-ordination of zinc ions [9,23], suggesting that the structures of the active site and of the zinc centre of the BlaB enzyme closely resemble those of the two enzymes of solved three-dimensional structures. Sequence comparison also indicated that the *C. meningosepticum* BlaB enzyme is a member of subclass B1, being overall more similar to Bc-II, CcrA and IMP-1 than to the *Aeromonas* (subclass B2) and *Stenotrophomonas* (subclass B3) enzymes. In fact BlaB could be aligned with other subclass B1 enzymes without introducing major gaps throughout the entire sequence, whereas it appeared to diverge more markedly from the *Aeromonas* and *Stenotrophomonas* enzymes with some deletions and insertions probably situated in loop structures (Figure 4).

According to phyletic analysis, the currently known metallo-β-lactamases of subclass B1 appear to be derived from two lineages, originated from a common ancestor, leading respectively to the endogenous *Bacillus* and *Chryseobacterium* enzymes and to the CcrA and IMP-1 enzymes (Figure 5). It should be noted that the latter enzymes are encoded by genes for which the possibility of horizontal transfer has been reported [4,6,7,45].

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