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Metallo-β-Lactamase Producers in Environmental Microbiota: New Molecular Class B Enzyme in *Janthinobacterium lividum*

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Eleven environmental samples from different sources were screened for the presence of metallo-β-lactamase-producing bacteria by using a selective enrichment medium containing a carbapenem antibiotic and subsequently testing each isolate for production of EDTA-inhibitable carbapenemase activity. A total of 15 metallo-β-lactamase-producing isolates, including 10 *Stenotrophomonas maltophilia* isolates, 3 *Chryseobacterium* spp., one *Aeromonas hydrophila* isolate, and one *Janthinobacterium lividum* isolate (a species in which production of metallo-β-lactamase activity was not previously reported), were obtained from 8 samples. In the *J. lividum* isolate, named JAC1, production of metallo-β-lactamase activity was elicited upon exposure to β-lactams. Screening of a JAC1 genomic library for clones showing a reduced imipenem susceptibility led to the isolation of a metallo-β-lactamase determinant encoding a new member (named THIN-B) of the highly divergent subclass B3 lineage of metallo-β-lactamases. THIN-B is most closely related (35.6% identical residues) to the L1 enzyme of *S. maltophilia* and more distantly related to the FEZ-1 enzyme of *Legionella gormanii* (27.8% identity) and to the GOB-1 enzyme of *Chryseobacterium meningosepticum* (24.2% identity). Sequences related to $bla_{\text{THIN-B}}$, and inducible production of metallo-β-lactamase activity, were also detected in the *J. lividum* type strain DSM1522. Expression of the $bla_{\text{THIN-B}}$ gene in *Escherichia coli* resulted in decreased susceptibility to several β-lactams, including penicillins, cephalosporins (including cephamycins and oxyimino cephalosporins), and carbapenems, revealing a broad substrate specificity of the enzyme. The results of this study indicated that metallo-β-lactamase-producing bacteria are widespread in the environment and identified a new molecular class B enzyme in the environmental species *J. lividum*.

Production of degrading enzymes (β-lactamases) is the most common mechanism of bacterial resistance to β-lactam antibiotics. The evolution of β-lactamase determinants started long before the introduction of β-lactams in clinical practice, presumably under the selective pressure of natural β-lactam compounds produced in various microbial ecosystems, while the recent (on an evolutionary timescale) exploitation of β-lactams for antimicrobial chemotherapy has strongly selected for spreading and evolution of similar resistance determinants among bacterial pathogens. In fact, it is well known that the continuous release of new β-lactams in clinical practice has invariably been followed by the appearance of new β-lactamases capable of degrading the newest compounds (7, 22).

Two different families of β-lactam-degrading enzymes, which catalyze the same reaction, i.e., the opening of the β-lactam ring by hydrolysis of the amide bond, but are structurally and mechanistically unrelated, have evolved in bacteria: (i) active-site serine-β-lactamases and (ii) metallo-β-lactamases (8, 13). The latter enzymes were identified some 25 years later than serine-β-lactamases and have remained less common among pathogenic bacteria (5, 8, 20). Nevertheless, they are potentially very dangerous as resistance effectors due to their efficient hydrolysis of carbapenem antibiotics, which are stable to hydrolysis by most β-lactamases and often represent the "last-resort" agents for chemotherapy of multidrug-resistant pathogens, and due to their lack of susceptibility to the serine-β-lactamase inhibitors such as clavulanic acid and penicillanic acid sulfones (2, 3; reference 5 and references therein; 18, 19, 26, 29, 31). The recent emergence of mobile metallo-β-lactamase genes capable of horizontal spreading among nosocomial strains of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and other gram-negative nonfermenters (reference 6 and references therein; 26, 30, 36, 37; G. Cornaglia, M. L. Riccio, A. Mazzariol, P. Piccoli, L. Lauretti, R. Fontana, and G. M. Rossolini, Letter, Lancet **353:**899–900, 1999) has considerably increased the attention to these enzymes, including them among the major threats for the 21st century in the field of microbial drug resistance (6).

Most known metallo-β-lactamases are encoded by chromosomal genes of some bacterial species that are primarily members of the environmental microbiota, such as *Bacillus cereus*, *Stenotrophomonas maltophilia*, *Aeromonas* spp., *Myroides odoratus* (formerly *Flavobacterium odoratum*), *Legionella gormanii* (reference 5 and references therein), and *Chryseobacterium* spp. (2, 3, 31), whereas some as yet unknown environmental species are the most likely sources of the mobile metallo-β-lactamase determinants that recently appeared among gram-

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TABLE 1. Environmental samples analyzed for the presence of metallo-β-lactamase-producing bacteria, and results of the screening

Sample (source and date)	No. of isolates growing on NACA medium (gram stain and cell morphology)	No. of metallo-β-lactamase producers (species)[a]
A (soil close to freshwater stream, hills of central Italy, Sept. 1997)	2 (gram-negative rods)	2 (*Stenotrophomonas maltophilia*)[b]
B (soil from cattle-breeding farm, contaminated with manure, Rome district, Oct. 1997)	3 (gram-negative rods)	2 (*Stenotrophomonas maltophilia*)[b]
C (beechwood soil, Appennine mountains [altitude ≅ 1,700 m], central Italy, Oct. 1997)	4 (gram-negative rods)	2 (1 *Stenotrophomonas maltophilia*, 1 *Aeromonas hydrophila*)
D (Oak-flex grove soil, hills of central Italy, Dec. 1997)	2 (gram-negative rods)	None
E (soil, campus of the University "La Sapienza," Rome, Jan. 1998)	3 (gram-negative rods)	2 (1 *Chryseobacterium* sp., 1 *Janthinobacterium lividum*)
F (soil from mountain plateau, Appennine Mountains [altitude ≅ 1,400 m], central Italy, Mar. 1998)	1 (gram-negative rod)	1 (*Stenotrophomonas maltophilia*)
G (soil from mountain pasture, Appennine Mountains [altitude ≅ 2,000 m], central Italy, Jun. 1998)	3 (gram-negative rods)	None
H (sandy beach, Tyrrhenian coast, central Italy, Oct. 1998)	1 (gram-positive staphylococcus)	None
I (flower bed soil, Rome, Dec. 1998)	3 (gram-negative rods)	2 (1 *Stenotrophomonas maltophilia*, 1 *Chryseobacterium indologenes*)
L (rotted celery, Jul. 1999)	3 (gram-negative rods)	2 (1 *Stenotrophomonas maltophilia*, 1 *Chryseobacterium* sp.)
M (rotted flowers, cemetery wastebasket, Sept. 1999)	2 (gram-negative rods)	2 (*Stenotrophomonas maltophilia*)[b]

[a] Carbapenemase activity was assayed in crude extracts as described in Materials and Methods. In isolates classified as metallo-β-lactamase producers, the carbapenemase activity ranged from 28 ± 2 to 153 ± 9 U/mg of protein, and the reduction of activity upon exposure to EDTA was in all cases >60%. In isolates classified as nonproducers, the carbapenemase activity was always lower than 5 U/mg of protein.

[b] The two *S. maltophilia* isolates exhibited different colonial morphology and different biochemical profiles.

negative pathogens. Therefore, environmental bacteria could be an important reservoir of similar resistance determinants.

In this work we carried out screening on various environmental samples for the presence of bacteria producing metallo-β-lactamase activity. In addition to several isolates belonging to various species that are known to produce similar enzymes, the screening also yielded a metallo-β-lactamase-producing isolate of *Janthinobacterium lividum*, a species in which production of metallo-β-lactamase activity has not been previously reported. Scanning for metallo-β-lactamase determinants carried by this isolate led to the identification of a new member of the highly divergent subclass B3 lineage of metallo-β-lactamases, named THIN-B, which appears to be resident in this species.

MATERIALS AND METHODS

Media, reagents and reference strain. Nutrient broth (NB), Nutrient agar (NA), and Mueller-Hinton (MH) medium (Difco Laboratories, Detroit, Mich.) were used for bacterial cultures. Antibiotics and other reagents were from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Imipenem was from Merck Research Laboratories (Rahway, N.J.), meropenem from Astra-Zeneca Pharmaceuticals (Macclesfield, Cheshire, United Kingdom), ceftazidime from Glaxo-Wellcome (Verona, Italy), aztreonam and cefepime from Bristol-Myers Squibb Co. (Wallingford, Conn.), and nitrocefin from Unipath (Milan, Italy). The *J. lividum* reference strain DSM1522^T was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Collection and analysis of environmental samples. Environmental samples were collected in 50-ml sterile screw-cap polypropylene tubes, from the different sources listed in Table 1. Each sample was collected, transferred to the laboratory, and processed on the same day. Samples were carefully resuspended in an approximately equal volume of sterile saline and left to decant at room temperature for 4 h. A 100-μl volume of the supernatant from each sample was then inoculated in 5 ml of NB containing imipenem (5 μg/ml) and amphotericin B (20 μg/ml) and was incubated at 25°C aerobically until the development of turbidity (usually evident after 24 to 48 h). Dilutions of each culture (in order to obtain isolated colonies) were then plated on NA containing imipenem (5 μg/ml) and amphotericin B (20 μg/ml) (NACA medium) and were incubated at 25°C until the appearance of colonies. A representative for each different colony morphology was reisolated on NACA medium, subjected to gram staining, and tested for production of metallo-β-lactamase activity. Metallo-β-lactamase producers were identified at the species or genus level according to the *Manual of Clinical Microbiology* (23) or, in the case of *J. lividum*, according to *Bergey's Manual of Systematic Bacteriology* (35). The API 20NE (Bio-Mérieux, Marcy-L'Etoile, France), ATB 32GN (Bio-Mérieux), and Crystal E/NF (Becton Dickinson Microbiology Systems, Cockeysville, Md.) systems were used for the identification of some gram-negative isolates.

β-Lactamase assays. Production of metallo-β-lactamase activity by the environmental isolates was assayed in crude cell extracts prepared from late-exponential-phase cells grown aerobically, at 25°C, in NB containing imipenem (5 μg/ml). Crude extracts were prepared as follows. Cells were collected by centrifugation, resuspended in 20 mM sodium phosphate buffer (PB) (pH 7.0) (1/10 of the original culture volume), and disrupted by sonication (six times for 15 s each time, at 50 W), and cell debris was removed by centrifugation at 10,000 × *g* for 10 min. β-Lactamase activity was assayed spectrophotometrically by monitoring imipenem hydrolysis at 299 nm (change in extinction coefficient, −9,000 M^{-1} cm^{-1}) at 25°C in PB. The initial substrate concentration was 150 μM. One unit of β-lactamase acivity was defined as the amount of enzyme hydrolyzing 1 nmol of imipenem per min under the above assay conditions. Inhibition of enzymatic activity by EDTA was assayed by measuring the residual carbapenemase activity after incubation of the crude extract, for 20 min at 25°C, in the presence of 5 mM EDTA. A control without EDTA was always carried out in parallel. Induction experiments with JAC1 and DSM1522^T were carried out as follows. Cells were grown aerobically, at 25°C, in MH broth until the mid-

TABLE 2. Carbapenemase activity in crude extracts of *J. lividum* JAC1, *J. lividum* DSM1522^T, and *E. coli* DH5α(pCIRO)[a]

Strain (inducer)[b]	Sp act (U/mg of protein)[c]	
	Without EDTA	With EDTA[d]
J. lividum JAC1 (none)	8 ± 0.8	2 ± 0.4
J. lividum JAC1 (IPM, 5 μg/ml)	47 ± 3	18 ± 2
J. lividum DSM 1522^T (none)	2 ± 0.2	ND
J. lividum DSM 1522^T (IPM, 5 μg/ml)	26 ± 2	8 ± 1
E. coli DH5α(pCIRO)	68 ± 5	21 ± 2
E. coli DH5α(pACYC184)	<1	ND

[a] The basal activity of *E. coli* DH5α carrying an empty vector is also shown for comparison.

[b] IPM, imipenem.

[c] Measured with 150 μM imipenem as the substrate (see Materials and Methods for experimental details). Values are means of three measurements with standard deviations. ND, not determined.

[d] Activity measured after exposure of the crude extract to EDTA as described in Materials and Methods.

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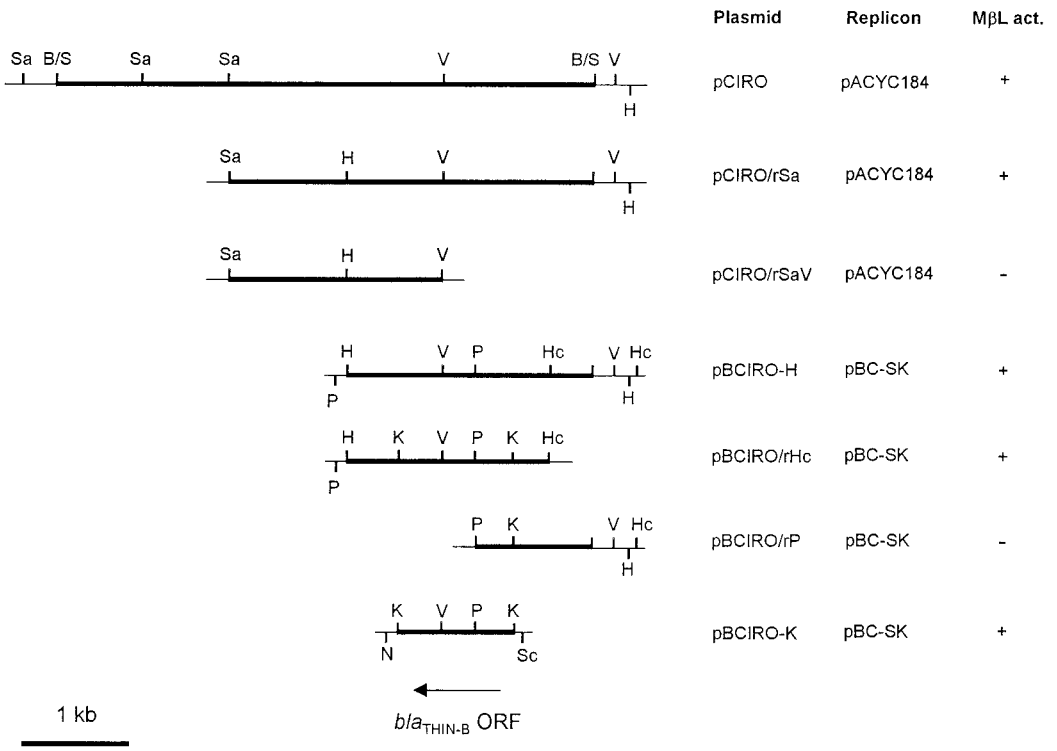

FIG. 1. Physical map of the insert of plasmid pCIRO, and subcloning strategy. Thick lines represent cloned DNA, while thin lines correspond to vector sequences. Production of metallo-β-lactamase activity (MβL act.) was assayed as described in Materials and Methods on crude extracts prepared from late-exponential-phase cultures. The location of the bla_{THIN-B} ORF is indicated. B/S, *Bam*HI/*Sau*3AI junction; H, *Hin*dIII; Hc, *Hin*cII; K, *Sac*II; N, *Not*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sac*I; V, *Eco*RV. In plasmid pBCIRO-K the orientation of the bla_{THIN-B} ORF is the same as that of the P_{lac} promoter flanking the polylinker.

exponential phase ($A_{600} \cong 0.3$ to 0.4). The culture was then split into two flasks, of which one was added with the inducer at the desired concentration. Cells were collected after a further 3-h incubation. Preparation of crude extracts and β-lactamase assays were carried out as described above. The protein concentration in solution was assayed with a commercial kit (Bio-Rad [Richmond, Calif.] protein assay), with bovine serum albumin as a standard.

Recombinant DNA methodology. Basic recombinant DNA procedures were performed as described by Sambrook et al. (33). Genomic DNA was extracted from *J. lividum* strains as described previously (16), with an additional extraction step with water-saturated ether before ethanol precipitation. For construction of the JAC1 genomic library, genomic DNA was partially digested with *Sau*3AI, and fragments in the 2- to 9-kb size range were purified by agarose gel electrophoresis using the Geneclean II kit (Bio 101, La Jolla, Calif.). The purified restriction fragments were ligated to *Bam*HI-linearized and dephosphorylated pACYC184 (33), and the ligation mixture was transformed into *Escherichia coli* DH5α (GIBCO-BRL, Gaithersburg, Md.) by electroporation using a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's instructions. The ratio of recombinant clones to those carrying an empty religated vector was >10, as shown by replica plating of transformants, selected on Luria-Bertani (LB) agar plates containing chloramphenicol (70 μg/ml), onto plates containing both chloramphenicol and tetracycline (20 μg/ml). Southern blot hybridization was performed as described previously (33) using a nylon membrane (Amersham Pharmacia Biotech, Milan, Italy) and a ^{32}P-labeled DNA probe.

DNA sequencing and computer analysis of sequence data. DNA sequences of both strands were determined on plasmid templates by the dideoxy-chain termination method using an automatic DNA sequencer (model 4000; LI-COR Inc., Lincoln, Nebr.), the Thermosequenase DNA sequencing kit (Amersham), and IRD 800-labeled custom sequencing primers (MWG Biotech, Munich, Germany). Similarity searches against sequence databases were performed using an updated version of the BLAST program at the BLAST interface of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Computer analysis of sequence data was performed using an updated version (8.1) of the Wisconsin Package (version 8.1; Genetics Computer Group Inc., Madison, Wis.) at the Italian EMBL Node of Bari. The multiple sequence alignment was generated with the help of the PILEUP program of the Wisconsin package and was manually refined by considering the information available on the three-dimensional structure of metallo-β-lactamases (9–12, 38). It essentially corresponds to that proposed for the definition of a standard numbering scheme for class B β-lactamases (14). The unrooted tree was constructed on the basis of the multiple sequence alignment with the help of the CLUSTAL W program at the Italian EMBL Node of Bari.

In vitro susceptibility testing. The vitro susceptibility of *E. coli* DH5α carrying the cloned bla_{THIN-B} gene was determined by a macrodilution broth method (24), using MH broth and a bacterial inoculum of 10^5 CFU per tube. Results were recorded after incubation at 28°C for 24 h.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank/DDBJ sequence databases and assigned accession number AJ250876.

RESULTS

Screening of environmental samples for metallo-β-lactamase-producing bacteria. Eleven environmental samples collected from different sources (Table 1) were screened for the presence of bacteria growing on a selective medium containing imipenem, a carbapenem antibiotic which is efficiently degraded by all known metallo-β-lactamases but resistant to hydrolysis by most active-site serine β-lactamases (7). A broad-spectrum antifungal agent was also added to the medium to prevent fungal overgrowth. Bacterial isolates growing on the selective enrichment medium were obtained from each sample, and a total of 27 different isolates were collected using this strategy (Table 1).

Production of carbapenemase activity was assayed in the 27

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isolates and detected in 15 of them (Table 1). In all cases the carbapenemase activity consistently decreased after exposure to EDTA (Table 1), suggesting that it was caused, at least in part, by metal-dependent enzymes.

Identification of the 15 metallo-β-lactamase producers showed that they belonged in the following species: *S. maltophilia*, *Aeromonas hydrophila*, *Chryseobacterium indologenes*, *Chryseobacterium* spp., and *J. lividum* (Table 1). *S. maltophilia* was the most common species, present in 7 of the 11 samples, either alone or in association with other species (Table 1).

Production of metallo-β-lactamase activity in *J. lividum*. Since production of metallo-β-lactamase activity has not been previously reported for *J. lividum*, the isolate of this species obtained from sample E, named JAC1, was subjected to further investigation.

The production of carbapenemase activity by JAC1 was studied in relation to exposure to imipenem. In cells growing in MH broth at 25°C a low level of activity was detectable, while exposure of the growing cells to subinhibitory concentrations (5 μg/ml) of imipenem significantly increased the production of EDTA-inhibitable carbapenemase activity (Table 2). Production of carbapenemase activity susceptible to inhibition by EDTA and regulated upon β-lactam exposure was also detected in the *J. lividum* type strain DSM1522 (Table 2), suggesting that metallo-β-lactamase production is a feature of the species rather than of the individual JAC1 isolate.

Cloning and characterization of a metallo-β-lactamase determinant from *J. lividum* JAC1. The genome of JAC1 was scanned for the presence of metallo-β-lactamase determinants by means of a shotgun cloning approach. For this purpose a genomic library of JAC1, constructed in the pACYC184 plasmid vector and transformed in *E. coli* DH5α, was replica plated on MH medium containing imipenem at a concentration of 5 μg/ml. One clone growing on this medium was obtained out of approximately 3,000 screened recombinants. The presence of carbapenemase activity susceptible to inhibition by EDTA was detectable in the crude extract of this clone (Table 2).

The recombinant plasmid harbored by the metallo-β-lactamase-producing clone, named pCIRO, contained a 5.2-kb DNA insert (Fig. 1). Subcloning analysis indicated that the metallo-β-lactamase gene was apparently located within a 1.1-kb *Sac*II fragment and was apparently interrupted by a *Pst*I site (Fig. 1). The origin of the cloned determinant was confirmed by a Southern blot experiment in which the 1.1-kb *Sac*II insert of plasmid pBCIRO-K (Fig. 1) was used to probe the JAC1 genomic DNA. The probe hybridized with the band of undigested chromosomal DNA, with single restriction fragments of 9.2, 1.1, and 5.8 kb after digestion with *Hin*dIII, *Sac*II, and *Sal*I, respectively, and with two restriction fragments of 3 and 2.2 kb after digestion with *Pst*I. The same probe also hybridized with the genomic DNA of *J. lividum* DSM1522^T in a Southern blot experiment (data not shown), revealing the presence of homologous sequences in the genome of the type strain.

The nucleotide sequence of the insert of plasmid pBCIRO-K was determined. Analysis of sequence data revealed the presence of an open reading frame (ORF) (Fig. 2) encoding a protein which, in a BLAST search, showed the highest sequence similarity with the L1 enzyme of *S. maltophilia* (39) and lower similarity scores with other molecular class B β-lactamases. Results of subcloning experiments (Fig. 1) were consistent with the identification of this ORF, named *bla*$_{THIN-B}$, as the metallo-β-lactamase determinant. Assuming the ATG trinucleotide at positions 37 to 39 as the most probable start codon, according to the presence of a putative ribosome-binding site 6 bp upstream (Fig. 2), the *bla*$_{THIN-B}$ ORF would encode a 316-amino-acid polypeptide whose amino-terminal sequence exhibits features typical of procaryotic signal peptides targeting protein secretion into the periplasmic space via the general secretory pathway (Fig. 2). According to known patterns (27), the cleavage site could be located after the alanine residue at position 18. In this case the calculated molecular mass and pI of the mature THIN-B protein would be 30,676 Da and 6.23, respectively. The high G+C content of the *bla*$_{THIN-B}$ locus (65.4%) is consistent with the value reported for the genome of *J. lividum* (35).

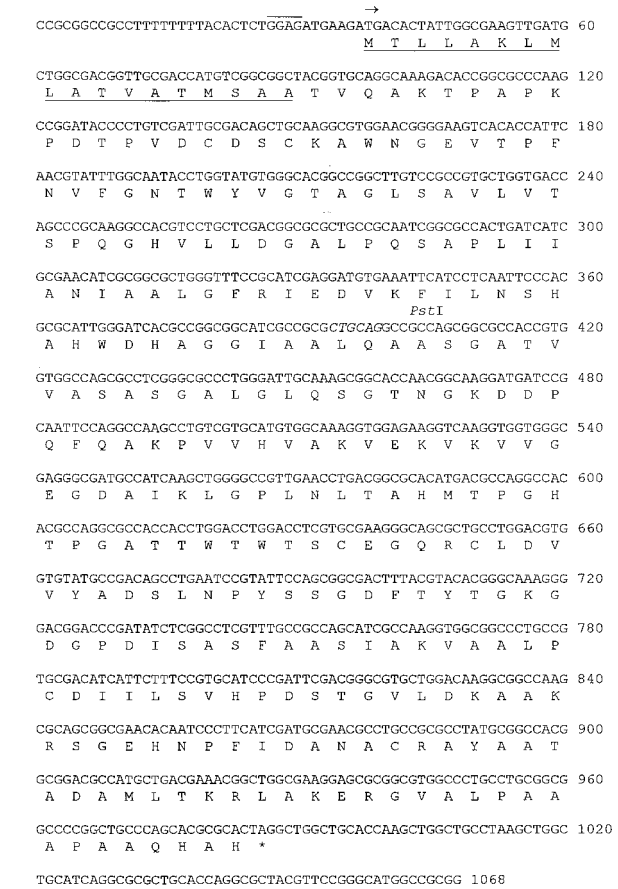

FIG. 2. Nucleotide sequence of the DNA insert of plasmid pBCIRO-K containing the *bla*$_{THIN-B}$ ORF and flanking regions. The putative ribosome-binding site is overlined. Protein translation is reported below the sequence, and the putative signal peptide for protein secretion is underlined.

Comparison of the THIN-B enzyme with other metallo-β-lactamases. A multiple sequence alignment analysis with other class B β-lactamases confirmed the closest similarity of THIN-B with the L1 enzyme of *S. maltophilia* and with the other enzymes of subclass B3. THIN-B could be aligned over the entire sequence with these enzymes without introducing major gaps (Fig. 3), and the percent identities among them (24.2 to 35.6%)

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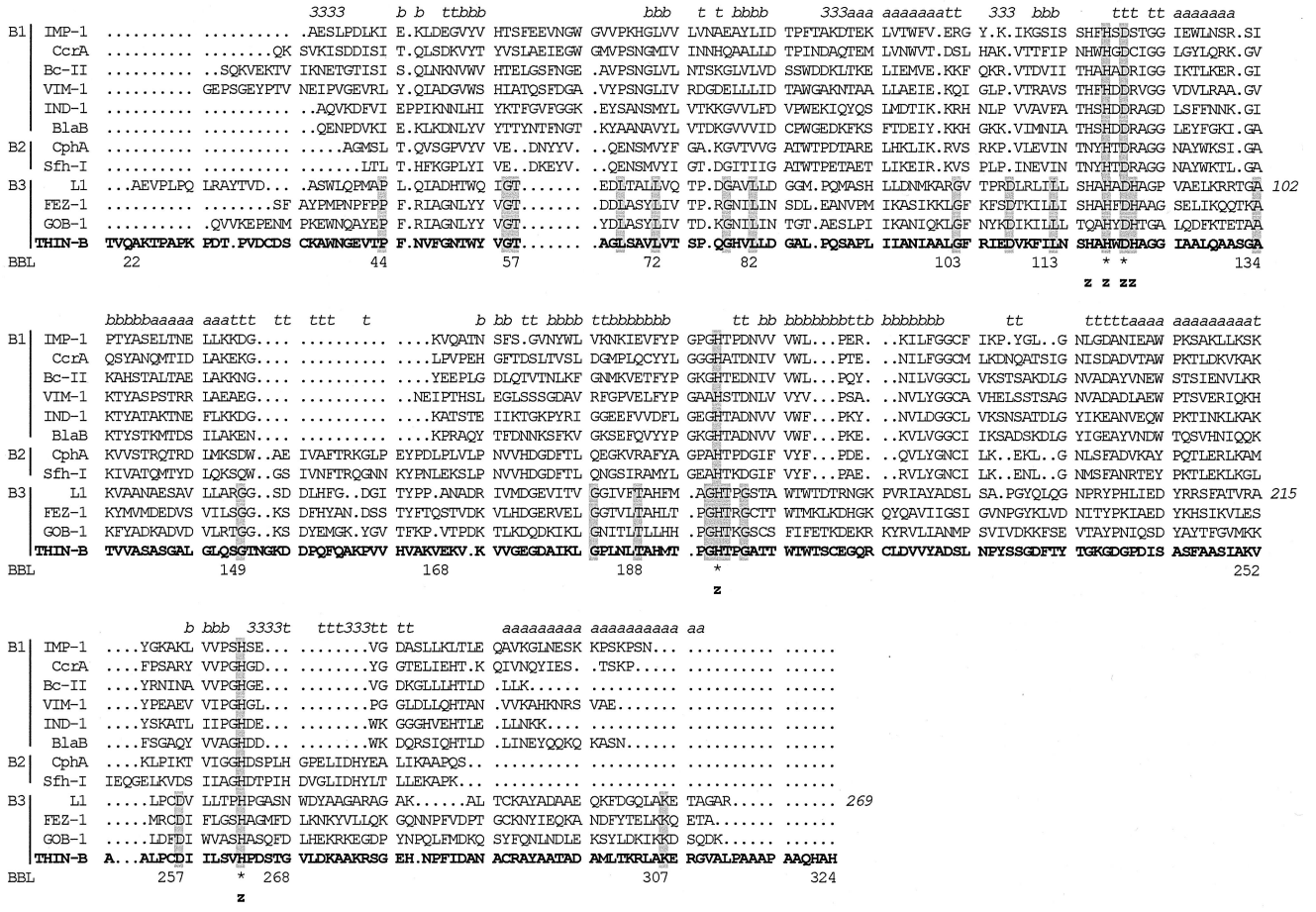

FIG. 3. Comparison of the THIN-B amino acid sequence (boldfaced) with those of other molecular class B β-lactamases of subclasses B1, B2, and B3. IMP-1, IMP-1 enzyme encoded by the bla_{IMP-1} gene cassette found in *Serratia marcescens* TN9106 (25) and in other gram-negative strains (1, 17); CcrA, CcrA enzyme from *Bacteroides fragilis* TAL3636 (28); Bc-II, β-lactamase II from *B. cereus* 569/H (15); VIM-1, VIM-1 enzyme encoded by the bla_{VIM-1} gene cassette found in *P. aeruginosa* VR-143/97 (19); IND-1, IND-1 enzyme from *C. indologenes* 001 (3); BlaB, BlaB enzyme from *C. meningosepticum* CCUG4310 (31); CphA, CphA enzyme from *A. hydrophila* AE036 (21); SfhI, SfhI enzyme from *Serratia fonticola* UTAD54 (EMBL/GenBank accession number AF197943); L1, L1 enzyme from *S. maltophilia* IID 1275 (39); FEZ-1, FEZ-1 enzyme from *L. gormanii* ATCC 33297^T (4); GOB-1, GOB-1 enzyme from *C. meningosepticum* PINT (2). The BBL numbering scheme (14) is indicated below the sequences; the numbering of the L1 enzyme (38) is also indicated, in italics. Identical residues are marked with an asterisk. Residues of the L1 enzyme involved in binding of Zn^{2+} are indicated by the letter z. Secondary structure elements of L1 (38) are also indicated above the sequences: *3*, 3_{10} helix; *b*, extended strand participating in β-ladder; *t*, hydrogen-bonded turn; *a*, α-helix. The invariant residues in all proteins, or in those of subclass B3, are shaded.

were considerably higher than those between THIN-B and the metallo-β-lactamases of subclasses B1 (9.6 to 17.8%) and B2 (15.7 to 16.5%) (Table 3).

Compared to L1, which is the closest THIN-B homolog, the major differences in THIN-B are represented by somewhat longer amino and carboxy termini, and by small insertions in some loops (those between α-helix 3 and β-strand 7, between α-helix 4 and β-strand 12, and between β-strand 12 and α-helix 5 [Fig. 3]). All the residues known to be involved in metal binding in the L1 enzyme (His-84, His-86, Asp-88, His-89, His-160, and His-225 in the numbering of the L1 enzyme of *S. maltophilia* IID 1275 [39]; His-116, His-118, Asp-120, His-121, His-196, and His-263 according to the BBL numbering scheme [14]) are conserved in THIN-B. THIN-B also contains a serine residue corresponding to Ser-185 of L1, unlike the enzymes of molecular subclasses B1 and B2 (Fig. 3).

Comparison with the other enzymes of subclass B3 showed that the following residues (in the BBL numbering) are conserved among members of this subclass: Pro-44, Gly-56, Thr-57, Leu-68, Leu-72, Gly-79, Leu-82, Gly-103, Asp-108, Leu-113, His-118, Asp-120, His-121, Ala-134, Gly-149, Gly-183, Thr-188, Gly-195, His-196, Thr-197, Gly-199, Asp-257, His-263, and Lys-307 (Fig. 3). Of these, four (His-118, Asp-120, His-196, and His-263) are conserved and five (Leu-72, Leu-82, Leu-113, Gly-195, and Thr-197) are conservatively subtituted in members of the other subclasses as well, while the remaining residues (Pro-44, Gly-56, Thr-57, Leu-68, Gly-79, Gly-103, Asp-108, His-121, Ala-134, Gly-149, Gly-183, Thr-188, Gly-199, Asp-257, and Lys-307) appear to be hallmarks of subclass B3 (Fig. 3).

Construction of an unrooted tree on the basis of the multiple sequence alignment showed that THIN-B and L1 apparently shared a common ancestry during the evolutionary history of the subclass B3 lineage of metallo-β-lactamases (Fig. 4).

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TABLE 3. Pairwise percent amino acid sequence identity between class B β-lactamases

Subclass	Enzyme[a]	% Amino acid sequence identity										
		CcrA	Bc-II	VIM-1	IND-1	BlaB	CphA	SfhI	L1	FEZ-1	GOB-1	THIN-B
B1	IMP-1	37.7	36.6	30.2	31.8	30.6	21.7	20.5	14.0	11.6	17.2	12.6
	CcrA		33.9	30.7	29.8	28.5	25.3	24.4	13.0	12.5	11.4	9.6
	Bc-II			36.1	35.0	36.4	26.5	21.8	18.6	12.9	14.9	17.8
	VIM-1				26.5	26.6	22.8	22.1	15.2	10.4	12.8	15.0
	IND-1					45.2	23.5	27.9	17.0	14.9	15.9	14.0
	BlaB						27.0	24.9	12.0	14.8	17.7	12.6
B2	CphA							63.0	16.2	12.4	12.9	16.5
	SfhI								15.4	13.5	16.8	15.7
B3	L1									31.0	23.5	35.6
	FEZ-1										37.1	27.8
	GOB-1											24.2

[a] The names of the enzymes of the three subclasses (B1, B2, and B3) are the same as in Fig. 3.

Influence of THIN-B production on β-lactam susceptibility. The substrate specificity of THIN-B was investigated by testing the susceptibility to various β-lactams of *E. coli* DH5α (pBCIRO-K), which carries a cloned copy of the $bla_{\text{THIN-B}}$ gene and produces the THIN-B enzyme (Fig. 1), in comparison with that of DH5α(pBC-SK), carrying the empty plasmid vector. THIN-B production was associated with a decrease in the in vitro susceptibility of the bacterial host to ampicillin, carbenicillin, piperacillin, cefotaxime, cephalothin, cefuroxime, cefoxitin, ceftazidime, cefepime, imipenem, and meropenem, while susceptibility to aztreonam was not affected (Table 4). The relative MIC increases were higher overall with cephalosporins (except for cefepime) and meropenem (Table 4).

DISCUSSION

Results of the screening performed in this work indicated that metallo-β-lactamase-producing bacteria are widespread in the environmental microbiota and that such an approach can be successful for their detection. In the screening procedure imipenem was used as a selective agent in consideration of the fact that carbapenemase activity is a constant feature of metallo-β-lactamases (2, 3; reference 5 and references therein; 18, 19, 26, 29, 31), but its concentration was kept relatively low in consideration of the low carbapenem MICs exhibited by some metallo-β-lactamase producers (32, 34). Nevertheless, the possibility that the selective conditions used in the medium might have biased the screening in favor of certain species cannot be excluded. On the other hand, the screening also yielded a consistent number of isolates that were able to grow on the selective enrichment medium while not producing detectable carbapenemase activity. In these cases resistance to the antibiotic present in the medium could be related to one or more of the following mechanisms: (i) low affinity of the β-lactam targets; (ii) production of enzymes with very low rates of turnover against carbapenems (undetectable under the assay conditions adopted in this study), and (iii) presence of permeability barriers or efflux systems. It would be interesting to evaluate whether modification of various parameters (nature and concentration of the selective agent, nature of the medium, temperature and atmosphere used for incubation) could increase the screening sensitivity, and also to analyze samples from

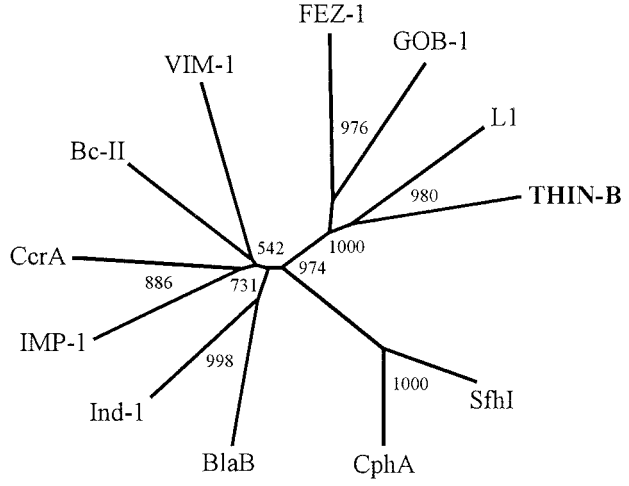

FIG. 4. Unrooted tree showing phylogenetic relationships among metallo-β-lactamases. The names of the enzymes are the same as in Fig. 3. Numbers at branching points indicate the number per 1,000 bootstrap trials returned for that point.

TABLE 4. β-Lactam susceptibility of *E. coli* DH5α(pBCIRO-K), carrying the cloned $bla_{\text{THIN-B}}$ gene and producing the THIN-B enzyme[a] compared to that of the *E. coli* host containing the plasmid vector pBC-SK

Antibiotic	MIC (μg/ml)	
	DH5α(pBCIRO-K)	DH5α(pBC-SK)
Ampicillin	16	1
Carbenicillin	32	4
Piperacillin	4	1
Cephalothin	64	4
Cefoxitin	128	2
Cefuroxime	64	4
Cefotaxime	4	0.06
Ceftazidime	32	0.12
Cefepime	0.12	0.03
Imipenem	0.50	0.12
Meropenem	0.50	0.015
Aztreonam	0.25	0.25

[a] For the clone, see Fig. 1.

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different sources (such as various types of aquatic environments).

Of the metallo-β-lactamase producers isolated in this study, most belonged to species that were already known for this trait, of which *S. maltophilia* was the most common. This suggests an overall broader diffusion and higher prevelance of this species, compared to the others, in the environmental microbiota, although an influence of the screening procedure on this result cannot be ruled out at this stage. The screening also yielded a metallo-β-lactamase-producing isolate of *J. lividum*, a species in which no similar activity has been reported previously. In *J. lividum*, production of metallo-β-lactamase activity is likely a species-related trait, since it was also detected in the type strain, and it is apparently regulated, since it is detectable at higher levels upon exposure to β-lactam compounds. Scanning the genome of the *J. lividum* isolate for carbapenemase determinants led to the isolation and identification of a chromosomal gene, named $bla_{\text{THIN-B}}$, encoding a new molecular class B enzyme. The chromosomal origin and base composition of this gene, together with the presence of closely related sequences in the *J. lividum* type strain, strongly suggest that $bla_{\text{THIN-B}}$ is a resident gene of this species.

Sequence analysis showed that the THIN-B enzyme belongs to the highly divergent subclass B3 lineage of metallo-β-lactamases and is most closely related to the L1 enzyme of *S. maltophilia*. With the addition of THIN-B, subclass B3, which until recently included only one member (29), now has four different enzymes, all from environmental species, clustered in two different evolutionary sublineages, one including the *L. gormanii* FEZ-1 (4) and *Chryseobacterium meningosepticum* GOB-1 (2) metallo-β-lactamases, and the other including L1 and THIN-B (Fig. 4). The almost 36% sequence identity to L1 and the complete conservation of the residues that in L1 are known to be directly or indirectly involved in metal coordination (38) suggest that the three-dimensional fold of THIN-B is likely very similar to that of L1 and that the geometry of zinc coordination in the active site of THIN-B is the same as that of L1 and different from those of the enzymes of subclass B1 (38; reference 40 and references therein). The conservation of the two cysteine residues that in L1 (at positions 256 and 290, in the BBL numbering) form an intramolecular disulfide bridge, which constrains the loop between β-strand 12 and the C-terminal α-helix 5 (38), suggests that a similar disulfide bridge is likely present also in THIN-B.

Production of the THIN-B enzyme in *E. coli* caused a decrease in host susceptibility to a broad array of β-lactams, including penicillins, narrow-spectrum cephalosporins, cephamycins, oxyimino cephalosporins, and carbapenems. Only aztreonam was apparently unaffected, in agreement with the properties of other metallo-β-lactamases (2, 3; reference 5 and references therein; 18, 19, 26, 29, 31). THIN-B, therefore, appears to be a class B enzyme of broad substrate specificity which can be assigned to group 3a in the functional classification of β-lactamases (8). A biochemical and structural analysis of THIN-B, in comparison with the other members of subclass B3, would be useful for acquiring a better understanding of the structure-function relationships of this emerging lineage of metallo-β-lactamases.

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