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Postgenomic Scan of Metallo-β-Lactamase Homologues in Rhizobacteria: Identification and Characterization of BJP-1, a Subclass B3 Ortholog from *Bradyrhizobium japonicum*

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The diffusion of metallo-β-lactamases (MBLs) among clinically important human pathogens represents a therapeutic issue of increasing importance. However, the origin of these resistance determinants is largely unknown, although an important number of proteins belonging to the MBL superfamily have been identified in microbial genomes. In this work, we analyzed the distribution and function of genes encoding MBL-like proteins in the class *Rhizobiales*. Among 12 released complete genomes of members of the class *Rhizobiales*, a total of 57 open reading frames (ORFs) were found to have the MBL conserved motif and identity scores with MBLs ranging from 8 to 40%. On the basis of the best identity scores with known MBLs, four ORFs were cloned into *Escherichia coli* for heterologous expression. Among their products, one (blr6230) encoded by the *Bradyrhizobium japonicum* USDA110 genome, named BJP-1, hydrolyzed β-lactams when expressed in *E. coli*. BJP-1 enzyme is most closely related to the CAU-1 enzyme from *Caulobacter vibrioides* (40% amino acid sequence identity), a member of subclass B3 MBLs. A kinetic analysis revealed that BJP-1 efficiently hydrolyzed most β-lactam substrates, except aztreonam, ticarcillin, and temocillin, with the highest catalytic efficiency measured with meropenem. Compared to other MBLs, BJP-1 was less sensitive to inactivation by chelating agents.

The production of β-lactamases is the most common cause of bacterial resistance to β-lactam antibiotics, particularly among gram-negative pathogens (7, 19). The recent emergence of acquired metallo-β-lactamases (MBLs), mainly of the IMP and VIM types, in important human pathogens (such as *Pseudomonas* spp., *Acinetobacter* spp., and members of the family *Enterobacteriaceae*) has been regarded as extremely worrisome, since these enzymes exhibit a very broad substrate profile, including carbapenems and expanded-spectrum cephalosporins, and are not susceptible to mechanism-based β-lactamase inactivators (6, 26, 31). Due to this, MBLs have become a subject of increasing interest in recent years. However, while a growing knowledge of their structure, catalytic mechanisms, and epidemiology in clinical settings has been achieved (31), the evolutionary history and natural distribution of these enzymes remain largely unclear.

The recent progress in large-scale genome sequencing revealed that genes encoding proteins sharing structural similarity with MBLs are widely distributed in prokaryotic and eukaryotic genomes (Pfam PF00753, http://www.sanger.ac.uk/Software/Pfam/). These proteins are part of a large superfamily of metallohydrolases that includes enzymes of different functions (e.g., glyoxalase II, aryl- and alkyl-sulfatase, cyclase, DNA repair, *N*-acyl homoserine lactone hydrolase) as well as several hypothetical proteins of unknown function (1, 8, 10). A postgenomic approach has been successful in identifying MBL orthologs encoded by microbial genomes (e.g., CAU-1 from *Caulobacter vibrioides* [formerly *Caulobacter crescentus*]) (13), and can be a powerful means to identify new MBLs and bacterial species that could play a role as reservoirs of similar resistance genes.

In this work, we analyzed the presence of putative proteins belonging to the MBL superfamily in the available complete genomes of various species in the order *Rhizobiales*. We also report on the identification and detailed biochemical characterization of a new subclass B3 MBL from *Bradyrhizobium japonicum*, named BJP-1.

MATERIALS AND METHODS

Bacterial strains and genetic vectors. *B. japonicum* USDA110 was kindly donated by the USDA Rhizobium Germplasm Resource Center. *Agrobacterium tumefaciens* C85, *Mesorhizobium loti* ATCC 33669, and *Sinorhizobium meliloti* ATCC 9930 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *Escherichia coli* DH5α (Gibco Life Technologies, Gaithersburg, Md.) was used as the host for genetic vectors and recombinant plasmids. *E. coli* BL21(DE3) (Stratagene, Inc., La Jolla, Calif.) was used as a host for T7 promoter-based expression vectors for enzyme production. Plasmid pBC-SK (Stratagene, Inc.) was used for cloning selected genes under the transcriptional control of the P_{lac} promoter to investigate the putative protein function and for in vitro susceptibility testing. Plasmid pET-9a (Novagen, Madison, Wis.) was used for high-level gene expression and recombinant enzyme production (Table 1).

Media and culture conditions. *B. japonicum*, *S. meliloti*, and *M. loti* were grown aerobically at 30°C in YM medium (yeast extract [3 g/liter], malt extract [3 g/liter], peptone [5 g/liter], glucose [10 g/liter]). *A. tumefaciens* was grown aerobically at 30°C in nutrient broth (peptone [5 g/liter], meat extract [3 g/liter]). Luria-Bertani (LB) medium was routinely used for propagation of *E. coli* DH5α derivatives. With BL21(DE3) derivatives, P-0.8G medium was used for routine propagation, while ZYP-0.8G and ZYP-5052 media were used for recombinant protein production (29).

Database screening and sequence analysis. Database searches were performed using BLAST 2.2.11 running at the National Center for Biological In-

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TABLE 1. Recombinant plasmids and primers used in this study

Plasmid	Genetic vector	Primer pair	Sequence	Target	Product size (bp)
pMS-AGR	pBC-SK	Agrob/fwd Agrob/rev	5′-GGTCTAGACATATGATAGGGCTTGGCGATTGTG 5′-CCGGATCCTCAGACCGGAAAATACTCGCC	*Agrobacterium tumefaciens* strain C58 AGR_C_1482	981
pET-AGR	pET-9a	Agrob/fwd Agrob/rev			
pMS-LOT	pBC-SK	Mes_loti/fwd Mes_loti/rev	5′-GCTCTAGACATATGGGATCTCTTGCGGTCAT 5′-CCGGATCCTCAGGGCCGCTGCCGCA	*Mesorhizobium loti* MAFF303099 mll0231	762
pET-LOT	pET-9a	Mes_loti/fwd Mes_loti/rev			
pMS-SIN	pBC-SK	Sin/fwd Sin/rev	5′-CGGGAGCTCCATATGCAAGCTCCGGAATTCGAC 5′-CCGAGCTCTCACGCCAGCCGGTACGCG	*Sinorhizobium meliloti* 1021 SMc00087	1,023
pET-SIN	pET-24a	Sin/fwd Sin/rev			
pLBII-BJP	pBC-SK	Brad/fwd Brad/rev	5′-GGTCTAGACATATGAGAAGGCTGACGGCC 5′-CCGGATCCCTATTTCTTCTCCAGCGCCG	*Bradyrhizobium japonicum* USDA 110 blr6230	885
pET-BJP	pET-9a	Brad/fwd Brad/rev			

formation (NCBI) (http://www.ncbi.nlm.nih.gov/). Multiple-sequence alignments were performed using ClustalX 1.83 (9) followed by manual adjustments using the BioEdit 7.0.5 package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees were generated using ClustalX 1.83 and TreeView 1.6.6 applications (9, 27). Theoretical calculations of protein molecular mass and pI were carried out using the software available at the ExPASy proteomic server (http://ca.expasy.org/), and leader peptide cleavage site was predicted using SignalP 3.0 (4).

Recombinant DNA methodologies and DNA analysis techniques. Basic recombinant DNA procedures were performed as described by Sambrook and Russell (28). Briefly, the selected putative open reading frames (ORFs) were amplified by PCR using specific custom primers (Table 1) with the Expand High Fidelity PCR system (Roche Biochemicals, Mannheim, Germany) as described previously (13) using the following cycling conditions: an initial denaturation step at 95°C for 3 min; 30 cycles, with 1 cycle consisting of denaturation at 95°C for 40 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min (10 s added to the extension step to each cycle in cycles 11 to 30); a final extension step at 72°C for 20 min. The amplification products were cloned into pBC-SK vector using XbaI and BamHI restriction sites (or SacI and XhoI for *S. meliloti* SMc00087 ORF). The NdeI-BamHI fragments from the various plasmids were subcloned into the pET-9a expression vector, except the NdeI-XhoI fragment of pBC-SIN, which was subcloned in pET-24a. All recombinant plasmids were sequenced to exclude the presence of any unwanted PCR-generated mutations.

Antimicrobial susceptibility testing. The in vitro susceptibility of *E. coli* strains carrying different plasmids and of *B. japonicum* USDA 110 was determined by the macrodilution broth method as recommended by the Clinical Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (25) using supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and YM broth, respectively, with an inoculum size of 10^5 CFU per tube. Results were recorded after incubation for 24 h at 37°C for *E. coli* and after incubation for 4 days at 30°C for *B. japonicum*.

Detection of β-lactamase activity and preparation of crude extracts. β-Lactamase activity in crude cell extracts and during the purification procedure was assayed spectrophotometrically by monitoring the hydrolysis of the following β-lactam substrates: 200 μM cephalothin at 260 nm ($\Delta\varepsilon = -6{,}300\ M^{-1} \cdot cm^{-1}$), 150 μM imipenem at 300 nm ($\Delta\varepsilon = -9{,}000\ M^{-1} \cdot cm^{-1}$), and 100 μM nitrocefin at 482 nm ($\Delta\varepsilon = 15{,}000\ M^{-1} \cdot cm^{-1}$). Reactions were performed in 50 mM HEPES-NaOH buffer (pH 7.5) containing 50 μM $ZnSO_4$ (HZN buffer) at 30°C in a total volume of 500 μl. Inhibition of enzymatic activity by EDTA was assayed by measuring the residual activity after incubation of the crude extract for 20 min at 25°C in the presence of 5 mM EDTA. Crude cell extracts were prepared from a culture grown aerobically in LB broth at 37°C. Cells were collected by centrifugation, resuspended in HZN buffer, and lysed by sonication (six times, for 15 s each time, at 50 W). The supernatant obtained after centrifugation at 10,000 × *g* for 10 min to remove cell debris provided the crude extract.

Purification of BJP-1. The BJP-1 enzyme was purified from a culture of *E. coli* BL21(DE3)/[pET-BJP] grown in 3 liters of ZYP-5052 medium for 24 h at 37°C. Cells were harvested by centrifugation (10,000 × *g*, 30 min, 4°C), resuspended in 100 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 50 μM $ZnSO_4$ (TZN buffer) and lysed using a cell disruption system (Constant Systems Ltd., Daventry, United Kingdom). Cellular debris was removed by centrifugation (12,000 × *g*, 40 min, 4°C), and the clarified supernatant was loaded on a column (1.6 × 40 cm) packed with 70 ml of DEAE-Sepharose FF (flow rate, 3 ml/min) previously equilibrated with TZN buffer. Under these conditions, the β-lactamase did not bind to the column but was eluted using 100 ml of TZN buffer. The active fractions were pooled, and the buffer exchanged to 20 mM ethanolamine (pH 9.2) containing 50 μM $ZnSO_4$ (EZN buffer) using a HiPrep 26/10 desalting column (Amersham Biosciences). The resulting sample was loaded (flow rate, 1 ml/min) on an HR 16/5 column packed with 10 ml of Source Q gel (Amersham Biosciences) previously equilibrated with EZN buffer. Again, the β-lactamase was only slightly retained by the column, and the enzyme was eluted using 40 ml of EZN buffer. All chromatography steps were performed using an Äkta purifier platform (Amersham Biosciences). Enzyme purity and authenticity were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and electrospray mass spectrometry (see below). The pure enzyme (4.4 mg/ml) was stored at −20°C until use.

Protein analysis techniques. Analytical isoelectric focusing and subsequent zymographic detection of β-lactamase were performed as described previously (23). SDS-PAGE was performed as described by Laemmli (21) using 12% and 5% for resolving and stacking gels, respectively. After electrophoresis, proteins were stained with SimplyBlue SafeStain (Invitrogen). The molecular mass of the native BJP-1 enzyme was estimated by size exclusion chromatography using a Superdex 75 HR 10/30 column (Amersham Biosciences) and HZN buffer supplemented with 150 mM NaCl. The enzyme was eluted in the same buffer at a flow rate of 0.4 ml/min. The column was calibrated with the low-range gel filtration calibration kit (Amersham Biosciences), and apparent partition coefficients were calculated as described previously (11). Protein concentration in solution was determined using a commercial kit (Bio-Rad [Richmond, Calif.] protein assay) with bovine serum albumin as the standard. The molecular mass of the enzymatic preparation of BJP-1 was measured by electrospray mass spectrometry as described previously (13), using a Finnigan LTQ mass spectrometer equipped with an ion spray source (Thermo Electron

TABLE 2. Distribution of MBL homologues among sequences of the genomes of *Rhizobiales* and investigation of carbapenem-hydrolyzing enzymes

Species and strain	Accession no.	Highest identity score with subclass B3 MBLs (%)	Closest enzyme	Code
Bradyrhizobium japonicum USDA 110	NP_772870[a]	39.5	CAU-1	BJP-1
	NP_766862	14.9	GOB-1	BJ1
	NP_766991	17.9	L1	BJ2
	NP_767540	18.3	FEZ-1	BJ3
	NP_769257	16.1	THIN-B	BJ4
	NP_770826	14.5	CAU-1	BJ5
	NP_771428	17.6	L1	BJ6
	NP_771689	17.4	GOB-1	BJ7
	NP_773001	16.3	L1	BJ8
	NP_773606	17.0	THIN-B	BJ9
	NP_774530	14.8	L1	BJ10
	NP_774533	15.6	THIN-B	BJ11
Agrobacterium tumefaciens C58	NP_353832[a]	17.5	CAU-1	AT1
	NP_396022	13.8	GOB-1	AT2
	NP_532874	15.5	L1	AT3
	NP_533962	14.5	THIN-B	AT4
Bartonella henselae strain Houston-1	YP_033938	16.7	FEZ-1	BH1
	YP_034354	14.6	GOB-1	BH2
Brucella melitensis 16M	NP_539047	15.3	FEZ-1	BM1
	NP_539413	16.6	CAU-1	BM2
	NP_540349	17.2	CAU-1	BM3
Mesorhizobium loti MAFF 303099	NP_102074[a]	17.3	GOB-1	ML1
	NP_102956	15.6	L1	ML2
	NP_103423	17.2	L1	ML3
	NP_103568	12.9	L1	ML4
	NP_103950	14.2	FEZ-1	ML5
	NP_104628	13.1	THIN-B	ML6
	NP_104783	16.3	GOB-1	ML7
	NP_105758	15.5	GOB-1	ML8
	NP_107838	16.7	THIN-B	ML9
Nitrobacter winogradskyi Nb-255	YP_316733	17.5	GOB-1	NH1
	YP_316992	16.4	THIN-B	NH2
	YP_317080	13.8	THIN-B	NH3
	YP_317232	18.0	THIN-B	NH4
	YP_317506	15.7	FEZ-1	NH5
	YP_319497	14.8	THIN-B	
Rhodopseudomonas palustris CGA009	NP_945718	15.3	FEZ-1	RP1
	NP_945921	18.2	GOB-1	RP2
	NP_945963	17.5	L1	RP3
	NP_946208	15.6	THIN-B	RP4
	NP_946446	16.4	L1	RP5
	NP_947830	15.8	CAU-1	RP6
	NP_949760	15.7	L1	RP7
Sinorhizobium meliloti 1021	NP_384865	15.1	L1	SM1
	NP_385042[a]	16.6	THIN-B	SM2
	NP_385810	15.9	L1	SM3
	NP_386438	16.5	FEZ-1	SM4
	NP_386770	14.1	THIN-B	SM5
	NP_435818	12.8	L1	SM6
	NP_436244	15.1	CAU-1	SM7

[a] Homologues cloned in *Escherichia coli* expression vectors (pBC-SK and pET derivatives, see Materials and Methods for details).

Co., Shaumberg, Ill.). The data were analyzed with the software delivered with the instrument.

Determination of steady-state kinetic parameters and inactivation by metal chelators. Substrate hydrolysis by the purified enzyme preparation was monitored at 30°C by measuring the absorbance variation using a Cary 100 UV-visible light spectrophotometer (Varian, Walnut Creek, Calif.). The wavelengths and changes in the extinction coefficients used were as described previously (22). Enzyme concentrations in the assays were 1.3 to 850 nM. The steady-state kinetic parameters (k_{cat} and K_m) were calculated after direct fit of initial rates versus substrate concentrations using the Henri-Michaelis equation. Inhibi-

FIG. 1. Unrooted tree showing the relationship between members belonging to the superfamily of Zn-dependent hydrolases of the β-lactamase fold (metallo-β-lactamase superfamily, Pfam PF00753). This figure includes proteins of unknown function identified in the 12 complete genomes of members of the order *Rhizobiales* (dashed lines) and proteins of the MBL superfamily for which function has been demonstrated on a biochemical basis (solid lines). The proteins of the MBL superfamily are as follows: L1, FEZ-1, GOB-1, THIN-B, and CAU-1, metallo-β-lactamases of *Stenotrophomonas maltophilia*, *Legionella gormanii*, *Chryseobacterium meningosepticum*, *Janthinobacterium lividum*, and *Caulobacter vibrioides* (formerly *crescentus*), respectively; LAC-Art, -Atu, and -Bth, *N*-acyl-homoserine lactone hydrolases of *Arthrobacter* sp. strain IBN 110, *Agrobacterium tumefaciens*, and *Bacillus thuringiensis*, respectively; CYC-Sar and -Sco, cyclases involved in the polyketide biosynthetic pathway of *Streptomyces arenae* and *Streptomyces coelicolor*, respectively; GLO-Ath, -Sce, and -Tbr, glyoxalase II of *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Trypanosoma brucei*, respectively; CNP-Cal, -Sce, and -Vfi, 3′-5′ cyclic nucleotide phosphodiesterase of *Candida albicans*, *S. cerevisiae*, and *Vibrio fischeri*, respectively; FLA-Meth and -Mth, flavoproteins of *Methanothermobacter thermautotrophicus* and *Moorella thermoacetica*, respectively; ROO-Dgi, rubredoxin-oxygen oxidoreductase of *Desulfovibrio gigas*; CBP-Spn, choline-binding protein of *Streptococcus pneumoniae*; CAR-Ach, carbofuran hydrolase of *Achromobacter* sp.; MPH-Ple, methyl parathion hydrolase of *Plesiomonas* sp. strain M6.

tion of enzymatic activity by divalent metal chelators was assayed as previously described (18) and by measuring the residual activity against 100 μM meropenem in 50 mM HEPES-NaOH buffer (pH 7.5) after incubation of the enzyme for 20 min at 25°C in the presence of different concentrations of inhibitors.

RESULTS AND DISCUSSION

MBL homologues in the *Rhizobiales* genomes. Microbial species belonging to the order *Rhizobiales* (class α-*Proteobacteria*) are the focus of great interest, representing possible human

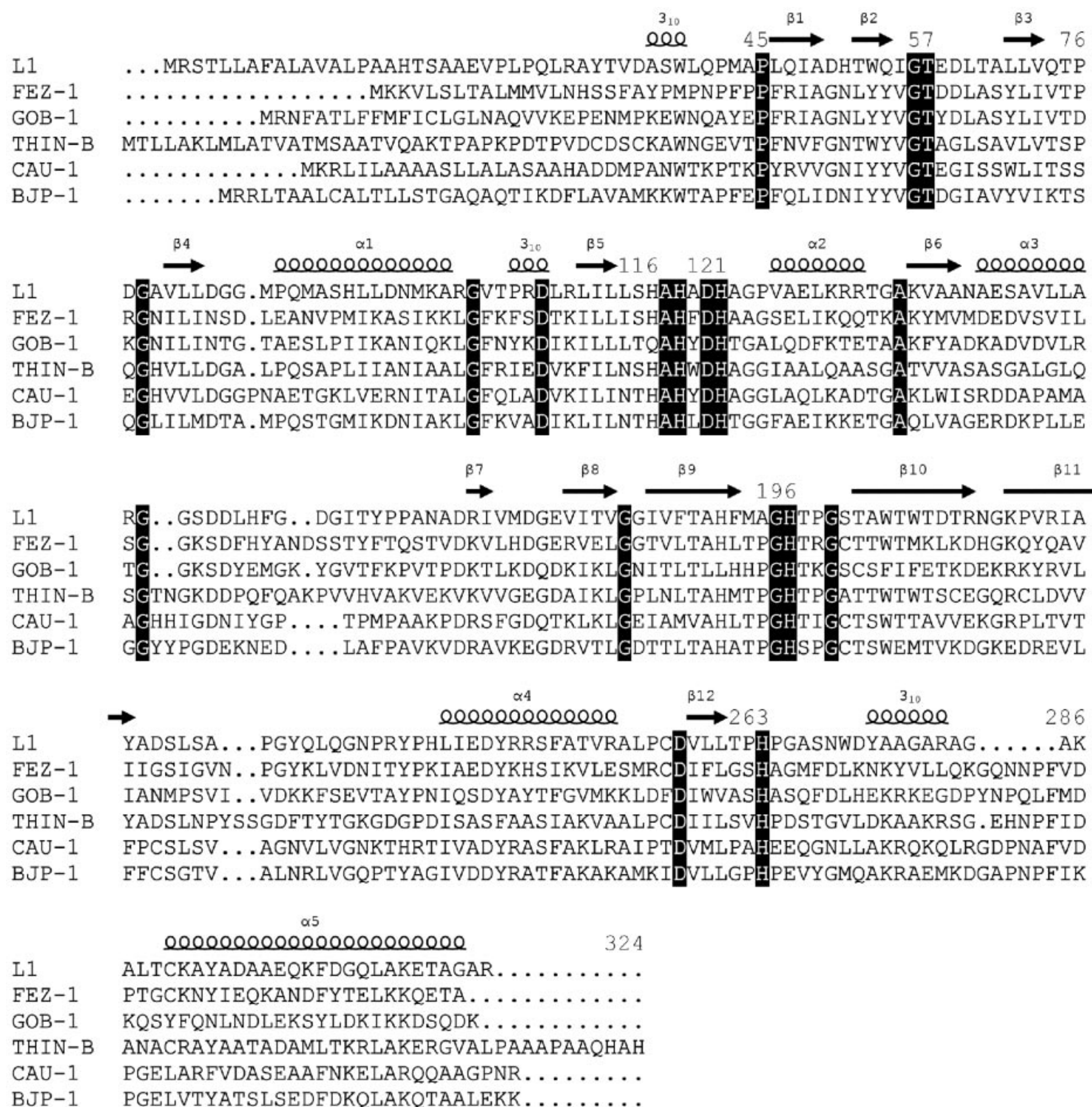

FIG. 2. Amino acid sequence alignment of BJP-1 in comparison with other enzymes of subclass B3. Residues identical in all sequences are shown in white type on a black background. Secondary structure elements (S, strands; 3_{10} and H, helices; L, loops) of the L1 enzyme are indicated above the sequences (30). Numbering is in accordance with the BBL scheme (16). Gaps introduced to maximize alignment are indicated by periods.

pathogens (e.g., *Bartonella henselae* and *Brucella* spp.), plant biotechnology tools (e.g., *Agrobacterium tumefaciens*), or important environmental factors in agriculture and ecology (e.g., nitrogen-fixing bacteria, such as *Bradyrhizobium japonicum*). Their importance is reflected by the 31 genome sequence projects currently listed at the NCBI, among which 12 have been completed (Table 2).

The presence of MBL homologues in these 12 genomes was investigated, and 57 proteins were found to belong to the MBL superfamily and exhibit amino acid sequence identities with members of subclass B3 MBLs ranging from 8% to 40% (Table 2 and Fig. 1). Among these, four homologues were selected for further expression analysis on the basis of their overall sequence similarity with subclass B3 MBLs and the presence of conserved zinc-binding motifs typical of MBLs, in particular the residues flanking His118, His121, and His196 (Fig. 2).

The open reading frames encoding the four homologues were cloned and expressed in two different *E. coli*-based systems, using two different promoters for the expression of cloned genes. The clones carrying the genes from *A. tumefaciens*, *Mesorhizobium loti*, and *Sinorhizobium meliloti* did not show any β-lactamase activity with either system (Table 3 and data not shown). However, the expression in *E. coli* of the gene encoding protein blr6230 from *B. japonicum* (accession no.

TABLE 3. Comparison of the specific activities of crude extracts prepared from different *E. coli* systems carrying different MBL homologues[a]

Plasmid carried by *E. coli* strain DH5α	Sp act (μmol/min · mg protein)	
	Cephalothin	Imipenem
pMS-AGR	<0.01	<0.005
pMS-LOT	<0.01	<0.005
pMS-SIN	<0.01	<0.005
pLBII-BJP	5.4	0.3
pBC-SK	<0.01	<0.005

[a] Reported values are the means of three measurements, and the standard deviations were below 10%. *E. coli* DH5α(pBC-SK) harboring the empty vector is shown for comparison.

TABLE 4. β-Lactam susceptibility profile of *B. japonicum* USDA 110 and *E. coli* DH5α(pLBII-BJP) carrying the cloned bla_{BJP-1} gene[a]

Antibiotic	MIC (μg/ml)		
	B. japonicum USDA 110	*E. coli* DH5α(pLBII-BJP)	*E. coli* DH5α(pBC-SK)
Ampicillin	4	2	2
Piperacillin	>128	2	2
Cephalothin	4	32	4
Cefuroxime	1	16	4
Cefotaxime	1	0.12	0.12
Ceftazidime	>16	0.5	0.5
Cefepime	ND[b]	0.06	0.06
Imipenem	0.5	0.5	0.12
Meropenem	16	0.5	0.03

[a] The *E. coli* strain containing the empty vector (pBC-SK) is shown for comparison.
[b] ND, not determined.

NP_772870) allowed the measurement of an EDTA-inhibited β-lactam-hydrolyzing activity (Table 3). This functional β-lactamase was named BJP-1 (after *B. japonicum*).

Contribution of BJP-1 to β-lactam resistance. No carbapenem-hydrolyzing activity was detected in crude extracts of *B. japonicum* USDA110 grown in liquid medium. The same result was obtained with samples prepared after growing the strain in medium containing subinhibitory concentrations of ampicillin or imipenem, used as potential β-lactamase inducers (data not shown). Despite this finding, *B. japonicum* exhibited rather high MICs for several β-lactams, including carbapenems (Table 4), suggesting that other resistance mechanisms might be involved in the overall poor susceptibility to β-lactams (e.g., low affinity of penicillin-binding proteins, permeability barriers or efflux, and/or the production of additional β-lactamases). In particular, the contribution of another putative class A β-lactamase (bll0941, accession no. NP_767581) encoded by the chromosome of *B. japonicum* and similar to the L2 β-lactamase of *Stenotrophomonas maltophilia* (38% identity) (accession no. CAA69869) could not be excluded, although this point was not specifically investigated.

E. coli DH5α carrying the recombinant plasmid for the expression of bla_{BJP-1} (pLBII-BJP) showed decreased susceptibility to cephalothin, cefuroxime, imipenem, and meropenem, but not to penicillins or expanded-spectrum cephalosporins (Table 4).

BJP-1 purification and biophysical characterization. BJP-1 enzyme was purified from a cell extract of *E. coli* BL21(DE3) [pET-BJP] obtained from a 3-liter culture in autoinducing medium ZYP-5052 grown at 37°C for 24 h. Purification was carried out in two steps of anion-exchange chromatography at pH 8.0 and pH 9.2, with a final yield of approximately 15 mg/liter of culture. The purification process is summarized in Table 5. The purity of the protein preparation was estimated to be >98% according to SDS-PAGE analysis (data not shown) with a protein size of approximately 30 kDa. Size exclusion chromatography yielded a molecular mass of 31 ± 3 kDa for BJP-1, indicating that, under the experimental conditions, the native enzyme is monomeric like most other subclass B3 MBLs (3, 12, 13, 24) except L1 (5, 30). Electrospray mass spectrometry yielded a mass value of 29,893 ± 8 Da, in good agreement with the theoretical mass of the mature protein (29,898.29 Da) obtained after the cleavage of a 20-residue N-terminal signal peptide, as predicted by the SignalP 3.0 algorithm (4). Analytical isoelectric focusing carried out on the purified protein and developed with the chromogenic substrate nitrocefin revealed the presence of a single β-lactamase band of pI 6.9 (data not shown), a value being slightly above the predicted pI (5.9).

Structural features of the BJP-1 enzyme. BJP-1 could be aligned over the entire sequence with other subclass B3 MBLs without introducing major gaps (Fig. 2). The closest similarity (pairwise amino acid sequence identity, 39.5%) was observed with the *C. vibrioides* (*C. crescentus*) CAU-1 enzyme. BJP-1 is slightly more divergent from the *Legionella gormanii* FEZ-1 (33% identity), *Janthinobacterium lividum* THIN-B (30.3% identity), *S. maltophilia* L1, and *Chryseobacterium meningosepticum* GOB-1 (28% identity) enzymes.

Although most conserved residues in enzymes of subclass B3 are also present in BJP-1 enzyme, a notable difference is represented by a threonine-to-serine substitution at position 197, just after one of the Zn-binding histidine residues (Fig. 2). Although the nature of these residues is similar, it might be interesting to investigate the role of this conserved residue in the structure and/or function of the enzyme. Similarly, at position 119 (sandwiched between three Zn-binding residues, His118, Asp120, and His121) there is usually an aromatic residue, except in the L1 and BJP-1 enzymes, where an alanine and a leucine are found, respectively.

The monomeric nature of the BJP-1 enzyme correlates with the replacement of the Met175 residue in the L1 enzyme by a lysine in BJP-1. Met175 is responsible for determinant intersubunit interactions with an hydrophobic pocket formed by residues Leu154, Pro198, and Tyr236 in the tetrameric L1 enzyme (17, 30). Interestingly, the two cysteine residues (at positions 256 and 290) involved in the disulfide bridge present in L1 (30), FEZ-1 (17), and probably also in THIN-B (12), are not conserved in BJP-1. In the latter and as in CAU-1, two cysteine residues found in positions 200 and 220 might form a disulfide bridge between the loop preceding strand S10 and that following strand S11, which could be located very close to the active site (Fig. 2) (13).

Kinetic properties of the BJP-1 β-lactamase. The kinetic parameters were determined for a representative set of β-lactam antibiotics, including penicillins, narrow- and expanded-spectrum cephalosporins, carbapenems, and aztreonam (Table 6). The results showed that BJP-1 exhibits a broad substrate profile, since

TABLE 5. Summary of the typical procedures of purification of BJP-1 metallo-β-lactamase produced in *E. coli* BL21(DE3)[pET-BJP]

Product of purification step	Vol (ml)	Total amt of protein (mg)	Total activity (U)[a]	Sp act (U/mg protein)	Recovery (%)	Purification (fold)
Cell extract	90	2,000	40,860	21	100	1
DEAE Sepharose FT[b]	160	109	27,500	252	67	12
Source Q FT	55	50	25,355	506	61	24

[a] One unit of activity is defined as the amount of enzyme hydrolyzing 1 μmol of cephalothin per min under the conditions described in Materials and Methods.
[b] FT, flowthrough.

most tested compounds were hydrolyzed, although with very different catalytic efficiencies (k_{cat}/K_m values ranging from 2×10^2 to 6×10^6 $M^{-1} \cdot s^{-1}$). Ticarcillin, temocillin, and aztreonam were not recognized by the enzyme. The best substrate of BJP-1 is CENTA, a chromogenic cephalosporin (2), for which the lowest K_m value (19 μM) and the highest turnover rate (k_{cat}, 114 s^{-1}) were measured, resulting in a catalytic efficiency at least 1 order of magnitude higher than those of other substrates. BJP-1 shows a higher activity against penicillin G, narrow-spectrum cephalosporins, cefotaxime, and meropenem, while ceftazidime, cefepime, and faropenem were poorly hydrolyzed (k_{cat}/K_m, $<10^4$ $M^{-1} \cdot s^{-1}$). BJP-1 exhibits overall high K_m values (K_m, >100 μM for most substrates, except CENTA), indicating a poor interaction with β-lactams, in particular with most penicillins, ceftazidime, and cefepime.

Overall, the functional properties of BJP-1 are very similar to those of some other subclass B3 enzymes, especially FEZ-1, CAU-1, and THIN-B. All these enzymes have a preference for meropenem over imipenem, narrow-spectrum cephalosporins over penicillins, and exhibit poor recognition of cefepime (a representative of zwitterionic expanded-spectrum cephalosporins).

Kinetic data are consistent with the limited effect of BJP-1 expression on *E. coli* susceptibility to some substrates (e.g., ceftazidime and cefepime, which are poor substrates for the enzyme), while this was less obvious with other β-lactams (e.g., piperacillin and cefotaxime, which are good substrates).

Inactivation of BJP-1 by metal chelators. In inactivation experiments where the enzyme activity was recorded in the presence of various concentrations of the metal chelators (up to 50 mM with EDTA) and using meropenem as the reporter substrate, complete enzyme inactivation was not achieved within 30 min, suggesting low inactivation efficiencies (k_{+2}/K) and, thus, a rather low susceptibility to these agents. In other experiments, where the enzyme activity was measured after preincubation with the metal chelators for 20 min, complete enzyme inactivation could not be observed, even at high inactivator concentrations. The most effective agent, *o*-phenanthroline, inhibited 80% of BJP-1 activity at a concentration of 500 μM, and no further inactivation was observed up to a concentration of 1.2 mM (Fig. 3). A similar behavior was observed for dipicolinic acid with a residual activity of 30% at 300 μM and no further changes up to 1.2 mM (Fig. 3). EDTA was the least efficient inactivator, and no more than 60% of the enzyme activity could be inhibited at concentrations as high as 50 mM (data not shown). This apparent poor sensitivity to chelating agents is a striking peculiarity of BJP-1, since all other enzymes of subclass B3 are readily inactivated by these agents (12, 13, 24). This behavior is apparently dependent on the protein structure and not on the presence of a metal co-

TABLE 6. Kinetic parameters of the purified BJP-1[a]

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)					
			BJP-1	L1	GOB-1	FEZ-1	CAU-1	THIN-B
Ampicillin	13	670	1.9×10^4	4.4×10^6	–[b]	1.1×10^4	5.0×10^5	3.7×10^5
Penicillin G	18	130	1.3×10^5	2.2×10^7	1.9×10^6	1.1×10^5	4.5×10^5	–
Piperacillin	47	700	6.7×10^4	7.0×10^6	1.7×10^6	1.2×10^4	5.7×10^5	2.0×10^5
Temocillin	NH[c]	ND[d]	ND	1.2×10^6	–	1.3×10^4	–	–
Ticarcillin	NH	ND	ND	2.7×10^6	5.2×10^5	1.3×10^4	–	–
Cephalothin	133	230	5.8×10^5	–	6.7×10^5	2.5×10^6	4.3×10^5	–
Cefoxitin	10	140	7.1×10^4	5.5×10^5	2.5×10^5	2.7×10^5	–	–
Cefuroxime	58	115	5×10^5	2.7×10^6	9.8×10^5	6.6×10^6	1.4×10^4	2.8×10^6
Cefotaxime	41	300	1.4×10^5	2.6×10^6	8.5×10^5	2.4×10^6	–	2.0×10^6
Ceftazidime	>3	>700	4.3×10^3	1.8×10^5	7.6×10^5	4.0×10^3	2.0×10^3	1.4×10^5
Ceftriaxone	>8	>80	9.6×10^4	–	–	–	–	–
Cefepime	>0.08	>400	2×10^2	1.9×10^4	2.0×10^5	6.0×10^3	–	7.9×10^3
CENTA	114	19	6×10^6	–	–	–	–	–
Imipenem	15	260	6×10^4	7.3×10^5	6.6×10^5	2.0×10^5	2.0×10^5	1.5×10^6
Meropenem	156	190	8.3×10^5	4.5×10^6	5.3×10^6	5.0×10^5	2.6×10^5	5.0×10^6
Faropenem	2	245	6.8×10^3	–	–	–	–	–
Aztreonam	NH	ND	ND	ND	ND	ND	ND	ND

[a] Kinetic parameters of the purified BJP-1 enzyme (standard deviations were below 10%). k_{cat}/K_m values of other subclass B3 enzymes are reported for comparison and are from the following references: L1 (14, 15), GOB-1 (3), FEZ-1 (24), CAU-1 (13), and THIN-B (12).
[b] –, data not available.
[c] NH, no hydrolysis detected with enzyme concentrations up to 850 nM.
[d] ND, data could not be determined.

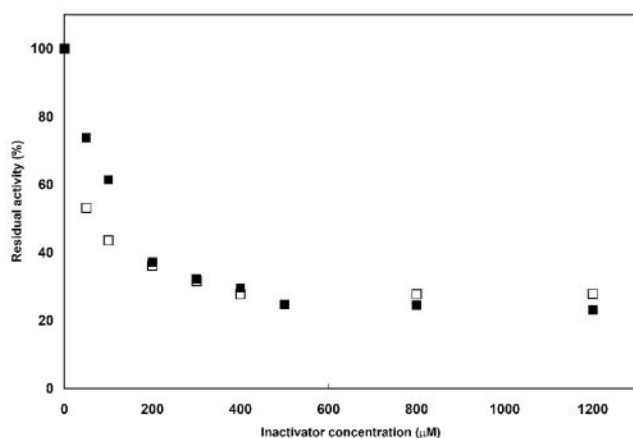

FIG. 3. Inactivation of BJP-1 with dipicolinic acid (empty squares) and *o*-phenanthroline (full squares).

factor other than zinc. A preliminary three-dimensional structure of the enzyme, solved using the anomalous signal present in the diffracted intensities of a data set collected with X-rays with a 1.2813-Å wavelength (corresponding to the zinc X-ray absorption edge peak), revealed the presence of the anomalous signal in the data, which clearly indicated that Zn ions were the metal cofactors in the enzyme. In addition, the anomalous difference electron density map computed from those data unambiguously showed that the enzyme possesses two zinc ions in the active site (S. Mangani, personal communication).

The structural basis for the slow and incomplete inactivation of BJP-1 by metal chelators remains unclear. With most subclass B3 enzymes, the inactivation by metal chelators was explained by a direct scavenging of the metal ions (the inactivation rate was independent of the inactivator concentration). One exception was represented by THIN-B, whose inactivation followed the formation of a enzyme-metal-inactivator ternary complex, as observed with subclass B1 and B2 enzymes (12). Considering this model, the behavior of BJP-1 might be explained by a rather high velocity constant for the formation of the ternary complex from the apoenzyme and the metal-inactivator complex (k_{-2}), with subsequent release of the active form of the enzyme. This would result in the establishment of an equilibrium between the apoenzyme, the ternary complex, and the active metalloenzyme, explaining the significant amount of residual activity observed.

Concluding remarks. Among 57 proteins encoded by the 12 complete genomes of *Rhizobiales*, four proteins were retained for analysis and only one was found to be a functional MBL, which also showed the highest similarity with subclass B3 enzymes. The other enzymes investigated (AT1, ML1, and SM2) did not exhibit β-lactamase activity when produced in *E. coli*. This suggests that functional MBLs might be present in a cluster of species but that this presence is not a conserved feature that might be extended to microorganisms belonging to the same order or family. The phylogenetic analysis presented in this study can give additional clues of the putative functions of some other MBL homologues, due to their relationships with enzymes for which biochemical function has been ascertained (e.g., BJ1 is related to glyoxalases II, BJ7 to *N*-acylhomoserine lactone hydrolases, BJ9 to methyl parathion hydrolases) (Fig. 1).

From the functional standpoint, BJP-1 and CAU-1 shared overall low affinities for β-lactam substrates. It has been hypothesized that CAU-1 might be involved in another metabolic process due to its peculiar genetic context characterized by overlapping ORFs, clearly showing its association with genes encoding proteins involved in methionine biosynthesis. However, the structure of the genetic locus flanking $bla_{\mathrm{BJP\text{-}1}}$ is not suggestive of a similar situation. Upstream of the $bla_{\mathrm{BJP\text{-}1}}$ gene, there are two ORFs that encode proteins of unknown function which do not exhibit any significant similarity with other known bacterial proteins, while a gene encoding a putative transmembrane signal transduction protein is found downstream. Moreover, it does not seem associated with any of the numerous insertion sequences found in the *B. japonicum* genome (20). The presence of a functional β-lactamase in a plant symbiont remains enigmatic and we cannot exclude the possibility that it might have another function.

In conclusion, BJP-1 is a new member of the growing subclass B3 of MBLs which exhibits peculiar functional features and represents an interesting model to further investigate the structure-function relationships among subclass B3 enzymes. This comparative analysis might lead to critical insights potentially useful for the design of inhibitors, and more generally, this approach could help to identify the structural features ruling the substrate specificity in the MBL superfamily, whose members evolved very different functions from a conserved structural topology.

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REFERENCES

1. **Aravind, L.** 1999. An evolutionary classification of the metallo-β-lactamase fold proteins. In Silico Biol. **1:**69–91.
2. **Bebrone, C., C. Moali, F. Mahy, S. Rival, J. D. Docquier, G. M. Rossolini, J. Fastrez, R. F. Pratt, J. M. Frère, and M. Galleni.** 2001. CENTA as a chromogenic substrate for studying β-lactamases. Antimicrob. Agents Chemother. **45:**1868–1871.
3. **Bellais, S., D. Aubert, T. Naas, and P. Nordmann.** 2000. Molecular and biochemical heterogeneity of class B carbapenem-hydrolyzing β-lactamases in *Chryseobacterium meningosepticum*. Antimicrob. Agents Chemother. **44:** 1878–1886.
4. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783–795.
5. **Bicknell, R., E. L. Emanuel, J. Gagnon, and S. G. Waley.** 1985. The production and molecular properties of the zinc β-lactamase of *Pseudomonas maltophilia* IID 1275. Biochem. J. **229:**791–797.
6. **Bush, K.** 1999. β-Lactamases of increasing clinical importance. Curr. Pharm. Des. **5:**839–845.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
8. **Callebaut, I., D. Moshous, J. P. Mornon, and J. P. De Villartay.** 2002. Metallo-β-lactamase fold within nucleic acids processing enzymes: the β-CASP family. Nucleic Acids Res. **30:**3592–3601.
9. **Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. **31:**3497–3500.

10. **Daiyasu, H., K. Osaka, Y. Ishino, and H. Toh.** 2001. Expansion of the zinc metallo-hydrolase family of the β-lactamase fold. FEBS Lett. **503:**1–6.
11. **Docquier, J. D., J. Lamotte-Brasseur, M. Galleni, G. Amicosante, J. M. Frère, and G. M. Rossolini.** 2003. On functional and structural heterogeneity of VIM-type metallo-β-lactamases. J. Antimicrob. Chemother. **51:**257–266.
12. **Docquier, J. D., T. Lopizzo, S. Liberatori, M. Prenna, M. C. Thaller, J. M. Frère, and G. M. Rossolini.** 2004. Biochemical characterization of the THIN-B metallo-β-lactamase of *Janthinobacterium lividum*. Antimicrob. Agents Chemother. **48:**4778–4783.
13. **Docquier, J. D., F. Pantanella, F. Giuliani, M. C. Thaller, G. Amicosante, M. Galleni, J. M. Frère, K. Bush, and G. M. Rossolini.** 2002. CAU-1, a subclass B3 metallo-β-lactamase of low substrate affinity encoded by an ortholog present in the *Caulobacter crescentus* chromosome. Antimicrob. Agents Chemother. **46:**1823–1830.
14. **Felici, A., and G. Amicosante.** 1995. Kinetic analysis of extension of substrate specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* metallo-β-lactamases. Antimicrob. Agents Chemother. **39:**192–199.
15. **Felici, A., G. Amicosante, A. Oratore, R. Strom, P. Ledent, B. Joris, L. Fanuel, and J. M. Frère.** 1993. An overview of the kinetic parameters of class B β-lactamases. Biochem. J. **291:**151–155.
16. **Galleni, M., J. Lamotte-Brasseur, G. M. Rossolini, J. Spencer, O. Dideberg, and J. M. Frère.** 2001. Standard numbering scheme for class B β-lactamases. Antimicrob. Agents Chemother. **45:**660–663.
17. **Garcia-Saez, I., P. S. Mercuri, C. Papamicael, R. Kahn, J. M. Frère, M. Galleni, G. M. Rossolini, and O. Dideberg.** 2003. Three-dimensional structure of FEZ-1, a monomeric subclass B3 metallo-β-lactamase from *Fluoribacter gormanii*, in native form and in complex with D-captopril. J. Mol. Biol. **325:**651–660.
18. **Hernandez-Valladeres, M., A. Felici, G. Weber, H. W. Adolph, M. Zeppezauer, G. M. Rossolini, G. Amicosante, J. M. Frère, and M. Galleni.** 1997. Zn(II) dependence of the *Aeromonas hydrophila* AE036 metallo-β-lactamase activity and stability. Biochemistry **36:**11534–11541.
19. **Jacoby, G. A., and L. S. Munoz-Price.** 2005. The new β-lactamases. N. Engl. J. Med. **352:**380–391.
20. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**189–197.
21. **Laemmli, U. K.** 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Laraki, N., N. Franceschini, G. M. Rossolini, P. Santucci, C. Meunier, E. de Pauw, G. Amicosante, J. M. Frère, and M. Galleni.** 1999. Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-β-lactamase IMP-1 produced by *Escherichia coli*. Antimicrob. Agents Chemother. **43:**902–906.
23. **Lauretti, L., M. L. Riccio, A. Mazzariol, G. Cornaglia, G. Amicosante, R. Fontana, and G. M. Rossolini.** 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.
24. **Mercuri, P. S., F. Bouillenne, L. Boschi, J. Lamotte-Brasseur, G. Amicosante, B. Devreese, J. Van Beeumen, J. M. Frère, G. M. Rossolini, and M. Galleni.** 2001. Biochemical characterization of the FEZ-1 metallo-β-lactamase of *Legionella gormanii* ATCC 33297^T produced in *Escherichia coli*. Antimicrob. Agents Chemother. **45:**1254–1262.
25. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial susceptibility testing; 14th informational supplement. NCCLS document M100-S14. National Committee for Clinical Laboratory Standards, Wayne, Pa.
26. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8:**321–331.
27. **Page, R. D.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
28. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, N.Y.
29. **Studier, F. W.** 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. **41:**207–234.
30. **Ullah, J. H., T. R. Walsh, I. A. Taylor, D. C. Emery, C. S. Verma, S. J. Gamblin, and J. Spencer.** 1998. The crystal structure of the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* at 1.7 A resolution. J. Mol. Biol. **284:**125–136.
31. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.