

## Molecular Characterization of a $\beta$ -Lactamase Gene, *bla*<sub>GIM-1</sub>, Encoding a New Subclass of Metallo- $\beta$ -Lactamase

Mariana Castanheira,<sup>1,2\*</sup> Mark A. Toleman,<sup>2</sup> Ronald N. Jones,<sup>3</sup> Franz J. Schmidt,<sup>4</sup> and Timothy R. Walsh<sup>2</sup>

*Disciplina de Doencas Infecciosas e Parasitarias, Universidade Federal de Sao Paulo, Sao Paulo, Brazil*<sup>1</sup>;  
*Department of Pathology and Microbiology, University of Bristol, Bristol, United Kingdom*<sup>2</sup>;  
*The JONES Group/JMI Laboratories, North Liberty, Iowa*<sup>3</sup>; and  
*Institut für Laboratoriumsmedizin, Mikrobiologie, Minden, Germany*<sup>4</sup>

Received 17 February 2004/Returned for modification 1 May 2004/Accepted 16 August 2004

As part of the SENTRY Antimicrobial Surveillance Program in 2002, five multidrug-resistant *Pseudomonas aeruginosa* clinical isolates were detected with metallo- $\beta$ -lactamase (MBL) activity. The isolates were recovered from different patients in a medical center located in Dusseldorf, Germany. The resistant determinant was isolated amplifying the region between the integrase and the *aacA4* gene cassette. Sequencing revealed a novel MBL gene, designated *bla*<sub>GIM-1</sub>. Additional analysis showed that GIM-1, comprising 250 amino acids and with a pI value of 5.4, differs in its primary sequence from that described for IMP, VIM, and SPM-1 enzymes by 39 to 43%, 28 to 31%, and 28%, respectively. The enzyme possesses unique amino acids within the major consensus sequence (HXHXD) of the MBL family. Kinetics analysis revealed that GIM-1 has no clear preference for any substrate and did not hydrolyze azlocillin, aztreonam, and the serine- $\beta$ -lactamase inhibitors. *bla*<sub>GIM-1</sub> was found on a 22-kb nontransferable plasmid. The new MBL gene was embedded in the first position of a 6-kb class 1 integron, In77, with distinct features, including an *aacA4* cassette downstream of the MBL gene that appeared to be truncated with *bla*<sub>GIM-1</sub>. The *aacA4* was followed by an *aadA1* gene cassette that was interrupted by a copy of the IS1394. This integron also carried an oxacillinase gene, *bla*<sub>OXA-2</sub>, before the 3'-CS region. GIM-1 appears to be a unique MBL, which is located in a distinct integron structure, and represents the fourth subclass of mobile MBL enzymes to be characterized.

*Pseudomonas aeruginosa* is a leading cause of nosocomial infections, giving rise to a wide range of life-threatening conditions. Its intrinsic resistance to many antimicrobial agents and its ability to develop multidrug resistance imposes a serious therapeutic problem (8). Carbapenems are very useful antimicrobial agents for the treatment of infections caused by *P. aeruginosa*; however, increasing use of these compounds has resulted in the development of carbapenem-resistant *P. aeruginosa*. Mechanisms of resistance to carbapenems in *P. aeruginosa* are associated with reduced uptake of the agent resulting from the loss or reduced expression of the OprD porin, combined with derepression of the chromosomal *ampC*  $\beta$ -lactamase gene (23); overexpression of an efflux pump system (22, 37); and production of a metallo- $\beta$ -lactamase (M $\beta$ L) (16).

The first mobile M $\beta$ L (IMP-1) was found in *P. aeruginosa* in Japan in the early 1990s (33). For many years, the occurrence of IMP-1-producing bacteria was confined to Japan. More recently, however, IMP-1 and IMP-variant enzymes have been reported from many other countries and across four continents (6, 9, 12, 13, 24, 27). Since the emergence of IMP-1, two other subclasses of clinically relevant M $\beta$ Ls have been described: the VIM series (15) and SPM-1 (29). VIM variants are now found throughout the world as well (2, 13, 17, 20, 21, 30, 32, 34, 35), whereas SPM-1 thus far seems to be restricted to Brazil.

We characterize here a novel subclass of Ambler class B

enzyme, GIM-1, which is encoded by a gene cassette located in the first position of a class 1 integron. The carbapenem-resistant *P. aeruginosa* clinical isolates that produce GIM-1 were recovered in Germany and were detected by The SENTRY Antimicrobial Surveillance Program in 2002.

### MATERIALS AND METHODS

**Bacterial strains and susceptibility testing.** In 2002, five clinical isolates of *P. aeruginosa* (73-5671, 73-12198, 73-15553, 73-15574, and 73-15480) were collected in a medical site located in Dusseldorf, Germany, and submitted to the SENTRY Program monitored in North Liberty, Iowa (JMI Laboratories). The clinical isolates were susceptibility tested by using the reference broth microdilution method as described by the National Committee for Clinical Laboratory Standards (19). Rifampin-resistant (Rif<sup>r</sup>) derivatives of *Escherichia coli* K-12 and *P. aeruginosa* PA01 (28) were used as recipients in conjugation experiments. *E. coli* DH5 $\alpha$  and Rif<sup>r</sup> PA01 were used for transformation.

**Phenotypic detection of M $\beta$ L.** M $\beta$ L Etest strips (AB Biodisk, Solna, Sweden) were used to screen for class B  $\beta$ -lactamase production according to the manufacturer's instructions. In addition, carbapenemase activities of cell sonicates from overnight broth cultures were determined by spectrophotometric assays. These were carried out with 150  $\mu$ M imipenem and meropenem as substrates at 299 nm. Assays were performed with or without EDTA (25 mM) to ascertain whether activity is inhibited by chelating agents.

**DNA analysis methodology.** Molecular screening for *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>SPM-1</sub> was performed by using PCR, as previously described (29), with primers targeting conserved regions of the MBL genes (Table 1). Strains known to harbor MBL genes were used as positive controls for the reactions. Since several  $\beta$ -lactamases are encoded on gene cassettes, embedded in class 1 integrons, primers targeting the 5' conserved sequence and the *aacA4* gene cassette were used in additional PCR screening reactions. The integron structure was revealed with a walking sequencing strategy, with the primers described in Table 1. Plasmid extraction from *P. aeruginosa* 73-5671 was undertaken with a QIAprep Spin Miniprep kit (Qiagen, West Sussex, United Kingdom). Restriction endonuclease analysis of the plasmid was carried out with seven different restriction enzymes,

\* Corresponding author. Mailing address: Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom. Phone: 44-117-9288819. Fax: 44-117-9287896. E-mail: maricastanh@yahoo.com.

TABLE 1. Oligonucleotides used as primers for PCR amplification and sequencing

Primer	Target	Sequence (5'-3')	Accession no.
IMP1F	<i>bla</i> <sub>IMP</sub> -like	TGAGCAAGTTATCTGTATTC	AJ223604
IMP1R	<i>bla</i> <sub>MP-1</sub> 59-be	GCTGCAACGACTTGTAG	AJ223604
ATT1F	<i>attI1</i>	TTATGGAGCAGCAACGATGT	AJ515707
VIMR	<i>bla</i> <sub>VIM</sub> -like	CGAATGCGGAGCACCAGG	AJ515707
SPM1F	<i>bla</i> <sub>SPM</sub> -like	CCTACAATCTAACGGCGACC	AJ492820
SPM1R	<i>bla</i> <sub>SPM</sub> -like	TCGCCGTGTCCAGGTATAAC	AJ492820
int11F	Integrase	GCCGTAGAAGAACAGCAAGG	AJ515707
int12F	Integrase	TCAATCTCCGCGAGAAGTGC	AJ515707
ATT1R	<i>attI1</i>	GCCTGTTCGGTTCGTAAGCT	AJ515707
GIMF	<i>bla</i> <sub>GIM-1</sub>	AGAACCCTTGACCGAACGCAG	This study
GIMR	<i>bla</i> <sub>GIM-1</sub>	ACTCATGACTCCTCACGAGG	This study
GIMFF	<i>bla</i> <sub>GIM-1</sub> flanking region	CTACGTGACCAACAGCAACG	This study
aacA4F	<i>aacA4</i>	TGCGATGCTCTATGAGTGGC	AJ515707
aacA4R	<i>aacA4</i>	ATGTACACGGCTGGACCATC	AJ515707
aacA4FF	<i>aacA4</i> flanking region	AACTTGCAGCGATCCGATG	AJ515707
aacA4FR	<i>aacA4</i> flanking region	AGCCAATCATAGAGCATCGC	AJ515707
aadA1F	<i>aadA1</i>	CGCCGAAGTATCGACTCAAC	AJ584652
IS1394R	IS1394	ACAGAGGTAGTGGCGTTGC	U37284
ISF	IS1394	CGGTCTTCTGGGTGATTTC	U37284
ISFR	IS1394	CGCGCTTAGCTGGATAACG	U37284
aadA1R	<i>aadA1</i>	GACTACCTTGGTGATCTCGC	AJ584652
aadA1FF	<i>aadA1</i> flanking region	GAGATCACC AAGGTAGTCGG	AJ584652
oxa2F	<i>bla</i> <sub>OXA-2</sub>	TCAAGCCAAAGGCACGATAG	AF300985
oxa2R	<i>bla</i> <sub>OXA-2</sub>	TCCGAGTTGACTGCCGGGTTG	AF300985
oxa2FF	<i>bla</i> <sub>OXA-2</sub> flanking region	AAGCCTTACC GCCCAACC	AF300985
oxa2FR	<i>bla</i> <sub>OXA-2</sub> flanking region	ATGCGCGAAAGTGGCAAGAG	AF300985
QACR	<i>qacEΔ1</i>	CGGATGTTGCGATTACTTCG	AJ515707
SUL2R	<i>sulI</i>	GGCTCTCATCGAAGAAGGAG	AJ515707
Sul1R	<i>sulI</i>	GGCTCTCATCGAAGAAGGAG	AJ515707

namely, BamHI, EcoRI, HindIII, HincII, SpeI, SmaI, and XbaI (Invitrogen, Carlsbad, Calif.), singly and in pairs, to determine the size of the plasmid. Southern blot analysis was performed on agarose gels by standard methods, and hybridization was undertaken with a *bla*<sub>GIM-1</sub> probe labeled with <sup>32</sup>P by using a random primer technique (25). Transformation was carried out by using electroporation, and selection for transformants was performed on nutrient agar plates containing ceftazidime at 4 μg/ml. Conjugation experiments were carried out in liquid medium, and selection was performed on nutrient agar with ceftazidime (4 μg/ml) and rifampin (200 μg/ml). Primers int11F and aacA4FR (Table 1) were used to amplify the integron structure harboring the resistant determinant, and the purified amplicons were cloned into PCRScriptCam SK+ (Stratagene Cloning Systems, La Jolla, Calif.). DHα was used as a host for the recombinant plasmid.

**DNA sequencing and computer analysis.** The PCR products were sequenced on both strands by using a Perkin-Elmer systems 377 DNA sequencer. The nucleotide sequences, deduced amino acid sequences, and phylogenetic relationships were analyzed by using the Lasergene software package (DNASTar, Madison, Wis.). Obtained sequences were compared to sequences available on the internet (<http://www.ebi.ac.uk/fasta33/>).

**Analytical IEF.** *P. aeruginosa* extracts showing β-lactamase activity were obtained by cell lyses with BugBuster (Novagen, Nottingham, United Kingdom). Isoelectric focusing (IEF) was performed with a pH 3 to 10 pre-cast vertical IEF gel with the Novex system (Invitrogen). The conditions applied were according to the manufacturer's instructions. The focused β-lactamases were detected by overlaying the gel with nitrocefin solution (Microbiology Systems, Cockeysville, Md.). Isoelectric points were estimated by using linear regression with Graf Prism software (GraphPad Software, Inc., San Diego, Calif.) and by comparison to reference proteins provided by a pI 4.5 to 9.5 Standard IEF Marker (Bio-Rad, Richmond, Calif.) and the TEM-1 β-lactamase (31).

**Pulsed-field gel electrophoresis (PFGE).** Genomic DNA was prepared in agarose blocks and digested in situ with the restriction enzyme SpeI (Invitrogen). DNA fragments were separated by electrophoresis in the CHEF-DR III apparatus (Bio-Rad). The band pattern was interpreted according to the recommendation of Tenover et al. (26).

**β-Lactamase purification.** Cultures of *P. aeruginosa* 73-5671 were grown overnight at 37°C in 4 liters of Terrific Broth (12% tryptone, 20% yeast extract, 0.4% glycerol, 0.17 M monopotassium phosphate, 0.72 M dipotassium phosphate). A

periplasmic protein preparation was obtained as previously described (1). This protein solution was treated with 60% saturated ammonium sulfate solution to precipitate most of the proteins, which were removed by centrifugation. The clarified supernatant was loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) preequilibrated with buffer A (50 mM cacodylate, 100 μM zinc chloride, 1 mM β-mercaptoethanol, 3 mM sodium azide [pH 6.8]). The proteins were eluted with a linear gradient of 300 mM to 800 mM sodium chloride. Fractions showing the highest degree of β-lactamase activity against nitrocefin were pooled, and proteins in the mix were subjected to a Superdex-200 (Amersham Pharmacia Biotech) gel filtration column at 0.3 ml/min. During the purification procedure, the β-lactamase activity was tracked by nitrocefin hydrolysis.

The protein recovered in the flowthrough was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and analytical IEF was applied to confirm the purity of the preparation.

**Kinetic measurements.** Purified β-lactamase was used to determine the kinetic parameters *k*<sub>cat</sub> and *K*<sub>m</sub>. Reactions were performed at 25°C in 2 ml of assay buffer (50 mM cacodylate, 100 mM sodium chloride, 100 μM zinc chloride [pH 7.0]). The rate of hydrolysis of each β-lactam was calculated with triplicate reactions for at least five different concentrations of substrate based on the extinction coefficients for each substrate. The assays were performed in a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, United Kingdom) by observing the changes in absorption resulting from the opening the β-lactam ring at the specific wavelengths for each of the 20 antimicrobial agents evaluated, as previously described (18).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in the present study have been assigned EMBL/GenBank nucleotide accession number AJ620678.

## RESULTS

**Properties of the *P. aeruginosa* clinical isolates.** The five carbapenem-resistant *P. aeruginosa* isolates were recovered from different patients in the same hospital ward in Dusseldorf, Germany, between February and July 2002. The index strain, 73-5671, was recovered from a 37-year-old male inten-

TABLE 2. Antimicrobial susceptibility patterns of the five GIM-1 producer isolates from Dusseldorf, Germany, submitted to the SENTRY Antimicrobial Surveillance Program in 2002

Antimicrobial agents	MIC ( $\mu\text{g/ml}$ ) for strain:							
	<i>P. aeruginosa</i> 73-5671	<i>P. aeruginosa</i> 73-12198	<i>P. aeruginosa</i> 73-15553	<i>P. aeruginosa</i> 73-15574	<i>P. aeruginosa</i> 73-15480	<i>E. coli</i> DH5 $\alpha$ (pGIM-1)	<i>E. coli</i> DH5 $\alpha$ (pSCRIPT)	
Piperacillin	>128	>128	>128	>128	>128	16	0.5	
Piperacillin-tazobactam	>64	>64	>64	>64	>64	–	–	
Ticarcillin	>128	>128	>128	>128	>128	–	–	
Ticarcillin-clavulanic acid	>128	>128	>128	>128	>128	–	–	
Ceftriaxone	>32	>32	>32	>32	>32	128	0.125	
Ceftazidime	>16	>16	>16	>16	>16	256	0.125	
Cefepime	16	16	>16	16	16	4	0.06	
Imipenem	>8	>8	>8	>8	>8	0.5	0.06	
Meropenem	>8	>8	>8	>8	>8	0.125	0.06	
Aztreonam	8	8	16	16	16	0.125	0.06	
Ciprofloxacin	>4	>4	>4	>4	>4	–	–	
Levofloxacin	>4	>4	>4	>4	>4	–	–	
Gatifloxacin	>4	>4	>4	>4	>4	–	–	
Amikacin	4	4	16	16	16	–	–	
Gentamicin	>8	>8	>8	>8	>8	–	–	
Tobramycin	16	16	>16	16	>16	–	–	
Netilmicin	>32	>32	>32	>32	>32	–	–	
Tetracycline	>8	>8	>8	>8	>8	–	–	
Trimethoprim-sulfamethoxazole	>2	>2	>2	>2	>2	–	–	
Polymyxin B	2	2	4	1	1	–	–	

<sup>a</sup> –, Not tested.

sive care unit patient with nosocomial pneumonia in February 2002. All five isolates were submitted to the SENTRY Program as respiratory tract specimens. The susceptibility patterns of the five isolates were found to be nearly identical. The isolates were highly resistant to 19 of the 20 antimicrobial agents tested (including all  $\beta$ -lactams, aminoglycosides, and quinolones), as shown in Table 2. The isolates showed particular resistance to imipenem (MIC, >8  $\mu\text{g/ml}$ ), meropenem (MIC, >8  $\mu\text{g/ml}$ ), ceftazidime (MIC, >16  $\mu\text{g/ml}$ ), cefepime (MIC,  $\geq$ 16  $\mu\text{g/ml}$ ), and piperacillin-tazobactam (MIC, >128  $\mu\text{g/ml}$ ). Of the antimicrobial agents tested, the five isolates were only susceptible to polymyxin B.

By PFGE analysis, the five *P. aeruginosa* isolates were indistinguishable (clonal). These strains were compared to six carbapenem-susceptible isolates recovered at the same time from the same medical site in Germany. All susceptible isolates were, by PFGE analysis, significantly different from the carbapenem-resistant isolates and also distinct from each other (data not shown).

**M $\beta$ L screening and PCR experiments.** Initial M $\beta$ L screening experiments showed that each of the five isolates produce an M $\beta$ L. Analysis with M $\beta$ L Etest (ABBIODISK) strips showed decreases in the MICs of imipenem in the presence of EDTA (from >256  $\mu\text{g/ml}$  for imipenem to 2  $\mu\text{g/ml}$  for the combination imipenem-EDTA). Spectrophotometric assays demonstrated carbapenemase activity against carbapenems (hydrolyzing activity against meropenem between 0.0113 and 0.0287 absorbance units/min), which was 85% EDTA inhibited in all five *P. aeruginosa* isolates.

Preliminary PCR amplification experiments failed to detect previously described M $\beta$ L genes, whereas controls with *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>SPM</sub>-like genes yielded PCR products of the expected sizes.

Considering that mobile M $\beta$ L genes were found in class I integron structures which also contain an *aacA4* cassette (6, 17,

32), a PCR was performed with specific primers for these elements. Amplicons of 900 bp were obtained from PCR with 3F1 and *aacA4FR* primer pair (Table 1). Sequence analysis of these fragments revealed an open reading frame of 753 bp coding for a protein of 250 amino acids with significant similarities to some class B M $\beta$ Ls. The new M $\beta$ L gene was named *bla*<sub>GIM-1</sub> (German imipenemase).

Independent PCRs with primers targeting different regions of the integrase and the *aacA4* cassette and reactions with primers specifically designed for the 753-bp open reading frame (Table 1) confirmed the presence of a novel M $\beta$ L gene.

**Sequence analysis and its deduced protein sequence.** The nucleotide sequence of *bla*<sub>GIM-1</sub> showed a GC content of 42.1% and encoded a mature protein of 25,501 Da with a theoretical pI of 5.3. Analytical IEF of *P. aeruginosa* revealed three  $\beta$ -lactamase bands, one with a pI of 5.4 and that likely corresponded to GIM-1 and two additional bands focused in pI 6.7 and 8.4. The additional band in pI 8.4 was probably the chromosomally encoded AmpC. The results obtained in the IEF showed that the pI value was 5.4, in agreement with the theoretical value. The identity of the  $\beta$ -lactamase focused in pI 6.7 could not be determined.

The amino acid sequence of GIM-1 had low identity with other clinically significant M $\beta$ L genes. The amino acid sequence displayed most identity with IMP variants IMP-6, IMP-1, and IMP-4 (43.5, 43.1, and 43.1%, respectively), followed by VIM variants ranging from a high of 31.2% with VIM-7 to 28.8% with VIM-1, VIM-4, and VIM-5 and only 28.0% similarity with SPM-1. A phylogenetic analysis placed the new enzyme in a new subclass of class B  $\beta$ -lactamases (Fig. 1).

The predicted protein sequence showed that in the active site, GIM-1 has amino acid motifs that are conserved in M $\beta$ L enzymes (4, 10, 11), namely, the consensus zinc binding motif HXHXD (residues 116 to 120) and the other residues involved in the coordination of the Zn<sup>2+</sup> ions (Hys196, Cys221, and

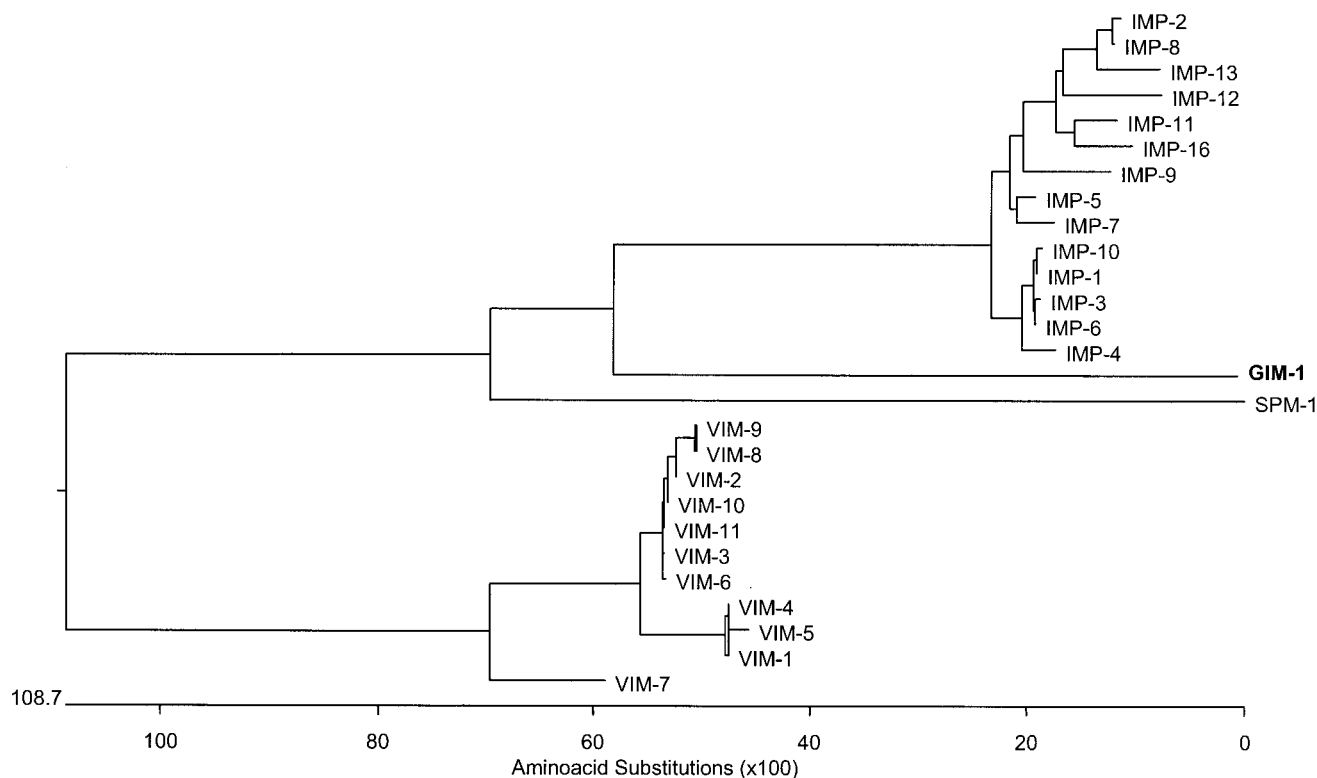


FIG. 1. Phylogenetic tree obtained for most of the mobile M $\beta$ Ls. The alignment used was prepared with CLUSTALW.

Hys293, according to the BBL numbering system). However, the zinc-binding motif of GIM-1 was unique in that there was a serine at position 117 and a glutamic acid residue at position 119, amino acid residues that were not found at these positions in other M $\beta$ L enzymes (Fig. 2).

**Genetic environment harboring *bla*<sub>GIM-1</sub>.** Plasmid DNA analysis showed that the five *P. aeruginosa* clinical isolates harbored plasmids of the same size. The plasmid obtained from strain 73-5671 was estimated to be ~22 kb in size, as determined from DNA fragment profiles generated with different restriction enzymes. Southern hybridization experiments showed that *bla*<sub>GIM-1</sub> is in the plasmid (Fig. 3), although transfer of the  $\beta$ -lactam resistance by electroporation and conjugation could not be demonstrated.

Extended analysis of the genetic environment of *bla*<sub>GIM-1</sub> revealed key genetic components found in class 1 integrons: the 5'-CS, containing the *intI1* integrase gene with its own promoter and the *attI1* recombination site, and the 3'-CS accommodating the fused structure *qacE $\Delta$ 1/sul1* (2). The M $\beta$ L gene is located in the first gene cassette position of this 6-kb class 1 integron, which was named In77. Two putative promoters (P1 and P2) precede the *bla*<sub>GIM-1</sub> start codon (ATG), both of which lie within the integrase structural gene. The primary promoter (P1) had all of the features of an intermediate strength promoter: the -35 box TGGACA and the -10 box TAAACT being separated by 17 bp (5). An insertion of three guanosine residues has been reported to activate the second promoter (P2) by spacing the -35 and -10 hexamers to 17 bp (4). This feature was not present in this integron, signaling that

only the P1 drives the expression of the gene cassettes embedded in the integron.

The M $\beta$ L gene has been inserted at the *attI1* recombination site and, although *bla*<sub>GIM-1</sub> is preceded by the expected core site (GTTAGAA), it is not immediately followed by an inverse core site and other elements of a 59-be (2L and 2R regions). The second cassette in In77 accommodates the *aacA4* gene; however, the expected 59-be between *bla*<sub>GIM-1</sub> and *aacA4* was absent, even within the M $\beta$ L gene. The *aacA4* allele in this integron encodes an AAC(6)-Ib aminoglycoside acetyltransferase that confers resistance to netilmicin, gentamicin, and tobramycin. The 3' end of the *aacA4* gene cassette leads to a 59-be that is 72 bp long, which is in turn followed by a second aminoglycoside-resistant determinant, *aadA1*. However, this gene is interrupted at nucleotide position 135 by a copy of IS1394 (GenBank accession no. U37284) (36), a 1,100-bp insertion sequence (IS) previously described in a strain of *Pseudomonas alcaligenes*. The IS1394 encodes a transposase that shows identity with those of the IS30 family of elements. As shown in Fig. 4, the transcriptional orientation of the IS1394 copy in In77 was opposite that of the genes acquired as cassettes. After the IS1394 was the remainder of the *aadA1* gene cassette. The 59-be of the *aadA1* gene cassette was 60 bp long and shows all consensus features of the recombination site structures (core site GTTRRRY, inverse core site RYYYAAC, and the internal domains 2L and 2R). Finally, the *aadA1* gene cassette is followed by another  $\beta$ -lactamase gene cassette accommodating *bla*<sub>OXA-2</sub>. This was followed by the fused gene cassette *qacE $\Delta$ 1/sul1*.



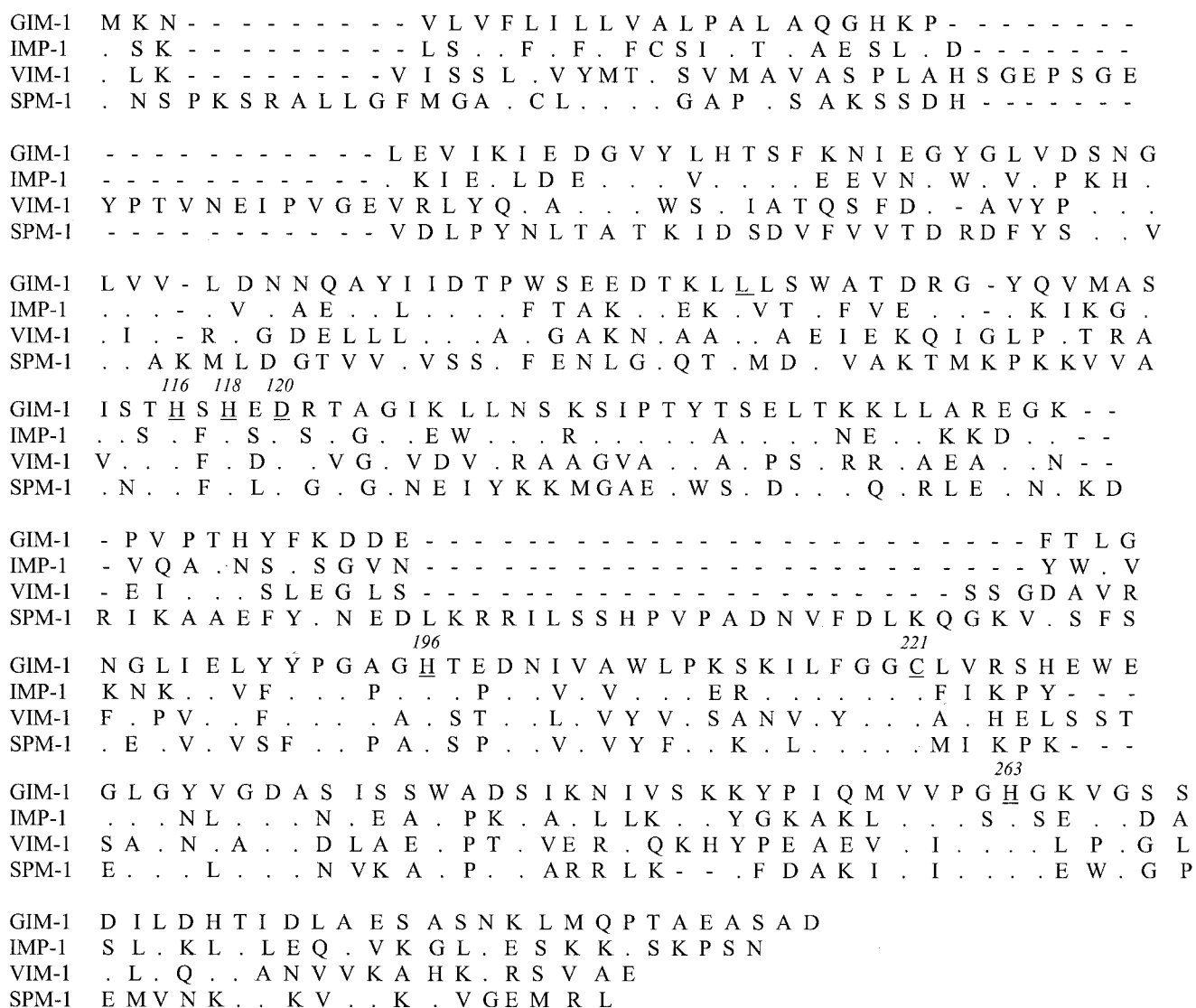


FIG. 2. Alignment of the amino acid sequence of GIM-1 with three representatives of the MβL groups IMP-1, VIM-1, and SPM-1. Differences in the amino acid sequences are noted by a single letter representing the amino acid change within a particular sequence. Residues involved in the coordination of the zinc iron are underlined and numbered according to the BBL system (in italics).

**Patterns of β-lactam susceptibility of *E. coli* producing the GIM-1 enzyme.** The substrate specificity of GIM-1 and its contribution to resistance were investigated by testing the susceptibility to β-lactams of *E. coli* DH5α carrying the recombinant plasmid containing the integron borne *bla*<sub>GIM-1</sub> (pGIM-1) and that produces GIM-1 enzyme in contrast to DH5α, which carries an empty vector (pSCRIPT). The GIM-1 production in the host strain was confirmed by measuring the hydrolysis rate of the *E. coli* DH5α carrying pGIM-1 compared to DH5α carrying the empty vector (0.0097 and 0.0011 Abs/min, respectively).

As shown in Table 2, GIM-1 production was associated with a decrease in the in vitro susceptibility of the *E. coli* host to ceftazidime, cefotaxime, cefepime, imipenem, meropenem, and ampicillin, indicating that the enzyme can contribute to broad-spectrum resistance in the microbial host.

**Kinetic properties of GIM-1.** Analysis of the purified preparation of GIM-1 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single 26-kDa band and was estimated to be >95% pure (data not shown). In addition, the pI of the protein was determined and, after nitrocefin staining, only one pI 5.4 band was observed in the gel.

The kinetic parameters of GIM-1, including  $k_{cat}$ ,  $K_m$ , and the  $k_{cat}/K_m$  ratio, were determined for several different β-lactam compounds, as presented in Table 3. Under the experimental conditions adopted, GIM-1 hydrolyzed most tested compounds, with the exception of aztreonam, azlocillin, and the serine β-lactamase inhibitors, clavulanic acid and tazobactam. Even after prolonged incubation (1 to 3 h) of the enzyme with these agents, enzyme activity was undetectable.

The kinetic parameters of the purified GIM-1 reveal a broad substrate profile but no clear preferences for any of the β-lac-

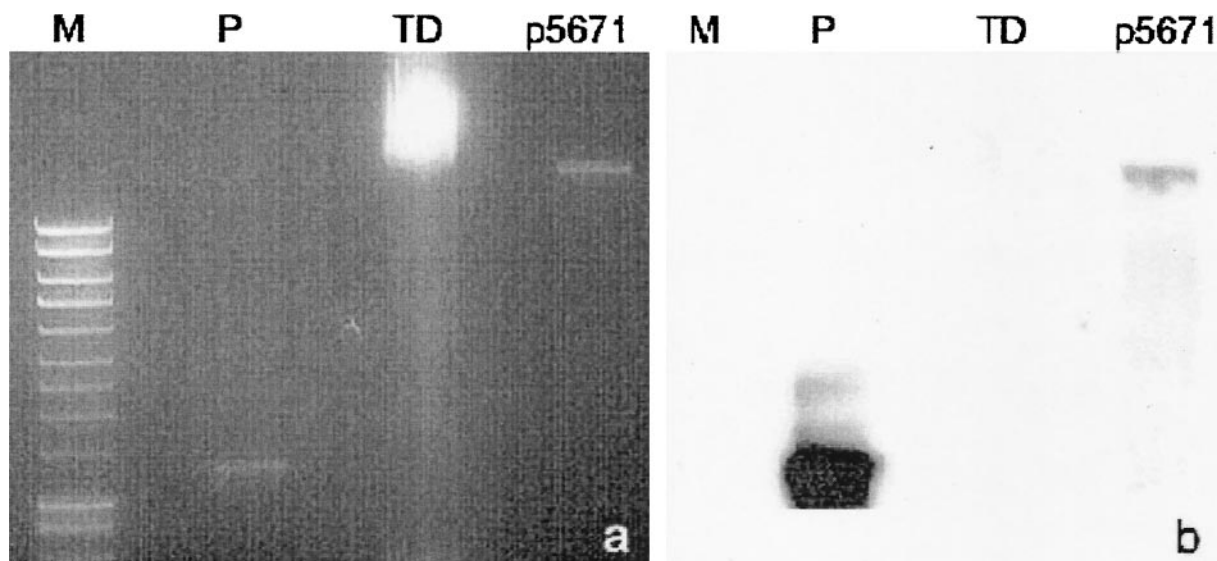


FIG. 3. (a) Agarose gel electrophoresis. Lanes: M, 1-kb plus ladder; P, probe; TD, genomic DNA; p5671, plasmid from strain 73-5671. (b) Results of Southern blot analysis showing the hybridization of *int11F/GIMR* marked probe with the probe fragment and the 22-kb plasmid from the index isolate harboring *bla*<sub>GIM-1</sub>.

tam subfamilies tested. Individual kinetic parameters for GIM-1 for different  $\beta$ -lactam agents differed considerably. The highest  $k_{cat}/K_m$  ratios were observed with cefuroxime, cephalothin, and ceftazidime (0.802, 0.718, and 0.577  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively). The lowest values were demonstrated with moxalactam, carbenicillin, and ticarcillin (0.014, 0.024, and 0.039  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively).

For the carbapenems, GIM-1 showed 10 times greater turnover of imipenem than meropenem ( $k_{cat}$  values of 27.1  $\text{s}^{-1}$  and 2.7  $\text{s}^{-1}$ , respectively). However, the affinity of the enzyme for imipenem is 10 times higher than for meropenem ( $K_m$  of 287.5 and 25.4  $\mu\text{M}$ , respectively), which makes the  $k_{cat}/K_m$  ratios for the two compounds very similar (0.094 for imipenem and 0.106  $\mu\text{M}^{-1} \text{s}^{-1}$  for meropenem). This finding contrasts those for other clinically important class B  $\beta$ -lactamases, which show larger  $k_{cat}/K_m$  ratios for imipenem than for meropenem (Table 3).

The comparison of GIM-1 kinetic values with the parameters reported for other clinically relevant M $\beta$ L (Table 3) showed that, in general, the  $k_{cat}/K_m$  ratios obtained for GIM-1 are lower than those of other M $\beta$ LS, specifically IMP-1, VIM-1, VIM-2, and SPM-1.

### DISCUSSION

The novel class B  $\beta$ -lactamase, GIM-1, is divergent from other class B  $\beta$ -lactamase enzymes and is the fourth subclass of

mobile M $\beta$ L thus far identified. The enzymes most closely related to GIM-1 are the IMP variants, although GIM-1 shares ca. 40% amino acid identity with this group. GIM-1 possesses the major consensus features of the M $\beta$ L family (8), such as the zinc-binding motif (HXHxD), with amino acids not previously reported at the variable positions in this motif likely to be relevant to the particular activity of the protein.

The kinetic properties of GIM-1 demonstrate that the enzyme has similar activity to other members of the class B  $\beta$ -lactamases (7, 14, 18, 21), although the kinetic parameters for GIM-1 show that these values are closer to those of IMP-1 than to those of VIM-1, VIM-2, and SPM-1. GIM-1, like IMP-1, prefers penicillin and ampicillin ( $k_{cat}/K_m = 0.142$  and 0.157  $\mu\text{M}^{-1} \text{s}^{-1}$ ) over other penam antimicrobial agents, such as carbenicillin and ticarcillin ( $k_{cat}/K_m$  0.024 and 0.039  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively). Although GIM-1 and IMP-1 exhibit similar kinetic properties when tested against penicillin and ampicillin, there are differences in the kinetic activities of these enzymes against cephalothin and cefoxitin as substrates ( $k_{cat}/K_m = 0.718$  and 0.040  $\mu\text{M}^{-1} \text{s}^{-1}$  for GIM-1 compared to 2.4 and 2.0  $\mu\text{M}^{-1} \text{s}^{-1}$  for IMP-1). Unlike the other class B  $\beta$ -lactamases, which show more significant activity against imipenem than against meropenem, GIM-1 demonstrates similar kinetic ratios with imipenem and meropenem ( $k_{cat}/K_m = 0.094$  and 0.106  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively). In conclusion, the low  $k_{cat}/K_m$  ratios determined for GIM-1 with most  $\beta$ -lactam antimicrobial

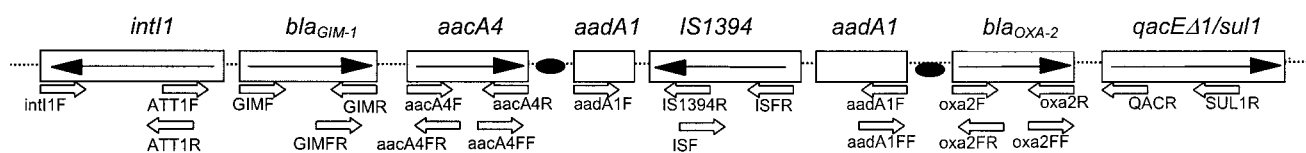


FIG. 4. Schematic representation of integron *ln77* carrying *bla*<sub>GIM-1</sub> (the arrows in the gene boxes indicating the direction of transcription). The black dots indicate 59-bes. In the third cassette position the *aadA1* is interrupted by a copy of the *IS1394*. Block arrows beneath the gene map indicate the positions of the primers used for PCRs and sequence analyses.

TABLE 3. Steady-state kinetic parameters of purified GIM-1 compared to those of IMP-1, VIM-1, VIM-2, and SPM-1

Antibiotic	Kinetic parameters <sup>a</sup> of:														
	GIM-1 <sup>b</sup>			IMP-1 <sup>c</sup>			VIM-1 <sup>d</sup>			VIM-2 <sup>e</sup>			SPM-1 <sup>f</sup>		
	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
Penicillin	6.6	46	0.14	320	520	0.62	29	841	0.034	55.8	49	1.14	108	38	2.8
Ampicillin	3.3	20	0.16	950	200	4.8	37	917	0.04	–	–	–	117	72	1.6
Carbenicillin	4.1	170	0.02	ND	ND	0.02	167	75	2.2	–	–	–	74	814	0.09
Azlocillin	ND	ND	ND	–	–	–	1,525	123	12	–	–	–	53	147	0.35
Piperacillin	6.9	69	0.10	ND	ND	0.72	1,860	3,500	0.53	32.7	72	0.45	117	59	2
Ticarcillin	2.3	57	0.04	1.1	740	0.0015	452	1,117	0.41	31.7	46	0.69	ND	<0.35	ND
Nitrocefin	5.8	12	0.47	63	27	2.3	95	17	5.6	–	–	–	0.53	4	0.12
Cephalothin	16	22	0.72	48	21	2.4	281	53	5.3	56.2	44	1.28	43	4	11.7
Cefuroxime	5.9	7	0.80	8	37	0.22	324	42	7.7	12.1	22	0.55	37	4	8.8
Cefoxitin	8.3	206	0.04	16	8*	2	26	131	0.2	3	24	0.12	8	2	4
Ceftazidime	18	31	0.58	8	44	0.18	60	794	0.076	89	98	0.90	28	46	0.6
Cefotaxime	1.1	4	0.24	1.3	4*	0.35	169	247	0.68	27.5	32	0.86	16	9	1.9
Cefepime	17	431	0.04	7	11*	0.66	549	145	3.8	4.7	184	0.03	18	18	1
Imipenem	27	287	0.09	46	39	1.2	2.0	1.5	1.3	9.9	10	0.99	33	37	1
Meropenem	2.7	25	0.11	50	10	0.12	13	48	0.27	1.4	5	0.28	63	281	0.22
Moxalactam	14	1,035	0.01	88	10*	8.8	–	–	–	14.8	80	0.18	13	97	0.13
Aztreonam	ND	ND	ND	>0.01	>1,000	<10 <sup>-5</sup>	<0.01	>1,000	<10 <sup>-5</sup>	<0.5	ND	ND	ND	<0.3	ND
Clavulanic acid	ND	ND	ND	–	–	–	–	–	–	–	–	–	ND	>0.1	ND
Tazobactam	ND	ND	ND	>1,000	>3.98	0.0039	5.3	337	0.016	–	–	–	0.6	3	0.2

<sup>a</sup> ND, data could not be determined; –, data not available; \*,  $K_m$  was obtained as the  $K_i$  value.

<sup>b</sup> As found in the present study.

<sup>c</sup> Laraki et al. (14).

<sup>d</sup> Franceschini et al. (7).

<sup>e</sup> Poirel et al. (21).

<sup>f</sup> Murphy et al. (18).

agents reflect high substrate affinities ( $K_m$ ) and low substrate turnover rates ( $k_{cat}$ ).

Like the majority of M $\beta$ L genes, *bla*<sub>GIM-1</sub> was found on a class 1 integron, In77, that is carried on a 22-kb plasmid in *P. aeruginosa* 73-5671. In addition to a novel M $\beta$ L gene, this integron harbors three other resistance genes, *aacA4*, *aadA1*, and *bla*<sub>OXA-2</sub>, in that order (Fig. 4). The *bla*<sub>GIM-1</sub> and *aacA4* genes appeared to be accommodated in a single gene cassette that has probably been generated from individual cassettes by deletion of most of the intervening 59-be. The *aadA1* cassette is bisected by an insertion element, *IS1394*, that inactivates this particular gene. In addition, the *IS1394* is oriented so that its transposase gene is transcribed toward the integrase gene of the In77. Since gene cassettes in class 1 integrons do not carry their own promoters (3) and thus are under the control of the integron promoter embedded in the *intI1*, in this integron structure, gene cassettes located downstream from the *IS1394*, such as *bla*<sub>OXA-2</sub>, are unlikely to be expressed.

The finding of *bla*<sub>GIM-1</sub> in what appears to be five clonal isolates of *P. aeruginosa* from different patients from the same hospital is evidence of a nosocomial outbreak. The production of a novel M $\beta$ L by these isolates could be a warning of future clinical problems with these and similar strains. However, in its present form, the GIM-1 gene may not be as mobile as those encoding IMP or VIM M $\beta$ Ls because the 22-kb plasmid carrying In77 is nonconjugative and appears to have a restricted host range. Only time will tell whether the two-gene cassette accommodating *bla*<sub>GIM-1</sub> and *aacA4* will move to another integron on a more easily transmissible plasmid, as happened with other cassette-borne M $\beta$ L genes.

Several important points arise from this and other studies of M $\beta$ L found in clinical isolates. (i) Of the four subclasses of

mobile M $\beta$ L gene identified to date, two were originally found in Europe, perhaps signaling future difficulties for empirical treatment with carbapenems in this region. (ii) Thus, it would be desirable that the incidence of M $\beta$ L production in clinical isolates of key gram-negative bacteria, such as *P. aeruginosa*, *Enterobacter cloacae*, *Serratia marcescens*, and *Klebsiella pneumoniae*, be carefully monitored. (iii) The results reported in this communication attest to the importance of global resistance surveillance programs such as the SENTRY Program in identifying the emergence and epidemic spread of new threats to the efficacy of antimicrobial therapy and beginning into genetic risk of further spread.

#### ACKNOWLEDGMENTS

We are grateful to Alan M. Simm and Tanya A. Murphy for their important advice during the purification of GIM-1 and to Peter M. Bennett, Ayman Hawrani, and Helio S. Sader for critically reviewing the manuscript.

This study was funded in part by EU grant LSHM-CT-2003-503335.

#### REFERENCES

1. Avison, M. B., C. S. Higgins, C. J. von Heldreich, P. M. Bennett, and T. R. Walsh. 2001. Plasmid location and molecular heterogeneity of the L1 and L2 beta-lactamase genes of *Stenotrophomonas maltophilia*. *Antimicrob. Agents Chemother.* **45**:413–419.
2. Bahar, G., A. Mazzariol, R. Koncan, A. Mert, R. Fontana, G. M. Rossolini, and G. Cornaglia. 2004. Detection of VIM-5 metallo-beta-lactamase in a *Pseudomonas aeruginosa* clinical isolate from Turkey. *J. Antimicrob. Chemother.* **54**:282–283.
3. Bennett, P. M. 1999. Integrons and gene cassettes: a genetic construction kit for bacteria. *J. Antimicrob. Chemother.*
4. Bush, K. 1998. Metallo- $\beta$ -lactamases: a class apart. *Clin. Infect. Dis.* **27** (Suppl. 1):S48–S53.
5. Collis, C. M., and R. M. Hall. 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Antimicrob. Agents Chemother.* **39**:155–162.
6. Da Silva, G. J., M. Correia, C. Vital, G. Ribeiro, J. C. Sousa, R. Leita, L.

- Peixe, and A. Duarte. 2002. Molecular characterization of *bla*<sub>IMP-5</sub>, a new integron-borne metallo-beta-lactamase gene from an *Acinetobacter baumannii* nosocomial isolate in Portugal. *FEMS Microbiol. Lett.* **215**:33–39.
7. Franceschini, N., B. Caravelli, J. D. Docquier, M. Galleni, J. M. Frere, G. Amicosante, and G. M. Rossolini. 2000. Purification and biochemical characterization of the VIM-1 metallo-beta-lactamase. *Antimicrob. Agents Chemother.* **44**:3003–3007.
  8. Gales, A. C., R. N. Jones, J. Turnidge, R. Rennie, and R. Ramphal. 2001. Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin. Infect. Dis.* **32**(Suppl. 2):S146–S155.
  9. Gales, A. C., M. C. Tognim, A. O. Reis, R. N. Jones, and H. S. Sader. 2003. Emergence of an IMP-like metallo-enzyme in an *Acinetobacter baumannii* clinical strain from a Brazilian teaching hospital. *Diagn. Microbiol. Infect. Dis.* **45**:77–79.
  10. Galleni, M., J. Lamotte-Brasseur, G. M. Rossolini, J. Spencer, O. Dideberg, and J. M. Frere. 2001. Standard numbering scheme for class B beta-lactamases. *Antimicrob. Agents Chemother.* **45**:660–663.
  11. Garau, G., I. Garcia-Saez, C. Bebrone, C. Anne, P. Mercuri, M. Galleni, J. M. Frere, and O. Dideberg. 2004. Update of the standard numbering scheme for class B beta-lactamases. *Antimicrob. Agents Chemother.* **48**:2347–2349.
  12. Gibb, A. P., C. Tribuddharat, R. A. Moore, T. J. Louie, W. Krulicki, D. M. Livermore, M. F. Palepou, and N. Woodford. 2002. Nosocomial outbreak of carbapenem-resistant *Pseudomonas aeruginosa* with a new *bla*(IMP) allele, *bla*(IMP-7). *Antimicrob. Agents Chemother.* **46**:255–258.
  13. Koh, T. H., G. C. Wang, and L. H. Sng. 2004. IMP-1 and a novel metallo-beta-lactamase, VIM-6, in fluorescent pseudomonads isolated in Singapore. *Antimicrob. Agents Chemother.* **48**:2334–2336.
  14. Laraki, N., N. Franceschini, G. M. Rossolini, P. Santucci, C. Meunier, E. de Pauw, G. Amicosante, J. M. Frere, and M. Galleni. 1999. Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-beta-lactamase IMP-1 produced by *Escherichia coli*. *Antimicrob. Agents Chemother.* **43**:902–906.
  15. 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-beta-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrob. Agents Chemother.* **43**:1584–1590.
  16. Livermore, D. M., and N. Woodford. 2000. Carbapenemases: a problem in waiting? *Curr. Opin. Microbiol.* **3**:489–495.
  17. Mendes, R. E., M. Castanheira, P. Garcia, M. Guzman, M. A. Toleman, T. R. Walsh, and R. N. Jones. 2004. First isolation of *bla*(VIM-2) in Latin America: report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob. Agents Chemother.* **48**:1433–1434.
  18. Murphy, T. A., A. M. Simm, M. A. Toleman, R. N. Jones, and T. R. Walsh. 2003. Biochemical characterization of the acquired metallo-beta-lactamase SPM-1 from *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **47**:582–587.
  19. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard M7–A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
  20. Patzer, J., M. A. Toleman, L. M. Deshpande, W. Kaminska, D. Dzierzanowska, P. M. Bennett, R. N. Jones, and T. R. Walsh. 2004. *Pseudomonas aeruginosa* strains harbouring an unusual *bla*VIM-4 gene cassette isolated from hospitalized children in Poland (1998–2001). *J. Antimicrob. Chemother.* **53**:451–456.
  21. Poirrel, L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J. D. Cavallo, and P. Nordmann. 2000. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob. Agents Chemother.* **44**:891–897.
  22. Poole, K., K. Krebes, C. McNally, and S. Neshat. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J. Bacteriol.* **175**:7363–7372.
  23. Quinn, J. P., A. E. Studemeister, C. A. DiVincenzo, and S. A. Lerner. 1988. Resistance to imipenem in *Pseudomonas aeruginosa*: clinical experience and biochemical mechanisms. *Rev. Infect. Dis.* **10**:892–898.
  24. Riccio, M. L., N. Franceschini, L. Boschi, B. Caravelli, G. Cornaglia, R. Fontana, G. Amicosante, and G. M. Rossolini. 2000. Characterization of the metallo-beta-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of *bla*(IMP) allelic variants carried by gene cassettes of different phylogeny. *Antimicrob. Agents Chemother.* **44**:1229–1235.
  25. Sambrook, J., P. MacCallum, and D. Russell. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  26. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233–2239.
  27. Toleman, M. A., D. Biedenbach, D. Bennett, R. N. Jones, and T. R. Walsh. 2003. Genetic characterization of a novel metallo-beta-lactamase gene, *bla*IMP-13, harboured by a novel *Tn5051*-type transposon disseminating carbapenemase genes in Europe: report from the SENTRY worldwide antimicrobial surveillance programme. *J. Antimicrob. Chemother.* **52**:583–590.
  28. Toleman, M. A., K. Rolston, R. N. Jones, and T. R. Walsh. 2003. Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d' beta-lactamase in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **47**:2859–2863.
  29. Toleman, M. A., A. M. Simm, T. A. Murphy, A. C. Gales, D. J. Biedenbach, R. N. Jones, and T. R. Walsh. 2002. Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. *J. Antimicrob. Chemother.* **50**:673–679.
  30. Tsakris, A., S. Pournaras, N. Woodford, M. F. Palepou, G. S. Babini, J. Douboyas, and D. M. Livermore. 2000. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. *J. Clin. Microbiol.* **38**:1290–1292.
  31. Walsh, T. R., D. J. Payne, A. P. MacGowan, and P. M. Bennett. 1995. A clinical isolate of *Aeromonas sobria* with three chromosomally mediated inducible beta-lactamases: a cephalosporinase, a penicillinase and a third enzyme, displaying carbapenemase activity. *J. Antimicrob. Chemother.* **35**:271–279.
  32. Walsh, T. R., M. A. Toleman, W. Hryniewicz, P. M. Bennett, and R. N. Jones. 2003. Evolution of an integron carrying *bla*VIM-2 in Eastern Europe: report from the SENTRY Antimicrobial Surveillance Program. *J. Antimicrob. Chemother.* **52**:116–119.
  33. Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi. 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **35**:147–151.
  34. Yan, J. J., P. R. Hsueh, W. C. Ko, K. T. Luh, S. H. Tsai, H. M. Wu, and J. J. Wu. 2001. Metallo-beta-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. *Antimicrob. Agents Chemother.* **45**:2224–2228.
  35. Yatsuyanagi, J., S. Saito, Y. Ito, K. Ohta, J. Kato, S. Harata, N. Suzuki, and K. Amano. 2004. Identification of *Pseudomonas aeruginosa* clinical strains harboring the *bla*(VIM-2) metallo-beta-lactamase gene in Akita prefecture, Japan. *Jpn. J. Infect. Dis.* **57**:130–132.
  36. Yeo, C. C., and C. L. Poh. 1996. IS1394 from *Pseudomonas alcaligenes* N.C.I.B. 9867: identification and characterization of a member of the IS30 family of insertion elements. *Gene* **175**:109–113.
  37. Zihni-Zarif, I., C. Llanes, T. Kohler, J. C. Pechere, and P. Plesiat. 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. *Antimicrob. Agents Chemother.* **43**:287–291.