ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2008, p. 3589–3596 Vol. 52, No. 10
0066-4804/08/$08.00+0 doi:10.1128/AAC.00465-08

Characterization of the New Metallo-β-Lactamase VIM-13 and Its Integron-Borne Gene from a *Pseudomonas aeruginosa* Clinical Isolate in Spain[▽]

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Received 8 April 2008/Returned for modification 25 June 2008/Accepted 14 July 2008

During a survey conducted to evaluate the incidence of class B carbapenemase (metallo-β-lactamase [MBL])-producing *Pseudomonas aeruginosa* strains from hospitals in Majorca, Spain, five clinical isolates showed a positive Etest MBL screening test result. In one of them, strain PA-SL2, the presence of a new bla_{VIM} derivative (bla_{VIM-13}) was detected by PCR amplification with bla_{VIM-1}-specific primers followed by sequencing. The bla_{VIM-13}-producing isolate showed resistance to all β-lactams (except aztreonam), gentamicin, tobramycin, and ciprofloxacin. VIM-13 exhibited 93% and 88% amino acid sequence identities with VIM-1 and VIM-2, respectively. bla_{VIM-13} was cloned in parallel with bla_{VIM-1}, and the resistance profile conferred was analyzed both in *Escherichia coli* and in *P. aeruginosa* backgrounds. Compared to VIM-1, VIM-13 conferred slightly higher levels of resistance to piperacillin and lower levels of resistance to ceftazidime and cefepime. VIM-13 and VIM-1 were purified in parallel as well, and their kinetic parameters were compared. The k_{cat}/K_m ratios for the antibiotics mentioned above were in good agreement with the MIC data. Furthermore, EDTA inhibited the activity of VIM-13 approximately 25 times less than it inhibited the activity of VIM-1. VIM-13 was harbored in a class 1 integron, along with a new variant (Ala108Thr) of the aminoglycoside-modifying enzyme encoding gene *aacA4*, which confers resistance to gentamicin and tobramycin. Finally, the VIM-13 integron was apparently located in the chromosome, since transformation and conjugation experiments consistently yielded negative results and the bla_{VIM-13} probe hybridized only with the genomic DNA.

Class B carbapenemases (metallo-β-lactamases [MBLs]) are the β-lactamases acquired by *Pseudomonas aeruginosa* that are of the most concern, since they are characterized by a very wide hydrolytic spectrum that affects all β-lactams except monobactams (17). MBLs need Zn^{2+} binding in their catalytic center to hydrolyze the β-lactam ring, and so they are inhibited by chelating agents like EDTA (23, 38). MBL genes are usually carried as cassettes in integrons along with other resistance determinants, such as aminoglycoside-modifying enzymes. Furthermore, the integrons involved are frequently located in plasmids or transposons, which certainly contribute to the global dissemination of these worrisome resistance mechanisms (2, 7, 11, 16, 26). Indeed, several outbreaks of MBL-producing *P. aeruginosa* strains have been described worldwide, and they are often amplified due to the horizontal transmission of the multiresistance determinants harbored in the cited integrons (13, 20, 21, 24, 32). There are two major groups of MBLs, IMP and VIM, which share only approximately 30% of their amino acid sequences. Among the VIM carbapenemases, there are two major phylogenetic lineages, the VIM-1 and VIM-2 clusters, whereas VIM-7 seems to be the single representative of a third phylogenetic cluster (35). The sequences of the VIM-1 and VIM-2 enzymes differ at 25 of 266 amino acids, although these differences confer remarkable changes in their functional behaviors (6, 8, 25). VIM-1 is characterized by high k_{cat}/K_m ratios (efficiency of hydrolysis [34]) for carbenicillin, cephaloridine, cephalothin, cefuroxime, cefepime, cefpirome, and imipenem and by the low level of inactivation caused by EDTA compared to those caused by other chelating agents, such as 1,10-*o*-phenanthroline (8). On the other hand, VIM-2 has many differences in k_{cat}/K_m ratios compared with those of VIM-1 for some antibiotics (e.g., a k_{cat}/K_m approximately 10-fold greater than that of VIM-1 for carbapenems) and seems to be much more susceptible than VIM-1 to inactivation by metal chelators, probably indicating a looser bond to the zinc ions (6). Polymorphisms in some of the active-site amino acids have been proposed as an explanation for these kinetic particularities and the reason for the differences in the substrate binding of each enzyme (6). Furthermore, the recently characterized VIM-12 enzyme, which seems to be a hybrid protein between VIM-1 and VIM-2, shows a narrow substrate specificity that is limited mainly to penicillin and imipenem, although it shows complete conservation of the active-site residues of the VIM enzymes (12, 27).

In this work, we describe a new integron-borne bla_{VIM} gene, bla_{VIM-13}, detected in a *P. aeruginosa* clinical isolate. Furthermore, VIM-13, which is found to be located closest to the VIM-1 cluster but which significantly diverges from it, was

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▽ Published ahead of print on 21 July 2008.

TABLE 1. Laboratory strains and plasmids used or constructed in this work

Strain or plasmid	Description	Reference or source
P. aeruginosa PAO1	Completely sequenced reference strain	Laboratory collection
E. coli		
XL1-Blue	F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15 *recA1 endA1 gyrA96* (Nal^r) *thi hsdR17*(r_K^- m_K^-) *mcrB1*	Laboratory collection
BL21	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*	Laboratory collection
Plasmids		
pGEM-T	PCR cloning vector	Promega
pUCP-24	Gm^r; pUC18-based *Escherichia-Pseudomonas* shuttle vector	39
pGEX-6P-1	Ap^r; GST-fusion purification vector	Amersham
pGTVIM-1	pGEM-T containing *bla*$_{VIM-1}$ gene from PA-SD2 strain	This work
pGTVIM-13	pGEM-T containing *bla*$_{VIM-13}$ gene from strain PA-SL2	This work
pUCPVIM-1	Gm^r; pUCP-24 containing *bla*$_{VIM-1}$ gene	This work
pUCPVIM-13	Gm^r; pUCP-24 containing *bla*$_{VIM-13}$ gene	This work
pGEXVIM-1	pGEX-6P-1 containing *bla*$_{VIM-1}$ gene without signal peptide; used for purification	This work
pGEXVIM-13	pGEX-6P-1 containing *bla*$_{VIM-13}$ gene without signal peptide; used for purification	This work
pGTA4	pGEM-T containing *aacA4* gene from strain PA-SD2	This work
pGTA4$_{A108T}$	pGEM-T containing *aacA4* gene from strain PA-SL2 (Ala108Thr variant)	This work

purified in parallel with VIM-1 and characterized biochemically.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. The laboratory strains and plasmids used in or constructed for this work are listed in Table 1. Five *Pseudomonas aeruginosa* clinical isolates resistant to imipenem and meropenem and showing a positive Etest MBL (AB Biodisk, Solna, Sweden) screening test result (imipenem and EDTA MICs at least 3 twofold dilutions lower than those of imipenem alone) were studied. These isolates were recovered during a survey conducted to evaluate the incidence of MBL-producing strains performed between August 2004 and December 2005 in two Majorcan hospitals (Hospital Son Dureta and Hospital Son Llàtzer). The MICs of piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, cefotaxime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, amikacin, and ciprofloxacin were determined by the Etest method (AB Biodisk), according to the manufacturer's recommendations. Breakpoints were applied according to the recommendations if Clinical and Laboratory Standards Institute (4).

Characterization of MBL-encoding genes. Following total DNA extraction with a DNeasy tissue kit (Qiagen, Hilden, Germany), the strains were evaluated for the presence of MBL-encoding genes by PCR amplification with primers (Table 2) specific for *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{VIM-1}$, and *bla*$_{VIM-2}$ or closely related genes, followed by DNA sequencing (9). Sequencing reactions were performed with a BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and the sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available in the GenBank database (www.ncbi.nih.gov/BLAST). Multiple-sequence alignments were performed and MBL phylograms were prepared with the ClustalW program (version 1.83).

Characterization of genetic elements harboring class B carbapenemases. The possible locations of MBL-encoding genes in self-transferable plasmids were evaluated in conjugation and transformation experiments performed by previously described procedures (9). To ascertain the location of *bla*$_{VIM-13}$, Southern blotting and hybridization experiments with a *bla*$_{VIM-13}$-specific PCR probe were performed with the chromosomal and plasmid DNA of strain PA-SL2 by using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, United Kingdom), according to the manufacturer's instructions. The integrons harboring the MBL-encoding genes were characterized by PCR, followed by DNA sequencing with specific primers (Table 2), to amplify *intI1*, *qacEΔ1*, and the DNA regions located between *intI1* or *qacEΔ1* and the corresponding MBL-encoding gene (9).

Cloning and characterization of the new VIM-13. The *bla*$_{VIM-1}$ and *bla*$_{VIM-13}$ genes were amplified in parallel by PCR with previously described primers VIM1-F and VIM1-R (Table 2). The purified PCR products were then ligated to plasmid pGEM-T to obtain plasmids pGTVIM-1 and pGTVIM-13, respectively, which were transformed into strain *Escherichia coli* XL1-Blue made competent with $CaCl_2$ (31). Transformants were selected in 50 μg/ml ampicillin MacConkey agar plates, and their sequences were checked by PCR amplification. The cloned *bla*$_{VIM-1}$ and *bla*$_{VIM-13}$ genes were sequenced to confirm that no mutations were produced during PCR amplification. The cloned *bla*$_{VIM-1}$ and *bla*$_{VIM-13}$ genes were then liberated from the corresponding pGEM-T plasmids by restriction with EcoRI and were further ligated to plasmid pUCP24 (digested with the same enzyme) to obtain pUCPVIM-1 and pUCPVIM-13, respectively. *E. coli* XL1-Blue transformants were then selected on 10 μg/ml gentamicin MacConkey agar plates. In both cases, recombinant plasmids with DNA inserts with the same orientation as the *lacZ* promoter (checked by PCR and sequencing) were selected for further study. Additionally, plasmids pUCPVIM-1 and pUCPVIM-13 were also electroporated as described previously (33) into strain *P. aeruginosa* PAO1, and transformants were selected in 50 μg/ml gentamicin Luria-Bertani agar plates. The MICs of piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, cefotaxime, aztreonam, imipenem, and meropenem were determined by Etest for the *E. coli* XL1-Blue and *P. aeruginosa* PAO1 transformants harboring pUCPVIM-1 or pUCPV-13. The Compute pI/M_w tool from www.expasy.org was used to predict the molecular mass of the new enzyme, VIM-13. To determine the pI of VIM-13 in parallel with that of VIM-1, isoelectric focusing of crude sonic extracts of strains PA-SL2 and PA-SD2 with Phast gels (pH gradient, 3 to 9) was performed in a Phast system apparatus (Pharmacia AB, Uppsala, Sweden).

Antibiotics and other chemicals. Ampicillin, benzylpenicillin, piperacillin, cephalothin, cefotaxime, ceftazidime, cefuroxime, and the chelating agents (EDTA, dipicolinic acid, and 1,10-*o*-phenanthroline) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Cefepime (Maxipime) was purchased from Bristol-Myers Squibb, imipenem was a gift from Merck (Whitehouse Station, NJ), and meropenem was a gift from AstraZeneca (London, United Kingdom).

Purification of VIM-1 and VIM-13 enzymes. To purify the VIM-1 and VIM-13 enzymes, the *bla*$_{VIM-1}$ and *bla*$_{VIM-13}$ genes were cloned into the pGEX-6P-1 vector (BamHI and EcoRI restriction sites) with the primers V1-F-BHI or V13-F-BHI (depending on the amplified *bla*$_{VIM}$ gene) and V1/13-R-ERI (Table 2). Cloning of the *bla*$_{VIM}$ genes allowed the creation of a fusion protein between glutathione *S*-transferase (GST) and the VIM-type enzymes lacking the signal peptide. The GST tag was then cleaved off and the β-lactamases were purified to homogeneity with the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH), in accordance with the manufacturer's instructions. *E. coli* BL21 was used for the expression and the purification of the VIM-1 and VIM-13 proteins (from plasmids pGEXVIM-1 and pGEXVIM-13, respectively). The purified proteins were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and appeared as a band of 26 kDa (purity, >99%). The concentrations of the purified proteins were determined by a protein assay (Bio-Rad, Richmond, CA). Finally, enzyme samples were equilibrated by dialyzing them overnight at 4°C in 50 mM HEPES (pH 7.5)–50 μM $ZnSO_4$. Purified samples of VIM-1 and VIM-13 supplemented with 20 μg/ml of bovine serum albumin (BSA) were stored at −80°C.

Kinetic parameters. The VIM-1 and VIM-13 MBLs were further used in biochemical studies with the β-lactam antibiotics mentioned above. The experiments were carried out at 25°C in a Nicolet Evolution 300 spectrophotometer

TABLE 2. Primers used in this work

Primer	Sequence (5′–3′)[a]	PCR product size (bp)	Use
VIM1-F VIM1-R	ATGTTAAAAGTTATTAGTAGTTTATTG CTACTCGGCGACTGAGC	801	Amplification and sequencing of bla_{VIM-1} and related genes
VIM2-F VIM2-R	ATGTTCAAACTTTTGAGTAAG CTACTCAACGACTGAGCG	801	Amplification and sequencing of bla_{VIM-2} and related genes
IMP1-F IMP1-R	ATGAGCAAGTTATCTGTATTC TTAGTTGCTTGGTTTTGATGG	741	Amplification and sequencing of bla_{IMP-1} and related genes
IMP2-F IMP2-R	ATGAAGAAATTATTTGTTTTATG TTAGTTACTTGGCTGTGATG	741	Amplification and sequencing of bla_{IMP-2} and related genes
VIM-F VIM-R	AGTGGTGAGTATCCGACAG ATGAAAGTGCGTGGAGAC		Sequencing of bla_{VIM}
INT-F INT-R	CTCTCACTAGTGAGGGGC ATGAAAACCGCCACTGCG	1,010	Amplification and sequencing of *intI*
INT-R-I VIM1-F-I	CGCAGTGGCGGTTTTCAT CAATAAACTACTAATAACTTTTAAC	210	Amplification and sequencing of gene(s) between *intI* and bla_{VIM}
qacE-F qacE-R	GAAAGGCTGGCTTTTTCTTG ATTATGACGACGCCGAGTC	210	Amplification of *qacEΔ1*
qacE-F-I VIM1-R-I	CAAGAAAAAGCCAGCCTTTC GCTCAGTCGCCGAGTAG	Variable	Amplification and sequencing of gene(s) between bla_{VIM} and *qacEΔ1*
aacA4-R	TTAGGCATCACTGCGTGTTC	697	Cloning of *aacA4* gene
V1-F-BHI	TCGGATCCGGGGAGCCGAGTGGTGAG	723	Cloning of bla_{VIM-1} and bla_{VIM-13} (without signal peptide) for purification
V13-F-BHI V1/13-R-ERI	TCGGATCCGGGGAGTCGAGAGGTGAG TCGAATTCCTACTCGGCGACTGAGCG		

[a] The underlined sequences represent sites for restriction enzymes.

(Thermo Electron Corporation, Waltham, MA), and the data obtained were analyzed with Vision Pro software (Thermo Electron Corporation). The wavelengths and molar extinction coefficients used were described previously (15). The tests were repeated three times in 50 mM HEPES (pH 7.5) with 50 μM $ZnSO_4$ and 20 μg/ml BSA. The representation of the substrate concentration ([*S*]) against the hydrolysis velocity (*V*) showed a Michaelis-Menten curve, and its Lineweaver-Burk conversion obtained by using $1/V$ and $1/[S]$ provided a straight line with an intersection at the abscissa of $-1/K_m$. The K_m and k_{cat} parameters were calculated from at least three independent experiments with 6 to 12 substrate concentrations, depending on the antibiotic (3, 29, 34).

Inactivation of the VIM-13 and VIM-1 enzymes by chelating agents. VIM-1 and VIM-13 inactivation by Zn^{2+} removal was studied at 20°C in 50 mM HEPES (pH 7.5) buffer supplemented with 20 μg/ml of BSA in the presence of different concentrations of EDTA, dipicolinic acid, and 1,10-*o*-phenanthroline. The reporter substrate was 100 μM imipenem, and the measurements were obtained after 10 min of incubation of the buffer mentioned above containing the enzyme and the corresponding chelating agent. The inhibitor concentration that gave a 50% reduction of the hydrolytic activity of each enzyme (IC_{50}) was determined for the chelating agents mentioned above (25).

Cloning and characterization of a new AAC(6′)-Ib variant (Ala108Thr). Primers VIM1-R-I and aacA4-R (Table 2) were used to amplify in parallel *aacA4* from the total DNA of strain PA-SD2 (which harbors the *aacA4* gene described by Lambert et al. [14] downstream of bla_{VIM-1}) and strain PA-SL2 (which harbors the new Ala108Thr variant). The PCR products were ligated to plasmid pGEM-T to obtain plasmids pGTA4 and $pGTA4_{A108T}$, respectively, which were transformed into *E. coli* XL1-Blue made competent with $CaCl_2$ (31). Transformants were selected in 50 μg/ml ampicillin MacConkey agar plates, and their sequences were checked by PCR amplification. The cloned *aacA4* genes were sequenced to confirm that no mutations were produced during PCR amplification. In both cases, recombinant plasmids with DNA inserts with the same orientation as that of the *lacZ* promoter (checked by PCR and sequencing) were selected for further study. The spectra of aminoglycosides affected by both AAC(6′)-Ib enzymes were assessed by determination of the MICs (Etest) of gentamicin, tobramycin, and amikacin for the selected transformants.

Nucleotide sequence accession numbers. The nucleotide sequences described in this work have been deposited in the GenBank database under accession numbers DQ365886 (bla_{VIM-13}), EF577407 (the VIM-13 integron from strain PA-SL2), and EF577408 (the VIM-1 integron from clone PA-SD2).

RESULTS

MBL-producing *P. aeruginosa* isolates from Majorcan hospitals: detection of the new bla_{VIM-13}. Five *P. aeruginosa* clinical isolates resistant to imipenem and meropenem and showing a positive Etest MBL screening test result were studied. These isolates were recovered during a survey to evaluate the incidence of MBL-producing strains performed between August 2004 and December 2005 in two Majorcan hospitals (Hospital Son Dureta and Hospital Son Llàtzer). The strains were evaluated for the presence of MBL-encoding genes by PCR amplification, followed by DNA sequencing. Three of the isolates showed positive PCR amplification with bla_{VIM-2}-specific primers, and sequencing of the PCR products confirmed the presence of bla_{VIM-2}. On the other hand, isolates PA-SD2 and

TABLE 3. MICs of *E. coli* and *P. aeruginosa* strains with cloned genes and MICs of clinical strains used in this work

Strain	Plasmid	Cloned gene	MIC (μg/ml)[a]											
			CIP	PIP	PIP-TZ	AZM	CAZ	FEP	CTX	IMP	MER	GEN	TOB	AMK
PA-SD2 (harboring bla_{VIM-1})			>32	>256	>256	24	>256	>256	>256	>32	>32	48	16	3
PA-SL2 (harboring bla_{VIM-13})			>32	>256	>256	4	32	32	>256	24	12	>256	48	16
P. aeruginosa PAO1	pUCPVIM-1	bla_{VIM-1}		64	48	1.5	>256	>256	>256	>32	>32			
P. aeruginosa PAO1	pUCPVIM-13	bla_{VIM-13}		192	192	1.5	128	64	>256	>32	>32			
P. aeruginosa PAO1			0.125	3	3	1	1	1	16	1.5	0.38			
E. coli XL1-Blue	pUCPVIM-1	bla_{VIM-1}		6	6	0.125	32	12	>256	1	0.25			
E. coli XL1-Blue	pUCPVIM-13	bla_{VIM-13}		8	8	0.125	6	1	>256	1	0.19			
E. coli XL_1 Blue	pGTA4	*aacA4*	0.064									1	2	0.75
E. coli XL1-Blue	$pGTA4_{A108T}$	*aacA4* variant (Ala108Thr)	0.064									1	2	0.75
E. coli XL1-Blue			0.064	1.5	1	0.19	0.38	0.19	0.19	0.25	0.064	0.19	0.25	0.75

[a] CIP, ciprofloxacin; PIP, piperacillin; PIP-TZ, piperacillin-tazobactam; AZM, aztreonam; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; IMP, imipenem; MER, meropenem; GEN, gentamicin; TOB, tobramycin; AMK, amikacin.

PA-SL2 showed positive PCR amplification with bla_{VIM-1}-specific primers. Sequencing of the PCR products confirmed the presence of bla_{VIM-1} in PA-SD2 but, interestingly, revealed the presence of a new bla_{VIM} derivative, designated bla_{VIM-13}, in PA-SL2. bla_{VIM-13}-producing isolate PA-SL2 was further characterized in this work, along with bla_{VIM-1}-producing isolate PA-SD2 for comparative purposes. Isolate PA-SL2 was recovered on 15 October 2005 from a skin ulcer of a patient admitted to the Nephrology Ward of Hospital Son Llàtzer. In addition to its high level of resistance to carbapenems, this isolate showed resistance to the other β-lactams tested (except aztreonam), gentamicin, tobramycin, and ciprofloxacin (Table 3). Isolate PA-SD2 was recovered from the peritoneal fluid of a patient admitted to the ICU of Hospital Son Dureta and showed a resistance phenotype similar to that of isolate PA-SL2, although it was additionally resistant to aztreonam (Table 3).

Characterization of the new VIM-13. The degrees of identity of bla_{VIM-13} with bla_{VIM-1}, bla_{VIM-2}, and bla_{VIM-7} were 93%, 88%, and 78%, respectively. The amino acid sequence of the enzyme (VIM-13) exhibited identities of 93%, 88%, and 76% with the amino acid sequences of VIM-1, VIM-2 and VIM-7,

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VIM-2    MFKLLSKLLVYLTASIMAIASPLAFSVDSSGEYPTVSEIPVGEVRLYQIADGVWSHIATQ 60
VIM-12   MLKVISSLLVYMTASVMAVASPLAHSGEPSGEYPTVNEIPVGEVRLYQIADGVWSHIATQ 60
VIM-1    MLKVISSLLVYMTASVMAVASPLAHSGEPSGEYPTVNEIPVGEVRLYQIADGVWSHIATQ 60
VIM-13   MLKVISSLLFYMTASLMAVASPLAHSGESRGFYPTVSEIPVGEVRLYQTDDGVWSHIATH 60
VIM-7    MFQIRS-FLVGISAFVMAVLGSAAYSAQPGGEYPTVDDIPVGEVRLYKIGDGVWSHIATQ 59
         *::: * :*. ::* :**: .. *.* :. ******.:*********:* *********:

VIM-2    SFDGAVYPSNGLCVRDGDELLLIDCAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRV 120
VIM-12   SFDGAVYPSNGLCVRDGDELLLIDCAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRV 120
VIM-1    SFDGAVYPSNGLCVRDGDELLLIDCAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRV 120
VIM-13   TFDGVVYPSNGLCVRDGDELLLIDCAWGTKNTVALLAEIEKQIGLPVTRSVSTHFHDDRV 120
VIM-7    KLGDTVYSSNGLCVRDADELLLIDCAWGAKNTVALLAEIEKQIGLPVTRSISTHFHDDRV 119
         .:...**.********.***********:***.****************::*********

VIM-2    GGVDVLRAAGVACYASPSTRRLAEVEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHS 180
VIM-12   GGVDVLRAAGVACYASPSTRRLAEAEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHS 180
VIM-1    GGVDVLRAAGVACYASPSTRRLAEAEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHS 180
VIM-13   GGVDALRAAGVACYASPSTRRLAEAEGNEVPTHSLEGLSSSGDAVRFGPVELFYPGAAHS 180
VIM-7    GGVDVLRAAGVACYTSPLTRQLAEAAGNEVPAHSLKALSSSGDVVRFGPVEVFYPGAAHS 179
         ****.*********:** **:***. ***:*:***:.******.*******:********

VIM-2    TDNLVVYVPSASVCYGGCAIYELSRTSAGNVADADLAEWPTSCERCQQHYPEAQFVIPGH 240
VIM-12   TDNLVVYVPSANVCYGGCAVHELSSTSAGNVADADLAEWPTSCERCQQHYPEAQFVIPGH 240
VIM-1    TDNLVVYVPSANVCYGGCAVHELSSTSAGNVADADLAEWPTSVERCQKHYPEAEVVIPGH 240
VIM-13   TDNLVVYVPSANVCYGGCAVLELSRTSAGNVADADLAEWPGSVERCQQHYPEAEVVIPGH 240
VIM-7    GDNLVVYVPAVRVCFGGCAVHEASRESAGNVADANLAEWPATCKRCQQRYPEAEVVIPGH 239
          ********:. **:****: * *  ********:***** :::***::****:.*****

VIM-2    GLPGGLDLLKRTCNVVKAHTNRSVVE 266
VIM-12   GLPGGLDLLKRTCNVVKAHTNRSVVE 266
VIM-1    GLPGGLDLLQRTANVVKAHKNRSVAE 266
VIM-13   GLPGGLDLLQRTANVVKAHTNRSVAE 266
VIM-7    GLPGGLELLQRTCNVVKTHKVRPVAE 265
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FIG. 1. Multiple-sequence alignment (performed with the ClustalW program, version 1.83) of the VIM-1, VIM-2, VIM-7, VIM-12, and VIM-13 carbapenemases. The signal peptide is shown in underlined boldface, and loop L3 (the active site of the enzyme [6]) is shown with a black background. Dashes indicate gaps. Asterisks, colons, and periods indicate identical, conserved, and semiconserved residues, respectively.

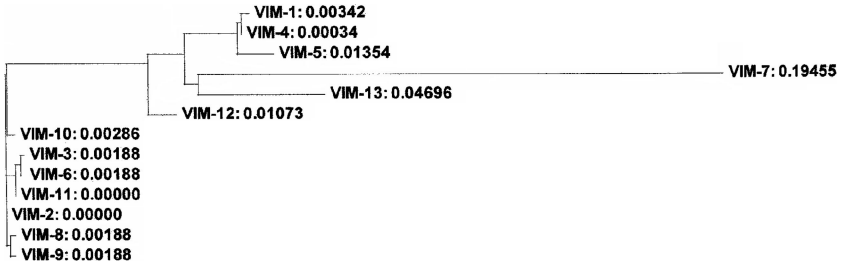

FIG. 2. Phylogram (performed with the ClustalW program, version 1.83) of VIM-type MBLs.

respectively. Figure 1 shows the multiple-sequence alignment of VIM-type MBLs, and Fig. 2 shows their phylograms (obtained with the ClustalW program, version 1.83). VIM-13 was found to be located the closest to the VIM-1 cluster, but it significantly diverged from it. In order to characterize this new VIM enzyme, bla_{VIM-13} and bla_{VIM-1} were cloned in parallel into plasmid pUCP24. The MICs conferred by the resulting plasmids in both *E. coli* XL1-Blue and *P. aeruginosa* PAO1 backgrounds are shown in Table 3. Compared to the levels of resistance conferred by VIM-1, VIM-13 conferred lower levels of resistance to ceftazidime and cefepime but higher levels of resistance to piperacillin. Additionally, VIM-13 showed a molecular mass of 28,220 Da. The results of isoelectric focusing showed a pI of approximately 5.1 for VIM-13, which is almost identical to that previously described for VIM-1 (8).

Characterization of the genetic element harboring bla_{VIM-13}. Several attempts to transfer the bla_{VIM-13} determinant by conjugation to *P. aeruginosa* PAO1 or *E. coli* HB101 and by electroporation to PAO1 consistently failed. Furthermore, the results of Southern blotting showed the hybridization of the bla_{VIM-13}-specific PCR probe with strain PA-SL2 genomic DNA but not with plasmid DNA (data not shown). Therefore, bla_{VIM-13} is apparently located in the chromosome of PA-SL2. The integron harboring bla_{VIM-13} in isolate PA-SL2 was characterized by a set of five PCR amplifications, followed by DNA sequencing, with specific primers for the amplification of *intI1*, *qacEΔ1*, and the DNA regions located between *intI1* or *qacEΔ1* and the VIM-13-encoding gene. Figure 3 shows the resulting integron structure. According to the *intI1* sequence, it is a class 1 integron and contains the 3-bp insertion (GGG) between the −10 and −35 boxes known to activate the P2 promoter, which was otherwise conserved in the integron (5). The sequence of the downstream region of bla_{VIM-13}, including the 59-bp element, showed only weak similarity to the sequences previously reported for bla_{VIM-1} and bla_{VIM-2} (data not shown) (16). The integron from VIM-13-producing strain PA-SL2 contained an *aacA4* gene cassette right after the VIM-13-encoding gene. Another remarkable finding was that the deduced amino acid sequence for the *aacA4* gene from PA-SL2 showed a polymorphism (Ala108Thr) not previously described for this aminoglycoside-modifying enzyme. With the exception of this change, this *aacA4* showed an amino acid sequence identical to that previously described by Lambert et al. (14). The integron harboring bla_{VIM-1} of isolate PA-SD2 was characterized in parallel for comparative purposes. As shown in Fig. 3, the structure was similar, but in this case, and additional gene (*aadA1*) was detected after *aacA4*. Furthermore, the *aacA4* gene did not contain the polymorphism described above.

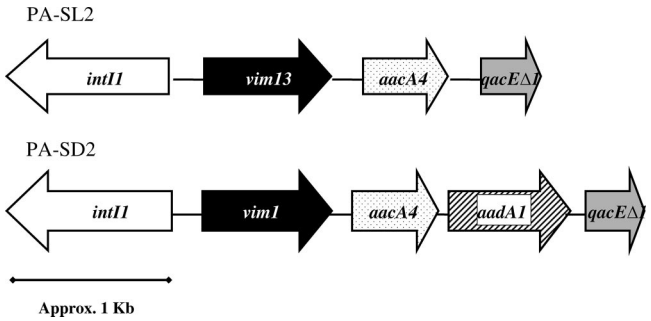

FIG. 3. Structure of the bla_{VIM-1} (strain PA-SD2) and bla_{VIM-13} (strain PA-SL2) carrying the integrons described in this work.

To explore whether the unique polymorphism detected in the VIM-13 integron has an effect on the spectra of aminoglycosides affected, *aacA4* and its Ala108Thr variant were cloned from PA-SD2 and PA-SL2, respectively. As shown in Table 3, the enzymes from both strains yielded the same MICs and conferred resistance to gentamicin and tobramycin but did not affect resistance amikacin, as described by Lambert et al. (14).

Kinetic parameters of VIM-13. Table 4 shows the kinetic parameters of VIM-13 with various β-lactams in comparison with those of VIM-1, which were obtained in parallel experiments. VIM-13 showed higher k_{cat}/K_m ratios (and, therefore, a greater efficiency of hydrolysis) than VIM-1 for all antibiotics tested, with the exceptions of ceftazidime and cefepime. It is remarkable that VIM-13 showed a poorer efficiency of hydrolysis (k_{cat}/K_m) for ceftazidime and cefepime than VIM-1: approximately 2- and 10-fold lower, respectively. Interestingly, there are two reasons for the lower k_{cat}/K_ms of VIM-13 for ceftazidime and cefepime (in fact, the two lowest values among the antibiotics studied). While VIM-13 showed a low k_{cat} for ceftazidime in comparison to that of VIM-1 (turnover rates, 10 and 42 s^{-1}, respectively), it showed an extremely high K_m (and, therefore, a lower affinity) for cefepime (1,870 and 337 μM, respectively). This lower efficiency of hydrolysis of VIM-13 for ceftazidime and cefepime was also in good agreement with the MIC data (Table 3), which show that either the XL1-Blue or the PAO1 pUCPVIM-13 transformants have lower levels of resistance than the pUCPVIM-1 transformants, particularly to cefepime. For the rest of the β-lactams tested, although VIM-13 tended to show a better efficiency of hydrolysis than VIM-1 (including the doubling of its efficiency of hydrolysis for imipenem and meropenem), no significant differences in the

TABLE 4. Kinetic parameters for VIM-13 enzyme with various β-lactams in comparison with those for VIM-1[a]

β-Lactam	k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m (M^{-1} s^{-1})	
	VIM-1	VIM-13	VIM-1	VIM-13	VIM-1	VIM-13
Penicillins						
Penicillin G	418 ± 116	757 ± 146	1,303 ± 393	1,127 ± 172	3.2E5	6.7E5
Ampicillin	307 ± 67	67 ± 9.5	1,737 ± 376	197 ± 48	1.8E5	3.4 E5
Piperacillin	374 ± 107	362 ± 15	1,377 ± 340	729 ± 96	2.7E5	4.9E5
Cephalosporins						
Cephalothin	449 ± 133	656 ± 143	135 ± 29	76 ± 16	3.3E6	8.6E6
Cefuroxime	239 ± 22	283 ± 99	148 ± 36	56 ± 5	1.6E6	5E6
Cefotaxime	204 ± 26	612 ± 120	285 ± 25	233 ± 46	7.18E5	2.6E6
Ceftazidime	42 ± 9.6	10 ± 1	1,132 ± 320	509 ± 120	3.7E4	1.9E4
Cefepime	119 ± 17	61 ± 21	337 ± 110	1,870 ± 569	3.5E5	3.3E4
Carbapenems						
Imipenem	3.4 ± 1.2	54 ± 17	4 ± 1.9	18.5 ± 2.2	8.38E5	2.92E6
Meropenem	20 ± 0.2	9 ± 1.5	72 ± 7.7	15.5 ± 2.3	2.8E5	5.92E5

[a] The k_{cat} and K_m values represent the means of three measurements ± standard deviations.

MICs were observed in either the *E. coli* or the *P. aeruginosa* background. Moreover, as has been observed for VIM-2 and the very recently characterized VIM-11, VIM-13 showed a higher catalytic efficiency for imipenem than for meropenem (6, 18).

Interaction of VIM-13 with metal-chelating agents. Data on the inhibition of hydrolysis activity of VIM-13 (compared to that of VIM-1) after incubation with EDTA, dipicolinic acid, or 1,10-*o*-phenanthroline are shown in Table 5. No significant differences between VIM-1 and VIM-13 were observed when they were incubated with dipicolinic acid or 1,10-*o*-phenanthroline, although dipicolinic acid showed a slightly greater ability to inactivate both enzymes. Interestingly, the data for EDTA revealed that this chelator inhibited the activity of VIM-13 approximately 25 times less than it inhibited the activity of VIM-1, which indicates a stronger binding of Zn ions in VIM-13 than in VIM-1.

DISCUSSION

The isolation of MBL-producing gram-negative bacilli, particularly *P. aeruginosa*, is a problem of growing concern in the nosocomial environment. Indeed, integrons encoding carbapenemases plus aminoglycoside-modifying enzymes are increasingly being reported (1, 13, 21, 22, 30, 37). Moreover, we are facing a marked increase in the diversity of MBLs: up to 25 IMP-type MBLs and 14 VIM-type MBLs have been reported so far (10, 23, 27, 38). In this work, we describe a new VIM-type MBL, designated VIM-13, that exhibits 93% and 88% amino acid sequence identities with VIM-1 and VIM-2, respectively. Additionally, we also report here for the first time the detection of a VIM-1-producing *P. aeruginosa* strain in Spain (VIM-2 had been found in *P. aeruginosa* and VIM-1 has been found only in members of the family *Enterobacteriaceae* [28, 36]).

TABLE 5. IC_{50}s of various chelating agents for VIM-1 and VIM-13 enzymes

Chelating agent	IC_{50} (μM)[a]	
	VIM-1	VIM-13
Dipicolinic acid	41.6 ± 2.9	48.0 ± 8.5
1,10-o-Phenanthroline	73.0 ± 7.2	69.3 ± 4.0
EDTA	9.3 ± 2.1	252.6 ± 42.4

[a] The IC_{50}s represent the means of three measurements ± standard deviations.

Several genes encoding aminoglycoside-modifying enzymes have been detected in integrons harboring bla_{VIM} (11, 19, 26, 30, 37). Strain PA-SL2, which is resistant to gentamicin and tobramycin, harbored an Ala108Thr variant of the previously described acetyltransferase encoded by *aacA4* (14). Its Leu83Ser change modifies its spectrum, affecting tobramycin, netilmicin, and gentamicin but not amikacin (14). As shown in this work, the Ala108Thr polymorphism that was detected seems not to affect further the spectrum of the enzyme.

The locations of several VIM-producing integrons have been traced to plasmids, although a chromosomal location has been suggested in other cases (38). The VIM-13 integron was apparently located in the chromosome of strain PA-SL2. Nevertheless, the potential location of this integron on a mobilizable transposon, which would therefore facilitate its dissemination, still needs to be explored.

Regarding the biochemical properties of VIM-13, the results of MIC and kinetics experiments clearly indicated that VIM-13 hydrolyzed piperacillin better than VIM-1 and hydrolyzed ceftazidime and cefepime worse than VIM-1. Furthermore, VIM-13 showed higher k_{cat}/K_m ratios than VIM-1 for the rest of the β-lactams, although no differences in MICs were documented, since pUCPVIM-1 already conferred very high levels of resistance (MICs > 256 μg/ml) to cefuroxime, cephalothin, cefotaxime, and ampicillin even in the *E. coli* background. Interesting particular properties were also observed when the individual kinetic parameters (k_{cat} or K_m) of both enzymes were compared. For instance, the high K_m of cefepime for VIM-13 appeared to be responsible for its low efficiency of hydrolysis. On the other hand, with other substrates, such as ampicillin or meropenem, notable differences in individual kinetic parameters between VIM-1 and VIM-13 balanced out, finally yielding similar efficiencies of hydrolysis, as observed by Docquier et al. when they compared VIM-1 and VIM-2 (6).

The 19-amino-acid difference between the two enzymes should explain the kinetic data for VIM-1 and VIM-13. As indicated by Docquier et al. (6), amino acid changes in loop L3 (the active enzyme site) could explain the distinct biochemical behaviors of VIM-1 and VIM-2 (6) because these changes affect substrate binding. Interestingly, VIM-13 showed two amino acid replacements, His201Leu and Ser205Arg, in the loop cited by Docquier et al. (6). Furthermore, the Ser205Arg polymorphism is also observed in VIM-2, whereas the histidine residue at position 201 is also modified in this enzyme, although in this case it is replaced by a tyrosine (Fig. 1). At position 205, VIM-13, like VIM-2, has an arginine instead of a serine. It has previously been speculated that this replacement may favor the interaction of the charged guanidinium group directly with the benzylpenicillin carboxylate (O-NH_2), as the distance of Ser is too short to create any interaction with the substrate (6). In agreement with that hypothesis, VIM-13 showed higher k_{cat}/K_m values than VIM-1 for penicillin G. At position 201, VIM-2 and VIM-13 showed the replacements His to Tyr and His to Leu, respectively (Fig. 1). The fact that these enzymes showed lower catalytic efficiencies against cefepime is interesting; therefore, it can be hypothesized that the His-imidazole group is somehow important in the direct interaction of this antibiotic with bulky substituents, although this hypothesis needs further confirmation. Hence, further site-directed mutagenesis studies are needed in order to demonstrate the involvement of the specific residues in the efficiencies of hydrolysis. Moreover, the recently characterized VIM-12 enzyme shows no amino acid changes in this loop in comparison with the amino acid sequence of VIM-1, although clear kinetic differences have been found for VIM-12 (12). This fact clearly indicates that the changes in the active center are not the only changes responsible for the different biochemical behaviors (18). Moreover, amino acid differences have also been shown to be responsible for the worse inhibitory power shown by EDTA against VIM-13 in comparison to that against VIM-1. Nevertheless, no amino acid changes were observed in VIM-13 zinc-ligand residues in comparison with the amino acid sequence of VIM-1 or VIM-2 (6). Interestingly, the IC_{50} of EDTA for VIM-2 from the work of Poirel et al. (25) was 50 μM, a value intermediate between our IC_{50} for VIM-1 (9.3 μM) and our IC_{50} for VIM-13 (252.6 μM). This fact seems to indicate that differences in the amino acid compositions and not only the differences related to the zinc-ligand residues are also responsible for the different levels of inhibition caused by the chelating agents against MBLs.

In summary, we describe a new integron-borne bla_{VIM} gene, bla_{VIM-13}, detected in a *P. aeruginosa* clinical isolate in Majorca, Spain. Although the encoded VIM-13 enzyme is found to be located the closest to the VIM-1 cluster, it shows remarkable differences from VIM-1 in its biochemical and microbiological behaviors.

ACKNOWLEDGMENTS

This work was supported by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, through the Spanish Network for the Research in Infectious Diseases (grants REIPI C03/14 and RD06/0008) and grant PI061368 and by the Govern de les Illes Balears (grant PROGECIC-4C).

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