Molecular Cloning and Biochemical Characterization of VIM-12, a Novel Hybrid VIM-1/VIM-2 Metallo-β-lactamase from a *Klebsiella pneumoniae* Clinical Isolate, Reveal Atypical Substrate Specificity†

Maria Kontou,‡ Spyros Pournaras,§ Ioulia Kristo,‡,§ Alexandros Ikonomidis,§ Antonios N. Maniatis,§ and Constantinos Stathopoulos*,‡,||

Department of Biochemistry and Biotechnology, University of Thessaly, 26 Ploutonos st., 41221 Larissa, Greece, Department of Microbiology, School of Medicine, University of Thessaly, 41222 Larissa, Greece, and Institute of Biomedical Research and Technology (BIOMED), 51 Papanastasiou st, 41222 Larissa, Greece

Received June 27, 2007; Revised Manuscript Received August 2, 2007

ABSTRACT: Metallo-β-lactamases (MBLs) are considered an emerging family of Zn^{2+}-dependent enzymes that significantly contribute to the resistance of many nosocomial pathogens against β-lactam antimicrobials. Since these plasmid-encoded enzymes constitute specific molecular targets for β-lactams, their exact mode of action is greatly important in deploying efficient anti-infective treatments and for the control of severe multi-resistant nosocomial infections, which becomes a global problem. A novel hybrid VIM-1/VIM-2-type β-lactamase (named VIM-12) has recently been identified in a clinical isolate of *Klebsiella pneumoniae* in Greece. The sequence of this enzyme is highly similar with that of VIM-1 at its N-terminal region and with that of VIM-2 at its C-terminal region, raising the question of whether this sequence similarity reflects also a similar functional role. Moreover, the possible contribution of this novel β-lactamase to the overall antibiotic resistance of this specific clinical isolate was investigated. The gene encoding VIM-12 was cloned and expressed, and the recombinant enzyme was used for detailed kinetic analysis, using a variety of β-lactam antibiotics. VIM-12 was found to exhibit narrow substrate specificity, compared to other known β-lactamases, limited mainly to penicillin and to a much lesser extent to imipenen. Interestingly, meropenem was found to act as a noncompetitive inhibitor of the enzyme, although the active site of VIM-12 exhibited complete conservation of residues among VIM enzymes. We conclude that VIM-12 represents a novel and unique member of the family of known metallo-β-lactamases, exhibiting atypical substrate specificity.

The production of β-lactamases is a predominant mechanism of bacterial resistance, and more than 470 β-lactamases, either chromosomally or plasmid-mediated, have been described to date (*1*). They are clustered into four classes: three serine-dependent [serine-β-lactamases (SBLs)] enzyme classes (A, C, and D) and one metal-dependent [metallo-β-lactamases (MBLs)] class (B) (*2*). The MBLs are zinc-dependent hydrolases and are completely distinct from their serine counterparts in terms of sequence, fold, and mechanism (*3*, *4*). They are small enzymes sharing a common four-layer $\alpha\beta/\beta\alpha$ fold, with a central β-sandwich and two α-helices on either side (*5*, *6*). This motif, arising possibly by a gene duplication event (*7*), is also found in other proteins (glyoxalases and certain flavoenzymes) (*8*, *9*) and exhibits an intrinsic metal-binding site, located at the edge of the β-sandwich (*10*, *11*). For the metallo-β-lactamases, this site is occupied by a divalent zinc ion having a tetrahedral array of three histidines and water. The role of zinc in the hydrolytic mechanism, beyond that of Lewis acid catalysis, is not certain. Nonetheless, there is a consensus that the water ligand of the zinc ion is the β-lactam ring opening nucleophile, via a mechanism parallel to that of the zinc metalloproteases (*12*−*15*).

Three MBL subclasses (B1−B3) exist (*16*). Despite a very low degree of sequence similarity among the three subclasses, the general structures and the relative positions of the secondary structure elements are similar, in particular among enzymes of subclasses B1 and B2 (*17*, *18*). Gram-negative microorganisms producing acquired MBLs are increasingly implicated in nosocomial infections. Two distinct MBL types, IMP (*19*) and VIM[1] (*20*), are more commonly detected worldwide, while two additional types, GIM (*21*) and SPM (*22*), have been sporadically detected. The sequences of the former MBL types, though, are less than 40% identical, and they exhibit similar kinetic properties, efficiently hydrolyzing most β-lactam compounds, including carbapenems (*23*). Additionally, both bla_{IMP}- and bla_{VIM}-type genes are carried as gene cassettes by class 1 integrons.

† This work was supported in part by a Research Grant from the University of Thessaly Research Committee (to C.S.).

* To whom correspondence should be addressed: Department of Biochemistry and Biotechnology, University of Thessaly, 26 Ploutonos st., 41221 Larissa, Greece. Telephone: +30-2410-565278. Fax: +30-2410-565290. E-mail: cstath@bio.uth.gr.

‡ Department of Biochemistry & Biotechnology, University of Thessaly.

§ Department of Microbiology, School of Medicine, University of Thessaly.

|| Institute of Biomedical Research & Technology (BIOMED).

[1] Abbreviation: VIM, Verona integron-encoded metallo-β-lactamase.

10.1021/bi701258w CCC: $37.00
Published on Web 10/18/2007

The VIM-type MBLs belong to subclass B1 of molecular class B. So far, 13 published VIM variants have been described that can be clustered into three groups on the basis of sequence similarity (*24*), represented by VIM-1 (*25*), VIM-2 (*26*), and VIM-7 (*27*). The VIM-1 group also comprises VIM-4, VIM-5, and VIM-11A (*28*−*30*). Six VIM variants (VIM-3, -6, -8, -9, -10, and -11B) are considered derivatives of VIM-2 (*31*−*33*). The amino acid sequence of VIM-2 is 90% identical with that of VIM-1 (*26*), and apparently, VIM-2 is the most widespread VIM-type MBL (*34*). VIM-type MBLs were first described in *Pseudomonas aeruginosa* (*25*) and thereafter in other Gram-negative nonfermenters and enterobacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (*32*, *35*−*38*). Recent studies indicate that VIM-producing microorganisms are endemic in various Mediterranean countries, including Greece, as well as in the Far East (*38*−*41*).

Despite the fact that the amino acid sequences of VIM-1 and VIM-2 diverge by only 7%, notable differences have been reported for their kinetic parameters using several β-lactam substrates, suggesting that the few structural differences between these two natural variants have functional significance (*42*). The detailed characterization and the elucidation of the catalytic mechanisms employed by MBLs are prerequisites for rational inhibitor design. In the challenging quest for new specific inhibitors, a detailed understanding of their biochemical properties and structure is desirable.

In this study, we focused on the biochemical characterization and kinetic analysis of a novel plasmid-encoded MBL (named VIM-12) from a *K. pneumoniae* clinical isolate, which is anchored by a gene cassette inserted into a class 1 integron (*43*). The clinical isolate was recovered in Thessaloniki, Greece, from blood cultures of a 67-year-old surgical patient. The same VIM-12 enzyme was recently identified from our group also in an *E. coli* clinical isolate (*44*). The sequence of the 266-amino acid VIM-12 polypeptide is 97.0 and 93.6% identical with those of VIM-1 and VIM-2, respectively (Figure 1a). Preliminary kinetic experiments assessing the enzyme's activity in the presence of various antibiotics showed interestingly that VIM-12 was hydrolyzing efficiently only penicillin and not other antibiotics. In an attempt to further characterize this unusual enzymatic behavior, we cloned the corresponding gene, purified the recombinant enzyme, and performed detailed kinetic analysis and competition experiments in the presence of various antibiotics (Figure 2). Our aim was to extract information about the mechanism of antibiotic resistance, which would be of great clinical importance.

MATERIALS AND METHODS

Bacterial Strains and Plasmids and MIC Determinations. *K. pneumoniae* 2873 was the clinical isolate which harbored the bla_{VIM-12} gene. *E. coli* DH5α (Invitrogen) was used as the host strain for recombinant plasmid pGEM-Teasy Vector (Promega), and *E. coli* BL21 (Novagen) was used for the overexpression of the VIM-12 enzyme. Plasmid pGEM-Teasy Vector was used for the cloning and sequencing of the PCR-amplified fragment of the bla_{VIM-12} gene. Plasmid pET15b (Novagen) was used as a T7-based expression vector for the overexpression of the bla_{VIM-12} gene. MICs for penicillin G, imipenem, meropenem, ceftazidime, aztreonam, cefotaxime, and cefoxitin were determined by Etest and Etest MBL (AB Biodisk, Solna, Sweden) in *E. coli* strain BL21 with and without the insertion of the bla_{VIM-12}-carrying plasmid pET15b, an *E. coli* transconjugant carrying bla_{VIM-12} in the originally described p2873 plasmid and clinical *E. coli* strain 28 from our collection carrying bla_{VIM-12}.

Cloning and Expression of the bla_{VIM-12} Gene. The bla_{VIM-12} gene was amplified by PCR using the following set of primers: VIM-12 F (5′-GCATATGTTAAAAGTTATTAGTAGTTTATTGG-3′) and VIM-12 R (5′-GGATCCCTACTCAACGACTGAG-3′) (the underline indicates the *Nde*I and *Bam*HI restriction sites, respectively). Plamid DNA extracted from *K. pneumoniae* 2873 (Qiagen DNA Miniprep Kit) was used as the template in the PCR which was performed under the following conditions: 2 min at 95 °C, 30 cycles of denaturation for 45 s at 95 °C, annealing for 1 min at 52 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR product was cloned via TA cloning into plasmid pGEM-Teasy Vector (Promega) and was sequenced to ensure that no mutations were incorporated. The recombinant plasmid was transformed into DH5α cells, and plasmid extract from the transformed cells was digested with *Nde*I and *Bam*HI enzymes. The restriction fragment was subcloned into the pET15b vector (Novagen) which was transformed into *E. coli* BL21 for overexpression. The strain was grown aerobically in 2 L of buffered Luria-Bertani medium containing chloramphenicol (100 μg/mL), at 37 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co.) was added to a final concentration of 0.5 mM when the culture reached an A_{600} of 0.6, and the incubation was continued for an additional 5 h at 20 °C.

Purification of the VIM-12 Metallo-β-lactamase. The VIM-12 enzyme was purified from *E. coli* BL21 carrying recombinant plasmid pET15b-bla_{VIM-12} as follows. Cells from a 1 L culture were harvested by centrifugation (4000*g* for 20 min), resuspended in 10 mL of 50 mM Hepes-KOH (pH 8.0) containing 100 μM $ZnSO_4$, 500 mM NaCl, 0.1% Triton, 5 mM β-mercaptoethanol, 10 μg/mL lysozyme, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5% glycerol, and disrupted by sonication. The lysate was clarified by centrifugation at 100000*g* for 1 h, and the supernatant was used for purification of the enzyme using an agarose Ni-NTA column (Qiagen) equilibrated in 50 mM Hepes-KOH buffer (pH 8.0) containing 100 μM $ZnSO_4$, 500 mM NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF, and 5% glycerol. The column was washed with the same buffer supplemented with 20 mM imidazole, and the enzyme was eluted with the same buffer including 500 mM imidazole. The fractions containing β-lactamase were detected after SDS−PAGE analysis and subsequent Coomassie Brilliant Blue or silver staining (>95% pure), followed by immunoblotting using an anti-His antibody which confirmed the presence of the recombinant protein in the elution fractions. To ensure that no contaminant proteins of the same molecular mass with VIM-12 were comigrating in the SDS−PAGE analysis, *E. coli* lysates from cultures without induction were used to perform the same affinity purification scheme. The active fractions containing recombinant VIM-12 were supplemented with 20% glycerol and were immediately stored at −20 °C to avoid any loss of enzymatic activity.

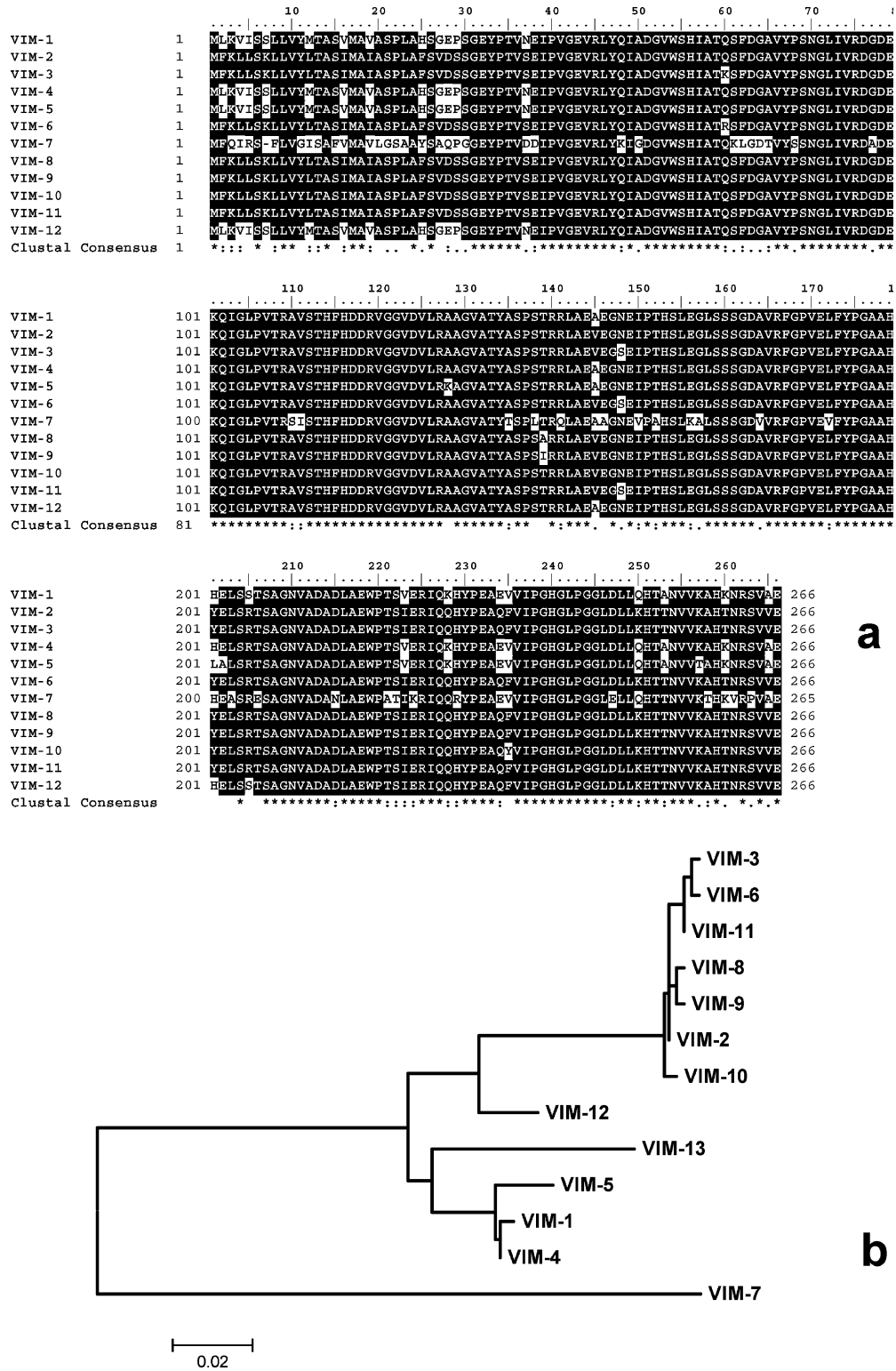

FIGURE 1: (a) Clustal-W protein sequence alignment of all published VIM-type MBLs generated by using the PAM350 matrix. (b) Neighbor-joining dendrogram showing the relatedness of VIM-12 and other acquired VIM-type MBLs. Calculation of the tree was based on panel a.

VIM-12 Activity Assay and Determination of Kinetic Parameters. In general, substrate hydrolysis by VIM-12 (1−5 μM) was monitored by following the absorbance variation at 40 °C, in 50 mM Hepes-KOH (pH 6.8) supplemented with 50 μM $ZnSO_4$ and 10 mM KCl in a 1 mL final volume of reaction mixture, using a lambda 2 spectrophotometer (Perkin-Elmer, Rahway, NJ) equipped with thermostatically controlled cells. The progress of the hydrolysis of antibiotics was monitored in 30 s intervals following the initial velocity of the reaction and stopped when it reached a plateau. The wavelengths used were 235 nm for penicillin G (10−500 μM), 299 nm for imipenem (10−500 μM) and meropenem (10−500 μM), 292 nm for aztreonam (50−500 μM), 237 nm for ceftazidime (10−200 μM), 257 nm for cefotaxime (50−100 μM), and 295 nm for cefoxitin (500 μM). For the determination of optimal assay conditions, we tested the

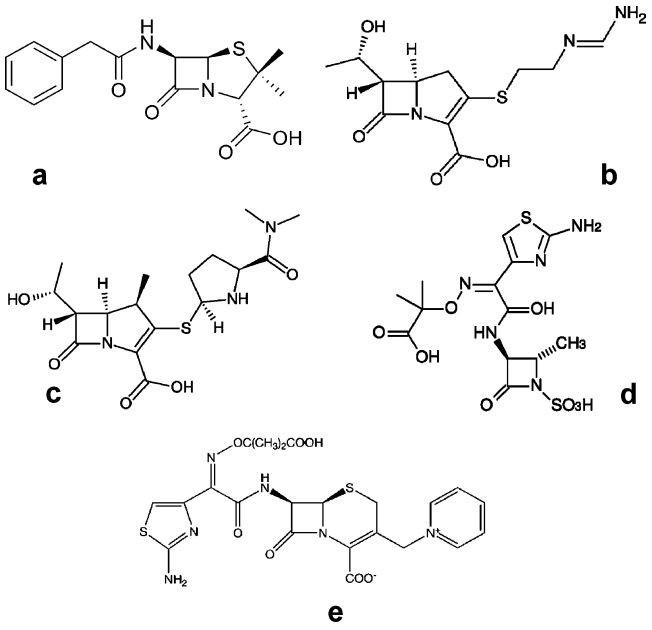

FIGURE 2: Chemical structures of all antibiotics used in this study as substrates of VIM-12: (a) penicillin G, (b) imipenem, (c) meropenem, (d) aztreonam, and (e) ceftazidime.

activity of VIM-12 at different pH values (range of 6.0−8.5), different temperatures (range of 25−50 °C), Zn^{2+} concentrations (0−500 μM), and KCl concentrations (0−100 mM). Since Zn^{2+} ions are important for optimal enzyme activity, we tried to replace them with either Mg^{2+} or Mn^{2+} metal ions (50−100 μM) to identify any effects on the enzyme's activity.

The steady-state kinetic parameters (K_m and V_{max}) were determined (under standard assay conditions) by analyzing β-lactam hydrolysis under initial rate conditions using the Lineweaver−Burk linearization of the Michaelis−Menten equation, with the statistic package GraFit (*45*). The k_{cat} values were calculated by dividing the V_{max} values by the concentration of the recombinant enzyme. Competition experiments were performed to study the interaction of imipenem (50−300 μM), meropenem (1−20 μM), ceftazidime (50−300 μM), and aztreonam (50−300 μM) with the enzyme, using penicillin G (50−300 μM) as the reporter substrate. The susceptibility to inhibition was determined with and without preincubation of the enzyme with various concentrations of each antibiotic for 10 min at 40 °C, before measuring the residual β-lactamase activity.

RESULTS

Purification of Recombinant VIM-12. In this study, a novel plasmid-encoded VIM-type metallo-β-lactamase from a clinical isolate of *K. pneumoniae* is described. The electrophoresis of the PCR product revealed a DNA band with an expected size of 801 bp. Sequencing of the PCR product verified the expected size and the fact that no mutations were incorporated. Subsequent cloning of the gene into the pET15b expression vector resulted in N-terminal His_6-tagged VIM-12 that was purified in one step on a Ni-NTA affinity chromatography column. VIM-12 was judged to be >95% pure by SDS−PAGE analysis followed by either Coomassie Brilliant Blue or silver staining. The same purification protocol was used for *E. coli* cell lysates that were not subjected to IPTG induction. There were no detectable contaminant protein bands migrating at the same molecular mass as recombinant VIM-12. Therefore, we concluded that the recombinant VIM-12 isolated from the elution fractions could be subsequently used for further biochemical analysis. In addition, an anti-His antibody was used to detect the presence of VIM-12 and to further ensure that the purification was not masked by contaminant proteins. VIM-12 was migrating with an apparent molecular mass of 28 kDa which was consistent with the predicted molecular mass of the protein (Figure 3). The sequence of VIM-12 polypeptide (266 amino acids, molecular weight of 28 120) was 97.0 and 93.6% identical with those of VIM-1 and VIM-2, respectively (Figure 1a). VIM-12 differs from VIM-1 by eight amino acid residues at positions 246, 251, 257, 258, 284, 287, 294, and 299 (standard class B metallo-β-lactamase numbering scheme) (*46*). All these residues are located at the protein surface and are distant from the active site. A dendrogram based on CLUSTAL W (*47*) multiple alignment of the VIM lactamases showed that VIM-12 was clustered between the VIM-1 and VIM-2 groups (Figure 1b).

Biochemical Characterization of VIM-12 Metallo-β-lactamase. On the basis of the high degree of sequence similarity between VIM-12 and VIM-1 and VIM-2 (Figure 1a), we expected that the substrate specificity of this hybrid metallo-β-lactamase would not be significantly different from that of VIM-1 or VIM-2, a hypothesis that was subsequently tested. The optimal conditions of the VIM-12 lactamase activity were determined by performing β-lactam antibiotic hydrolysis assays, in 50 mM Hepes-KOH buffer, at different pH values, different temperatures, different metal ion concentrations (Zn^{2+}, Mg^{2+}, and Mn^{2+}), and different KCl concentrations, using as a substrate penicillin G. The hydrolytic activity of VIM-12 was optimal at pH 6.7 and 40 °C (see the Supporting Information). Addition of zinc ions to a final concentration of 50 or 100 μM did not affect the hydrolytic activity of the enzyme, while concentrations higher than 100 μM had a considerable negative effect. Concentrations of KCl higher than 20 mM weakened slightly the hydrolytic ability of VIM-12. The substitution of zinc ions with magnesium and manganese ions did not support the activity of the enzyme.

Determination of Kinetic Parameters of VIM-12. The kinetic parameters of the purified VIM-12 were determined in the presence of several β-lactam substrates (Figure 2). Surprisingly, the biochemical assays revealed that VIM-12 exhibited a narrow substrate profile, as only penicillin G was efficiently hydrolyzed (Figure 4a). VIM-12 also exhibited a moderate rate of hydrolysis of imipenem (Figure 4b), with a relative k_{cat}/K_m value of 0.0003 μM^{-1} s^{-1}. Also, hydrolysis of meropenem, ceftazidime, cefoxitin, and cefotaxime was not observed, in contrast to other VIM-type lactamases (Figure 4b). Moreover, we did not observe hydrolysis with the monobactam aztreonam. The following kinetic parameters were determined: $V_{max} = 616.07 \pm 21.37$ μM/min and $K_m = 280.11 \pm 28.04$ μM for penicillin G (Figure 5a), and $V_{max} = 0.507 \pm 0.030$ μM/min and $K_m = 93.62 \pm 11.1$ μM for imipenem (Figure 5b). Although VIM-12 hydrolyzes imipenem very slowly, this specific antibiotic represents a true substrate for VIM-12 and in the subsequent experimentation was manipulated as such. The k_{cat} values were calculated by dividing the respective V_{max} value by the concentration of

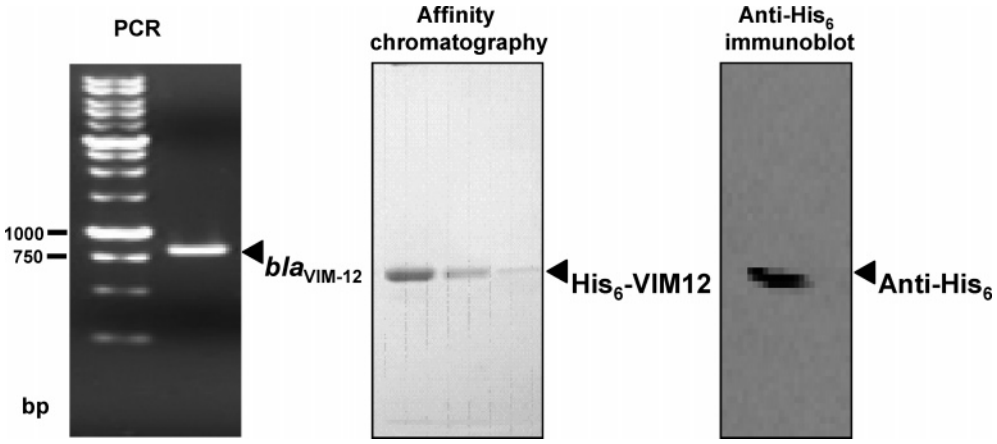

FIGURE 3: PCR amplification of bla_{VIM-12} (left), purification on a Ni-NTA affinity chromatography column (center), and immunoblot analysis of a representative fraction containing purified His_6-VIM-12 (right).

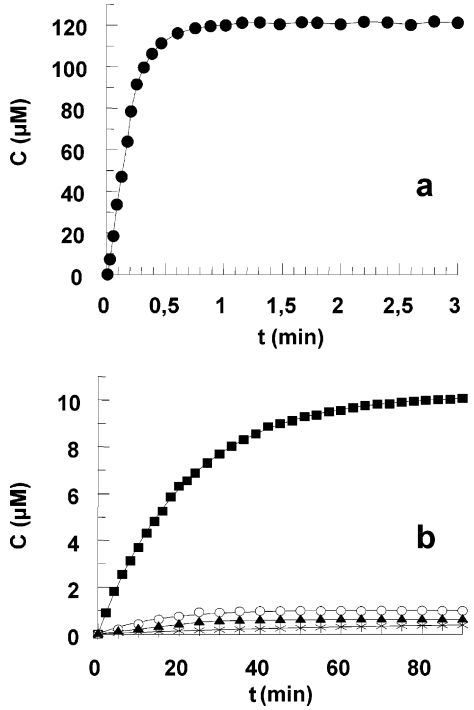

FIGURE 4: Representative hydrolysis curves of various β-lactam compounds in the presence of recombinant VIM-12: (a) penicillin G (●) and (b) imipenem (■), meropenem (○), aztreonam (×) and ceftazidime (▲). All reactions were carried out as described in Materials and Methods.

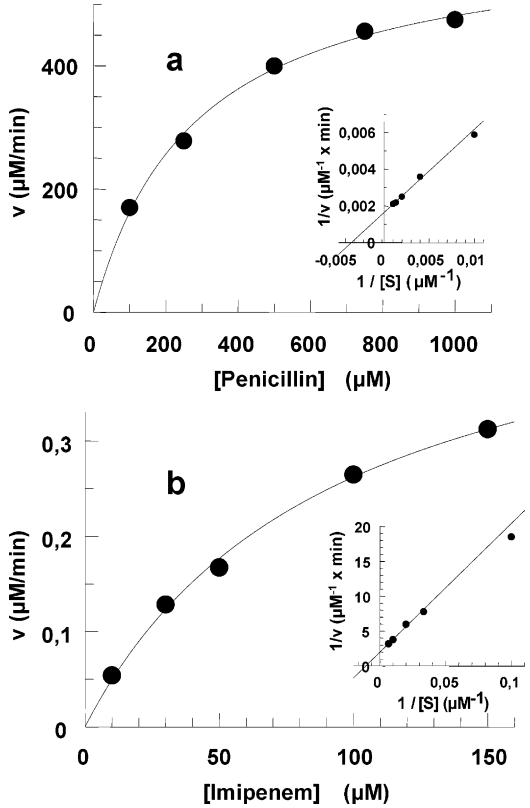

FIGURE 5: Penicillin and imipenem hydrolysis by recombinant VIM-12. (a) Michaelis−Menten plot of penicillin hydrolysis. The inset is a double-reciprocal plot ($1/v$ vs 1/[penicillin]). (b) Michaelis−Menten plot of imipenem hydrolysis. The inset is a double-reciprocal plot ($1/v$ vs 1/[imipenem]).

the recombinant VIM-12. This calculation was based on the assumption that all the enzyme molecules were active. In addition, we did not observe any decrease in the enzymatic activity during the experimental procedure.

To study the mode of interaction of VIM-12 with the antibiotics that were not hydrolyzed, competition experiments were conducted using penicillin G as the reporter substrate. These inhibition studies would reveal whether the β-lactamase was able to bind these antibiotics via its active site. The initial velocity in the presence or absence of inhibitors was determined from the initial slopes of time plots. Data obtained from these assays were used to create the corresponding Lineweaver−Burk diagrams and to calculate the inhibition constants for each antibiotic. Figure 6a shows double-reciprocal plots with increasing concentrations of meropenem. The slope of the line was replotted against the concentrations of meropenem, and the results are shown in the top panel. The linearity of this plot is indicative of simple noncompetitive inhibition and led to the determination of a K_i value of 4.2 μM. Further evidence for simple noncompetitive kinetics for meropenem comes from the Dixon plot (Figure 7a). The same detailed kinetic analysis was performed for imipenem, ceftazidime, and aztreonam. In contrast with meropenem, imipenem exhibits simple competitive inhibition (Figure 6b). The slope of the line was replotted against the concentrations of imipenem, and the results are shown in the top panel. The linearity of this plot is indicative of simple competitive inhibition with a calculated K_i value of 80.6 μM. The Dixon plot (Figure 7b)

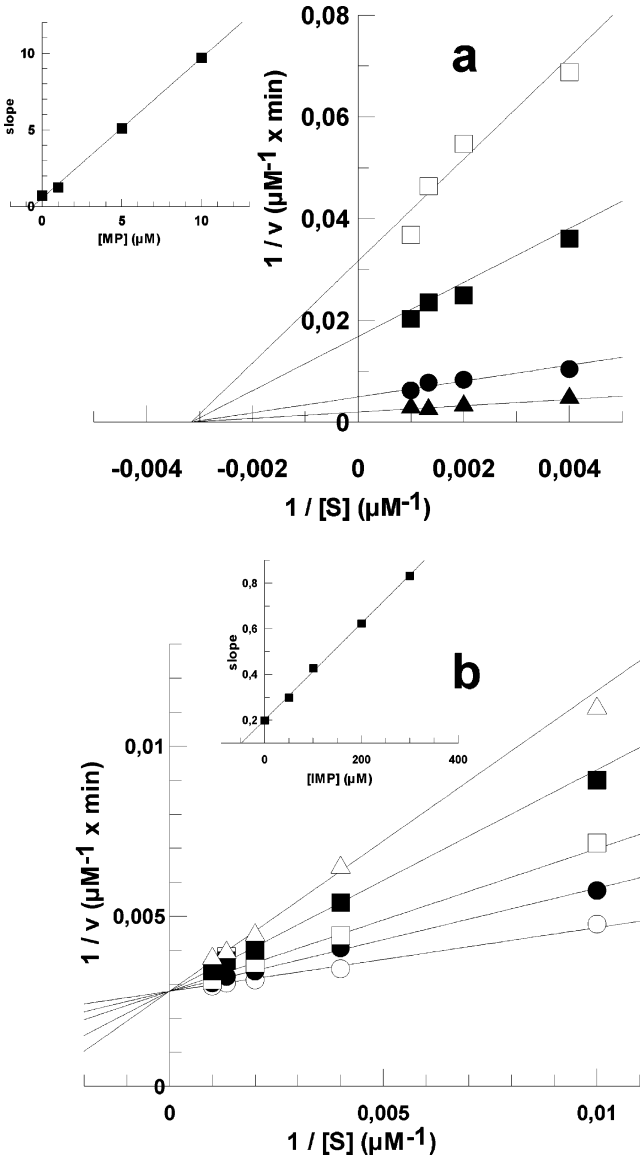

FIGURE 6: (a) Double-reciprocal plot ($1/v$ vs 1/[penicillin]) for the hydrolysis of penicillin by VIM-12 in the presence of meropenem. The reaction was carried out at each one of the indicated concentrations in the presence or absence of inhibitor (meropenem). All reactions were carried out at 40 °C as described in Materials and Methods: without meropenem (▲) or with 1 (●), 5 (■), or 10 μM meropenem (□). The inset is a replot of the slopes of the double-reciprocal lines vs meropenem (MP) concentration. (b) Double-reciprocal plot ($1/v$ vs 1/[penicillin]) for the hydrolysis of penicillin by VIM-12 in the presence of imipenem. The reaction was carried out at each one of the indicated concentrations in the presence or absence of inhibitor (imipenem). All reactions were carried out at 40 °C in 1 mL of 50 mM Hepes buffer (pH 6.8) without imipenem (○) or with 50 (●), 100 (□), 200 (■), or 300 μM imipenem (△). The inset is a replot of the slopes of the double-reciprocal lines vs imipenem concentration.

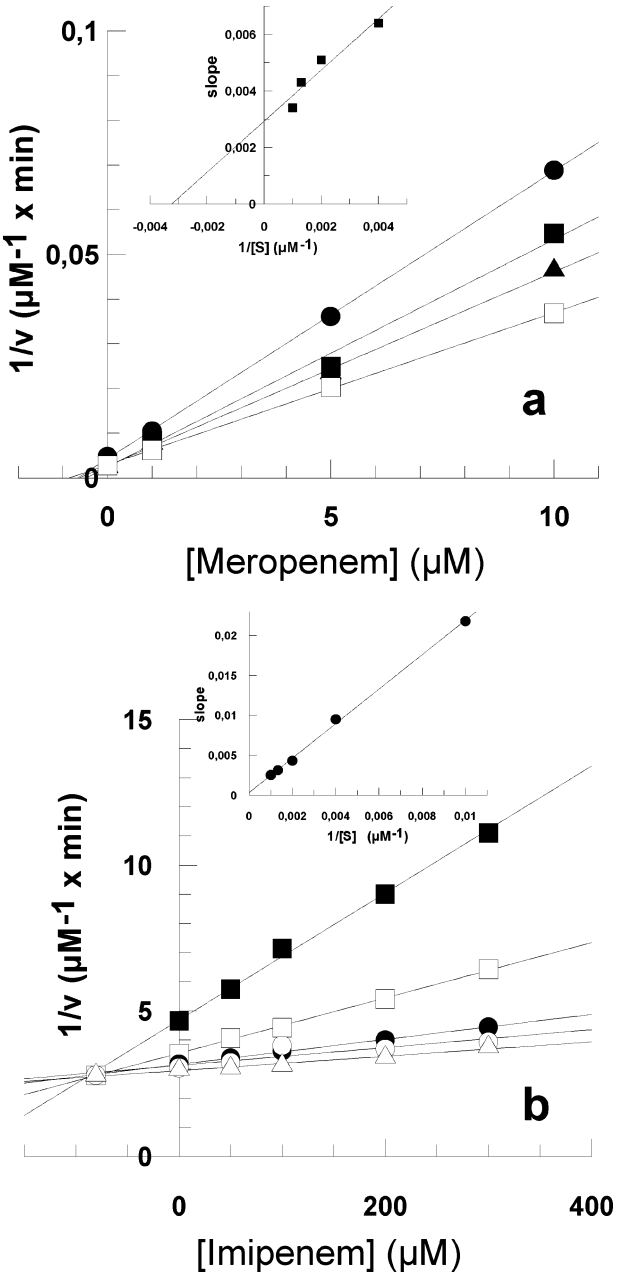

FIGURE 7: (a) Dixon plot of the hydrolysis of penicillin by VIM-12 in the presence of meropenem (MP). The penicillin concentrations were 250 (●), 500 (■), 750 (▲), and 1000 μM (□). The inset is a replot of the slopes of the Dixon lines vs 1/[penicillin] (1/[S]). (b) Dixon plot of the hydrolysis of penicillin by VIM-12 in the presence of imipenem (IMP). The penicillin concentrations were 100 (■), 250 (□), 500 (●), 750 (○), and 1000 μM (△). The inset is a replot of the slopes of the Dixon lines vs 1/[penicillin] (1/[S]).

confirmed this mode of inhibition. Ceftazidime acts as a very weak simple noncompetitive inhibitor, while aztreonam exhibited no inhibitory effect whatsoever. The inhibition constants determined for each antibiotic in this study were compared with previously reported values and are summarized in Table 1. The results that we obtained with and without preincubation of the enzyme with each antibiotic were identical.

The kinetic analysis showing that VIM-12 exhibits narrow substrate specificity was confirmed by in vivo experiments that tested the sensitivity of bla_{VIM-12}-overexpressing *E. coli* strain BL21 against the spectrum of antibiotics that were tested in vitro. The MIC values that were observed (Table 2), and in particular the very low MICs for meropenem of the strains that were tested, were consistent with the fact that VIM-12 does not seem to contribute significantly to carbapenem resistance, besides its prominent function of hydrolyzing penicillin (which is the natural substrate). In addition, we tested under the same conditions an *E. coli* clinical isolate that was recently published and encompasses a VIM-12 enzyme. Again, this strain proved to be almost as sensitive as bla_{VIM-12}-overexpressing strain *E. coli* BL21,

Table 1: Kinetic Parameters of the Purified VIM-12 Metallo-β-lactamase, Compared with Those of VIM-1 and VIM-2[a]

enzyme	antibiotic	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_i (μM)
VIM-12	penicillin	280	34	0.121	–
	imipenem	94	0.028	0.0003	80.6
	meropenem	>1000	<0.01	$<10^{-5}$	4.2
	ceftazidime	>1000	<0.01	$<10^{-5}$	728
	aztreonam	>1000	<0.01	$<10^{-5}$	ND[b]
VIM-1	penicillin	841	29	0.034	ND[b]
	imipenem	1.5	2	1.3	ND[b]
	meropenem	48	13	0.27	ND[b]
	ceftazidime	794	60	0.076	ND[b]
	aztreonam	>1000	<0.01	$<10^{-5}$	ND[b]
VIM-2	penicillin	49	55.8	1.14	ND[b]
	imipenem	10	9.9	0.99	ND[b]
	meropenem	5	1.4	0.28	ND[b]
	ceftazidime	98	89	0.9	ND[b]
	aztreonam	ND[b]	<0.5	ND[b]	ND[b]

[a] The kinetic data for VIM-1 are from ref *20*, and those for VIM-2 are from ref *1*. [b] Not determined.

Table 2: Susceptibility Status of Competent Cells with and without bla_{VIM-12} Inserts and Other bla_{VIM-12}-Carrying Strains Tested in This Study[a]

	MIC (μg/mL)				
	IMP	MP	PEN G	ATM	CEF
E. coli DH5α	0.25	0.064	> 32	0.047	0.125
DH5α-VIM-12	0.38	0.094	> 32	0.094	1
E. coli BL21	0.25	0.064	≥ 32	0.016	0.064
BL21-VIM-12	0.38	0.064	> 32	0.023	0.125
Trcs *E. coli* 26R793 (bla_{VIM-12})	2	0.5	>256	4	> 128
E. coli 28 (VIM-12)	1	0.25	> 32	0.5	> 256

[a] Trcs, transconjugant strain; IMP, imipenem; MP, meropenem; PENG, penicillin G; ATM, aztreonam; and CEF, ceftazidime.

thus consolidating our results about this peculiar behavior of VIM-12 against antibiotics other than penicillin.

DISCUSSION

The spread of plasmid-encoded MBL genes among opportunistic and pathogenic bacteria, together with the lack of clinically useful inhibitors, is becoming a serious and yet unsolved clinical problem. The recombination system based on integrons and mobile gene cassettes plays a major role in the dissemination of these genes among various pathogens. This study identified the emergence of a novel class B plasmid-encoded metallo-β-lactamase, VIM-12, anchored by a gene cassette inserted into a class 1 integron from a clinical isolate of *K. pneumoniae*.

VIM-12 is a new member of the VIM lineage, which exhibits narrow substrate specificity, limited to penicillins. The sequence of VIM-12 polypeptide was 97.0 and 93.6% identical with those of VIM-1 and VIM-2, respectively. However, the dendrogram that was produced on the basis of all known VIM protein sequences indicates that VIM12 clusters between VIM-1 and VIM-2 enzymes, which is clearly indicated by the respective sequence alignments (Figure 1). Moreover, VIM-12 bears at its N-terminal region the SGEPS amino acid signature which is characteristic of the VIM-1 lactamases [the corresponding signature for VIM-2 is SVDSS (Figure 1a)]. A possible explanation for the origin of VIM-12 might have been a recombination event between VIM-1 and VIM-2 that facilitated the anchoring of this novel hybrid VIM-type lactamase in the clinical isolate strain of *K. pneumoniae*, although this hypothesis is difficult to prove and this gene might have also arisen independently. Should bla_{VIM-12} be an evolutionarily new recombinant gene, it may have not yet acquired its full functional potential. Although the amino acid differences between these enzymes were relatively few in number and located distant from the active site, it seems that they could influence substrate binding, a fact that was partially confirmed in the case of VIM-1 and VIM-2 which, despite their high degree of sequence similarity, exhibit quite different kinetic properties. In terms of structure−function relationships, further research is required to determine the significance of these amino acid changes.

The fact that VIM-12 is limited so far to this specific clinical isolate and the fact that at least its recombinant form that we investigated has limited substrate specificity could support this hypothesis. However, we cannot exclude the possibility that VIM-12 is a novel and genuine MBL that has been elusive so far in many bacterial strains. In fact, a recent report on the identification of the same plasmid-encoded gene in an *E. coli* clinical isolate (*E. coli* strain 28, recovered February 2006 in Thessaloniki, Greece) (*44*) indicates that either VIM-12 already started to spread among Gram-negative bacteria or it has been elusive so far among clinical isolates and its identification requires further investigation. Nevertheless, the amino acids that configure the active site of VIM-12 were identical with those encountered in all VIM active sites (*48*). In addition, all the amino acid residues that have been reported to play important role in Zn^{2+} binding (*48*, *49*) were present in the VIM-12 sequence, including an asparagine at position 120 that, as recently reported, places Zn^{2+} for optimal MBL activity. Taking into account this structural similarity of the active site of VIM-12 when compared to the available structural data for VIM-2 (PDB entries 1KO2 and 1KO3), we would expect similar substrate hydrolysis profiles. However, recent studies have proposed that besides the conserved residues that seem to participate in Zn^{2+} coordination in MBLs, the active site is probably modulated by a hydrogen bonding network of residues located along the MBLs' primary structure (*48*). The fact that the kinetic properties of VIM-12 were found to be atypical compared to those of other VIM-type metallo-β-lactamases raises the question of whether the N-terminal and C-terminal residues that exhibit homology with residues in VIM-1 and VIM-2, respectively, could contribute in such a bonding network that results in this atypical specificity of VIM-12. This hypothesis requires further experimentation with extensive mutational analysis and support by crystallographic data. All other lactamases of this group hydrolyze most β-lactam compounds, except aztreonam, far more effectively.

A reason that cannot be excluded when we try to explain the ability of VIM-12 to efficiently hydrolyze only penicillin is the stereochemistry of the antibiotics that were used in this study (Figure 2). All of them were synthetic derivatives of penicillin bearing different side groups and a lactamic ring in the opposite direction. However, imipenem bears a rather flexible side chain that could facilitate binding to the active site and efficient, albeit slower, hydrolysis. On the other hand, meropenem contains bulky rings that contribute to a less

flexible overall structure. This structural difference could explain why this antibiotic is not suitable for correct recognition and processing by VIM-12, although it has been reported as an appropriate substrate for other VIM-type lactamases. Interestingly, it seems that VIM-12 contains allosteric sites for binding of meropenem (and possibly for ceftazidime and other antibiotics that were not tested in this study) and these sites favor inactivation of VIM-12 in a noncompetitive mode, something that has never been reported before for VIM-type lactamases. If we assume VIM-12 is a rather new entry in the expanding family of MBLs, then this enzymatic behavior appears to be quite reasonable, from an evolutionary point of view. Nevertheless, the fact that the *K. pneumoniae* clinical isolate that encompasses this enzyme with limited ability to hydrolyze lactam antibiotics exhibits at the same time remarkable antibiotic resistance points toward the possibility that this organism possibly possesses alternative mechanisms against antibiotic action (such as a different porin profile, different penicillin binding proteins, or different antibiotic efflux pumps) that require further investigation.

The individual kinetic parameters of VIM-12 are listed in Table 1. Aztreonam was not hydrolyzed by VIM-12, a fact consistent with the behavior of all VIM-type metallo-β-lactamases. In addition, we could not observe any detectable hydrolysis of caftazidime, cefotaxime, and cefotoxin either in vitro or in vivo. Finally, only slow hydrolysis of imipenem was detected, but not of meropenem. The very low imipenem hydrolytic efficiency of VIM-12 compared with those of other VIM-type lactamases resulted from a combination of a relatively higher K_m value with an extremely low turnover rate (Table 1).

The competition experiments that were conducted to clarify the mode of recognition of these β-lactam compounds from the VIM-12 enzyme showed that quite low K_i values were determined with carbapenems, especially in the case of meropenem, despite the lack of its hydrolysis. The detailed kinetic analysis showed that imipenem acts as competitive inhibitor, as expected, with respect to the fact that imipenem represents a true substrate of VIM-12 and not a dead-end competitive inhibitor. It also important to mention that the kinetic analysis presented in this study is supportive of the observation that this competitive inhibition comes as a result of displacement of penicillin from the active site of VIM-12, rather than a competitive binding from the accumulation of hydrolysis product. Although this latter effect cannot be entirely excluded, all the competition experiments that we performed point toward the first possibility.

On the other hand, meropenem cannot be hydrolyzed but can bind the enzyme acting as a noncompetitive inhibitor. This observation is indicative of the possible presence in the enzyme structure of an allosteric site that facilitates the binding of this antibiotic. Interestingly, the decrease in susceptibility to carbapenems conferred by VIM-12 in *K. pneumoniae* and its transformant, although not very high, is significant, strongly supporting as we mentioned above the possibility that additional mechanisms are responsible for this resistance. This finding is also supported by the very low meropenem MIC (0.25 μg/mL) of the bla_{VIM-12}-bearing clinical isolate *Escherichia coli* 28 (Table 2). Nevertheless, in view of the kinetic data shown, the inhibitory effect of carbapenems on this enzyme might play a role in determining in the future antibiotic combinations capable of synergistically improving the treatment of nosocomial infections.

From the results of this study, we conclude that VIM-12 is the first of a new subgroup of class B1 metallo-β-lactamases to emerge in *K. pneumoniae*. Its biochemical properties further underscore the notion that considerable functional and structural diversity can be encountered among class B β-lactamases. Additional investigation of enzymes of this class, which are becoming increasingly clinically important, will contribute to the understanding of the mechanistic properties which remain poorly understood.

ACKNOWLEDGMENT

We thank Dr. Leonidas S. Tzouvelekis for helpful discussions and Stamatina Giannouli and Zoi Karoulia for suggestions, technical help, and encouragement.

SUPPORTING INFORMATION AVAILABLE

Optimization of VIM-12 activity assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Poirel, L., and Nordmann, P. (2002) Acquired carbapenem-hydrolyzing β-lactamases and their genetic support, *Curr. Pharm. Biotechnol. 3*, 117−127.
2. Ambler, R. P. (1980) The structure of β-lactamases, *Philos. Trans. R. Soc. London, Ser. B 289*, 321−331.
3. Fisher, J. F., Meroueh, S. O., and Mobashery, S. (2005) Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity, *Chem. Rev. 105*, 395−424.
4. Carfi, A., Pares, S., Duee, E., Galleni, M., Duez, C., Frere, J. M., and Dideberg, O. (1995) The 3-D structure of a zinc metallo-β-lactamase from *Bacillus cereus* reveals a new type of protein fold, *EMBO J. 14*, 4914−4921.
5. Fabiane, S. M., Sohi, M. K., Wan, T., Payne, D. J., Bateson, J. H., Mitchell, T., and Sutton, B. J. (1998) Crystal structure of the zinc-dependent β-lactamase from *Bacillus cereus* at 1.9 Å resolution: Binuclear active site with features of a mononuclear enzyme, *Biochemistry 37*, 12404−12411.
6. Concha, N. O., Janson, C. A., Rowling, P., Pearson, S., Cheever, C. A., Clarke, B. P., Lewis, C., Galleni, M., Frere, J. M., Payne, D. J., Bateson, J. H., and Abdel-Meguid, S. S. (2000) Crystal structure of the IMP-1 metallo β-lactamase from *Pseudomonas aeruginosa* and its complex with a mercaptocarboxylate inhibitor: Binding determinants of a potent, broad-spectrum inhibitor, *Biochemistry 39*, 4288−4298.
7. Fast, W., Wang, Z., and Benkovic, S. J. (2001) Familial mutations and zinc stoichiometry determine the rate-limiting step of nitrocefin hydrolysis by metallo-β-lactamase from *Bacteroides fragilis*, *Biochemistry 40*, 1640−1650.
8. Cameron, A. D., Ridderstrom, M., Olin, B., and Mannervik, B. (1999) Crystal structure of human glyoxalase II and its complex with a glutathione thiolester substrate analogue, *Structure 7*, 1067−1078.
9. Frazão, C., Silva, G., Gomes, C. M., Matias, P., Coelho, R., Sieker, L., Macedo, S., Liu, M. Y., Oliveira, S., Teixeira, M., Xavier, A. V., Rodrigues-Pousada, C., Carrondo, M. A., and Le Gall, J. (2000) Structure of a dioxygen reduction enzyme from *Desulfovibrio gigas*, *Nat. Struct. Biol. 7*, 1041−1045.
10. Gomes, C. M., Frazao, C., Xavier, A. V., Legall, J., and Teixeira, M. (2002) Functional control of the binuclear metal site in the metallo-β-lactamase-like fold by subtle amino acid replacements, *Protein Sci. 11*, 707−712.
11. Schilling, O., Wenzel, N., Naylor, M., Vogel, A., Crowder, M., Makaroff, C., and Meyer-Klaucke, W. (2003) Flexible metal binding of the metallo-β-lactamase domain: Glyoxalase II incorporates iron, manganese, and zinc in vivo, *Biochemistry 42*, 11777−11786.
12. Spencer, J., Clarke, A. R., and Walsh, T. R. (2001) Novel mechanism of hydrolysis of therapeutic β-lactams by *Stenotrophomonas maltophilia* L1 metallo-β-lactamase, *J. Biol. Chem. 276*, 33638−33644.

13. Garau, G., Bebrone, C., Anne, C., Galleni, M., Frere, J. M., and Dideberg, O. (2005) A metallo-β-lactamase enzyme in action: Crystal structures of the monozinc carbapenemase CphA and its complex with biapenem, *J. Mol. Biol. 345*, 785−795.
14. Wommer, S., Rival, S., Heinz, U., Galleni, M., Frere, J. M., Franceschini, N., Amicosante, G., Rasmussen, B., Bauer, R., and Adolph, H. W. (2002) Substrate-activated zinc binding of metallo-β-lactamases: Physiological importance of mononuclear enzymes, *J. Biol. Chem. 277*, 24142−24147.
15. Matagne, A., Lamotte-Brasseur, J., and Frere, J. M. (1998) Catalytic properties of class A β-lactamases: Efficiency and diversity, *Biochem. J. 330*, 581−598.
16. Rasmussen, B. A., and Bush, K. (1997) Carbapenem-hydrolyzing β-lactamases, *Antimicrob. Agents Chemother. 41*, 223−232.
17. Hall, B. G., Salipante, S. J., and Barlow, M. (2003) The metallo-β-lactamases fall into two distinct phylogenetic groups, *J. Mol. Evol. 57*, 249−254.
18. Hall, B. G., Salipante, S. J., and Barlow, M. (2004) Independent origins of subgroup Bl + B2 and subgroup B3 metallo-β-lactamases, *J. Mol. Evol. 59*, 133−141.
19. Osano, E., Arakawa, Y., Wacharotayankun, R., Ohta, M., Horii, T., Ito, H., Yoshimura, F., and Kato, N. (1994) Molecular characterization of an enterobacterial metallo β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance, *Antimicrob. Agents Chemother. 38*, 71−78.
20. Franceschini, N., Caravelli, B., Docquier, J. D., Galleni, M., Frere, J. M., Amicosante, G., and Rossolini, G. M. (2000) Purification and biochemical characterization of the VIM-1 metallo-β-lactamase, *Antimicrob. Agents Chemother. 44*, 3003−3007.
21. Castanheira, M., Toleman, M. A., Jones, R. N., Schmidt, F. J., and Walsh, T. R. (2004) Molecular characterization of a β-lactamase gene, blaGIM-1, encoding a new subclass of metallo-β-lactamase, *Antimicrob. Agents Chemother. 48*, 4654−4661.
22. Murphy, T. A., Simm, A. M., Toleman, M. A., Jones, R. N., and Walsh, T. R. (2003) Biochemical characterization of the acquired metallo-β-lactamase SPM-1 from *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother. 47*, 582−587.
23. Livermore, D. M., and Woodford, N. (2000) Carbapenemases: A problem in waiting? *Curr. Opin. Microbiol. 3*, 489−495.
24. Walsh, T. R., Toleman, M. A., Poirel, L., and Nordmann, P. (2005) Metallo-β-lactamases: The quiet before the storm? *Clin. Microbiol. Rev. 18*, 306−325.
25. Lauretti, L., Riccio, M. L., Mazzariol, A., Cornaglia, G., Amicosante, G., Fontana, R., and Rossolini, G. M. (1999) Cloning and characterization of blaVIM, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate, *Antimicrob. Agents Chemother. 43*, 1584−1590.
26. Poirel, L., Naas, T., Nicolas, D., Collet, L., Bellais, S., Cavallo, J. D., and Nordmann, P. (2000) Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France, *Antimicrob. Agents Chemother. 44*, 891−897.
27. Toleman, M. A., Rolston, K., Jones, R. N., and Walsh, T. R. (2004) blaVIM-7, an evolutionarily distinct metallo-β-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States, *Antimicrob. Agents Chemother. 48*, 329−332.
28. Pournaras, S., Tsakris, A., Maniati, M., Tzouvelekis, L. S., and Maniatis, A. N. (2002) Novel variant (bla(VIM-4)) of the metallo-β-lactamase gene bla(VIM-1) in a clinical strain of *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother. 46*, 4026−4028.
29. Bahar, G., Mazzariol, A., Koncan, R., Mert, A., Fontana, R., Rossolini, G. M., and Cornaglia. G. (2004) Detection of VIM-5 metallo-β-lactamase in a *Pseudomonas aeruginosa* clinical isolate from Turkey, *J. Antimicrob. Chemother. 54*, 282−283.
30. Pasteran, F., Faccone, D., Petroni, A., Rapoport, M., Galas, M., Vazquez, M., and Procopio, A. (2005) Novel variant (bla(VIM-11)) of the metallo-β-lactamase bla(VIM) family in a GES-1 extended-spectrum-β-lactamase-producing *Pseudomonas aeruginosa* clinical isolate in Argentina, *Antimicrob. Agents Chemother. 49*, 474−475.
31. Yan, J. J., Hsueh, P. R., Ko, W. C., Luh, K. T., Tsai, S. H., Wu, H. M., and Wu, J. J. (2001) Metallo-β-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.
32. Koh, T. H., Wang, G. C., and Sng, L. H. (2004) IMP-1 and a novel metallo-β-lactamase, VIM-6, in fluorescent pseudomonads isolated in Singapore, *Antimicrob. Agents Chemother. 48*, 2334−2336.
33. Crespo, M. P., Woodford, N., Sinclair, A., Kaufmann, M. E., Turton, J., Glover, J., Velez, J. D., Castaneda, C. R., Recalde, M., and Livermore, D. M. (2004) Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing VIM-8, a novel metallo-β-lactamase, in a tertiary care center in Cali, Colombia, *J. Clin. Microbiol. 42*, 5094−5101.
34. Walsh, T. R. (2005) The emergence and implications of metallo-β-lactamases in Gram-negative bacteria, *Clin. Microbiol. Infect. 11*, 2−9.
35. Giakkoupi, P. A., Xanthaki, M., Kanelopoulou, A., Vlahaki, V., Miriagou, S., Kontou, E., Papafraggas, H., Malamou-Lada, H., Tzouvelekis, L. S., Legakis, N. J., and Vatopoulos, A. C. (2003) VIM-1 metallo-β-lactamase-producing *Klebsiella pneumoniae* strains in Greek hospitals, *J. Clin. Microbiol. 41*, 3893−3896.
36. Jeong, S. H., Lee, K., Chong, Y., Yum, J. H., Lee, S. H., Choi, H. J., Kim, J. M., Park, K. H., Han, B. H., Lee, S. W., and Jeong, T. S. (2003) Characterization of a new integron containing VIM-2, a metallo-β-lactamase gene cassette, in a clinical isolate of *Enterobacter cloacae*, *J. Antimicrob. Chemother. 51*, 397−400.
37. Miriagou, V., Tzelepi, E., Gianneli, D., and Tzouvelekis, L. S. (2003) *Escherichia coli* with a self-transferable, multiresistant plasmid coding for metallo-β-lactamase VIM-1, *Antimicrob. Agents Chemother. 47*, 395−397.
38. Tsakris, A. S., Pournaras, N., Woodford, M.-F., Palepou, I., Babini, G. S., Douboyas, J., and Livermore, D. M. (2000) Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece, *J. Clin. Microbiol. 38*, 1290−1292.
39. Lee, K., Ha, G. Y., Shin, B.-M., Kim, J. J., Kang, J. O., Jang, S. J., Yong, D., and Chong, Y. [The Korean Nationwide Surveillance of Antimicrobial Resistance (KONSAR) group] (2004) Metallo-β-lactamase-producing gram-negative bacilli in Korean nationwide surveillance of antimicrobial resistance group hospitals in 2003: Continued prevalence of VIM-producing *Pseudomonas* spp. and increase of IMP-producing *Acinetobacter* spp., *Diagn. Microbiol. Infect. Dis. 50*, 51−58.
40. Nordmann, P., and Poirel, L. (2002) Emerging carbapenemases in gram-negative aerobes, *Clin. Microbiol. Infect. 8*, 321−331.
41. Pournaras, S., Maniati, M., Petinaki, E., Tzouvelekis, L. S., Tsakris, A., Legakis, N. J., and Maniatis, A. N. (2003) Hospital outbreak of multiple clones of *Pseudomonas aeruginosa* carrying the unrelated metallo-β-lactamase gene variants blaVIM-2 and blaVIM-4, *J. Antimicrob. Chemother. 51*, 1409−1414.
42. Docquier, J. D., Lamotte-Brasseur, J., Galleni, M., Amicosante, G., Frere, J. M., and Rossolini, G. M. (2003) On functional and structural heterogeneity of VIM-type metallo-β-lactamases, *J. Antimicrob. Chemother. 51*, 257−266.
43. Pournaras, S., Ikonomidis, A., Tzouvelekis, L. S., Tokatlidou, D., Spanakis, N., Maniatis, A. N., Legakis, N. J., and Tsakris, A. (2005) VIM-12, a novel plasmid-mediated metallo-β-lactamase from *Klebsiella pneumoniae* that resembles a VIM-1/VIM-2 hybrid, *Antimicrob. Agents Chemother. 49*, 5153−5156.
44. Ikonomidis A., Labrou, M., Afkou, Z., Maniatis, A. N., Sofianou, D., Tsakris, A., and Pournaras, S. (2007) First occurrence of an *Escherichia coli* clinical isolate producing the VIM-1/VIM-2 hybrid metallo-β-lactamase, VIM-12, *Antimicrob. Agents Chemother.* (in press).
45. Leatherbarrow, R. J. (2001) *GraFit*, version 5, Erithacus Software Ltd., Horley, U.K.
46. Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O., and Frere, J. M. (2001) Metallo-β-lactamases Working Group. Standard numbering scheme for class B β-lactamases, *Antimicrob. Agents Chemother. 45*, 660−663.
47. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res. 22*, 4673−4680.
48. Murphy, T. A., Catto, L. E., Halford, S. E., Hadfield, A. T., Minor, V., Walsh, T. R., and Spencer, J. (2006) Crystal structure of *Pseudomonas aeruginosa* SPM-1 provides insights into variable zinc affinity of metallo-β-lactamases, *J. Mol. Biol. 357*, 890−903.
49. Garau, G., Garcia-Saez, I., Bebrone, C., Anne, C., Mercuri, P., Galleni, M., Frere, J. M., and Dideberg, O. (2004) Update of the standard numbering scheme for class B β-lactamases, *Antimicrob. Agents Chemother. 48*, 2347−2349.

BI701258W