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Biochemical Characterization of Metallo-β-Lactamase VIM-11 from a *Pseudomonas aeruginosa* Clinical Strain[▽][†]

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**A detailed biochemical characterization of the *Pseudomonas aeruginosa* VIM-11 metallo-β-lactamase (MβL)
is reported. The only substitution differentiating VIM-11 from VIM-2 (N165S) promoted a slightly improved
catalytic efficiency of the former on 3 out of 12 substrates, notably the bulky cephalosporins. Thus, MβL-
mediated resistance also may be modulated by remote mutations.**

Acquired metallo-β-lactamases (MβLs) are emerging resistance determinants in numerous bacterial species of clinical relevance (3, 17). These enzymes pose particular threats, including their capacity to confer broad-spectrum β-lactam resistance and the unavailability of clinically useful inhibitors. In addition, their potential for rapid and generalized dissemination represents a great concern (2, 17). In recent years, two major groups of MβLs, corresponding to IMP and VIM types, increasingly have been identified in gram-negative pathogens (2). Recently, a novel variant of the VIM family encoded by bla_{VIM-11} was reported from a *Pseudomonas aeruginosa* clinical strain that differs from the bla_{VIM-2} sequence in a unique nonsynonymous mutation (N165S) (13). Despite their clinical relevance, studies addressing the impact of bla_{VIM} allelic mutations on kinetic performance still are scarce. We report here the cloning, expression, purification, and biochemical characterization of VIM-11 as well as a comparison of the properties of VIM-11 to those of VIM-2. We also studied the contribution of each of these enzymes to in vitro resistance to different β-lactams when produced in *Escherichia coli*.

A VIM-11-producing *P. aeruginosa* clinical strain, M5109, recovered from Hospital de Niños Ricardo Gutierrez, Buenos Aires, Argentina (13), and *P. aeruginosa* COL-1, a VIM-2-producing strain (14), were used as sources of genomic DNA for the isolation of the cognate genes. Bacterial DNA was obtained essentially as described previously (15).

The contribution of VIM-11 or VIM-2 to bacterial β-lactam resistance was investigated by testing the susceptibility of *E. coli* DH5α harboring plasmids directing the production of these enzymes to various β-lactams. For this purpose, we designed the primers CIM-F (5′-GCCGGATCCCATAGTTAA GTAGCACTCACC-3′) and CIM-R (5′-ATCGGATCCCTAC TCAACGACTGAGC-3′), both containing BamHI restriction sites, which allow the amplification of the complete VIM-2 or VIM-11 coding sequences, including the corresponding signal peptides. PCR products then were cloned into the corresponding sites of the broad-host-range expression plasmid pαΩ (5). As a control, we also employed *E. coli* DH5α cells carrying the empty vector. MICs were determined by the standard agar macrodilution method according to the guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS) (1). As shown in Table 1, the production of VIM-11 or VIM-2 in *E. coli* DH5α decreased the susceptibility of the host cells to all β-lactam substrates tested, with the expected exception of aztreonam (14). These results demonstrate that either enzyme can confer broad-spectrum β-lactam resistance to the bacterial host. Moreover, the inhibition of imipenem hydrolysis by EDTA using a microbiological assay confirmed the presence of MβL activity (9).

Primers VIM-C (5′-CTCGCTGGATCCGTAGATTCTAG CGGTGAG-3′) and VIM-N (5′-GCTCAGTCGTTGAGTAG AAGCTTGATGCG-3′) were designed after the corresponding VIM gene sequences (11, 13). These primers contain an additional BamHI and HindIII restriction site, respectively, and were used to amplify and clone the DNA regions encoding the corresponding mature VIM sequences into the corresponding sites of the pET-Term expression vector (12). The resulting pET-TermVIM-2 and pET-TermVIM-11 plasmids direct the production of the corresponding enzymes as C-terminal fusions to glutathione *S*-transferase (12). By using these plasmids, the corresponding fusion proteins were over-produced in *E. coli* BL21(DE3) pLysS and purified to homogeneity essentially as described previously (12), with the following modifications: (i) expression was induced at 20°C during 6 h, and (ii) the purified fusion proteins were dialyzed against 100 mM Tris-HCl (pH 8.0) to remove glutathione, digested with thrombin, and finally loaded onto a glutathione-agarose column (GE) equilibrated with 100 mM Tris-HCl (pH

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TABLE 1. MICs of β-lactams for *E. coli* DH5α harboring recombinant plasmids[a]

β-Lactam	MIC (μg/ml) for:		
	E. coli DH5α pαΩ	*E. coli* DH5α pαΩ *bla*VIM-11	*E. coli* DH5α pαΩ *bla*VIM-2
Benzylpenicillin	32	128	128
Ampicillin	8	64	256
Cephalothin	4	64	64
Cefuroxime	4	64	128
Cefoxitin	4	32	64
Ceftazidime	0.125	8	8
Cefotaxime	0.03	4	8
Cefepime	0.03	0.5	0.5
Cefpirome	0.015	0.5	0.5
Aztreonam	0.25	0.25	0.25
Imipenem	0.125	0.5	1
Meropenem	0.03	0.06	0.125

[a] *E. coli* DH5α pαΩ, *E. coli* DH5α containing pαΩ (as a control); *E. coli* DH5α pαΩ *bla*VIM-11 or *bla*VIM-2, *E. coli* DH5α harboring recombinant plasmid pαΩ *bla*VIM-11 or pαΩ *bla*VIM-2, respectively.

8.0) to separate the cleaved proteins from glutathione *S*-transferase. The eluted proteins were more than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown) and were used for the further comparative studies detailed below.

Metal content analysis of the purified proteins, as determined by using 4-(2-pyridylazo)-resorcinol (PAR) (16), revealed that both enzymes contain two bound Zn(II) ions.

To gain insight into the active sites of VIM-11 and VIM-2, we produced the corresponding dicobalt(II) derivatives as described elsewhere (12). Both Co(II)-substituted VIM-11 and VIM-2 displayed four ligand field bands in the visible region (see Fig. S1 in the supplemental material) that were similar to those reported for Co(II)-BcII from *Bacillus cereus* (12) and other MβLs (3). This shows that the Co1 sites are structurally similar among different Co(II) derivatives. On the contrary, a charge transfer band attributed to a Cys-Co(II) moiety, a probe of the Co2 site, is found on both VIM-11 and VIM-2 at 383 and 380 nm, respectively (see Fig. S1 in the supplemental material). A comparison of these spectral features with the

charge transfer band of Co(II)-substituted BcII (340 nm) reveals a slightly different Co2 site (8a, 12).

Kinetic parameters with several representative β-lactam compounds, including penicillins, cephalosporins, carbapenems, and aztreonam (Table 2), were determined as previously reported (10, 16). β-Lactamase activity was determined by monitoring the changes in absorbance in a Jasco V-550 spectrophotometer. $\Delta\varepsilon_{lmax}$ values for the different substrates were detailed previously (8, 10, 16). As seen in Table 2, all tested β-lactams were hydrolyzed by these two enzymes, with the exception of aztreonam. The highest catalytic efficiencies for both enzymes were observed for benzylpenicillin, cephalothin, cefuroxime, cefotaxime, imipenem, and nitrocefin, while cefepime was a rather poor substrate. Between the two carbapenem substrates tested, VIM-11 showed greater hydrolytic efficiency on imipenem (Table 2), a result also reported for VIM-2 by other authors (4). As seen in Table 2, VIM-11 displayed a slightly better catalytic efficiency on 3 of the 12 hydrolyzed substrates compared to that of VIM-2 when tested under similar experimental conditions. Remarkably, these substrates (i.e., ceftazidime, cefepime, and cefpirome) are cephalosporins with bulky substituents at the C-3 position. The differences for these substrates are reflected in fourfold (ceftazidime), threefold (cefepime), and twofold (cefpirome) increases in k_{cat}/K_m values of VIM-11 relative to those of VIM-2 (Table 2).

These minor differences in catalytic efficiency for the purified enzymes were not reflected in the MICs for ceftazidime, cefepime, or cefpirome measured for the *E. coli* host producing the corresponding recombinant enzymes (Table 1). This also could result from differential intrinsic properties between these enzymes, such as different levels of stability in vivo. In this context, the measurements of MβL-specific activity on periplasmic extracts (using a saturated concentration of substrates) indicated that *E. coli* cells producing VIM-11 contained severalfold lower levels of activity than those producing VIM-2 on all β-lactam substrates tested (e.g., 0.9 versus 5.5 μmol ceftazidime, 5.4 versus 141 μmol cephalothin, or 1.3 versus 27 μmol imipenem hydrolyzed/min/mg total periplasmic

TABLE 2. Kinetic parameters for the hydrolysis of different β-lactam substrates of VIM-11 and VIM-2 enzymes

β-Lactam	VIM-11[a]			VIM-2[a]			k_{cat}/K_m (s⁻¹ M⁻¹) for VIM-11/VIM-2 ratio
	K_m (μM)	k_{cat} (s⁻¹)	k_{cat}/K_m (s⁻¹ M⁻¹)	K_m (μM)	k_{cat} (s⁻¹)	k_{cat}/K_m (s⁻¹ M⁻¹)	
Benzylpenicillin	15.3	113	7.4×10^6	19.1	141	7.4×10^6	1.0
Ampicillin	109	190	1.8×10^6	136	302	2.2×10^6	0.81
Cephalothin	4.5	81	1.8×10^7	6.2	96.4	1.6×10^7	1.12
Cefuroxime	5.5	27	4.9×10^6	5.5	30.5	5.5×10^6	0.89
Cefoxitin	7.4	6.4	8.6×10^5	9.9	10	1.0×10^6	0.86
Ceftazidime	110	14	1.3×10^5	88.2	2.8	3.2×10^4	4.06
Cefotaxime	11.4	41.3	3.6×10^6	13.4	61	4.6×10^6	0.78
Cefepime	>1,200	>100	8.3×10^4	>1,200	>33	2.8×10^4	2.9
Cefpirome	183	102	5.6×10^5	171	48.1	2.8×10^5	2.0
Nitrocefin	8.2	129	1.6×10^7	13.8	206	1.5×10^7	1.06
Imipenem	9.4	20	2.1×10^6	9.9	30.2	3.1×10^6	0.67
Meropenem	22.7	3.2	1.4×10^5	24.5	3.5	1.4×10^5	1.0
Aztreonam	NH[b]			NH			

[a] The K_m and k_{cat} values represent the means of two independent measurements. Standard deviation values were less than 5%.
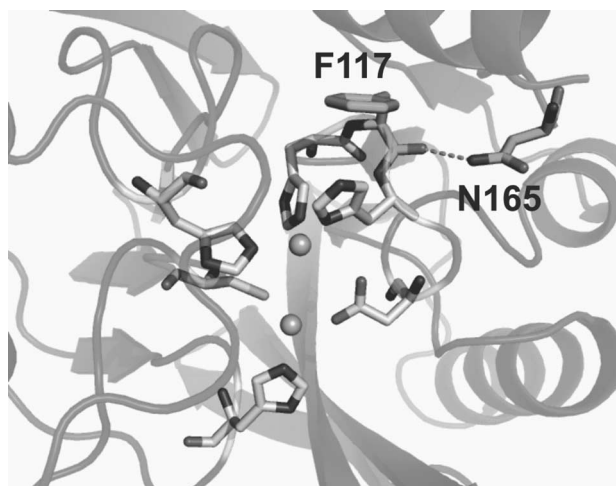[b] NH, no hydrolysis was detected.

FIG. 1. Upper view of the active site of VIM-2 (Protein Data Bank entry 1ko3) (6). The protein backbone is shown in the cartoons. Residues of the active site and residues F117 and N165 (involved in the mentioned H-bond interaction) are indicated by sticks. Zn(II) ions are shown as spheres, and the H bond is indicated with dashed lines.

protein for VIM-11- and VIM-2-producing bacteria, respectively).

The crystal structure of dizinc(II) VIM-2 (6) revealed that the Asn165 side chain forms an H bond with the peptide carbonyl of Phe117, flanked in the protein sequence by two Zn ligands (His116 and His118) (Fig. 1). The N165S mutation is expected to remove the interaction between loops L7 and L8, possibly enhancing loop flexibility and resulting in a relatively wider active-site groove, a situation that would better accommodate cephalosporins with bulkier substituents at C-3. In line with these observations, a number of pieces of evidence account for the impact of second-shell ligands in enzymatic activity (7, 16), suggesting that mutations outside the active site also contribute to tuning the MβL catalytic performance.

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