ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2008, p. 2977–2979 Vol. 52, No. 8
0066-4804/08/$08.00+0 doi:10.1128/AAC.00175-08

VIM-15 and VIM-16, Two New VIM-2-Like Metallo-β-Lactamases in *Pseudomonas aeruginosa* Isolates from Bulgaria and Germany▽

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Received 7 February 2008/Returned for modification 27 March 2008/Accepted 28 May 2008

Two *Pseudomonas aeruginosa* urine isolates from Bulgaria and Germany produced two new VIM-2 variants. VIM-15 had one amino acid substitution (Tyr218Phe) which caused a significant increase in hydrolytic efficiency. The substitution Ser54Leu, characterizing VIM-16, showed no influence on enzyme activity. Both genes were part of class I integrons located in the chromosome.

VIM-type β-lactamases are commonly acquired metallo-β-lactamases (MBLs) mostly found in *Pseudomonas aeruginosa* (11). They contribute significantly to the resistance of nonfermenting gram-negative organisms to carbapenems. Furthermore, as all bla_{VIM}s are part of integrons, their acquisition is often linked with resistance to other compounds, e.g., aminoglycosides.

We analyzed the MBLs of two *P. aeruginosa* strains that were isolated in Bulgaria and Germany.

(Part of this work was presented at the 17th European Congress of Clinical Microbiology and Infectious Diseases, Munich, Germany, 2007 [10].)

P. aeruginosa 166301 was isolated in April 2005 at the Medizinisches Versorgungszentrum, Munich, Germany, from the urine of a 76-year-old male patient. *P. aeruginosa* 9551 was recovered in March 2006 at the Medical Institute, Ministry of the Interior, Sofia, Bulgaria, from the urine of a 49-year-old male patient treated at the nephrology ambulatory clinic.

MICs, determined by the agar dilution technique following Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (6), are shown in Table 1. Both *P. aeruginosa* strains were resistant to carbapenems, suggesting the presence of carbapenemases. The results of a Hodge test and a double-disk synergy test with EDTA, carried out as described previously (5), demonstrated the production of carbapenem-hydrolyzing enzymes susceptible to EDTA inhibition, thereby confirming the presence of MBLs.

A PCR with bla_{VIM}-specific oligonucleotides (VIM-F, 5′-TTGGTCGCATATCGCAAC-3′, and VIM-R, 5′-CGCAGCACCRGGATAGAA-3′) was positive. We used combinations of VIM-F and VIM-R with oligonucleotides binding to conserved regions of class I integrons (qacEΔ1, 5′-GCCAACTATTGCGATAAC-3′, and IntIa-attI, 5′-TCTATGCCTCGGGCATCC-3′) to sequence the whole gene and its environment. The sequences revealed close homology to bla_{VIM-2}, although one nucleotide substitution causing an amino acid to differ from

TABLE 1. Antibiotic susceptibilities of wild-type and transformant strains

Antibiotics	MIC (μg/ml) for:					
	P. aeruginosa		*E. coli* DH5α			
	9551 (VIM-15)	166301 (VIM-16)	pBC-VIM-15	pBC-VIM-16	pBC-VIM-2	Host strain
Amoxicillin	512	>512	256	256	256	4
Piperacillin-tazobactam[a]	128	128	8	8	4	1
Ceftazidime	64	64	0.5	0.5	1	0.13
Cefotaxime	>256	>256	4	2	1	0.03
Cefepime	32	32	0.03	0.03	0.03	0.016
Aztreonam	16	32	0.016	0.016	0.016	0.016
Meropenem	128	>128	0.03	0.03	0.03	0.016
Imipenem	>128	>128	0.5	0.25	0.25	0.25
Gentamicin	0.5	>128	0.13	0.13	0.13	0.13
Tobramycin	8	>128	0.25	0.25	0.25	0.25

[a] Tazobactam was used at a fixed concentration of 4 μg/ml.

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▽ Published ahead of print on 2 June 2008.

that in VIM-2 was found for both strains. The nucleotide substitution A584T leading to a tyrosine 218 phenylalanine substitution characterized the VIM enzyme of *P. aeruginosa* 9551 (MBL numbering according to Garau et al. [2]). At that position, all VIM-type MBLs described so far have a tyrosine, except for VIM-7, which also shows a phenylalanine but shares only 74% amino acid identity with VIM-2. The bla_{VIM} of *P. aeruginosa* 166301 had one nucleotide substitution (C164T), causing a serine 54 leucine substitution. At that position, all other VIM variants carry a serine. These new VIM-type MBLs were named VIM-15 (*P. aeruginosa* 9551) and VIM-16 (*P. aeruginosa* 166301).

To explore whether the amino acid substitutions influence hydrolytic activity, we cloned the bla_{VIM} genes of *P. aeruginosa* strain 9551, strain 166301, and the VIM-2-producing reference strain *P. aeruginosa* 98/10/U1315 in an isogenic background. Cloning was performed as described previously using the vector pBC and *Escherichia coli* DH5α (9). Primers VIM-2-EcoRI-V (5′-AGGAATTCCTAGTGCCGCACTCACC-3′) and VIM-2-BamHI-R (5′-CAGGATCCTTCATGTTATGCCG-3′) were used to amplify the gene, including 27 bp of the upstream and 18 bp of the downstream region. The expression of the bla_{VIM} genes in the transformants led to significant increases in the MICs for amoxicillin (64-fold) and cefotaxime (32- to 128-fold), and to moderate increases in the MICs for piperacillin-tazobactam and ceftazidime (4- to 8-fold) (Table 1). The MICs of cefepime (2-fold increase), meropenem (2-fold increase), imipenem (1- to 2-fold increase), and aztreonam were not or only slightly affected. The absence of the original promoter, which was removed during cloning, caused MICs for ceftazidime that were lower than values found in the literature (7). Interestingly, the production of VIM-15 caused slightly higher MICs for cefotaxime and imipenem than the production of VIM-2 or VIM-16. Increased activity of VIM-15 in comparison to the activity of VIM-2 and VIM-16 against cefotaxime, meropenem, and imipenem could be seen by the results of a Hodge test using crude strain homogenates (data not shown).

The purification of β-lactamases from transformants producing VIM-15 and VIM-16 and the determination of kinetic parameters were carried out as described previously (8). Overnight cultures were centrifuged, washed with phosphate buffer, and subjected to five freeze-thaw cycles. Following centrifugation, supernatants were filtered and passed through a Superdex 100 gel filtration column. Active fractions were further purified by using HiTrap SP cation and Q anion exchange columns. The data for VIM-2, previously obtained using the same procedure, were taken from Queenan et al. (8).

Both VIM-15 and VIM-16 showed a higher turnover rate (k_{cat}) for cefotaxime than for ceftazidime and cefepime, resulting in higher hydrolytic efficiencies (k_{cat}/K_m) for cefotaxime (Table 2). All three enzymes hydrolyzed imipenem faster than meropenem and, as is typical for MBLs, aztreonam was hydrolyzed only very slowly.

VIM-16 presented k_{cat} and K_m values very similar to those for VIM-2, resulting in similar hydrolytic efficiencies. The amino acid substitution from serine to leucine at position 54 is located on the second β-strand of the enzyme (3). Although this is a nonconservative change, because of its remote position

TABLE 2. Kinetic parameters of VIM-15, VIM-16, and VIM-2[a]

Substrate	VIM-15					VIM-16					VIM-2				
	k_{cat} (s^{-1})	Relative[b] k_{cat}	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	Relative[b] k_{cat}/K_m	k_{cat} (s^{-1})	Relative[b] k_{cat}	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	Relative[b] k_{cat}/K_m	k_{cat} (s^{-1})	Relative[b] k_{cat}	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	Relative[b] k_{cat}/K_m
Cephaloridine	190 ± 4	100	83 ± 2	2.3	100	89 ± 2	100	1400 ± 53	0.064	100	120 ± 5	100	1400 ± 6	0.086	100
Benzylpenicillin	240 ± 3	130	25 ± 0.5	9.6	420	230 ± 24	260	450 ± 8	0.51	800	73 ± 0.4	61	150 ± 1	0.49	570
Cefepime	9.5 ± 0.1	5.0	130 ± 2	0.076	3.3	0.31 ± 0.03	0.35	210 ± 1	0.0015	2.3	0.38 ± 0.02	0.32	110 ± 6	0.0035	4.1
Ceftazidime	1.0 ± 0.04	0.53	37 ± 2	0.027	1.2	0.22 ± 0.00	0.25	150 ± 2	0.0014	2.2	0.23 ± 0.01	0.19	150 ± 6	0.0015	1.7
Cefotaxime	90 ± 7	47	13 ± 1	6.9	300	81 ± 0.2	91	240 ± 9	0.34	530	NT	NT	NT	NT	NT
Imipenem	61 ± 2	32	7.3 ± 0.5	8.4	370	45 ± 2	51	89 ± 2	0.51	800	20 ± 0.3	17	60 ± 2	0.33	380
Meropenem	6.5 ± 0.05	3.4	3.4 ± 0.5	1.9	83	8.4 ± 0.3	9.4	120 ± 5	0.070	110	2.1 ± 0.1	1.8	40 ± 2	0.053	62
Aztreonam[c]	≤0.031	≤0.016	ND	ND	ND	≤0.11	≤0.12	ND	ND	ND	NT	NT	NT	NT	NT

[a] NT, not tested; ND, not determined as hydrolysis was too slow to determine K_m.
[b] The cephaloridine value was taken as 100%.
[c] Hydrolysis of aztreonam was very slow; V_{max} was estimated as 2 times the maximum hydrolysis rate observed.

in relation to the active site, no influence on the hydrolytic activity is expected.

VIM-15 showed hydrolytic efficiencies about 1 order of magnitude higher than those of VIM-2 and VIM-16 for all substrates tested. This is caused by lower K_m values for cephaloridine, cefotaxime, imipenem, and meropenem, indicating a stronger affinity of VIM-15 for those substrates. In contrast, the increased activity for cefepime was caused by an elevated turnover rate. The higher efficiencies for ceftazidime and benzylpenicillin were a result of both increased turnover rate and decreased K_m values. The change from tyrosine to phenylalanine at position 218 is located on β-strand 11 (3) and has a conservative character. However, this substitution is near the cysteine zinc binding site (Cys221) and apparently affected the binding properties or catalytic activity of VIM-15.

Both $bla_{\text{VIM-15}}$ and $bla_{\text{VIM-16}}$ were part of class I integrons. The integron of *P. aeruginosa* 9551 harbored the $bla_{\text{VIM-15}}$ cassette only. This structure is identical to that of the VIM-2-producing *P. aeruginosa* COL-1 isolated in 1996 in France (7). The $bla_{\text{VIM-16}}$ cassette of *P. aeruginosa* 166301 was flanked by two *aac(6′)-Ib′* cassettes coding for an aminoglycoside acetyltransferase. This structure is identical to that of the VIM-2-producing *P. aeruginosa* B63230 isolated in 2003 in Germany, except that the integron of *P. aeruginosa* B63230 additionally harbored *cmlA* and *ant(3″)-Ib* cassettes (4). For *P. aeruginosa* 9551, the absence of cassettes coding for aminoglycoside-modifying enzymes is in accordance with the susceptibility to aminoglycosides, while *P. aeruginosa* 166301, harboring two genes of an aminoglycoside acetyltransferase, was highly resistant to aminoglycosides (Table 1).

To test whether the bla_{VIM}-containing integrons reside on plasmids, we tried transferring them by conjugation to *E. coli* C600 R^- (1) and by electroporation to *E. coli* DH5α. Both attempts failed. The bla_{VIM} localization was further analyzed by performing Southern blotting of plasmid preparations (Qiagen plasmid midi kit; Qiagen, Hilden, Germany) followed by hybridization with probes amplified from bla_{VIM} (VIM-F and VIM-2-BamHI-R) and 16S rRNA (616V, 5′-AGAGTTTGATCMKGGCTCAG-3′, and 610R, 5′-CAGGATCCTTCATGTTATGCCG-3′) using a Gene Images AlkPhos direct labeling and detection system (GE Healthcare, Little Chalfont, United Kingdom). In addition, we treated plasmid DNA preparations with Plasmid-safe (Epicenter Biotechnologies, Madison, WI), a DNase which selectively digests linear DNA, but not circular DNA. For both *P. aeruginosa* strains, a DNA band which was recognized by the VIM probe as well as by the 16S rRNA probe and which was affected by treatment with Plasmid-safe was found. Assuming that those bands are chromosomal DNA, the bla_{VIM} genes appear to be chromosomally located.

In conclusion, the isolation of two new VIM-type MBLs in Bulgaria and Germany highlights the ongoing spread and evolution of this group of β-lactamases. VIM-type MBLs have already been described in Germany (4, 12), although no report on VIM MBLs in Bulgaria was found. While the Ser54Leu substitution has no influence on hydrolytic activity, the amino acid change from tyrosine to phenylalanine at position 218 enhances enzymatic activity.

Nucleotide sequence accession numbers. The nucleotide sequences of $bla_{\text{VIM-15}}$ and $bla_{\text{VIM-16}}$ have been deposited in the GenBank database under accession numbers EU419745 and EU419746.

We thank Wenchi Shang, Johnson & Johnson Pharmaceutical Research and Development, Raritan, NJ, for purification of the VIM β-lactamases and Yunsop Chong and Kyungwon Lee, Yonsei University College of Medicine, Seoul, Korea, for providing us with the VIM-2 reference strain *P. aeruginosa* 98/10/U1315.

REFERENCES

1. **Bauernfeind, A., S. Wagner, R. Jungwirth, I. Schneider, and D. Meyer.** 1997. A novel class C β-lactamase (FOX-2) in *Escherichia coli* conferring resistance to cephamycins. Antimicrob. Agents Chemother. **41:**2041–2046.
2. **Garau, G., I. García-Sáez, C. Bebrone, C. Anne, P. Mercuri, M. Galleni, J. M. Frère, and O. Dideberg.** 2004. Update of the standard numbering scheme for class B β-lactamases. Antimicrob. Agents Chemother. **48:**2347–2349.
3. **Garcia-Saez, I., J. D. Docquier, G. M. Rossolini, and O. Dideberg.** 2008. The three-dimensional structure of VIM-2, a Zn-β-lactamase from *Pseudomonas aeruginosa* in its reduced and oxidised form. J. Mol. Biol. **375:**604–611.
4. **Henrichfreise, B., I. Wiegand, K. J. Sherwood, and B. Wiedemann.** 2005. Detection of VIM-2 metallo-β-lactamase in *Pseudomonas aeruginosa* from Germany. Antimicrob. Agents Chemother. **49:**1668–1669.
5. **Lee, K., Y. Chong, H. B. Shin, Y. A. Kim, D. Yong, and J. H. Yum.** 2001. Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Clin. Microbiol. Infect. **7:**88–91.
6. **National Center for Clinical and Laboratory Standards.** 2002. Performance standards for antimicrobial susceptibility testing, 12th informational supplement. M100-S12. National Center for Clinical and Laboratory Standards, Wayne, PA.
7. **Poirel, L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J. D. Cavallo, and P. Nordmann.** 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.
8. **Queenan, A. M., W. Shang, M. Kania, M. G. P. Page, and K. Bush.** 2007. Interactions of ceftobiprole with β-lactamases from molecular classes A to D. Antimicrob. Agents Chemother. **51:**3089–3095.
9. **Schneider, I., R. Markovska, E. Keuleyan, M. Sredkova, K. Rachkova, I. Mitov, and A. Bauernfeind.** 2007. Dissemination and persistence of a plasmid-mediated TEM-3-like β-lactamase, TEM-139, among *Enterobacteriaceae* in Bulgaria. Int. J. Antimicrob. Agents **29:**710–714.
10. **Schneider, I., E. Keuleyan, R. Rasshofer, and A. Bauernfeind.** 2007. New VIM-2-like metallo-beta-lactamases in *P. aeruginosa* from Germany and Bulgaria. Clin. Microbiol. Infect. **13**(Suppl. 1)**:**S107.
11. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
12. **Weile, J., H. Rahmig, S. Gfröer, K. Schroeppel, C. Knabbe, and M. Susa.** 2007. First detection of a VIM-1 metallo-β-lactamase in a carbapenem-resistant *Citrobacter freundii* clinical isolate in an acute hospital in Germany. Scand. J. Infect. Dis. **39:**264–266.