ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2002, p. 2014–2016 Vol. 46, No. 6
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.6.2014–2016.2002

Detection of a Variant Metallo-β-Lactamase, IMP-10, from Two Unrelated Strains of *Pseudomonas aeruginosa* and an *Alcaligenes xylosoxidans* Strain

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Received 21 June 2001/Returned for modification 5 October 2001/Accepted 16 March 2002

The gene bla_{IMP-10} of a variant metallo-β-lactamase, IMP-10, had a single base replacement of G by T at nucleotide 145, which led to an amino acid alteration of Val49 to Phe compared to the IMP-1 enzyme, indicating that IMP-10 was a point mutation derivative of IMP-1. Highly purified enzymes revealed that IMP-10 was different from IMP-1 in its extremely low hydrolyzing activities for penicillins, such as benzylpenicillin, ampicillin, and piperacillin.

The metallo-β-lactamase IMP-1, which belongs to molecular class B (2), is clinically important because of its broad substrate specificity and its efficient hydrolysis of almost all of the β-lactam antibiotics, such as cephems, penicillins, and especially carbapenems (15). Since the IMP-1-encoding gene, bla_{IMP-1}, was cloned and sequenced, bla_{IMP-1} or bla_{IMP-1}-like genes have been detected with imipenem-resistant bacteria of clinical origin by the PCR method with primers constructed for the bla_{IMP-1} sequence (12, 19). Furthermore, sequence analysis of the bla_{IMP-1}-like genes has so far discerned nine kinds of variant IMP-type β-lactamases (3, 6, 8, 17, 23, 24) (GenBank accession numbers AF290912, AF318077, AY033653, and AB074436). Among IMP-type enzymes, kinetic parameters were reported for five enzymes, IMP-1 (8, 11, 13, 14, 22, 24), IMP-2 (17), IMP-3 (8), IMP-4 (3), and IMP-6 (24). Carbapenems and cephems are good substrates for these enzymes, and penicillin-hydrolyzing activities were obviously low in IMP-3 and IMP-6 compared to the other enzymes. The number of amino acid substitutions in IMP-2, IMP-4, IMP-3, and IMP-6 compared to those in IMP-1 were 36, 10, 2, and 1, respectively. Variant β-lactamases, such as IMP-3 and IMP-6, were detectable by estimating the relative activities of enzymes obtained from PCR-positive strains, by hydrolyzing the substrates cephaloridine, benzylpenicillin, and imipenem.

We examined β-lactamase activities by the UV spectrophotometric method (22) using crude cell extracts from PCR-positive strains of 44 *Pseudomonas aeruginosa* and 2 *Alcaligenes xylosoxidans* isolates, which were collected in Gunma University Hospital, Maebashi, Japan, over the past 7 years with no duplication among the patients. Among these, we found one *A. xylosoxidans* strain, isolated in March 2000 from a urine specimen, and two *P. aeruginosa* strains, isolated in February 1997 from a stool specimen and in August 1998 from a urine specimen, whose benzylpenicillin-hydrolyzing activity was scarcely detectable.

None of the imipenem-resistant genes was transferred by conjugation from these three strains to a *P. aeruginosa* recipient, ML5017, as seen in the gene bla_{IMP-1} reported previously (22). Plasmid DNA was prepared from each of the strains by the alkaline-sodium dodecyl sulfate method and analyzed by agarose gel electrophoresis using size marker plasmids of 8.7 (RSF1010), 35 (pSa), 56 (RP4), and 105 (R100-1) kb (1). Plasmid DNAs of different sizes were revealed from each strain: a ca. 56-kb plasmid from *P. aeruginosa* strain PAI134, ca. 135- and ca. 6.6-kb plasmids from *P. aeruginosa* strain PAI97, and a ca. 27-kb plasmid from *A. xylosoxidans* strain AXI2. Southern hybridization was carried out with these DNA preparations using the PCR-amplified 587-bp segment of bla_{IMP-1} as the probe, which was labeled with digoxigenin-11-dUTP. Hybridization was positive with the plasmid DNA of PAI134 but negative with the plasmid DNAs of PAI97 and AXI2, for which a trace of chromosomal DNA contained in the preparation was hybridized instead (data not shown).

The PAI134 plasmid was introduced by transformation into a laboratory strain of *P. aeruginosa* ML5017 by selection with 8-μg/ml ceftazidime. The 56-kb plasmid, named pMS455, was isolated from the transformant and digested with the restriction enzyme *Bam*HI. Next, the 4.5-kb *Bam*HI segment was cloned in an *Escherichia coli* strain, K-12 JM83, using the multicopy vector plasmid pHSG399 (21), constructing the recombinant plasmid pMS600. The nucleotide sequence of a ca. 1,100-bp region including the predicted bla_{IMP-1} gene was determined by the PCR product direct sequencing method with an ABI377 autosequencer. The gene from PAI134 was identical in sequence to bla_{IMP-1}, but with one base replacement of G by T at nucleotide 145, which led to an amino acid alteration of Val49 to Phe. Subsequently, we subcloned the 4.5-kb *Bam*HI segment into a shuttle vector, pMS360 (9), and introduced the resultant plasmid, pMS364, by transformation into each of the following: *P. aeruginosa* strain PAO4141 (a β-lac-

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TABLE 1. Resistance levels conferred by IMP-10 metallo-β-lactamase[a]

Strain and plasmid	MIC (μg/ml)								
	CTX	CAZ	AMP	TIC	TIM	PIP	ATM	IPM	MEM
P. aeruginosa									
PAI134	>128	>128	>128	128	128	4	4	>128	>128
PAO4141, pMS455	>128	>128	128	64	64	4	2	8	64
PAO4141, pMS364	>128	>128	>128	128	128	4	2	32	128
PAO4141, pMS360	4	0.5	16	8	8	1	1	<0.5	0.5
PAO4141	4	0.5	16	8	8	1	1	<0.5	0.5
E. coli									
K-12 χ1037, pMS364	64	128	16	32	32	1	<0.5	1	8
K-12 χ1037, pMS360	<0.5	<0.5	8	2	2	1	<0.5	<0.5	<0.5
K-12 χ1037	<0.5	<0.5	4	2	2	1	<0.5	<0.5	<0.5

[a] pMS455, plasmid-bearing bla_{IMP-10} gene; pMS364, recombinant plasmid-bearing bla_{IMP-10} gene; pMS360, vector plasmid. Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; AMP, ampicillin; TIC, ticarcillin; TIM, ticarcillin-clavulanic acid; PIP, piperacilin; ATM, aztreonam; IPM, imipenem; MEM, meropenem.

tamase-deficient mutant of the PAO line) and *E. coli* strain K-12 χ1037 (9).

The MICs of various β-lactam antibiotics for pMS455- or pMS364-possessing strains are shown in Table 1. These plasmids conferred resistance to cephems (cefotaxime and ceftazidime) and carbapenems (imipenem and meropenem) on both the *E. coli* K-12 and *P. aeruginosa* hosts. The increased MIC levels of the penicillins (ampicillin, piperacillin, and ticarcillin) mediated by pMS455 and pMS364 were relatively low, especially in the *E. coli* host. The MICs of ticarcillin were not affected by the inhibitor clavulanic acid, and no increase in MICs of aztreonam was observed in pMS455- or pMS364-bearing strains.

The bla_{IMP-1}-like genes from the other two strains, *P. aeruginosa* PAI97 and *A. xylosoxidans* AXI2, were sequenced by the PCR product direct sequencing method using primers constructed from the conserved sequences of the integron structure, which is known in most cases to flank the bla_{IMP-1} gene (12). Their nucleotide sequences were identical to that of the PAI134 strain, having a single base replacement of G by T at position 145 of the bla_{IMP-1} gene. The gene identified in the three different strains and the enzyme it encodes were designated bla_{IMP-10} and IMP-10 β-lactamase, respectively.

We replaced the *Hpa*I segment consisting of nucleotides from position 127 to 415 of the bla_{IMP-1} gene in a previously constructed recombinant plasmid, pMS501 (8), with that of the bla_{IMP-10} gene of pMS600 and named the resultant plasmid pMS601. Accordingly, pMS601 had bla_{IMP-10} instead of bla_{IMP-1} in the same background plasmid.

β-Lactamase IMP-10 was prepared from the pMS601-bearing *E. coli* K-12 JM83 strain, purified to a 305-fold purity with a yield of 36.8% (specific activity, 301 U/mg of protein for 100 μM cephalothin) according to the method described previously (8). The kinetic parameters were determined with the highly purified enzyme. Statistical data were obtained by online analysis (UV absorption method and Lineweaver-Burk plot analysis with the computer system) by measuring the rate of hydrolysis (k_{cat}) and K_m values more than three times. When K_m values were too large for this system, they were obtained from Michaelis-Menten graphs by measuring the initial hydrolysis rates. The kinetic parameters were compared with those of IMP-1 from the pMS501-bearing strain (Table 2). The kinetic parameters with IMP-1 so far reported were not necessarily in good agreement each other (8, 11, 13, 14, 22, 24). Our data were almost comparable to those of Laraki et al. (11) with the exception for cephalothin and cefotaxime, which had values that were rather close to that described by Marumo et al. (13) or Watanabe et al. (22).

The hydrolyzing activities of IMP-10 for penicillins except for carbenicillin were very low compared to those of IMP-1, indicating that a single amino acid alteration resulting from a single point mutation caused a decrease in penicillin-hydrolyz-

TABLE 2. Kinetic parameters for hydrolysis of various β-lactam antibiotics by IMP-10 and IMP-1 metallo-β-lactamases

Antibiotic	Kinetic parameter for β-lactamase (source)					
	IMP-10 (pMS601)			IMP-1 (pMS501)		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Cephaloridine	140 ± 18	28 ± 0.8	5.0	62 ± 3.0	7 ± 0.4	8.9
Cephalothin	230 ± 7	4.9 ± 0.3	47	65 ± 2.9	2 ± 0.1	32.5
Cefotaxime	74 ± 6	5.7 ± 0.3	13	23 ± 1.9	1.4 ± 0.1	16
Ceftazidime	51 ± 4	51 ± 4.5	1.0	16 ± 0.9	46 ± 1	0.3
Benzylpenicillin	ND[a]	ND	0.07	460 ± 17	240 ± 10	1.9
Ampicillin	ND	ND	0.06	160 ± 15	140 ± 9	1.1
Carbenicillin	ND	ND	0.18	15 ± 2	390 ± 12	0.04
Piperacillim	ND	ND	0.04	200 ± 9	280 ± 10	0.7
Imipenem	220 ± 11	60 ± 3	3.7	130 ± 11	30 ± 3.9	4.3
Meropenem	64 ± 2	47 ± 1.5	1.4	13 ± 0.5	7 ± 1.2	1.9

[a] ND, not determined.

ing ability in this variant IMP-10 metallo-β-lactamase. The position of the altered amino acid corresponded to 31 in the mature enzyme (15). As previously reported, a single amino acid substitution of Ser196 (IMP-1 β-lactamase) for Gly196 (IMP-3 and IMP-6 β-lactamases) resulted in a markedly low level of penicillin-hydrolyzing activity and more efficient hydrolysis of meropenem than imipenem (24). In contrast, the substitution of Phe for Val at position 31 had little effect on the kinetic parameters for both imipenem and meropenem, as well as the cephalosporins (cephaloridine, cephalothin, cefotaxime, and ceftazidime). As the crystal structure of IMP-1 revealed that amino acid residues 22 to 31 constituted a flexible loop structure called a flap, which affected the affinity of the enzyme to substrates (4), it was predicted that the replacement of residue 31, which was positioned at the root of the flap, affected the substrate specificity especially for penicillins by altering the three-dimensional structure of the enzyme protein.

We used the PCR method with specific primers to identify the integron structure (18) and revealed that the gene $bla_{\text{IMP-10}}$ in three different strains was located in class 1 integrons (5). In all cases, the $bla_{\text{IMP-10}}$-containing cassette that was inserted into the specific target, GTTRRRY, on the integrons was identical in its nucleotide sequence (GenBank accession numbers AB074433 to -35) to that of the $bla_{\text{IMP-1}}$-containing cassette (10, 16). This finding suggested that $bla_{\text{IMP-10}}$ as well as $bla_{\text{IMP-1}}$ were movable among replicons in different strains, causing disseminations of different species of bacteria producing IMP-type class B β-lactamases, especially in Japan (7, 19, 20).

We thank Y. Yamaguchi, Chiba University, and S. Murayama, Teikyo University School of Medicine, for technical assistance.

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