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Characterization of a Novel IMP-28 Metallo-β-Lactamase from a Spanish *Klebsiella oxytoca* Clinical Isolate

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An isolate of *Klebsiella oxytoca* carrying a novel IMP metallo-β-lactamase was discovered in Madrid, Spain. The bla_{IMP-28} gene is part of a chromosomally located class I integron. The IMP-28 k_{cat}/K_m values for ampicillin, ceftazidime, and cefepime and, to a lesser extent, imipenem and meropenem, are clearly lower than those of IMP-1. The His306Gln mutation may induce important modifications of the L3 loop and thus of substrate accessibility and hydrolysis and be the main reason for this behavior.

The emergence of metallo-β-lactamases (MBL) in members of the family *Enterobacteriaceae* is a problem of major concern for clinicians worldwide (11). These enzymes can hydrolyze most β-lactams, including carbapenems, and are not susceptible to conventional β-lactamase inhibitors (2).

The IMP family has at least 33 unique IMP variants (http://www.lahey.org/Studies), which may differ widely in regard to the primary sequence and biochemical activity. However, some allelic variants with the following mutations are associated with decreased overall activity (particularly against penicillins), i.e., Ser62 in IMP-12 (3), Ser196 in IMP-3 (6) and IMP-6 (17), and Gly242 in IMP-18 (1).

Here we describe the genetic context and kinetic parameters of the new MBL IMP-28, which was first described in a *Klebsiella oxytoca* isolate from Spain, and in addition, we consider the possible cause of its poor overall activity.

K. oxytoca HGUGM21530 was isolated from a lip wound patient seropositive for human immunodeficiency virus diagnosed with progressive multifocal leukoencephalopathy in the Gregorio Marañon Hospital (Madrid, Spain) in 2009.

Pulsed-field gel electrophoresis (PFGE) with S1 nuclease digestion of whole-genome DNA (S1-PFGE) and PCR-based replicon typing (PBRT) were used to characterize plasmids as described previously (4). The S1-PFGE-I gel was transferred and hybridized with IMP and Inc A/C probes (the only amplicon obtained by PBRT). The results showed one band of 340 kb that hybridized only with the A/C probe. PFGE with I-CeuI digestion of whole-genome DNA, as described by Liu et al. (9), was used to determine whether the bla_{IMP-28} gene was located in the chromosome. The PFGE-I-CeuI gel was transferred and hybridized with 16S rRNA and IMP probes. The results showed one band that hybridized with both the 16S rRNA and IMP probes. These data suggest that the bla_{IMP-28} gene is located in the chromosome (data not shown).

The genetic context of the bla_{IMP-28} gene was elucidated by PCR and sequencing. The bla_{IMP-28} gene is located in a class I integron, designated In767 (http://integrall.bio.ua.pt/), that displays more structural differences from (1, 5, 7, 14, 15, 17) than similarities to (12, 18) the other integrons published for IMP-encoding genes in the last decade. The structure consists of two aminoglycoside resistance genes, *aacA44* and *aadA13*, just downstream of bla_{IMP-28}. The *aacA44* gene codes for a newly described aminoglycoside-(6′)-acetyltransferase variant showing 86% sequence identity with AacA4.

The bla_{IMP-1} and bla_{IMP-28} genes were cloned into plasmid pBGS18-pCTX under the control of the promoter of the gene for the CTX-M-14 β-lactamase and then transformed into *Escherichia coli* strain TG1. The bla_{IMP-28} gene was obtained from *K. oxytoca* HGUGM21530 by PCR and cloned into plasmid pBGS18 harboring the $bla_{CTXM-14}$ promoter, which was previously used in similar studies of β-lactamase expression (10). The primers used for cloning were 5′AAAAGGTACCATGAGCAAGTTATTTGTATTCTTTATG (forward) and 5′ AAAAGAATTCTTAGTTACTTGGTTTTGATGGTTTTTTA (reverse). The bla_{IMP-1} gene was obtained by PCR from plasmid pET-28a(+) with bla_{IMP-1} as an insert (obtained from Y. Ishii [Toho University School of Medicine, Tokyo, Japan]) and cloned into the same plasmid as the bla_{IMP-28} gene. The primers used for cloning in this case were 5′AAAGATCCATGAGCAAGTTATCTGTA (forward) and 5′AAAGAATTCTTAGTTGCTTGGTTTTGA(reverse). Microbiological analysis showed show a 4-fold minimum decrease in the MICs of all of the antibiotics, except cefotaxime and aztreonam, for bacteria expressing IMP-28 relative to those for bacteria expressing IMP-1 (Table 1). These data suggest that the IMP-28 enzyme displays lower activity than IMP-1. In order to confirm this point, we purified both enzymes and obtained the corresponding kinetic data.

To purify IMP-28, the bla_{IMP-1} gene was cloned into the pGEX-6P-1 expression vector with primers 5′AAAAGAATTCAGCGGGGAGGCCCCGC (forward) and 5′AAAAGTCGACTCACTCGGCCAACTGACTCAG (reverse). The construct was transformed into *E. coli* M15 and produced a fusion protein consisting of glu-

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TABLE 1 MICs of ampicillin, oxyiminocephalosporins, carbapenems, and other antibiotics for *E. coli* TG1 and the bacterial clinical isolate expressing the IMP-1 and IMP-28 β-lactamases

Antibiotic	MIC (μg/ml)[a] for:			
	K. oxytoca HGUGM21530 (IMP-28)	*E. coli* TG1 PBGS18-pCTX IMP-28	*E. coli* TG1 PBGS18-pCTX IMP-1	*E. coli* TG1 PBGS18-pCTX
Ampicillin	32	64	512	2
Cefoxitin	256	256	1,028	1
Cefotaxime	64	256	256	0.06
Ceftazidime	64	128	512	0.06
Cefepime	2	32	128	<0.12
Aztreonam	<0.25	<0.25	<0.25	<0.25
Imipenem	1	0.5	2	0.12
Meropenem	1	4	16	<0.03

[a] The results were confirmed in three independent experiments.

tathione *S*-transferase (GST) and the IMP-28 enzyme without its signal peptide. The β-lactamase was purified to homogeneity according to the manufacturer's instructions for the GST gene fusion system (Amersham Pharmacia Biotech, Europe, GmbH). After the cleavage of GST from IMP-28, the purified (≥99% pure) protein appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Hydrolysis of the antibiotics by the IMP-28 β-lactamase was monitored by recording the variation in absorbance resulting from the opening of the β-lactam ring. The kinetic parameters of nitrocefin were determined from the initial rates by Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For the other antibiotics, the K_m value was measured as the K_i in a competition experiment with nitrocefin as the reporter substrate (16). The k_{cat} values were then obtained by monitoring the hydrolysis of the antibiotic at a concentration >10 times the K_m.

The results revealed a systematic decrease in the k_{cat} values of IMP-28 relative to those of IMP-1. This decrease was not very significant with nitrocefin and cefotaxime and was largest for ampicillin (>1,000), for which IMP-1 displays the highest k_{cat} value. Comparison of the K_m values of IMP-1 and IMP-28 did not reveal any significant modifications; the greatest difference was a 6-fold increase in the K_m of IMP-28 for cefepime. The catalytic efficiency of IMP-28 was relatively poor against the other antibiotics tested, especially ampicillin, ceftazidime, and cefepime, and much lower than that of IMP-1 against these antibiotics (Table 2). Although the reduced K_m contributed to some extent, the most important factor in this behavior was the significantly lower k_{cat}. The overall kinetic data were consistent with a general decrease in the MICs when the enzymes were expressed in *E. coli* TG-1. Therefore, the data confirmed that IMP-28 has a lower hydrolytic capacity than IMP-1. To rule out a loss of activity linked with this lower activity, stability studies by thermal denaturation were performed. The overall data showed the two enzymes to be similarly stable (data not shown).

It is difficult to assign any mutation to this general low activity since IMP-28 differs in 6 amino acids from IMP-5, its most similar counterpart, and in 15 residues from IMP-1 (Fig. 1). An alignment of the amino acid sequences of representative IMP β-lactamases, including those with lower activity toward beta-lactams, revealed that there are no modifications in the catalytic residues of IMP-28. Moreover, IMP-28 does not contain the mutations that some groups have associated with decreased overall activity (particularly against penicillins). We also observed three unique replacements in IMP-28, namely, Arg47Lys, Gly174Ser, and His306Gln, none of which was repeated in any other enzyme of this group.

The impact of the 15 amino acids modified in IMP-28 relative to IMP-1 was therefore assessed by examining the various IMP-1 structures available. Among the 15 mutations differentiating IMP-28 from IMP-1, the 10 involving surface residues and the homologous Val216Ile mutation are not expected to affect substrate hydrolysis (Fig. 2A). The Asn208Arg mutation, which we identified as potentially being involved in protein dynamics, is also unlikely to play a major role because it would probably have affected the hydrolysis of the various substrates more uniformly. The remaining 3 amino acid differences between IMP-28 and IMP-1 are related to the L3 loop, which defines one side of the MBL active site and is known to be important for efficient hydrolysis (13). The Val223Ile and Leu241Ile differences found at both

TABLE 2 Kinetic data for the pure IMP-28 and IMP-1 β-lactamases[a]

Antibiotic	k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	
	IMP-1	IMP-28	IMP-1	IMP-28	IMP-1	IMP-28
Nitrocefin	63 ± 10	35.86 ± 12	27 ± 3	17.6 ± 4	2.3	2.03
Ampicillin	950 ± 50	0.649 ± 0.14	200 ± 25	359 ± 172	4.8	$1.8\ 10^{-3}$
Cefoxitin	16 ± 1	0.75 ± 0.07	8 ± 1	7 ± 0.3	2	0.1
Cefotaxime	1.3 ± 5	0.98 ± 0.042	4 ± 0.5	9.4 ± 0.5	0.35	0.104
Ceftazidime	8 ± 1	0.35 ± 0.046	44 ± 3	112 ± 10	0.18	$3\ 10^{-3}$
Cefepime	7 ± 0.5	0.145 ± 0.01	11 ± 1	72 ± 9	0.66	$2\ 10^{-3}$
Imipenem	46 ± 3	8.66 ± 0.17	39 ± 4	90 ± 21	1.2	0.096
Meropenem	50 ± 5	3.05 ± 0.1	10 ± 2	14.4 ± 0.4	5	0.21

[a] Data are means ± standard deviations (where applicable) and are from Laraki et al. (8).

FIG 1 Alignment of the amino acid sequences of some representative members of the IMP β-lactamase family (IMP-1, GenBank accession number, S71932; IMP-3, AB010417; IMP-5, 290912; IMP-6, AB040994; IMP-12, AJ420864; IMP-18, AY780674; IMP28, JQ407409; IMP-29, HQ438058). Asterisks indicate strictly conserved amino acids. The catalytic residues are shown in blue. Amino acids of IMP-28 differing from those of IMP-1 are shown in red. Residues of IMP enzymes known to affect substrate hydrolysis (relative to IMP-1) are highlighted in yellow. Alignment was performed with the CLUSTAL W program of EMBL-EBL.

ends of this loop are not specific to the IMP-28–IMP-1 pair, unlike the His306Gln mutation.

The His306Gln mutation is of the greatest interest. In IMP-1, the glutamine side chain is involved in two hydrogen bonds with residues of the L3 loop, which defines one side of the active-site cleft (Fig. 2B). Therefore, replacement of this amino acid with a shorter bulkier histidine may induce significant modifications of the L3 loop and thus of substrate accessibility and hydrolysis. In

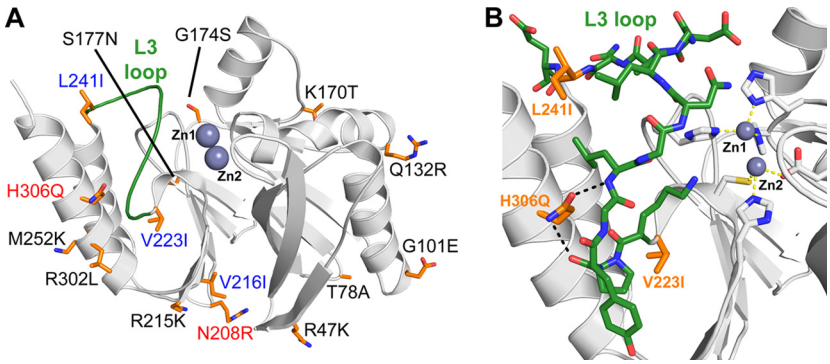

FIG 2 (A) Molecular representation of the structure of IMP-1 (Protein Data Bank code 1DDK). The 15 residues that are different in IMP-28 are shown as orange sticks, surface residues are black, internal hydrophobic residues are blue, and polar internal residues are red. (B) Enlarged view of the L3 loop of IMP-1 (green).

summary, a new IMP variant has been characterized that shows low overall activity, probably due to a His306Gln modification. Laboratory studies are under way to clarify this point.

Nucleotide sequence accession numbers. The nucleotide sequence of the class 1 integron harboring bla_{IMP-28} in *K. oxytoca* strain HGUGM21530 has been deposited in the GenBank database under accession number JQ407409.

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