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Genetic Context and Biochemical Characterization of the IMP-18 Metallo-β-Lactamase Identified in a *Pseudomonas aeruginosa* Isolate from the United States▽

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The production of metallo-β-lactamase (MBL) is an important mechanism of resistance to β-lactam antibiotics, including carbapenems. Despite the discovery and emergence of many acquired metallo-β-lactamases, IMP-type determinants (now counting at least 27 variants) remain the most prevalent in some geographical areas. In Asian countries, and notably Japan, IMP-1 and its closely related variants are most widespread. Some other variants have been detected in other countries and show either an endemic (e.g., IMP-13 in Italy) or sporadic (e.g., IMP-12 in Italy or IMP-18 in the United States) occurrence. The IMP-18-producing *Pseudomonas aeruginosa* strain PS 297 from the southwestern United States carried at least two class 1 integrons. One was identical to In*51*, while the other, named In*133* and carrying the *bla*$_{IMP-18}$ gene cassette in the third position, showed an original array of five gene cassettes, including *aacA7*, *qacF*, *aadA1*, and an unknown open reading frame (ORF). Interestingly. In*133* differed significantly from In*96*, the *bla*$_{IMP-18}$-carrying integron identified in a *P. aeruginosa* isolate from Mexico. The meropenem and ertapenem MIC values were much lower for *Escherichia coli* strains producing IMP-18 (0.06 and 0.12 μg/ml, respectively) than for strains producing IMP-1 (2 μg/ml for each). Kinetic data obtained with the purified enzyme revealed lower turnover rates of IMP-18 than of other IMP-type enzymes with most substrates.

IMP-type enzymes were the first acquired metallo-β-lactamases (MBLs) to be detected in clinically relevant Gram-negative pathogens such as *Enterobacteriaceae* and *Pseudomonas aeruginosa* (15, 19). IMP-1 was first described in imipenem-resistant isolates from Japan in the 1990s (19). Other IMP-type variants were later identified in Europe (5). Although other types of acquired subclass B1 MBLs subsequently appeared (e.g., VIM-type MBLs, SPM-1, GIM-1, SIM-1, KHM-1, TMB-1, NDM-1, and DIM-1) (23, 25, 27, 29, 32), IMP-type enzymes remain among the most prevalent and widely spread MBL determinants, especially in some geographical settings (25). The clinical relevance of these determinants is also shown by the relatively high prevalence of MBL producers among carbapenem-resistant organisms. More specifically, IMP-type enzymes represent the major acquired MBL in Japan (almost 80% of MBL-producing *P. aeruginosa* strains in some regions [13]) and are most common in the Asian-Pacific region, while they remain only sporadically encountered in North America and in some European countries (e.g., Romania and Italy) (7, 9, 10, 17, 20, 24, 30). Recent reports also documented the import of *bla*$_{IMP}$-carrying strains from Asia (a region of higher endemicity of carbapenem-resistant strains) to northern Europe, where antimicrobial resistance is limited (26).

The genes encoding IMP-type MBLs, as well as some other subclass B1 enzymes (e.g., VIM-type enzymes), are found in gene cassettes inserted in plasmid- or chromosome-borne integron/transposon structures. These latter structures play an important role in the spread of these resistance determinants among bacterial isolates, often resulting in a multidrug resistance profile, as integrons frequently carry various kinds of resistance determinants (15, 22).

The IMP sublineage has at least 27 unique variants (http://www.lahey.org/studies) differing by up to 22% amino acid sequence divergence (between IMP-9 and IMP-19) that exhibit important structural and functional differences from each other or from enzymes of other sublineages. IMP-type MBL determinants are mostly found in *P. aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae* isolates, although they have also been sporadically identified in other organisms (e.g., *Pseudomonas putida*) (25). They confer resistance to most β-lactam antibiotics, including β-lactamase inhibitor–β-lactam combinations, oxyiminocephalospsorins, and carbapenems. However, these enzymes differ from other acquired MBLs in that they are apparently unable to hydrolyze temocillin, a derivative of ticarcillin presenting an α-methoxy substituent (7, 14).

The IMP-18 determinant was first identified in the Unites States and then in Mexico and, more recently, in Puerto Rico (10, 11, 31). In *P. aeruginosa* isolate 4660 recovered from Mexico, the *bla*$_{IMP-18}$ determinant was found in the *bla*$_{IMP-18}$-*aadA1b* array (In*96*). To our knowledge, no detailed biochemical characterization of IMP-18 is currently available. However, a rationale for a detailed biochemical characterization of IMP-18 could be found in the sequence comparison of

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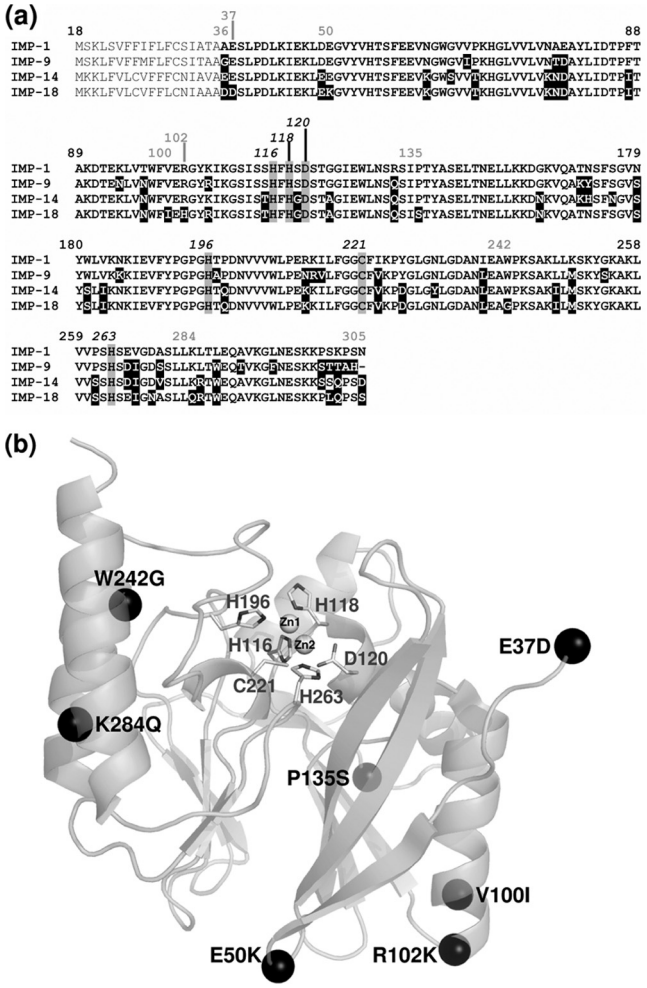

FIG. 1. (a) Amino acid sequence alignment of selected IMP variants. Substitutions with IMP-1 are shown with a black background. The sequence corresponding to the mature enzyme is boldfaced (the first 18 amino acids being the signal peptide). The numbering is according to the standard scheme proposed for class B β-lactamases (8), and amino acid substitutions that are unique in IMP-18 are indicated by gray numbers. (b) Cartoon representation of the IMP-1 X-ray crystal structure (Protein Data Bank code 1DDK), showing the locations of the unique amino acid substitutions found in IMP-18. Substitutions A36D and N305S, located at the amino- and carboxy-terminal extremities of the protein, are not shown because the corresponding residues are not included in the IMP-1 structure.

the various IMP-type enzymes (Fig. 1a). Indeed, such an alignment highlights that IMP-18 was divergent from the other IMP-type MBLs by at least 8.9% (i.e., 22 amino acids) and that some substitutions occurs in unique positions (Fig. 1b). Specifically, one of these substitutions affects position 242, where a tryptophan (replaced by a glycine in IMP-18) was invariantly found not only in all other IMP-type variants but also in all subclass B1 enzymes reported thus far. In this work, we investigated the genetic context of the bla_{IMP-18} determinant identified in *P. aeruginosa* isolate PS 297 recovered in the United States and determined the biochemical properties of its gene product.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* PS 297, used as the source of the MBL gene, was isolated from a tracheal aspirate in the southwestern United States; the properties of this strain have been described previously (11). *Escherichia coli* DH5α (Gibco Life Technologies, Gaithersburg, MD) was used as a host for recombinant plasmids, and *E. coli* BL21(DE3) (Stratagene, La Jolla, CA) was used for overproduction of the IMP-18 enzyme by using a T7 promoter-based expression system. Bacteria were always grown aerobically at 37°C. *E. coli* DH5α derivative strains were cultured in Mueller-Hinton broth (Oxoid Ltd., Basingstoke, United Kingdom), and *E. coli* BL21(DE3) was grown in ZYP-5052 medium for production of the recombinant protein (28). Medium, when necessary, was supplemented with the appropriate antibiotics (kanamycin at 50 μg/ml or chloramphenicol at 85 μg/ml).

Determination of cassette array sequences. PCR amplification of class 1 integron variable regions (cassette arrays) was performed using primers designed to target the 5′-CS and 3′-CS regions as previously described (24). Five hundred nanograms of purified total DNA of *P. aeruginosa* PS 297 was used as the template. The resulting PCR products were purified and completely sequenced on both strands by using a primer walking approach.

Cloning of the MBL gene. The complete bla_{IMP-18} gene from *P. aeruginosa* PS 297 was amplified by PCR using oligonucleotides IMP-18-EXP-Fw (5′-AGGG TACATATGAAAAAATTATTTGTTTTATGTG) and IMP-18-EXP-Rev (5′-C CGGATCCTTAGCTACTTGGCTGTAACG) (the underlines indicate the NdeI and BamHI restriction sites, respectively) as previously reported (6, 7). The amplified DNA was digested with NdeI and BamHI restriction enzymes and cloned into vector pLB-II (a pBC-SK derivative [Stratagene, La Jolla, CA] modified in our laboratory [1]) and into the T7 promoter-based expression vector pET-9a (Novagen, Madison, WI) previously digested with the same enzymes. The resulting constructs were named pLBII-IMP-18 and pET-IMP-18, respectively. A strain producing the IMP-1 MBL [*E. coli* DH5α(pLB-II-IMP-1)] was also prepared by adopting the same cloning strategy and using PCR primers IMP-1-EXP-Fw (5′-CG**GAATT**CATATGAGCAAGTTATCTGTATTCTTTATA, which includes restriction sites for EcoRI [boldface] and NdeI [underlined]) and IMP-1-EXP-Rev (5′-CCGGATCCTTAGTTGCTTGGTTTTGATGG, which includes a BamHI restriction site [underlined]).

Antimicrobial susceptibility testing. The *in vitro* antimicrobial susceptibility profiles of *E. coli* DH5α derivatives were determined with the microdilution broth method as recommended by the Clinical and Laboratory Standards Institute (3), using Mueller-Hinton broth with a bacterial inoculum of 5×10^4 CFU/well. MICs were recorded after 18 h at 37°C.

Production and purification of IMP-18. IMP-18 was produced from a culture of *E. coli* BL21(DE3)(pET-IMP-18) grown for 24 h at 37°C. Bacterial cells were harvested by centrifugation (10,000 × *g*, 30 min, 4°C) and lysed using a cell disruption system (Constant Systems Ltd., Daventry, United Kingdom). The sample was first clarified by centrifugation in order to eliminate the cellular debris and then desalted using a HiPrep 26/10 desalting column (GE Healthcare, Uppsala, Sweden) with 20 mM HEPES containing 50 μM $ZnSO_4$ (pH 7.0) buffer. The resulting sample was loaded at a flow rate of 2 ml/min onto an SP Sepharose high-performance column (bed volume, 5 ml) (GE Healthcare), previously equilibrated with the same buffer, and bound proteins were eluted using a linear NaCl gradient (0 to 1 M in 100 ml). β-Lactamase-containing fractions were pooled, concentrated to 1.2 mg/ml, and stored at −20°C.

Protein analysis techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to the method of Laemmli (13a), using final acrylamide concentrations of 12% and 5% (wt/vol) for the separating and the stacking gels, respectively. After electrophoresis, the protein bands were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA).

Determination of kinetic parameters and inactivation by chelating agents. The hydrolysis of β-lactam substrates was monitored by measuring the absorbance variation under the experimental conditions reported previously (6, 14). All measurements were performed on a Cary 100 UV-Vis spectrophotometer (Varian, Walnut Creek, CA) at 30°C in 10 mM HEPES buffer (pH 7.5) containing 50 μM $ZnSO_4$, using a reaction volume of 500 μl. The purified IMP-18 was diluted in the same buffer supplemented with 20 μg/ml bovine serum albumin (BSA) to prevent enzyme denaturation. The steady-state kinetic parameters (k_{cat} and K_m) were calculated after direct fit of the initial rates to the Henri-Michaelis-Menten equation or using the Hanes-Woolf linearization. K_m values lower than 10 μM were measured as inhibition constants by using a competitive inhibition model and 100 μM nitrocefin as the reporter substrate. The inactivation of IMP-18 by chelating agents was investigated as previously described (12).

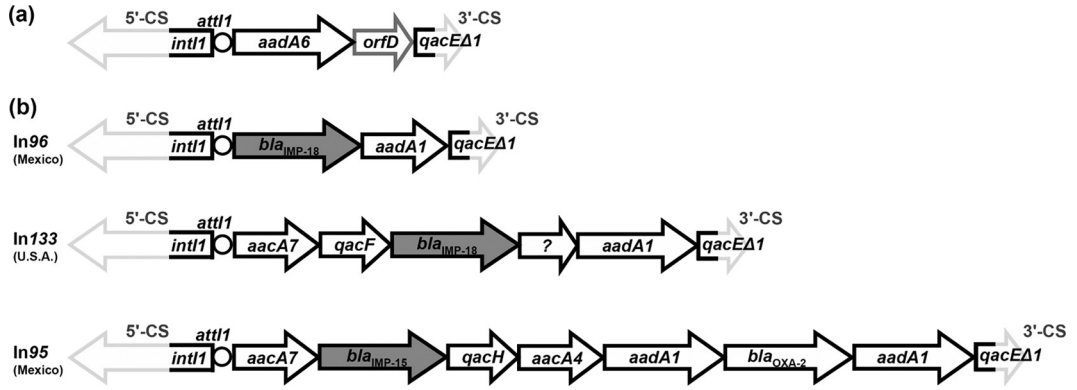

FIG. 2. Structure of the cassette arrays of the two class 1 integrons found in *P. aeruginosa* PS 297. Gene cassettes are represented by arrows (the sequences of the gray-outlined portions of *intI1* and *qacEΔ1* were not determined). (a) Structure of the shorter cassette array (accession no. FN556189), identical to In*51*. (b) Structure of the bla_{IMP-18}-carrying cassette array (In*133*, accession no. FN556190). The putative gene cassette of unknown function (fourth position) is indicated by a question mark. The structure of In*133* is compared to that of other bla_{IMP}-containing cassette arrays (In*95* and In*96*) identified in clinical isolates from Mexico, highlighting the differences between In*133* and In*96* (both carrying a bla_{IMP-18} gene cassette) and the presence of similar gene cassettes in In*133* and In*95* (which carries a bla_{IMP-15} determinant) (10, 11).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank/DDBL sequence databases and assigned the accession numbers FN556189 and FN556190.

RESULTS AND DISCUSSION

Genetic context of the bla_{IMP-18} determinant in *P. aeruginosa* strain PS 297. PCR experiments designed to amplify the variable region of class 1 integrons yielded two amplification products that significantly differed in size (3.9 and 1.5 kb).

The smaller cassette array was identical to the *aadA6-orfD* array (In*51*) previously found in a VEB-1-producing *P. aeruginosa* isolate from France (18). This array seems to be widespread and has been found in, e.g., Brazil, Mexico, Costa Rica, India, Iran, and China (http://www.ncbi.nlm.nih.gov), in some cases with minor differences. An *aadA6-orfD* cassette array (In*97*, 4 nucleotide differences with the In*51* cassette array) was also found in *P. aeruginosa* strain 4677 from Mexico, which also carries the IMP-15-encoding array designated In*95* (Fig. 2) (9).

The larger cassette array (named In*133*), *aacA7-qacF-*bla_{IMP-18}-unknown gene cassette-*aadA1b*, has not been reported before (Fig. 2). It was associated with the strong version of the P_c promoter located in the integrase gene (4). In agreement with the resistance to gentamicin and amikacin exhibited by isolate PS 297, two cassettes (in first and last positions) were found to encode aminoglycoside resistance factors (the AACA7 acetyltransferase and the AADA1 adenylyltransferase), the first being compatible with the resistance to gentamicin and amikacin exhibited by strain PS 297. The *qacF*-like gene encodes a quaternary ammonium compound resistance protein variant identical to a QacF variant reported only once in the database (accession no. DQ149925) encoded by a plasmid-borne cluster of three integrons in several isolates of *Vibrio cholerae* (2) and showing two amino acid substitutions (L7M and S10A) compared with the QacF determinant encoded by In*40* from *Enterobacter aerogenes* BM2688 (21). It encodes an efflux protein belonging to the small multidrug resistance family and shows sequence similarity with other Qac/Erm-type drug:proton antiporters that have been reported in other integrons or hosts (sequence identity ranging from 79.3 to 35.1% with QacG and QacC, respectively) (15, 16).

The IMP-18-encoding cassette is found in the third position, which suggests that other cassette integration events occurred after the insertion of the bla_{IMP} determinant in this integron. This observation is rather unusual, as MBL-encoding gene cassettes are more frequently found in either first or second position in the integron structure (25, 30).

The *attC* site (59-base element) of the bla_{IMP-18} gene cassette is 134 bp long and thus closer to the *attC* sites found associated with most bla_{IMP} alleles (e.g., $bla_{IMP-1-like}$, bla_{IMP-4}, bla_{IMP-7}, and bla_{IMP-16}) compared to those of the bla_{IMP-2} and bla_{IMP-8} gene cassettes, the latter exhibiting a much shorter *attC* site (7). Compared to In*96*, the bla_{IMP-18} gene cassette *attC* site differs by a single deletion (T_{1291} of sequence accession no. EF184215). The bla_{IMP-18} *attC* site also exhibits the largest divergence with other bla_{IMP}-associated *attC* sites of similar lengths (nucleotide identities range from 69 to 81%, while other *attC* sites share identities in the range of 75 to 98%).

The nature of the putative gene cassette found in the fourth position remains unknown. Indeed, the corresponding 411-bp DNA fragment did not show any similarity with sequences present in the NCBI/EMBL/DDJB databases, nor does it contain any open reading frame, but the sequence does present the typical features of a gene cassette with its *attC* site that includes both the core and inverse core site for recombination.

Molecular cloning of bla_{IMP-18} and contribution to β-lactam resistance. In order to compare the contributions of IMP-18 and IMP-1 to β-lactam resistance, laboratory strains where the MBL gene was cloned under the transcriptional control of the P_{lac} promoter were obtained. The production of IMP-18 in *E. coli* resulted in higher MIC values for most tested antibiotics but did not affect aztreonam (which is not a substrate of MBLs), piperacillin, and temocillin, the latter apparently behaving as poor substrates of IMP-18 (Table 1). A similar behavior was also observed, to some extent, with the IMP-1-producing recombinant strain, indicating that rather low MICs

TABLE 1. *In vitro* susceptibility profiles of *E. coli* DH5α derivatives carrying the cloned bla_{IMP-18} genes and *E. coli* DH5α carrying the empty plasmid[a]

β-Lactam	MIC (μg/ml)		
	E. coli DH5α (pLBII-IMP-18)	*E. coli* DH5α (pLBII-IMP-1)	*E. coli* DH5α (pBC-SK)
Ampicillin	64	256	4
Piperacillin	1	1	1
Ticarcillin	256	512	8
Temocillin	16	32	16
Cephalothin	32	256	4
Cefoxitin	256	>256	4
Cefuroxime	64	128	4
Cefotaxime	32	8	0.25
Ceftazidime	32	32	0.25
Ceftriaxone	4	8	0.25
Cefepime	2	4	0.25
Cefotetan	32	>32	0.25
Imipenem	1	2	0.06
Meropenem	0.06	2	≤0.015
Ertapenem	0.12	2	≤0.015
Aztreonam	0.12	0.12	0.12

[a] Strains producing the IMP-1 enzyme or carrying the empty vector are shown for comparison.

are commonly observed with these agents when IMP-type determinants are transferred in *E. coli* laboratory strains. The MIC values for oxyiminocephalosporins were increased 16- to 128-fold, the major changes being observed with cefotaxime, ceftazidime, and cefotetan.

As already reported in previous studies, the production of either IMP-1 or IMP-18 did not confer full resistance to carbapenems (per CLSI breakpoints) but resulted in increases in MIC values of up to seven 2-fold dilutions. Interestingly, the contributions of IMP-1 and IMP-18 to carbapenem MICs were very different (Table 1). While IMP-1 yields similar MIC values with all tested carbapenems, the production of IMP-18 was associated with much lower MICs for meropenem and ertapenem. This most likely reflects a difference in the hydrolytic activity toward these compounds (Table 2).

Purification and biochemical characterization of IMP-18. Thanks to the high yield of MBL production (approximately 100 mg/liter of culture), a purified IMP-18 preparation (purity, >98%) could be obtained after a single cation exchange chromatography step. In kinetic assays, IMP-18 was able to efficiently hydrolyze most tested β-lactam compounds except aztreonam (which is not recognized by MBLs) and temocillin, which typically is a poor substrate for IMP-type enzymes (7, 14). Most observed catalytic efficiencies were greater than 10^5 $M^{-1} \cdot s^{-1}$, except for piperacillin, meropenem, and ertapenem (Table 2). Surprisingly, the lowest turnover rates and catalytic efficiencies measured were those for the latter carbapenems. These data represent a striking feature of IMP-18 compared with other IMP variants. These data are also in agreement with the low MIC values exhibited by the recombinant *E. coli* clone producing IMP-18 for these compounds. K_m values were overall lower for cephalosporins and carbapenems (K_m, 0.1 to 11 μM) than for penicillin substrates (K_m, ≥90 μM) (Table 2). Although the highest turnover rates were measured using benzylpenicillin, ampicillin, ticarcillin, and imipenem, IMP-18 shows strikingly low k_{cat} values for the other substrates, including expanded-spectrum cephalosporins (e.g., cefotaxime and cefepime) and 1-β-methyl carbapenems (meropenem and ertapenem). The turnover rate of IMP-18 with these substrates was 2 orders of magnitude lower than that of IMP-1 or IMP-12. A similarly low k_{cat} value was measured for cephalothin, although a concomitant decrease of the K_m value of this substrate yielded a catalytic efficiency close to that of IMP-1. However, the comparison of the MIC values of the strains producing IMP-1 and IMP-18 would indicate that, at least with this compound, the k_{cat} value (rather than k_{cat}/K_m) would influence the resulting susceptibility of the strain. Cefoxitin was also hydrolyzed by IMP-18, although less efficiently than by IMP-1. It remains unclear why this substrate is overall a good

TABLE 2. Kinetic parameters of selected IMP-type MBLs for the hydrolysis of various β-lactam substrates[d]

Substrate	k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	
	IMP-1	IMP-18	IMP-1	IMP-18	IMP-1	IMP-18
Ampicillin	950	65	200	103	4.8×10^6	6.3×10^5
Benzylpenicillin	320	54	520	90	6.2×10^5	6.2×10^5
Piperacillin[a]		>10		>200	7.2×10^5	5.1×10^4
Ticarcillin	1.1	30	740	180	1.5×10^3	1.5×10^5
Temocillin		>0.75	>2,000	>1,000	$<10^2$	7.5×10^{2b}
Cephalothin	48	0.4	21	0.1	2.4×10^6	4.0×10^6
Cefoxitin	16	2	8	11	2.0×10^6	1.8×10^5
Cefuroxime	8	0.9	37	7	2.2×10^5	1.3×10^5
Cefotaxime	1.3	0.7	4	3	3.5×10^5	2.3×10^5
Ceftazidime	8	1	44	1.3	1.8×10^5	7.7×10^5
Cefepime	7	0.35	11	0.8	6.6×10^5	4.4×10^5
Nitrocefin	63	55	27	9	2.3×10^6	6.1×10^6
Imipenem	46	17	39	7	1.2×10^6	2.4×10^6
Meropenem	5	0.05	10	8.4	5.0×10^5	6.0×10^3
Ertapenem	16	0.03	21	2.6	7.6×10^5	1.2×10^4
Aztreonam	<0.01	<0.01[c]	>1,000	>1,000		

[a] First-order kinetics were observed with this substrate.
[b] With temocillin, the standard deviation on kinetic parameters was 20%.
[c] No hydrolysis was detected by using an enzyme concentration of 1.8 μM in the reaction mixture.
[d] Data for IMP-1 (except ertapenem [this study]) are from reference 14. Individual kinetic parameters are the means of three measurements, and standard deviations were always <10%, unless otherwise specified.

substrate for IMP-type enzymes, while no interaction with temocillin was observed even though this compound shows the same typical α-methoxy substituent.

IMP-18 was efficiently inactivated by metal chelators, with dipicolinic acid showing higher pseudo-first-order inactivation rates than those of EDTA (k_2/K, >40 and 0.15 $M^{-1} \cdot s^{-1}$ for dipicolinic acid and EDTA, respectively). This behavior is consistent with that observed with other IMP-type enzymes and, generally, with all other subclass B1 MBLs.

Concluding remarks. The present study further highlights the heterogeneity and complexity shown by the cassette arrays of MBL-encoding class 1 integrons. Although the epidemiological relationship between the recently reported IMP-18-producing isolates has not been specifically investigated, it is noteworthy that the same MBL determinant has completely different genetic backgrounds, suggesting different sources of acquisition of the MBL gene cassette. Moreover, the structure of In*133* is quite unusual as the MBL-encoding cassette was not found at the first or second position as in most MBL-encoding integrons, reflecting the possibility that current selective pressure might have induced the acquisition of other resistance traits. This finding indicates that this integron substantially evolved its structure after the acquisition of the MBL gene, as gene cassettes appear to integrate at the front of the array (recombination events could also alter cassette order). The complexity of this cassette array might also have played a crucial role in the coselection of resistance genes, including bla_{IMP-18}, which conferred carbapenem resistance. Therefore, the selective pressure driving the acquisition of bla_{IMP-18} may not be a consequence of direct selection by β-lactam agents but possibly a consequence of selection by other antimicrobial agents or disinfectants. These data raise an important question on how to practically avoid such resistance phenotypes in the clinical setting, if such clinical strains that are resistant to last-resort drugs could be maintained by rather ordinary and commonly used antimicrobial agents and/or disinfectants.

From a functional standpoint, IMP-18 is characterized by overall lower turnover rates than those of other IMP-type variants, especially toward the carbapenem meropenem. The unprecedented replacement of the highly conserved Trp242 residue with a glycine might impact on the overall structure and stability of IMP-18, thus contributing to its lower reactivity. Further investigation of its structural and biochemical properties should be carried out to elucidate this point.

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