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Biochemical Characterization of the *Pseudomonas aeruginosa* 101/1477 Metallo-β-Lactamase IMP-1 Produced by *Escherichia coli*

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The *bla*$_{IMP}$ gene coding for the IMP-1 metallo-β-lactamase produced by a *Pseudomonas aeruginosa* clinical isolate (isolate 101/1477) was overexpressed via a T7 expression system in *Escherichia coli* BL21(DE3), and its product was purified to homogeneity with a final yield of 35 mg/liter of culture. The structural and functional properties of the enzyme purified from *E. coli* were identical to those of the enzyme produced by *P. aeruginosa*. The IMP-1 metallo-β-lactamase exhibits a broad-spectrum activity profile that includes activity against penicillins, cephalosporins, cephamycins, oxacephamycins, and carbapenems. Only monobactams escape its action. The enzyme activity was inhibited by metal chelators, of which 1,10-*o*-phenanthroline and dipicolinic acid were the most efficient. Two zinc-binding sites were found. The zinc content of the *P. aeruginosa* 101/1477 metallo-β-lactamase was not pH dependent.

The ingenuity of chemists has produced a large number of new potent antimicrobial agents, but resistant bacterial strains have consistently emerged. The case of the β-lactam compounds is a good example. Bacteria have developed several strategies to escape the activities of these lethal molecules: synthesis of β-lactamases, enzymes which hydrolyze the β-lactam antibiotics, decreased target sensitivity, the development of efflux systems, and/or modification of the diffusion barrier (14). The production of β-lactamase is the most prevalent mechanism of resistance to β-lactams. To date, more than 200 different β-lactamases have been described and characterized (3). Their catalytic mechanisms involve either an active-site serine residue (serine β-lactamase) or a divalent transition metal ion (metallo-β-lactamase) (27).

Metallo-β-lactamases are endogenously produced by some gram-positive and gram-negative species including *Bacillus cereus* (17), a cluster of *Bacteroides fragilis* (9), *Stenotrophomonas maltophilia* (25, 28), some *Aeromonas* spp. (20), and *Chryseobacterium meningosepticum* (24). Recently, a metallo-β-lactamase (IMP-1), encoded by a mobile gene (*bla*$_{IMP}$) located on an integron-like element, was found in some clinical isolates of *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Alcaligenes xylosoxidans* that acquired the gene by horizontal transfer (18, 19, 29, 30). From the clinical point of view and due to the ability of the *bla*$_{IMP}$ gene to rapidly spread among such major pathogens, the IMP-1 enzyme currently represents the most dangerous metallo-β-lactamase.

Metallo-β-lactamases constitute a heterogeneous family. Although their primary structures exhibit a relatively low degree of sequence isology (23) (most often less than 40% and sometimes less than 11% identical residues), their three-dimensional structures show a high degree of similarity (4–6) and they share four main characteristics: (i) inactivation of the carbapenem antibiotics, (ii) no interaction with monobactams, (iii) susceptibility to chelating agents such as EDTA and dipicolinic acid, and (iv) the presence of Zn^{2+} ions as the naturally occurring cations, although the Cd^{2+} and Co^{2+} derivatives are enzymatically active in vitro (27). Two zinc-binding sites are present in the molecule (4, 6, 8). The three-dimensional structure of the *B. cereus* 569H metallo-β-lactamase shows that the side chains of residues His86, His88, and His149 and a water molecule provide the metal ligands in the first binding site. The second involves the side chains of residues Asp90, Cys168, and His210 (4, 6). By one possible catalytic mechanism, the zinc ion acts as a Lewis acid to stabilize the transient tetrahedral intermediate formed by the nucleophilic attack of a hydroxide ion on the carbonyl group of the β-lactam ring. The hydroxide ion is provided by the water molecule present in the active site which interacts with Asp90. This residue might act as a general base by subtracting a proton from the water molecule (2).

Metallo-β-lactamases are not sensitive to the conventional β-lactamase inhibitors of their active-site serine counterparts and, except for the enzymes produced by *Aeromonas* species, exhibit a broad-spectrum activity profile against β-lactams (11, 12, 13). This also appears to be the case for the IMP-1 enzyme, according to a previous study performed with a limited number of substrates (21, 22). However, a detailed analysis of the IMP-1 properties has not yet been performed.

In this study, the enzymatic activity of the IMP-1 metallo-β-lactamase was determined on a large number of substrates. A system for the overproduction of the enzyme in *Escherichia coli* and a simplified purification protocol were developed. The properties of the recombinant IMP-1 enzyme produced in *E. coli* were shown to be identical to those of the original enzyme produced by the *P. aeruginosa* clinical isolate. The effects of various chelating agents on the enzyme activity and the pH dependence of the zinc content were also measured.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* 101/1477 (a clinical isolate from Japan producing IMP-1) was a gift of D. Livermore (Antibiotic Reference Unit, Central Public Health Laboratory, London, United Kingdom). *E. coli* DH5α [*supE4 ΔlacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used as the host for recombinant plasmids, and *E. coli* BL21(DE3) [*hsdS gal*(λcIts857 *ind1 Sam7 nin5 lac*UV5-T7 gene 1)] was used for the over-expression of the IMP-1 enzyme via the T7 expression system (Novagen Inc., Madison, Wis.). The bacteria were grown aerobically at 37°C in Luria-Bertani medium supplemented, when necessary, with the appropriate antibiotics (kana-mycin at 50 μg/ml or ampicillin at 100 μg/ml) for plasmid selection. Plasmid PCR II (Invitrogen BV, NV Leek, The Netherlands) was used as the vector for the cloning and sequencing of a PCR-amplified fragment of the *bla*$_{IMP}$ gene. Plasmid pET9a (Novagen Inc.) was used as a T7-based expression vector for the over-expression of the *bla*$_{IMP}$ gene in *E. coli*.

Antibiotics and other chemicals. Benzylpenicillin, ampicillin, cephalothin, and cephaloridine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nitro-cefin was purchased from Unipath (Milan, Italy). Cefoxitin and imipenem were gifts of Merck Sharp & Dohme Research Laboratories (Rahway, N.J.). Pipera-cillin was a gift of Lederle Wyeth (Catania, Italy). Cefpirome, cefotaxime, and desacetyl-cefotaxime were gifts of Hoechst AG (Frankfurt, Germany). Az-treonam was a gift of the Squibb Institute for Medical Research (Princeton, N.J.). Cefuroxime and ceftazidime were gifts of Glaxo Wellcome (Verona, Italy). Carumonam was a gift of Hoffmann-La Roche (Basel, Switzerland). Temocillin, carbenicillin, and ticarcillin were gifts of SmithKline Beecham Pharmaceuticals (Brentford, United Kingdom). Moxalactam and loracarbef were gifts of Ely Lilly & Co. (Indianapolis, Ind.). Cefepime was a gift of Bristol Meyers (Wallingford, Conn.). Meropenem was a gift of Zeneca Pharmaceuticals (Cheshire, United Kingdom). Biapenem was a gift of Cyanamid (Catania, Italy). Panipenem was a gift of Sankyo Co. Ltd., Biological Research Laboratories (Tokyo, Japan). EDTA, EGTA, pyridine-2,6-dicarboxylic acid (dipicolinic acid), and 1,10-*o*-phenanthro-line were purchased from Sigma, kanamycin was purchased from Merck (Darm-stad, Germany), and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Eurogentech (Liège, Belgium).

Recombinant DNA methodology. Plasmid pBCAM-52R, a pBCSK vector con-taining a cloned copy of the *bla*$_{IMP}$ gene of *P. aeruginosa* 101/1477 (19), was used as a source of the gene for PCR amplification of the moiety of the *bla*$_{IMP}$ gene corresponding to the NH$_2$-terminal sequence of the protein. A new restriction site (*Nde*I) was introduced directly before the *bla*$_{IMP}$ gene by PCR. The oligo-nucleotide primers used for the PCR were *imp-Nde*I (5′-CATATGAGCAAGT TATCTGTATTCTTTATA-3′) and *imp-Sma*I (5′-AGTGTGTCCCGGGCCTG G-3′) (the underscores indicate the *Nde*I and *Sma*I restriction sites, respectively).

The PCR was performed with 25 ng of pBCAM-52R and 5 U of *Taq* DNA polymerase, which were incubated in the reaction buffer proposed by the *Taq* manufacturer. After 4 min at 95°C, 30 cycles of amplification were performed under the following conditions: denaturation for 30 s at 95°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C. The PCR product was cloned into plasmid PCR II to yield pCIP2 and was sequenced by the Sanger method with a fluorescent universal primer on an ALF DNA sequencer (Pharmacia, Uppsala, Sweden) (1). A *Sma*I-*Bam*HI fragment was isolated from pBCAM-52R and was subcloned into pCIP2 to yield pCIP3. The *Nde*I-*Bam*HI fragment of pCIP3 containing the entire *bla*$_{IMP}$ open reading frame was subcloned into pET 9a, which had been digested with the same enzymes, to obtain pCIP4, and pCIP4 was transformed into *E. coli* BL21(DE3).

Expression and purification of the IMP-1 β-lactamase. The zinc β-lactamase was produced by *E. coli* BL21(DE3) carrying pCIP4 in Luria-Bertani medium at 37°C under orbital agitation. Kanamycin (50 μg/ml) was used as the selecting agent during the growth of the bacteria. At an A_{600} of 0.6, IPTG (final concen-tration, 1 mM) was added, and the culture was further incubated for 2 h and centrifuged at 5,000 × *g* for 10 min. The pellet was resuspended in 50 mM HEPES (pH 7.5) containing 50 μM ZnSO$_4$ (buffer H), and the cells were disrupted with the help of cell disrupter equipment (Basic Z Model; Constant Systems Ltd., Warwick, United Kingdom). The lysate was clarified by centrifu-gation at 30,000 × *g* for 20 min in order to eliminate the cell debris. The solution was loaded onto an S-Sepharose FF column (2 by 30 cm; Pharmacia) equili-brated in 50 mM HEPES (pH 7.5)–50 μM ZnSO$_4$ (buffer H). The column was washed with the same buffer, and the enzyme was eluted with the help of a linear NaCl gradient (0 to 0.5 M). The active fractions were pooled and concentrated by ultrafiltration with a YM-10 membrane (Amicon, Beverly, Mass.). The protein solution was dialyzed against buffer H and was loaded onto a MonoS column (0.5 by 5 cm; Pharmacia) that had been prequilibrated in buffer H. The enzyme was eluted with the help of a linear salt gradient (0 to 0.5 M). The active fractions were collected, pooled, and concentrated to 1 mg/ml by ultrafiltration as de-scribed above. The enzymatic solution was stored at −70°C.

Purification of the IMP-1 enzyme from *P. aeruginosa* 101/1477 was done as described by Watanabe et al. (29).

Protein analysis techniques. The N-terminal sequence was determined with the help of a gas-phase sequencer (Prosite; 492 protein sequencer; Applied Biosystems, Foster City, Calif.). The M_r value of the enzyme was determined on an electrospray mass spectrometer (VG Platform; Micromass, Fisons, United Kingdom) in acetonitrile-water (50:50 [vol/vol]; pH 6.5). The source temperature

was kept at 80°C. The sample was directly introduced into the ionization cham-ber (at atmospheric pressure) through a steel capillary at a flow rate of 40 μl/min. The sampling cone voltage was maintained at 40 V.

Kinetic parameters. The hydrolysis of the antibiotics by the IMP-1 metallo-β-lactamase was monitored by monitoring the variation in absorbance resulting from the opening of the β-lactam ring under the experimental conditions re-ported in Table 1. All the measurements were made on an Uvikon 80 spectro-photometer connected to a personal computer via an RS232C interface. All the reactions were performed in a total volume of 500 μl. All the solutions were made in 50 mM HEPES (pH 7.5)–50 μM ZnSO$_4$. Bovine serum albumin (final concentration, 20 μg/ml) was added to the diluted solutions of β-lactamase to prevent enzyme denaturation. The steady-state kinetic parameters (K_m and k_{cat}) were determined by analyzing the complete hydrolysis time courses as described by De Meester et al. (10). When the K_m value was too small ($K_m \leq 20$ μM), it was measured as a K_i in a competition experiment with 100 μM nitrocefin as the reporter substrate (15). The initial rate of hydrolysis of the reporter substrate was measured in the presence of different concentrations of the compounds that were studied. The k_{cat} values were then obtained by monitoring the hydrolysis of the antibiotic at a concentration ≥10 times the K_m.

Inactivation of the metallo-β-lactamase by chelating agents. The loss of β-lactamase activity was monitored in the presence of EDTA, EGTA, dipicolinic acid, and 1,10-*o*-phenanthroline. The progressive inactivation of the enzyme (2.5 nM) by EDTA, EGTA, 1,10-*o*-phenanthroline, and dipicolinic acid was moni-tored in the presence of 100 μM nitrocefin (reporter substrate) in a total volume of 500 μl. All the solutions were prepared in 50 mM HEPES (pH 7.5). The rate constants characterizing the inactivation of the enzyme were derived from the dependence of the pseudo-first-order constant k_i upon the chelating agent con-centration, on the basis of scheme 1, in which E.Zn, C, E.Zn.C, E, and Zn.C are the metalloenzyme, the chelator, a ternary metalloenzyme-chelator complex, the apoenzyme, and the metal-chelator complex, respectively, as follows:

$$\text{E.Zn} + \text{C} \underset{}{\overset{K}{\rightleftharpoons}} \text{E.Zn.C} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{E} + \text{Zn.C}$$
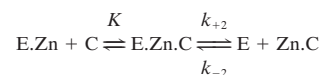
K represents the dissociation constant of the E.Zn.C ternary complex, k_{+2} is the individual rate constant for the dissociation of E.Zn.C, and k_{-2} is the second-order rate constant for the formation of the ternary complex. Because [Zn.C] was higher than [E], the formation of the ternary complex from the apoenzyme and the Zn-chelator complex was characterized by the pseudo-first-order rate con-stant $k'_{-2} = k_{-2}$ [C]. The values of the individual rate constants were obtained with the help of the following equation, in which $K_{m,S}$ and [S] are the K_m of the reporter substrate and the concentration of the reporter substrate, respectively:

$$k_i = \frac{k_{+2}[C]}{K[(K_{m,S} + [S])/K_{m,S}] + [C]} + k_{-2}' \qquad (1)$$

TABLE 1. Experimental conditions for kinetic studies

Antibiotic	Concn range (μM)	Δε (M^{-1} cm^{-1})	λ (nm)	Enzyme (μg)
Benzylpenicillin	500–1,000	−775	235	0.5–1
Ampicillin	250–1,000	−820	235	1–2
Carbenicillin	800–1,500	−780	235	1–2
Piperacillin	500–2,000	−820	235	1–10
Temocillin	100–1,200	−660	235	5–10
Ticarcillin	100–1,200	−660	235	<10
Imipenem	50–120	−9,000	300	0.02–0.05
Meropenem	100–200	−6,500	300	0.5–2
Panipenem	50–100	−7,400	300	0.02–0.5
Biapenem	100–200	−7,600	293	1–2
Nitrocefin	50–150	+15,000	482	0.05–0.5
Cephaloridine	25–150	−10,000	260	0.5–1
Cephalothin	25–150	−6,500	260	0.5–1
Cefuroxime	50–150	−7,600	260	0.5–1
Ceftazidime	50–120	−9,000	260	5–10
Cefotaxime	50–100	−7,500	260	1–5
Desacetyl-cefotaxime	50–100	−7,250	260	0.05–1
Cefoxitin	25–100	−7,700	260	0.2–0.5
Cefpirome	50–100	−4,500	260	0.5–2
Cefepime	25–75	−10,000	260	1–3
Loracarbef	50–100	−10,600	260	0.05–0.5
Moxalactam	50–100	−4,000	260	0.5–1
Aztreonam	1,000	−700	320	5
Carumonam	1,000	−810	310	5

TABLE 2. Summary of purification of metal-dependent
β-lactamase of *P. aeruginosa* 101/1477 produced
in *E. coli* BL21(DE3)[a]

Product or purification step	Vol (ml)	Amt of protein (mg)	Total activity (μmol min^{-1})	Yield (%)
Crude extract	150	380	41,800	100
S-Sepharose FF column elution	80	49	40,800	97
MonoS column elution	20	35	39,000	93

[a] Standard deviations were ±10%.

When the *K* value is much larger than the chelator concentration, this equation simplifies to the following:

$$K_i = \frac{k_{+2}\,[C]}{K[(K_{m,S} + [S])/K_{m,S}]} + k_{-2}' \qquad (2)$$

Determination of the metal pH dependence of the metal content of the IMP-1 metallo-β-lactamase. Inductively coupled plasma mass spectrometry (ICPMS) was used to determine the pH dependence of the metal content of the metallo-β-lactamase. All the determinations were done after an overnight dialysis at 4°C against 300 volumes of buffer. The enzyme concentration was 38 μM. The following buffers were used: citric acid-sodium citrate (pH 5), acetic acid-sodium acetate (pH 5), sodium cacodylate-HCl (pH 6, 6.5, and 7), and HEPES-HCl (pH 7.5 and 8). The concentration of the buffering component was 50 mM in all cases. The catalytic efficiencies of the various dialyzed enzyme solutions were determined against imipenem.

RESULTS AND DISCUSSION

Overexpression and purification of the IMP-1 β-lactamase. The protocol originally used for the purification of the IMP-1 metallo-β-lactamase involved an ammonium sulfate precipitation step followed by two column chromatographies (23, 29). In our hands, with *P. aeruginosa* 101/1477 as the enzyme source, the yield of the procedure was low (<40%). In order to facilitate the study of the enzyme, the *bla*$_{IMP}$ gene was cloned into a T7 expression vector (pET9a) and was overexpressed in *E. coli* BL21(DE3). In this strain, under the conditions described in the Materials and Methods section, high levels of enzyme accumulated in the periplasmic space. As a consequence, the purification protocol was simplified and avoided the ammonium sulfate precipitation step. Moreover, the purification yield was increased to >93%, as reported in Table 2. By using this system and the simplified purification protocol the average yield of pure enzyme was 35 mg per liter of culture. The specific activity of the purified enzyme toward nitrocefin was 19.5 μmol s^{-1} mg^{-1}. The pI value was 9 ± 0.2, which was not significantly different from that observed with the enzyme purified from the original *P. aeruginosa* strain.

The N-terminal sequence of the IMP-1 β-lactamase produced by *E. coli* was NH$_2$-AESLP, showing that the processing of the proenzyme was identical in *E. coli* and *P. aeruginosa* 101/1477 (19). Moreover, the N-terminal sequence was identical to that reported by Muramo et al. (21) and Osano et al. (22). These data suggested that the enzymes produced by different *S. marcescens* strains and *P. aeruginosa* were closely related.

Mass spectrometry confirmed the homogeneity of the preparation (data not shown). The measured M_r was estimated to be 25,103 ± 10, which is in good agreement with that deduced from the amino acid sequence (M_r, 25,111).

Kinetic parameters of the IMP-1 enzyme. The values of k_{cat} and K_m for a representative set of antibiotics are presented in Table 3. The enzyme exhibited a broad-spectrum activity profile but did not significantly hydrolyze the monobactam compounds aztreonam and carumonam ($k_{cat}/K_m < 0.0001$ μM^{-1} s^{-1}). A high concentration (1 mM) of the various monobac-

tams did not affect the rate of hydrolysis of 100 μM nitrocefin. A prolonged incubation (3 h) of the enzyme in the presence of 1 mM monobactams did not modify its activity.

For penicillins, the structure of the C-6 side chain played an important function. Ampicillin behaved as a good substrate ($k_{cat}/K_m = 5$ μM^{-1} s^{-1}). Substitution of the amino group by a bulky group (for piperacillin, $k_{cat}/K_m = 0.72$ μM^{-1} s^{-1}) or a carboxylic function (for carbenicillin, $k_{cat}/K_m = 0.02$ μM^{-1} s^{-1}) decreased the catalytic efficiency. For carbenicillin, the large K_m value did not allow the determination of the individual kinetic parameters. Temocillin was neither recognized nor hydrolyzed by the IMP-1 β-lactamase, as observed for the Zn β-lactamase produced by *B. cereus* 569H (11), while ticarcillin was poorly hydrolyzed ($k_{cat}/K_m = 0.0015$ μM^{-1} s^{-1}). This difference may be explained by the presence of an α-methoxy group at position C-6 of temocillin. A 3-h incubation of the enzyme in a 2 mM solution of temocillin did not affect the activity of IMP-1.

All the cephalosporin derivatives tested were well recognized and hydrolyzed by the IMP-1 enzyme. The catalytic efficiency varied from 0.2 to 3 μM^{-1} s^{-1}. Except for the K_m and k_{cat} values for desacetyl-cefotaxime, the values of K_m and k_{cat} were small compared to those for the best penicillins. Interestingly, the modification of the C-3 side chain affected the K_m value, which was larger for desacetyl-cefotaxime than for cefotaxime. The comparison of the kinetic constant for the hydrolysis of cephalothin and cefoxitin indicated that the presence of an α-methoxy group at position C-7 did not affect the activity of the metallo-β-lactamase. Both compounds are good substrates for IMP-1, and their kinetic parameters are similar. Moxalactam (an oxacephem compound) is also a good substrate. Oximinocephalosporins (cefotaxime, ceftazidime) were efficiently recognized and relatively well hydrolyzed by the β-lactamase, with catalytic efficiencies of >0.1 μM^{-1} s^{-1}. Loracarbef was well hydrolyzed ($k_{cat}/K_m = 0.9$ μM^{-1} s^{-1}).

TABLE 3. Kinetic parameters of the *P. aeruginosa*
metallo-β-lactamase at pH 7 and 30°C

Antibiotics	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
Benzylpenicillin	320 ± 30	520 ± 30	0.62
Ampicillin	950 ± 50	200 ± 25	4.8
Carbenicillin	ND[a]	ND	0.02
Piperacillin	ND	ND	0.72
Temocillin	ND	>2,000	<0.00010
Ticarcillin	1.1	740	0.0015
Imipenem	46 ± 3	39 ± 4	1.2
Meropenem	50 ± 5	10 ± 2	0.12
Panipenem	44 ± 6	30 ± 3	1.5
Biapenem	160 ± 20	28 ± 2	6
Nitrocefin	63 ± 10	27 ± 3	2.3
Cephaloridine	53 ± 2	22 ± 2	2.4
Cephalothin	48 ± 4	21 ± 2	2.4
Cefuroxime	8 ± 1	37 ± 3	0.22
Ceftazidime	8 ± 1	44 ± 3	0.18
Cefotaxime	1.3 ± 0.5	4 ± 0.5[b]	0.35
Desacetyl-cefotaxime	ND	ND	0.72
Cefpirome	9 ± 1	14 ± 3[b]	0.64
Cefepime	7 ± 0.5	11 ± 1[b]	0.66
Cefoxitin	16 ± 1	8 ± 1[b]	2
Moxalactam	88 ± 10	10 ± 2[b]	8.8
Loracarbef	ND	ND	0.9
Aztreonam	>0.01	>1,000	<0.0001
Carumonam	>0.01	>1,000	<0.0001

[a] ND, not determined.
[b] K_m was obtained as the K_i value.

TABLE 4. Individual parameters for inactivation of *P. aeruginosa* 101/1477 metallo-β-lactamase by chelating agents

Chelating agent	k_{+2} (s^{-1})	k_{-2}' (s^{-1})	K (mM)	k_{+2}/K (M^{-1} s^{-1})
EDTA	ND[a]	ND	>10	ND
EGTA	ND	ND	>10	ND
1,10-*o*-Phenanthroline	ND	0.032 ± 0.004	ND	120 ± 20
Dipicolinic acid	0.024 ± 0.004	ND	0.3 ± 0.03	380 ± 50

[a] ND, not determined.

All the carbapenem compounds tested (imipenem, biapenem, meropenem, and panipenem) behaved as good substrates ($k_{cat}/K_m = 0.12$ to 6 μM^{-1} s^{-1}).

The kinetic parameters of the enzymes produced by *P. aeruginosa* 101/1477 and *E. coli* BL21(DE3) against a panel of selected substrates were quite similar in our laboratory. For the enzyme purified from *P. aeruginosa* 101/1477, the indicated k_{cat}, K_m, and k_{cat}/K_m values were found for benzylpenicillin ($k_{cat} = 280 \pm 50$ s^{-1}, $K_m = 600 \pm 30$ μM, $k_{cat}/K_m = 0.47$ μM^{-1} s^{-1}), cefuroxime ($k_{cat} = 10 \pm 2$ s^{-1}, $K_m = 45 \pm 9$ μM, $k_{cat}/K_m = 0.22$ μM^{-1} s^{-1}), ceftazidime ($k_{cat} = 8 \pm 2$ s^{-1}, $K_m = 35 \pm 5$ μM, $k_{cat}/K_m = 0.22$ μM^{-1} s^{-1}), cefotaxime ($k_{cat} = 2 \pm 0.5$ s^{-1}, $K_m = 4 \pm 0.5$ μM, $k_{cat}/K_m = 0.5$ μM^{-1} s^{-1}), cephaloridine ($k_{cat} = 50 \pm 10$ s^{-1}, $K_m = 20 \pm 10$ μM, $k_{cat}/K_m = 2.5$ μM^{-1} s^{-1}), and imipenem ($k_{cat} = 40 \pm 4$ s^{-1}, $K_m = 40 \pm 5$ μM, $k_{cat}/K_m = 1$ μM^{-1} s^{-1}).

Our kinetic data are in good agreement with the data reported by Muramo et al. (21), confirming that our enzyme was similar to the carbapenemase from *S. marcescens* FHSM4055. Surprisingly, we found major discrepancies with the kinetic data obtained by Osano et al. (22) with the metallo-β-lactamase of *S. marcescens* TN9106. The differences observed for the K_m values might be due to different assay conditions.

The catalytic properties of IMP-1 toward β-lactam antibiotics were similar to those exhibited by two other clinically relevant metallo-β-lactamases, those of *B. fragilis* CcrA (31) and *S. maltophilia* (7, 11, 12, 13). IMP-1 is the most active against moxalactam and cefoxitin. These compounds are poorly hydrolyzed by the *B. cereus* enzyme and are poor inactivators of the *Aeromonas* β-lactamase. Among all the studied Zn β-lactamases, the *Aeromonas* enzyme (CphA) can be considered a strict carbapenemase since it is significantly active only against carbapenems. Cephaloridine and cefoxitin are poorly hydrolyzed or behave as inactivators, respectively. All the other known metalloenzymes exhibit broad spectra of activity. For example, the oximinocephalosporins cefotaxime and ceftazidime are well hydrolyzed by these enzymes. By contrast, none of the Zn β-lactamases could hydrolyze monobactams such as aztreonam.

Influence of the zinc ion concentration on the enzyme activity. At pH 7.5, when increasing Zn^{2+} ion concentrations were added to the enzyme, the initial rate of nitrocefin hydrolysis was not affected. The k_{cat} and K_m values in the absence ($K_m = 63 \pm 10$ μM, $k_{cat} = 27 \pm 3$ s^{-1}, $k_{cat}/K_m = 0.4$ μM^{-1} s^{-1}) and in the presence ($K_m = 60 \pm 15$ μM, $k_{cat} = 30 \pm 5$ s^{-1}, $k_{cat}/K_m = 0.5$ μM^{-1} s^{-1}) of 100 μM Zn^{2+} were not significantly different.

Inactivation by Zn-chelating agents. Of the four chelating agents tested, EDTA and EGTA behaved as poor inactivators (Table 4). Even at final concentrations of 10 mM and after 1 h, the activity was reduced by only 10%. For 1,10-*o*-phenanthroline and dipicolinic acid, a time-dependent pseudo-first-order inactivation was observed. The value of the pseudo-first-order rate constant k_i increased with the concentration of the che-

lating agent in a hyperbolic manner with dipicolinic acid and linearly with 1,10-*o*-phenanthroline. These data indicate that the chelators do not act by scavenging the free metal but act via the formation of a transient enzyme-metal-chelator ternary complex. In the case of dipicolinic acid, the curve was analyzed on the basis of equation 1 assuming that k_2' was negligible. With 1,10-*o*-phenanthroline, on the basis of equation 2, k_{-2}' was obtained by extrapolation of the line to the ordinate.

1,10-*o*-Phenanthroline and dipicolinic acid inactivated IMP-1 with a rather good efficiency ($k_{+2}/K > 100$ μM^{-1} s^{-1}). The Zn enzyme produced by *A. hydrophila* was more efficiently inhibited by the chelating agents than IMP-1 (16). For example, the K values for dipicolinic acid were 5 and 300 μM for CphA and IMP-1, respectively.

pH dependence of activity and zinc content of IMP-1. The pH dependence of the Zn content of the metallo-β-lactamase of *P. aeruginosa* was measured by ICPMS (Table 5). Cd(II), Co(II), Ca(II), Cu(II), Mn(II), and Ni(II) were not found. In the absence of excess of metal and between pH 6 and pH 8, the enzyme contained almost two zinc ions per molecule. At pH 5 the nature of the buffer influenced the [Zn]/[enzyme] molar ratio. In citrate buffer, the ratio decreased to a value of 0.3, while in acetate buffer, the ratio was close to 2. Finally, the pH dependence of the catalytic efficiency of the enzyme against imipenem in the absence of added Zn^{2+} was determined. The IMP-1 metallo-β-lactamase exhibited a maximum activity against imipenem between pH 6.5 and pH 7.5. All metallo-β-lactamases studied exhibit two Zn-binding sites. However, the occupancy of the second binding site has a different effect on the catalytic activities of the enzymes. For the *B. cereus* 569H enzyme, the catalytic efficiency is not strongly modified by the presence of a second zinc ion (2). The *A. hydrophila* AE036 enzyme is fully active with one zinc, but its activity is inhibited by the binding of a second zinc ion (16). The *B. fragilis* metallo-β-lactamase tightly binds to two zinc ions at pH 7 (7), but the activity of the mono zinc form is unknown at present, and the same situation appears to prevail for the IMP-1 enzyme.

Conclusions. The spread of the IMP-1 metallo-β-lactamase in major nosocomial strains is one of the most powerful responses toward the anti-infective strategies widely used in several hospitals in Japan. In the study described in this report, a recombinant *E. coli* strain was used to produce large amount of IMP-1. The enzyme produced in *E. coli* and *P. aeruginosa* exhibited the same properties. The metal content of the protein was not significantly affected by the pH. Under all conditions tested except when citrate was used as the buffer, the metallo-β-lactamase contained two zinc ions. As expected, the enzyme was inactivated by metal chelating agents. These compounds did not act by scavenging the free metal, and therefore, the transient formation of a ternary enzyme-metal-chelator complex is proposed. Finally, at pH 7.5, the enzymatic activity

TABLE 5. Metal/enzyme ratios and catalytic efficiencies of the metallo-β-lactamase at different pH values[a]

pH	Buffer	[Zn]/[enzyme]	k_{cat}/K_m (μM^{-1} s^{-1})
5	Acetate	1.8 ± 0.2	0.8
5	Citrate	0.3 ± 0.1	<0.01
6	Cacodylate	1.9 ± 0.2	1
6.5	Cacodylate	1.8 ± 0.2	1.2
7	Cacodylate	1.9 ± 0.2	1.2
7.5	HEPES	1.7 ± 0.2	1.2
8	HEPES	1.8 ± 0.2	0.5

[a] The substrate was imipenem.

was not influenced by the presence of increasing concentration of free zinc ions.

The di-zinc form of the enzyme exhibited a broad-spectrum activity profile. Only the monobactam compounds and temocillin seemed to escape the hydrolytic action of the metallo-β-lactamase. A comparison of the activities of the mono-zinc and di-zinc forms of IMP-1 is being performed. Furthermore, detailed structural and mechanistic studies of IMP-1 are in progress.

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