teicoplanin with three amino acid substitutions in the *vanS* gene.

The same amino acid substitutions are found in the *vanS* gene of VanA-type VRE strains isolated from Thai chicken, which also exhibit high-level resistance to vancomycin and low-level resistance to teicoplanin (Ozawa *et al.* 2002). The VanA-type resistance of KC122.1, 122.3 and VRE from Thai chicken transferred to FA2-2 in filter mating at a frequency of 10^{-5} and 10^{-4} per donor cell respectively (Ozawa *et al.* 2002). In both cases, the vanA cluster resides on a conjugative plasmid and it is evident that conjugative plasmids play a major role in the dissemination of VRE.

By sequencing and comparison of the *ddl* gene of KC122.1, KC122.3 and *E. faecalis* FA2-2, it was suggested that amino acid substitution (Gly9–Asp) resulted in the inactivation of *ddl* and the requirement of *vanA* ligase activity for growth. It has been shown that the vancomycin-dependent strain is associated with mutation(s) in the *ddl* gene in the host chromosome, resulting in the synthesis of an inactive D-Ala : D-Ala ligase and the requirement of the D-Ala : D-Lac ligase encoded in the glycopeptide-resistance determinant (Sifaoui and Gutmann 1997). In addition, this mutation is located close to the conserved amino acid predicted to play a key role in enzymatic activity (Fig. 1) (Gholizadeh *et al.* 2001). It is quite likely that this amino acid substitution has an effect on the activity of the D-Ala : D-Ala ligase.

All vancomycin-dependent strains that have been isolated to date originate from situations in which vancomycin was used intensively. KC122.1 is the first vancomycin-dependent strain isolated from a nonhuman source. It has been suggested that the use of avoparcin in animal food results in an increased incidence of acquired vancomycin resistance (van den Bogaard *et al.* 1997; Ike *et al.* 1999). Avoparcin was used in Japan between 1989 and 1996, but has since been prohibited. So far, VRE has not been isolated from Japanese chicken meat. It is important to review the use of antibiotics as a growth promoter in poultry and food animals, otherwise poultry farming and animal husbandry might become a major reservoir of antibiotic resistant bacteria.

ACKNOWLEDGEMENTS

REFERENCES

Arthur, M., Molinas, C. and Courvalin, P. (1992) The VanS–VanR two component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* 174, 2582–2591.

Arthur, M., Reynolds, P.E. and Courvalin, P. (1996) Glycopeptide resistance in enterococci. *Trends Microbiol* 4, 401–407.

Baptista, A.E., Fantin, B., Depardieu, F., Arthur, M., Courvalin, P. and Carbon, C. (1997) Selection of glycopeptide-resistant mutants of VanB-type *Enterococcus faecalis* BM4281 *in vitro* and in experimental endocarditis. *J Infect Dis* 175, 598–605.

van den Bogaard, A.E., Jensen, L.B. and Stobberingh, E.E. (1997) Vancomycin-resistant enterococci in turkeys and farmers. *N Engl J Med* 337, 1558–1559.

Clewell, D., Tomich, P., Gawron-Burke, M., Franke, A.E., Yagi, A. and An, F. (1982) Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. *J Bacteriol* 152, 1220–1230.

Dever, L.L., Smith, S.M., Handwerger, S. and Eng, R.H. (1995) Vancomycin-dependent *Enterococcus faecium* isolated from stool following oral vancomycin therapy. *J Clin Microbiol* 33, 2770–2773.

Dunny, G.M., Craig, R.A., Carron, R.L. and Clewell, D.B. (1979) Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. *Plasmid* 2, 454–465.

Farrag, N., Eltringham, I. and Liddy, H. (1996) Vancomycin-dependent *Enterococcus faecalis*. *Lancet* 348, 1581–1582.

Fraimow, H.S., Jungkind, D.L., Lander, D.W., Delso, D.R. and Dean, J.L. (1994) Urinary tract infection with an *Enterococcus faecalis* isolate that requires vancomycin for growth. *Ann Intern Med* 121, 22–26.

Gholizadeh, Y., Prevost, M., Van Bambeke, F., Casadewall, B., Tulkens, P.M. and Courvalin, P. (2001) Sequencing of the *ddl* gene and modeling of the mutated D-alanine:D-alanine ligase in glycopeptide-dependent strains of *Enterococcus faecium*. *Protein Sci* 10, 836–844.

Green, M., Shlaes, J.H., Barbadora, K. and Shlaes, D.M. (1995) Bacteremia due to vancomycin-dependent *Enterococcus faecium*. *Clin Infect Dis* 20, 712–714.

Hashimoto, Y., Tanimoto, K., Ozawa, Y., Murata, T. and Ike, Y. (2000) Amino acid substitutions in the VanS sensor of the VanA-type vancomycin-resistant enterococcus strains result in high-level vancomycin resistance and low-level teicoplanin resistance. *FEMS Microbiol Lett* 185, 247–254.

Ike, Y. and Clewell, D.B. (1984) Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn917 as an insertional mutagen. *J Bacteriol* 158, 777–783.

Ike, Y., Tanimoto, K., Ozawa, Y., Nomura, T., Fujimoto, S. and Tomita, H. (1999) Vancomycin-resistant enterococci in imported chickens in Japan. *Lancet* 353, 1854.

Kak, V. and Chow, J.W. (2002) Acquired antibiotic resistances in Enterococci. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* ed. Gilmore, M.S. pp. 355–383. Washington, DC: American Society for Microbiology.

Korten, V. and Murray, B.E. (1993) The nosocomial transmission of enterococci. *Curr Opin Infect Dis* 6, 498–505.

Murray, B.E. (1990) The life and times of *Enterococcus*. *Clin Microbiol Rev* 3, 46–65.

National Committee for Clinical Laboratory Standards. (2000) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 5th edn. Approved standard M7-A4. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Nosocomial Infections Surveillance System Report. (1999) Data summary from January 1990–May 1999. *Am J Infect Control* **27**, 520–532.

Ozawa, Y., Tanimoto, K., Nomura, T., Yoshinaga, M., Arakawa, Y. and Ike, Y. (2002) Vancomycin-resistant enterococci in humans and imported chickens in Japan. *Appl Environ Microbiol* **68**, 6457–6461.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sifaoui, F. and Gutmann, L. (1997) Vancomycin dependence in a VanA-probing *Enterococcus avium* strain with a nonsense mutation in the natural D-Ala : D-Ala ligase gene. *Antimicrob Agents Chemother* **41**, 1409.

219

Gene Mutations Responsible for Overexpression of AmpC β-Lactamase in Some Clinical Isolates of *Enterobacter cloacae*

Kenichi Kaneko,[1] Ryoichi Okamoto,[1,2] Ryuichi Nakano,[1] Sayoko Kawakami,[3] and Matsuhisa Inoue[1,2]*

Department of Environmental Infectious Diseases, Graduate School of Medical Sciences,[1] and Department of Microbiology, School of Medicine,[2] Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa, and Department of Central Laboratory, School of Medicine, Teikyo University, 2-11-1, Kaga, Itabashi-ku, Tokyo,[3] Japan

AmpC regulatory genes in 21 ceftazidime-resistant clinical isolates of *Enterobacter cloacae* (MICs of ≥ 16 µg/ml) were characterized. All isolates exhibited AmpC overproduction due to AmpD mutation. Additionally, we found two AmpR mutants among the isolates. This is the first report of chromosomal *ampR* mutation in clinical isolates of *E. cloacae*.

Resistance of members of the family *Enterobacteriaceae* (e.g., *Enterobacter cloacae* and *Serratia marcescens*) to expanded-spectrum cephalosporins is a serious clinical problem (14). The resistance of these bacteria is principally due to constitutive overexpression of chromosomal *ampC* (which encodes AmpC, a class C β-lactamase) (5, 33). The *ampC* gene is located on the chromosome of many gram-negative bacilli and is inducible in the presence of β-lactam antibiotics (9).

The mechanism of AmpC induction is closely linked to a cell wall recycling system (8, 12). The cell walls of gram-negative bacilli must be reconstituted during growth. *Escherichia coli* (and presumably *E. cloacae*) degrades 40 to 50% of the cell wall peptidoglycans during each generation, and 90% of the degradation products are recycled (28). Peptidoglycan degradation products (anhydromuropeptides) released into the periplasm are transported into the cytoplasm by AmpG, a transmembrane permease (6, 16, 22). Subsequently, these anhydromuropeptides are hydrolyzed by AmpD, which is a cytoplasmic *N*-acetyl-anhydromuramyl-L-alanine amidase that specifically hydrolyzes several anhydromuropeptides such as (GlcNAc-) anhMurNAc tripeptide and GlcNAc-anhMurNAc tetrapeptide (11). This action of AmpD prepares anhydromuropeptides for further recycling. In the presence of β-lactams, however, anhydromuropeptides increase in both the periplasm and cytoplasm. Cytoplasmic anhydromuropeptides that have avoided hydrolysis by AmpD bind to AmpR, a transcriptional regulator of *ampC* (9, 19, 21). In the absence of an activating ligand, AmpR represses *ampC* expression with UDP-MurNAc pentapeptide, but cell wall degradation products [(GlcNAc-) anhMurNAc tripeptides] may be an AmpR-activating ligand that causes the activation of *ampC* expression. In AmpD-defective bacterial strains, unhydrolyzed anhydromuropeptides accumulate in the cytoplasm (7, 13), and this causes activation of AmpR, which results in the semiconstitutive or constitutive expression of *ampC*. The role of AmpD in the induction of AmpC in gram-negative bacilli has been studied in detail (15, 18, 20).

AmpD-dependent constitutive expression of AmpC occurs in vitro at a frequency of 10^{-5} to 10^{-7} (19, 29). In addition, we previously reported that AmpR-dependent constitutive overproduction of AmpC occurred at a frequency of 10^{-6} in AmpD-defective strains (17). However, the isolation rate of AmpD mutants among cephalosporin-resistant clinical strains has not been determined. In addition, there have been no reports describing the isolation of chromosomal AmpR mutants from clinical isolates.

Twenty-one ceftazidime-resistant strains of *E. cloacae* KU6323 to KU6332, KU6334, KU6336 to KU6341, and KU6343 to KU6346 were isolated during a 1-year period in 1999 from patients attending a university-affiliated hospital in Tokyo, Japan. KU4892, KU4894, and KU1917 were positive-control strains ($bla_{\text{TEM-1}}$, $bla_{\text{TOHO-1}}$, and $bla_{\text{IMP-1}}$, respectively) and were created by introduction of plasmids pKU507, pMTY010, and pMS361, respectively (10, 34). *E. cloacae* KU3262 (wild-type AmpD) was a clinical isolate and used for complementation assay of *ampR* (17). The MICs were determined by the agar dilution method according to CLSI (formerly NCCLS) guidelines (25). β-Lactamase activity was detected by a colorimetric assay using cephalothin as a substrate (17, 27). For inhibition of class A β-lactamase, clavulanic acid (at a final concentration of 5 µg/ml) was added to either substrate solution or extracted enzyme solution. Results were analyzed by Student's *t* test, and values were expressed as the means ± standard deviations (SDs). A P of <0.001 was defined as indicating statistical significance.

To detect β-lactamase genes, except for chromosomal *ampC*, PCR amplification was performed. Template DNA solution was prepared by the boiling preparation method (32). PCR was performed in a final volume of 100 µl containing 1× PCR buffer, 200 µM deoxynucleoside triphosphate, 0.2 µM of each primer, 2.5 U of HotStarTaq DNA polymerase (QIA-GEN, Tokyo, Japan), and 5-µl aliquot of a template DNA solution. After initial incubation at 95°C for 15 min, DNA was amplified by 25 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The final cycle included additional incubation at 72°C for 7 min. The primers used to amplify each *bla* gene were as follows: TEM#1 (5'-AAGCCA TACCAAACGACGAG-3') and TEM#2 (5'-ATTGTTGCC GGGAAGCTAGA-3') for bla_{TEM} (108 bp, a PCR product

---

* Corresponding author. Mailing address: Department of Environmental Infectious Diseases, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan. Phone: 81-42-778-9349. Fax: 81-42-778-9350. E-mail: matsu @kitasato-u.ac.jp.

TABLE 1. β-Lactamase activities of *E. cloacae* clinical isolates and MICs of ceftazidime

Strain	β-Lactamase activity (U/mg protein)[a]		MIC of ceftazidime (μg/ml)		β-Lactamase profile[b]
	Clinical isolates	Transformants[c]	Clinical isolates	Transformants[c]	
KU6344	37.02 ± 0.59	15.66 ± 0.2	32	0.25	
KU6345	24.13 ± 0.44	1.82 ± 0.04	>128	>128	bla_{IMP}
KU6339	14.64 ± 1.21	<0.01	64	0.125	
KU6329	12.63 ± 0.13	0.05 ± 0.01	>128	1	
KU6326	12.18 ± 0.43	<0.01	>128	8	
KU6331	11.95 ± 0.47	0.33 (0.07 ± 0.04)	128	0.5	bla_{TEM}
KU6332	11.50 ± 0.22	0.30 ± 0.01 (0.06 ± 0.01)	128	0.5	bla_{TEM}
KU6330	9.31 ± 0.07	0.02	64	0.5	
KU6325	7.56 ± 0.25	<0.01	32	0.125	
KU6340	7.28 ± 0.13	0.05 ± 0.01	64	0.25	
KU6328	6.13 ± 0.09	0.05 ± 0.01	128	0.5	
KU6323	6.03 ± 0.14	<0.01	32	0.125	
KU6341	5.76 ± 0.28	<0.01	32	1	
KU6343	3.41 ± 0.23	2.97 ± 0.11 (1.43 ± 0.08)	128	64	bla_{TOHO}
KU6334	2.55 ± 0.08	0.34 ± 0.02	32	1	
KU6346	0.88 ± 0.01	0.39 ± 0.01 (0.21 ± 0.02)	64	1	
KU6327	0.51 ± 0.04	<0.01	16	0.5	
KU6338	0.41 ± 0.01	0.02 ± 0.01	32	0.5	
KU6336	0.39 ± 0.01	0.03 ± 0.01	16	0.25	
KU6324	0.31 ± 0.03	<0.01	16	0.5	
KU6337	0.14	<0.01	32	0.25	
KU3262	0.07 ± 0.01	NT[d]	0.25	NT	NT

[a] Values are the means ± SDs for three independent experiments. One unit of β-lactamase activity was defined as the amount that hydrolyzed 1 mmol of cephalothin in 1 min at 30°C. Values in parentheses indicate the activity determined in the presence of clavulanic acid (5 μg/ml).
[b] bla_{TEM}, bla_{TOHO}, and bla_{IMP} indicate β-lactamase genotypes detected by PCR amplification in addition to chromosomal *ampC*.
[c] Strains transformed with plasmid pKU420 containing the wild-type *ampD* gene from *E. coli*.
[d] NT, not tested.

length), TOHO#1 (5′-TGGAAGCCCTGGAGAAAAGT-3′) and TOHO#2 (5′-CTTATCGCTCTCGCTCTGTT-3′) for bla_{TOHO} (448 bp), and IMP#1 (5′-CATGGTTTGGTGGTTC TTGT-3′) and IMP#2 (5′-ATAATTTGGCGGACTTTGGC-3′) for bla_{IMP} (164 bp). Amplified PCR products were confirmed by agarose gel electrophoresis.

Transformation of *ampD* mutants with wild-type *ampD* caused a decrease of AmpC expression (1). Therefore, complementation analysis of all isolates was performed using pKU420 (a plasmid containing wild-type *ampD*). pKU420 was constructed by blunt end cloning of a 994-bp PCR product into the SmaI site of pMW219 (Nippon Gene, Tokyo, Japan). The 994-bp PCR product, including the promoter and *ampD*-coding region of *E. coli* K-12 χ1037, was amplified by PCR using *Pyrobest* DNA polymerase (Takara, Kyoto, Japan) and the following pair of primers: ampD#1 (5′-ACGTCGGGTGTCAGGGTTAT-3′) and ampD#2 (5′-GTGGCACGGGCATGGCTATCA-3′). The PCR product was purified using a Qiaquick PCR purification kit (QIAGEN) and was subsequently ligated with SmaI-digested pMW219. *E. coli* K-12 XL1-Blue was used as the host strain for cloning. Transformation with the ligated plasmid was performed by electroporation as described previously (1). *ampD* clones were selected by incubation in LB agar containing 50 μg/ml of kanamycin, 100 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Takara), and 50 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Takara). The transcriptional orientation of *ampD* was opposite to the orientation of *lacZ*. Isolation of plasmids was performed using a QIAprep Spin Miniprep kit (QIAGEN). For the selection of clinical isolates transformed by pKU420, butirosin A (Sigma-

Aldrich, St. Louis, Mo.) (at a final concentration of 50 μg/ml) was used.

Ceftazidime MICs and β-lactamase activities of clinical isolates of *E. cloacae* are shown in Table 1. The clinical isolates exhibited various levels of β-lactamase activity, suggesting a diversity of genetic changes in *ampD*. After transformation with pKU420, all of the clinical isolates showed a dramatic decrease of β-lactamase activity. This indicated that all clinical isolates resistant to ceftazidime harbor AmpD mutations. However, seven transformed strains (KU6331, KU6332, KU6334, KU6343, KU6344, KU6345, and KU6346) exhibited residual β-lactamase activity comparable to that of KU3262, a ceftazidime-susceptible strain. bla_{TEM}, bla_{TOHO}, and bla_{IMP} were detected in these strains, except for KU6334, KU6344, and KU6346 (Table 1). Additionally, β-lactamase activity was inhibited by clavulanic acid in these strains, except for KU6334, KU6344, and KU6345. Since these β-lactamases can hydrolyze cephalosporins, such as ceftazidime, residual β-lactamase activity and the high MIC of ceftazidime in *ampD* transformants seem to be principally due to these β-lactamases (10, 26). However, the β-lactamase activities of KU6343 (2.97 U/mg protein) and KU6346 (0.39 U/mg protein) were only partially inhibited (to 1.43 and 0.21 U/mg protein, respectively). Thus, we confirmed the presence of residual AmpC activity in the KU6334, KU6343, KU6344, and KU6346 strains.

The DNA sequence of the *ampD* region was determined in six selected strains (KU6324, KU6325, KU6327, KU6328, KU6329, and KU6339). DNA sequencing was performed with an ABI PRISM 310 DNA sequencer (Applied Biosystems, Tokyo, Japan) using a BigDye Terminator v1.1 cycle sequenc-

TABLE 2. β-Lactamase activities and MICs of ceftazidime for
E. cloacae bearing *ampR* genes from clinical isolates

Strain	β-Lactamase activity (U/mg protein)[a]		MIC (μg/ml) of ceftazidime
	Noninduced	Induced[b]	
KU3262	0.07 ± 0.01	9.97 ± 0.23	0.25
KU3262/pKU430	1.56 ± 0.11[c]	14.40 ± 0.70[c]	2
KU3262/pKU431	1.35 ± 0.12[c]	13.98 ± 0.30[c]	8
KU3262/pKU432	0.08 ± 0.02	12.15 ± 2.67	0.125

[a] Values are the means ± SDs for three independent experiments. One unit of β-lactamase activity was defined as the amount that hydrolyzed 1 mmol of cephalothin in 1 min at 30°C.

[b] β-Lactamase was induced with imipenem at 1/4 of the MIC for KU3262 (0.125 μg/ml).

[c] $P < 0.001$ versus KU3262.

ing kit (Applied Biosystems) according to the manufacturer's instructions. A homology search of the database was performed using the BLASTN program. All of the strains had mutations of AmpD compared with the wild-type sequence (GenBank accession no. Z14003). These amino acid substitutions were as follows: Ala-94 to Pro in strain KU6339; Asn-150 to Ile in KU6325; Leu-38 to Phe and Ala-60 to Val in KU6327 and KU6324; and Phe-63 to Tyr, Glu-96 to Gln, Ile-106 to Val, Arg-108 to Leu, and Ala-122 to Gly in KU6329 and KU6328. It has already been reported that not only mutations of the active-site core residues but also mutations of structurally independent residues can cause constitutive expression of AmpC (29). Therefore, we could not find any substitutions in the active-site core residues of AmpD, but each mutation seemed to cause overproduction of AmpC in the isolates.

Bartowsky et al. previously reported that a mutation of AmpR resulted in the semiconstitutive expression of AmpC (3, 4). In a previous study, we confirmed that introduction of a plasmid harboring mutant *ampR* into wild-type *E. cloacae* resulted in the constitutive expression of AmpC (17). Therefore, we used *E. cloacae* KU3262 with wild-type *ampD* to assess the effect of *ampR* mutation. The *ampR* region, including its promoter region and the partial *ampC* region, was amplified by PCR as described above. The primers were C#7 (5'-GCCTT GCCAAACGTGTAATAGTGC-3') and R#2 (5'-AAATCCA TGTGCCAAGCGGTAAAT-3') for KU6334 and KU6343, while they were 6344C#1 (5'-GTTTGTGCAGTGACGGGT GTG-3') and 6344R#2 (5'-TCCTGACGGTGGTTACGCTG AT-3') for KU6344. Amplified PCR products (1,323 bp for KU6334 and KU6343 and 1,311 bp for KU6344) were purified and subsequently cloned using a PCR cloning kit (QIAGEN) according to the manufacturer's instructions. Although strain KU6346 exhibited residual AmpC activity, cloning of its *ampR* was not performed because there were no amino acid substitutions in the coding region. Transformation, clonal selection, and plasmid extraction were done as described above for *ampD*. The clonal *ampR* genes were subcloned into the BamHI and HindIII sites of pMW219.

The AmpC activity of *E. cloacae* KU3262 harboring these recombinant plasmids is shown in Table 2. Introduction of pKU430 and pKU431 into strain KU3262, significantly increased AmpC activity by 21-fold (1.56 U/mg protein) and 19-fold (1.35 U/mg protein) above the basal level (0.07 U/mg protein), respectively. Similarly, the MICs of ceftazidime also

increased 16-fold (2 μg/ml) and 64-fold (8 μg/ml) above the basal value (0.25 μg/ml). In contrast, transformants of KU3262 harboring pKU432 did not exhibit any increase in the MIC of ceftazidime or AmpC activity. By comparison with wild-type *ampR* in MHN1 (GenBank accession no. X04730), amino acid substitutions were identified in all strains. The amino acid substitutions were Arg-86 to Ser in KU6334 and Thr-64 to Ile in KU6343. As for Arg-86, we have previously reported that introduction of a plasmid expressing this mutation of AmpR could increase AmpC activity (17). Additionally, the same phenomenon was observed by introducing pKU431 into KU3262. Considering these findings, mutations of residues 64 and 86 alter the conformational change of AmpR triggered by binding to anhydromuropeptides (the AmpR-activating ligand). On the other hand, the amino acids affecting constitutive AmpC expression (Arg-86, Gly-102, and Asp-135) were conserved in AmpR from KU6344, and introduction of pKU432 did not significantly induce expression of AmpC. Therefore, the residual AmpC activity of KU6344 appears to be related to unknown factors.

In the present study, we found that the resistance of *E. cloacae* clinical isolates to ceftazidime was principally due to AmpD-dependent constitutive expression of AmpC. On the other hand, we identified chromosomal AmpR mutants among clinical isolates. One of the problems in clinical practice is the spread of plasmid-mediated AmpC (30). In particular, strains expressing inducible plasmid-mediated AmpC with functional AmpR have been isolated (2, 23, 24, 31). Among those, bla_{CFE-1}, a plasmid-encoded AmpC that we reported previously, seems to be involved in AmpR-dependent constitutive expression of AmpC (24). Our results suggest that chromosomal AmpR mutations may have the potential to confer not only high-level constitutive expression of chromosomal AmpC but also plasmid-mediated constitutive AmpC overproduction to clinical isolates.

Nucleotide sequence accession numbers. The nucleotide sequence data in this study will appear in the EMBL/GenBank/ DDBJ nucleotide sequence databases. The accession numbers are as follows: AY789446 for *ampR* from *E. cloacae* KU6334, AY789447 for *ampR* from KU6343, and AY789448 for *ampR* from KU6344.

REFERENCES

1. **Bagge, N., O. Ciofu, M. Hentzer, J. I. A. Campbell, M. Givskov, and N. Høiby.** 2002. Constitutive high expression of chromosomal β-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS*1669*) located in *ampD*. Antimicrob. Agents Chemother. **46:**3406–3411.
2. **Barnaud, G., G. Arlet, C. Verdet, O. Gaillot, P. H. Lagrange, and A. Philippon.** 1998. *Salmonella enteritidis*: AmpC plasmid-mediated inducible β-lactamase (DHA-1) with an *ampR* gene from *Morganella morganii*. Antimicrob. Agents Chemother. **42:**2352–2358.
3. **Bartowsky, E., and S. Normark.** 1991. Purification and mutant analysis of *Citrobacter freundii* AmpR, the regulator for chromosomal AmpC β-lactamase. Mol. Microbiol. **5:**1715–1725.
4. **Bartowsky, E., and S. Normark.** 1993. Interactions of wild-type and mutant AmpR of *Citrobacter freundii* with target DNA. Mol. Microbiol. **10:**555–565.
5. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification

scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.

6. **Cheng, Q., and J. T. Park.** 2002. Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides. J. Bacteriol. **184:** 6434–6436.

7. **Dietz, H., D. Pfeifle, and B. Wiedemann.** 1996. Location of *N*-acetylmuramyl-L-alanyl-D-glutamylmesodiaminopimelic acid, presumed signal molecule for β-lactamase induction, in the bacterial cell. Antimicrob. Agents Chemother. **40:**2173–2177.

8. **Hanson, N. D., and C. C. Sanders.** 1999. Regulation of inducible AmpC beta-lactamase expression among *Enterobacteriaceae.* Curr. Pharm. Des. **5:** 881–894.

9. **Honoré, N., M. H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli.* EMBO J. **5:** 3709–3714.

10. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajyo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli.* Antimicrob. Agents Chemother. **39:**2269–2275.

11. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. V. Beeumen, D. Mengin-Lecreulx, J. V. Heijenoort, J. T. Park, S. Normark, and J. M. Frère.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15:**553–559.

12. **Jacobs, C., J. M. Frère, and S. Normark.** 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell **88:**823–832.

13. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.

14. **Jones, R. N.** 1998. Important and emerging β-lactamase-mediated resistance in hospital-based pathogens: the AmpC enzymes. Diagn. Microbiol. Infect. Dis. **31:**461–466.

15. **Kopp, U., B. Wiedemann, S. Lindquist, and S. Normark.** 1993. Sequences of wild-type and mutant *ampD* genes of *Citrobacter freundii* and *Enterobacter cloacae.* Antimicrob. Agents Chemother. **37:**224–228.

16. **Korfmann, G., and C. C. Sanders.** 1989. *ampG* is essential for high-level expression of AmpC β-lactamase in *Enterobacter cloacae.* Antimicrob. Agents Chemother. **33:**1946–1951.

17. **Kuga, A., R. Okamoto, and M. Inoue.** 2000. *ampR* gene mutations that greatly increase class C β-lactamase activity in *Enterobacter cloacae.* Antimicrob. Agents Chemother. **44:**561–567.

18. **Langaee, T. Y., L. Gagnon, and A. Huletsky.** 2000. Inactivation of the *ampD* gene in *Pseudomonas aeruginosa* leads to moderate-basal-level and hyperinducible AmpC β-lactamase expression. Antimicrob. Agents Chemother. **44:** 583–589.

19. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii ampC* β-lactamase induction. Proc. Natl. Acad. Sci. USA **82:**4620–4624.

20. **Lindberg, F., S. Lindquist, and S. Normark.** 1987. Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. J. Bacteriol. **169:**1923–1928.

21. **Lindquist, S., F. Lindberg, and S. Normark.** 1989. Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. J. Bacteriol. **171:** 3746–3753.

22. **Lindquist, S., K. Weston-Hafer, H. Schmidt, C. Pul, G. Korfmann, J. Erickson, C. Sanders, H. H. Martin, and S. Normark.** 1993. AmpG, a signal transducer in chromosomal β-lactamase induction. Mol. Microbiol. **9:**703–715.

23. **Miriagou, V., L. S. Tzouvelekis, L. Villa, E. Lebessi, A. C. Vatopoulos, A. Carattoli, and E. Tzelepi.** 2004. CMY-13, a novel inducible cephalosporinase encoded by an *Escherichia coli* plasmid. Antimicrob. Agents Chemother. **48:** 3172–3174.

24. **Nakano, R., R. Okamoto, Y. Nakano, K. Kaneko, N. Okitsu, Y. Hosaka, and M. Inoue.** 2004. CFE-1, a novel plasmid-encoded AmpC β-lactamase with an *ampR* gene originating from *Citrobacter freundii.* Antimicrob. Agents Chemother. **48:**1151–1158.

25. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., vol. 17, no. 2. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.

26. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in gram-negative aerobes. Clin. Microbiol. Infect. **8:**321–331.

27. **Okamoto, R., T. Okubo, and M. Inoue.** 1996. Detection of genes regulating β-lactamase production in *Enterococcus faecalis* and *Staphylococcus aureus.* Antimicrob. Agents Chemother. **40:**2550–2554.

28. **Park, J. T.** 1995. Why does *Escherichia coli* recycle its cell wall peptides? Mol. Microbiol. **17:**421–426.

29. **Petrosino, J. F., A. R. Pendleton, J. H. Weiner, and S. M. Rosenberg.** 2002. Chromosomal system for studying AmpC-mediated β-lactam resistance mutation in *Escherichia coli.* Antimicrob. Agents Chemother. **46:**1535–1539.

30. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.

31. **Reisbig, M. D., and N. D. Hanson.** 2002. The ACT-1 plasmid encoded AmpC β-lactamase is inducible: detection in a complex β-lactamase background. J. Antimicrob. Chemother. **49:**557–560.

32. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

33. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23:**918–925.

34. **Yano, H., A. Kuga, R. Okamoto, H. Kitasato, T. Kobayashi, and M. Inoue.** 2001. Plasmid-encoded metallo-β-lactamase (IMP-6) conferring resistance to carbapenems, especially meropenem. Antimicrob. Agents Chemother. **45:** 1343–1348.

the bla_{TEM-21} gene that encodes a β-lactamase of pI 6.4.[7] We have recently reported an endemic situation in this nursing home owing to the dissemination of TEM-21-producing strains of *P. aeruginosa* and enterobacteria, and a TEM-21-encoding plasmid.[4,6] Conjugation experiments by a filter mating technique between the clinical strain Af1930 and the azide-resistant *Escherichia coli* C600, the rifampicin- and nalidixic acid-resistant *E. coli* K12 or the rifampicin-resistant mutant *P. aeruginosa* ATCC 27853 as recipients, did not yield any ESBL-producing transconjugant ($<10^{-8}$). Plasmid DNA extract showed the presence of a large plasmid, but ESBL transfer by electroporation into *E. coli* DH5α remained unsuccessful. Moreover, the restriction profile of this large plasmid, obtained with the *Eco*RI enzyme, was different from that of the epidemic plasmid encountered in the enterobacteria strains of the nursing home (data not shown). Finally, a Southern-blot hybridization using a bla_{TEM} probe gave a smear of high molecular weight with the unrestricted whole-cell DNA, and an absence of signal with the unrestricted or *Nru*I-digested plasmid DNA of Af1930 (data not shown). These data strongly argued for a chromosomal location of the bla_{TEM-21} gene in *A. faecalis* Af1930 as in the epidemic *P. aeruginosa* strain.[6] Indeed, differences in origins of replication may lead to the elimination of plasmids from Enterobacteriaceae in glucose-non-fermenting, Gram-negative bacilli. Therefore, the persistence of the resistance genes in strains of *P. aeruginosa* or *A. faecalis* may have required their chromosomal integration. Using several laboratory-designed primers [C6T7, C6T7bis, aac(3)-IIb, TnpA4R and IRL4321],[6] DNA amplifications showed that in Af1930, as in enterobacteria, the bla_{TEM-21} gene was part of a Tn*801* transposon disrupted by IS*4321* and adjacent to the *aac(3)-II* gene. In addition, the *aac(6')-I* gene, present on the resistance plasmid epidemic among enterobacteria, was detected by PCR in Af1930, suggesting the complete chromosomal integration of the plasmid in Af1930, in contrast with the *P. aeruginosa* strains, in which this gene was lacking. In the previous report on the PER-1-producing *A. faecalis*, the ESBL-encoding gene was associated with a transposon-like element of the Tn*3* family located on a large non-conjugative plasmid and the acquisition of the bla_{PER-1} determinant was probably related to an outbreak of PER-1-expressing *P. aeruginosa* in the same hospital.[3,8]

The present work is the first report of a TEM-type ESBL in *A. faecalis*, highlighting the possible spread of these enzymes from Enterobacteriaceae to saprophytic and taxonomically distant Gram-negative bacilli, when the epidemic pressure becomes intense and providing that the epidemic plasmid may persist. However, chromosomal insertion should allow limited further dissemination of the ESBL.

References

1. Bizet J, Bizet C. Strains of *Alcaligenes faecalis* from clinical material. *J Infect* 1997; 35:167–9.

2. Bizet C, Tekaia F, Philippon A. In-vitro susceptibility of *Alcaligenes faecalis* compared with those of other *Alcaligenes* spp. to antimicrobial agents including seven β-lactams. *J Antimicrob Chemother* 1993; 32: 907–10.

3. Pereira M, Perilli M, Mantengoli E *et al.* PER-1 extended-spectrum β-lactamase production in an *Alcaligenes faecalis* clinical isolate resistant to expanded-spectrum cephalosporins and monobactams from a hospital in Northern Italy. *Microb Drug Resist* 2000; 6: 85–90.

4. Arpin C, Dubois V, Coulange L *et al.* Extended-spectrum β-lactamase-producing Enterobacteriaceae in community and private health care centers. *Antimicrob Agents Chemother* 2003; 47: 3506–14.

5. Jarlier V, Nicolas MH, Fournier G *et al.* Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988; 10: 867–78.

6. Dubois V, Arpin C, Noury P *et al.* Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. *J Clin Microbiol* 2005; 43: 4129–38.

7. Tessier F, Arpin C, Allery A *et al.* Molecular characterization of a TEM-21 β-lactamase in a clinical isolate of *Morganella morganii*. *Antimicrob Agents Chemother* 1998; 42: 2125–7.

8. Mantengoli E, Rossolini GM. Tn5393d, a complex Tn5393 derivative carrying the PER-1 extended-spectrum β-lactamase gene and other resistance determinants. *Antimicrob Agents Chemother* 2005; 49: 3289–96.

AmpC β-lactamase-mediated cefpodoxime-resistant *Escherichia coli* isolated from faecal samples of healthy volunteers

Kenichi Kaneko[1], Yoshinori Sato[1], Shu-ko Tokunaga[2]†, Sho-ko Tamaki[2]†, Ryoichi Okamoto[1,2] and Matsuhisa Inoue[1,2]*

[1]*Department of Environmental Infectious Diseases, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa, Japan;* [2]*Department of Microbiology, School of Medicine, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan*

Keywords: *E. coli*, AmpC promoter, CMY-2, resistance

*Corresponding author. Tel: 81-42-778-9349; Fax: 81-42-778-9350; E-mail: matsu@kitasato-u.ac.jp

†These authors contributed equally to this work.

Sir,
Escherichia coli is one of the most common pathogens causing nosocomial infections. In recent years, clinical reports of *E. coli* with extended-spectrum cephalosporin (ESC) resistance, particularly mediated by AmpC β-lactamase, have been increasing rapidly.[1–3] This indicates the spread of either plasmid-borne

Table 1. MIC values and β-lactamase activity of the *E. coli* isolates

| Strain | MIC (mg/L) | | | | | | | | | | β-Lactamase activity (U/mg of protein)[a] |
	AMP	AMP/CLA	CEF	FOX	FLO	CPD	CTX	CAZ	ATM	IPM	
χ1037	8	4	4	16	0.25	1	0.06	0.25	0.125	0.25	0.01
KU6763	>128	128	>128	64	16	128	4	16	4	0.125	1.50 ± 0.70
χ1037/pKU433	>128	>128	>128	128	32	>128	8	64	8	0.5	1.32 ± 0.70
KU6764	128	64	>128	32	0.5	16	0.5	2	2	0.125	0.99 ± 0.03

AMP, ampicillin; CLA, clavulanic acid; CEF, cefalotin; FOX, cefoxitin; FLO, flomoxef; CPD, cefpodoxime; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem.
[a]Values are the means ± SD from three independent experiments. One unit of β-lactamase activity was defined as the amount that hydrolysed 1 mmol of cefalotin in 1 min at 30°C.

AmpC or chromosomal AmpC mutants among clinical isolates. In order to analyse the population of drug-resistant microorganisms among normal microflora and detect microorganisms with the potential to cause pandemics in the future, extensive surveillance of drug-resistant microorganisms has been performed in healthy humans and animals. However, little is known about the existence of *E. coli* with AmpC-mediated ESC resistance in the normal intestinal microflora of healthy humans. Here, we report that two strains of ESC-resistant *E. coli* were detected in the faeces of healthy volunteers. These two isolates had different resistance mechanisms, which were CMY-2 (a plasmid-borne AmpC) and a mutation in the *ampC* promoter, respectively.

In 2003, 63 *E. coli* strains were isolated from faecal samples of healthy third-year medical students. Each bacterial strain was isolated from an individual student, and none of the students had received antibiotic therapy within 1 month before the sampling. MICs of nine β-lactams and a combination with β-lactam-inhibitor were determined by the agar dilution method according to CLSI (formerly NCCLS) guidelines (Table 1).

Two cefpodoxime-resistant *E. coli* strains (KU6763 and KU6764) were detected (Table 1). KU6763 and KU6764 both exhibited resistance to cefpodoxime (MIC = 128 and 16 mg/L, respectively). Measurement of AmpC activity and detection of plasmid-borne bla_{AmpC} were performed by a colorimetric assay and multiplex PCR, respectively as described previously.[4,5] The β-lactamase activity of both strains was >99-fold higher than that of χ1037, a cefpodoxime-susceptible control (1.5 ± 0.7 and 0.99 ± 0.03 U/mg of protein, respectively). Detection of plasmid-borne AmpC by multiplex PCR revealed a 462 bp PCR amplicon in KU6763. Cefpodoxime resistance of KU6763 was transmissible to χ1037 at a frequency of 1.1×10^{-7}, and this resistance was mediated by a 100 kb plasmid (designated as pKU433). After conjugating pKU433 to χ1037, the MICs of cefoxitin and flomoxef were increased by 8- and 128-fold, respectively. In parallel, the β-lactamase activity of the transconjugant also increased 132-fold compared with that of the recipient strain. Extraction of plasmids from KU6763 was done with a HiSpeed Plasmid Midi kit (Qiagen, Tokyo, Japan). pKU433 thus obtained was digested with *Bam*HI, and then was cloned into the *Bam*HI site of the pHSG398 cloning vector (Takara, Kyoto, Japan). Finally, a cefpodoxime-resistant clone carrying a 4 kb fragment from pKU433 was obtained by selection with cefpodoxime (at a concentration of 25 mg/L). DNA sequence analysis of pHSG398 carrying the *Bam*HI-digested fragment of pKU433 was done with an ABI PRISM 310 DNA sequencer (Applied Biosystems, Tokyo,

Japan). Identification of the β-lactamase gene using the BLASTN database search program showed that the plasmid-borne bla_{AmpC} gene was identical to bla_{CMY-2} (GenBank accession no. X91840). It was previously demonstrated that CMY-2 conferred resistance to cephamycins in Gram-negative bacilli.[2] These findings indicated that the ESC resistance of KU6763 was mediated by CMY-2.

KU6764 also exhibited ESC resistance and high β-lactamase activity compared with χ1037, although plasmid-borne AmpC was not detected by PCR amplification. The MICs of cefpodoxime and ceftazidime were increased by 16- and 8-fold, respectively, compared with those for χ1037. In parallel, AmpC activity was increased by 99-fold compared with that of χ1037. Previous studies have shown that insertion of one or two bases between −35 and −10 in the *E. coli ampC* promoter causes overexpression of $ampC.$[3] To confirm this, KU6764 chromosomal *ampC* was amplified by PCR using HotStar Taq (Qiagen) with the following primers: ampC Up#1, 5′-AATGTTTCCTTACTGGTTTTT-3′; and ampC Low#0, 5′-GGTCGCGTATTCCTGTTCCTGATG-3′. DNA sequence analysis of chromosomal *ampC* revealed insertion of T at a site 73 bp upstream of the *ampC* coding region, and this mutation corresponded with the *ampC* promoter mutant reported by Forward *et al.*[3] Therefore, we concluded that AmpC overproduction by KU6764 was due to this mutation of the promoter.

In this study, an *E. coli* strain harbouring bla_{CMY-2} and another strain with an *ampC* promoter region mutation were isolated from the faecal samples of healthy volunteers. To our knowledge, this is the first report concerning bla_{CMY-2} isolation from healthy volunteers in Japan. CMY-2 is one of the most widely disseminated plasmid-borne AmpCs among patients and animals.[6] The isolation of *E. coli* with ESC resistance due to *ampC* promoter mutation has also been increasing among both patients and healthy animals.[1,3,6] Our findings suggest that chromosomal or plasmid-borne AmpC-mediated ESC resistance is already widely disseminated among *E. coli*, even in the normal human intestinal flora. We conclude that further surveillance to detect microorganisms with ESC resistance in the human intestinal flora is necessary.

Technology; the COE Program of the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare (the Research Project of Emerging and Establishment of Rapid Identification Methods, H15-Sinko-9); and the Charitable Trust Clinical Pathology Research Foundation of Japan.

Transparency declarations

We have no conflicts to declare.

References

1. Tracz DM, Boyd DA, Bryden L *et al.* Increase in *ampC* promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT–PCR. *J Antimicrob Chemother* 2005; **55**: 768–72.

2. Bauernfeind A, Stemplinger I, Jungwirth R *et al.* Characterization of the plasmidic β-lactamase CMY-2, which is responsible for cephamycin resistance. *Antimicrob Agents Chemother* 1996; **40**: 221–4.

3. Forward KR, Willey BM, Low DE *et al.* Molecular mechanisms of cefoxitin resistance in *Escherichia coli* from the Toronto area hospitals. *Diagn Microbiol Infect Dis* 2001; **41**: 57–63.

4. Kaneko K, Okamoto R, Nakano R *et al.* Gene mutations responsible for overexpression of AmpC β-lactamase in some clinical isolates of *Enterobacter cloacae*. *J Clin Microbiol* 2005; **43**: 2955–58.

5. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; **40**: 2153–62.

6. Briñas L, Zarazaga M, Sáenz Y *et al.* β-Lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrob Agents Chemother* 2002; **46**: 3156–63.

371

227

Probing the Role of Asp-120(81) of Metallo-β-lactamase (IMP-1) by Site-directed Mutagenesis, Kinetic Studies, and X-ray Crystallography*

Yoshihiro Yamaguchi‡§, Takahiro Kuroki‡, Hisami Yasuzawa‡, Toshihiro Higashi‡, Wanchun Jin‡, Akiko Kawanami‡, Yuriko Yamagata¶, Yoshichika Arakawa‖, Masafumi Goto‡, and Hiromasa Kurosaki‡

From the Departments of ‡Structure-Function Physical Chemistry and ¶Structural Biology, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862-0973 and the ‖Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan

Metallo-β-lactamase IMP-1 is a di-Zn(II) metalloenzyme that efficiently hydrolyzes β-lactam antibiotics. Wild-type (WT) IMP-1 has a conserved Asp-120(81) in the active site, which plays an important role in catalysis. To probe the catalytic role of Asp-120(81) in IMP-1, the IMP-1 mutants, D120(81)A and D120(81)E, were prepared by site-directed mutagenesis, and various kinetics studies were conducted. The IMP-1 mutants exhibited 10^2–10^4-fold drops in k_{cat} values compared with WT despite the fact that they contained two Zn(II) ions in the active site. To evaluate the acid-base characteristics of Asp-120(81), the pH dependence for hydrolysis was examined by stopped-flow studies. No observable pK_a values between pH 5 and 9 were found for WT and D120(81)A. The rapid mixing of equimolar amounts of nitrocefin and all enzymes failed to result in the detection of an anion intermediate of nitrocefin at 650 nm. These results suggest that Asp-120(81) of IMP-1 is not a factor in decreasing the pK_a for the water bridging two Zn(II) ions and is not a proton donor to the anionic intermediate. In the case of D120(81)E, the nitrocefin hydrolysis product, which shows a maximum absorption at 460 nm, was bound to D120(81)E in the protonated form. The three-dimensional structures of D120(81)A and D120(81)E were also determined at 2.0 and 3.0 Å resolutions, respectively. In the case of D120(81)E, the Zn-Zn distance was increased by 0.3 Å compared with WT, due to the change in the coordination mode of Glu-120(81)OE1 and the positional shift in the conserved His-263(197) at the active site.

β-Lactam antibiotics are effective chemotherapeutic agents for the treatment of infectious diseases caused by bacteria. The

mechanism of the antibacterial activity of β-lactams involves the inhibition of the biosynthesis of the bacterial cell wall peptidoglycan. Since the introduction of β-lactams into clinical use, bacteria continue to evolve and to defend themselves from antibiotics by means of several strategies. One of these adaptations is the production of β-lactamases, which hydrolyze β-lactams to biologically inactive compounds. As of this writing, more than 300 β-lactamases have been identified (1).

β-Lactamases are classified into four molecular classes, A–D, according to their amino acid sequences (2). Until recently, β-lactamases of clinical concern were classes A, C, and D, which contain a serine residue at the active site. However, since 1990, as the use of carbapenems such as imipenem, which are often very stable against serine β-lactamases, has expanded, bacterial pathogens producing class B β-lactamases (so-called metallo-β-lactamases (MBLs)[1] which require Zn(II) ion(s) and hydrolyze almost all clinically useful β-lactams) have emerged (3–9). Today, more than 30 types of MBLs are known, but despite numerous efforts, no clinically relevant inhibitors for MBLs have yet been developed (10–19).

A subdivision of class B β-lactamases based on primary amino acid sequences has been proposed (20). Those belonging to subclass B1 have the amino acid sequence HXHXD, including β-lactamase II (BcII) from *Bacillus cereus* (21), CcrA from *Bacteroides fragilis* (22), IMP-1 found in some clinical isolates of *Serratia marcescens* (4) and *Pseudomonas aeruginosa* (23), and VIM-2 found in some clinical isolates of *P. aeruginosa* (5). Those belonging to subclass B2 have the amino acid sequence NXHXD, including CphA and ImiS from *Aeromonas* (24, 25). Finally, those belonging to subclass B3 have the amino acid sequence H(Q)XHXDH, including L1 from *Stenotrophomonas maltophilia* (3) and THIN-B from *Janthinobacterium lividum* (26). Therefore, the identification of common structural and mechanistic features of metallo-β-lactamases constitutes a clinically important issue.

IMP-1, first isolated from *S. marcescens* in Japan, is a single chain peptide composed of 246 amino acids (4). The *IMP-1* gene is mainly encoded in a plasmid and is generally thought to be the most dangerous enzyme. Several bacteria have been reported to produce this enzyme (27). The three-dimensional structure of IMP-1 has been reported by Concha *et al.* (14), and IMP-1 was found to possess two Zn(II) ions in the active site for

---

---

[1] The abbreviations used are: MBL, metallo-β-lactamase; Mes, 2-morpholinoethansulfonic acid; Me₂SO, dimethyl sulfoxide; WT, wild type; BBL, class B β-lactamase; PDB, Protein Data Bank.
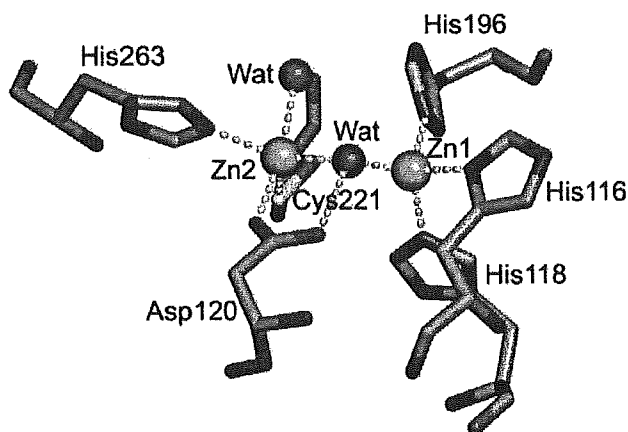
FIG. 1. **Schematic representation of the active site of CcrA (PDB code 1ZNB) from *B. fragilis* (28).** The residues are labeled according to the BBL standard numbering (20). The Zn(II) atoms are shown in *green*, oxygen atoms in *red*, nitrogen atom in *blue*, and sulfur atoms in *yellow*.

activity, as has been reported for CcrA.

Mechanistic studies of di-Zn(II) MBLs have been carried out with a CcrA belonging to subclass B1 (28–33, 47). In the three-dimensional structure of CcrA, reported in 1996, Concha *et al.* (28) concluded that Zn1 (the two Zn(II) ions are termed Zn1 and Zn2, respectively) is coordinated by three His(s) and a bridging OH_2 or OH^-, whereas Zn2 is coordinated by Asp, His, Cys, a bridging OH_2 or OH^-, and another OH_2 or OH^- (Fig. 1). OH_2 or OH^- bridging between the two Zn(II) ions in the active site is proposed to act as the deprotonated nucleophile to attack the carbonyl carbon of the β-lactam ring with the assistance of Asp-120(103) (following BBL numbering),[2] which is conserved in all MBLs (20, 28). Wang *et al.* (29, 30, 47) observed a transient increase in the intermediate in the hydrolysis of nitrocefin by CcrA in a pre-steady-state study and reported that the decay of this intermediate, a ring-opened form of nitrocefin with an anionic nitrogen, is rate-limiting. Yanchak *et al.* (31) reported that Asp-120(103) in the active site of CcrA plays a role in generating an intermediate but does not donate a proton to the intermediate during the rate-limiting step in the catalysis. In a quantum chemical study, Díaz *et al.* (32) predicted that the Zn2-bound Asp-120 in CcrA could participate as a proton shuttle or proton donor. Moreover, Suárez *et al.* (33) proposed that the unprotonated Asp-120 results in the rapid formation of a rigid Zn1-OH-Zn2 linkage, whereas the neutral Asp-120 is compatible with a fluctuating Zn1-Zn2 distance through the breaking and/or formation of the Zn1-OH-Zn2 bridge.

To investigate the role of Asp-120(81) in catalysis by IMP-1, we prepared two mutants in which this amino acid is replaced with Ala and Glu, and we carried out a steady-state kinetics study with four β-lactams and pre-steady-state experiments with nitrocefin as a reporter substrate. Moreover, x-ray crystallographic studies of the mutants were performed to elucidate the correlation between the reactivity with respect to the hydrolysis of β-lactams and the coordination structure at the active site.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The bla_{IMP} gene cloned from strain *S. marcescens* TN9160 was amplified using two primers, IMP-PCR-F (5'-CCgATACAAgAACAACAg-3') and IMP-PCR-R (5'-gTAgTggCgT-

gCTgCAAC-3') (4). The PCR products of the 1.2-kb DNA fragment were digested with SmaI and subcloned into a vector pKF19k that had been digested with SmaI in advance. The ligated pKF19k was named pKF19k/IMP. Site-directed mutagenesis was performed with the help of a Takara Mutan™-Super Express Kit (Tokyo, Japan) by PCR. The PCRs were performed with the selection primer attached to the kit and mutagenic primers for D120(81)A (5'-CCgTgCTggCgCTAT-3') and D120(81)E (5'-ATTCCgCCCgTCCTgTCgCTATg-3'). After the PCR products were transformed into *Escherichia coli* MV1184 cells, the base sequences of the two IMP-1 mutants were confirmed by DNA sequencing by using two primers IMP_{for} (5'-CggTCTgTAggCTgTAATgC-3') and IMP_{rev} (5'-TgTggAATTgTgAgCgg-3').

Enzyme Preparation—WT was purified as described previously (4, 34). The mutagenic plasmids were transformed into *E. coli* JM109. The IMP-1 mutants were also purified according to the procedure of WT with the modification of the conditions as follows. 1) The antibiotic for selection was changed to 50 μg/ml kanamycin. 2) The elution buffers for the SP-Sepharose Fast Flow column and Sephadex G-75 column were Tris-HCl buffer (50 mM, pH 7.4, 10 μM $Zn(NO_3)_2$ for D120(81)A or 100 μM $Zn(NO_3)_2$ for D120(81)E).

Circular Dichroism—The conservation of secondary structures was observed by CD spectroscopy. The proteins were diluted to a concentration of 1.5 μM in phosphate buffer (10 mM, pH 7.0). The concentrations of WT and the IMP-1 mutants were determined by measuring the absorbance at 280 nm using an extinction coefficient of ϵ_{280} = 49,000 M^{-1} cm^{-1} (34). CD spectra were recorded on a Jasco J-720 circular dichrograph (Tokyo, Japan) at 25 °C over 200–250 nm.

Metal Contents—Before the analyses of metal content, the buffer used for the protein samples was changed from phosphate buffer (50 mM, pH 7.0) to Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl), and the solution was applied to an Amersham Biosciences PD-10 column (inner diameter, 15 × 50 mm) and eluted with metal-free Tris-HCl buffer (50 mM, pH 7.4). Each of the IMP-1 mutants was diluted to give the predetermined protein concentrations (2.0 and 4.0 μM) with 0.2% HNO_3. The determination of Zn(II), by atomic absorption, was conducted on a Hitachi Z8000 atomic absorption spectrophotometer (Tokyo, Japan) in the flame mode.

Steady-state Kinetic Studies—The hydrolysis of cepharolidine ($\Delta\epsilon_{260}$ = -10,500 M^{-1} cm^{-1}), imipenem ($\Delta\epsilon_{298}$ = -9,650 M^{-1} cm^{-1}), nitrocefin ($\Delta\epsilon_{491}$ = 20,000 M^{-1} cm^{-1}), and benzyl penicillin ($\Delta\epsilon_{230}$ = -1,070 M^{-1} cm^{-1}) was monitored photometrically at 30 °C in Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl). Kinetic experiments were conducted using Hitachi U-1500 (Tokyo, Japan) or Shimadzu UV-2200 UV-visible spectrophotometers (Kyoto, Japan) equipped with thermostated cells. Enzyme parameters K_m and k_{cat} were obtained by fitting the data to the Michaelis-Menten equation using a nonlinear regression analysis with KaleidaGraph (Synergy software).

Zn(II) Ion Dissociation from the IMP-1 Mutants—The rate of hydrolysis of nitrocefin by the IMP-1 mutants was monitored in varying concentrations of $Zn(NO_3)_2$ (0–6000 μM) in acetic acid/sodium acetate buffers (0.2 and 0.5 M NaCl) adjusted to pH 4.4–5.6 as described previously (34). Buffer solutions were prepared using ultrapure water from Wako (Osaka, Japan). The saturated activity, k_{max}, and the dissociation constant of Zn(II) ion from the IMP-1 mutants (Equation 1) were obtained by fitting v_{init} to Equation 2 by using a nonlinear regression analysis with KaleidaGraph,

$$K_d = [E][Zn]/[E \cdot Zn] \qquad \text{(Eq. 1)}$$

$$v_{init} = \frac{k_{max}[E]_T[Zn]}{K_d + [Zn]} + v_0 \qquad \text{(Eq. 2)}$$

where v_{init} is the initial velocity; $[E]_T$ is the total concentration of enzyme, and v_0 is the velocity without the addition of Zn(II) ion.

The pH Dependence of Hydrolysis under Conditions of Excess Zn(II)—The pH dependence studies were carried out with MTEN buffer (50 mM Mes, 25 mM Tris, 25 mM 2-aminoethanol, 100 mM NaCl) adjusted to pH 5.6–9.0, and with acetic acid/sodium acetate buffers (0.2 and 0.5 M NaCl) adjusted to pH 4.6–5.6. The concentration of $Zn(NO_3)_2$ was adjusted to 25 times the K_d at each pH. Before measurement of the rate of hydrolysis of nitrocefin by the IMP-1 mutants, the enzyme solution was preincubated for 10 min at 30 °C in the adjusted buffers. The enzymatic parameters, K_m and k_{cat}, were obtained by fitting to the Michaelis-Menten or the Lineweaver-Burk equations.

Pre-steady-state Kinetic Studies—Stopped-flow spectrophotometry was carried out with Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl and 3.23% Me_2SO) on a Photal RA-401 UV-visible spectrophotometer

---

[2] In this paper, the amino acid residues of IMP-1 and its mutants are designated with the BBL number and amino acid sequence number by putting the latter in parentheses.

Asp-120 Mutants of Metallo-β-lactamase IMP-1

TABLE I

Kinetic parameters for the hydrolysis of various β-lactam antibiotics by WT and the IMP-1 mutants D120(81)A and D120(81)E

The concentration of added $Zn(NO_3)_2$ is 1 mM. ND indicates not determined.

Antibiotic	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	μM	$\mu M^{-1} min^{-1}$
WT			
Cephaloridine	5400 ± 350	8.0 ± 1.7	680 ± 190
Imipenem	7400 ± 450	45 ± 8.2	93 ± 14
Nitrocefin	28,000 ± 2000	20 ± 3	1400 ± 300
Benzyl penicillin	39,000 ± 7000	800 ± 300	50 ± 30
D120(81)A			
Cephaloridine	0.21 ± 0.03	150 ± 40	0.0016 ± 0.00014
(Added Zn^{2+})	1.3 ± 0.083	200 ± 40	0.0065 ± 0.00085
Imipenem	190 ± 40	3100 ± 900	0.07 ± 0.0048
(Added Zn^{2+})	ND	ND	ND
Nitrocefin	450 ± 20	27 ± 3	17 ± 1.1
(Added Zn^{2+})	250 ± 20	50 ± 10	5.2 ± 0.6
Benzyl penicillin	10.0 ± 5.0	8000 ± 5000	0.003 ± 0.00016
(Added Zn^{2+})	ND	ND	ND
D120(81)E			
Cephaloridine	14.0 ± 1.0	270 ± 60	0.05 ± 0.0071
(Added Zn^{2+})	13.3 ± 0.9	200 ± 38	0.3 ± 0.13
Imipenem	122 ± 7.4	640 ± 100	0.2 ± 0.02
(added Zn^{2+})	220 ± 60	750 ± 500	0.3 ± 0.13
Nitrocefin	16 ± 0.8	1.3 ± 0.2	12 ± 1.3
(Added Zn^{2+})	33 ± 2.3	1.2 ± 0.3	28 ± 5.3
Benzyl penicillin	32 ± 3.6	2600 ± 700	0.012 ± 0.0002
(Added Zn^{2+})	ND	ND	ND

(Otsuka Electronics, Osaka, Japan) at 25 °C. The WT (9 μM) or a IMP-1 mutant was rapidly mixed with nitrocefin (9 μM) followed by monitoring the absorption at 491 nm (formation of nitrocefin product), 391 nm (depletion of nitrocefin), and 650 nm (formation of an assumed intermediate from nitrocefin) (29, 30, 35, 47).

Rapid-scan studies were carried out with Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl and 3.23% Me$_2$SO) on Photal RA-415S rapid-scan spectrophotometer at 25 °C. The spectra were collected between 325 and 525 nm and monitored at time intervals of 2 s (gate time 200 ms, and 16 repeats).

Spectroscopic Titration of the Nitrocefin Hydrolyzed Product by Hydrochloric Acid and D120(81)E Mutant—A solution of the nitrocefin hydrolysis product was prepared by mixing 100 μl of a Me$_2$SO solution of nitrocefin (1240 μM) and 100 μl of an IMP-1 solution (54.6 μM) with 2.9 ml of Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl) followed by preincubation for 30 min at 30 °C. Spectroscopic titration of the nitrocefin hydrolysis product (10 μM) produced using WT was performed by the stepwise addition of hydrochloric acid (0.2 M) and the D120(81)E mutant (56 μM) in Tris-HCl buffer (50 mM, pH 7.4, and 0.5 M NaCl) at room temperature.

Inhibition of D120(81)A Mutant by Anions and Anion Dependence on the Kinetics of the Hydrolysis—The change in the catalytic activity of D120(81)A by the presence of phosphate anion was examined by monitoring the change in absorbance at 491 nm with nitrocefin as a substrate for 5 min at 30 °C in Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl) at various concentrations of phosphate ion. Under the same conditions, the effects of NaClO$_4$, Na$_2$SO$_4$, K$_3$[Co(CN)$_6$], and K$_4$[Fe(CN)$_6$] were also examined.

Crystallization—Crystals of the IMP-1 mutants for x-ray crystallography were prepared by the vapor diffusion (hanging drop method), analogous to the method reported by Concha *et al.* (14). The drops were prepared by mixing 5 μl of protein solution (10 mg/ml in 20 mM Hepes-NaOH, pH 7.5) and 5 μl of reservoir solution containing 0.2 M sodium acetate, 0.1 M sodium citrate buffer, pH 6.5, 30% PEG 4000 against 500 μl of reservoir solution. The crystals grew as thin plates after ~3 weeks at 20 °C.

X-ray Data Collection and Processing—All diffraction data were collected at 100 K without additional cryoprotectants. The data sets for D120(81)A and D120(81)E were collected at the SPring-8 on beamline 40B2 and 41XU (Harima, Japan) at 2.0 and 3.0 Å resolutions with a wavelength of λ = 1.00 and 0.98 Å, respectively. The data for the both crystals were integrated, merged, and scaled using HKL2000 (36).

Structure Determination, Model Building, and Refinement—The structure of IMP-1 complexed with the inhibitor from *P. aeruginosa* at 2.0 Å resolution (PDB code 1DD6) (14) with neither Zn(II) ions nor water molecules nor inhibitor was used as the model for molecular replacement using the AMoRe software program (37). The CNS software program was used to refine the structures by positional and

B-factor refinement (38). The interactive graphics program O was used to build the structures of the IMP-1 mutants (39). Topology and parameter files of oxidized Cys were utilized in the HIC-UP (x-ray.bmc.uu.se/hicup) (40).

RESULTS

Construction of Recombinant Plasmids and the Mutation of Asp-120(81)—Site-directed mutagenesis was carried out with *Pfu* Turbo™ polymerase using pKF19k/IMP as a template. The CD spectra of the IMP-1 mutants showed the same pattern as that for WT between 250 and 200 nm, indicating that no major structural change occurred as the result of mutation (data not shown). The Zn(II) contents of the D120(81)A and D120(81)E mutants were 2.2 and 2.3 per enzyme molecule, respectively, as determined by atomic absorption spectroscopy. A smaller binding affinity had been expected, but the replacement of Asp-120(81) with Ala or Glu did not significantly change the affinity of the protein for Zn(II) ion.

Steady-state Kinetics of Substrate Hydrolysis—The K_m and k_{cat} values for the hydrolysis of four β-lactams, cephaloridine, imipenem, nitrocefin, and benzyl penicillin, which are catalyzed by WT and the IMP-1 mutants, are shown in Table I. The inclusion of 1 mM Zn(NO$_3$)$_2$ in the buffer resulted in a slight change in K_m and k_{cat}. For all substrates measured, the IMP-1 mutants exhibited a reduced k_{cat} compared with WT. D120(81)A and D120(81)E exhibited the k_{cat} values that were 25–41,000 times and 40–1750 times lower than those of WT, respectively. The IMP-1 mutants exhibited larger K_m values for almost all the substrates examined than those of WT, except for nitrocefin, which showed a smaller K_m value for D120(81)E. The hydrolysis of benzyl penicillin with D120(81)A and D120(81)E and of imipenem with D120(81)A in a buffer containing 1 mM Zn(NO$_3$)$_2$ could not be measured because the Zn(NO$_3$)$_2$ solution hydrolyzed these substrates very rapidly in the absence of the IMP-1 mutants.

pH Dependence of Hydrolysis under Conditions of Excess Zn(II) Ion—We reported previously that the activity of IMP-1 was restored by the addition of Zn(II) ions in an acidic buffer (34). The hydrolysis was measured in acidic buffer using nitrocefin (100 μM) as the substrate, and the Zn(II) dissociation constant K_d, based on Equation 2 of the IMP-1 mutants was evaluated by varying the concentrations of Zn(NO$_3$)$_2$ in acidic

TABLE II

The dissociation constant of Zn(II) from WT and the IMP-1 mutants D120(81)A and D120(81)E with concentration of Zn(II) determined using nitrocefin as the substrate

pH	D120(81)A		D120(81)E	
	K_d	k_{max}	K_d	k_{max}
	μM	min^{-1}	μM	min^{-1}
4.4	2900 ± 480	58 ± 4.5	970 ± 180	3.3 ± 0.18
4.6	420 ± 54	72 ± 3.5	76 ± 15	3.6 ± 0.14
4.8	77 ± 3.5	80 ± 0.9	11 ± 1.1	3.3 ± 0.078
5.0	22 ± 1.1	96 ± 1.1	11 ± 1.5	4.1 ± 0.11
5.2	5.8 ± 0.27	92 ± 1.3	6.5 ± 1.2	4.7 ± 0.12
5.4	3.3 ± 0.3	94 ± 2.4	2.5 ± 0.42	5.3 ± 0.096
5.6	1.6 ± 0.18	76 ± 2.6	0.84 ± 0.18	5.7 ± 0.11

pH	WT(34)	
	K_d	k_{max}
	μM	min^{-1}
4.33	840	990
4.45	180	1210
4.55	84	1370
4.73	16	1450
5.0	2.6	1670
5.21	1.3	1790
5.41	0.38	1910
5.61	0.70	2660

buffers with pH from 4.4 to 5.6. The kinetic parameters, K_d and k_{max}, are summarized in Table II. The dissociation constants, K_d, of the Zn(II) ion from D120(81)A and D120(81)E increased with decreasing pH. The values of K_d were generally larger for D120(81)A than for WT and D120(81)E.

The pH dependence of the steady-state kinetic constant, k_{cat}, was determined for the IMP-1 mutants with nitrocefin as a substrate. Two buffers were used as follows: acetic acid/sodium acetate buffers (0.2 and 0.5 M NaCl) (below pH 5.6) under the concentrations of $Zn(NO_3)_2$ at 25 times the K_d in the range between pH 4.6 and 5.6, and MTEN buffer (pH 5.6–9.0) containing $Zn(NO_3)_2$ (40 μM for D120(81)A, 20 μM for D120(81)E) in the range between pH 6.0 and 9.0. Plots of the logarithm of k_{cat} for WT and the IMP-1 mutants, D120(81)A and D120(81)E, against pH are shown in Fig. 2. When the pH values of the buffers were in the range 4.6–5.0, k_{cat} and K_m values were obtained from Lineweaver-Burk plots because nitrocefin precipitated below pH 4.8. The plots for WT and D120(81)A showed no inflections over the pH range measured. However, the plots for D120(81)E were increased slightly in the pH range from 5.2 to 9.0. Moreover, the logarithm of k_{cat} of D120(81)E in the pH range from 5.2 to 4.6 was largely decreased.

Pre-steady-state Kinetics for the Hydrolysis of Nitrocefin Using WT and the IMP-1 Mutants—The reactions of nitrocefin with WT or D120(81)A were monitored by stopped-flow spectrophotometry at 391, 491, and 650 nm; the decrease in absorption at 391 nm and the increase in absorption at 491 nm were almost synchronous. No significant transient increase in the absorbance around 650 nm was detected. The time scale for the reaction of D120(81)A was 1/1000 slower than for WT.

However, the experiment with D120(81)E showed a nonsynchronous decrease and an increase in the absorbances at 391 and 491 nm, respectively, as shown in Fig. 3a. The increase in product appeared to reach completion more rapidly than the decrease in reactant. To solve this puzzle, rapid-scan spectroscopy was employed. When nitrocefin (8 μM) and D120(81)E (8 μM) were mixed in Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl, and 3.23% Me_2SO), the absorption at 460 nm increased instead to 491 nm for the normal hydrolyzed nitrocefin as shown in Fig. 3b. This absorption at 460 nm showed no change for at least 50 min after the completion of the reaction of equimolar D120(81)E and nitrocefin (data not shown). However, for the
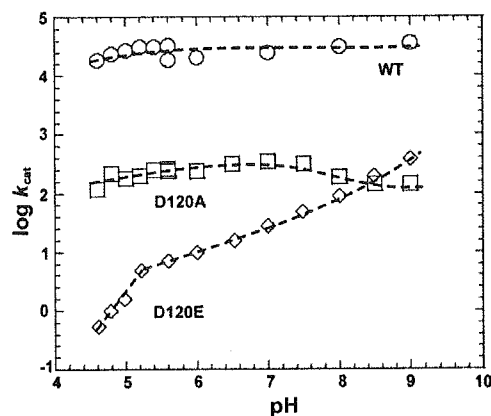

FIG. 2. **pH dependence plots for the hydrolysis of nitrocefin at 30 °C for WT and the IMP-1 mutants D120(81)A and D120(81)E.** Data points were obtained in MTEN buffer (pH 5.6–9.0) and acetic acid/sodium acetate buffer (pH 4.6–5.6) in the presence of an excess of Zn(II) ion.

reaction of nitrocefin (40 μM) with D120(81)E (4 μM), an ordinary absorption increase at 491 nm was observed (data not shown). To investigate the nature of this species having an absorbance maxima at 460 nm, the nitrocefin hydrolysis product was spectrophotometrically titrated with 0.2 M hydrochloric acid. The addition of hydrochloric acid caused a blue shift of 31 nm, and the absorption maxima was shifted to 460 nm with an ϵ of 18,000 M^{-1} cm^{-1} when the pH of the medium reached a value of 2.3 (data not shown). The nitrocefin hydrolysis product was also titrated with D120(81)E. By the successive addition of D120(81)E to the nitrocefin hydrolysis product, the absorption spectra of the product was altered to that having an absorbance maxima at 460 nm (data not shown). Thus, the 460 nm species appears to be a protonated form of the nitrocefin hydrolysis product bound to D120(81)E.

Three-dimensional Structures of D120(81)A and D120(81)E— The three-dimensional structures of the D120(81)A and D120(81)E mutants were determined by the molecular replacement method using the known structure of WT complexed with the inhibitor (PDB code 1DD6) (14). Both mutants crystallize with a space group of P1, and four independent proteins, termed A–D, were located in the crystal lattice. The data collection and refinement statistics are summarized in Table III. The structures of D120(81)A and D120(81)E were refined to a final $R_{working}$ of 21.2 and 22.4% and a final R_{free} of 21.8 and 29.5% at 2.0 and 3.0 Å resolutions, respectively. The atomic coordinates of D120(81)A and D120(81)E structures have been deposited in the Protein Data Bank accession codes 1WUO and 1WUP, respectively.

The overall structures of the IMP-1 mutants have the well conserved architecture of metallo-β-lactamases, being composed of $\alpha\beta/\beta\alpha$ domains, and are almost identical to the structure of WT (14). The four molecules of D120(81)A included the following residues: Ser-38(3)–Val-61(25) and Gly-65(29)–Lys-298(221) for molecule A; Ser-38 (3)–Val-61(25) and Gly-65(29)–Lys-298(221) for molecule B; Ser-38(3)–Val-61(25) and Val-66(30)–Lys-298(221) for molecule C; and Ser-38(3)–Asn-62(26) and Gly-65(29)–Lys-298 (221) for molecule D, respectively, whereas those of D120(81)E included residues Leu-39(4)–Asn-62(26) and Val-66(30)–Ser-297(220) for molecule A; Leu-39(4)–Asn-62(26) and Gly-65(29)–Glu-296(219) for molecule B; Ser-38(3)–Gly-63(27) and Val-66(30)–Glu-296(219) for molecule C; and Ser-38(3)–Gly-63(27) and Gly-65(29)–Glu-296(219) for molecule D, respectively. Other residues, except those listed above, were disordered. The superposition of the C-α atoms of
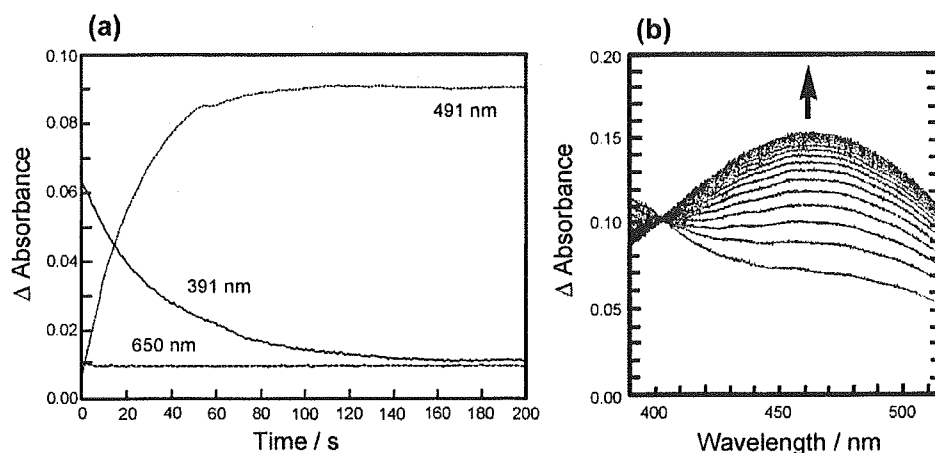
FIG. 3. *a*, pre-steady-state studies by stopped-flow for D120(81)E using nitrocefin as a substrate at 25 °C. The spectra of the substrate, product, and intermediate were determined at 391, 491, and 650 nm, respectively. *b*, rapid-scan studies for D120(81)E at 25 °C. The spectra were collected with the following experimental parameters: center, 420 nm; scan range, 325–525 nm; gate time, 200 ms; time interval, 2 s; number of spectra, 16.

TABLE III
X-ray data collection and structure refinement.

	D120(81)A	D120(81)E
Data collection statistics		
Unit cell parameter		
a (Å)	49.7	49.2
b (Å)	73.0	73.1
c (Å)	82.5	80.9
α (°)	85.4	84.8
β (°)	75.5	76.1
γ (°)	73.6	74.0
Space group	P1	P1
Molecules/asymmetric unit	4	4
Resolution (Å) (outer shell)	50.0–2.01 (2.08–2.01)	99.0–2.97 (3.08–2.97)
No. observed reflections	226,690	32,258
No. unique reflections	67,829	19,685
Overall completeness (%) (outer shell)	94.5 (67.4)	85.7 (62.3)
$R_{sym}{}^{a}$ (%) (outer shell)	6.0 (18.1)	5.8 (11.9)
Refinement statistics		
$R_{working}{}^{b}$ (%)	21.2	22.4
R_{free} (%)	21.8	29.5
r.m.s.d. from ideal		
Bonds (Å)	0.023	0.008
Angles (°)	2.00	1.60

[a] $R_{sym} = \Sigma|I - \langle I \rangle|/\Sigma I$.
[b] $R_{working} = \Sigma\|F_{o}| - |F_{c}\|/\Sigma|F_{o}|$, where F_{o} and F_{c} are the observed and calculated structure factors, respectively. The R_{free} was determined from 5% of the data.

the four independent molecules in the asymmetric unit for D120(81)A and D120(81)E gave root mean square deviations (r.m.s.d) of 0.23 and 0.49 Å, respectively. The IMP-1 mutants (molecule A was selected for each mutant) and WT were also superimposed, except for residues 62(26)–65(29), which are disordered in WT, D120(81)A, and D120(81)E. The r.m.s.d. for the C-α atoms between the IMP-1 mutants D120(81)A and D120(81)E and WT were 0.35 and 0.56 Å, respectively. In a comparison of the deviations of the C-α atom of each residue between the IMP-1 mutants and WT, the residues moved most (>1 Å) were Glu-50(14) (1.8 Å), Val-61(25) (1.1 Å), Glu-210(150) (1.1 Å), and His-263(197) (1.2 Å) for D120(81)A; and Asp-49(13) (1.1 Å), Glu-50(14) (2.2 Å), Val-61(25) (2.1 Å), Pro-68(32) (1.0 Å), Val-76(40) (1.2 Å), Asp-77(41) (1.2 Å), Lys-186(129) (1.0 Å), Ile-187(130) (1.1 Å), Glu-210(150) (1.1 Å), His-263(197) (1.3 Å), Glu-296(219) (1.5 Å), and Ser-297(220) (2.9 Å) for D120(81)E.

A comparison of Zn(II) ion(s) and amino acid residue(s) as ligand(s) in the active sites is shown in Fig. 4, and the distances of the Zn(II) ion(s), ligands of the IMP-1 mutants, along with those of WT are summarized in Table IV. The active site in D120(81)A contains only one Zn(II) ion that is coordinated to

His-116(77), His-118(79), and His-196(139) and two oxygen atoms of an acetate ion, a bidentate external ligand (Fig. 4*b*). There is no spherical electron density in the space corresponding to a Zn(II) ion, but a tetragonal electron density corresponding to cysteine-*s*-dioxide (Csw221) was derived from Cys-221 (158). The active site of D120(81)E contains two Zn(II) ions, referred to as Zn1 and Zn2. Zn1 is coordinated by His-116(77), His-118(79), and His-196(139), and Zn2 is coordinated by Glu-120(81), Cys-221(158), His-263(197), and one oxygen atom of an acetate ion (Fig. 4*c*). The bridging OH_2 or OH^- and a water molecule coordinating to Zn2 are not positioned in the active sites of both mutants, as was found for CcrA. In WT, Asp-120(81)OD1 is considered to bind via a hydrogen bond with OH_2 or OH^-, although OH_2 or OH^- was not observed in the three-dimensional structure of WT as a bridging ligand between Zn1 and Zn2 as CcrA (28). However, in D120(81)E, the distances from Glu-120(81)OE1 to Zn1 and Zn2 are 2.5 ± 0.6 and 2.5 ± 0.3 Å, respectively; therefore, it is presumed that this oxygen atom binds both Zn1 and Zn2 as a bridging ligand. The distance between Zn1 and Zn2 in D120(81)E is 3.6 ± 0.1 Å, somewhat longer than that of WT (3.3 Å).
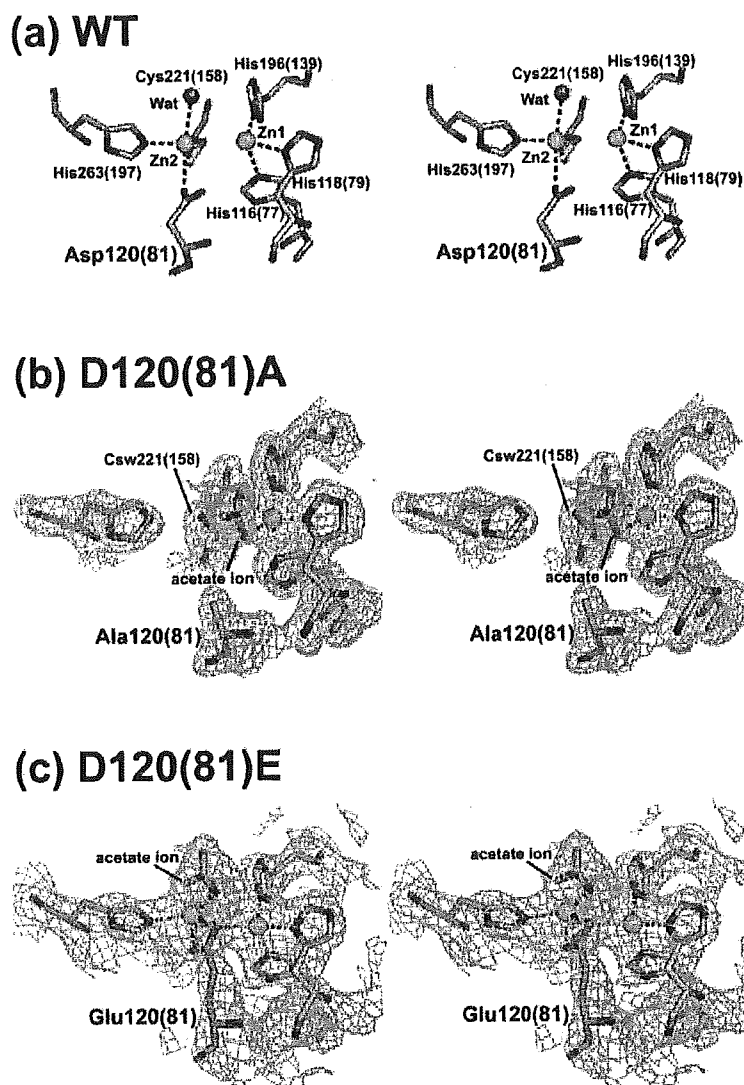
(a) WT

(b) D120(81)A

FIG. 4. **Comparison of the active site of WT (a) and the IMP-1 mutants D120(81)A (b) and D120(81)E (c).** The calculated $2|F_o| - |F_c|$ maps (*magenta*) are represented at 1.5 and 1.0 σ levels for *b* and *c*, respectively. Zn(II) ions and a water molecule are represented by *green* and *red spheres*, respectively. Amino acid residues are displayed as *sticks* (carbon, oxygen, nitrogen, and sulfur atoms colored in *gray, red, blue,* and *yellow*, respectively). In WT, a bridging OH_2 or OH^- is not observed.

(c) D120(81)E

TABLE IV

Comparison of Zn(II)-ligand distance for WT and the IMP-1 mutants, D120(81)A and D120(81)E

The deviations in the distance arise from the average Zn(II)-ligand distance of molecules A–D in the structure of the IMP-1 mutants.

	WT	Distance (Å)	
		D120(81)A	D120(81)E
Zn1-His-116(77)	2.3	2.3 ± 0.041	2.2 ± 0.17
Zn1-His-118(79)	2.3	2.0 ± 0.051	2.0 ± 0.068
Zn1-His-196(139)	2.1	2.0 ± 0.046	2.1 ± 0.15
Zn1-ACY_OXT	2.9	2.1 ± 0.12	2.9 ± 0.081
Zn1-ACY_O		2.4 ± 0.27	
Zn1-Asp-120(81)OD1 (Glu-120(81)OE1)	4.2		2.5 ± 0.58
Zn2-Asp-120(81)OD2 (Glu-120(81)OE2)	2.5		2.0 ± 0.18
Zn2-Cys-221(158)	2.4		2.3 ± 0.18
Zn2-His-263(197)	2.3		2.1 ± 0.16
Zn2-ACY_OXT	2.8		2.5 ± 0.35
Zn2-Asp-120(81)OD1 (Glu-120(81)OE1)	3.2		2.5 ± 0.30
Zn1-Zn2	3.3		3.6 ± 0.081

Inhibition of D120(81)A Mutant by Anion and Anion Dependence on the Kinetics of the Hydrolysis—D120(81)A was inhibited by phosphate ion after its purification by column chroma-

tography using phosphate buffer. The catalytic activities of WT and the IMP-1 mutants for nitrocefin hydrolysis were dependent on the concentration of phosphate ion as shown in Fig. 6. The activity of WT was decreased by 30% at a phosphate ion concentration of 50 mM, but D120(81)A lost 90% of its activity at a concentration of 5 mM. The activity of D120(81)E, on the other hand, was increased by 30% at a concentration of 50 mM. This inhibitory action of phosphate ion against D120(81)A was analyzed as a competitive binding of phosphate ion with the enzymes,

$$v_{init} = v_{o,init}/(1 + [\text{phosphate}]/K_i) \qquad (\text{Eq. 3})$$

where $v_{o,init}$ is the initial velocity in the absence of an inhibitor. The inhibition constants, K_i, were estimated to be 144 and 0.32 mM for WT and D120(81)A, respectively. Moreover, in order to examine the effect of other anions, the hydrolysis activity for D120(81)A was measured in Tris-HCl buffer (50 mM, pH 7.4, 0.5 M NaCl) containing $NaClO_4$, Na_2SO_4, $K_3[\text{Co(CN)}_6]$ or $K_4[\text{Fe(CN)}_6]$. The effect of phosphate ion on D120(81)A was also measured in a buffer solution containing 100 μM $Zn(NO_3)_2$. The activity of D120(81)A was inhibited by phosphate ion, $[\text{Co(CN)}_6]^{3-}$ ($K_i = 1.1$ mM) and $[\text{Fe(CN)}_6]^{4-}$ ($K_i = 4.0$ mM) but not by ClO_4^- and SO_4^{2-} (data not shown). Moreover, when 100

Asp-120 Mutants of Metallo-β-lactamase IMP-1

μM Zn(NO$_3$)$_2$ was added to the reaction buffer, the inhibition of D120(81)A by phosphate ion was suppressed (K_i = 1.8 mM; data not shown).

DISCUSSION

In this study, we performed a detailed analysis of the role of Asp-120, the conserved amino acid in the active site of all metallo-β-lactamases and which is thought to play an important role in enzymatic activity.

However, the critical role of Asp-120 in the mechanism of hydrolysis of di-Zn(II) metallo-β-lactamases is not clear to date. In this study, Asp-120(81) of a metallo-β-lactamase, IMP-1, was replaced with Ala or Glu by site-directed mutagenesis, and the point mutated enzymes, D120(81)A and D120(81)E, were prepared and characterized by spectroscopic and kinetic studies and by x-ray crystallography. The introduction of a mutation at Asp-120(81) caused no modification in the overall structure, based on the CD spectra, compared with that of WT, and two Zn(II) ions were retained in the proteins, as evidenced by atomic absorption spectroscopy.

The effect of the substitution of Asp-120(81) with Ala or Glu on steady-state kinetics was examined using four β-lactams as substrates. In Table I, D120(81)A and D120(81)E showed a remarkable reduction in activity, *i.e.* a decrease in k_{cat} by 10^2–10^4-fold and an increase in K_m by severalfold to 10^2-fold, although the effect was dependent on the β-lactam used. Haruta *et al.* (41) reported that the substitutions of His-116(77), His-118(79), or His-196(139), which act as Zn(II) ligands, with Ala caused a decrease in IMP-1 activity compared with that of WT, due to the loss of Zn(II) ion. However, the activity of the IMP-1 mutants, D120(81)A and D120(81)E, was not recovered, even in the presence of an excess of Zn(II) ion. These results clearly indicate that Asp-120 in the MBLs is important for the hydrolysis of β-lactams, including those that are in widespread clinical use. The increased values of K_m of the IMP-1 mutants also indicate that Asp-120(81) plays an important role in substrate binding. The strong binding of nitrocefin by WT, D120A(81), and D120E(81), as indicated by the small K_m values, is probably due to the dinitrophenyl substituent of nitrocefin, which is oriented in a local hydrophobic environment of IMP-1.

Wang *et al.* (29, 30, 47) have shown that an intermediate is produced during the course of the hydrolysis of nitrocefin by the metallo-β-lactamase from *B. fragilis* (CcrA) based on the observation of the transient appearance of an absorption band around 665 nm. Essentially the same change was observed for a metallo-β-lactamase from *S. maltophilia* (L1) (35). Moali *et al.* (44) reported that this intermediate does not accumulate in the case of IMP-1. We carried out a pre-steady-state kinetic study using nitrocefin as a substrate, to determine whether an intermediate could be detected under equimolar conditions for D120(81)A and D120(81)E. However, the transient increase in the absorption at 650 nm was not observed for WT or the IMP-1 mutants. Kaminskaia *et al.* (42) reported that a di-Zn(II) complex, which was a model of metallo-β-lactamase, showed an "anionic intermediate" of nitrocefin at 640 nm in 80% Me$_2$SO. The pre-steady-state experiments for WT and the IMP-1 mutants were again repeated with reaction medium containing up to 80% glycerin, but no indication of a transient absorption at 650 nm was found. From these results, the rate-determining step for the IMP-catalyzed reaction of nitrocefin appears to proceed without the significant accumulation of an intermediate having an absorption maximum around 650 nm. This suggests that Asp-120(81) is not the proton donor to the anionic intermediate, and no accumulation of intermediate occurs because of the ease of proton transfer to the presumed intermediate or the operation of other mechanisms for IMP-1.

Pre-steady-state experiments of nitrocefin with D120(81)E gave strange results. The two curves (391 and 491 nm, Fig. 3a) were not synchronized, and a new band having a λ_{max} at 460 nm appeared. This band was rather stable in an experiment using equimolar conditions (Fig. 3b). Bicknell *et al.* (45) reported that an intermediate having an absorption maximum at 450 nm could be detected under the conditions of temperatures lower than -58 °C with a BcII enzyme. The species having an absorption maxima at 460 nm found in our study is probably identical to those reported by Bicknell *et al.* (45). The visible absorption of the nitrocefin hydrolysis product originates from the conjugated system that includes a dinitrophenyl substituent. A shorter wavelength than 491 nm would arise from a reduction in conjugation, which is supported by the spectroscopic titration of the nitrocefin hydrolysis product with hydrochloric acid. The species with an absorption maximum at 460 nm is probably formed by the binding of the nitrocefin hydrolysis product to D120(81)E and is not an intermediate in the reaction. This behavior was observed only for D120(81)E. One of the reasons for this is thought to be the shift in the location and orientation of the carboxylate side chain of Glu-120(81) from that of the Asp-120(81).

To probe the role of Asp-120(81) in the mechanism of hydrolysis of IMP-1, the pH dependence of hydrolysis was examined using nitrocefin as a substrate. We reported previously that IMP-1 is inactivated in acidic medium as the result of the dissociation of Zn(II) from the holoenzyme (34). Therefore, prior to the examination of pH dependence of the IMP-1 mutants, the K_d was obtained as reported previously, and these values are similar to those reported for WT (Table II) (34). The pH dependence of the hydrolysis for the IMP-1 mutants was then measured under conditions of excess Zn(II) by the method reported previously (34). The K_m and k_{cat} values for WT and D120(81)A were almost constant between pH 4.6 and 9.0, but the k_{cat} for D120(81)E increased by 10^2-fold when the pH was increased from 5.2 to 9.0 (Fig. 2). These results suggest that Asp-120(81) is not a factor in decreasing the pK_a for the water bridging two Zn(II) ions, because the substitution of Asp-120(81) with Ala do not show any pH dependence between pH 4.6 and 9.0. It is likely that the positive charges of the two Zn(II) ions are the main cause for lowering the pK_a for the water bridging two Zn(II) ions. These results are supported by pH dependence studies of the Asp-mutants, D120(103)C, of CcrA from *B. fragilis* reported by Yanchak *et al.* (31) and D120(88)C and D120(88)S of L1 from *S. maltophilia* reported by Garrity *et al.* (43); a water bridging two Zn(II) ions has a pK_a < 5.0. The logarithm of k_{cat} of D120(81)E decreased with decreasing pH from 5.2 to 4.6. This indicates the possibility of the participation of an acid-base group with pK_a values between pH 4.6 and 5.2, but we were unable to identify which residue is responsible for this pK_a value at present.

To investigate the reasons for why the kinetics studies of the IMP-1 mutants showed strange results, crystals of the IMP-1 mutants were prepared, and x-ray crystallography was carried out. The overall structure was conserved despite the mutations of Asp-120(81), but the local structure at the active center was changed (Fig. 4). The flaps, residues 62(26)–65(29) of the IMP-1 mutants, were also disordered in the crystal structures, similar to WT. This is probably due to the high flexibility of the flaps. C-α atoms of the IMP-1 mutants were superimposed on that of WT, and each r.m.s.d. for C-α atoms was investigated. The residues moved more than 1 Å in r.m.s.d., Asp-49(13), Glu-50(14), Val-61(25), Pro-68(32), Val-76(40), Asp-77(41), Lys-186(129), Ile-187(130), and Glu-210(150) positioned on hairpin loops exposed to solvent and residues, and Glu-296(219) and Ser-297(220) positioned at the C terminus. Most interestingly,
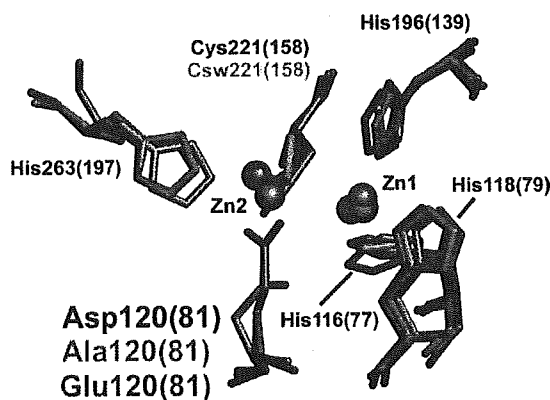
FIG. 5. **Superposition of the active site of WT and the IMP-1 mutants D120(81)A and D120(81)E.** WT, D120(81)A, and D120(81)E are shown in *gray, red,* and *blue,* respectively.
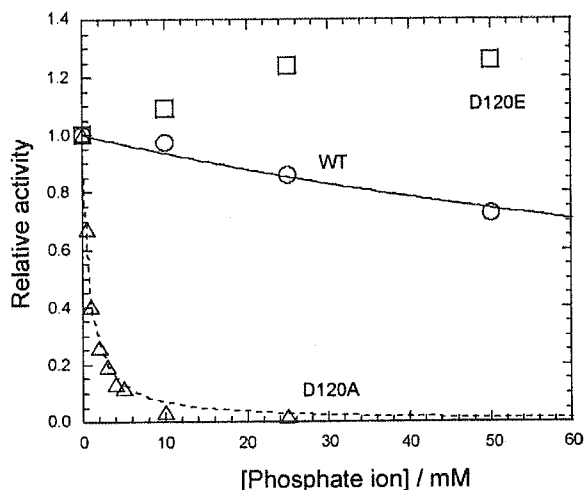

FIG. 6. **Plots of relative activity of nitrocefin hydrolysis by WT and the IMP-1 mutants D120(81)A and D120(81)E against the concentrations of phosphate ion at 30 °C.**

the C-α atoms of His-263(197) in the active sites of both structures of D120(81)A and D120(81)E were moved 1.2 and 1.3 Å away from the zinc-binding site compared with those in WT, respectively (Fig. 5). Moreover the Zn-Zn distance of D120(81)E is also longer by 0.3 Å than that of WT (Table IV). His-263(197) is conserved in the active sites of all known MBLs and is the residue that coordinates to Zn2. Suárez *et al.* (33) and Oelschlaeger *et al.* (46) reported that the Zn-Zn distance could possibly increase to 4.5–5.0 and 4.3–5.2 Å by using CcrA and IMP-1 in the substrate complex as a model in molecular dynamics simulations, respectively. This suggests that His-263(197) is flexible, to some extent, and this flexibility may cause an increase in the Zn-Zn distance.

The active site in D120(81)A was compared with that of WT (Fig. 4b). The active site in WT contains two Zn(II) ions, but the active site in D120(81)A contains only one Zn(II) ion, despite the fact that the Zn(II) content, as determined by atomic absorption spectroscopy, was two per protein molecule. This is probably due to the removal of Zn2 by chelation by the excess acetate ion present, because of the length of time required for crystal growth and the oxidation of Cys-221(158) to SO_2, resulting in a modified Cys-221(158) and Csw221(158), buried in the Zn2 coordination sphere. Zn1 was coordinated by an acetate ion acting as a bidentate ligand and three His(s) and was not coordinated by H_2O. These results are likely due to the increased positive charge because of the substitution of Asp with Ala at the 120 position (81 residue). This increase in positive charge could be responsible for the inhibition of D120(81)A by phosphate ion.

D120(81)E has two Zn(II) ions at the active site, as does WT, but its coordination environment is quite different from that of WT (Fig. 4c). It is assumed that an oxygen atom of Asp-120(81)OD1 of WT is not coordinated to Zn2 but binds to the bridging OH_2 or OH^- by a hydrogen bond, similar to CcrA (Fig. 1) (14, 28). In D120(81)E, one oxygen atom (Glu-120(81)OE1) would be able to bridge both Zn1 and Zn2 because the distances of Zn1-Glu-120(81)OE1 and Zn2-Glu-120(81)OE1 are 2.5 ± 0.6 and 2.5 ± 0.3 Å, respectively (Table IV). The Zn-Zn distance of D120(81)E (3.6 ± 0.1 Å) is longer by 0.3 Å than that of WT (3.3 Å). This indicates that the dissociation of H^+ from OH_2 is suppressed in D120(81)E because OH_2 would not bridge the two Zn(II) ions but was disordered in the structure, and the positive charges of the two Zn(II) ions would be dispersed among the two coordinated oxygen atoms of Glu. This type of coordination is in agreement with the pH dependence for hydrolysis for the reaction catalyzed by D120(81)E, *i.e.* the pK_a of the Zn1-bound H_2O is increased since it is not bridged. This

decrease in positive charge in the active site of D120(81)E is also in agreement with the binding of the nitrocefin hydrolysis product to D120(81)E.

D120(81)A was strongly inhibited by phosphate ion with nitrocefin as a reporter substrate, as shown in Fig. 6. This inhibition was weak for WT. The substitution of Asp with Ala changes the local charge in the active site, and a plausible cause for this is the increase in positive charge around the substrate-binding site and is in agreement with the x-ray structure of D120(81)A. The binding of phosphate ion to a Zn(II) ion or the catalytic active site would result in inhibition by lowering the extent of substrate binding. If this inhibition occurs through the binding of phosphate with the active site, the dissociation constants, assuming a competitive inhibition, are $K_i = 144$ and 0.32 mM for WT and D120(81)A, respectively. The effect of several anions on the inhibition of D120(81)A mutant was also evaluated. Whereas ClO_4^- and SO_4^{2-} had no effect, $[Co(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ showed K_i values of 1.1 and 4.0 mM, respectively. Moreover, when 100 μM $Zn(NO_3)_2$ was added to the reaction buffer, the inhibition of D120(81)A by phosphate ion was suppressed. From these results, strong inhibition of D120(81)A by phosphate ion occurred after the removal of the Zn(II) ion from the active site by chelation. The anion would bind to the active site, since the local positive electric charge of the active site in D120(81)A is increased.

CONCLUSION

The role of Asp-120 in IMP-1, which is a conserved amino acid in the active site of all MBLs, was investigated by the preparation of some Asp-120-mutants, kinetic studies, and x-ray crystallographies. The following four points can be made. (i) Asp-120 in IMP-1 plays the important roles in catalysis and the substrate recognition, as evidenced by steady-state kinetics studies. (ii) Asp-120 in IMP-1 does not act as a proton donor to the anion intermediate of nitrocefin and a proton shuttle, as evidenced by pH dependence studies of the hydrolysis. (iii) The hydrolytic mechanism for IMP-1 is different from those for CcrA and L1, and one of main reasons is the ease of proton transfer because there is no accumulation of the anion intermediate of nitrocefin, as evidenced by pre-steady-state kinetics studies using IMP-1 (44) and the IMP-1 mutants. (iv) From the results of x-ray crystallography, the structure of D120(81)E (the oxygen atom of Glu-120(81)OE1 is presumably not coordinated to bridging H_2O or OH), the oxygen atom of the carboxylate of Asp-120 is essential to coordinate the bridging H_2O or

OH⁻ by a hydrogen bond to achieve the catalytic activity. Thus, the critical role of Asp-120 is to orientate H_2O or OH^-, which acts as a nucleophile to the carbonyl carbon on the β-lactam ring. The crystal structures also show that Asp-120 is important not only for orienting water but also for positioning the Zn(II) ions and His-263.
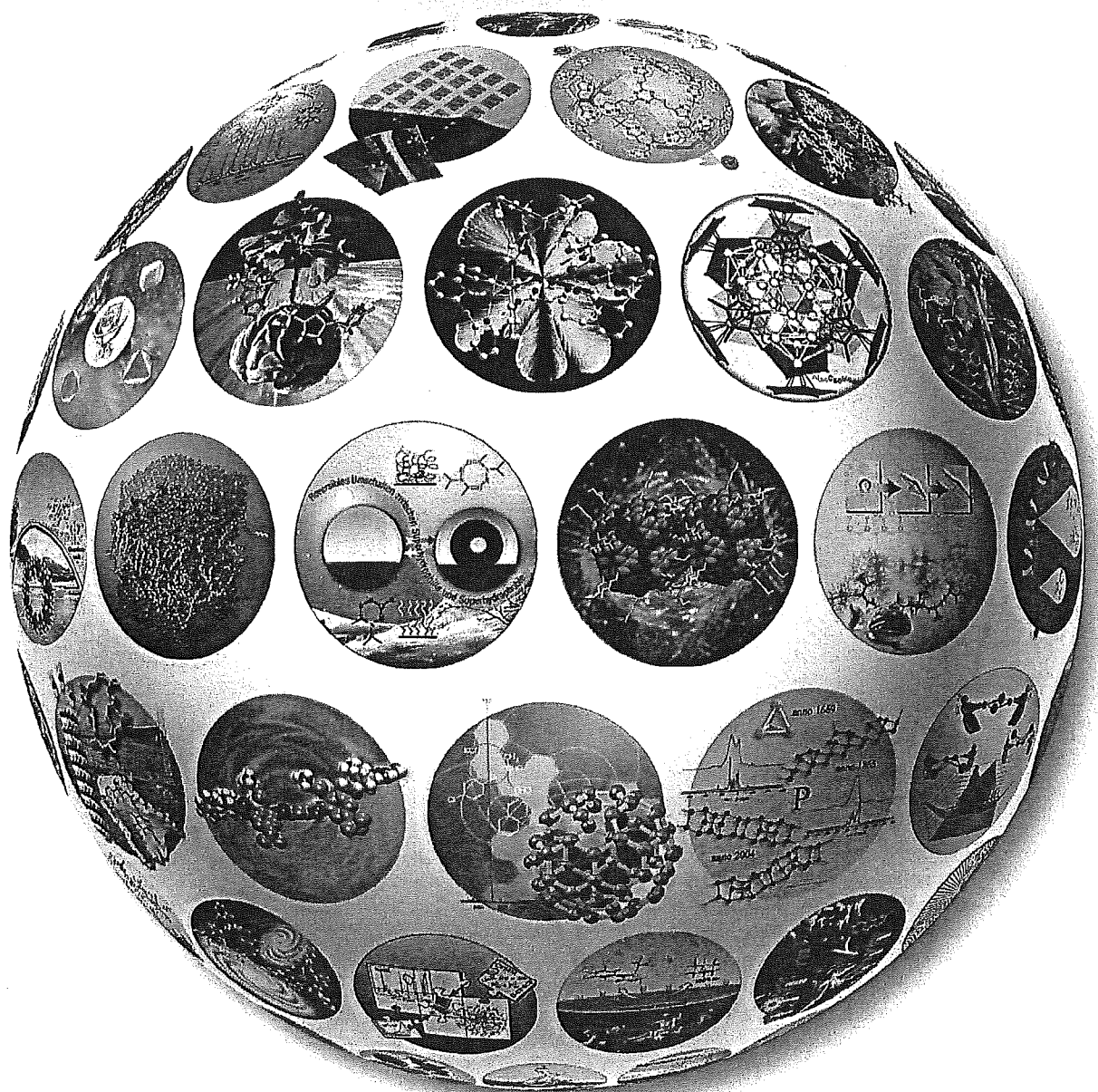
REFERENCES

1. Bush, K. (1998) *Clin. Infect. Dis.* **27**, Suppl. 1, 48–53
2. Ambler, R. P. (1980) *Philos. Trans. R. Soc. Lond. B Biol. Ser.* **289**, 321–331
3. Felici, A., Amicosante, G., Oratore, A., Strom, R., Ledent, P., Joris, B., Fanuel, L., and Frère, J.-M. (1993) *Biochem. J.* **291**, 151–155
4. Osano, E., Arakawa, Y., Wacharotayankun, R., Ohta, M., Horii, T., Ito, H., Yoshimura, F., and Kato, N. (1994) *Antimicrob. Agents Chemother.* **38**, 71–78
5. Poirel, L., Naas, T., Nicolas, D., Collet, L., Bellais, S., Cavallo, J.-D., and Nordmann, P. (2000) *Antimicrob. Agents Chemother.* **44**, 891–897
6. Rossolini, G. M., Franceschini, N., Riccio, M. L., Mercuri, P. S., Perilli, M., Galleni, M., Frère, J.-M., and Amicosante, G. (1998) *Biochem. J.* **332**, 145–152
7. Bellais, S., Aubert, D., Naas, T., and Nordmann, P. (2000) *Antimicrob. Agents Chemother.* **44**, 1878–1886
8. Docquier, J.-D., Pantanella, F., Giuliani, F., Thaller, M. C., Amicosante, G., Galleni, M., Frère, J.-M., Bush, K., and Rossolini, G. M. (2002) *Antimicrob. Agents Chemother.* **46**, 1823–1830
9. Mercuri, P. S., Bouillenne, F., Boschi, L., Lamotte-Brasseur, J., Amicosante, G., Devreese, B., Beeumen, J. V., Frère, J.-M., Rossolini, G. M., and Galleni, M. (2001) *Antimicrob. Agents Chemother.* **45**, 1254–1262
10. Goto, M., Takahashi, T., Yamashita, F., Koreeda, A., Mori, H., Ohta, M., and Arakawa, Y. (1997) *Biol. Pharm. Bull.* **20**, 1136–1140
11. Nagano, R., Adachi, Y., Imamura, H., Yamada, K., Hashizume, T., and Morishima, H. (1999) *Antimicrob. Agents Chemother.* **43**, 2497–2503
12. Greenlee, M. L., Laub, J. B., Balkovec, J. M., Hammond, M. L., Hammond, G. G., Pompliano, D. L., and Epstein-Toney, J. H. (1999) *Bioorg. Med. Chem. Lett.* **9**, 2549–2554
13. Toney, J. H., Cleary, K. A., Hammond, G. G., Yuan, X., May, W. J., Hutchins, S. M., Ashton, W. T., and Vanderwall, D. E. (1999) *Bioorg. Med. Chem. Lett.* **9**, 2741–2746
14. Concha, N. O., Janson, C. A., Rowling, P., Pearson, S., Cheever, C. A., Clarke, B. P., Lewis, C., Galleni, M., Frère, J.-M., Payne, D. J., Bateson, J. H., and Abdel-Meguid, S. S. (2000) *Biochemistry* **39**, 4288–4298
15. Mollard, C., Moali, C., Papamicael, C., Damblon, C., Vessilier, S., Amicosante, G., Schofield, C. J., Galleni, M., Frère, J.-M., and Roberts, G. C. K. (2001) *J. Biol. Chem.* **276**, 45015–45023
16. Toney, J. H., Hammond, G. G., Fitzgerald, P. M. D., Sharma, N., Balkovec, J. M., Rouen, G. P., Olson, S. H., Hammond, M. L., Greenlee, M. L., and Gao, Y.-D. (2001) *J. Biol. Chem.* **276**, 31913–31918
17. Siemann, S., Evanoff, D. P., Marrone, L., Clarke, A. J., Viswanatha, T., and Dmitrienko, G. I. (2002) *Antimicrob. Agents Chemother.* **46**, 2450–2457
18. Payne, D. J., Hueso-Rodríguez, J. A., Boyd, H., Concha, N. O., Janson, C. A., Gilpin, M., Bateson, J. H., Cheever, C., Niconovich, N. L., Pearson, S., Rittenhouse, S., Tew, D., Díez, E., Pérez, P., de la Fuente, J., Rees, M., and Rivera-Sagredo, A. (2002) *Antimicrob. Agents Chemother.* **46**, 1880–1886
19. Kurosaki, H., Yasuzawa, H., Yamaguchi, Y., Jin, W., Arakawa, Y., and Goto, M. (2003) *Org. Biomol. Chem.* **1**, 17–20
20. Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O., and Frère, J.-M. (2001) *Antimicrob. Agents Chemother.* **45**, 660–663
21. Hussain, M., Carlino, A., Madonna, M. J., and Lampen, J. O. (1985) *J. Bacteriol.* **164**, 223–229
22. Rasmussen, B. A., Gluzman, Y., and Tally, F. P. (1990) *Antimicrob. Agents Chemother.* **34**, 1590–1592
23. Laraki, N., Franceschini, N., Rossolini, G. M., Santucci, P., Meunier, C., de Pauw, E., Amicosante, G., Frère, J.-M., and Galleni, M. (1999) *Antimicrob. Agents Chemother.* **43**, 902–906
24. Massidda, O., Rossolini, G. M., and Satta, G. (1991) *J. Bacteriol.* **173**, 4611–4617
25. Walsh, T. R., Gamblin, S., Emery, D. C., MacGowan, A. P., and Bennett, P. M. (1996) *J. Antimicrob. Chemother.* **37**, 423–431
26. Rossolini, G. M., Condemi, M. A., Pantanella, F., Docquier, J.-D., Amicosante, G., and Thaller, M. C. (2001) *Antimicrob. Agents Chemother.* **45**, 837–844
27. Arakawa, Y., Murakami, M., Suzuki, K., Ito, H., Wacharotayankun, R., Ohsuka, S., Kato, N., and Ohta, M. (1995) *Antimicrob. Agents Chemother.* **39**, 1612–1615
28. Concha, N. O., Rasmussen, B. A., Bush, K., and Herzberg, O. (1996) *Structure (Lond.)* **4**, 823–836
29. Wang, Z., Fast, W., and Benkovic, S. J. (1998) *J. Am. Chem. Soc.* **120**, 10788–10789
30. Wang, Z., Fast, W., and Benkovic, S. J. (1999) *Biochemistry* **38**, 10013–10023
31. Yanchak, M. P., Taylor, R. A., and Crowder, M. W. (2000) *Biochemistry* **39**, 11330–11339
32. Díaz, N., Suárez, D., and Merz, K. M., Jr., (2000) *J. Am. Chem. Soc.* **122**, 4197–4208
33. Suárez, D., Brothers E. N., and Merz, K. M., Jr. (2002) *Biochemistry* **41**, 6615–6630
34. Goto, M., Yasuzawa, H., Higashi, T., Yamaguchi, Y., Kawanami, A., Mifune, S., Mori, H., Nakayama, H., Harada, K., and Arakawa, Y. (2003) *Biol. Pharm. Bull.* **26**, 589–594
35. McManus-Munoz, S., and Crowder, M. W. (1999) *Biochemistry* **38**, 1547–1553
36. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307–326
37. Navaza, J. (1994) *Acta Crystallogr. Sect. A* **50**, 157–163
38. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kruszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 905–921
39. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* **47**, 110–119
40. Kleywegt, G. J., and Jones, T. A. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 1119–1131
41. Haruta, S., Yamaguchi, H., Yamamoto, E. T., Eriguchi, Y., Nukaga, M., O'Hara, K., and Sawai, T. (2000) *Antimicrob. Agents Chemother.* **44**, 2304–2309
42. Kaminskaia, N. V., Spingler, B., and Lippard, S. J. (2000) *J. Am. Chem. Soc.* **122**, 6411–6422
43. Garrity, J. D., Carenbauer, A. L., Herron, L. R., and Crowder, M. W. (2004) *J. Biol. Chem.* **279**, 920–927
44. Moali, C., Anne, C., Lamotte-Brasseur, J., Groslambert, S., Devreese, B., Beeumen, J. V., Galleni, M., and Frère, J.-M. (2003) *Chem. Biol.* **10**, 319–329
45. Bicknell, R., and Waley, S. G. (1985) *Biochemistry* **24**, 6876–6887
46. Oelschlaeger, P., Schmid, R. D., and Pleiss, J. (2003) *Protein Eng.* **16**, 341–350
47. Wang, Z., and Benkovic, S. J. (1998) *J. Biol. Chem.* **273**, 22402–22408