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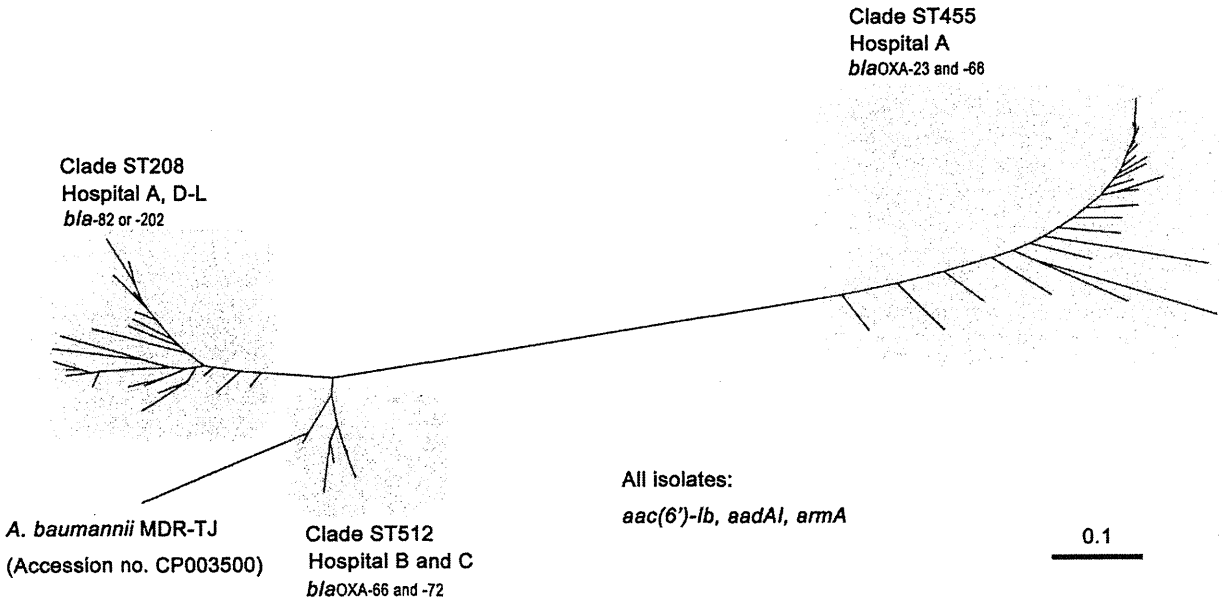

Jpn. J. Infect. Dis., 67, 120-121, 2014

Short Communication

Isolation of OXA-48 Carbapenemase-Producing *Klebsiella pneumoniae* ST101 from an Overseas Traveler Returning to Japan

Aki Hashimoto[1], Maki Nagamatsu[2,3], Norio Ohmagari[2], Kayoko Hayakawa[2]*, Yasuyuki Kato[2], and Teruo Kirikae[3]

[1]*Medical Mycology Research Center, Chiba University, Chiba 260-8673; and* [2]*Disease Control and Prevention Center and* [3]*Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, Tokyo162-0052, Japan*

(Received August 1, 2013. Accepted October 2, 2013)

SUMMARY: OXA-48 carbapenemase-producing organisms have emerged rapidly worldwide and may be transmitted through patients who receive medical care abroad. To our knowledge, this is the second case of OXA-48-producing *Klebsiella pneumoniae* isolated from a patient who had returned to Japan after receiving treatment abroad.

Infections by multidrug-resistant gram-negative rods present a great public health concern and have continued to spread worldwide (1). Receiving medical care abroad has been suggested as a potential route of transmission of multidrug-resistant organisms (MDROs) (2). β-Lactamase genes, particularly those coding for carbapenemase, have a high transmissibility rate and play a significant role in the development of multidrug resistance. The OXA-48 carbapenemase was first isolated from *Enterobacteriaceae* in Istanbul, Turkey in 2001 (3). Since then, outbreaks with enormous clinical impact have been reported worldwide (4–7). Furthermore, the isolation of OXA-48-producing organisms from patients transferred from foreign countries to their native countries has also been increasingly reported (8,9). The first case of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* was reported in Japan in December 2012 (9). Here we report the second case of an OXA-48-producing *K. pneumoniae* isolate from a clinical sample obtained from a patient returning to Japan.

An 84-year-old Japanese man with no significant past medical history or exposure to antimicrobial agents went on a 15-day tour to Egypt and Turkey in April 2012. On the 14th day of his trip, he presented at a hospital in Cairo, Egypt with vomiting, diarrhea, fever, and jaundice. He was subsequently diagnosed with traveler's diarrhea, septic shock, and obstructive jaundice and admitted to the intensive care unit (ICU), where administration of meropenem, ciprofloxacin, and metronidazole elicited a prompt response. On day 8, he was transported to our hospital in Tokyo, Japan, where abdominal ultrasoundgraphy and a computed tomography (CT) scan revealed a liver abscess. Due to the patient's history of receiving medical care outside Japan, he was considered to be at risk of infection by antimicrobial-resistant organisms. Therefore, he was kept in a single room with contact precaution. Screening results of a stool culture to identify MDROs were positive for *K. pneumoniae*, which was found to be resistant to third and fourth generation cephalosporins and levofloxacin. The minimum inhibitory concentration (MIC) of imipenem was 4 mg/L as measured using the MicroScan WalkAway™ system (Siemens AG, Munch, Germany). The MIC was also determined using the manual broth microdilution method as per the Clinical and Laboratory Standards Institute (CLSI) criteria (Table 1) (10). Polymerase chain reaction (PCR) with specific primers was used to detect genes encoding plasmid-mediated AmpC β-lactamases (bla_{ACC}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{FOX}, and bla_{MOX}), metallo-β-lactamases (bla_{AIM}, bla_{DIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, and bla_{SPM}) (11), carbapenemases (bla_{BIC}, bla_{KPC}, bla_{OXA-10}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, and bla_{OXA-51}) (2,12), and extended-spectrum β-lactamases (ESBL) (bla_{CTX-M}, bla_{PER}, bla_{SHV}, and bla_{TEM}) (12,13). DNA sequences of open reading frames of the drug-resistant PCR-positive genes were determined. The multidrug-resistant *K. pneumoniae* isolate harbored 3 ESBL-encoding genes (bla_{TEM-1}, bla_{SHV-1}, and $bla_{CTX-M-14}$) and a carbapenemase-encoding gene (bla_{OXA-48}), but no genes encoding plasmid-mediated AmpC β-lactamases or metallo-β-lactamase. Multilocus sequence typing (MLST) was performed as described in the *K. pneumoniae* MLST Database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). The sequence type (ST) of the isolate was ST101.

Blood culture test results for this patient upon admission were negative. The patient responded well to empiric treatment with 750 mg/day of levofloxacin and 1000 mg/day of metronidazole. Although the liver abscess was not drained, based on the clinical response to levofloxacin, OXA-48-producing *K. pneumoniae* was thought to be only colonizing organism, which was not contributing to the infectious clinical syndrome in this patient. He was discharged 21 days after arrival.

To our knowledge, this is the second case of isolation of OXA-48-producing *K. pneumoniae* in Japan. The first involved a man who had been hospitalized in a

*Corresponding author: Mailing address: Disease Control and Prevention Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo, 162-0052, Japan. Tel: +81-3-3202-7181, Fax: +81-3-3207-1038, E-mail: kayokohayakawa@gmail.com

Table 1. Minimum inhibitory concentrations (μg/mL) of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* isolate

	PIP	CEZ	CTX	CAZ	CMZ	IPM/CS	MEPM	MINO	LVFX	AMK	GM	ABK	AZT	T/S
W/A	≥128	≥32	≥64	≥16	≥64	4	NA	2	≥8	≥64	≥16	NA	≥32	2
BMD	>2048	NA	2048	32	64	NA	32	NA	16	64	16	≤0.25	128	NA

ABK, arbekacin; AMK, amikacin; AZT, aztreonam; BMD, broth microdilution; CAZ, ceftazidime; CEZ, cefazolin; CMZ, cefmetazole; CTX, cefotaxime; GM, gentamicin; IPM/CS, imipenem/cilastatin; LVFX, levofloxacin; MEPM, meropenem; MINO, minocycline; NA, data not available; PIP, piperacillin; T/S, trimethoprim/sulfamethoxazole; W/A, MicroScan WalkAway™.

Southeast Asian country. Multidrug-resistant (resistant to third and fourth generation cephalosporins, aminoglycosides, and quinolones; MIC of imipenem was 2 mg/L) *K. pneumoniae* and *E. coli* were isolated from the sputum and/or feces, and PCR analyses of the carbapenemase genes revealed the presence of a bla_{OXA-48}-like gene in these isolates (9).

Our patient had traveled to Turkey and received medical care at an ICU in Egypt. OXA-48-producing organisms have been reported in both countries (14). The patient had no other history of travel to a foreign country for 1 year prior to this episode; therefore, it is likely that he acquired OXA-48-producing *K. pneumoniae* while receiving medical care at the ICU in Egypt. Drug-resistant *K. pneumoniae* ST101 has been reported as a causative agent of outbreaks or as a predominant clone of nosocomial pathogens in medical settings in several Mediterranean countries, including Greece (15), Italy (16,17), Libya (18), and Spain (19). The isolate from our patient was identified as ST101 by MLST, and thus, it was considered not to be of the K1 serotype, which is associated with liver abscess (20).

Of particular concern, OXA-48 carbapenemase-producing organisms may not necessarily be reported as carbapenem-resistant based on the MIC, as most microbiology laboratories in Japan continue to use the former CLSI criteria, in which *Enterobacteriaceae* samples with an MIC for imipenem of ≤4 mg/L are categorized as susceptible to carbapenem (10). Clinical isolates that show resistance to third generation cephalosporins and/or other classes of antibiotics (e.g., aminoglycoside and quinolone) and reduced susceptibility (MIC >1 mg/L) to carbapenems should be carefully considered and analyzed. Screening for carbapenemase with the modified Hodge test and PCR analyses for such isolates is strongly recommended.

For patients at potential risk of infection, such as those with a history of hospitalization abroad, a proactive approach is necessary to control the spread of MDROs. Thus, screening of all patients with a history of hospitalization abroad, as well as those transferred from other hospitals and nursing homes, should be considered.

Acknowledgments This study was supported by a grant from the Ministry of Health, Labour and Welfare of Japan (H24-Shinko-Ippan-010).

Conflict of interest None to declare.

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Tada et al. BMC Infectious Diseases 2014, 14:56
http://www.biomedcentral.com/1471-2334/14/56

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

NDM-1 Metallo-β-Lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal

Tatsuya Tada[1], Tohru Miyoshi-Akiyama[1], Rajan K Dahal[3], Manoj K Sah[3], Hiroshi Ohara[2], Kayo Shimada[1], Teruo Kirikae[1*] and Bharat M Pokhrel[3]

Abstract

Background: Drug-resistant *Providencia rettgeri* producing metallo-β-lactamase and 16S rRNA methylase has been reported in several countries. We analyzed *P. rettgeri* clinical isolates with resistance to carbapenems and aminoglycosides in a hospital in Nepal.

Methods: Five clinical isolates of multidrug-resistant *P. rettgeri* were obtained in a hospital in Nepal. Antimicrobial susceptibilities were determined using the microdilution method and entire genomes were sequenced to determine drug-resistant genes. Epidemiological analysis was performed by pulsed-field gel electrophoresis.

Results: Four of the 5 isolates were resistant to carbapenems (imipenem and meropenem), with MICs ≥16 mg/L, with the remaining isolate showing intermediate resistance to imipenem, with an MIC of 2 mg/L and susceptibility to meropenem with an MIC ≤1 mg/L. All 5 isolates had bla_{VEB-1}. Of the 4 carbapenem-resistant strains, 3 had bla_{NDM-1} and 1 had bla_{OXA-72}. All isolates were highly resistant to aminoglycosides (MICs ≥1,024 mg/L) and harbored *armA*. As the result of pulsed-field gel electrophoresis pattern analysis in the 5 *P. rettgeri* isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity.

Conclusions: This is the first report describing multidrug-resistant *P. rettgeri* strains harboring bla_{NDM-1} or bla_{OXA-72} and *armA* isolated from patients in Nepal.

Keywords: NDM-1, OXA-72, 16S rRNA methylase, *Providencia rettgeri*, Molecular epidemiology

Background

Providencia rettgeri has been associated with hospital acquired infections, including catheter-related urinary tract infections, bacteremia, skin infections, diarrhea, and gastroenteritis [1,2]. To date, there have been 5 reports of *P. rettgeri* isolates harboring metallo-β-lactamase (MBL) encoding genes, including IMP-type MBL producers in Japan [3,4]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [5]; and NDM-type MBL in Israel [6] and Brazil [7].

NDM-type MBL was initially identified in *Klebsiella pneumoniae* and *Escherichia coli* in 2009 in Sweden [8]. Since then, NDM-1-producing *Enterobacteriaceae* have been isolated in various parts of the world [9,10].

Exogenously acquired 16S rRNA methylase genes responsible for very high levels of resistance to various aminoglycosides are widely distributed among *Enterobacteriaceae* and glucose-nonfermentative microbes [11]. Gram-negative pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [11].

Although co-production of several resistance determinants is not rare in *Enterobacteriaceae* [12-16], it is less common in *P. rettgeri* [5]. We describe here *P. rettgeri* clinical isolates from Nepal that produce carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA).

* Correspondence: tkirikae@ri.ncgm.go.jp
[1]Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan
Full list of author information is available at the end of the article

Table 1 Summary of the characteristics of the 5 *P. rettegeri* strains, including antimicrobial resistance profiles and resistant genes

Strains	Tissue sources	Infection	MIC (mg/L)														Antibiotics resistant genes
			PIP	TZP	CAZ	FEP	IPM	DPM	MEM	ATM	ABK	AMK	GEN	CIP	CST	FOF	TIG	
IOMTU1	Pus	SSI	1,024	512	>1,024	64	32	16	64	1,024	>1,024	>1,024	>1,024	128	>128	512	4	bla_{NDM-1}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*, *aadA2*
IOMTU4	Sputum	NLRTI	1,024	128	>1,024	256	16	16	32	1,024	>1,024	>1,024	>1,024	>256	>128	512	4	bla_{OXA-72}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU91	Sputum	NLRTI	>1,024	1,024	>1,024	1,024	64	32	64	1,024	>1,024	>1,024	>1,024	256	128	128	4	bla_{NDM-1}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU94	Pus	SSI	1,024	4	>1,024	256	2	1	1	>1,024	1,024	1,024	>1,024	256	>128	1,024	4	bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU99	Sputum	NLRTI	>1,024	512	>1,024	128	64	32	64	1,024	>1,024	>1,024	>1,024	>256	>128	1,024	4	bla_{NDM-1}, bla_{VEB-1}, bla_{OXA-10}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*

SSI, surgical site infection; NLRTI, nosocomial lower respiratory tract infection PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; DPM, doripenem; MEM, meropenem; ATM, aztreonam; ABK, arbekacin; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; CST, colistin; FOF, fosfomycin; TIG, tigecycline.

Methods

Bacterial strains

Five *P. rettgeri* clinical isolates were obtained from May to July 2012 from 5 patients at Tribhuvan University Teaching Hospital in Kathmandu, Nepal. Three isolates were from sputum and 2 from pus at surgical sites. Samples were obtained as part of standard patient care. Phenotypical identification [17] was confirmed by API 32GN (BioMérieux, Mercy l'Etoile, France) and 16S rRNA sequencing (1,497 bp) [18,19].

Antimicrobial susceptibilities

MICs were determined using the microdilution method, according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [20]. Breakpoints to antibiotics were determined. The modified Hodge test, the meropenem-sodium mercaptoacetic acid double-disk synergy test (Eiken Chemical, Tokyo, Japan) and E-test (imipenem/EDTA) (AB Biodisk, Solna, Sweden) were performed.

Entire genome sequencing

The entire genomes of these isolates were extracted and sequenced by MiSeq (Illumina, San Diego, CA). CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used for de novo assembly of reads and to search for 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acethyl/adenylyltransferases; point mutations in the *gyrA*, *parC* and *pmrCAB* operons; and point mutations in the *fos* genes, including *fosA*, *fosA2*, *fosA3*, *fosC* and *fosC2*.

Pulsed-field gel electrophoresis (PFGE) and southern hybridization

PFGE analysis was performed as described [3]. An 813 bp probe for bla_{NDM-1} was synthesized by PCR amplification using the primers 5′-atggaattgcccaatattatg-cac-3′ (forward) and 5′-tcagcgcagcttgtcggccatgcggg-3′ (reverse), and a 780 bp probe for bla_{OXA-72} was synthesized using the primers 5′-agtttctctcagtgcatgttcatctat-3′ (forward) and 5′-agaaccagacattccttctttcatttc-3′ (reverse). Southern hybridization to detect bla_{NDM-1} and bla_{OXA-72} was performed using these probes, which were detected using DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics, Mannheim, Germany).

Nucleotide sequence accession numbers

The nucleotide sequences surrounding bla_{NDM-1} and bla_{OXA-72} have been deposited in GenBank with the accession number AB828598 and AB857844, respectively.

Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of the Institute of Medicine, Tribhuvan University (ref. 6-11-E) and the Biosafety Committee, National Center for Global Health and Medicine (approval number: 23-M-49).

Results

Antimicrobial susceptibilities

Four of the 5 isolates were resistant to carbapenems (doripenem, imipenem and meropenem) and piperacillin/tazobactam, whereas the fifth was susceptible to piperacillin/tazobactam, doripenem and meropenem and showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cefepime), aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin, and all 5 showed intermediate resistance to tigecycline. The four isolates resistant to carbapenems were negative with the modified Hodge test, but three of the four isolates were positive with the meropenem-sodium mercaptoacetic acid double-disk synergy test and E-test/EDTA.

Drug-resistant genes

All 5 isolates tested had several genes associated with β-lactam and aminoglycoside-resistance (Table 1). These isolates had bla_{VEB-1}, bla_{OXA-10}, bla_{TEM-1}, bla_{ADC-67} (*ampC*), *armA* and *aadA1*; 3 had bla_{NDM-1}; and 1 had bla_{OXA-72}. None of these isolates had any other β-lactamase encoding genes, including the class A genes bla_{SHVs} and bla_{CTX-Ms}; the class B genes bla_{AIM}, bla_{DIM}, bla_{FIM}, bla_{GIM}, bla_{IMPs}, bla_{INDs}, bla_{KHM}, bla_{SIM}, bla_{SMB}, bla_{SPM}, bla_{TMBs}, and bla_{VIMs}; or the class D gene bla_{OXAs} except for bla_{OXA-10} and bla_{OXA-72}. None had other genes encoding 16S rRNA methylases or aminoglycoside acetyl/adenylyltransferases. All 5 isolates had point mutations in the quinolone-resistance-determining regions of *gyrA* and *parC*, with amino acid substitutions of S83I and D87E in GyrA and S80I in ParC, but none had any mutations in the *pmrCAB* operon and *fos* genes. All sequences of the drug-resistant genes tested were identical to those registered in GenBank.

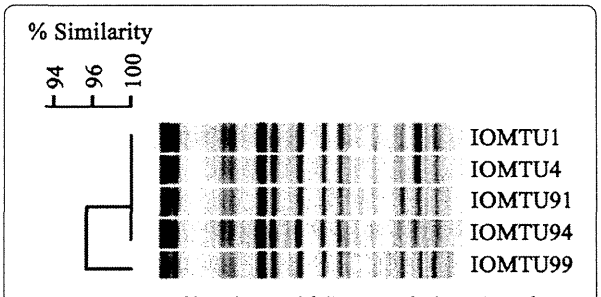

Figure 1 PFGE profiles obtained following *SfiI* digestion of *P. rettgeri* chromosomes.

PFGE and southern hybridyzation

Of the 5 *P. rettgeri* isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity (Figure 1). Three of these isolates had a plasmid harboring bla_{NDM-1} and one had a plasmid harboring bla_{OXA-72}, with plasmid sizes ranging from 9.42 to 23.1 kbp (data not shown).

Genomic structures surrounding bla_{NDM-1} and bla_{OXA-72}

The genetic environments surrounding bla_{NDM-1} (Accession no. AB828598) was bla_{NDM-1}-ble_{MBL}-*trpF-dsbC-cutA1*. All 3 isolates harboring bla_{NDM-1} (IOMTU1, 91 and 99) had the same genetic environments. The bla_{OXA-72} gene was flanked by conserved inverted repeats at the XerC/XerD binding sites [21], indicating mobilization by site-specific recombination mechanisms. The *repl* gene was located downstream of bla_{OXA-72} (Accession no. AB857844).

Discussion

The relatively high MICs to piperacillin/tazobactam and carbapenems of the five *P. rettgeri* isolates were likely due to the presence of bla_{NDM-1} or bla_{OXA-72}. The enzymatic activities of metallo-β-lactamases, including NDM-1, were not inhibited by tazobactam [22], a β-lactamase inhibitor, in agreement with the MIC profiles of these isolates to piperacillin/tazobactam. The high MICs of all 5 isolates to ceftazidime, cefepime and aztreonam were likely due to the presence of bla_{VEB-1} [23], and the presence of *armA* in these isolates was likely associated with their extremely high resistance to all aminoglycosides tested [11]. Point mutations in the quinolone-resistance-determining regions of *gyrA* and *parC* have been associated with high resistance to quinolones [24]. Point mutations in *pmrCAB* operon have been associated with the resistance of *Acinetobacter* spp. [25] and *Pseudomonas aeruginosa* [26] to polymxyin and colistin; and the presence of *fos* genes, including *fosA*, *fosA2*, *fosA3*, *fosC* and *fosC2*, has been associated with resistance to fosfomycin in Gram-negative bacteria [27-29].

Plasmids containing bla_{NDM-1} or bla_{OXA-72} may be disseminated among Gram-negative pathogens in Nepal. The genetic environments surrounding bla_{NDM-1} in our *P. rettgeri* strains (bla_{NDM-1}-ble_{MBL}-*trpF-dsbC-cutA1*) were also observed in other plasmids, including *A. baumannii* plasmid pAbNDM-1 from China (Accession no. JN377410), *Citrobacter freundii* plasmid pYE315203 from China (Accession no. JX254913), *E. coli* plasmid pNDM102337 from Canada (Accession no. JF714412), *K. pneumoniae* plasmid pKP-NCGM18-1 from Nepal (Accession no. AB824738) [30], *K. pneumoniae* plasmids pKPX-1, pKPN5047 and pNDM-HN380 from China (Accession nos. AP012055, KC311431 and JX104760, respectively), and *P. rettgeri* plasmid pFR90 (Accession no. JQ362415) from China. In addition, the genetic structures of OXA-72 producing *Acinetobacter* spp [31-34] and *K. pneumoniae* (Accession no. JX268653 and AB825955 deposited in 2012 and 2013, respectively) had the same genetic structure (bla_{OXA-72}-*repl*) as our strain of *P. rettgeri*.

Conclusions

To our knowledge, this is the first report describing *P. rettgeri* strains harboring bla_{NDM-1} or bla_{OXA-72} and *armA* isolated from patients in Nepal. These 5 strains were highly resistant to both β-lactams and aminoglycosides and expanded in a clonal manner in the hospital.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

TT: Performed PCR and sequencing, analyzed data and drafted the manuscript. TMA: Performed entire genome sequencing. RKD and MKS: Performed drug susceptibility tests. HO: Supervised this study. KS: Performed pulsed-field gel electrophoresis and its pattern analysis. TK and BMP: Designed protocols and supervised this study. All authors read and approved the final manuscript.

Acknowledgements

The authors thank emeritus professor Masayasu Nakano, Jichi Medical University, for comments on the manuscript. This study was supported by grants [International Health Cooperation Research (23-A-301 and 24-S-5), a grant from the Ministry of Health, Labor and Welfare of Japan (H24-Shinko-Ippan-010), and JSPS KAKENHI Grant Number 24790432].

Author details

[1]Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. [2]Department of International Medical-Cooporation, National Center for Global Health and Medicine, Shinjuku, Tokyo, Japan. [3]Department of Microbiology, Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu, Nepal.

Received: 1 May 2013 Accepted: 27 January 2014
Published: 3 February 2014

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doi:10.1186/1471-2334-14-56
Cite this article as: Tada *et al.*: NDM-1 Metallo-β-Lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal. *BMC Infectious Diseases* 2014 **14**:56.

Regular Article

Crystal Structure of IMP-2 Metallo-β-lactamase from *Acinetobacter* spp.: Comparison of Active-Site Loop Structures between IMP-1 and IMP-2

Yoshihiro Yamaguchi,*[,a,#] Satoshi Matsueda,[b] Kazuyo Matsunaga,[b] Nobutoshi Takashio,[b] Sachiko Toma-Fukai,[c] Yuriko Yamagata,[b] Naohiro Shibata,[d] Jun-ichi Wachino,[e] Keigo Shibayama,[d] Yoshichika Arakawa,[e] and Hiromasa Kurosaki*[,b,#]

[a] *Environmental Safety Center, Kumamoto University; 2–39–1 Kurokami, Chuo-ku, Kumamoto 860–8555, Japan:* [b] *Graduate School of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Chuo-ku, Kumamoto 862–0973, Japan:* [c] *Graduate School of Pharmaceutical Sciences, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan:* [d] *Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases; 4–7–1 Gakuen, Musashi-Murayama, Tokyo 208–0011, Japan: and* [e] *Department of Bacteriology, Nagoya University Graduate School of Medicine; 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan.*
Received August 18, 2014; accepted October 30, 2014

IMP-2, a subclass B1 metallo-β-lactamase (MBL), is a Zn(II)-containing hydrolase. This hydrolase, involved in antibiotic resistance, catalyzes the hydrolysis of the C–N bond of the β-lactam ring in β-lactam antibiotics such as benzylpenicillin and imipenem. The crystal structure of IMP-2 MBL from *Acinetobacter* spp. was determined at 2.3 Å resolution. This structure is analogous to that of subclass B1 MBLs such as IMP-1 and VIM-2. Comparison of the structures of IMP-1 and IMP-2, which have an 85% amino acid identity, suggests that the amino acid substitution at position 68 on a β-strand (β3) (Pro in IMP-1 *versus* Ser in IMP-2) may be a staple factor affecting the flexibility of loop 1 (comprising residues at positions 60–66; EVNGWGV). In the IMP-1 structure, loop 1 adopts an open, disordered conformation. On the other hand, loop 1 of IMP-2 forms a closed conformation in which the side chain of Trp64, involved in substrate binding, is oriented so as to cover the active site, even though there is an acetate ion in the active site of both IMP-1 and IMP-2. Loop 1 of IMP-2 has a more flexible structure in comparison to IMP-1 due to having a Ser residue instead of the Pro residue at position 68, indicating that this difference in sequence may be a trigger to induce a more flexible conformation in loop 1.

Key words antibiotic resistance; β-lactam antibiotic; metallo-β-lactamase; X-ray crystallography

β-Lactamases catalyze the hydrolysis of β-lactams, opening the β-lactam ring and rendering the antibiotics inactive. β-Lactamases are classified into four classes, A–D[1,2]: Classes A, C, and D are serine enzymes that use a serine residue as a nucleophile, whereas class B consists of metallo enzymes whose active sites contain one or two Zn(II) ion(s) and are referred to as metallo-β-lactamases (MBLs). MBLs are divided into three subclasses (B1, B2, B3) based on the sequence of the Zn(II) ligands.[3] MBLs hydrolyze most β-lactams used currently, such as cephems and carbapenems, but not monobactam such as aztreonam. MBLs are hardly blocked by the inhibitors for serine β-lactamases, including clavulanate, sulbactam and, tazobactam.

In 1994, IMP-1 MBL, belonging to subclass B1, was first identified from *Serratia marcescens* and *Pseudomonas aeruginosa* in Japan.[4,5] Its gene, bla_{IMP}, encodes the IMP-1 enzyme and is integrated as a gene cassette into integrons carried by transferable plasmids.[6] Therefore, the bla_{IMP} gene can spread among different nosocomial pathogens horizontally. To date, at least 48 variants of IMP-type MBLs have been deposited (http://www.lahey.org/Studies) by the end of July 2014.

In 1997, an IMP-2 MBL was identified from an *Acinetobacter baumannii* clinical isolate AC-54/97 in Italy,[7] followed by the isolation of IMP-2-producing *A. baumannii*, *A. lowffii*, and *P. aeruginosa* in Japan.[8] The IMP-2 gene (bla_{IMP-2}) is also carried as an integron-bone gene cassette, similar to the IMP-1 gene (bla_{IMP}).[6,7] IMP-2 possesses approximately an 85% amino acid identity with IMP-1, and differs in 36 amino acids from IMP-1: 10 amino acid residues are clustered within the signal peptide region and the remaining 26 amino acid residues are found in the mature protein[7] (Fig. 2C). The structure of IMP-1 suggests that 4 of 26 amino acid residues predicted to be involved in substrate recognition in IMP-2 (Ser68, Gln198, Asp227, and Ser261; the amino acid residues of IMP-1 and IMP-2 are designated by their BBL number[3]) are located in the neighborhood at its active site within a distance of *ca.* 9 Å (Fig. 1). The remaining 22 amino acid residues are located at the protein surface or are far from the active site.

The kinetic parameters of the hydrolysis of several β-lactams by IMP-2 are overall similar to those by IMP-1, but the catalytic efficiency values of the two enzymes (k_{cat}/K_m) for ampicillin are different[7]: the k_{cat}/K_m values are 4.8 $\mu M^{-1} s^{-1}$ for IMP-1 and 0.21 $\mu M^{-1} s^{-1}$ for IMP-2.[7] The k_{cat}/K_m value of IMP-1 to IMP-2 increases 23-fold, so IMP-1 hydrolyses ampicillin more efficiently than IMP-2. These differences in kinetic parameters might be related to the subtle structural changes arising from the different amino acid sequences of the enzymes, even though the 6 amino acid residues (His116, His118, Asp120, His196, Cys221, and His263) which construct the active site of the enzyme are conserved between IMP-1 and IMP-2.

Therefore, determination of the fine three-dimensional

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: yyamagu@gpo.kumamoto-u.ac.jp; ayasaya@gpo.kumamoto-u.ac.jp

structure of IMP-2 would be very useful for elucidating the mechanism underlying the difference in the substrate specificity between IMP-1 and IMP-2 in order to develop inhibitors specific for MBLs. Here, we describe the crystal structure of IMP-2 MBL from *Acinetobacter* spp.

MATERIALS AND METHODS

Plasmid and Reagents The pBC SK(+) plasmid vector was purchased from Agilent Technologies, Inc. (Santa Clara, CA, U.S.A.). Ampicillin and zinc(II) nitrate hexahydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was purchased from Dojindo Laboratories (Kumamoto, Japan). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Nacalai Tesque (Kyoto, Japan). Polyethylene glycol 4000 (PEG 4000) was purchased from Hampton Research (Aliso Viejo, CA, U.S.A.). All other reagents were of the highest grade commercially available.

Expression and Purification The IMP-2 enzyme was expressed in *Escherichia coli* HB101 harboring pBC SK(+) vector carrying the bla_{IMP-2} gene; pBC SK(+)/bla_{IMP-2}. The cells were cultured in 2 L of LB broth containing ampicillin (50 μg/mL) for 14 h at 37°C, then centrifuged at 6000×g for 15 min at 4°C. The pellet was resuspended in 30 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 10 μM $Zn(NO_3)_2$. The cells were disrupted by sonication, then centrifuged at 105000×g for 75 min at 4°C. The supernatant was purified by column chromatography. Cation exchange chromatography was performed using a SP Sepharose Fast Flow column (ϕ26 mm×100 cm, GE Healthcare UK Ltd., Little Chalfont, U.K.) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 10 μM $Zn(NO_3)_2$. Bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in 50 mM sodium phosphate buffer (pH 7.0) containing 10 μM $Zn(NO_3)_2$. Fractions exhibiting β-lactamase activity were collected, pooled, and concentrated by ultrafiltration with an Amicon YM-10 (Merck KGaA, Darmstadt, Germany). Then, the sample buffer was exchanged with 50 mM Tris–HCl buffer (pH 7.4) containing 0.3 M NaCl, followed by concentration by ultrafiltration with a Centricon YM-10 (Merck KGaA) to 2 mL. The concentrated samples were applied to a gel filtration column (Sephacryl HR-100, ϕ16 mm×80 cm, GE Healthcare), pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.4) containing 0.3 M NaCl. Fractions exhibiting β-lactamase activity were collected, pooled, concentrated by ultrafiltration using an Amicon YM-10 (Merck KGaA), and then stored at −80°C. The purity of the preparation was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); the final preparation showed a single band using Comassie Brilliant Blue (CBB) dye, indicating more than 95% purity. For crystallization of the purified IMP-2 enzyme, the protein buffer was exchanged with 20 mM HEPES–NaOH (pH 7.5) using an Amicon Ultra (Merck KGaA).

Crystallization Initial screening of IMP-2 crystallization conditions was performed using the hanging drop method at 293 K by referring to the IMP-1 crystallization conditions.[9] Drops prepared by mixing 3 μL of protein solution (5 mg/mL) with 3 μL of reservoir solution, and were equilibrated against 350 μL of reservoir solution in the well. Crystals of IMP-2 were appeared after one month using a reservoir solution consisting of 30% (w/v) PEG 4000, 0.1 M citric acid/sodium citrate buffer containing 0.2 M sodium acetate (pH 6.0).

Data Collection and Refinement X-Ray diffraction data

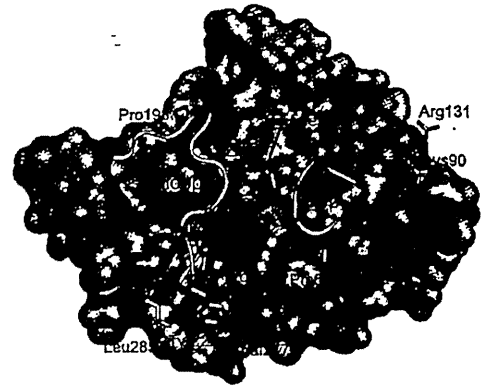

Fig. 1. Molecular Surface Representation of IMP-1

Zn(II) ions are shown as green spheres, and loop 1 and loop 2 are shown as yellow ribbon models. The acetate ion and the mutated amido acid residues as compared to the IMP-2 sequence are shown as sticks. The amino acid residues of IMP-1 are designated by their BBL number.[3]

Table 1. Crystallographic Data Collection and Refinement Statistics for IMP-2

Data collection	
Resolution (Å)	44.2–2.30 (2.38–2.30)[a]
Wavelength (Å)	1.5418
Cell dimensions	
a, b, and c (Å)	37.9, 68.5, 88.3
α, β, and γ (°)	90.0, 90.0, 90.0
Space group	$P2_12_12_1$
Redundancy	6.81 (6.62)
Completeness (%)	99.8 (100.0)
R_{merge}[b]	0.095 (0.255)
No. of observed reflections	73284 (7065)
No. of unique reflections	10767 (1068)
$\langle I/(\sigma)\rangle$	6.6 (2.3)
Refinement statistics	
σ Cutoff	None
Resolution (Å)	44.2–2.30 (2.63–2.30)
No. of reflections used	9994 (751)
B factors (Å^2)	
Average	31.9
Protein	31.8
Ligand	26.6
Water	34.1
No. of non-H atoms[c]	
Protein	1717
Ligand	5
Water	113
R.m.s.d deviation from ideal[d]	
Bond lengths (Å)	0.009
Angles (deg.)	1.17
$R_{working}$[e]	0.232 (0.276)
R_{free}[f]	0.299 (0.265)

a) Values in parentheses are for the highest resolution shell. *b*) $R_{merge}=\Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity for reflection hkl and $\langle I(hkl)\rangle$ is the average intensity calculated for reflection j from replicate data. *c*) Per asymmetric unit. *d*) R.m.s.d: root-mean-square-deviation. *e*) $R_{working}=\Sigma_{hkl}||F_o|-|F_c||/\Sigma_{hkl}|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. *f*) $R_{free}=\Sigma_{hkl}||F_o|-|F_c||/\Sigma_{hkl}|F_o|$ for 5% of the data not used at any stage of structural refinement.

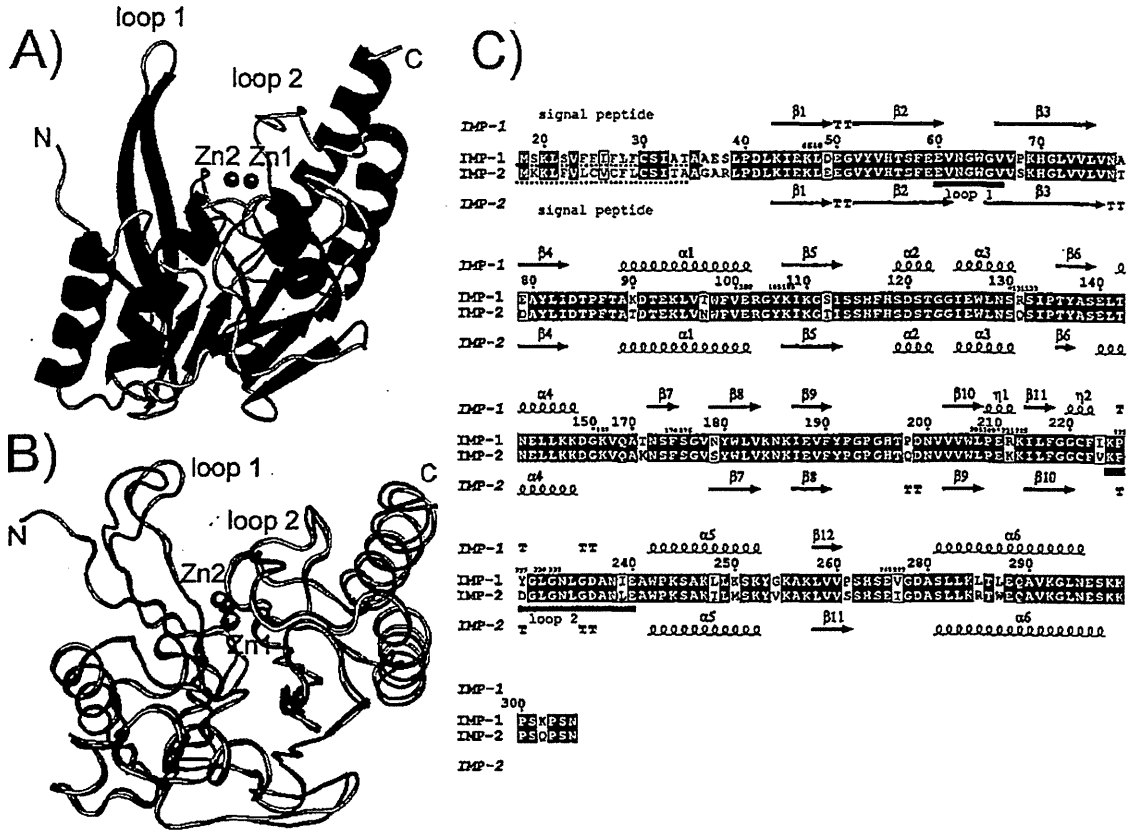

Fig. 2. A) Overall Structure of IMP-2 from *Acinetobacter* spp.

α-Helices, β-strands, loops, and Zn(II) ions are shown in red, green, yellow, and orange, respectively. B) Superposition of IMP-1 (orange) and IMP-2 (beige) structures. Considerable differences are observed in the loop 1 motifs in both IMP-1 and IMP-2 structures. C) Sequence alignment and secondary structures of IMP-2 from *Acinetobacter* spp. with that of IMP-1 from *Serratia marcescens* using the PDB file, 1DDK (IMP-1), and the structure from this study (IMP-2). References for each sequence are as follows: IMP-1 (EMBL/GenBank/DDBJ accession number: IMP-1 (S71932)) and IMP-2 (AB182996). The figure was produced using the ESPript 3.0 program (http://espript.ibcp.fr).[10] The BBL number is indicated above the sequences.[3] The dashed lines indicate the signal peptide sequences. Invariant residues are shown in red columns and conserved residues are shown in boxes. The arrows indicate β-sheets, the coils indicate α-helices, TT indicates β turns, and η indicates 3_{10} helices. The loop 1 and loop 2 regions in IMP-1 and IMP-2 are underlined in blue.

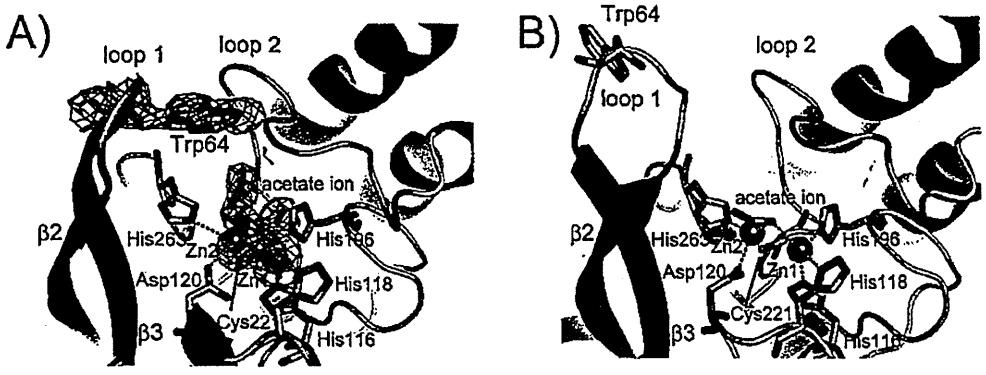

Fig. 3. Comparison of the IMP-2 Structure and the IMP-1 Structure

α-Helices, β-strands, and loops are shown in red, green, and yellow, respectively. Zn(II) ions are shown as orange spheres. Trp64, His116, His118, Asp120, His196, Cys221, and His263 residues and an acetate ion are represented as sticks (carbon, gray; nitrogen, blue; oxygen, red; and sulfur, light green). A) Structure of the active site in IMP-2. The electron density map (cyan mesh) is shown contoured at the 1.0σ level in the $2|F_o|-|F_c|$ map. B) Structure of the active site in IMP-1.

were collected in house X-ray diffraction system. Cu*Kα* X-ray radiation from a rotating-anode X-ray generator (Rigaku Micro Max007, Rigaku Corporation, Tokyo, Japan) and an imaging-plate detector (Rigaku R-AXIS VII) were used. Crystals could be flash-cooled at 100 K in a stream of cold nitrogen without cryoprotectant to avoid crystal cracking. Diffraction data from IMP-2 crystals were collected to 2.30 Å resolution. The diffraction data sets were processed using program CrystalClear (Rigaku Corporation). The crystallographic statistics of the collected data are summarized in Table 1.

The structure of IMP-2 was solved by molecular replacement using the program Morlep[10] of the CCP4 suite ver. 6.3[11] using the structure of IMP-1 from *P. aeruginosa* (PDB code: 1DD6) as a search model. The initial model was refined

with REFMAC 5.5[12] in the CCP4 suite[11] using resolution limits of 44.2–2.30 Å. Water molecules were added using Coot 0.7[13] selected from peaks in the $2|F_o|-|F_c|$ difference density map (σ=1.8). The final model had an $R_{working}$ factor of 23.2% and an R_{free} factor of 29.9%. The quality of the final model was checked with RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).[14] The Ramachandran plot showed 95.8% (207 residues) of the residues in the favoured region and 3.7% (8 residues) of the residues in the allowed region, while 0.5% (1 residue; Asp84) was in the outlier region.

All structural figures were prepared using the program PyMOL v0.99rc6.[15]

PDB Accession Code Coordinates and structural factors have been deposited in the PDB under the accession code: 4UBQ

RESULTS AND DISCUSSION

Overall Structure of IMP-2 The final refined model of IMP-2 per asymmetric unit included one IMP-2 molecule consisting of residues Leu39–Lys298, two Zn(II) ions, 113 water molecules, and one acetate ion. The overall structure of IMP-2 adopts an $\alpha\beta/\beta\alpha$ sandwich structure, with an interface comprising two central antiparallel β-strands surrounded by two α-helices (Fig. 2A), similar to the structural fold in other subclass B1 MBLs such as IMP-1,[9] CcrA,[16] IND-7,[17] and VIM-2.[18] The N-terminal domain consists of four α-helices (α1–α4) and six antiparallel β-strands (β1–β6), whereas the C-terminal domain is formed by two α-helices (α5 and α6) and five antiparallel β-strands (β7–β11). The active site of IMP-2 contains two Zn(II) ions (Zn1, Zn2) separated by 3.2 Å and is located at the bottom of a wide, shallow cleft enclosed by two extended loops (loop 1, loop 2, Fig. 2A). Loop 1, a β-turn connected by two antiparallel β-strands (β2, β3), comprises residues 60–66 (EVNGWGV) (Fig. 2C). Loop 1 is likely involved in the binding of substrates or inhibitors.[19,20] Loop 2, which connects a strand (β10) and a helix (α5), is composed of residues 224–240 (Fig. 2) and is located on approximately the opposite side of loop 1 centered around the Zn(II) ion-binding site. Lys224 and Asn233 on loop 2 participated in substrate and inhibitor binding.[9,21,22]

Asp84 in IMP-2 is the outlier in the Ramachandran plot and has a sterically strained main chain conformation, with ϕ and ψ angles of 59° and 150°, respectively. The carboxylate oxygen, OD1, is hydrogen bonded to Ser115OG (3.0 Å) and Ser115N (2.8 Å), whereas OD2 is hydrogen bonded to Lys69NZ (2.7 Å), Ser115OG (3.1 Å), and Ser121OG (2.7 Å). In IMP-1, Asp84 also has a sterically strained main chain conformation in both the native and in the inhibitor complex, 2-[5-(1-tetrazoylmethyl)thien-3-yl]-*N*-[2-(mercaptomethyl)-4-(phenylbutyrylglycine)] with mean ϕ and ψ angles of 81° and 148°, respectively.[9] The carboxylate oxygen atoms of Asp84 in IMP-1 form hydrogen bonds to Lys69NZ (2.8 Å), Ser115N (2.8 Å), Ser115OG (2.8 Å), and Ser121OG (2.7 Å).[9] Asp84 has a common strained conformation not only in IMP-1 and IMP-2, but also in other subclass B1 MBLs.[9,16,23] Therefore, Asp84 likely plays an important role in the folding of MBLs.

Structural Comparison with IMP-1 The overall structure of IMP-2 superposed on IMP-1 (PDB code: 1DDK the structure discussed here) with a root-mean-square deviation (rmsd) of 0.55 Å (for the Cα atoms of Leu39–Gly293, Fig. 2B). Significant differences were located in loop 1 of the IMP-1 and IMP-2 structures. Different conformations for the loop 1 were observed between the two enzymes, even though there was an acetate ion in the active site of both enzymes (see discussion below).

In the IMP-1 structure, Gly63–Trp64–Gly65 (the GWG portion) located near the apex of loop 1, are disordered, and Trp64 is positioned away from the active site groove, towards the solvent.[9] NMR studies on CcrA by Scrofani *et al.* suggest that Trp64 of IMP-1 plays a role in recruiting and stabilizing the substrate ligand.[19] The conformational flexibility of the GWG portion likely creates an open cavity in the active site, allowing the accommodation of a variety of bulky substrates. In contrast, judging from the $2|F_o|-|F_c|$ electron density map, the backbone of the GWG portion in IMP-2 is in a single conformation with a well-defined electron density (Fig. 3A). Two antiparallel β-strands (β2, β3) in IMP-2 extend perpendicularly to the active site cleft, where the indole ring of Trp64 is situated, thus covering the active site from the upper part (Fig. 3A). Residues 60–66 in loop 1 of IMP-2 are transformed from an open conformation, as seen in the IMP-1 structure (Fig. 3B),[9] to a closed conformation (Fig. 3A), resulting in a tunnel-shaped cavity in the active site.

Interestingly, this closed conformation of loop 1 in IMP-2 is similar to those found in the crystal structures of IMP-1 complexed with inhibitors.[9,24,25] However, the active site cleft showed no major difference between IMP-1 and IMP-2 (Fig. 2B). One structural factor that may be triggering the conformational change of loop 1 may be the nature of the residue at position 68, located between Val67 and Lys69 on a β-strand (β3) that creates part of the hydrophobic pocket for the substrate of loop 1. Position 68 in IMP-1 is Pro, which is conformationally rigid, whereas that of IMP-2 is a Ser residue. Substitution of the residue at position 68 led to changes in the dihedral angle of the adjacent Val67 (for the Cα atom of Val67: ϕ −147°, φ 122°, and ω 180° for IMP-1 and ϕ −95°, φ 149°, and ω 174° for IMP-2) and to changes in hydrogen bond formation of loop 1 between IMP-1 and IMP-2. Rotational transfer of Vla67 (ϕ: −147°; IMP-1 to −95°; IMP-2) may influence interaction with substrates. Palzkill *et al.* analyze the residues in or near the active site of IMP-1 by codon randomization and selection experiments[22,26,27] and suggest that Val67 is essential for ampicillin hydrolysis.[26,27]

Loop 1 of IMP-2 seems more flexible due to the lack of steric hindrance with the cyclic side chain of Pro, compared with IMP-1. Borra *et al.* pointed out that loop 1 of VIM-7 MBL with Ser at position 68 is more flexible than that of VIM-2, with Pro at position 68.[28] The crystal structures of IMP-1 with and without a mercaptocarboxylate inhibitor indicate that IMP-1 takes an open conformation without an inhibitor and converts to a closed conformation upon binding of the inhibitor to the active site.[9] Such an observation is found in X-ray crystal structures of unliganded MBL from *Bacteroides fragilis* (CcrA) and its 4-morpholinoethanesulfonic acid (MES) complex.[29]

From the results of the IMP-2 structure although there is only one case, it is thought that IMP-2 can take a closed conformation, even when a substrate or an inhibitor is not present in the active site, because of the conformational flexibility.

Comparison of the Active Site Structure between IMP-1 and IMP-2 Zn1 in IMP-2 showed a very clear electron

density and was coordinated by three His residues (His116, His118, and His196) and one acetate ion. The average bond distance between Zn1–His and the average angle for His–Zn1–His were 2.3 Å and 105°, respectively, which are almost identical to those found in IMP-1 (2.3 Å, 94°). An acetate ion in the active site of IMP-2 exhibited two alternate conformations with half-occupancy (ACTA, ACTB), with one of the two oxygen atoms in ACTA located 2.7 Å from Zn1 (IMP-1; 2.9 Å). No apparent electron density for a bridging water molecule/hydroxide ion in between Zn1 and Zn2 was observed, in contrast with the majority of other MBL structures. The coordination environment around Zn1 can be described as a distorted tetrahedral geometry, as can be seen in the Zn1 site of IMP-1.

The coordination geometry of Zn2 in IMP-2 is different from that of IMP-1. Unlike Zn1, the $2|F_o|-|F_c|$ electron density map at Zn2 showed the existence of partially dissociated Zn(II) ion from the active site. The occupancies for Zn1 and Zn2 were set to 1.0 and 0.3, respectively, for subsequent refinement. As a result, the final *B*-factors approached 33.6 Å^2 for Zn1 and 38.3 Å^2 for Zn2 (*B*-factor average: 35.8 Å^2). This result indicates that the Zn(II) binding affinity of the Zn2 site is lower than that of the Zn1 site. Moreover, the side chain of Cys221 adopted alternate conformations, where the occupancy of Cys221A was refined by 0.3, and that of Cys221B was refined by 0.7. The former conformer was the Zn2-bound form, whereas the latter was the Zn2-unbound form. The Zn2–Cys221A and Zn2–His263 bond distances were 2.3 Å and 3.0 Å, respectively. Thus, the Zn2–Cys221A bond distance was similar to that of IMP-1 but the Zn2–His263 bond distance in IMP-2 was much longer by 0.6 Å than that of IMP-1. The side chain of Asp120 in IMP-2, the Zn2 ligand, displayed a well-defined single conformation and the Zn2–Asp120 bond distance in IMP-2 is 2.6 Å, very similar to that of IMP-1 (2.6 Å). One of the two oxygen atoms in ACTB is located 3.1 Å from Zn2, which is the same position as the apical water of plane in IMP-1. The IMP-2 ligand–Zn2–ligand bond angle of 74–104° is close to the optimal tetrahedral angles, although those of IMP-1 are 64–88°. Thus, the coordination environment around Zn2 can be described as a distorted tetrahedral geometry. In the IMP-1 structure, Zn2 is coordinated with Asp120, His196, Cys221, and one water molecule, and a bridging water/hydroxide ion (but not seen due to a low resolution), forming a trigonal bipyramidal geometry.[9] In addition, an acetate ion in IMP-1 is positioned 2.8 Å from Zn2. Thus, there is a considerable difference in the coordination geometry of the Zn2 sites between IMP-1 and IMP-2.

Another interesting difference in and near the active site is the portion of residues 261–263: IMP-2 harbors two contiguous (Ser261–Ser262) residues adjacent to the Zn2 ligand His263, whereas IMP-1 harbors Pro261–Ser262 adjacent to His263. In the crystal structure of IMP-2, the hydroxyl oxygen atom of Ser261 is hydrogen bonded to the main chain carbonyl of Ser264 (3.0 Å), indicating that the conformational freedom of this portion of the protein by the participation of this hydorgen bond is decreased relative to IMP-1. In addition, His263Nδ1 in IMP-1 and IMP-2 is hydrogen bonded to the main chain carbonyl of the residue at position 68. From these findings, we propose that the conformational flexibility of residues 261–263 may well also influence the position, mobility, or affinity of Zn2.

CONCLUSION

In conclusion, we have determined the crystal structure of a subclass B1 MBL, IMP-2. Comparison of the structures of IMP-1 and IMP-2 revealed that the substitution of the amino acid residue at position 68 (Pro in IMP-1, Ser in IMP-2) causes conformational flexibility of loop 1 (comprising residues at positions 60–66) in IMP-2 that may be responsible for substrate binding. Our data will help elucidate the correlation between substrate specificity and structural polymorphism among MBLs belonging to the IMP family. Crystallographic studies of IMP-2 complexed with the hydrolyzed product of ampicillin are in progress in order to quantitatively analyze the structure–activity relationship of IMP-2.

Acknowledgments This work was supported by H24-Shinkou-Ippan-010 from the Ministry of Health, Labour and Welfare of Japan, and in part by JSPS KAKENHI Grant Numbers 24659059 and 10363524.

Conflict of Interest The authors declare no conflict of interest.

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