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IMP-43 and IMP-44 Metallo-β-Lactamases with Increased Carbapenemase Activities in Multidrug-Resistant *Pseudomonas aeruginosa*

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Two novel IMP-type metallo-β-lactamase variants, IMP-43 and IMP-44, were identified in multidrug-resistant *Pseudomonas aeruginosa* isolates obtained in medical settings in Japan. Analysis of their predicted amino acid sequences revealed that IMP-43 had an amino acid substitution (Val67Phe) compared with IMP-7 and that IMP-44 had two substitutions (Val67Phe and Phe87Ser) compared with IMP-11. The amino acid residue at position 67 is located at the end of a loop close to the active site, consisting of residues 60 to 66 in IMP-1, and the amino acid residue at position 87 forms a hydrophobic patch close to the active site with other amino acids. An *Escherichia coli* strain expressing bla_{IMP-43} was more resistant to doripenem and meropenem but not to imipenem than one expressing bla_{IMP-7}. An *E. coli* strain expressing bla_{IMP-44} was more resistant to doripenem, imipenem and meropenem than one expressing bla_{IMP-11}. IMP-43 had more efficient catalytic activities against all three carbapenems than IMP-7, indicating that the Val67Phe substitution contributed to increased catalytic activities against carbapenems. IMP-44 had more efficient catalytic activities against all carbapenems tested than IMP-11, as well as increased activities compared with IMP-43, indicating that both the Val67Phe and Phe87Ser substitutions contributed to increased catalytic activities against carbapenems.

Metallo-β-lactamases (MBLs) confer resistance to all β-lactams, except the monobactams, and are characterized by their efficient hydrolysis of carbapenems (1). Acquired MBLs are produced by Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp., and several enterobacteria (1). MBLs are categorized by their amino acid sequences into various types (2–4), including AIM (5), DIM (6), FIM (7), GIM (8), IMPs (9), KHM (10), NDMs (11), SMB (12), SIM (13), SPM (14), TMBs (15) and VIMs (16). The most prevalent types of MBLs are IMP-, VIM-, and NDM-type enzymes (1, 2, 17). We describe here the bacteriological and biochemical characterization of two novel IMP-type MBL variants in multidrug-resistant *P. aeruginosa* isolates obtained in medical settings in Japan.

MATERIALS AND METHODS

Bacterial strains. A total of 161 clinical isolates of multidrug-resistant *P. aeruginosa*, which were resistant to IPM (MIC ≥ 16 μg/ml), AMK (MIC ≥ 32 μg/ml), and CIP (MIC ≥ 4 μg/ml), were obtained between July and September 2011 from 161 hospitals located in 30 of 47 prefectures in Japan by BML Biomedical Laboratories R&D Center (Kawagoe, Saitama, Japan). These strains were isolated from urinary tracts ($n = 93$), respiratory tracts ($n = 62$), and other tissues of patients ($n = 6$). An isolate of *P. aeruginosa* harboring bla_{IMP-7} (NCGM1438) was used to clone bla_{IMP-7}, and *Enterobacter cloacae* harboring bla_{IMP-11} (NCGM5) (18) was used to clone bla_{IMP-11}. *E. coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of bla_{IMP-7}, bla_{IMP-11}, bla_{IMP-43}, and bla_{IMP-44}, respectively.

Drug susceptibility tests. MICs of amikacin, cefoxitin, ceftazidime, cefuroxime, cephradine, colistin, piperacillin, ticarcillin, tigecycline (Sigma-Aldrich, St. Louis, MO), ampicillin, gentamicin, penicillin G (Nacalai Tesque, Kyoto, Japan), arbekacin, fosfomycin (Meiji Seika Pharma, Tokyo, Japan), aztreonam (Eizai, Tokyo, Japan), cefepime (Bristol-Myers Squibb, New York, NY), cefotaxime, ceftriaxone (Chugai Pharmaceutical, Tokyo, Japan), cefmetazole, ciprofloxacin, panipenem (Daiichi-Sankyo Pharmaceutical Co, Tokyo, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), meropenem (Sumitomo Pharmaceutical, Osaka, Japan), doripenem, moxalactam (Shionogi, Osaka, Japan), piperacillin-tazobactam (Toyama Pure Chemical Industries, Tokyo, Japan), cefozopran, cefsulodin (Takeda Pharmaceutical, Tokyo, Japan), cefoselis (Fujisawa Pharmaceutical, Tokyo, Japan), ampicillin-sulbactam (Pfizer Pharmaceutical, Tokyo Japan), and cefpirome (Chemix, Kanagawa, Japan) were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (19).

Detection and typing of IMP-type MBLs. IMP-type enzymes were detected using an immunochromatographic assay kit (Mizuho Medy Co., Saga, Japan) (20). The *bla*IMP genes were amplified using PCR primers as described previously (20). All PCR products were sequenced using an ABI Prism 3130 sequencer (Applied Biosystems, Foster City, CA).

Pulsed-field gel electrophoresis (PFGE). PFGE analysis was performed as described previously (21). Fingerprinting patterns were analyzed by the unweighted-pair-group method by using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA) to create an average linkage-based dendrogram.

Whole-genome sequencing. The entire genomes of NCGM1496 and NCGM1663 were sequenced by Illumina GAIIx (Illumina, San Diego, CA). We obtained 764,108 reads and 6,811,220 bp from 1,006 contigs in NCGM1496, and 2,762,006 reads and 6,911,518 bp from 1,532 contigs in NCGM1663. The multilocus sequence types (MLSTs) according to the *P. aeruginosa* MLST Database website (http://pubmlst.org/paeruginosa/) and the genetic environments surrounding *bla*IMP genes, β-lactamase encoding genes, and efflux pump encoding genes were determined using

Received 9 April 2013 Returned for modification 11 May 2013
Accepted 22 June 2013
Published ahead of print 8 July 2013
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doi:10.1128/AAC.00716-13

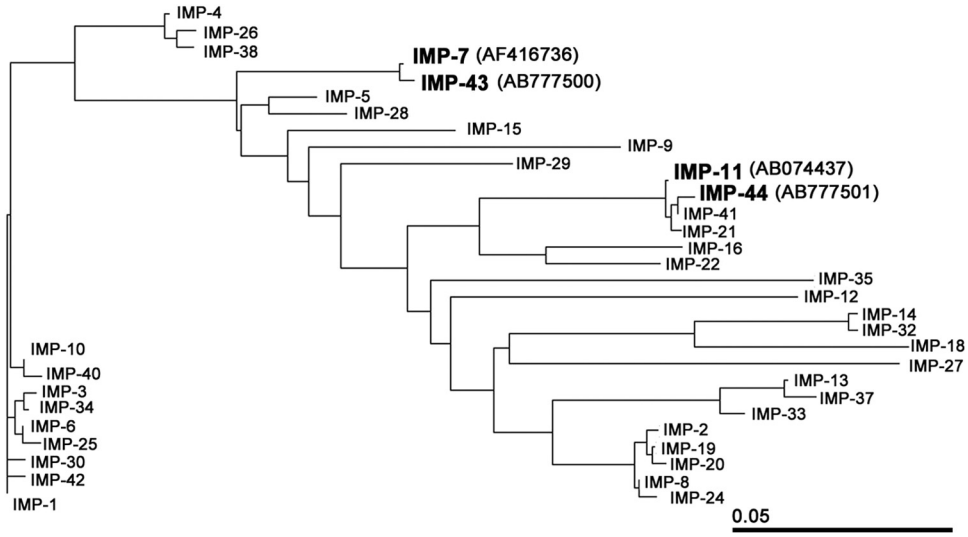

FIG 1 Dendrogram of 43 IMP-type MBLs for comparison with IMP-43 and IMP-44. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for IMP-type enzymes.

the entire genome data. β-Lactamase- and efflux pump-encoding genes were found and sequenced in the genomes.

Cloning of bla_{IMP-7}, bla_{IMP-11}, bla_{IMP-43}, and bla_{IMP-44}. The open reading frames (ORFs) of bla_{IMP-7} and bla_{IMP-43} were PCR amplified using the primers EcoRI-IMP-7/43-F (5′-CCGCATGCGATGAAAAAGTTATC AGTATTC-3′) and PstI-IMP-7/43-R (5′-GGGCGGCCGCTTAGTTACT TGGTTTTGAT-3′), and the ORFs of bla_{IMP-11} and bla_{IMP-44} were amplified using the primers EcoRI-IMP-11/44-F (5′-CCGCATGCGATGAAA AAACTATTTGTTTTA-3′) and PstI-IMP-11/44-R (5′-GGGCGGCCGC TTAATGAACAGTGTTACTTT-3′). The PCR products of each were digested with EcoRI and PstI and ligated into pHSG398 (TaKaRa Bio, Shiga, Japan). The plasmids were used to transform DH5α, transformants were selected on LB agar containing 100 μg/ml of chloramphenicol, and their susceptibility to various β-lactams was assayed.

Purification of recombinant IMP-7, IMP-11, IMP-43, and IMP-44. To determine the kinetic parameters of these IMP-type enzymes, the ORFs of IMP-7, IMP-11, IMP-43, and IMP-44 without signal peptide regions were amplified by PCR. IMP-7, and IMP-43 were amplified using the primers BamHI-IMP-7/43-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGC GGAGAGGCTTTGCCAGATTT-3′) and XhoI-IMP-7/43-R (5′-ATCCT CGAGTTAGTTACTTGGTTTTGATAG-3′), whereas IMP-11 and IMP-44 were amplified using the primers BamHI-IMP-11/44-F (5′-ATG GATCCGAAAACCTGTATTTCCAAGGCGGAGCGTCTTTGCCTGAT TT-3′) and XhoI-IMP-11/44-R (5′-ATCCTCGAGTTAATGAACAGTG TTACTTT-3′). These PCR products were digested with BamHI and XhoI and ligated into pET28a (Novagen, Inc., Madison, WI). The plasmids were used to transform *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA), and transformants were selected on LB agar containing 20 μg/ml of kanamycin. The bacterial cells were lysed by sonication and the recombinant IMP proteins were purified from the soluble fraction on nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen, Tokyo, Japan). His-tagged proteins were digested with TurboTEV protease (Accelagen, San Diego, CA), and both the His tag and the protease were removed on Ni-NTA agarose. SDS-PAGE analysis showed that each target protein was over 90% pure. During the purification procedures, the presence of β-lactamase activities was monitored with 100 μM nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Kinetic analysis was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 μM $Zn(NH_3)_2$ at 37°C using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m, k_{cat} and k_{cat}/K_m ratio of each enzyme were determined by analyzing β-lactam hydrolysis under initial-rate conditions using Lineweaver-Burk plots (22–24).

Nucleotide sequence accession numbers. The nucleotide sequences of class I integrons, including bla_{IMP-43} and bla_{IMP-44}, have been deposited in GenBank with the accession numbers AB777500 and AB777501, respectively.

RESULTS

Identification of bla_{IMP-43} in *P. aeruginosa* NCGM1496 and bla_{IMP-44} in NCGM1663. Of the 161 *P. aeruginosa* clinical isolates, 101 were positive for IMP-type enzymes using an immunochromatographic assay kit to detect IMP-type enzymes. Of these, 61 had bla_{IMP-1}, 20 had bla_{IMP-10}, 12 had bla_{IMP-7}, 5 had a new bla_{IMP-7} variant, 2 had bla_{IMP-6}, and 1 had a new bla_{IMP-11} variant. The new bla_{IMP-7} and bla_{IMP-11} variants were designated bla_{IMP-43} and bla_{IMP-44}, respectively. Dendrograms of IMP-type enzymes, including IMP-43 and IMP-44, are shown in Fig. 1. Analysis of the amino acid sequences revealed that IMP-43 had one amino acid substitution (Val67Phe) compared with IMP-7 and IMP-44 had two substitutions (Val67Phe and Phe87Ser) compared with IMP-11. To clarify whether these amino acid substitutions affect enzymatic activities, IMP-43 was compared with IMP-7 and IMP-44 with IMP-11, due to their similar amino acid sequences (Fig. 1). The amino acid sequences of IMP-7 and IMP-11 were 85% identical, with differences in 36 amino acids, and those of IMP-43 and IMP-44 were also 85% identical, with differences in 37 amino acids.

Whole-genome analysis revealed that both NCGM1496 and NCGM1663 did not have any other genes encoding β-lactamase except for those encoding PDC-11 (an AmpC variant) and PoxB (an intrinsic β-lactamase). They had efflux pump-encoding genes, including *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY*, and had a point mutation in *mexR* resulting in an amino acid substitution of Val126Glu. The mutation is known to be associated with the overexpression of the MexAB-OprM efflux pump (25).

The MLSTs of both NCGM1496 and NCGM1663 were ST357 (*Pseudomonas aeruginosa* MLST Database [http://pubmlst.org/paeruginosa/]). Four isolates harboring bla_{IMP-43} except for

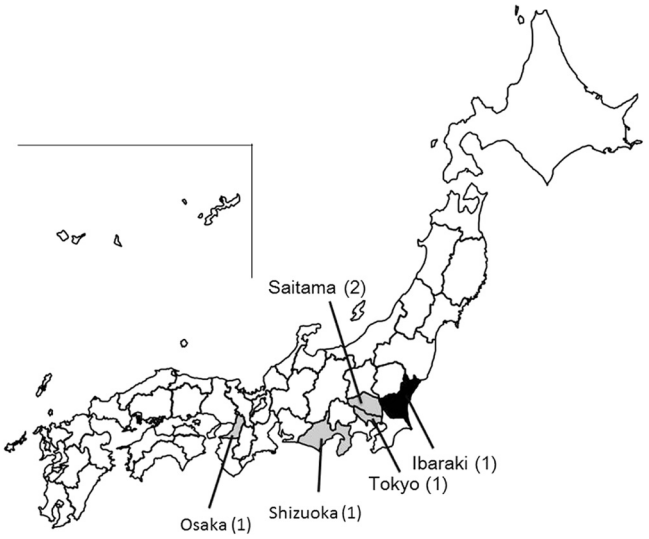

FIG 2 Geographical distribution of *P. aeruginosa* isolates producing IMP-43 and IMP-44 in Japan. The 5 isolates producing IMP-43 were obtained from prefectures marked in gray, and the isolate producing IMP-44 was obtained from the prefecture marked in black. The numbers of isolates are in parentheses.

NCGM1496 were ST235. The PFGE pattern of NCGM1496 had 66.7% similarity to that of NCGM1663 (data not shown).

***bla*$_{IMP-43}$ and *bla*$_{IMP-44}$ in class I integrons.** *bla*$_{IMP-43}$ and *bla*$_{IMP-44}$ were located in class I integrons. These integrons had the different structures; i.e., one had a unique gene cassette array of *aac*(6′)-*Ib*, *bla*$_{IMP-43}$, *aac*(6′)-*Ib*, and *bla*$_{OXA-2}$ (accession no. AB777500), whereas the other had an array similar (with more than 96% identity) to that in a *P. aeruginosa* isolate (accession no. AJ628135) obtained in Italy (26).

Geographic distribution. A total of 5 isolates harboring *bla*$_{IMP-43}$ and 1 harboring *bla*$_{IMP-44}$ were detected in this study. The isolates harboring *bla*$_{IMP-43}$ were obtained in Osaka, Shizuoka, Tokyo, and Saitama prefectures, whereas the isolate harboring *bla*$_{IMP-44}$ was from Ibaraki prefecture (Fig. 2). Although isolates harboring *bla*$_{IMP-7}$ were obtained in these prefectures, these isolates were not obtained at the hospitals where the isolates harboring *bla*$_{IMP-43}$ were obtained.

Drug susceptibility of *E. coli* DH5α expressing IMP-43 and IMP-44. *P. aeruginosa* NCGM1496 harboring *bla*$_{IMP-43}$ and NCGM1663 harboring *bla*$_{IMP-44}$ were resistant to all antibiotics tested, except for colistin (MICs of 2 and 0.5 μg/ml, respectively). The MICs of β-lactams in NCGM1496 and NCGM1663 are shown in Table 1; the MICs of other antibiotics in these two strains were 32 and 32 μg/ml, respectively, for arbekacin; 32 and 32 μg/ml, respectively, for amikacin; 64 and 512 μg/ml, respectively, for gentamicin; 16 and 64 μg/ml, respectively, for ciprofloxacin; and 4 and 4 μg/ml, respectively, for tigecycline.

When the drug susceptibility of *E. coli* DH5α expressing IMP-43, an IMP-7 variant, was compared with that of the same strain expressing IMP-7, the MICs of doripenem and meropenem were each 4-fold higher for *E. coli* expressing IMP-43 than *E. coli* expressing IMP-7, whereas the MICs of cefmetazole, cefoxitin, ticarcillin, and ticarcillin-clavulanic acid were 8-fold lower for *E. coli* expressing IMP-43 than IMP-7 (Table 1). When drug susceptibility of *E. coli* expressing IMP-44, an IMP-11 variant, was compared with that expressing IMP-11, the MICs of doripenem, imipenem, meropenem, and panipenem were at least 4-fold higher for *E. coli* expressing IMP-44 than IMP-11, whereas the MICs of ampicillin, ampicillin/sulbactam, cefmetazole, cefoselis, cefoxitin, cefpirome, cephradine, ticarcillin, and ticarcillin-clavulanic acid were at least 4-fold lower for *E. coli* expressing IMP-44 than IMP-11 (Table 1).

Catalytic activities of IMP-43 and IMP-44. All four recombinant IMP-type enzymes tested, including IMP-7, IMP-11, IMP-

TABLE 1 MICs of β-lactams for *P. aeruginosa* NCGM1496 and NCGM1663 and *E. coli* strains transformed with IMP-7, IMP-43, IMP-11, and IMP-44

Antibiotic(s)[a]	MIC (μg/ml)						
	P. aeruginosa NCGM1496	*P. aeruginosa* NCGM1663	*E. coli* DH5α(pHSG398/IMP-7)	*E. coli* DH5α(pHSG398/IMP-43)	*E. coli* DH5α(pHSG398/IMP-11)	*E. coli* DH5α(pHSG398/IMP-44)	*E. coli* DH5α(pHSG398)
Ampicillin	>512	>512	16	8	32	8	2
Ampicillin-sulbactam	>512	>512	8	4	16	4	1
Aztreonam	32	64	<0.25	<0.25	<0.25	<0.25	<0.25
Cefepime	>512	>512	4	4	16	16	<0.25
Cefmetazole	>512	>512	256	32	256	64	1
Cefoselis	>512	>512	8	8	32	4	<0.25
Cefotaxime	>512	>512	32	32	128	128	<0.25
Cefoxitin	>512	>512	512	64	512	128	2
Cefozopran	512	>512	8	8	32	32	<0.25
Cefpirome	64	>512	32	32	4	1	<0.25
Cefsulodin	>512	>512	>512	>512	>512	>512	256
Ceftazidime	>512	>512	512	512	256	256	<0.25
Ceftriaxone	>512	>512	64	64	128	256	<0.25
Cefuroxime	>512	>512	512	512	512	>512	4
Cephradine	>512	>512	512	512	512	128	16
Doripenem	>512	>512	0.5	2	8	32	<0.25
Imipenem	>512	>512	0.5	0.5	2	8	<0.25
Meropenem	>512	>512	0.5	2	16	64	<0.25
Moxalactam	>512	>512	256	256	256	>512	<0.25
Panipenem	>512	>512	1	1	2	16	<0.25
Penicillin G	>512	>512	32	32	64	32	32
Piperacillin	64	64	2	2	4	2	2
Piperacillin-tazobactam	32	32	2	2	1	1	1
Ticarcillin	256	>512	512	64	512	128	2
Ticarcillin-clavulanic acid	256	>512	256	64	512	128	2

[a] The ratio of ampicillin to sulbactam was 2:1. The ratio of piperacillin to tazobactam was 4:1. The ratio of ticarcillin to clavulanic acid was 15:1.

TABLE 2 Kinetic parameters of β-lactamases IMP-7, IMP-11, IMP-43, and IMP-44 with various substrates

Substrate	*Km* (μM)[a]				*kcat* (s^{-1})[a]				*kcat*/*Km* ($\mu M^{-1} \cdot s^{-1}$)[a]			
	IMP-7	IMP-43	IMP-11	IMP-44	IMP-7	IMP-43	IMP-11	IMP-44	IMP-7	IMP-43	IMP-11	IMP-44
Penicillin G	207 ± 19	3176 ± 236	574 ± 18	482 ± 53	25.3 ± 0.7	64 ± 3	36 ± 2	14 ± 1	0.12	0.02	0.063	0.029
Ampicillin	180 ± 10	494 ± 40	230 ± 21	627 ± 80	9.1 ± 0.6	3.5 ± 0.2	7.4 ± 0.6	11 ± 1	0.051	0.0072	0.032	0.017
Cephradine	27 ± 2	69 ± 5	39 ± 4	119 ± 8	8.0 ± 0.3	10.3 ± 0.8	14.6 ± 0.7	14.1 ± 0.2	0.29	0.15	0.38	0.12
Cefoxitin	33 ± 1	55 ± 5	4.4 ± 1.2	53 ± 5	4.18 ± 0.05	3.48 ± 0.05	3.2 ± 0.1	12 ± 1	0.13	0.062	0.76	0.22
Cefotaxime	24 ± 2	6.8 ± 0.9	35 ± 4	27 ± 3	1.88 ± 0.07	7.2 ± 0.1	4.3 ± 0.2	44 ± 1	0.08	1.1	0.12	1.6
Ceftazidime	59 ± 4	14 ± 2	29 ± 3	63 ± 4	0.89 ± 0.05	1.9 ± 0.1	1.33 ± 0.09	5.6 ± 0.2	0.015	0.14	0.046	0.089
Cefepime	50 ± 6	30 ± 4	40 ± 5	64 ± 5	1.34 ± 0.04	4.6 ± 0.1	2.00 ± 0.04	19.4 ± 0.7	0.027	0.16	0.05	0.31
Aztreonam	NH[b]	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH
Doripenem	63 ± 5	59 ± 7	101 ± 10	257 ± 27	6.8 ± 0.2	10.0 ± 0.4	11.3 ± 0.6	589 ± 40	0.11	0.17	0.11	2.3
Imipenem	254 ± 20	268 ± 23	142 ± 7	119 ± 11	20 ± 1	49 ± 2	19.2 ± 0.5	165 ± 5	0.078	0.18	0.13	1.4
Meropenem	59 ± 7	24 ± 2	50 ± 5	137 ± 16	2.6 ± 0.2	8.2 ± 0.3	5.9 ± 0.2	335 ± 13	0.044	0.34	0.12	2.5

[a] The K_m and k_{cat} values are the means ± standard deviations from three independent experiments.
[b] NH, no hydrolysis detected with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

43, and IMP-44, hydrolyzed all β-lactams tested except for aztreonam (Table 2). The k_{cat}/K_m ratios of IMP-43 and IMP-44 against cefotaxime, ceftazidime, cefepime, doripenem, imipenem, and meropenem were higher than those of IMP-7 and IMP-11, respectively, whereas the k_{cat}/K_m ratios of IMP-43 and IMP-44 against penicillin, ampicillin, cephradine, and cefoxitin were lower than those of IMP-7 and IMP-11, respectively. The K_m value of IMP-43 for penicillin was markedly higher than that of other IMP-type enzymes tested (Table 2). The k_{cat}/K_m ratios for IMP-44 against all carbapenems tested were higher than those of the other IMP-type enzymes, and its k_{cat} values were 9 to 57 times higher than those of IMP-11 (Table 2).

DISCUSSION

This is the first report of *P. aeruginosa* ST357 isolates found in Japan, although these isolates have been found in other countries. To date, 5 *P. aeruginosa* ST357 isolates from France, Nigeria, Poland, Senegal, and Singapore have been registered on the PubMLST website (http://pubmlst.org/paeruginosa/). Regional spread of *P. aeruginosa* ST357 producing IMP-7 has been reported in central Europe (27). The two isolates of NCGM1496 and NCGM1663 from different regions belonged to ST357, indicating that ST357 isolates will exist widely in Japan, although PFGE analysis revealed that the isolates were not very close to each other (with 66.7% similarity).

The Val67Phe amino acid substitution seems to have a significant impact on the catalytic efficiency for meropenem, whereas the efficiencies for imipenem and doripenem are slightly influenced in IMP-43. The presence of both the amino acid substitutions Val67Phe and Phe87Ser results in a significant increase of catalytic efficiencies for all the carbapenems in IMP-44. The amino acid residue 67 in IMP-1 is located at the end of a loop close to the active site consisting of residues 60 to 66 (28). This loop may be a major determinant for the tight binding of substrates in the active site (28). The active-site loop of MBL BcII has been reported to contribute to substrate binding by providing hydrophobic interactions between the phenyl and methyl groups of penicillin G and residues located at both ends of the loop (Phe61 and Val67) (29). The Phe87Ser amino acid substitution observed in IMP-44 could also contribute to enzymatic activity against carbapenems. Mutational analysis of VIM-2 revealed that the aromatic residue Trp87 is critical for the stability and folding of this protein (30). Residues Phe-61, Tyr-67, and Trp-87 of VIM-2, which form a hydrophobic patch close to the active site, were close to the second Zn binding site in VIM-2 (28). Two aromatic side chains in the hydrophobic pocket (the phenyl groups of Phe61 and Trp87) interact with the phenyl group of penicillin G (29, 31). The Phe87Ser amino acid substitution may therefore affect stability and folding, resulting in changes in substrate specificities due to the absence of a benzene ring.

The amino acid substitutions Val67Phe and Phe87Ser affected catalytic activities of β-lactams other than the carbapenems. The Val67Phe amino acid substitution reduced affinity with penicillin G and the Phe87Ser marginally affected the affinity (Table 2). Both amino acid substitutions increased catalytic activities against cefotaxime, ceftazidime, and cefepime, although the MICs of these antibiotics are intrinsically high and did not change regardless of substitutions. These substitutions may specifically affect their affinity for carbapenems. These IMP-type enzymes did not show catalytic activities against aztreonam and did not confer aztreonam resistance on *E. coli* strains expressing these IMP-type enzymes. Nevertheless, NCGM1496 and NCGM1663 were resistant to aztreonam, perhaps due to the presence of genes encoding efflux pumps and PDC-11, an AmpC variant (32). Both strains had 4 operons encoding efflux pump systems (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY*) as described for *P. aeruginosa* (33). Among these systems, *mexAB-oprM* and a point mutation in *mexR* may be associated with aztreonam resistance in these strains (34).

IMP-7 and IMP-11 also showed epidemiological differences. IMP-7 producers were observed globally, whereas IMP-11 producers have been reported only in Japan. IMP-7 was originally isolated from a patient in Canada (35) and IMP-11 from a patient in Japan (accession no. AB074437). IMP-7 has been detected in patients in Canada (35), the Czech Republic (36), Japan (37), Malaysia (38), and Slovakia (39).

In conclusion, multidrug-resistant *P. aeruginosa* strains producing IMP-43 and IMP-44 are emerging in medical settings in Japan. In particular, IMP-43-producing *P. aeruginosa* isolates were disseminated throughout Japan. The amino acid substitutions Val67Phe and Phe87Ser found in IMP-44 appear to significantly increase the hydrolytic efficiency of IMP-type enzymes. Therefore, it is necessary to carefully investigate these isolates.

ACKNOWLEDGMENTS

This study was supported by grants from the International Health Cooperation Research (23-A-301) and JSPS KAKENHI (grant 24790432) and by a grant from the Ministry of Health, Labor and Welfare of Japan (H24-Shinko-Ippan-010).

REFERENCES

1. **Bush K.** 2001. New beta-lactamases in Gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin. Infect. Dis. **32:**1085–1089.
2. **Walsh TR, Toleman MA, Poirel L, Nordmann P.** 2005. Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
3. **Bush K, Jacoby GA.** 2010. Updated functional classification of beta-lactamases. Antimicrob. Agents Chemother. **54:**969–976.
4. **Cornaglia G, Giamarellou H, Rossolini GM.** 2011. Metallo-beta-lactamases: a last frontier for beta-lactams? Lancet Infect. Dis. **11:**381–393.
5. **Yong D, Toleman MA, Bell J, Ritchie B, Pratt R, Ryley H, Walsh TR.** 2012. Genetic and biochemical characterization of an acquired subgroup B3 metallo-beta-lactamase gene, *bla*AIM-1, and its unique genetic context in *Pseudomonas aeruginosa* from Australia. Antimicrob. Agents Chemother. **56:**6154–6159.
6. **Rogalski TM, Gilbert MM, Devenport D, Norman KR, Moerman DG.** 2003. DIM-1, a novel immunoglobulin superfamily protein in *Caenorhabditis elegans*, is necessary for maintaining bodywall muscle integrity. Genetics **163:**905–915.
7. **Pollini S, Maradei S, Pecile P, Olivo G, Luzzaro F, Docquier JD, Rossolini GM.** 2013. FIM-1, a new acquired metallo-beta-lactamase from a *Pseudomonas aeruginosa* clinical isolate from Italy. Antimicrob. Agents Chemother. **57:**410–416.
8. **Castanheira M, Toleman MA, Jones RN, Schmidt FJ, Walsh TR.** 2004. Molecular characterization of a beta-lactamase gene, *bla*GIM-1, encoding a new subclass of metallo-beta-lactamase. Antimicrob. Agents Chemother. **48:**4654–4661.
9. **Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, Yoshimura F, Kato N.** 1994. Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
10. **Sekiguchi J, Morita K, Kitao T, Watanabe N, Okazaki M, Miyoshi-Akiyama T, Kanamori M, Kirikae T.** 2008. KHM-1, a novel plasmid-mediated metallo-beta-lactamase from a *Citrobacter freundii* clinical isolate. Antimicrob. Agents Chemother. **52:**4194–4197.
11. **Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR.** 2009. Characterization of a new metallo-beta-lactamase gene, *bla*NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother. **53:**5046–5054.
12. **Wachino J, Yoshida H, Yamane K, Suzuki S, Matsui M, Yamagishi T, Tsutsui A, Konda T, Shibayama K, Arakawa Y.** 2011. SMB-1, a novel subclass B3 metallo-beta-lactamase, associated with ISCR1 and a class 1 integron, from a carbapenem-resistant *Serratia marcescens* clinical isolate. Antimicrob. Agents Chemother. **55:**5143–5149.
13. **Lee K, Yum JH, Yong D, Lee HM, Kim HD, Docquier JD, Rossolini GM, Chong Y.** 2005. Novel acquired metallo-beta-lactamase gene, *bla*SIM-1, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob. Agents Chemother. **49:**4485–4491.
14. **Zavascki AP, Gaspareto PB, Martins AF, Goncalves AL, Barth AL.** 2005. Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing SPM-1 metallo-beta-lactamase in a teaching hospital in southern Brazil. J. Antimicrob. Chemother. **56:**1148–1151.
15. **El Salabi A, Borra PS, Toleman MA, Samuelsen O, Walsh TR.** 2012. Genetic and biochemical characterization of a novel metallo-beta-lactamase, TMB-1, from an *Achromobacter xylosoxidans* strain isolated in Tripoli, Libya. Antimicrob. Agents Chemother. **56:**2241–2245.
16. **Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, Rossolini GM.** 1999. Cloning and characterization of *bla*VIM, a new integron-borne metallo-beta-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.
17. **Jacoby GA, Munoz-Price LS.** 2005. The new beta-lactamases. N. Engl. J. Med. **352:**380–391.
18. **Hamada Y, Watanabe K, Tatsuya T, Mezaki K, Takeuchi S, Shimizu T, Kirikae T, Ohmagari N.** 2012. Three cases of IMP-type metallo-beta-lactamase-producing *Enterobacter cloacae* bloodstream infection in Japan. J. Infect. Chemother. doi:10.1007/s10156-012-0520-6.
19. **Clinical and Laboratory Standards Institute.** 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 9th ed. Approved standard M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
20. **Kitao T, Miyoshi-Akiyama T, Tanaka M, Narahara K, Shimojima M, Kirikae T.** 2011. Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-beta-lactamases that mediate carbapenem resistance in *Pseudomonas*. J. Microbiol. Methods. **87:**330–337.
21. **Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Kasai A, Mizuguchi Y, Araake M, Fujino T, Kikuchi H, Sasaki S, Watari H, Kojima T, Miki H, Kanemitsu K, Kunishima H, Kikuchi Y, Kaku M, Yoshikura H, Kuratsuji T, Kirikae T.** 2007. Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. J. Clin. Microbiol. **45:**979–989.
22. **Boschi L, Mercuri PS, Riccio ML, Amicosante G, Galleni M, Frere JM, Rossolini GM.** 2000. The *Legionella* (*Fluoribacter*) *gormanii* metallo-beta-lactamase: a new member of the highly divergent lineage of molecular-subclass B3 beta-lactamases. Antimicrob. Agents Chemother. **44:**1538–1543.
23. **Crowder MW, Walsh TR, Banovic L, Pettit M, Spencer J.** 1998. Overexpression, purification, and characterization of the cloned metallo-beta-lactamase L1 from *Stenotrophomonas maltophilia*. Antimicrob. Agents Chemother. **42:**921–926.
24. **Queenan AM, Shang W, Flamm R, Bush K.** 2010. Hydrolysis and inhibition profiles of beta-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. Antimicrob. Agents Chemother. **54:**565–569.
25. **Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plesiat P.** 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob. Agents Chemother. **48:**1797–1802.
26. **Pagani L, Colinon C, Migliavacca R, Labonia M, Docquier JD, Nucleo E, Spalla M, Li Bergoli M, Rossolini GM.** 2005. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-beta-lactamase. J. Clin. Microbiol. **43:**3824–3828.
27. **Hrabak J, Cervena D, Izdebski R, Duljasz W, Gniadkowski M, Fridrichova M, Urbaskova P, Zemlickova H.** 2011. Regional spread of *Pseudomonas aeruginosa* ST357 producing IMP-7 metallo-beta-lactamase in Central Europe. J. Clin. Microbiol. **49:**474–475.
28. **Moali C, Anne C, Lamotte-Brasseur J, Groslambert S, Devreese B, Van Beeumen J, Galleni M, Frere JM.** 2003. Analysis of the importance of the metallo-beta-lactamase active site loop in substrate binding and catalysis. Chem. Biol. **10:**319–329.
29. **Prosperi-Meys C, Wouters J, Galleni M, Lamotte-Brasseur J.** 2001. Substrate binding and catalytic mechanism of class B beta-lactamases: a molecular modelling study. Cell. Mol. Life Sci. **58:**2136–2143.
30. **Borgianni L, Vandenameele J, Matagne A, Bini L, Bonomo RA, Frere JM, Rossolini GM, Docquier JD.** 2010. Mutational analysis of VIM-2 reveals an essential determinant for metallo-beta-lactamase stability and folding. Antimicrob. Agents Chemother. **54:**3197–3204.
31. **Docquier JD, Lamotte-Brasseur J, Galleni M, Amicosante G, Frere JM, Rossolini GM.** 2003. On functional and structural heterogeneity of VIM-type metallo-beta-lactamases. J. Antimicrob. Chemother. **51:**257–266.
32. **Jacoby GA.** 2009. AmpC beta-lactamases. Clin. Microbiol. Rev. **22:**161–182.
33. **Schweizer HP.** 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet. Mol. Res. **2:**48–62.
34. **Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**3322–3327.
35. **Gibb AP, Tribuddharat C, Moore RA, Louie TJ, Krulicki W, Livermore DM, Palepou MF, Woodford N.** 2002. Nosocomial outbreak of carbapenem-resistant *Pseudomonas aeruginosa* with a new *bla*IMP allele, bla(IMP-7). Antimicrob. Agents Chemother. **46:**255–258.
36. **Hrabak J, Fridrichova M, Stolbova M, Bergerova T, Zemlickova H, Urbaskova P.** 2009. First identification of metallo-beta-lactamase-

producing *Pseudomonas aeruginosa* in the Czech Republic. Euro Surveill. **14:**19102.
37. **Kouda S, Kuwahara R, Ohara M, Shigeta M, Fujiwara T, Komatsuzawa H, Usui T, Sugai M.** 2007. First isolation of bla_{IMP-7} in a *Pseudomonas aeruginosa* in Japan. J. Infect. Chemother. **13:**276–277.
38. **Ho SE, Subramaniam G, Palasubramaniam S, Navaratnam P.** 2002. Carbapenem-resistant *Pseudomonas aeruginosa* in Malaysia producing IMP-7 beta-lactamase. Antimicrob. Agents Chemother. **46:**3286–3287.
39. **Ohlasova D, Kmet V, Niks M.** 2007. First report of the carbapenem-resistant *Pseudomonas aeruginosa* producing IMP-7 metallo-beta-lactamase in Slovakia. Int. J. Antimicrob. Agents. **30:**370–371.