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MECHANISMS OF RESISTANCE

Emergence of Carbapenem-Resistant *Providencia rettgeri* and *Providencia stuartii* Producing IMP-Type Metallo-β-Lactamase in Japan

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ABSTRACT Four *Providencia rettgeri* isolates and one *Providencia stuartii* isolate were obtained from urine samples of five patients in 2018 in Japan. All of the isolates were resistant to imipenem and meropenem, and three were highly resistant to both carbapenems, with MICs of 512 μg/ml. The three highly carbapenem-resistant isolates harbored bla_{IMP-70}, encoding a variant of IMP-1 metallo-β-lactamase with two amino acid substitutions (Val67Phe and Phe87Val), and the other two harbored bla_{IMP-1} and bla_{IMP-11}, respectively. Whole-genome sequencing revealed that an isolate harbored two copies of bla_{IMP-1} on the chromosome and that the other four harbored a copy of bla_{IMP-11} or bla_{IMP-70} in a plasmid. Expression of bla_{IMP-70} conferred carbapenem resistance in *Escherichia coli*. Recombinant IMP-70 and an IMP-1 variant with Val67Phe but without Phe87Val had significant higher hydrolytic activities against meropenem than recombinant IMP-1, indicating that an amino acid substitution of Val67Phe affects increased activities against meropenem in IMP-70. These results suggest that *Providencia* spp. become more highly resistant to carbapenems by acquisition of two copies of bla_{IMP-1} or by mutation of bla_{IMP} genes with amino acid substitutions, such as bla_{IMP-70}.

KEYWORDS *Providencia rettgeri*, *Providencia stuartii*, IMP-70, metallo-β-lactamase

Providencia is a genus of Gram-negative bacteria belonging to the family *Enterobacteriaceae*. Among *Providencia* spp., *Providencia rettgeri* and *Providencia stuartii* are the common causes of hospital-acquired infections (1, 2). They mainly cause travelers' diarrhea and catheter-related urinary tract infections (3, 4). However, there are several case reports of other infections with *P. rettgeri* and *P. stuartii*, including neonatal sepsis caused by *P. rettgeri* (5) and conjunctivitis (6), meningitis after neurosurgery (7), and endocarditis (8) caused by *P. stuartii*, indicating that these pathogens have the potency to cause serious infections.

P. rettgeri clinical isolates producing IMP-1 metallo-β-lactamase (MBL) were first obtained in 2000 during a laboratory-based surveillance in western Japan (9). A *P. stuartii* clinical isolate producing VIM-19 MBL was first reported in 2008 in Algeria (10). Since then, there have been at least 12 reports of carbapenem-resistant *P. rettgeri* isolates and 5 reports of carbapenem-resistant *P. stuartii* isolates (Table 1). All of these isolates produced MBLs, and the majority of carbapenem-resistant *P. rettgeri* isolates produced IMP-type or NDM-type MBLs (Table 1), i.e., IMP-type MBL-producing isolates were detected in Japan, the Republic of Korea, and the United States, and NDM-type MBL-producing isolates were detected worldwide. The majority of carbapenem-resistant *P. stuartii* isolates, however, produced VIM-type MBLs, and these were detected in Algeria and Greece (Table 1). There is a report describing an NDM-type MBL-producing *P. stuartii* isolate in Afghanistan (Table 1).

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TABLE 1 Previous reports of *P. rettgeri* and *P. stuartii* isolates producing MBLs

Species	Metallo-β-lactamase	Location of MBL-encoding gene[a]	Yr(s) of isolation	Country of isolation	Reference
P. rettgeri	IMP-1	–	2000	Japan	9
	IMP-1	Plasmid (2.5 kb)	2002	Japan	30
	IMP-27	Plasmid	2016	USA	31
	IMP-27	–	2018	Republic of Korea	32
	NDM-1	–	2008, 2011	Israel	33
	NDM-1	Plasmid	2012	Nepal	34
	NDM-1	Plasmid (178 kb)	2012	Mexico	35
	NDM-1	Plasmid (190 kb)	2014	China	36
	NDM-1	–	2017	Bulgaria	37
	NDM	–	2013	Brazil	38
	NDM-18	Plasmid	2017	South Africa	39
P. stuartii	VIM-2	–	2004	Republic of Korea	40
	NDM-1	Plasmid (178kb)	2012	Afghanistan	41
	VIM-1	–	2011	Greece	42
	VIM-1	Plasmid (180 kb, IncA/C)	2012	Greece	43
	VIM-1	Plasmid	2013	Greece	44
	VIM-19	Plasmid (180 kb)	2008	Algeria	10

[a]A dash (–) indicates that the location of MBL-encoding genes was unspecified.

IMP-type MBL was first detected in a clinical isolate of *Pseudomonas aeruginosa* in Japan in 1991 (11). Since then, IMP-type MBL producers have been spreading nationwide in Japan and in east and southeast Asian countries, although they have been globally detected (12).

This report describes the detection of 5 clinical isolates of carbapenem-resistant *P. rettgeri* and *P. stuartii* producing IMP-type metallo-β-lactamase in 3 hospitals located in different regions in Japan.

RESULTS

Drug susceptibility. As shown by the drug susceptibility profiles of 5 *Providencia* sp. isolates shown in Table 2, *P. rettgeri* BML2496 was susceptible to amikacin and aztreonam but resistant to amoxicillin, amoxicillin-clavulanate, ceftazidime, cephradine, ciprofloxacin, and meropenem. The MICs of imipenem and meropenem were 16 and 64 μg/ml, respectively. *P. rettgeri* BML2531 showed a similar profile of drug susceptibility to that of BML2496 (Table 2). However, against *P. rettgeri* BML2526 and BML2576, MICs of imipenem and meropenem (512 μg/ml) were much higher than those against BML2496 and BML2531 (Table 2). BML2526 and BML2576 were highly resistant to ciprofloxacin (MICs of 512 μg/ml and 256 μg/ml, respectively). *P. stuartii* BML2537 was intermediate to amikacin but resistant to amoxicillin, amoxicillin-clavulanate, aztreonam, ceftazidime, cephradine, ciprofloxacin, and meropenem, and MICs of imipenem and meropenem (>512 μg/ml and 512 μg/ml, respectively)

TABLE 2 Drug susceptibility profiles of *Providencia* sp. clinical isolates

	MIC (μg/ml) of antibiotic against:				
	P. rettgeri				*P. stuartii*
Antibiotic(s)	BML2496	BML2531	BML2526	BML2576	BML2537
Arbekacin	4	8	2	8	2
Amikacin	8	4	32	32	32
Amoxicillin	512	>512	>512	>512	>512
Amoxicillin-clavulanate[a]	256	128	128	128	512
Aztreonam	2	<0.25	8	8	32
Ceftazidime	128	256	128	128	512
Cephradine	>512	>512	>512	>512	>512
Ciprofloxacin	32	64	512	256	16
Colistin	>64	>64	>64	>64	>64
Imipenem	16	32	512	512	>512
Meropenem	64	32	512	512	512

[a]The compound ratio of amoxicillin versus clavulanate is 1:5 (wt/wt).

were much higher (Table 2). For all isolates tested, arbekacin MICs were low (2 to 8 μg/ml), but colistin MICs were high (64 μg/ml) (Table 2).

Drug-resistant genes. All five isolates had several genes and mutations associated with resistance to aminoglycosides, β-lactams, and fluoroquinolones (Table 3). All had one or two of five genes encoding aminoglycoside-modifying enzymes, namely *aac*(*2′*)-*Ia*, *aac*(*6′*)-*Iae*, *aac*(*6′*)-*Ib4*, *aac*(*6′*)-*Il*, and/or *aadA1* (Table 3). The *aac*(*6′*)-*Iae* gene may be associated with the increased susceptibility to amikacin of BML2526, BML2537, and BML 2576 (Table 3). Isolates had one of three genes encoding metallo-β-lactamases, namely bla_{IMP-1}, bla_{IMP-11}, or bla_{IMP-70} (Table 3). In addition, they had one or two of four other genes encoding β-lactamases, namely $bla_{CTX-M-2}$, bla_{OXA-1}, bla_{OXA-2}, and/or bla_{TEM-1B} (Table 3). All isolates had three or four mutations in the quinolone resistance-determining regions of *gyrA*, *parC*, and *gyrB* with amino acid substitutions that included Ser83Ile, Asp87Ala, and Asp87Glu in *gyrA*; Ser87Ile and Ser87Arg in *parC*; and Glu468Asp in *gyrB* (Table 3).

Complete whole-genome sequences. Analysis using MiSeq and MinION platforms revealed the complete whole-genome sequences of all five isolates (Table 3). Four isolates of *P. rettgeri* had a 4.3- to 4.7-Mbp chromosome with a GC content of 49.6 to 51.0%, and an isolate of *P. stuartii* had a 4.4-Mbp chromosome with a GC content of 49.8% (Table 3). BML2496 did not have any plasmid, but the others had one or two plasmid(s) of 30 to 200 kbp (Table 3). BML2526 had two plasmids of 70 kbp and 200 kbp, and BML2576 had two with the same sizes as those of BML2526. The sequences of the two 70-kbp and 200-kbp plasmids were identical between the two isolates.

Locations of bla_{IMP} genes and their genetic environments. The bla_{IMP-1} gene was located on the chromosome of BML2496 (Table 3). The bla_{IMP-11} gene was located in an 85-kbp plasmid of BML2531. The bla_{IMP-70} gene was located in a 200-kbp plasmid of BML2526/BML2576 and in a 150-kbp plasmid of BML2537 (Table 3).

Two copies of bla_{IMP-1} were detected on the chromosome of BML2496, and the two copies existed in a row at nucleotide positions 3,384,049 to 3,384,789 and 3,387,611 to 3,387,958, i.e., the following genetic structure of *int1*Δ-bla_{IMP-70}-*qacE*Δ*1-sul1* was repeated (Fig. 1). To confirm the existence of the two copies of bla_{IMP-1}, PCR was conducted using a primer set (IMP-1 DF and IMP-1 DR) targeting the two copies, as shown in Fig. 1. Consequently, a 3.5-kbp PCR product was detected as expected based on the whole-genome sequence of BML2496 (Fig. 1).

Drug susceptibility of *Escherichia coli* DH5α expressing bla_{IMP-1}, bla_{IMP-70}, and two variants of bla_{IMP-1}. IMP-70 is a variant of IMP-1 with two amino acid substitutions, Val67Phe and Phe87Val (GenBank accession no. LC348383.1). A variant of bla_{IMP-1} encoding an amino acid substitution of Val67Phe was bla_{IMP-10} (accession no. NG_049173). Another variant of bla_{IMP-1} encoding an amino acid substitution of Phe87Val [$bla_{IMP-1(Phe87Val)}$] was prepared as described in Materials and Methods. *E. coli* DH5α expressing bla_{IMP-1}, bla_{IMP-10}, $bla_{IMP-1(Phe87Val)}$, and bla_{IMP-70} showed significantly increased MICs for all antibiotics tested, including four carbapenems, compared with those of a vector control (Table 4). Compared with *E. coli* expressing bla_{IMP-1}, *E. coli* expressing bla_{IMP-70} showed slightly, but not significantly, increased MICs of doripenem and meropenem, while it showed the same MICs of imipenem and panipenem (Table 4). However, *E. coli* expressing bla_{IMP-10} showed a significantly higher MIC of doripenem and a slightly increased MIC of meropenem (Table 4). *E. coli* expressing $bla_{IMP-1(Phe87Val)}$, in contrast, showed no increased MIC for all carbapenems tested (Table 4).

Enzymatic activities of IMP-1, IMP-70, and two variants of IMP-1. IMP-70 is a variant of IMP-1 with Val67Phe and Phe87Val. Two variants of IMP-1 with an amino acid substitution of Val67Phe (IMP-10) and that of Phe87Val [IMP-1(Phe87Val)] were prepared as described in Materials and Methods. Enzymatic activities of recombinant IMP-70, IMP-10, and IMP-1(Phe87Val) were compared with those of recombinant IMP-1. IMP-70 and IMP-10 showed greater hydrolytic activities than IMP-1 against meropenem, i.e., the k_{cat}/K_m values of IMP-70 and IMP-10 were 2.3- and 3.4-fold higher, respectively, than those of IMP-1. However, IMP-70 showed similar enzyme activities against dorip-

TABLE 3 Genetic characterization of carbapenem-resistant *Providencia* sp. isolates

Isolate	Genome	Plasmid type	Size (bp)	GC content (%)	Antibiotic resistance gene(s) encoding:		Amino acid substitution(s) in quinolone resistance gene:		
					Aminoglycoside(s)	β-lactam(s)	*GyrA*	*ParC*	*GyrB*
P. rettgeri BML2496	Chromosome		4,651,003	49.6	*aac(6′)-Ib4*	bla_{IMP-1}, bla_{OXA-1}	Ser83Ile, Asp87Ala	Ser87Ile	
P. rettgeri BML2526	Chromosome		4,342,905	51.0	*aadA1*				
	Plasmid	IncA/C2	204,791	50.2	*aac(6′)-Iae*	bla_{IMP-70}, $bla_{CTX-M-2}$, bla_{TEM-1B}	Ser83Ile, Asp87Glu	Ser87Ile	Glu468Asp
	Plasmid	Nontypeable	71,575	50.5					
P. rettgeri BML2531	Chromosome		4,696,377	50.1					
	Plasmid	IncT	84,930	50.3	*aac(6′)-Il*	bla_{IMP-11}, bla_{OXA-2}	Ser83Ile, Asp87Glu	Ser87Ile	
P. rettgeri BML2576	Chromosome		4,351,300	51.0	*aadA1*				
	Plasmid	IncA/C2	204,791	50.2%	*aac(6′)-Iae*	bla_{IMP-70}, $bla_{CTX-M-2}$, bla_{TEM-1B}	Ser83Ile, Asp87Glu	Ser87Ile	Glu468Asp
	Plasmid	Nontypeable	71,575	50.5%					
P. stuartii BML2537	Chromosome		4,418,649	49.8%	*aac(2′)-Ia*				
	Plasmid	IncA/C	152,754	50.0%	*aac(6′)-Iae*	bla_{IMP-70}	Ser83Ile, Asp87Glu	Ser87Arg	
	Plasmid	Nontypeable	30,292	50.9%		$bla_{CTX-M-2}$			

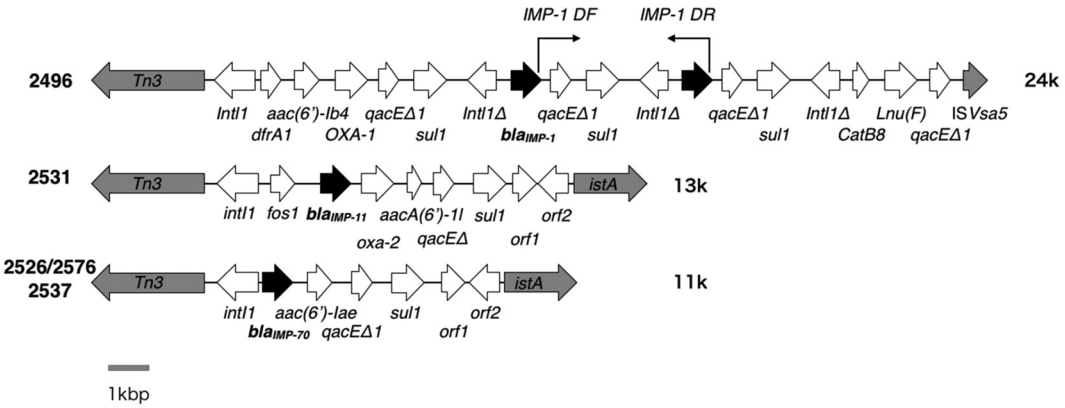

FIG 1 Genomic environments of bla_{IMP-1} and bla_{IMP-70} in *P. rettgeri* and *P. stuartii* clinical isolates. Genes are represented as arrows, which indicate their transcription orientations and relative lengths. MBL genes, *tnp* genes, and truncated genes are shown as black arrows, gray arrows, and Δ, respectively. *orf1* is a gene encoding a hypothetical protein, and *orf2* is a gene encoding an ATP-binding protein.

enem, imipenem, and panipenam to those of IMP-1 (Table 5). IMP-1(Phe87Val) showed similar or reduced enzymatic activities against all carbapenems tested (Table 5).

Production of IMP-type MBL in clinical isolates of *P. rettgeri* and *P. stuartii*. As shown by Western blotting in Fig. 2, all five isolates tested (BML2496, BML2526, BML2531, BML2537, and BML2576) produced IMP-type MBLs (IMP-1 was produced by BML2496, and IMP-70 was produced by the other three isolates). *P. rettgeri* BML2496, an IMP-1 producer, produced the largest amount of IMP-type MBL among all isolates tested (Fig. 2). *P. rettgeri* BML2526 and BML2576 and *P. stuartii* BML2537 produced relatively large amounts of IMP-type MBLs, whereas BML2531 produced a smaller amount of IMP-type MBL (IMP-11) than did the other four isolates tested. IMP-type MBL (IMP-11) produced by BML2531 was detected as a band with a lower molecular weight than those of other bands produced by the remaining four isolates, which correspond to their molecular weights calculated with amino acid sequences (27.06 kDa and 27.12 kDa, respectively).

DISCUSSION

Two copies of bla_{IMP-1} in *P. rettgeri* BML2496 may be necessary for effective IMP-1 production and high hydrolytic activities against carbapenems. Western blot analysis revealed that BML2496 produced a relatively high level of IMP-1 (Fig. 2). There are at least four reports describing carbapenem-resistant Gram-negative pathogens harboring two copies of genes encoding carbapenemases (13–16). An isolate of *P. aeruginosa* obtained from a patient in Serbia harbored two bla_{NDM-1} copies in the genome on

TABLE 4 Drug susceptibility profiles of *E. coli* isolates expressing IMP-1, IMP-10, a variant of IMP-1 with an amino acid substitution (Phe87Val), and IMP-70

Antibiotic(s)	Antibiotic MIC (μg/ml) against:				
	E. coli DH5α (pHSG398)	*E. coli* DH5α (pHSG398/IMP-1)	*E. coli* DH5α (pHSG398/IMP-10)[a]	*E. coli* DH5α [pHSG398/IMP-1(Phe87Val)]	*E. coli* DH5α (pHSG398/IMP-70)[b]
Amoxicillin	4	512	32	32	16
Amoxicillin-clavulanate[c]	4	128	32	16	8
Ceftazidime	≤0.25	128	256	16	64
Cephradine	16	256	128	128	64
Doripenem	≤0.06	2	8	1	4
Imipenem	0.125	1	1	1	1
Meropenem	≤0.06	4	8	2	8
Panipenem	0.125	2	2	1	2

[a]IMP-10 is a variant of IMP-1 with an amino acid substitution of Val67Phe.
[b]IMP-70 is a variant of IMP-1 with two amino acid substitutions, Val67Phe and Phe87Val.
[c]The compound ratio of amoxicillin versus clavulanate is 1:5 (wt/wt).

TABLE 5 Kinetic parameters of β-lactamases IMP-1 and IMP-70 with substrates

	Kinetic parameter for β-lactamase:											
	K_m (μM)[a]				K_{cat} (s^{-1})[a]				K_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)[a]			
Substrate	IMP-1	IMP-10	IMP-1(F87V)	IMP-70	IMP-1	IMP-10	IMP-1(F87V)	IMP-70	IMP-1	IMP-10	IMP-1(F87V)	IMP-70
Penicillin G	941 ± 53	305 ± 17	818 ± 48	203 ± 6	390 ± 11	14 ± 1	13 ± 1	7 ± 0	0.42	0.047	0.016	0.036
Ceftazidime	71 ± 3	259 ± 5	363 ± 5	106 ± 6	7 ± 0	43 ± 1	4 ± 0	2 ± 0	0.1	0.17	0.0097	0.021
Doripenem	32 ± 3	10 ± 1	2626 ± 61	27 ± 0	4 ± 0	8 ± 0	239 ± 5	5 ± 0	0.13	0.82	0.091	0.18
Imipenem	205 ± 14	472 ± 18	534 ± 26	88 ± 6	46 ± 2	112 ± 3	92 ± 3	21 ± 0	0.23	0.24	0.17	0.24
Meropenem	40 ± 2	79 ± 3	1799 ± 67	36 ± 2	6 ± 0	40 ± 2	310 ± 11	13 ± 0	0.15	0.51	0.17	0.35
Panipenem	60 ± 6	46 ± 2	744 ± 19	83 ± 6	24 ± 1	16 ± 0	178 ± 4	19 ± 1	0.4	0.35	0.24	0.23

[a]K_m and k_{cat} were calculated as means ± SD from three independent experiments.

the chromosome (13). Carbapenem-resistant *P. aeruginosa* harboring two copies of bla_{IMP-34} on the chromosome was isolated from a patient in Japan (14). A clinical isolate of *E. coli* obtained from a patient in China harbored bla_{NDM-1} on the chromosome along with two tandem copies of an IS*CR1* element (15). An *E. coli* isolate obtained from a patient in China had an IncF plasmid carrying two copies of bla_{NDM-5} (16). Our present study, together with these reports, strongly suggests that acquisition of two copies of a gene encoding carbapenemase is a strategy of Gram-negative pathogens to obtain high resistance to carbapenems.

The amino acid substitution of Val67Phe, but not that of Phe87Val, in IMP-70 seems to play a crucial role in a significant increase compared with IMP-1 of enzymatic activities against meropenem. Amino acid residue 67 in IMP-1 is located at the end of a loop close to the active site that consists of residues 60 to 66 (17). This loop is a major determinant for the tight binding of substrates in the active site (17). A Val67Phe amino acid substitution in IMP-43, a variant of IMP-7, increased catalytic activities against imipenem and meropenem (18). Amino acid substitutions at position 67 in IMP-1 MBLs affect the ability to hydrolyze and confer resistance to β-lactams (19). Residue 67 was

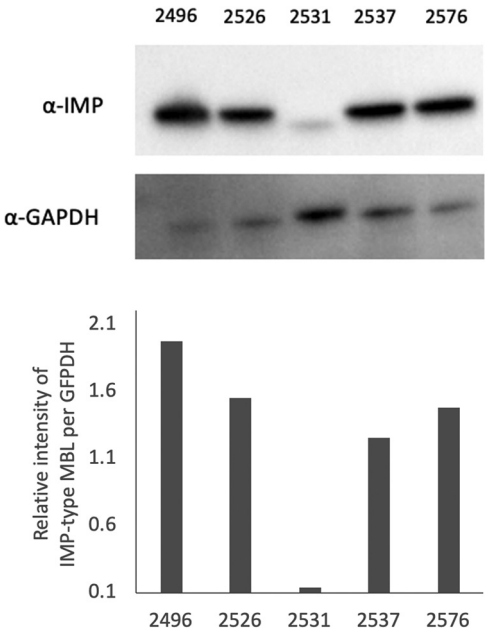

FIG 2 IMP-type metallo-β-lactamase expression in carbapenem-resistant clinical isolates of *P. rettgeri* and *P. stuartii*. Bacterial cells of *P. rettgeri* BML2496, BML2526, BML2531,and BML2576 and of *P. stuartii* BML2537 were solubilized in 2×10^7 CFU, which was applied to an SDS-PAGE gel and transferred onto Immobilon-P. IMP-type MBL and GAPDH were visualized using monoclonal antibodies (upper panel), respectively, as described in Materials and Methods. Relative intensity of IMP-type MBL per GAPDH was calculated using NIH ImageJ software (https://imagej.nih.gov/ij/) (lower).

important for substrate binding in VIM-type MBLs (20). Residue 87 plays a critical role in the stability and folding of VIM-2 (21).

IMP-44, a variant of IMP-11 with two substitutions (Val67Phe and Phe87Ser), had more efficient catalytic activities against carbapenems than those of IMP-11 (18), suggesting that coexistence of two amino acid substitutions at positions 67 and 87 may be necessary to increase enzymatic activities and change substrate profiles in IMP-44. However, a substitution of Phe87Val in IMP-70 did not affect enzymatic activities (Table 5). A substitution of Phe87 to a polar amino acid such as Ser, but not to a hydrophobic amino acid such as Val, may affect enzymatic activities.

Regarding the naming of IMP-70, two sequences of IMP-70 were registered at the NCBI database (GenBank accession no. LC348383 and AUM56801). One sequence (accession no. LC348383) was registered in December 2017, and the other (accession no. AUM56801) in January 2018. We describe here IMP-70, a variant of IMP-1 in *P. rettgeri* and *P. stuartii* isolates, the sequences of which were deposited in the NCBI database (accession no. LC507075 and AP022377, respectively). These sequences correspond to that of IMP-70 (accession no. LC348383). To avoid confusion, IMP-70 sequences should be mentioned with their accession numbers, such as IMP-70 (accession no. LC348383).

MATERIALS AND METHODS

Bacterial strains and identification. Four *P. rettgeri* isolates and a *P. stuartii* isolate were obtained from urine samples of five patients (76.6 ± 7.6 years old) collected from October to September 2018 in 3 hospitals in Japan (BML Biomedical Laboratories R&D Center, Kawagoe, Saitama, Japan). *P. rettgeri* BML2496 and BML2531 were isolated at two hospitals in Saitama and Kochi, Japan, respectively. Isolates of *P. rettgeri* BML2526 and BML 2576 and of *P. stuartii* BML2537 were obtained at a hospital in Osaka, Japan. Bacterial species were identified by WalkAway System (Beckman Coulter, Brea, CA), and confirmed by 16S RNA sequencing. A clinical isolate of *Pseudomonas aeruginosa* harboring bla_{IMP-10} was used to clone the bla_{IMP-10} gene (18).

Drug susceptibility testing. MICs of ceftazidime, aztreonam, imipenem, meropenem, arbekacin, amikacin, and cephalosporin were determined using the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (22). MICs of colistin were determined using the microdilution method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (23).

Whole-genome sequencing. DNA of these isolates was extracted using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan), and their complete genomes were sequenced using the MiSeq platform (Illumina, San Diego, CA). The raw reads were assembled using CLC Genomics Workbench v10.0.1 (CLC bio, Aarhus, Denmark). The sequences of drug resistance genes, including those encoding β-lactamase and aminoglycoside resistance, were determined using ResFinder v3.1 (https://cge.cbs.dtu.dk/services/ResFinder/). The complete genome sequences of all 5 isolates were determined using MinION (Oxford Nanopore Technologies, Oxford, UK). The raw data were base called by Albacore v2.3.1, and adapters were removed by Porechop v0.2.3 (https://github.com/rrwick/Porechop). The long reads generated by MinION and the short reads generated by MiSeq were assembled using Unicycler. Drug resistance genes encoding β-lactamases, aminoglycoside modification enzymes, and 16S rRNA methylases (24) were detected using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/). The sequences of the quinolone resistance genes (25) *gyrA*, *parC*, and *gyrB* were determined using CLC Genomics Workbench v8.0. Multilocus sequence types (MLSTs) were deduced as described in the protocols of the PubMLST database (https://pubmlst.org/paeruginosa/).

PCR. PCR was conducted using a set of primers, IMP-1 DF (5′-GTAAGCCTCAGCATTTACAAGAACC-3′) and IMP-1DR (5′-CAAGTCACAGTGAAGTTGGAGAC-3′), to determine a distance between these sequences of bla_{IMP-1} on the genome of an isolate (BML2496).

Cloning of bla_{IMP-1}, bla_{IMP-10}, and bla_{IMP-70}. The open reading frames (ORFs) of bla_{IMP-1}, bla_{IMP-10}, and bla_{IMP-70} were PCR amplified using the primers EcoRI-IMP-1/70-F (5′-GGGGAATTCATGAGCAAGTTAT CTGTATTC-3′) and PstI-IMP-1/70-R (5′-AAACTGCAGTTAGTTGCTTGGTTTTGATGG-3′), and 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. A mutant clone of bla_{IMP-1} (TTT→GTT at nucleotide positions 205 to 207) encoding IMP-1with an amino acid substitution of Phe87Val [IMP-1(Phe87Val)] was introduced into plasmid pHSGS398-IMP-1 and pET28a-IMP-1 using the KOD Plus mutagenesis kit (Toyobo, Osaka, Japan) using a set of primers, Phe87Val F (5′-TAATTGAC ACTCCAGTTACGGCTAAAGAT-3′) and Phe87Val R (5′-ATCTTTAGCCGTAACTGGAGTGTCAATTA-3′).

Purification of recombinant IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val). To determine the kinetic parameters of these IMP-type enzymes, the ORFs of IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val) without signal peptide regions were amplified by PCR. IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val) were amplified using the primers BamHI-IMP-1/70-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCGCAGAGTCT TTGCCAGATTTAA-3′) and XhoI-IMP-1/70-R (5′-ATCTCGAGTTAGTTGCTTGGTTTTGATGGT-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 transformants were incubated overnight in 200 ml of LB broth containing 20 μg/ml of kanamycin, and the progeny were transferred to 2 liters of the same broth, and further incubated for 3 h. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the bacteria at a final concentration of 0.5 mM/ml, and further incubation was allowed for 2 h. The bacterial cells were collected and 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). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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, UK). These enzymes were prepared in 50 mM Tris-HCl buffer (pH 7.5) in the range of 0.41 to 0.51 mg/ml. Kinetic analysis was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 μM $Zn(NO_3)_2$ (Nakalai Tesque, Inc., Kyoto, Japan) at 37°C using a UV-visible spectrophotometer (V-730; 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 (26–28).

Western blot analysis. Bacterial cells of *P. rettgeri* BML2496, BML2526, BML2531, and BML2576 and of *P. stuartii* BML2537 growing in the log phase were collected and solubilized in 2×10^7 CFU per 10 μl of SDS-PAGE sample buffer. These solubilized bacterial cells were applied to an SDS-PAGE gel and transferred onto Immobilon-P (Merk Millipore Ltd., Cork, Ireland). IMP-type MBL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on Immobilon-P were visualized using a rat monoclonal antibody against IMP-type MBL (4E7) (29) and a mouse monoclonal antibody against GAPDH (Proteintech, Cambridge, UK), respectively. As secondary antibodies, horseradish peroxidase (HRP)-linked goat anti-rat IgG (SouthernBiotech, Birmingham, Alabama) and goat anti-mouse IgG (Abcam, London, UK), respectively, were used and were detected by chemiluminescence (Western BLoT chemiluminescence HRP substrate; TaKaRa Bio, Shiga, Japan). Relative intensities of IMP-type MBL per GAPDH (log relative intensity values) were calculated using NIH ImageJ software (https://imagej.nih.gov/ij/).

Ethics approval. The study protocol was approved by the ethics committee of Juntendo University (approval number 809) and by the Biosafety Committee, Juntendo University (approval number BSL2/29-1). Allowed information about patients included age, gender, and sample tissues.

Data availability. The genome sequence data generated by MiSeq and MinION were deposited in the DNA Data Bank of Japan (DDBJ). The data of assembled and annotated nucleotide sequences were deposited in the GenBank database with the accession numbers AP022371 to AP022377 and LC507075, respectively.

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Miho Ogawa are Masahiro Shimojima are employed by BMI, Inc. There are no patents, products in development, or marketed products to declare.

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