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Novel 6′-*N*-Aminoglycoside Acetyltransferase AAC(6′)-Iaj from a Clinical Isolate of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **NCGM1588 has a novel chromosomal class 1 integron, In151, which includes the *aac(6′)-Iaj* gene. The encoded protein, AAC(6′)-Iaj, was found to consist of 184 amino acids, with 70% identity to AAC(6′)-Ia. *Escherichia coli* transformed with a plasmid containing the *aac(6′)-Iaj* gene acquired resistance to all aminoglycosides tested except gentamicin. Of note, *aac(6′)-Iaj* contributed to the resistance to arbekacin. Thin-layer chromatography revealed that AAC(6′)-Iaj acetylated all aminoglycosides tested except gentamicin. These findings indicated that AAC(6′)-Iaj is a functional acetyltransferase that modifies the amino groups at the 6′ positions of aminoglycosides and contributes to aminoglycoside resistance of *P. aeruginosa* NCGM1588, including arbekacin.**

The major mechanism of resistance to aminoglycosides is the production of aminoglycoside-modifying enzymes (1). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides, including amikacin, gentamicin, netilmicin, and tobramycin. The AAC(6′)-I type confers resistance to amikacin through acetylation of the drug, whereas the AAC(6′)-II type acetylates gentamicin. To date, 43 genes, designated *aac(6′)-Ia* to *aacA43*, which encode AAC(6′)-I enzymes, have been cloned and characterized (1–3). Genes encoding aminoglycoside-modifying enzymes are often located on integrons (4), sequences that can integrate gene cassettes through site-specific recombination (5), in both plasmid and genomic DNA (4). Class 1 integrons participate in multidrug resistance in *Pseudomonas aeruginosa* (6–8).

Pseudomonas aeruginosa is a nosocomial pathogen that exhibits a remarkable ability to acquire resistance to several antibiotics. In Japan, the most serious problem has been the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains, which are defined as having resistance to carbapenems, amikacin, and fluoroquinolones (9, 10).

Previously, we described a nosocomial outbreak caused by an MDR *P. aeruginosa* strain, IMCJ2.S1 (present name, NCGM2.S1) in a hospital in the eastern part of Japan (10). IMCJ2.S1 was found to harbor an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, in a chromosomal integron (9). A study in Japan in 2008 revealed two MDR *P. aeruginosa* clinical isolates harboring *aac(6′)-Iaf* (11). In 2011, a clinical isolate of MDR *P. aeruginosa* negative for *aac(6′)-Iae* and *aac(6′)-Iaf* was found. The isolate contained a novel aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iaj*. Here, we report the structure of this gene and the properties of its product.

MATERIALS AND METHODS

Bacterial strains and plasmids. A clinical isolate of *P. aeruginosa*, NCGM1588, was obtained from the respiratory tract of a patient in 2011 in a hospital in Osaka, Japan. *P. aeruginosa* IMCJ2.S1, which is a representative strain of a cluster endemic to Japan, was used as a control (10, 12). *Escherichia 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 *aac(6′)-Iaj*, respectively. Plasmids pSTV28 and pQE2 were used for cloning of *aac(6′)-Iaj* and purification of recombinant AAC(6′)-Iaj, respectively (11).

Antimicrobial agents. Amikacin (AMK), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CST), lividomycin A (LIV), piperacillin (PIP), polymyxin B (PMB), sisomicin (SIS), and tobramycin (TOB) were obtained from Sigma-Aldrich (St. Louis, MO), arbekacin (ABK), dibekacin (DIB), and kanamycin A (KAN) were purchased from Meiji Seika Pharma Co. (Tokyo, Japan), aztreonam (ATM) was obtained from Eizai (Tokyo, Japan), cefepime (FEP) was obtained from Bristol-Myers Squibb Co. (New York, NY), gentamicin (GEN) and neomycin B and C mixtures (NEO) were obtained from Nacalai Tesque (Kyoto, Japan), imipenem (IPM) was obtained from Banyu Pharmaceutical Co. (Tokyo, Japan), isepamicin (ISP) was obtained from Nichi-Iko Co. (Toyama, Japan), meropenem (MEM) and netilmicin (NET) were obtained from Sumitomo Pharmaceutical Co. (Osaka, Japan), ofloxacin (OFX) was obtained from LKT laboratories (St. Paul, MN), and piperacillin-tazobactam (TZP) was obtained from Toyama Pure Chemical Industries (Tokyo, Japan).

***In vitro* susceptibility tests.** MICs were determined using a microdilution method according to the protocols recommended by the Clinical and Laboratory Standards Institute (13).

Serotyping. The O serotypes of isolates were determined with a slide agglutination test kit (Denka Seiken Co., Tokyo, Japan) and sequence analysis of serotype-specific genes (14).

MLST. Multilocus sequence typing (MLST) was performed according to the protocols described on the *P. aeruginosa* MLST database website (http://pubmlst.org/paeruginosa/). PCR and sequencing were performed for 7 chromosomal genes (*acsA*, *aroE*, *guaA*, *nuoL*, *nuoD*, *ppsA*, and *trpE*). The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allele numbers and sequence types (STs).

PCR amplification of a class 1 integron. Genomic DNA was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, WI). A class 1 integron was detected by PCR using 5′-CS and 3′-CS primers as described previously (9) and genetically mapped using the primers listed

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TABLE 1 PCR primers used in this study

Primer	Sequence[a] (5′ to 3′)	Description
5′-CS	GGCATCCAAGCAGCAAG	5′-end common segment of class 1 integrons
3′-CS	AAGCAGACTTGACCTGA	3′-end common segment of class 1 integrons
intI-F	CTACCTCTCACTAGTGAGGGG	Positions 1–21 in *intI1*
intI-R	TGCGTGTAAATCATCGTCGT	Positions 196–177 in *intI1*
aac(6′)-Iaj-41F	ATCAGATCGATGCTGCAAGAATTC	Positions 41–64 in *aac(6′)-Iaj*
aac(6′)Iaj-543R	ACTTTTCCACATCCAAATATCGGG	Positions 543–520 in *aac(6′)-Iaj*
qacEdelta-F	TGAAAGGCTGGCTTTTTCTT	Positions 2–21 in *qacEΔ1*
qacEdelta-R	GCAATTATGAGCCCCATACC	Positions 268–287 in *qacEΔ1*
sul1-R	GGGTTTCCGAGAAGGTGATT	Positions 768–787 in *sul1*
sul1-F	TCACCGAGGACTCCTTCTTC	Positions 29–48 in *sul1*
*IS*6100-R	GGCTCTGTTGCAAAGATTGGC	Sequence 34–54 downstream of IS*6100*
PstI-aac-F	aactgcagGGCTTGTTATGACTGTTTTT	Sequence in the 180- to 161-bp upstream region of *aac(6′)-Iaj* with PstI site
SalI-aac-R	ggtcgacTCAATTGAGTAGACTTTTCCAC	Positions 555–534 in *aac(6′)-Iaj* with SalI
SphI-aac-F	ccgcatgcgATGGAATATTCAATTATCAAT	Positions 1–21 in *aac(6′)-Iaj* with SphI
NotI-aac-R	gggcggccgcTCAATTGAGTAGACTTTTCC	Positions 555–536 in *aac(6′)-Iaj* with NotI
23S-rRNA-F	CGAGGACAGTGTATGGTGGGCAGT	Positions 2207–2231 in 23S rRNA gene
23S-rRNA-R	CTCAACGCCTCACAACGCTTACACA	Positions 2856–2832 in 23S rRNA gene

[a] Lowercase letters represent restriction enzyme recognition sites attached on the 5′ ends of primers.

in Table 1. The Expand High-Fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany) was used for PCR amplification. All PCR products were sequenced to identify genes and their orders in the integron.

DNA sequencing. DNA sequences were determined using an ABI PRISM3130 sequencer (Applied Biosystems, Foster City, CA). Homology searches of nucleotide and translated protein sequences were performed using BLAST. Multiple-sequence alignments, searches for open reading frames (ORFs), and dendrograms for AACs were performed using Genetyx software (Genetyx, Tokyo, Japan).

PFGE and Southern hybridization. DNA plugs were prepared as described previously (5) and digested overnight at 37°C with SpeI (TaKaRa Bio) or I-CeuI (New England BioLabs, Ipswich, MA). Pulsed-field gel electrophoresis (PFGE) analysis was performed as described previously (9). Southern hybridization was performed using an enhanced chemiluminescence direct nucleic acid-labeling and detection system according to the manufacturer's instructions (GE Healthcare, Tokyo, Japan), as described previously (11), to determine whether the novel class 1 integron identified in the *P. aeruginosa* isolates has a chromosomal location. Probes for *aac(6′)-Iaj* and 23S rRNA genes from NCGM1588 were amplified by PCR using the primer sets aac(6′)-Iaj-41F/aac(6′)Iaj-543R and 23S-rRNA-F/23S-rRNA-R, respectively (Table 1).

Cloning of *aac(6′)-Iaj* gene. The ORF of *aac(6′)-Iaj* and 180 bp of the upstream region of the gene, including the promoter, were amplified by PCR from *P. aeruginosa* NCGM1558 using the primer set PstI-aac-F and SalI-aac-R (Table 1). The PCR products were digested with PstI and SalI and ligated into the PstI and SalI sites of pSTV28. The plasmids were used to transform DH5α, and transformants were selected on LB agar containing 100 μg/ml of chloramphenicol. To determine the MICs of aminoglycosides, *E. coli* DH5α was transformed with pSTV28-*aac(6′)-Iaj*.

Purification of recombinant AAC(6′)-Iaj. The *aac(6′)-Iaj* gene from *P. aeruginosa* NCGM1588 was amplified by PCR using the primer set SphI-aac-F and NotI-aac-R (Table 1), and the product was digested with SphI and NotI and ligated into pQE2 (Invitrogen, Carlsbad, CA), which had been digested with the same restriction enzymes. The plasmid was used to transform DH5α, and the transformants were selected on LB agar containing 100 μg/ml of ampicillin. The resulting plasmid, pQE-*aac(6′)-Iaj*, was used to transform BL21-CodonPlus (DE3)-RIP (Agilent Technologies), which was used for recombinant protein purification. BL21-CodonPlus (DE3)-RIP carrying plasmid pQE2-*aac(6′)-Iaj* was grown in LB medium containing 200 μg/ml ampicillin at 37°C until the A_{600} reached 0.3. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 0.1 mM to induce expression of AAC(6′)-Iaj, and the culture was incubated for 4 h at 37°C. The soluble fraction of six-histidine-tagged AAC(6′)-Iaj was obtained from the bacterial cells lysed by sonication in buffer A (20 mM Tris, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The AAC(6′)-Iaj was purified from the soluble fraction using Ni-NTA agarose according to the manufacturer's instruction (Qiagen, Tokyo, Japan).

TLC analysis of acetylated aminoglycosides. Mixtures containing 2 mM aminoglycoside, 2 mM acetyl coenzyme A (acetyl-CoA), and 50 μg/ml AAC(6′)-Iaj in 20 μl of phosphate buffer (pH 7.4) were incubated for 16 h at 37°C. Aliquots of 3 μl of each aminoglycoside mixture were spotted onto the surface of a Silica Gel 60 thin-layer chromatography (TLC) plate containing a fluorescence indicator with an excitation wavelength of 254 nm (Merck, Darmstadt, Germany), and the results were developed with a 5% phosphate potassium solution. The aminoglycosides and their acetylated products were detected with 0.2% ninhydrin in acetone.

Nucleotide sequence accession number. The nucleotide sequence of In151 determined in this study has been deposited in the EMBL and GenBank databases and the DDBJ and assigned the accession number AB709942.

RESULTS AND DISCUSSION

Characterization of *P. aeruginosa* NCGM1588. The MICs of antibiotics for NCGM1588 were as follows: PIP, 32 μg/ml; TZP, 32 μg/ml; CAZ, 8 μg/ml; FEP, 16 μg/ml; IPM, 32 μg/ml; MEM, 16 μg/ml; ATM, 32 μg/ml; AMK, 128 μg/ml; ABK, 32 μg/ml; GEN, 8 μg/ml; CIP, 16 μg/ml; OFX, 32 μg/ml; PMB, 4 μg/ml; and CST, 4 μg/ml. NCGM1588 showed high levels of AMK resistance. In particular, it showed high levels of ABK resistance, whereas the representative epidemic strain of MDR *P. aeruginosa* IMCJ2.S1 in Japan was susceptible to ABK (9, 15). The serotype of NCGM1588 was O7, and the MLST was ST560. NCGM1588 showed different PFGE patterns from that of MDR *P. aeruginosa* IMCJ2.S1, with similarity of 46.2%.

P. aeruginosa NCGM1588 is an emerging MDR pathogen in Japan. Therefore, it is necessary to carefully investigate whether the NCGM1588 will expand in medical settings. NCGM1588 seems to be quite different from the epidemic strain of MDR *P. aeruginosa* IMCJ2.S1, which is widespread in Japan (9, 10, 12), because of different PFGE patterns, MLSTs (ST560 versus ST235), and serotypes (O7 versus O11). IMCJ2.S1 causes mainly urinary

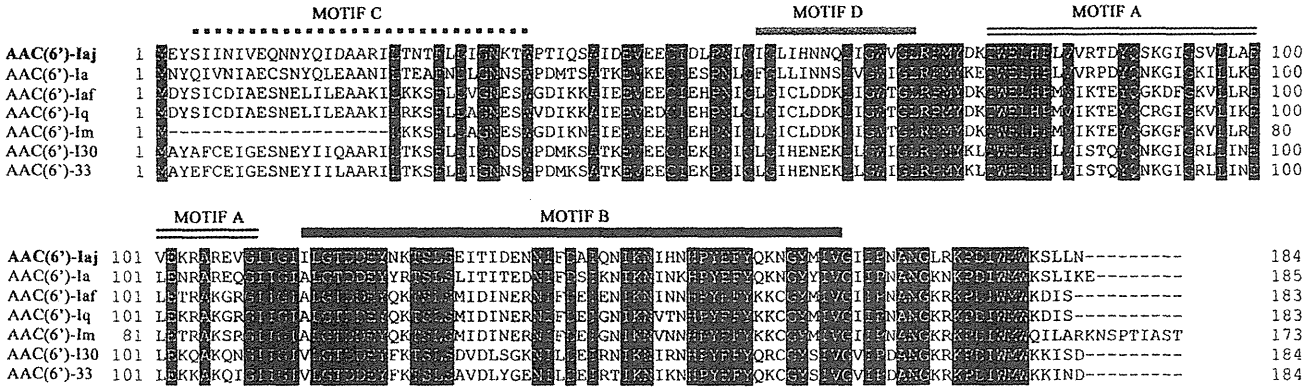

FIG 1 Alignment of the AAC(6′)-Iaj amino acid sequence with those of six members of AAC(6′)-I subfamily. Identical residues are marked with black boxes. Four motifs, C, D, A, and B, are indicated by a dotted line, a gray line, double lines, and a black line, respectively.

tract infections (9), whereas NCGM1588 caused respiratory infection. During our surveillance from 2009 to 2010, we found 16 isolates of MDR *P. aeruginosa*, including a novel MDR *P. aeruginosa* strain, NCGM1179 (16), which had identical PFGE patterns and were ST991 and serotype O18; all of these isolates were isolated from the respiratory tract (17). To date, 14 strains of *P. aeruginosa* showing ST560, including NCGM1588, have been reported—8 in Australia, 3 in China, 1 in the Netherlands, and 1 in Spain (http://pubmlst.org/paeruginosa/). This is the first report of the isolation of *P. aeruginosa* showing ST560 in Japan.

***aac(6′)-Iaj* in the class 1 integron.** To identify the drug resistance genes of NCGM1588, the variable regions of class 1 integrons were amplified with the primers 5′-CS and 3′-CS (Table 1). PCR products of 1.1 kb were generated from this strain. DNA sequence analysis revealed a variable region containing a cassette of a novel *aac(6′)* gene. Based on the standard nomenclature (18), we named this ORF *aac(6′)-Iaj*. The novel gene consisted of an ORF of 555 bp, and its sequence showed 70% identity to that of *aac(6′)-Ia* from *Corynebacterium resistens* (accession number FN825254) (19). The *aac(6′)-Iaj* gene had a G+C content of 31.2%.

We designated the gene *aac(6′)-Iaj* according to a system of nomenclature proposed by Shaw et al. (1), which is easy to understand and indicates the functional properties of the enzymes in a straightforward manner as follows: numbers in parentheses, e.g., (1), (2), (3), and (6′), etc., for the site of modification; roman numerals, e.g., I, II, IV, etc., for unique resistance profiles; and lowercase letters, e.g., a, b, c, etc., for unique protein designations (1, 20).

The structure of the class 1 integron harboring *aac(6′)-Iaj* was determined using external primers (Table 1). The sequence of the integron was not found in any database; therefore, it was named In151. In151 had a structure similar to that of In4 integron (accession number U12338) except for the gene cassette array (21). Between the 5′-CS and 3′-CS, In151 had one gene cassette that contained *aac(6′)-Iaj* and a 60-nt 59-base element, which is known as *attC*, located 11 bp downstream of *aac(6′)-Iaj* (22).

In151 could be derived from the same origin as In4 of *P. aeruginosa* plasmid R1033. In4 was found in plasmid R1033 of a *P. aeruginosa* strain isolated in 1975 (23). The In151 backbone differed from that of In4 by the presence of a partial copy of *IS6100*; i.e., In151 had the 5′-CS, 3′-CS, and a complete copy of *IS6100* located downstream of the 3′-CS.

Amino acid sequence of AAC(6′)-Iaj enzyme. AAC(6′)-Iaj consists of 184 amino acids. Multiple sequence alignments among AAC(6′) enzymes revealed that AAC(6′)-Iaj had 70% identity to AAC(6′)-Ia from *Shigella sonnei* (24), 66% to AAC(6′)-Iaf from *P. aeruginosa* (11), 65% to AAC(6′)-Iq from *Klebsiella pneumoniae* (25), 64% to AAC(6′)-Im from *Citrobacter freundii* (26), 63% to AAC(6′)-33 from *P. aeruginosa* (27), and 63% to AAC(6′)-I30 from *Salmonella enterica* (28). Based on the work of Neuwald and Landsman (29), four motifs in the amino acid sequences of the subfamily proteins belonging to AAC(6′)-Iaj were designated motifs C, D, A, and B (Fig. 1). Comparison of amino acid sequences of members of the AAC(6′)-I subfamily with that of AAC(6′)-Iaj revealed that motifs C, D, A, and B, which are found in most

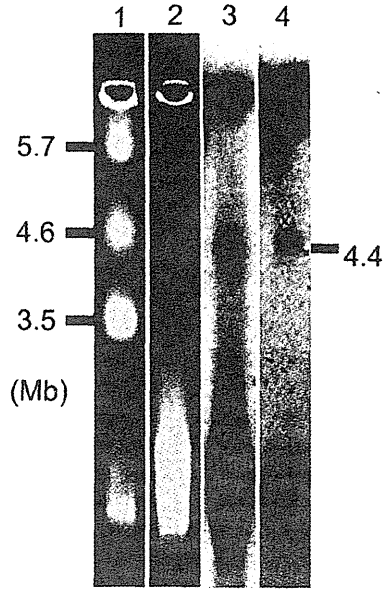

FIG 2 Localization of the *aac(6′)-Iaj* gene on I-CeuI-digested total DNA of *P. aeruginosa* strain NCGM1588 separated by PFGE. *P. aeruginosa* NCGM1588 genomic DNA digested by I-CeuI was done as previously described (11). Lane 1, molecular standard of *Schizosaccharomyces pombe* chromosomal DNA; lane 2, I-CeuI-digested total DNA of *P. aeruginosa* strain NCGM1588 with ethidium bromide; lane 3, Southern hybridization was performed with probes for 23S rRNA gene; lane 4, Southern hybridization was performed with probes for *aac(6′)-Iaj*.

TABLE 2 MICs of various aminoglycosides for *P. aeruginosa* NCGM1588 and *E. coli* strains transformed with *aac(6′)-Iaj*

Strain[a]	MIC[b] (μg/ml)									
	AMK	ABK	DIB	GEN	ISP	KAN	NEO	NET	SIS	TOB
NCGM1588	128	32	1,024	8	512	1,024	256	>1,024	1,024	128
E. coli DH5α/pSTV28	0.5	0.5	0.5	0.5	0.5	0.5	2	0.25	1	0.25
E. coli DH5α/pSTV28-*aac(6′)-Iaj*	16	4	16	0.5	4	32	8	32	4	16

[a] The MICs for *E. coli* strains were determined with Mueller-Hinton broth preparations containing chloramphenicol (30 μg/ml) and individual aminoglycosides.
[b] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

GCN5-related *N*-acetyltransferases (GNATs) (29, 30), were conserved in AAC(6′)-Iaj (Fig. 1). A large motif at the C terminus, motif B (30), was 77.6% identical between AAC(6′)-Ia (24) and AAC(6′)-Iaj.

Localization of the *aac(6′)-Iaj* gene. PFGE analysis and Southern hybridization using NCGM1588 genomic DNA digested by I-CeuI revealed that the probes specific for the 23S rRNA and *aac(6′)-Iaj* were detected in a chromosomal fragment of about 4.4 Mb (Fig. 2). These results indicate that *aac(6′)-Iaj* was located on the chromosomal DNA. Lower DNA bands were observed in lanes 2 to 4 of Fig. 2. They were probably due to nonspecific cleavage during DNA preparation and enzyme digestion. However, we cannot exclude the possibility that NCGM1588 has multiple copies of *aac(6′)-Iaj*.

Drug resistance mediated by AAC(6′)-Iaj enzyme. *P. aeruginosa* NCGM1588 was resistant to all aminoglycosides tested except GEN (Table 2). A vector control of *E. coli* DH5α/pSTV28 was susceptible to all aminoglycosides tested, whereas *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* was resistant to all aminoglycosides, including ABK, except GEN, with 4- to 128-fold-higher MIC values than those of the vector control (Table 2). The MIC of GEN in *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* was the same as that in the vector control.

To examine the acetylase activity of AAC(6′)-Iaj to aminoglycosides, we performed thin-layer chromatography using the purified recombinant AAC(6′)-Iaj. LIV, an aminoglycoside compound, was used as a negative control. LIV has a hydroxyl group instead of an amino group at the 6′ position and therefore cannot be acetylated by AAC(6′). As shown in Fig. 3, all of these aminoglycosides, except GEN, were acetylated by AAC(6′)-Iaj. The acetylation rates were only 2% for GEN when estimated with the ImageJ analyzer (http://rsbweb.nih.gov/ij/index.html). The TLC data for GEN were consistent with the MICs of GEN for *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* and *E. coli* DH5α/pSTV28 (Table 2). The reason for the incomplete acetylation is that commercially available gentamicin is a mixture of its derivatives; some of them have a methyl group on N-6′ and are refractory to AAC(6′)-I enzymes (1).

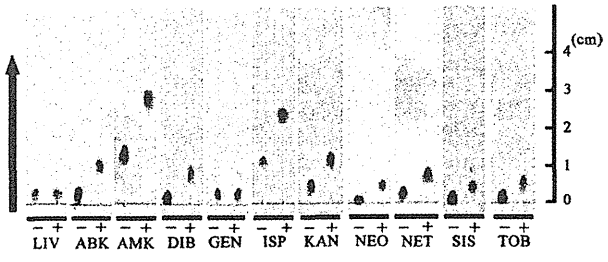

FIG 3 Analysis of acetylated aminoglycosides by TLC. AAC(6′)-Iaj and various aminoglycosides were incubated in the absence (−) or presence (+) of acetyl coenzyme A. The arrow indicates the direction of development.

AAC(6′)-Iaj-producing *P. aeruginosa* NCGM1588 was more resistant to ABK (MIC, 32 μg/ml) than AAC(6′)-Iae-producing IMCJ2.S1 (MIC, 2 μg/ml) (see Table 2 in reference 9), a representative epidemic MDR *P. aeruginosa* strain in Japan, indicating that AAC(6′)-Iaj could inactivate ABK more effectively than AAC(6′)-Iae. *E. coli* DH5α producing AAC(6′)-Iaj was relatively resistant to ABK compared to *E. coli* DH5α producing AAC(6′)-Iae (compare Table 2 in this paper and Table 3 in reference 9). As demonstrated by TCL analyses, both AAC(6′)-Iaj and AAC(6′)-Iae catalyzed inactivation of ABK (compare Fig. 3 in this paper and Fig. 6 in reference 9). The enzymatic activity of AAC(6′)-Iaj against ABK may be stronger than that of AAC(6′)-Iae, although further kinetic studies of both enzymes and chemical analysis of the products of acetylation by both enzymes will be necessary. The chemical structures of AMK and ABK are nearly identical, with only a few differences at the 2′, 3′, and 4′ positions in ring I; that is, AMK has 2′-, 3′-, and 4′-hydroxyl groups, whereas ABK has a 2′-amino group (31). The different substitutions at the 2′, 3′, and 4′ positions in ring I would be responsible for the different levels of ABK resistance between NCGM1179 and IMCJ2.S1.

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REFERENCES

1. Shaw KJ, Rather PN, Hare RS, Miller GH. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. 57:138–163.
2. Rather PN, Munayyer H, Mann PA, Hare RS, Miller GH, Shaw KJ. 1992. Genetic analysis of bacterial acetyltransferases: identification of amino acids determining the specificities of the aminoglycoside 6′-N-acetyltransferase Ib and IIa proteins. J. Bacteriol. 174:3196–3203.
3. Vakulenko SB, Mobashery S. 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. 16:430–450.
4. Hall RM, Collis CM. 1998. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. Drug Resist. Updat. 1:109–119.
5. Grundmann H, Schneider C, Hartung D, Daschner FD, Pitt TL. 1995. Discriminatory power of three DNA-based typing techniques for Pseudomonas aeruginosa. J. Clin. Microbiol. 33:528–534.
6. Laraki N, Galleni M, Thamm I, Riccio ML, Amicosante G, Frere JM, Rossolini GM. 1999. Structure of In31, a blaIMP-containing Pseudomonas aeruginosa integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. 43:890–901.
7. Lee K, Lim JB, Yum JH, Yong D, Chong Y, Kim JM, Livermore DM. 2002. bla(VIM-2) cassette-containing novel integrons in metallo-beta-lactamase-producing Pseudomonas aeruginosa and Pseudomonas putida isolates disseminated in a Korean hospital. Antimicrob. Agents Chemother. 46:1053–1058.

8. Partridge SR, Hall RM. 2003. In34, a complex In5 family class 1 integron containing orf513 and dfrA10. Antimicrob. Agents Chemother. 47:342–349.
9. Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Fujino T, Kobayashi I, Morita K, Kikuchi Y, Kuratsuji T, Kirikae T. 2005. Multidrug-resistant Pseudomonas aeruginosa strain that caused an outbreak in a neurosurgery ward and its aac(6′)-Iae gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. 49:3734–3742.
10. 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.
11. Kitao T, Miyoshi-Akiyama T, Kirikae T. 2009. AAC(6′)-Iaf, a novel aminoglycoside 6′-N-acetyltransferase from multidrug-resistant Pseudomonas aeruginosa clinical isolates. Antimicrob. Agents Chemother. 53: 2327–2334.
12. Miyoshi-Akiyama T, Kuwahara T, Tada T, Kitao T, Kirikae T. 2011. Complete genome sequence of highly multidrug-resistant Pseudomonas aeruginosa NCGM2.S1, a representative strain of a cluster endemic to Japan. J. Bacteriol. 193:7010. doi:10.1128/JB.06312-11.
13. 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.
14. Raymond CK, Sims EH, Kas A, Spencer DH, Kutyavin TV, Ivey RG, Zhou Y, Kaul R, Clendenning JB, Olson MV. 2002. Genetic variation at the O-antigen biosynthetic locus in Pseudomonas aeruginosa. J. Bacteriol. 184:3614–3622.
15. Kitao T, Miyoshi-Akiyama T, Shimada K, Tanaka M, Narahara K, Saito N, Kirikae T. 2010. Development of an immunochromatographic assay for the rapid detection of AAC(6′)-Iae-producing multidrug-resistant Pseudomonas aeruginosa. J. Antimicrob. Chemother. 65:1382–1386.
16. Tada T, Kitao T, Miyoshi-Akiyama T, Kirikae T. 2011. Genome sequence of multidrug-resistant Pseudomonas aeruginosa NCGM1179. J. Bacteriol. 193:6397. doi:10.1128/JB.06129-11.
17. Kitao T, Tada T, Tanaka M, Narahara K, Shimojima M, Shimada K, Miyoshi-Akiyama T, Kirikae T. 2012. Emergence of a novel multidrug-resistant Pseudomonas aeruginosa strain producing IMP-type metallo-beta-lactamases and AAC(6′)-Iae in Japan. Int. J. Antimicrob. Agents 39: 518–521.
18. Vanhoof R, Hannecart-Pokorni E, Content J. 1998. Nomenclature of genes encoding aminoglycoside-modifying enzymes. Antimicrob. Agents Chemother. 42:483.
19. Schroder J, Maus I, Meyer K, Wordemann S, Blom J, Jaenicke S, Schneider J, Trost E, Tauch A. 2012. Complete genome sequence, lifestyle, and multi-drug resistance of the human pathogen Corynebacterium resistens DSM 45100 isolated from blood samples of a leukemia patient. BMC Genomics 13:141. doi:10.1186/1471-2164-13-141.
20. Ramirez MS, Tolmasky ME. 2010. Aminoglycoside modifying enzymes. Drug Resist. Updat. 13:151–171.
21. Partridge SR, Brown HJ, Stokes HW, Hall RM. 2001. Transposons Tn1696 and Tn21 and their integrons In4 and In2 have independent origins. Antimicrob. Agents Chemother. 45:1263–1270.
22. Stokes HW, O'Gorman DB, Recchia GD, Parsekhian M, Hall RM. 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. 26:731–745.
23. Smith DI, Lus RG, Rubio Calvo MC, Datta N, Jacob AE, Hedges RW. 1975. Third type of plasmid conferring gentamicin resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 8:227–230.
24. Tenover FC, Filpula D, Phillips KL, Plorde JJ. 1988. Cloning and sequencing of a gene encoding an aminoglycoside 6′-N-acetyltransferase from an R factor of Citrobacter diversus. J. Bacteriol. 170:471–473.
25. Centron D, Roy PH. 1998. Characterization of the 6′-N-aminoglycoside acetyltransferase gene aac(6′)-Iq from the integron of a natural multiresistance plasmid. Antimicrob. Agents Chemother. 42:1506–1508.
26. Hannecart-Pokorni E, Depuydt F, de Wit L, van Bossuyt E, Content J, Vanhoof R. 1997. Characterization of the 6′-N-aminoglycoside acetyltransferase gene aac(6′)-Im [corrected] associated with a sulI-type integron. Antimicrob. Agents Chemother. 41:314–318.
27. Viedma E, Juan C, Acosta J, Zamorano L, Otero JR, Sanz F, Chaves F, Oliver A. 2009. Nosocomial spread of colistin-only-sensitive sequence type 235 Pseudomonas aeruginosa isolates producing the extended-spectrum beta-lactamases GES-1 and GES-5 in Spain. Antimicrob. Agents Chemother. 53:4930–4933.
28. Mulvey MR, Boyd DA, Baker L, Mykytczuk O, Reis EM, Asensi MD, Rodrigues DP, Ng LK. 2004. Characterization of a Salmonella enterica serovar Agona strain harbouring a class 1 integron containing novel OXA-type beta-lactamase (blaOXA-53) and 6′-N-aminoglycoside acetyltransferase genes [aac(6′)-I30. J. Antimicrob. Chemother. 54:354–359.
29. Neuwald AF, Landsman D. 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. Trends Biochem. Sci. 22:154–155.
30. Dyda F, Klein DC, Hickman AB. 2000. GCN5-related N-acetyltransferases: a structural overview. Annu. Rev. Biophys. Biomol. Struct. 29:81–103.
31. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. 1999. Aminoglycosides: activity and resistance. Antimicrob. Agents Chemother. 43:727–737.

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ERRATUM

Erratum to: Three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* bloodstream infection in Japan

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The correct name of the third author should be given as Tatsuya Tada, not Tada Tatsuya.

We sincerely apologize for the error.

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CASE REPORT

Three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* bloodstream infection in Japan

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Abstract We report three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* bloodstream infection, which showed minimum inhibitory concentration values for imipenem with 2 μg/ml in all isolates. Although carbapenems were initiated empirically in all cases, two of three cases died. The Clinical and Laboratory Standards Institute lowered the breakpoints of carbapenems for *Enterobacteriaceae* in 2010. However, the previous breakpoints are still used in many clinical laboratories, which can result in failure to detect carbapenem-resistant *Enterobacteriaceae*. Therefore, lower breakpoints of carbapenems should be used in clinical settings, and alternative tests for detecting metallo-β-lactamase such as polymerase chain reaction and immunochromatographic assays may contribute to better detection of carbapenem-resistant isolates.

Keywords Metallo-β-lactamase · Carbapenemase · *Enterobacter cloacae* · Bloodstream infection

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Introduction

Since metallo-β-lactamase (MBL)-producing *Enterobacter cloacae* was first reported in 2000, there have been increasing reports of carbapenem-resistant *E. cloacae* worldwide [1, 2]. MBL-producing *Enterobacteriaceae*, including *E. cloacae*, often exhibited relatively low minimum inhibitory concentrations (MIC) of carbapenems (0.06–2 μg/ml) [3]. Recently, the Clinical and Laboratory Standards Institute (CLSI) lowered the breakpoints of carbapenems against *Enterobacteriaceae* [4]. However, higher MIC breakpoints are still used in many clinical laboratories because Food and Drug Administration (FDA)-approved breakpoints have not been changed, which can result in failure to detect carbapenem-resistant *Enterobacteriaceae* [5]. Furthermore, it is unclear whether carbapenems are effective against MBL-producing *E. cloacae* [2]. Clinical studies to determine appropriate chemotherapeutic regimens against MBL-producing *E. cloacae* infection are required. Here, we report three cases including two with unsuccessful outcomes of IMP-type MBL-producing *E. cloacae* bloodstream infections in a hospital in Japan.

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Case reports

Case 1 was a 91-year-old man who was admitted to the hospital for cerebral infraction. He developed aspiration pneumonia on day 20 and was treated with ampicillin/sulbactam (ABPC/SBT) for 10 days. He developed septic shock consequent to peripheral venous catheter infection on day 33, and administration of meropenem (MEM) was initiated. However, hemodynamic instability persisted, and blood culture revealed *E. cloacae* with elevated MIC for imipenem (IPM) (MIC = 2 μg/ml) and *Proteus vulgaris*. MEM was changed to levofloxacin (LVFX) based on the results of susceptibility testing on day 37. Furthermore, MBL production was tested using Cica-β-test [6] and an immunochromatographic assay [7] because of the elevated MIC for imipenem, which revealed positive. IMP-1 was confirmed by polymerase chain reaction and sequencing in our research institute. Although an additional blood culture was negative for *E. cloacae*, the patient died on day 40.

Case 2 was a 77-year-old man with type 2 diabetes receiving insulin therapy. He was admitted to the hospital for esophageal cancer, and subtotal esophageal resection and subcutaneous reconstruction were performed. He developed infection in the cervical wound because of leakage and received vancomycin and MEM for 36 days, with subsequent oral LVFX for 1.5 months. Although the surgical wound infection improved, he had recurrent aspiration pneumonia. On postoperative day (POD) 105, he developed bacteremia caused by *E. cloacae*, which was possibly caused by central venous catheter infection, and MEM was commenced on POD 106. The central venous catheter was removed on POD 109. *E. cloacae* was still isolated from blood culture despite 3 days of antibiotic therapy, and MEM was considered to be ineffective. The isolate was revealed to be a MBL producer and thus gentamycin was added on POD 116. However, the patient died on POD 117.

Case 3 was an 88-year-old man with an abdominal artery aneurysm for which an endovascular graft was inserted 6 months before admission. He was admitted to the hospital for colon cancer, and right hemicolectomy was performed. On POD 3, MEM was initiated for postoperative fever because of surgical site infection. Although his fever improved on POD 5, blood culture revealed MBL-producing *E. cloacae* and *Bacteroides* sp. Thus, MEM was switched to LVFX + ABPC/SBT on POD 7. Bacterial clearance was documented by a follow-up blood culture. The patient completed a 2-week course of intravenous antibiotic therapy followed by 2 weeks of oral LVFX + metronidazole and was discharged.

Drug susceptibility profiles are shown in Table 1. MIC values for both IPM and MEM were 2–4 μg/ml in all isolates, which were reported as susceptible in our clinical laboratory. All the isolates were positive for MBL by phenotypic, immunochromatographic, and polymerase chain reaction (PCR) assays. Two isolates produced IMP-1 and the remaining isolate produced IMP-11. There was no epidemiological link among the three patients.

Discussion

The reduced breakpoints of carbapenems for *Enterobacteriaceae* as revised recently by CLSI should be applied in clinical laboratories [4]. The MICs of IPM for all *Enterobacter cloacae* isolates from these cases were within the susceptible range according to the criteria recommended by CLSI in 2009 [8]. Nevertheless, these isolates were MBL producers. The breakpoint should have been ≤1 μg/ml for IPM in the present cases. CLSI recently recommended lowering the breakpoints for *Enterobacteriaceae* to improve the detection of carbapenemase producers [4]. However, higher MIC breakpoints are still used in many clinical laboratories, including those in Japan, because FDA-approved breakpoints have not been changed [5]. These higher breakpoints can lead to underestimation of the resistance, which may result in inadequate treatment. Yan et al. [9] recently reported that MBL production was not correlated with clinical outcomes and thus it was unnecessary to test MBL routinely. However, they did not analyze the association between MBL production and mortality by multivariate analysis. Information is still

Table 1 Susceptibility profiles of MBL-producing *Enterobacter cloacae* isolates

Isolates	MBL typing	MIC (μg/ml)									
		IPM	MEM	CTX	CAZ	CPR	AZT	P/T	CIP	AMK	CLS
1	IMP-1	2	2	512	512	6	64	64	1	1	2
2	IMP-11	2	2	32	64	4	0.5	64	32	8	2
3	IMP-1	2	4	256	512	16	32	64	0.5	1	2

IMP subtyping was performed by polymerase chain reaction and sequencing

MBL metallo-β-lactamase, *IPM* imipenem, *MEM* meropenem, *CTX* ceftriaxone, *CAZ* ceftazidime, *CPR* cefpirome, *AZT* aztreonam, *P/T* piperacillin/tazobactam, *CIP* ciprofloxacin, *AMK* amikacin, *CLS* colistin

scarce on this point, and further studies are needed to clarify whether MBL production is truly associated with poor outcome and should be tested routinely in clinical settings.

Tests for detecting MBLs may contribute to improved treatment of infections with carbapenem-resistant *Enterobacteriaceae*. These rapid tests include SMA Eiken (SMA, disk diffusion; Eiken Chemical) [10], Cica-β-test [6], PCR [11], and immunochromatographic assays [7]. Infections with MBL producers that have lower breakpoints than those presented by CLSI have been reported [12]. Therefore, additional methods may be required to accurately diagnose infections caused by MBL producers.

To our knowledge, this is the first report of IMP-type MBL-producing *E. cloacae* bloodstream infection in Japan, although a number of VIM-type MBL-producing *E. cloacae* infections have been reported in European countries [2, 3]. The Center for Disease Control and Prevention recommends active surveillance following isolation of carbapenemase-producing *Klebsiella* spp. or *Escherichia coli* because these isolates represent the majority of carbapenemase-producing *Enterobacteriaceae* in the United States [13]. However, active surveillance of *Enterobacter cloacae* is not included in this recommendation. More information is required to determine the validity of active surveillance of MBL-producing *E. cloacae* in healthcare facilities in Japan.

It is unclear whether carbapenems are effective against infections caused by IMP type MBL-producing *E. cloacae* showing MIC within the susceptible range. Two of our three cases were refractory to MEM, suggesting clinical inefficacy of carbapenems against MBL-producing *E. cloacae* regardless of their MIC. Falcone et al. [3] described seven cases of VIM-1-type MBL-producing *E. cloacae* infections: these cases were difficult to diagnose because of apparent susceptibility to carbapenems and were associated with high relapse rate and a prolonged duration of antibiotic therapy. Clinical studies on appropriate chemotherapies against MBL-producing *E. cloacae* infections will be required.

We reported three cases of MBL-producing *E. cloacae* showing relatively low MICs around the breakpoints for carbapenems. Effective testing strategies should be urgently implemented in medical facilities to adequately detect carbapenem-resistant *E. cloacae*.

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Conflict of interest None declared.

Ethical approval Approved by Human Research Ethics Committee of National Center for Global Health and Medicine (NCGM/G/001232/00).

References

1. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J Clin Microbiol. 2000;38:40–3.
2. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-beta-lactamases: a last frontier for beta-lactams? Lancet Infect Dis. 2011;11:381–93.
3. Falcone M, Mezzatesta ML, Perilli M, Forcella C, Giordano A, Cafiso V, et al. Infections with VIM-1 metallo-β-lactamase-producing *Enterobacter cloacae* and their correlation with clinical outcome. J Clin Microbiol. 2009;47:3514–9.
4. Clinical and Laboratory Standards Institute (CLSI) Performance standards for antimicrobial susceptibility testing. Twentieth Informational Supplement (June 2010 update). CLSI document M100-S20-U. Wayne: CLSI; 2010.
5. Gupta N, Limbago BM, Patel JB, Kallen AJ. Carbapenem-resistant *Enterobacteriaceae*: epidemiology and prevention. Clin Infect Dis. 2011;53:60–7.
6. Livermore DM, Warner M, Mushtaq S. Evaluation of the chromogenic Cica-beta-Test for detecting extended-spectrum, AmpC and metallo-beta-lactamases. J Antimicrob Chemother. 2007;60:1375–9.
7. Kitao T, Miyoshi-Akiyama T, Tanaka M, Narahara K, Shimojima M, Kirikae T. Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-beta-lactamases that mediate carbapenem resistance in *Pseudomonas*. J Microbiol Methods. 2011;87:330–7.
8. Clinical and Laboratory Standards Institute (CLSI) Performance standards for antimicrobial susceptibility testing. 19th Informational Supplement. CLSI document M100-S19. Wayne: CLSI; 2009.
9. Yan JJ, Lee NY, Chen HM, Wang MC, Ko WC, Tsai LH, et al. Bloodstream infections caused by IMP-8-producing *Enterobacteriaceae* isolates: the need for clinical laboratory detection of metallo-beta-lactamases? Eur J Clin Microbiol Infect Dis. 2012.
10. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol. 2003;41:4623–9.
11. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis. 2011;70:119–23.
12. Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis. 2011;17:1791–8.
13. Guidance for control of infections with carbapenem-resistant or carbapenemase-producing *Enterobacteriaceae* in acute care facilities. MMWR Morb Mortal Wkly Rep 2009,58:256–260.

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Note

Development of an immunochromatographic assay for rapid detection of AAC(6′)-Ib-producing *Pseudomonas aeruginosa*

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ABSTRACT

To detect aminoglycoside 6′-*N*-acetyltransferase-Ib [AAC(6′)-Ib]-producing, *Pseudomonas aeruginosa* isolates which are a frequent cause of nosocomial infections in Japan, an immunochromatographic assay was developed using two kinds of monoclonal antibodies (mAbs) recognizing AAC(6′)-Ib. The results of the assessment were fully consistent with those of *aac(6′)-Ib* PCR analyses.

The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains, defined as having resistance to all carbapenems, amikacin, and fluoroquinolones, has become a serious problem in medical settings in Japan (Kirikae et al., 2008; Sekiguchi et al., 2007; Sekiguchi et al., 2005; Kitao et al., 2012).

The major mechanism of aminoglycoside resistance is the production of aminoglycoside-modifying enzymes (Shaw et al., 1993). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of interest because they can modify a number of clinically important aminoglycosides.

More than 20% of MDR *P. aeruginosa* isolates in Japan produced the aminoglycoside-modifying enzyme 6′-*N*-acetyltransferase-Ib [AAC(6′)-Ib], which will be reported elsewhere. Approximately 70% of MDR *P. aeruginosa* isolates in Japan produced AAC(6′)-Iae (Kitao et al., 2012). We first developed an immunochromatographic assay to detect AAC(6′)-Iae (Kitao et al., 2010). In this study, an immunochromatographic assay using mAbs to AAC(6′)-Ib was developed.

P. aeruginosa NCGM509 producing AAC(6′)-Ib was obtained from a respiratory tract of a patient in a hospital located in Tokyo in 2005. NCGM2.S1 producing AAC(6′)-Iae (Sekiguchi et al., 2005), NCGM798 producing AAC(6′)-Iaf (Kitao et al., 2009) and NCGM1588 producing AAC(6′)-Iaj (Tada et al., unpublished results) were obtained. Two hundred seventeen *P. aeruginosa* isolates were obtained from BML. Of those, 98 were PCR-positive for *aac(6′)-Ib*, and the remaining 119 were PCR-negative.

The *aac(6′)-Ib* was PCR amplified from *P. aeruginosa* NCGM509 using the primer sets SphI-aac(6′)-Ib-F (5′-ccccgcatgcgATGACCAACAG-CACCGATTCCGTCACA-3′) and NotI-aac(6′)-Ib-R (5′-gggggcggccgcTTA-GGCATCACTGCGTGTTCGCTCGAA-3′). Recombinant His-tagged AAC(6′)-Ib, AAC(6′)-Iae (Sekiguchi et al., 2005), AAC(6′)-Iaf (Kitao et al., 2009), and AAC(6′)-Iaj (Tada et al., unpublished results), purified by Protein G Sepharose 4 Fast Flow (GE Healthcare Bio-Science, Tokyo, Japan) were prepared as described previously (Kitao et al., 2010). Anti-AAC(6′)-Ib monoclonal antibodies (mAbs) were prepared as described previously (Kitao et al., 2010). The animal experiments were approved by the Ethics Committee for Animal Experiments at NCGM (approval number: NCGM23-C-1).

The immunochromatographic assay was assembled as described previously (Miyoshi-Akiyama et al., 2010). Nitrocellulose membranes were coated with 0.76 mg of rat mAbs and 0.2 mg of anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) per test at positions 30 and 39 mm from the sample application area, respectively. Pads were prepared by soaking glass filters with rat mAb and rabbit IgG, each conjugated with colloidal gold.

To determine the epitopes for anti-AAC(6′)-Ib antibody, 3F9 and 3A9; recombinant peptides having the same amino acid sequences of regions in AAC(6′)-Ib were prepared using PCR cloning procedure. We designed 4 long peptides consisting of 55–61-mers covering from

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positions 1 to 184-end region of AAC(6′)-1b, and 13 short peptides consisting of 14–15-mers covering from the positions 51 to 184-end region (Table 1).

The immunochromatographic assay was evaluated using 217 clinical isolates of *P. aeruginosa*, and NCGM2.S1 producing AAC(6′)-Iae, NCGM798 producing AAC(6′)-Iaf, NCGM1588 producing AAC(6′)-Iaj, and *Escherichia coli* harboring pQE2-*aac(6′)-Ib*. Bacterial colonies grown on Mullar–Hinton agar plates were picked with a swab and suspended in soft test tubes containing 0.4 M Tris–HCl buffer containing 1.0% Triton X-100 (pH 7.5). Three drops of the bacterial lysate were added to the test plate. The results were analyzed by visual inspection 15 min after addition of the sample.

The *aac(6′)-Ib* was amplified with primers as follows; 5′-ATGACTGAGCATGACCTTGCGAT-3′ and 5′-GGCATCACTGCGTGTTCGCTCGAAT-3′. PCR amplification started at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR procedure took 90 min.

We obtained 8 mAbs with reactivities to recombinant His-AAC(6′)-Ib NCGM509 in ELISA. Fifty-six immunochromatographic assay prototypes were constructed using these 8 mAbs and examined for reactivities to His-AAC(6′)-Ib (data not shown). The strongest intensity of the test line was obtained with the prototype consisting of 3F9/F1 immobilized on the membrane and 3A9/F1 labeled with colloidal gold (data not shown). Therefore, these two mAbs were utilized in the development of an immunochromatographic assay for detection of AAC(6′)-Ib (Fig. 1A). The sensitivity was 8 ng per test or 7.6×10^6 cfu per test (Fig. 1B). Other aminoglycoside acetyltransferases, including AAC(6′)-Iae, AAC(6′)-Iaf and AAC(6′)-Iaj, were not detected using the assay (data not shown).

Competition assays using 4 long peptides covering the whole region of AAC(6′)-Ib indicated that 3A9/F1 bound to two peptides, 91–145 and 130–184, whereas 3F9/F1 bound to peptide 45–105 (Fig. 2A). Further competition assays were performed using 13 short peptides. As shown in Fig. 2B, 3A9/F1 bound to peptide 171–184 consisting of VQTRQAFERTRSDA, whereas 3F9/F1 bound to peptide 61–75 consisting of TPYIAMLNGEPIGYA.

Of all 217 *P. aeruginosa* isolates tested, 98 were positive by the immunochromatographic assay. These results were fully consistent with those of PCR for *aac(6′)-Ib* showing 100% specificity and 100% sensitivity.

Here, we demonstrated that the newly developed immunochromatographic assay was a rapid and easy-to-use kit to detect AAC(6′)-Ib-producing *P. aeruginosa* isolates. The sensitivity is sufficient to use for bacteria isolated in clinical laboratories, although the sensitivity was lower than that of PCR (≤10 cfu, data not shown).

The 3A9/F1 and 3F9/F1 antibodies were likely to bind to the opposite sites of AAC(6′)-Ib and capture the molecule effectively on the

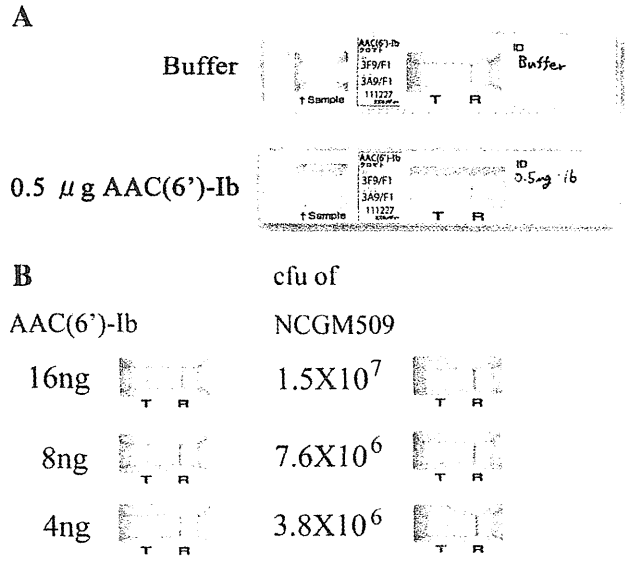

Fig. 1. (A) Immunochromatography using mAbs 3A9/F1 and 3F9/F1. In negative cases, a line appears only at the reference line (R). In positive cases, another line also appears at the positive test line (T) in addition to the reference line. The arrow indicates the direction of lateral flow. (B) Sensitivity testing using AAC(6′)-Ib or the positive control strain NCGM509.

immunochromatographic assay. The three-dimensional structure of AAC(6′)-Ib (PDB ID: 2PRB) (Vetting et al., 2008) indicated that AAC(6′)-Ib is composed of 7 β-strands and 5 α-helices (Fig. 2C). The aminoglycoside binding site is constructed as a pocket with β2–4 (Vetting et al., 2008). The 3F9/F1 seems to bind to β2, β3, and a loop formed by both β strands, whereas 3A9/F1 seems to bind to α5 in the C-terminal region (Fig. 2C).

The immunochromatographic assay to detect AAC(6′)-Ib will be useful when applied together with immunochromatographic assays to detect isolates of AAC(6′)-Iae (Kitao et al., 2010) and iMP (Kitao et al., 2011), to detect MDR *P. aeruginosa* isolates effectively in medical settings in Japan.

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Table 1
Peptides used in epitope mapping were expressed and purified recombinant peptides.

Number	Region in AAC(6′)-Ib	Sequence
1	1–60	VTNSTDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESV
2	45–105	DVQEQYLPSVLAQESVTPYIAMLNGEPIGYAQSYVALGSGDGWWEEETDPGVRGIDQLLAN
3	91–145	ETDPGVRGIDQLLANASQLGKGLGTKLVRALVELLFNDPEVTKIQTDPSPSNLRA
4	130–184	VTKIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA
5	51–65	LPSVLAQESVTPYIA
6	61–75	TPYIAMLNGEPIGYA
7	71–85	PIGYAQSYVALGSGD
8	81–95	LGSGDGWWEEETDPG
9	91–105	ETDPGVRGIDQLLAN
10	101–115	QLLANASQLGKGLGT
11	111–125	KGLGTKLVRALVELL
12	121–135	LVELLFNDPEVTKIQ
13	131–145	VTKIQTDPSPSNLRA
14	141–155	SNLRAIRCYEKAGFE
15	151–165	KAGFERQGTVTTPDG
16	161–175	TTPDGPAVYMVQTRQ
17	171–184	VQTRQAFERTRSDA

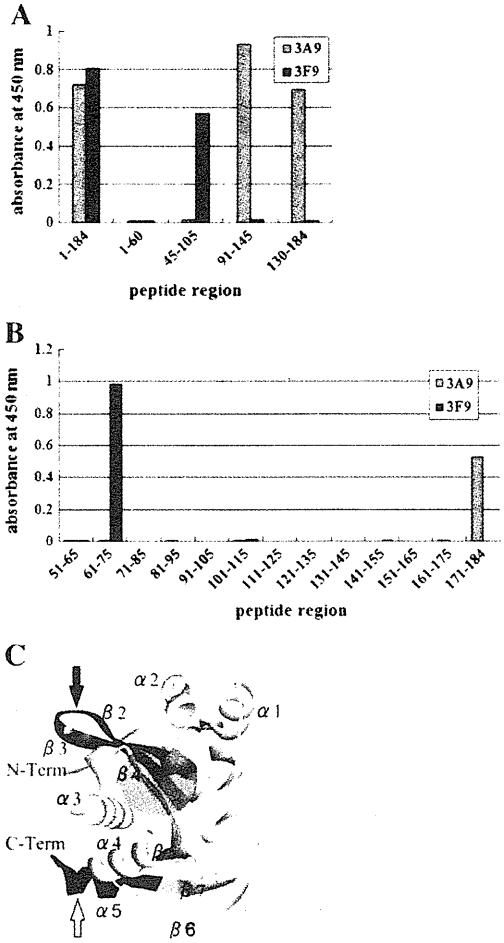

Fig. 2. Determination of epitopes by ELISA. (A) Competition assays using 4 long peptides covering the whole region of AAC(6')-Ib revealed that 3A9/F1 bound to two peptides, 91–145 and 130–184, whereas 3F9/F1 bound to peptide 45–105. (B) Competition assays using 13 short peptides covering the region of peptide 51–184 of AAC(6')-Ib revealed that 3A9/F1 bound to peptide 171–184, whereas 3F9/F1 bound to peptide 61–75. (C) Epitope regions of 3A9/F1 and 3F9/F1 on AAC(6')-Ib crystal structure (PDB ID: 2PRB) (Vetting et al., 2008). The white arrow indicates the epitope region of 3A9/F1, which consists of amino acid residues corresponding to positions 171–184 of AAC(6')-Ib. The black arrow indicates the epitope region of 3F9/F1, which consists of amino acid residues corresponding to positions 61–75 of AAC(6')-Ib. The gray disk indicates the location of the aminoglycoside binding pocket.

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References

Kirikae, T., Mizuguchi, Y., Arakawa, Y., 2008. Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan. J. Antimicrob. Chemother. 61, 612–615.

Kitao, T., Miyoshi-Akiyama, T., Kirikae, T., 2009. AAC(6')-Iaf, a novel aminoglycoside 6'-*N*-acetyltransferase from multidrug-resistant *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. 53, 2327–2334.

Kitao, T., Miyoshi-Akiyama, T., Shimada, K., Tanaka, M., Narahara, K., Saito, N., et al., 2010. Development of an immunochromatographic assay for the rapid detection of AAC(6')-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 65, 1382–1386.

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.

Kitao, T., Tada, T., Tanaka, M., Narahara, K., Shimojima, M., Shimada, K., Miyoshi-Akiyama, T., Kirikae, T., 2012. Emergence of a novel multidrug-resistant *Pseudomonas aeruginosa* strain producing IMP-type metallo-b-lactamases and AAC(6')-Iae in Japan. Int. J. Antimicrob. Agents 39 (6), 518–521.

Miyoshi-Akiyama, T., Narahara, K., Mori, S., Kitajima, H., Kase, T., Morikawa, S., et al., 2010. Development of an immunochromatographic assay specifically detecting pandemic H1N1 (2009) influenza virus. J. Clin. Microbiol. 48, 703–708.

Sekiguchi, J., Asagi, T., Miyoshi-Akiyama, T., Fujino, T., Kobayashi, I., Morita, K., et al., 2005. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. 49, 3734–3742.

Sekiguchi, J., Asagi, T., Miyoshi-Akiyama, T., Kasai, A., Mizuguchi, Y., Araake, M., et al., 2007. Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. J. Clin. Microbiol. 45, 979–989.

Shaw, K.J., Rather, P.N., Hare, R.S., Miller, G.H., 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. 57, 138–163.

Tada, T., Miyoshi-Akiyama, T., Shimada, K., Shimojima, M., Kirikae, T., Novel 6'-*N*-aminoglycoside acetyltransferase, AAC(6')-Iaj, from a clinical isolates of *Pseudomonas aeruginosa*. Unpublished results.

Vetting, M.W., Park, C.H., Hegde, S.S., Jacoby, G.A., Hooper, D.C., Blanchard, J.S., 2008. Mechanistic and structural analysis of aminoglycoside *N*-acetyltransferase AAC(6')-Ib and its bifunctional, fluoroquinolone-active AAC(6')-Ib-cr variant. Biochemistry 47, 9825–9835.

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Short communication

Emergence of a novel multidrug-resistant *Pseudomonas aeruginosa* strain producing IMP-type metallo-β-lactamases and AAC(6′)-Iae in Japan

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ABSTRACT

The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases (MBLs) and aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] has become a serious problem in medical settings in Japan. A total of 217 MDR *P. aeruginosa* isolates were obtained from August 2009 to April 2010 from patients at 144 hospitals in Japan, of which 145 (66.8%) were positive for IMP-type MBLs and AAC(6′)-Iae when tested with an immunochromatographic assay. Polymerase chain reaction (PCR) showed that these isolates were also positive for *bla*IMP and *aac(6′)-Iae* genes. When these IMP-type MBL- and AAC(6′)-Iae-producing isolates were analysed by pulsed-field gel electrophoresis (PFGE), two clusters (I and II) were detected. Most of the isolates (88.3%; 128/145) were grouped under cluster I and had multilocus sequence type ST235 and serotype O11, except for one isolate that was ST991 and serotype O3. The isolates were mainly isolated from the urinary tract (82/145; 56.6%) and respiratory tract (58/145; 40.0%). The epidemiological properties of the isolates belonging to cluster I were similar to those of MDR *P. aeruginosa* isolates that have been previously reported in Japan. The remaining 16 isolates belonged to cluster II, had identical PFGE patterns and were multilocus sequence type ST991 and serotype O18; all of these isolates were isolated from the respiratory tract. The properties of isolates belonging to cluster II have not been previously described, indicating that a novel IMP-type MBL- and AAC(6′)-Iae producing *P. aeruginosa* strain is emerging in Japan. Isolates belonging to both clusters were isolated from different parts of the country.

1. Introduction

The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates, which are resistant to all β-lactams, aminoglycosides and fluoroquinolones, is a serious medical problem in Japan. MDR *P. aeruginosa* are defined as strains showing resistance to carbapenems [minimum inhibitory concentration (MIC) ≥ 16 μg/mL], amikacin (AMK) (MIC ≥ 32 μg/mL) and fluoroquinolones (MIC ≥ 4 μg/mL) based on the criteria specified by the Ministry of Health, Labour, and Welfare of Japan [1].

MDR *P. aeruginosa* isolates in Japan frequently produce IMP-type metallo-β-lactamases (MBLs) and aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] [2–4]. Therefore, we recently designed immunochromatographic assay kits for the detection of IMP-type MBL- [4] and AAC(6′)-Iae producing *P. aeruginosa* [3].

In this study, 145 isolates of MDR *P. aeruginosa* were randomly obtained from 89 medical settings to perform a nationwide epidemiological study on IMP-type MBL- and AAC(6′)-Iae producing MDR *P. aeruginosa* in Japan.

2. Materials and methods

2.1. Bacterial strains

A total of 217 clinical isolates of *P. aeruginosa* resistant to imipenem (IPM) (MIC ≥ 16 μg/mL), AMK (MIC ≥ 32 μg/mL) and ciprofloxacin (CIP) (MIC ≥ 4 μg/mL) were obtained from 144 hospitals located in 31 of the 47 prefectures in Japan from August 2009 to April 2010. The strains were isolated from the urinary tract (n = 111), respiratory tract (n = 94) and other systems of patients (n = 12). MDR *P. aeruginosa* strains NCGM2.S1 [5] and NCGM1179 were used as reference strains. NCGM1179 [6] strain was one of the 217 isolates.

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2.2. Detection of IMP-type metallo-β-lactamases and AAC(6′)-Iae

IMP-type MBLs and AAC(6′)-Iae were detected using an immunochromatographic assay kit (Mizuho Medy Co., Saga, Japan) designed for the detection of these enzymes [3,4].

2.3. Antimicrobial susceptibility

MICs of IPM (Banyu Pharmaceutical Co., Tokyo, Japan), AMK (Banyu Pharmaceutical), CIP (Daiichi Pharmaceutical Co., Tokyo, Japan) and colistin (Sigma-Aldrich, St Louis, MO) were determined using the microdilution method as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [7]. Values of MICs at which 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}, respectively) were determined. Isolates were tested for the presence of MBL by a double-disk synergy test with disks containing sodium mercaptoacetic acid (SMA) as described previously [8].

2.4. Detection of antibiotic resistance genes

The bla_{IMP} and *aac(6′)-Iae* genes were amplified using polymerase chain reaction (PCR) primers as described previously [9]. All of the PCR products were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA). The class 1 integron was amplified using the PCR primer set of 5′CS and 3′CS. All of the PCR products were sequenced to identify the contents of the genes [10].

2.5. Pulsed-field gel electrophoresis (PFGE)

DNA plugs were prepared and digested overnight at 37 °C with *Spe*I (Takara Bio, Otsu, Japan). PFGE analysis was performed as described previously [8]. Fingerprinting patterns were analysed by the unweighted pair-group method using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA) to create an average linkage-based dendrogram.

2.6. Multilocus sequence typing (MLST)

MLST was performed according to the protocols described on the *P. aeruginosa* MLST Database website (http://pubmlst.org/paeruginosa/). PCR and sequencing were performed for seven chromosomal genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*). The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allelic numbers and sequence types (STs).

2.7. Serotyping

Serotypes of the isolates were determined using a slide agglutination test kit (Denka Seiken Co., Tokyo, Japan). Serotype O18 was determined using a sequence-based method [11].

3. Results

3.1. Screening of multidrug-resistant Pseudomonas aeruginosa producing IMP-type metallo-β-lactamases and AAC(6′)-Iae

In total, 217 MDR *P. aeruginosa* isolates were screened for production of IMP-type MBLs and AAC(6′)-Iae using an immunochromatographic assay. Of these, 145 isolates (66.8%) produced both IMP-type MBLs and AAC(6′)-Iae, 29 (13.4%) produced IMP-type MBLs but did not produce AAC(6′)-Iae and 6 (2.8%) produced AAC(6′)-Iae but did not produce IMP-type MBLs. The six isolates producing AAC(6′)-Iae but not IMP-type MBLs were negative for MBL by the SMA double-disk synergy test. Results of the immunochromatographic assay were consistent with those of the PCR for bla_{IMP} and *aac(6′)-Iae* genes.

3.2. Genetic context of the IMP-type metallo-β-lactamases and AAC(6′)-Iae

DNA sequencing showed that the 145 isolates producing both IMP-type MBLs and AAC(6′)-Iae did not have a mutation in the *aac(6′)-Iae* gene. Of these, 125 isolates had bla_{IMP-1}, 6 had bla_{IMP-6} and 14 had bla_{IMP-10}.

PCR showed that of the 145 isolates producing IMP-type MBLs and AAC(6′)-Iae, 142 were positive for a class I integron. Of these 142 isolates, 124 had integron In*113*, which carried bla_{IMP-1} [9]; the remaining 18 isolates had In*113*-like integrons, which have the same structure as integron In*113* but the bla_{IMP-1} is replaced by IMP-6 (4 isolates) and IMP-10 (14 isolates).

3.3. Pulsed-field gel electrophoresis analysis, multilocus sequence typing and serotyping

The 145 isolates of MDR *P. aeruginosa* producing both IMP-type MBLs and AAC(6′)-Iae were analysed by PFGE. Analysis showed two clusters with >60% similarity (clusters I and II) (Fig. 1). Cluster I comprised 128 isolates and cluster II comprised 16 isolates; 1 isolate did not belong to any cluster. Cluster I included the NCGM2.S1 strain, which was the first reported MDR *P. aeruginosa* strain producing IMP-type MBLs and AAC(6′)-Iae [9]. The PFGE patterns of all of the isolates belonging to cluster II were identical (Fig. 1).

Of the 128 isolates belonging to cluster I, 127 were ST235 (STs: *acsA* 38, *aroE* 11, *guaA* 3, *mutL* 13, *nuoD* 1, *ppsA* 2 and *trpE* 4) and serotype O11, and 1 isolate was ST991 (STs, *acsA* 6, *aroE* 3, *guaA* 12, *mutL* 3, *nuoD* 3, *ppsA* 6 and *trpE* 7) and serotype O3. All 16 isolates belonging to cluster II were ST991 and serotype O18. ST991 does not appear to be related to ST235 because all the STs of the housekeeping genes are different.

3.4. Antimicrobial susceptibility

All of the isolates belonging to clusters I and II were highly resistant to IPM, AMK and CIP; there was no difference in the MIC profiles of these two groups (Table 1). Of the 16 isolates belonging to cluster II, 15 were susceptible to colistin (MIC = 2 μg/mL) and 1 was intermediately susceptible (MIC = 4 μg/mL). One isolate belonging to cluster I and one isolate not belonging to any cluster were susceptible to colistin (MIC = 2 μg/mL).

3.5. Geographical distribution

MDR *P. aeruginosa* isolates producing IMP-type MBLs and AAC(6′)-Iae were obtained from 89 medical settings located in 22 prefectures in Japan (Fig. 2). Of these, isolates belonging to cluster I were obtained from 17 prefectures distributed along the northern to southern region of Japan. Isolates belonging to cluster II were obtained from nine prefectures that were also distributed along the northern to southern region of Japan (Fig. 2). The MDR *P. aeruginosa* isolates were obtained from relatively many medical settings in the Kanto area of Japan, e.g. 19 in Saitama, 15 in Tokyo and 9 in Chiba (Fig. 2). These findings suggest that MDR *P. aeruginosa* isolates belonging to both the clusters were spread throughout Japan.

4. Discussion

This study showed that IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa* ST235, serotype O11, which belong to cluster I (Fig. 1), have undergone clonal expansion in medical settings in Japan. NCGM2.S1 strain, which belongs to cluster I, was

Table 1
Minimum inhibitory concentrations (MICs) and percent antimicrobial resistance for IMP-type metallo-β-lactamase- and AAC(6′)-Iae-producing *Pseudomonas aeruginosa* isolates belonging to clusters I and II.

Antimicrobial agent	Breakpoint for resistance (μg/mL)	Cluster I (n = 128)				Cluster II (n = 16)			
		%R	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	%R	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
IPM	≥16	100	32 to >128	128	>128	100	128	128	128
AMK	≥32	100	32 to >128	128	>128	100	64	64	64
CIP	≥8	100	8 to >128	64	>128	100	>128	>128	>128

%R, percent resistance; $MIC_{50/90}$, MIC at which 50% and 90% of the isolates were inhibited, respectively; IPM, imipenem; AMK, amikacin; CIP, ciprofloxacin.

determined to be the cause of an outbreak of catheter-associated urinary tract infections in the neurosurgery ward of a hospital in Miyagi [8], Japan. Further epidemiological studies found that clonal expansion of this strain had also occurred in community hospitals in Kanto region [3] and Hiroshima [2]. Clonal expansion of MBL-producing *P. aeruginosa* ST235, serotype O11 has also been reported in South Korea [12] and Scandinavia [13].

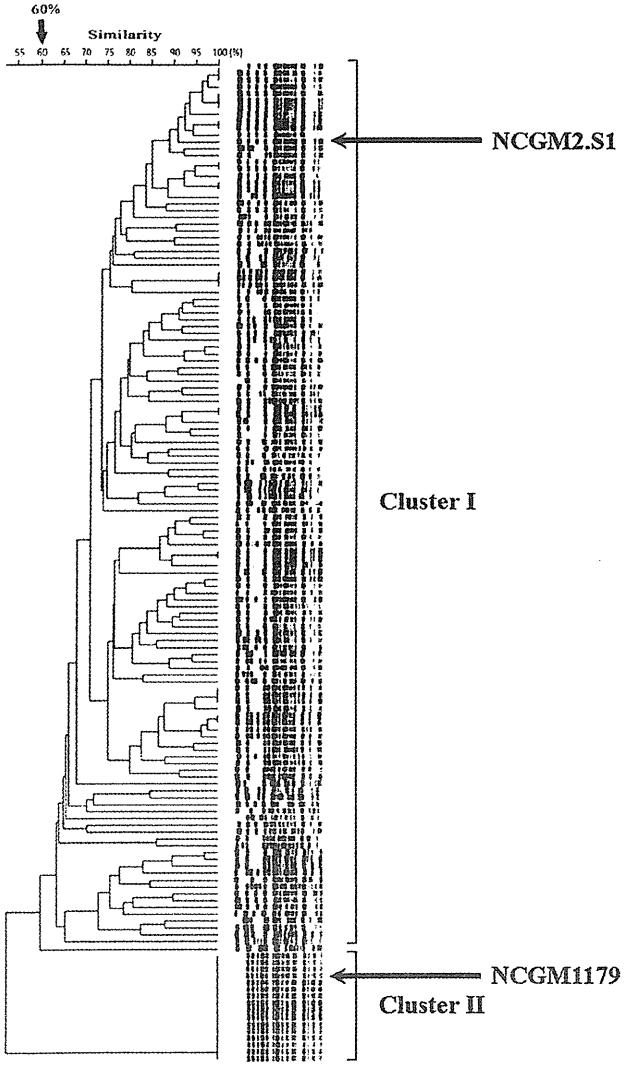

Fig. 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of 145 multidrug-resistant *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae. Two clusters (I and II) were detected. Of the 128 isolates belonging to cluster I, 127 isolates were ST235 and serotype O11 and 1 isolate was ST991 and serotype O3. All of the 16 isolates belonging to cluster II were ST991 and serotype O18.

The isolates belonging to cluster I were mainly obtained from the urinary and respiratory tracts; the percentage of isolates from the urinary tract was markedly higher. A surveillance study of *P. aeruginosa* clinical isolates with and without multidrug resistance showed that MDR isolates were particularly increased in the urinary tract of Japanese individuals [1]. The increase in the number of MDR isolates in the urinary tract may be related to the epidemic of IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa* in Japan.

This is the first report describing MDR *P. aeruginosa* ST991, serotype O18, which belonged to cluster II (Fig. 1) and is a recent emerging strain in medical settings in Japan. ST991 was originally registered by C. Giske at Karolinska University Hospital, Sweden in 2010 in the *P. aeruginosa* MLST Database (http://pubmlst.org/paeruginosa/). However, to the best of our knowledge, there are no reports on the association of ST991 and multidrug resistance in *P. aeruginosa*. All of the isolates belonging to cluster II were obtained from the respiratory tract. In contrast, 32.8% of the isolates belonging to cluster I (42/128) were obtained from the respiratory tract. MDR *P. aeruginosa* ST991 dominantly causes respiratory infections. MDR isolates of *P. aeruginosa* serotype O18 have not been previously reported. Most of the MDR clinical isolates of *P. aeruginosa* exhibit serotype O11 or O12 [11].

We have reported the complete genome sequences of NCGM2.S1 [5] and NCGM1179 [6]. Integron In*113* was inserted in the *oprD* gene and disrupted it in NCGM2.S1; integron In*113* was located downstream of the *tnpA* gene that codes for

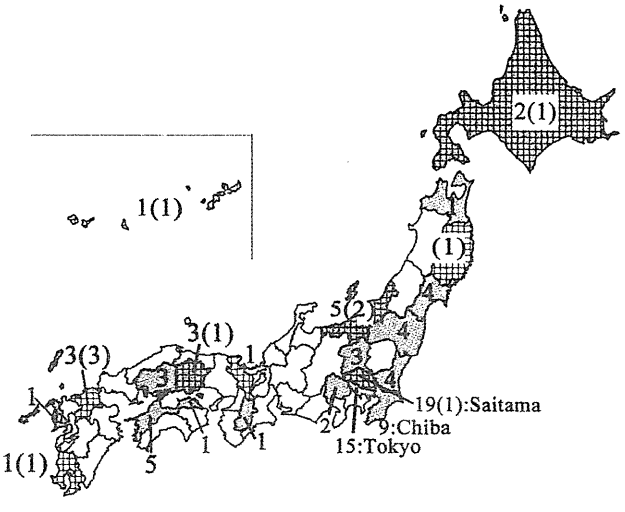

Fig. 2. Geographical distribution of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae in Japan. Isolates belonging to cluster I (Fig. 1) were obtained from prefectures marked in grey; isolates belonging to cluster II were obtained from prefectures marked in a checked pattern. Isolates belonging to both clusters were obtained from prefectures marked in a grey checked pattern. The number and the number in parenthesis represent the number of medical settings in the prefecture where MDR *P. aeruginosa* isolates belonging to cluster I and cluster II, respectively, were obtained.

transposase of Tn*4380* of the mercury transposon Tn*3* family and the *tnpR* gene that codes for serine-base site-specific recombinase of Tn*6050*. However, the *oprD* was found to be intact in the NCGM1179 strain. *oprD* codes for a specialised pore protein, OprD, which allows selective permeation of basic amino acids and their structural analogues such as carbapenems, including IPM and meropenem [14]. It is unclear whether OprD affects the MIC of carbapenems in IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa*. The details of the comparative genome analysis of the two MDR strains will be reported elsewhere.

Of the 217 MDR *P. aeruginosa* isolates tested in this study, 72 did not produce IMP-type MBLs and/or AAC(6′)-Iae. At present, we are looking for genes conferring high resistance to all β-lactams, aminoglycosides and fluoroquinolones.

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Competing interests: None declared.

Ethical approval: This study was approved in 2010 by the Biosafety Committee, National Center for Global Health and Medicine (Tokyo, Japan) (approval no. 23-M-58).

References

[1] Kirikae T, Mizuguchi Y, Arakawa Y. Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan. J Antimicrob Chemother 2008;61:612–5.

[2] Kouda S, Ohara M, Onodera M, Fujiue Y, Sasaki M, Kohara T, et al. Increased prevalence and clonal dissemination of multidrug-resistant *Pseudomonas aeruginosa* with the bla_{IMP-1} gene cassette in Hiroshima. J Antimicrob Chemother 2009;64:46–51.

[3] Kitao T, Miyoshi-Akiyama T, Shimada K, Tanaka M, Narahara K, Saito N, et al. Development of an immunochromatographic assay for the rapid detection of AAC(6′)-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*. J Antimicrob Chemother 2010;65:1382–6.

[4] Kitao T, Miyoshi-Akiyama T, Tanaka M, Narahara K, Shimojima M, Kirikae T. Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-β-lactamases that mediate carbapenem resistance in *Pseudomonas*. J Microbiol Methods 2011;87:330–7.

[5] Miyoshi-Akiyama T, Kuwahara T, Tada T, Kitao T, Kirikae T. Complete genome sequence of highly multidrug-resistant *Pseudomonas aeruginosa* NCGM2.S1, a representative strain of a cluster endemic to Japan. J Bacteriol 2011; 193:7010.

[6] Tada T, Kitao T, Miyoshi-Akiyama T, Kirikae T. Genome sequence of multidrug-resistant *Pseudomonas aeruginosa* NCGM1179. J Bacteriol 2011;193:6397.

[7] Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 6th ed. Document M7-A7. Wayne, PA: CLSI; 2003.

[8] Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Kasai A, Mizuguchi Y, Araake M, et al. Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. J Clin Microbiol 2007;45:979–89.

[9] Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Fujino T, Kobayashi I, Morita K, et al. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6′)-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob Agents Chemother 2005;49:3734–42.

[10] Levesque C, Piche L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother 1995;39:185–91.

[11] Raymond CK, Sims EH, Kas A, Spencer DH, Kutyavin TV, Ivey RG, et al. Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. J Bacteriol 2002;184:3614–22.

[12] Seok Y, Bae IK, Jeong SH, Kim SH, Lee H, Lee K. Dissemination of IMP-6 metallo-β-lactamase-producing *Pseudomonas aeruginosa* sequence type 235 in Korea. J Antimicrob Chemother 2011;66:2791–6.

[13] Samuelsen O, Toleman MA, Sundsfjord A, Rydberg J, Leegaard TM, Walder M, et al. Molecular epidemiology of metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolates from Norway and Sweden shows import of international clones and local clonal expansion. Antimicrob Agents Chemother 2010;54:346–52.

[14] Trias J, Nikaido H. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1990;34:52–7.

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Identification of VanN-Type Vancomycin Resistance in an *Enterococcus faecium* Isolate from Chicken Meat in Japan

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Five VanN-type vancomycin-resistant *Enterococcus faecium* strains were isolated from a sample of domestic chicken meat in Japan. All isolates showed low-level resistance to vancomycin (MIC, 12 mg/liter) and had the same pulsed-field gel electrophoresis profile. The vancomycin resistance was encoded on a large plasmid (160 kbp) and was expressed constitutively. The VanN-type resistance operon was identical to the first resistance operon to be reported, with the exception of a 1-bp deletion in $vanT_N$ and a 1-bp substitution in $vanS_N$.

Since the first reports of vancomycin resistance in *Enterococcus faecium* in 1988 (12, 20), the glycopeptide-resistant enterococci (GRE) have become increasingly widespread throughout the world and are found as multiresistant opportunistic pathogens in hospitals and also in the environment (food animals). To date, nine types of operon structure conferring resistance to glycopeptides have been reported (5, 11). They are designated according to the characteristics of a key ligase gene that encodes either a D-alanyl–D-lactate or a D-alanyl–D-serine ligase (2). The D-alanyl–D-lactate ligase group includes the *vanA*, *vanB*, *vanD*, and *vanM* genes. The D-alanyl–D-serine group includes the *vanC1*, *vanC2*, *vanC3*, *vanE*, *vanG*, *vanL*, and *vanN* genes. Except for *vanC*-type resistance, which is intrinsic to *Enterococcus gallinarum* and *Enterococcus casseliflavus*, all resistance types are acquired externally. The D-Ala–D-Lac-type operons may be located on either plasmids or the chromosome. While the D-Ala–D-Ser-type *vanG*, *vanE*, and *vanL* operons have been detected only in the chromosome of *Enterococcus faecalis*, the location of VanN in *E. faecium* has not been clearly identified to date. VanG and VanN are transferable D-Ala–D-Ser resistance-type operons. The recently reported VanN type was identified in *Enterococcus faecium* isolated from a blood culture, showed a low level of vancomycin resistance (MIC, 16 mg/liter), and was susceptible to teicoplanin (0.5 mg/liter) (11). The *vanN* resistance operon was reported to be found on a transferable element. VanN-type resistance was detected in *E. faecium* strains isolated from a patient in France in 2008, which is the only report of VanN-type GRE to date. So far, there have not been any epidemiological data reported for VanN-type GRE strains obtained anywhere in the world and the significance of the acquisition of VanN-type resistance by enterococci is not clear.

In Japan, there is a lower incidence of GRE in humans and animals than in other countries (16). However, there are several reports showing the possible transmission of GRE or glycopeptide resistance between humans and food animals through food products such as chicken meat (8, 16). More than 10 years ago, we isolated GRE strains from both imported and domestic meats, including chicken meat (9). Since then, we have examined both imported and domestic meat samples as part of a surveillance program looking at GRE contamination (16, 19). While VanA- and VanB-type GRE strains, which show high-level resistance to glycopeptides, are occasionally detected in the samples, most of the GRE isolates from the meat samples had VanC-type resistance, which is carried naturally by enterococci that show low-level resistance to glycopeptides. We recently identified VanN-type GRE strains isolated from a sample of domestic chicken meat. Here we present the results of the analysis of those strains.

During the period from February to May 2011, a total of 322 meat and swab samples from meat destined for consumption in Japan were collected and investigated. The samples were obtained from two major national quarantine stations (Yokohama and Kobe) and from three meat inspection offices (Gunma, Kagoshima, and Miyazaki) in Japan. They included samples from 90 domestic chickens, 45 domestic pork meat samples, 85 imported chickens, and 102 imported pork meat samples. The country or region of origin of each sample is listed in Table 1. A hundred grams of each meat sample (mincemeat) was smashed and homogenized using an EXNIZER 400 (Organo, Japan) in 150 ml of buffered peptone water (Nissui, Japan). Eight milliliters of the supernatant from the homogenized meat sample was mixed with 32 ml of esculin saline buffer containing vancomycin at a concentration of 6 mg/liter and preincubated for 48 h at 37°C. After preincubation, 0.1 ml of each culture broth was spread on bile esculin azide agar (BEAA) plates containing vancomycin at a concentration of 6 mg/liter. The meat swab samples were preincubated for 48 h in 40 ml of brain heart infusion broth (Difco, Detroit, MI) containing vancomycin at a concentration of 6 mg/liter, and then 0.1 ml of each culture broth was spread on BEAA plates containing vancomycin at a concentration of 6 mg/liter. In the

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TABLE 1 GRE isolates from meat samples[a]

Country (prefecture) of origin	No. of chicken, pig samples	Corresponding no. of GRE strains/samples[b]		
		vanC1	*vanC2*	*vanN*
Japan (Gunma)	30, 15	62/21, 6/3	3/1, 3/1	0, 0
Japan (Miyazaki)	30, 15	77/27, 0	0, 0	5/1, 0
Japan (Kagoshima)	30, 15	15/5, 0	3/1, 0	0, 0
United States	3, 44	3/1, 0	0, 0	0, 0
Brazil	71, 0	173/59, 0	0, 0	0, 0
France	6, 0	0, 0	0, 0	0, 0
Philippines	5, 0	13/5, 0	0, 0	0, 0
Canada	0, 25	0, 0	0, 3/1	0, 0
Denmark	0, 19	0, 0	0, 2/1	0, 0
Mexico	0, 7	0, 0	3/1, 0	0, 0
Chile	0, 3	0, 0	0, 0,	0, 0
Spain	0, 2	0, 0	0, 0	0, 0
Hungary	0, 1	0, 0	0, 0	0, 0
Netherlands	0, 1	0, 0	0, 0	0, 0
Chicken, pig totals	175, 147	343/118, 6/3	9/3, 8/3	5/1, 0

[a] The vancomycin MICs of GRE isolates were more than 12 mg/liter.
[b] Neither VanA-type nor VanB-type GRE was detected in this study. VanC1-type and VanC2-type resistances are carried naturally by *E. gallinarum* and *E. casseliflavus*.

present study, enterococci showing a vancomycin MIC of >12 mg/liter were isolated and further analyzed as GRE, because the VanA-type GRE strain occasionally shows low-level resistance to vancomycin (18). Two colonies were picked at random from each GRE-positive sample giving multiple colonies on the selective plate. A total of 128 samples (40%) were positive for GRE, and 371 GRE isolates were obtained in total. To genotype the vancomycin resistance of the GRE isolates, multiplex PCR of the key ligase genes was performed (6). Three hundred forty-nine VanC1-type GRE strains, 17 VanC2-type GRE strains, and 5 unknown-type GRE strains were isolated (Table 1). It was considered that the VanC1-type GRE isolates would be *E. gallinarum* strains and the VanC2-type GRE isolates would be *E. casseliflavus* strains, as both species naturally exhibit low-level resistance to glycopeptides (5). At first, two unknown-type GRE strains were isolated from one domestic chicken that had been bred in Miyazaki Prefecture, Kyusyu Island, Japan. During this study, an additional three isolates of the unknown-type GRE strain were picked up from the master plate which produced the original two unknown-type GRE strains to give five isolates in total. These five isolates were then used to examine whether they arose from a single clone or from multiple clones. The five GRE isolates, named GU121-1, GU121-2, GU121-3, GU121-4, and GU121-5, were all identified as *E. faecium* strains on the basis of their *ddl* gene sequences (15). They showed low-level resistance to vancomycin (MIC, 12 mg/liter) and were susceptible to teicoplanin (MIC, 1.5 mg/liter). The five isolates were susceptible or showed intermediate resistance to other antibiotics (MICs [mg/liter]: ampicillin, ≤4; chloramphenicol, ≤4, ciprofloxacin, 1; erythromycin, 2; fosfomycin, 32; gentamicin, ≤125; kanamycin, ≤125; linezolid, ≤1; rifampin, 2; tetracycline, ≤2). Pulsed-field gel electrophoresis (PFGE) analysis confirmed that they were indistinguishable and were from a single GRE clone (see Fig. S1 in the supplemental material) (16). Of the five isolates, *E. faecium* GU121-1 was picked for further analysis as the representative strain in this study.

Multilocus sequence typing (MLST) analysis showed that one of seven genes, *purK*, was a new allele (number 58), and *E. faecium* isolate GU121-1 was categorized as a new sequence type (ST) designated ST669 (gene, allele number: AtpA, 9; Ddl, 8; Gdh, 14; PurK, 58; Gyd, 6; PstS, 27; Adk, 6). This new type showed the greatest similarity to previously reported ST329 (gene, allele number: AtpA, 9; Ddl, 8; Gdh, 14; PurK, 8; Gyd, 10; PstS, 27; Adk, 6) on the basis of information in the database (MLST database [http://efaecium.mlst.net/]). ST669 was a double-locus variant of ST329 (we found two nucleotide substitutions in *purK* and one in *gyd*). The VanA-type GRE strain categorized as ST329 was isolated from a blood sample from a patient hospitalized in the Netherlands in 1999. Both ST669 and ST329 do not belong to well-characterized clonal complex 17, which is found in the hospital-adapted and epidemic *E. faecium* strain cluster. ST669 was grouped as a satellite sequence type on the basis of the MLST analysis (10).

The specific PCR primer sets used to amplify the internal region of the reported resistance ligase genes (*vanA*, *vanB*, *vanC1*, *vanC2*, and *vanC3*) did not work, and no PCR product was obtained (6). Previously reported primers oligo V1 and oligo V2, which were designed on the basis of the ligase amino acid sequences, were then used for PCR amplification of the unknown ligase gene (7). The PCR product successfully amplified using this primer set was analyzed by direct DNA sequencing. The DNA sequence obtained for the ligase gene was homologous to the *vanL* ligase (around 70% identity at the base pair level) (4). On the basis of the DNA sequence obtained for the ligase gene, a pair of primers for inverse PCR was designed in order to examine the entire resistance gene cluster (operon) around the ligase gene. Several repeated inverse PCR amplifications with restriction enzymes HindIII, BamHI, and SalI were performed to determine the entire DNA sequence of the *vanL*-like resistance operon structure. A DNA region covering 12,344 bp that was located between a SalI site and an EcoRI site and included the predicted vancomycin resistance operon (including the *vanL*-like ligase gene) was determined in this study (Fig. 1). Analyses of the DNA sequence data showed that the unknown resistance operon structure containing the *vanL*-like gene was almost identical to the newly reported D-Ala–D-Ser VanN-type ligase and was composed of five genes designated *vanN*, $vanXY_N$, $vanT_N$, $vanR_N$, and $vanS_N$ (11). Compared to the previously reported VanN-type operon found in the *E. faecium* UCN71 strain, there was a 1-bp insertion and a 1-bp substitution in the VanN-type operon of *E. faecium* GU121-1. A thymidine residue was inserted at bp 1891 in the $vanT_N$ gene. The insertion resulted in a frameshift in the C-terminal region of $vanT_N$ and the production of an elongated $VanT_N$ peptide compared with the prototype protein (a length increase from 656 to 700 amino acids) (Fig. 1). A 1-bp substitution was located in $vanS_N$ gene at 466 bp. This caused an amino acid change of Ser to Pro at residue 156 of the $VanS_N$ protein.

Both the upstream and downstream regions of the *vanN* operon structure of *E. faecium* GU121-1 were examined in the present study. A DNA sequence of about 3,400 bp located upstream of the *vanN* ligase gene and a 3,400-bp DNA sequence located downstream of $vanS_N$ were determined (Fig. 1). Five open reading frames (ORFs) were identified in these regions; two ORFs were located upstream and three ORFs were downstream of the *van* resistance operon. These ORFs were transcribed in the opposite direction from the resistance genes. Homology analysis showed that most of the ORFs encoded unknown hypothetical

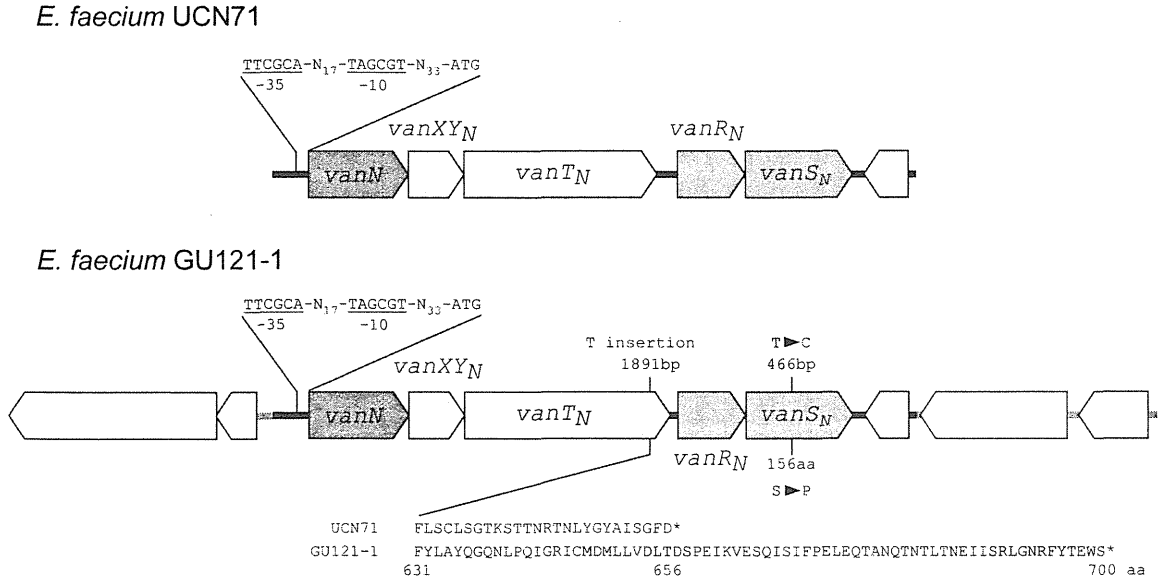

FIG 1 The VanN-type resistance operon structure and the predicted ORFs found around the resistance region of *E. faecium* GU121-1. The upper panel shows the operon structure located on the conjugative plasmid found in the first reported VanN-type vancomycin-resistant *E. faecium* UCN71 clinical isolate in France (GenBank accession number: JF802084). The lower panel shows the operon structure and the predicted ORFs located on the chromosomal DNA of *E. faecium* GU121-1 (AB701345). The horizontal open arrows indicate ORFs and their direction of transcription. The gray-colored ORFs show the VanN-type resistance gene clusters. The thick horizontal lines behind the ORFs show the DNA sequence regions determined. The thick black lines indicate regions corresponding to the published DNA sequence data of UCN71 (6,750 bp). The thick gray lines indicate the plasmid DNA sequence regions determined in this study (12,344 bp). The promoter regions (−35 and −10 sequences) for *vanN* genes are shown in detail.

proteins. It was obvious that there is no predicted gene related to mobile elements, including insertion sequence-related structures such as transposase and inverted repeat sequences. The ORF located downstream of the *vanN* operon was identical to that found in the reported DNA sequence, though its function is unknown (Fig. 1).

The first reported D-Ala–D-Ser VanN-type resistance was transferable between *E. faecium* strains at a low frequency (on the order of 10^{-10}) by filter mating experiments. We examined whether the VanN-type resistance could be transferred by conjugation *in vitro*. Vancomycin-resistant transconjugants could not be obtained from the five VanN-type GRE isolates by solid-surface mating experiments with *E. faecium* BM4105RF and *E. faecalis* FA2-2 as the recipient strains. Although no transconjugant was obtained in this study, we cannot dismiss the possibility of transferability of the VanN-type resistance of the GU121 isolates. If the resistances were transferable, the transfer frequencies were less than 4×10^{-8} per donor cell, which was the limit of detection of transconjugants in the present study. Further experimental studies and DNA sequence analysis are needed to clarify this point.

The plasmid DNAs of VanN-type strains were isolated by the alkaline-lysis method and analyzed (17). All five VanN-type *E. faecium* isolates showed the same plasmid profiles in agarose gel electrophoresis analysis (data not shown). The result of PFGE analyses using the S1 nuclease (Promega) showed that VanN-type strains harbor at least four plasmids (Fig. 2) (3). The conditions for electrophoresis were as follows: 19.5 h at 6 V/cm, 5.3 to 66.0 s nonlinear 21% (for 50 to 1,000 kbp), 0.5× Tris-borate-EDTA buffer, and 1% agarose gel. Four plasmids of approximately 160, 70, 60, and 40 kbp were identified. Standard PFGE analysis with the I-Ceu I endonuclease enzyme and Southern blotting hybridization using the specific probe for 23S rRNA confirmed that the four bands did not correspond to chromosomal DNA but were plasmid DNA (see Fig. S2 in the supplemental material) (13). The location of the VanN-type operon of the strains was also determined by Southern blotting hybridization using the *vanN* probe (Fig. 2; see Fig. S2 in the supplemental material). The 1,315-bp *vanN*-specific probe was constructed by PCR amplification using forward primer 5′-AGGAACATCACACTTCGAGG-3′ and reverse primer 5′-CGCATAGGTCGCTTGAACAA-3′. Hybridization analyses of PFGE showed that the *vanN* probe hybridized to the largest plasmid DNA band. The results clearly indicated that the *vanN*-type operon was located on the 160-kbp plasmid.

Gene expression in the VanN-type resistance operon was examined by the real-time PCR (RT-PCR) method to detect the transcription of the *vanN* ligase gene. An Applied Biosystems 7500 Fast Real-Time PCR System machine and SYBR premix EX *Taq* II (TaKaRa, Tokyo, Japan) were used for the RT-PCR experiment (1). The expression of *rrsA* was used as the endogenous control. The chromosomal D-Ala–D-Ala ligase gene of *E. faecium* BM4105S was also used as an internal control. The transcription level of *vanN* was measured in the presence of different concentrations of vancomycin in the culture (0, 1, 2, and 4 mg/liter). The transcription of *vanN* was detected without vancomycin in the medium, and the expression levels remained unchanged after the addition of vancomycin (see Fig. S3 in the supplemental material). These data indicated that the VanN-type vancomycin resistance gene was expressed constitutively and was not induced by the addition of glycopeptide to the culture medium. This observation is consistent with the report describing the VanN-type *E. faecium* UCN71 strain, in which the resistance operon is considered to be expressed constitutively and which has the same promoter sequence for the VanN-type operon (*vanN* ligase gene) (Fig. 1) (11).

In the present study, VanN-type vancomycin resistance en-