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Ⅲ. 研究成果の刊行物・別刷

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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).

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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 2011in 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*, *mutL*, *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*
IS6100-R	GGCTCTGTTGCAAAGATTGGC	Sequence 34–54 downstream of *IS6100*
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

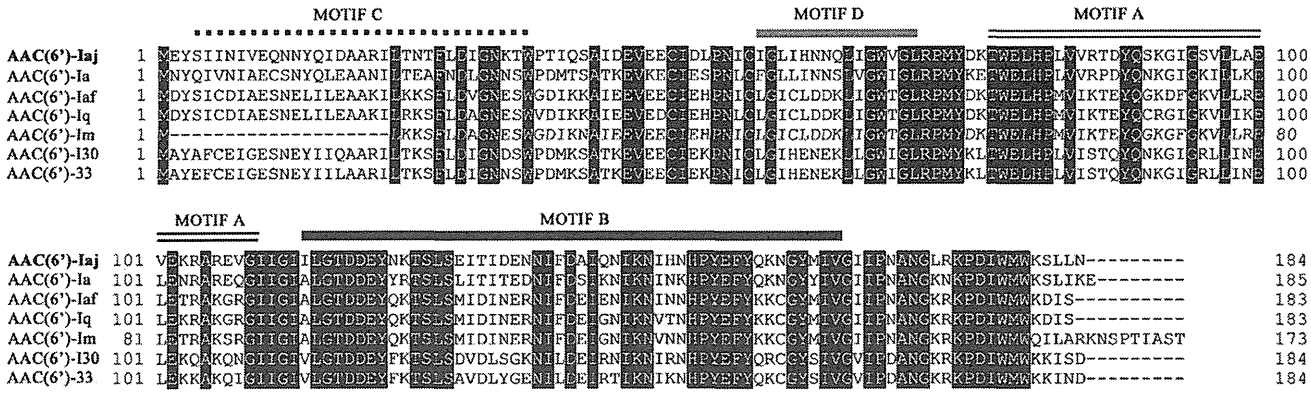

FIG 1 Alignment of the AAC(6′)-Iaj amino acid sequence with those of six members of AAC(6′)-I subfamily. Identical residues are marked with black boxes. Four motifs, C, D, A, and B, are indicated by a dotted line, a gray line, double lines, and a black line, respectively.

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 IS*6100*; i.e., In151 had the 5′-CS, 3′-CS, and a complete copy of IS*6100* 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

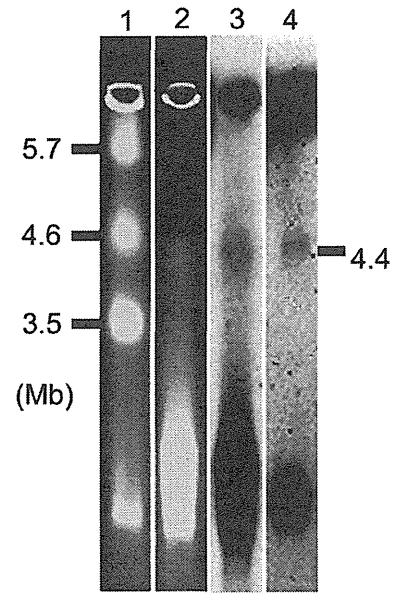

FIG 2 Localization of the *aac(6′)-Iaj* gene on I-CeuI-digested total DNA of *P. aeruginosa* strain NCGM1588 separated by PFGE. *P. aeruginosa* NCGM1588 genomic DNA digested by I-CeuI was done as previously described (11). Lane 1, molecular standard of *Schizosaccharomyces pombe* chromosomal DNA; lane 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).

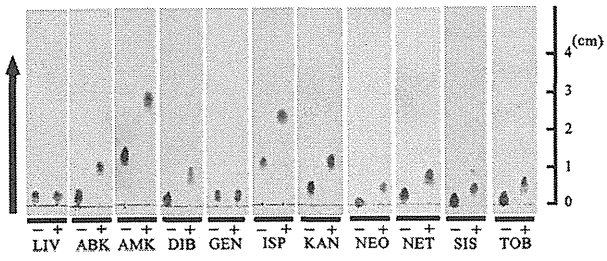

FIG 3 Analysis of acetylated aminoglycosides by TLC. AAC(6′)-Iaj and various aminoglycosides were incubated in the absence (−) or presence (+) of acetyl coenzyme A. The arrow indicates the direction of development.

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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