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Spread of Novel Aminoglycoside Resistance Gene *aac*(6′)-*Iad* among *Acinetobacter* Clinical Isolates in Japan

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A novel aminoglycoside resistance gene, *aac*(6′)-*Iad*, encoding aminoglycoside 6′-*N*-acetyltransferase, was identified in *Acinetobacter* genospecies 3 strain A-51. The gene encoded a 144-amino-acid protein, which shared modest identity (up to 36.7%) with some of the aminoglycoside 6′-*N*-acetyltransferases. The results of high-pressure liquid chromatography assays confirmed that the protein is a functional aminoglycoside 6′-*N*-acetyltransferase. The enzyme conferred resistance to amikacin, tobramycin, sisomicin, and isepamicin but not to gentamicin. The prevalence of this gene among *Acinetobacter* clinical isolates in Japan was then investigated. Of 264 *Acinetobacter* sp. strains isolated from geographically diverse areas in Japan in 2002, 16 were not susceptible to amikacin, and *aac*(6′)-*Iad* was detected in 7. Five of the producers of aminoglycoside 6′-*N*-acetyltransferase type Iad were identified as *Acinetobacter baumannii*, and two were identified as *Acinetobacter* genospecies 3. These results suggest that *aac*(6′)-*Iad* plays a substantial role in amikacin resistance among *Acinetobacter* spp. in Japan.

Acinetobacter spp., especially *Acinetobacter baumannii*, are emerging pathogens responsible for causing a variety of nosocomial infections, including pneumonia, urinary tract infections, and septicemia (1). Outbreaks have been increasingly reported in the past 2 decades, particularly from intensive care units, where patients undergo invasive procedures and receive broad-spectrum antimicrobial agents, resulting in higher mortality rates (5, 27). Furthermore, because *Acinetobacter* spp. have an ability to readily accept foreign DNA, including genetic determinants for antimicrobial resistance, so as to adapt to and survive in environments that are hazardous to bacterial growth (6, 17), they have a propensity for developing resistance to multiple classes of useful antimicrobial agents, including broad-spectrum cephalosporins, fluoroquinolones, and aminoglycosides (1).

Aminoglycosides are widely used to treat infections caused by gram-negative bacilli, including *Acinetobacter* spp. (1). However, resistance rates to classic aminoglycosides such as gentamicin and kanamycin are now high among *Acinetobacter* spp. in many geographic regions (15). The mechanisms of *Acinetobacter* sp. resistance to newer semisynthetic aminoglycosides such as amikacin, tobramycin, sisomicin, and isepamicin are diverse and commonly involve production of aminoglycoside-modifying enzymes such as aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT, or AAD), and/or aminoglycoside phosphotransferases (APH). Production of AAC(3)-I, APH(3′)-VI, and ANT(3″)-I was reported to be predominant by worldwide surveys on *Acinetobacter* spp., but there were considerable regional differences in their genotypes (14, 15, 21). In Japan, although the prevalence of amikacin resistance was estimated to be high, especially among non-carbapenem-susceptible *Acinetobacter* strains (25), the overall prevalence of aminoglycoside resistance and the mechanisms of resistance among *Acinetobacter* spp. have not been elucidated to date.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. In March 2002, 264 nonrepetitive strains identified as belonging to *Acinetobacter* spp. were collected from 88 hospitals located in geographically diverse areas in Japan. Among these, 16 strains (6.1%) that were not susceptible to amikacin (MICs, >16 μg/ml) by preliminary susceptibility testing were selected for further study. Species identification was carried out with API 20NE (bioMérieux Japan, Ltd., Tokyo, Japan) complemented by a carbon source utilization test and growth at 41 and 44°C (2). *Escherichia coli* XL1-Blue was used as the host for cloning experiments with vector pBCSK+ (Stratagene, La Jolla, Calif.). *E. coli* BL21(DE3)pLysS was used with vector pET29a(+) (Novagen, Madison, Wis.) for expression of *aac*(6′)-*Iad*. The strains were grown in Luria-Bertani (LB) broth or medium (Becton Dickinson Diagnostic Systems, Sparks, Md.) supplemented with appropriate antimicrobial agents, unless described otherwise.

Antimicrobial agents and susceptibility testing. Antimicrobial agents were obtained from the following sources: amikacin, Bristol Pharmaceuticals K. K., Tokyo, Japan; arbekacin, kanamycin, ribostamycin, and streptomycin, Meiji Seika Kaisha Ltd., Tokyo, Japan; chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; gentamicin and sisomicin, Schering-Plough K. K., Osaka, Japan; isepamicin, Asahi Kasei Corporation, Tokyo, Japan; neomycin, Nippon Kayaku Co., Ltd., Tokyo, Japan; rifampin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; tobramycin, Shionogi Pharmaceutical Co., Osaka, Japan.

MICs were determined by the agar dilution method with Mueller-Hinton agar (Becton Dickinson Diagnostic Systems) according to the protocol recommended by the National Committee for Clinical Laboratory Standards (16).

Transfer of aminoglycoside resistance genes. Conjugation experiments were conducted by using rifampin-resistant *E. coli* CSH2 and *Acinetobacter calcoaceticus* DU1, a rifampin-resistant derivative of *A. calcoaceticus* ATCC 33305, as the recipients by the broth mating method (7). Transconjugants were selected on LB agar supplemented with rifampin (50 μg/ml) and kanamycin (10 μg/ml).

Cloning and sequencing of the aminoglycoside resistance gene. The genomic DNA of *Acinetobacter* genospecies 3 strain A-51 was partially digested with Sau3AI, and the resultant fragments were ligated to the BamHI-cleaved cloning site of plasmid vector pBCSK+ (Stratagene). Electrocompetent *E. coli* XL1-Blue was transformed with these recombinant plasmids carrying total-DNA restriction fragments of various sizes prepared from the aminoglycoside-resistant

TABLE 1. Susceptibilities of *Acinetobacter* spp. and *E. coli* strains with *aac(6′)-Iad* to various aminoglycosides

Strain	Hospital	Specimen	MIC (μg/ml) of the following aminoglycoside[a]:								
			KAN	TOB	AMK	ABK	GEN	SISO	ISP	NEO	STR
Acinetobacter genomic species 3, strain A-51	A	Sputum	>1,024	>1,024	1,024	1,024	>1,024	>1,024	>1,024	64	>1,024
A. baumannii A-67	B	Urine	>1,024	64	128	32	8	1,024	256	8	256
A. baumannii A-74	B	Pus	>1,024	512	128	32	8	512	256	8	256
A. baumannii A-87	C	Sputum	512	128	32	16	4	256	256	4	256
A. baumannii A-88	C	Sputum	256	64	128	32	4	128	128	8	256
Acinetobacter genomic species 3, strain A-178	D	Sputum	128	16	32	8	1	64	64	1	64
A. baumannii A-260	E	Sputum	512	256	128	16	4	256	128	8	128
E. coli XL1-Blue(pA51S3)			256	64	128	16	1	64	64	4	4
E. coli XL1-Blue(pA51SG5)			512	32	1	0.13	32	32	0.13	0.25	2
E. coli XL1-Blue(pBCSK+)			0.5	0.25	0.5	0.13	0.13	0.13	0.25	0.25	1

[a] KAN, kanamycin; TOB, tobramycin; AMK, amikacin; ABK, arbekacin; GEN, gentamicin; SISO, sisomicin; ISP, isepamicin; NEO, neomycin; STR, streptomycin.

strain. Transformants were selected by their resistance to chloramphenicol (30 μg/ml) and kanamycin (25 μg/ml). The enzymes used for gene manipulation were purchased from New England Biolabs, Inc. (Beverly, Mass.), or TAKARA Bio, Inc. (Ohtsu, Japan). The DNA sequences were determined on both strands by using BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequence analyzer (Applied Biosystems, Foster City, Calif.). Alignments of nucleotide and amino acid sequences were performed with the GENETYX-MAC computer program (version 10.1.1; Software Development Co., Ltd., Tokyo, Japan).

Purification of the acetyltransferase. For use in N-terminal sequencing and high-pressure liquid chromatography (HPLC) assays, AAC(6′)-Iad was purified by using a histidine tag purification system. The entire coding region of *aac(6′)-Iad* and its upstream sequence were amplified by PCR with primers AAC-F (5′-GCT CTA GAA GAC TGA CTT CGC ATT G-3′) and AAC-R (5′-CCC AAG CTT GAG CTG CTT TGT AAA AC-3′). The product was double digested with XbaI and HindIII and then ligated with pET29a(+) (Novagen) digested with the same enzymes. Electrocompetent *E. coli* XL1-Blue was transformed with the recombinant plasmids, and transformants were selected on LB agar containing kanamycin (25 μg/ml). Several of the colonies obtained were found to harbor plasmids with inserts encoding AAC(6′)-Iad tagged with six histidine residues at the C-terminal end. *E. coli* BL21(DE3)pLysS (Novagen) was transformed with one such plasmid, pA51H7. The transformants were cultured in 1 liter of LB broth supplemented with kanamycin (25 μg/ml) to an A_{620} of approximately 0.7. The pellet was washed once with 50 mM phosphate buffer (pH 7.0) and suspended in 20 mM phosphate buffer (pH 7.4) containing 10 mM of imidazole. The suspension was passed twice through a French pressure cell (Ohtake Works Co., Ltd., Tokyo, Japan) at 120 MPa and then centrifuged at 30,000 × *g* for 30 min. Histidine-tagged AAC(6′)-Iad contained in the supernatant was purified by using HiTrap Chelating HP, included in the HisTrap kit (Amersham Biosciences, K. K., Tokyo, Japan), according to the manufacturer's instructions. It was eluted at an imidazole concentration of 300 mM and was estimated to be more than 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Finally, the enzyme was dialyzed twice at 4°C against 500 volumes of 50 mM phosphate buffer (pH 7.4) and was stored in aliquots at −80°C until use. N-terminal sequencing of the purified enzyme was performed by Shimadzu Corporation (Kyoto, Japan).

Preparation of crude extracts. As positive controls for acetylation reactions and HPLC assays, the following strains were used: AAC(2′)-producing *Streptomyces lividans* TK21/pANT12-1′, AAC(3)-producing *S. lividans* TK21/pANT3-1, and AAC(6′)-producing *S. lividans* TK21/pANTS-2 (8). They were cultured in 100 ml of TS medium containing 10 μg of ribostamycin/ml and 10 μg of thiostrepton/ml (Sigma-Aldrich Japan K. K., Tokyo, Japan) for 48 h. The cells were then harvested, washed once with 50 mM phosphate buffer (pH 7.0), and suspended in the same buffer. The suspension was passed twice through a French pressure cell (Ohtake Works) at 120 MPa and then centrifuged at 30,000 × *g* for 30 min. The supernatant was used as the crude enzyme.

Acetylation. Reaction mixtures for acetylation contained 25 μmol of Tris-hydrochloride buffer (pH 7.6), 7.5 μmol of $MgCl_2$, 200 nmol of acetyl coenzyme A (acetyl-CoA), and 50 μmol of either tobramycin or neomycin in a final volume of 500 μl. Acetylation was initiated by adding 50 μl of the enzyme and was carried out at 37°C for 30 min. *ortho*-Phthalaldehyde derivatization was then performed by adding equal volumes of 2-propanol and the derivatization reagent to the reaction mixture and heating at 60°C for 10 min. The derivatization reagent consisted of 80 mM *o*-phthalaldehyde, 1 M boric acid, and 250 mM thioglycolic acid with the pH adjusted to 10.4 with 40% potassium hydroxide.

HPLC assay. HPLC was performed to identify the site of acetylation of substrate aminoglycosides according to the methods described by Lovering et al. (12). The system consisted of a Separations module 2690 (Waters Corporation, Milford, Mass.), a Dual λ absorbance detector set at 330 nM (Waters), and a Chemcobond 5-ODS-H column (4.6 by 100 mm; Chemco Scientific Co., Ltd., Osaka, Japan). The mobile phase consisted of methanol-water-acetic acid (61.25:33.75:5) plus 5 g of 1-heptanesulfonic acid sodium salt per liter at a flow rate of 2 ml/min.

PCR amplification. PCR analysis was performed for the 16 non-amikacin-susceptible *Acinetobacter* strains with primers ABA-F (5′-TTT GGC TAT GAT CCT ATG-3′) and ABA-R (5′-CAT GTC GAA CAA GTA CGC-3′) to amplify an internal fragment of the *aac(6′)-Iad* gene. The conditions used have been described previously (7). When amplicons were obtained, they were directly sequenced with the same primers.

Nucleotide sequence accession number. The nucleotide sequence of *aac(6′)-Iad* will appear in GenBank under accession no. AB119105.

RESULTS

Prevalence and resistance profile of *Acinetobacter* strains with *aac(6′)-Iad*. Of the 16 non-amikacin-susceptible *Acinetobacter* strains included in this study, 7 were PCR positive for *aac(6′)-Iad*. Five were phenotypically identified as *A. baumannii*, whereas the remaining two were identified as *Acinetobacter* genospecies 3. When the amplicons were sequenced, all were identical to *aac(6′)-Iad*. The MICs of aminoglycosides for *Acinetobacter* strains possessing *aac(6′)-Iad* are shown in Table 1. All the strains studied were resistant to kanamycin, amikacin, tobramycin, sisomicin, isepamicin, and streptomycin. In addition, strain A-51 was resistant to all of the aminoglycosides tested, including arbekacin, gentamicin, and neomycin.

Molecular characterization of aminoglycoside resistance genes. Several transformants were obtained by selection with kanamycin and chloramphenicol. When these colonies were inoculated onto plates containing either amikacin (5 μg/ml) or gentamicin (5 μg/ml), they grew only on one or the other plate. The colonies on the plates containing amikacin or gentamicin were found to harbor recombinant plasmids of various sizes with inserts originating from the genomic DNA of strain A-51. Among these, the smallest plasmids (pA51S3 from an amikacin-resistant colony and pA51SG5 from a gentamicin-resistant colony) were selected out for further study. The MICs of aminoglycosides for *E. coli* XL1-Blue(pA51S3) and XL1-Blue (pA51SG5) are listed in Table 1. pA51S3 conferred resistance to kanamycin, amikacin, tobramycin, sisomicin, and isepami-

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AAC(6')-Iad              MIRKATVQDPPLLARLAMNVWKESSLKELVAEFEQMTKSND---AVAFILFIED   51
AAC(6')-Ic               MIVICDHDNLDAWLALRTALWPSGSPEDHRAEMREILASPH---HTAFMARGLD   51
AAC(6')-Id               MIEACHSVECPGWLQLRFLLWPQDSADEHLAEMAIFVAEPNR--FAQFIAYDEA   52
AAC(6')-If                   MDEASLSMWVGLRSQLWPDHSYEDHILDSQHILSCPDK--YVSFLAINNQ   48
AAC(6')-Ig             MNIKPASEASLKDWLELRNKLWS-DSEASHLQEMHQLLAEKY---ALQLLAYSD-   50
AAC(6')-Ih             MNIMPISESQLSDWLALRCLLWP-DHEDVHLQEMRQLITQAH---RLQLLAYTDT   51
AAC(6')-Ij             MNIMPVSESLMADWLGLRKLLWP-DHDEAHLQEMQRLLQQTQ---SLQLLAYSDT   51
AAC(6')-Ik             MNIKPASEASLKDWLKLRIKLWN-DLEESHLQEMHQLLAEKH---ALQLLVYSD-   50
AAC(6')-Il          MDSSPLVRPVETTDSASWLSMRCELWPDGTCQEHQSEIAEFLSGKVARPAAVLIAVAPD   59
AAC(6')-Ir             MKIMPVSEPFLADWLQLRILLWP-DHEDAHLLEMRQLLEQPH---TLQLLSYNDQ   51
AAC(6')-Is             MNIMPISESQLSDWLALRSLLWP-DHEDAHLLEMRHVLKQTD---TLQLLVYSET   51
AAC(6')-It             MHIMPITESQLSDWLVLRCLLWP-DHEDADLQEMRQLITQAH---CLQLLAYTNT   51
AAC(6')-Iu             MNILPISESQLSDWLALRSLLWP-DHEEAHLQEMRQLLKQTD---TLQLLAYSET   51
AAC(6')-Iv             MKIMPISESQLSDWLVLRCLLWP-DHEEQHLQEMRQLITQAH---CLQLLAYTDT   51
AAC(6')-Iw             MKIMPISEALLADWLQLRILLWP-DHEDAHLLEMRQLLTRTD---SLQLLAYSET   51
AAC(6')-Ix             MNIMPISESQLSDWLALRSLLWP-DHDDAHLLEMHQLLKQTD---TLQLLAYTDS   51
AAC(6')-Iy             MDIRQMNKTHLEHWRGLRKQLWPGHPDDAHLADGEEILQA-DH--LASFIAMADG   52
AAC(6')-Iz          MIASAPTIRQATPADAAAWAQLRLGLWP--DADDPLEELTQSLADAE---GAVFLACAAD   55
                                          :   :*          :               ::

AAC(6')-Iad          -QAVGFAQCQLRHDYVEGTNTSPVGYLEGIFVEKEFRHRGYASELLLKCEDWVKTKGCLQ  110
AAC(6')-Ic           GAFVAFAEVALRYDYVNGCESSPVAFLEGIYTAERARRQGWAARLIAQVQEWAKQQGCSE  111
AAC(6')-Id           NKPLGFVEAALRSDYVNGTNSSPVAFLEGVYVLPEARRRGIAHALVGAVEIWARNRACTE  111
AAC(6')-If           SQAIAFADAAVRHDYVNGCESSPVVYLEGIFVIPEQRGHGVAKLLVAAVQDWGVAKGCTE  108
AAC(6')-Ig           HQAIAMLEASIRFEYVNGTETSPVGFLEGIYVLPAHRRSGVATMLIRQAEVWAKQFSCTE  110
AAC(6')-Ih           QQAIAMLEASIRYEYVNGTQTSPVAFLEGIFVLPEYRRSGIATGLVQQVEIWAKQFACTE  111
AAC(6')-Ij           QQAIAMLEASIRYEYVNGTQTSPVAFLEGIYVLPDYRRSGIATHLVQQVEAWAKPFGCIE  111
AAC(6')-Ik           DHAVGMLEASIRYEYVNGTETSPVAFLEGIYVLPEYRRLGVATLLVRQVEAWAKQFSCTE  110
AAC(6')-Il           GEALGFAELSIR-PYAEECYSGNVAFLEGWYVVPSARRQGVGVALVKAAEHWARGRGCTE  118
AAC(6')-Ir           QQAVAMLEASIRYEYVNGQQSSPVAFLEGIYVLPEYRRLGVASTLVQQVEHWAKQFACTE  111
AAC(6')-Is           QLAIAMLEASIRHEYVNGTQTSPVAFLEGIYVLPEYRRSGIATQLVQCVEEWAKQFACTE  111
AAC(6')-It           QKAIGMLEASIRYEYVNGTQTSPVAFLEGIYVLPEYRRSGIATGLVQHVEIWAKQFACTE  111
AAC(6')-Iu           QHAIAMLEASIRHEYVNGTQTSPVAFLEGIYVLPEYRRSGIATQLVQCVEEWAKQFACTE  111
AAC(6')-Iv           QQAIAMLEASIRYEYVNGTQTSPVAFLEGIYVLPEYRRSGIATGLVQHVEIWAKQFSCTE  111
AAC(6')-Iw           QQPIAMLEASIRHEYVNGTQTSPVAFLEGIYVLPEHRRSGIATQLVQQVEQWAKQYACTE  111
AAC(6')-Ix           QQAVAMLEASIRHEYVNGTQTSPVAFLEGIYILPEYRRSGIATQLVQYVEEWAKQFACTE  111
AAC(6')-Iy           -VAIGFADASIRHDYVNGCDSSPVVFLEGIFVLPSFRQRGVAKQLIAAVQRWGTNKGCRE  111
AAC(6')-Iz           GETVGFAEVRLRHDYVNGTESSPVGFLEGWYVQPQWQGSGVGRALLAAVQAWTRDAGCRE  115
                        :.: :  :*  *.:   :. * :*** :     :  * .  *:   : *    .* :

AAC(6')-Iad          FASDCELDNIDSLAFHLKVGFTEANRMICFTKQL                           144
AAC(6')-Ic           LASDTDIANLDSQRLHAALGFAETERVVFYRKTLG                          146
AAC(6')-Id           FASDASTDNPESHRFHQSLGFKETERVVYFRKMLAPE                        149
AAC(6')-If           MASDAALDNHISYQMHQALGFEETERVVFFRKRIAG                         144
AAC(6')-Ig           FASDAALDNVISHAMHRSLGFQETEKVVYFSKKID                          145
AAC(6')-Ih           FASDAALDNQISHAMHQALGFHETERVVYFKKNIG                          146
AAC(6')-Ij           FASDAALDNRISHAMHQALGFHETERVVYFKKHIG                          146
AAC(6')-Ik           FASDAALDNVISHAMHRALGFQETERVVYFSKKID                          145
AAC(6')-Il           FASDTQLTNSASTSAHLAAGFTEVAQVRCFRKPL                           152
AAC(6')-Ir           FASDAALDNTISHAMHRALGFQETECVVYFKKNIS                          146
AAC(6')-Is           FASDAALENTISHAMHRALGFHETERVVYFKKNIG                          146
AAC(6')-It           FASDATLDNQISHAMHRALGFHETERVVYFKKNIG                          146
AAC(6')-Iu           FASDAALDNTISHAMHRALGFHETERVVYFKKNIS                          146
AAC(6')-Iv           FASDAALDNQITHAMHQALGFQETERVVYFKKNIG                          146
AAC(6')-Iw           FASDAAIDNTISHAMHQALGFHETERVVYFKKNIS                          146
AAC(6')-Ix           FASDAAIDNTISHAMHRALGFHETERVVYFKKNIG                          146
AAC(6')-Iy           MASDTSPENTISQKVHQALGFEETERVIFYRKRC                           145
AAC(6')-Iz           LASDSRVEDVQAHAAHRACGFEETERVVYFRMPLEPSA                       153
                     :***    :  :   *   ** *.  :  :
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FIG. 1. Alignment of the deduced amino acid sequences of AAC(6′)-Iad and other aminoglycoside acetyltransferases, including AAC(6′)-Ic (GenBank accession no. M94066), AAC(6′)-Id (X12618), AAC(6′)-If (X55353), AAC(6′)-Ig (L09246), AAC(6′)-Ih (L29044), AAC(6′)-Ij (L29045), AAC(6′)-Ik (L29510), AAC(6′)-Il (Z54241, U13880), AAC(6′)-Ir (AF031326), AAC(6′)-Is (AF031327), AAC(6′)-It (AF031328), AAC(6′)-Iu (AF031329), AAC(6′)-Iv (AF031330), AAC(6′)-Iw (AF031331), AAC(6′)-Ix (AF031332), AAC(6′)-Iy (AF144880), and AAC(6′)-Iz (AF140221). Asterisks indicate identical amino acids. Conservative amino acid substitutions are indicated by dots.

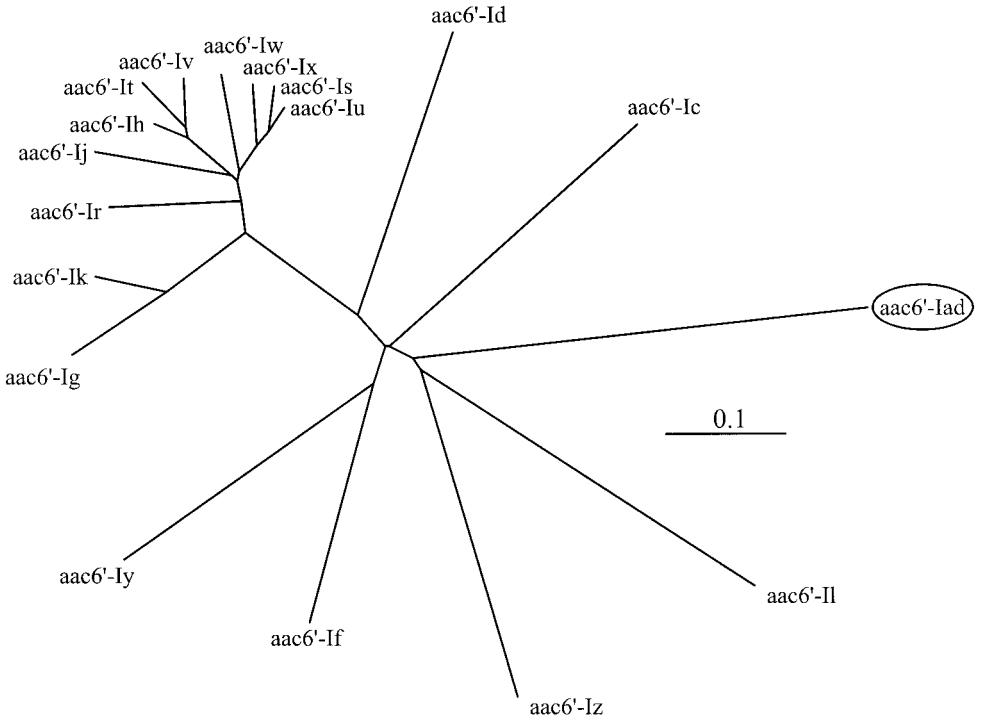

FIG. 2. Dendrogram for aminoglycoside 6′-*N*-acetyltransferases belonging to the subfamily represented by AAC(6′)-Ic. The dendrogram was calculated by the ClustalW computer program, available on the National Institute of Genetics website (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html), and illustrated with the TreeViewPPC computer program (version 1.6.5 for Macintosh). Branch lengths correspond to the numbers of amino acid exchanges.

cin, while pA51SG5 conferred resistance to kanamycin, gentamicin, tobramycin, and sisomicin. Neither plasmid conferred resistance to streptomycin or neomycin.

pA51S3 contained a 1.0-kb insert with one open reading frame, though several possible start codons were recognized. Therefore, N-terminal sequencing of the purified protein was carried out. Consequently, it was confirmed that the open reading frame encodes 144 amino acids and has a G+C content of 36.1%. The deduced amino acid sequence displayed the highest identity with that of AAC(6′)-Iy (36.7%) (13). It also showed moderate identities with the sequences of other aminoglycoside acetyltransferases [35.2% with AAC(6′)-If, 34.6% with AAC(6′)-Ic, 33.3% with AAC(6′)-Iz, and 29.7% with AAC(6′)-Il] (3, 11, 23, 26). The motifs that are conserved among the aminoglycoside 6′-*N*-acetyltransferases (24) were also found in the newly identified enzyme. This novel aminoglycoside acetyltransferase gene was thus designated *aac*(*6′*)-*Iad*. The deduced amino acid sequence of AAC(6′)-Iad is shown in Fig. 1, along with those of known aminoglycoside acetyltransferases. The dendrogram of phylogenetic relationships among aminoglycoside acetyltransferases is shown in Fig. 2. The 1.1-kb insert of pA51SG5 contained an aminoglycoside (2′) adenylyltransferase gene, *ant*(*2″*)-*Ia* (4).

Identification of site of modification. The results of HPLC assays are shown in Table 2. The retention times of *o*-phthalaldehyde derivatives of tobramycin and neomycin after the acetylation reaction with AAC(6′)-Iad coincided only with those of positive controls for AAC(6′), confirming that AAC(6′)-Iad is a functional acetyltransferase and modifies position 6′ of aminoglycosides.

Transfer of aminoglycoside resistance. The amikacin resistance determinant of *A. baumannii* A-67 and A-74 could be transferred to the recipient *A. calcoaceticus* DU1 by conjugation at a frequency of approximately 5×10^{-4} to 1×10^{-3} and was confirmed by PCR to be *aac*(*6′*)-*Iad*. It was not transferred to *E. coli* CSH2. For the rest of the strains, amikacin resistance was not transferable to *A. calcoaceticus* DU1 or *E. coli* CSH2. The DNA probes for detection of *aac*(*6′*)-*Iad* hybridized with the large plasmids (>50 kb) harbored by all seven strains (data not shown).

DISCUSSION

A variety of aminoglycoside 6′-*N*-acetyltransferase genes from *Acinetobacter* species have been described to date (Fig. 2). *aac*(*6′*)-*Ib* and *aac*(*6′*)-*Ih* have been identified previously as

TABLE 2. Retention times of aminoglycoside modification products after acetylation reactions

Aminoglycoside acetyltransferase	Retention time (min) of aminoglycoside modification product			
	Tobramycin		Neomycin	
	With acetyl-CoA	Without acetyl-CoA	With acetyl-CoA	Without acetyl-CoA
AAC(6′)-Iad	3.3	17.0	4.9	11.8
Positive controls				
AAC(6′)	3.3	16.9	4.9	11.8
AAC(2′)	11.3	16.9	10.5	11.9
AAC(3)	4.4	16.9	6.7	11.9

the most prevalent plasmid-mediated *aac*(6′)-*I* genes among *A. baumannii* strains (18), while other genes have been associated with specific species. *aac*(6′)-*Ig* is specific to *Acinetobacter haemolyticus* (10), whereas *aac*(6′)-*Ij* and *aac*(6′)-*Ik* are specific to *Acinetobacter* genospecies 13 and 6, respectively (9, 19). *aac*(6′)-*Ir*, *aac*(6′)-*Is*, *aac*(6′)-*It*, *aac*(6′)-*Iu*, *aac*(6′)-*Iv*, *aac*(6′)-*Iw*, and *aac*(6′)-*Ix* have also been described for various *Acinetobacter* species (20). However, *aac*(6′)-*Iad* demonstrated considerable phylogenetic distance from these aminoglycoside-modifying enzymes (as shown in Fig. 2), suggesting the emergence of a novel subgroup of aminoglycoside 6′-*N*-acetyltransferases.

In the present study, we report identification of a novel aminoglycoside 6′-*N*-acetyltransferase gene, *aac*(6′)-*Iad*, in seven clinical isolates belonging to *A. baumannii* and *Acinetobacter* genospecies 3. The spectrum of resistance conferred by the gene product included kanamycin, tobramycin, amikacin, isepamicin, and sisomicin, a pattern typical of AAC(6′)-I (22). Preliminary sequencing results suggest that *aac*(6′)-*Iad* is located on a transposon (data not shown); in view of this possibility, along with the fact that the gene is transferable by conjugation in some of the producers of the enzyme, it is likely that *aac*(6′)-*Iad* is carried by a plasmid.

Three subgroups have been identified among aminoglycoside 6′-*N*-acetyltransferases (22). AAC(6′)-Iad is closest to the largest subfamily, which contains the proteins mentioned above as identified in *Acinetobacter* species, but the amino acid sequence identity between AAC(6′)-Iad and these proteins is limited (≤36.7%) (Fig. 1). Considering the low G+C content (36.1%) of *aac*(6′)-*Iad* for *Acinetobacter* species, we may speculate that the gene was acquired from some environmental species with an intrinsically low G+C content.

PFGE of the seven strains that produce AAC(6′)-Iad showed five distinct digestion patterns, except for those isolated from the same hospital (data not shown). Taken together, it is likely that *aac*(6′)-*Iad* was disseminated among *Acinetobacter* spp. via plasmid- and transposon-mediated lateral transfer, which is now responsible for reduced susceptibility to amikacin among *Acinetobacter* spp. in nearly half of the cases (7 out of 16 non-amikacin-susceptible strains) in Japan.

When the susceptibilities of the AAC(6′)-Iad producers to other classes of antimicrobial agents were tested, we found that none were susceptible to ceftazidime, moxalactam, or aztreonam, and two were resistant to ciprofloxacin as well. Only imipenem and meropenem were uniformly effective in vitro among the agents tested. The emergence and spread of plasmid-mediated *aac*(6′)-*Iad* genes could contribute to further acquisition of a multidrug-resistant phenotype among *Acinetobacter* spp. in Japan, thus limiting the treatment options in clinical settings in the near future.

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