TABLE 1. Results of antibiotic susceptibility testing

β-Lactam	MIC (μg/ml)			
	E. coli HKY28	*E. coli* XL1-Blue(pBE28 W)	*E. coli* XL1-Blue(pBE28 R)	*E. coli* XL1-Blue
Amoxicillin	128	>128	>128	4
Amoxicillin-clavulanate[a]	128	>128	>128	4
Ampicillin	>128	>128	>128	2
Ampicillin-sulbactam[b]	32	64	>128	2
Piperacillin	8	8	8	0.5
Piperacillin-tazobactam[c]	4	2	4	0.5
Cefotaxime	16	32	8	0.06
Cefotaxime-clavulanate[a]	8	8	2	0.06
Cefotaxime-sulbactam[b]	1	1	2	0.06
Cefotaxime-tazobactam[c]	2	4	4	0.06
Ceftazidime	32	128	16	0.06
Ceftazidime-clavulanate[a]	16	32	8	0.13
Ceftazidime-sulbactam[b]	4	8	8	0.06
Ceftazidime-tazobactam[c]	16	8	8	0.13
Cephaloridine	64	128	128	4
Cefminox	32	32	32	0.5
Cefoxitin	16	32	>128	8
Cefmetazole	16	32	128	1
Moxalactam	8	4	8	0.25
Cefpirome	2	4	0.03	0.015
Cefepime	2	4	0.03	0.015
Aztreonam	8	16	16	0.06
Imipenem	0.13	0.13	0.13	0.13

[a] Fixed concentration of clavulanate, 4 μg/ml.
[b] Fixed concentration of sulbactam, 4 μg/ml.
[c] Fixed concentration of tazobactam, 4 μg/ml.

for the revertant clone. On the other hand, the degree of resistance to cefoxitin and cefmetazole conferred by the revertant clone was significantly higher than that conferred by the HKY28 clone. When various β-lactam–β-lactamase inhibitor combinations were tested, the piperacillin, cefotaxime, and ceftazidime MICs for the HKY28 clone were reduced by up to 16-fold. The degree of reduction was the greatest when sulbactam was used as the inhibitor. The reductions in the MICs of the three inhibitors for the revertant clone were fourfold or less.

Isoelectric focusing. The isoelectric points were estimated to be 9.9 for the HKY28 AmpC ($AmpC^D$) and 9.8 for the revertant AmpC ($AmpC^R$). When the crude extract of *E. coli* HKY28 was subjected to analytical isoelectric focusing, only one band corresponding to $AmpC^D$ was visualized with nitrocefin, confirming that $AmpC^D$ is the only β-lactamase produced by *E. coli* HKY28 (data not shown).

Enzyme assays. The specific activites of $AmpC^D$ and $AmpC^R$ were 88 and 220 U/mg of protein, respectively. The kinetic parameters (K_m and k_{cat}) and hydrolytic efficiencies (k_{cat}/K_m) of $AmpC^D$ and $AmpC^R$ against various β-lactams are given in Table 2. The k_{cat} values of $AmpC^D$ were greater than those of $AmpC^R$ for cefpirome and cefepime but lower for the rest of the substrates tested. However, for all substrates with the exception of cefotaxime, $AmpC^D$ exhibited lower K_m values than $AmpC^R$. This difference was approximately 100-fold for ceftazidime, and overall, $AmpC^D$ showed a 2.5-fold greater hydrolytic efficiency for ceftazidime than $AmpC^R$, despite the much poorer k_{cat}. $AmpC^D$ exhibited much lower K_m values and higher k_{cat} values for cefpirome and cefepime than $AmpC^R$, resulting in approximately 40- and 20-fold greater hydrolytic efficiencies, respectively.

The IC_{50}s of the β-lactamase inhibitors for $AmpC^D$ and $AmpC^R$ and the K_i values of the enzymes against the inhibitors are listed in Table 3. $AmpC^D$ exhibited approximately 5- to 10-fold lower K_i values than $AmpC^R$ against all three inhibitors. Tazobactam was the best inhibitor and had the lowest IC_{50} for $AmpC^D$.

TABLE 2. Kinetic activity of $AmpC^D$ and $AmpC^R$

Substrate	$AmpC^D$			$AmpC^R$		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
Cephaloridine	100 ± 10	64 ± 4	6.4×10^5	780 ± 40	300 ± 10	3.9×10^5
Ampicillin	6.2 ± 1.6	0.43 ± 0.06	7.0×10^4	13 ± 3	4.0 ± 0.3	3.2×10^5
Cefoxitin	1.2 ± 0.3	0.043 ± 0.006	3.8×10^4	3.9 ± 0.1	0.35 ± 0.01	9.1×10^4
Ceftazidime	5.7 ± 0.8	0.084 ± 0.006	1.5×10^4	550 ± 10	3.5 ± 0.1	6.4×10^3
Cefotaxime	31 ± 8	0.37 ± 0.02	1.2×10^4	13 ± 2	1.2 ± 0.1	9.7×10^4
Cefpirome	21 ± 1	1.5 ± 0.1	7.1×10^4	120 ± 20	0.21 ± 0.03	1.8×10^3
Cefepime	49 ± 5	1.0 ± 0.1	2.1×10^4	200 ± 40	0.21 ± 0.02	1.1×10^3

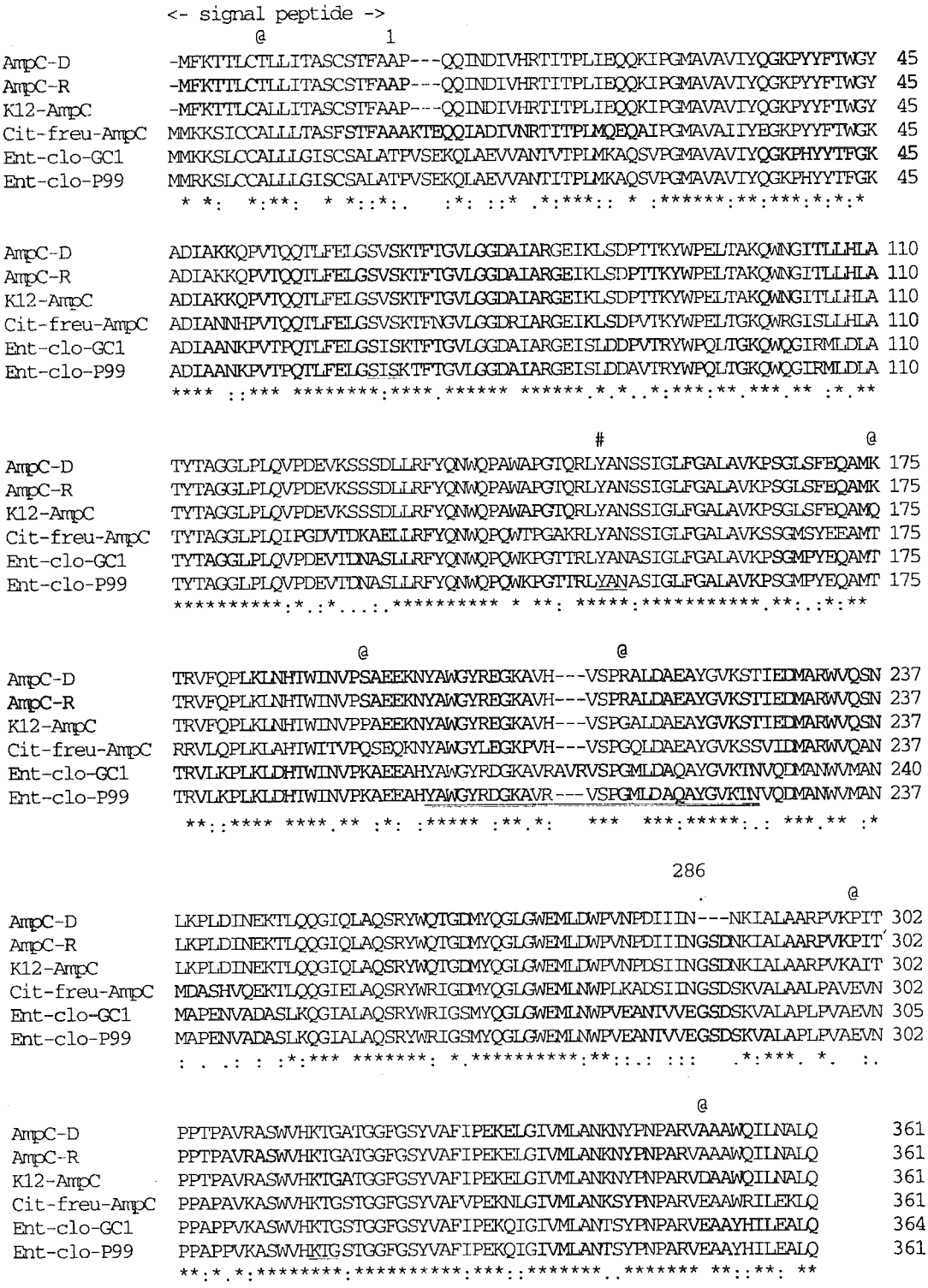


FIG. 1. Predicted amino acid sequence of the AmpC β-lactamase of *E. coli* HKY28 aligned with that of *E. coli* K-12 (10). The 3-amino-acid deletion in the HKY28 AmpC is shaded. Underlines, the β-lactamase active site SVSK, the conserved tripeptide KTG, and the class C motif YXN; #, position of Tyr150; @, positions of the amino acid substitutions observed between the AmpC[1] of strain HKY28 and the AmpC of strain K-12; numbers on the right, numbers of amino acid residues from the N terminus of each mature protein; *, amino acid residues conserved among the six AmpC-type enzymes; colons and dots, amino acid substitutions that result in homologous amino acid residues: Cit-freu, *Citrobacter freundii*; Ent-clo, *E. cloacae*; double underline, AmpC Ω-loop domain.

TABLE 3. IC_{50}s and K_i values of β-lactamase inhibitors for $AmpC^D$ and $AmpC^R$

β-Lactamase	Clavulanic acid		Sulbactam		Tazobactam	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
$AmpC^D$	19 ± 1	320 ± 30	3.9 ± 0.2	9.2 ± 0.2	1.4 ± 0.1	8.7 ± 2.4
$AmpC^R$	140 ± 20	4,100 ± 1,600	24 ± 4	780 ± 150	25 ± 1	1,100 ± 120

Molecular modeling study. A molecular modeling study was conducted to elucidate the mechanism for the lower K_m of $AmpC^D$ for ceftazidime (Fig. 2). In the AmpC of *E. coli* K-12, the tripeptide Gly286-Ser287-Asp288 loops out in the direction of ceftazidime (Fig. 2B). Conversely, the tripeptide deletion in $AmpC^D$ creates an open site in the vicinity of the R-2 side chain of ceftazidime (Fig. 2C). Similar models were obtained for cefpirome and cefepime (data not shown).

DISCUSSION

E. coli HKY28 produced an AmpC β-lactamase which conferred resistance to ceftazidime and reduced susceptibility to cefotaxime (MICs, 32 and 16 μg/ml, respectively). This resistance was significantly compromised by the β-lactamase inhibitors sulbactam and tazobactam and to some extent by clavulanic acid. This was an uncommon finding, since *E. coli* rarely acquires resistance to ceftazidime solely by the production of chromosomal β-lactamase. Also, the AmpC β-lactamase, which belongs to Ambler class C β-lactamases, is not usually inhibited well by β-lactamase inhibitors. We therefore investigated the AmpC β-lactamase of the strain.

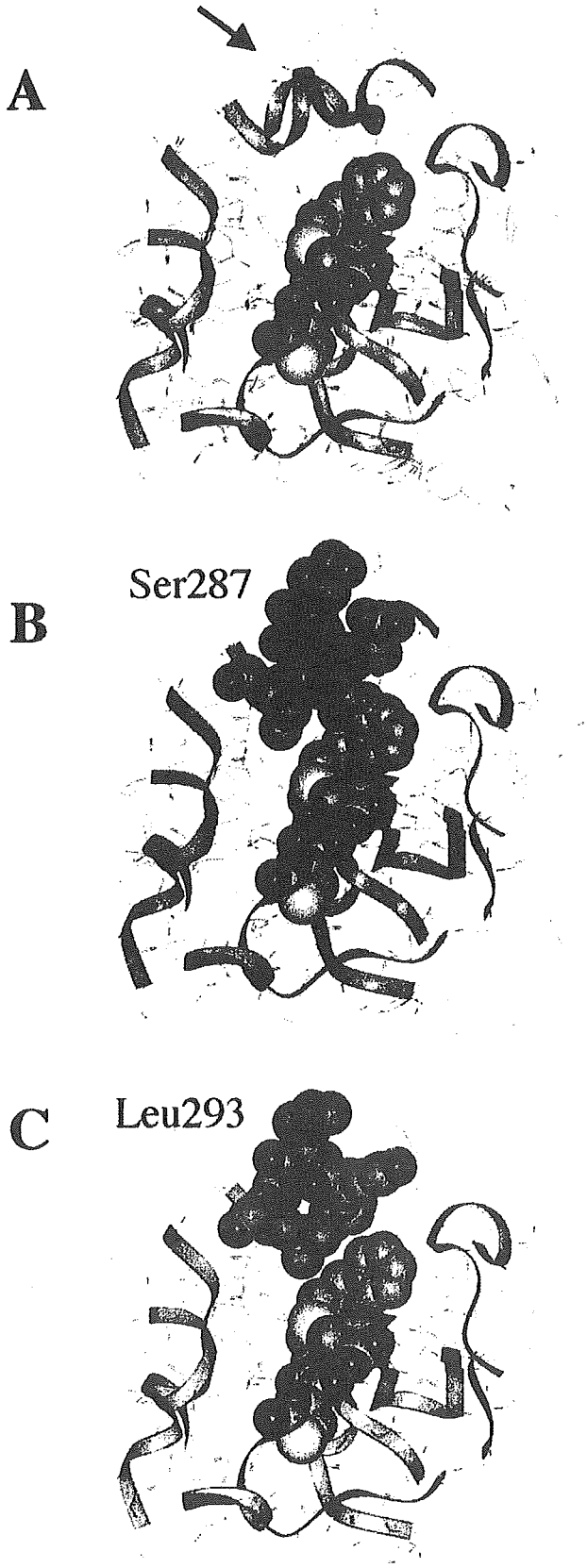
When the *ampC* gene was cloned and expressed in *E. coli* XL1-Blue, it conferred resistance to ceftazidime and cefotaxime, and the resistance could be reversed by any of the three commercially available β-lactamase inhibitors. Sulbactam and tazobactam were much more potent inhibitors in terms of lowering the MICs than clavulanic acid, a distinct profile compared with those of class A ESBLs, which are generally inhibited well by any of the three inhibitors (4).

Sequencing of the entire *ampC* structural gene of *E. coli* HKY28 revealed the presence of seven amino acid alterations and a tripeptide deletion at positions 286 to 288 corresponding to Gly-Ser-Asp in the deduced amino acid sequence of AmpC (Fig. 1). None of the substituted residues has been implicated in playing a functional role in the hydrolysis of β-lactams (23, 25). On the other hand, residues 287 to 289, which overlap the residues deleted from $AmpC^D$, is known to be positioned in close proximity to R-2 substituents of β-lactams (16). The levels of resistance to ceftazidime and cefotaxime were reduced by 4-fold or more, while those of newer oxyiminocephalosporins, such as cefepime and cefpirome, were also reduced by 64-fold for the revertant clone producing $AmpC^R$. The three β-lactamase inhibitors no longer reversed resistance to cefotaxime and ceftazidime in the revertant clone. $AmpC^R$ has a G214R substitution in the so-called Ω loop, and this substitution may have some influence on the expansion of substrate specificity, especially for cephamycins such as cefoxitin and cefmetazole. The kinetic values of $AmpC^R$ for broad-spectrum cephalosporins, including cefotaxime, ceftazidime, cefepime, and cefpirome, as well as cephamycins, such as cefoxitin, indicate that $AmpC^R$ certainly has some unusual properties. Some of the five amino acid substitutions found in $AmpC^R$ might contribute to such a phenotype. In addition, the three amino acid deletions at the H-10 domain observed in $AmpC^D$ might provide this enzyme with a special characteristic, such as enhanced susceptibility to β-lactamase inhibitors and an augmented ability to hydrolyze ceftazidime, cefepime, and cefpirome. However, the deletion might result in a decrease in the ability to hydrolyze cephamycins.

The results of the kinetics studies were very much in accordance with the susceptibility profiles. $AmpC^D$ generally exhibited lower K_m values than $AmpC^R$ against all substrates tested except cefotaxime. These reductions in K_m values were accompanied by compromised k_{cat} values, with the exception of those for cefpirome and cefepime. $AmpC^D$ showed both lower K_m values and greater k_{cat} values for these two agents, resulting in 40- and 20-fold better hydrolytic efficiencies, respectively, compared with those of $AmpC^R$. The kinetic data for cefotaxime did not correlate well with the MICs. A similar observation was reported for an atypical AmpC of an *Enterobacter cloacae* clinical isolate lacking 6 amino acids at positions 289 to 294, located adjacent to the deletion identified in $AmpC^D$ (2). By consideration of the fact that these data were obtained for two clones which differed only by the presence and the absence of the 3 amino acids in AmpC, one possibility is that $AmpC^D$ is unstable.

The results of inhibition studies confirmed the role of the Gly286-Ser287-Asp288 deletion in the increased sensitivity of $AmpC^D$ to all three commercially available β-lactamase inhibitors. The tripeptide deletion in $AmpC^D$ was shown to lower the K_i values against the inhibitors by approximately 10- to 100-fold. In terms of IC_{50}s, sulbactam and tazobactam were potent inhibitors of $AmpC^D$, whereas clavulanic acid only mildly inhibited the enzyme. The AmpC β-lactamase of *E. cloacae* P99 is inhibited well by tazobactam but is inhibited only modestly by sulbactam and is hardly inhibited at all by clavulanic acid (4). In this respect, $AmpC^D$ is an AmpC β-lactamase that is unusually sensitive, especially to sulbactam.

Gly286-Ser287-Asp288 is located in the H-10 helix of AmpC (16). While the functional roles of these residues in the catalytic mechanism have not been clearly elucidated, Asp288 of the *E. coli* AmpC has been suggested to play a role in recognizing the carboxylate group of β-lactams (23, 25). In native AmpC, Ser287 forms hydrogen bonds with Asn346 and Arg349 (23), but these bonds are lost in $AmpC^D$, along with the deletion of Asp288. The result of the molecular modeling study provided a structural explanation for the lowered K_m of $AmpC^D$ for ceftazidime, as shown in Fig. 2. In the *E. coli* K-12 AmpC, the tripeptide Gly286-Ser287-Asp288 impeded access of ceftazidime to the active site of the enzyme, resulting in high K_m values, whereas the tripeptide deletion in $AmpC^D$ was



found to provide an open site where the R-2 side chain of ceftazidime could readily be accommodated. This explains the significantly lower K_m for AmpC^D compared with that for AmpC^R.

While inducible chromosomal AmpC β-lactamases are known to confer resistance to oxyiminocephalosporins and cephamycins by mutations in their regulator genes that lead to derepressed production of the enzymes in many species of gram-negative bacteria, only a few AmpC enzymes with altered substrate specificities have been reported to date (17). The extended-spectrum AmpC produced by *E. cloacae* GC1 contained a tripeptide insertion of a tandem repeat, Ala211-Val212-Arg213, in the Ω loop (6, 20). It was suggested that the conformational flexibility in the expanded Ω loop facilitates hydrolysis of oxyiminocephalosporins (6). It is noteworthy that an AmpC with extended resistance to cefepime and cefpirome was recently described from an *E. cloacae* clinical isolate, as mentioned above (2). A deletion of 6 amino acids (Ser, Lys, Val, Ala, Leu, and Ala) from positions 289 to 294 was likely responsible for the extension of the spectrum of activity. The enzyme showed approximately 10 times higher hydrolytic efficiency for the oxyiminocephalosporins than the P99 β-lactamase did, mostly due to lower K_m values. This amino acid deletion is in close proximity to that in the AmpC^D studied here, both of which are located in the H-10 helix. Therefore, it is not surprising that the two enzymes share similar kinetic characteristics.

E. coli is known to constitutively produce only an insignificant amount of chromosomal AmpC β-lactamase, due to relatively weak promoter activity and the presence of a transcriptional attenuator (11, 13). However, occasional isolates produce large amounts of the enzyme and become resistant to various β-lactams, including ceftazidime. This overproduction could result from gene amplification (8) or the acquisition of a stronger promoter region (21, 22); but most commonly it results from mutations that take place in the promoter region at positions such as −42, −32, and +24, which lead to enhanced transcription of *ampC* (5, 19). These modifications in transcription typically lead to moderately elevated ceftazidime MICs (13). The nucleotide sequence of the promoter and attenuator regions of the *ampC* gene of *E. coli* HKY28 revealed the presence of three mutations (a C-to-T change at position −73, a C-to-T change at position +6, and a G-to-A change at position +34) and a T insertion between positions −14 and −13. The first two mutations have not been implicated as a cause of increased *ampC* transcription, while a nucleotide insertion between −35 and −10 hexamers is known to enhance AmpC transcription, possibly by bringing the distance between the hexamers to the optimal 17 bp (5, 11). Therefore, it is likely that the insertion of a T residue between positions −14 and

FIG. 2. Optimized ribbon structures of ceftazidime docked in the active site of HKY28 AmpC (gray) compared with that of *E. coli* K-12 AmpC (light blue) (10). (A) The two structures are superimposed. The Gly286-Ser287-Asp288 deletion in the HKY28 AmpC creates an open space at the top of the binding site (arrow) that allows the accommodation of the R-2 side chain of ceftazidime in the *E. coli* K-12 AmpC and the R-2 side chain of ceftazidime collides with Ser287(B), but in the HKY28 AmpC (C) it comes near Leu293 but does not make direct contact.

−13 caused the hyperproduction of AmpC[D], explaining in part the ceftazidime resistance displayed by *E. coli* HKY28.

Class A ESBLs are inhibited well by the β-lactamase inhibitors clavulanic acid and sulbactam, a characteristic that serves to differentiate them from other β-lactamases, including AmpC (15). The K_i values of class A enzymes for the inhibitors are in the nanomolar range, but those of the AmpC[D] enzyme reported in the present study are in the micromolar range. Anyway, AmpC[D] acquired considerable sensitivity to inhibition by sulbactam and tazobactam but acquired sensitivity to inhibition by clavulanic acid to a much lower degree, as it extended its spectrum to cephalosporins, including ceftazidime. A few other studies have also reported on the isolation of *E. coli* strains displaying similar inhibitor-sensitive phenotypes, but their mechanisms remain to be described (1, 26). It would be interesting to know if they produce AmpC variants with characteristics similar to those of the AmpC described in the present study.

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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 amjkacin, 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--PAQFIAYDEA   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.

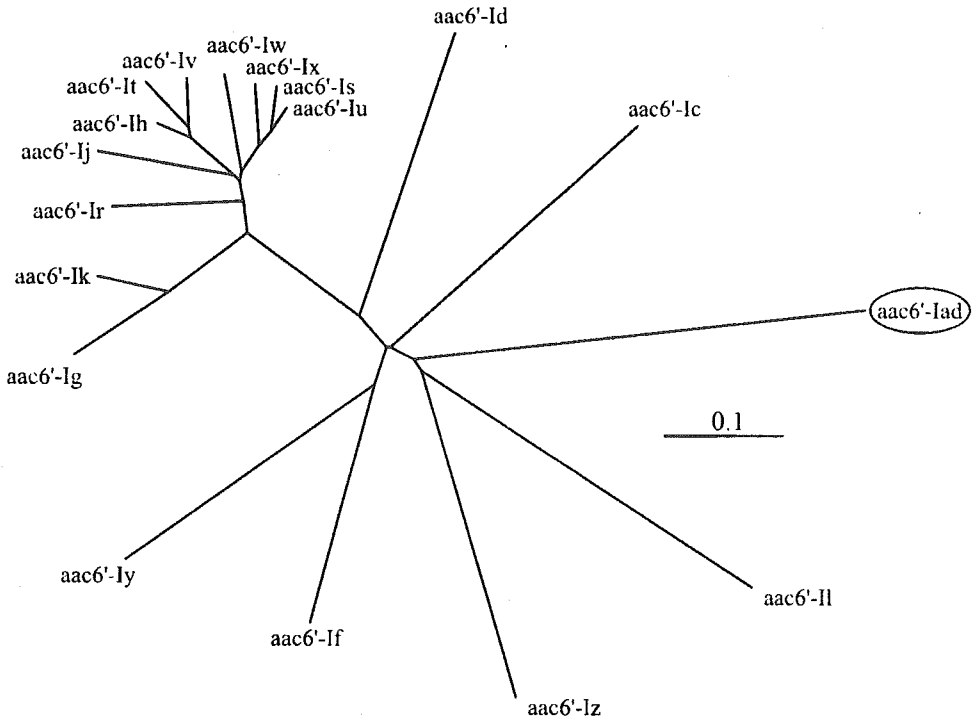

FIG. 2. Dendrogram for aminoglycoside 6′-*N*-acetyltransferases belonging to the subfamily represented by AAC(6′)-Ic. The dendrogram was calculated by the ClustalW computer program, available on the National Institute of Genetics website (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html), and illustrated 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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Genetic Environments of the *rmtA* Gene in *Pseudomonas aeruginosa* Clinical Isolates

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Nine *Pseudomonas aeruginosa* strains showing very high levels of resistance to various aminoglycosides have been isolated from clinical specimens in seven separate Japanese hospitals in five prefectures since 1997. These strains harbor the newly identified 16S rRNA methylase gene (*rmtA*). When an *rmtA* gene probe was hybridized with genomic DNAs of the nine strains digested with EcoRI, two distinct patterns were observed. The 11.1- and 15.8-kb regions containing the *rmtA* genes of strains AR-2 and AR-11, respectively, were sequenced and compared. In strain AR-2, a transposase gene-like sequence (sequence 1) and a probable tRNA ribosyltransferase gene (*orfA*) were located upstream of *rmtA*, and a Na^+/H^+ antiporter gene-like sequence (sequence 2) was identified downstream of *rmtA*. This 6.2-kbp insert (the *rmtA* locus) was flanked by 262-bp κγ elements. Part of the *orfQ* gene adjacent to an inverted repeat was found outside of the *rmtA* locus. In strain AR-11, the *rmtA* gene and sequence 2 were found, but the 5′ end of the *orfA* gene was truncated and replaced with IS*6100*. An *orfQ-orfI* region was present on each side of the *rmtA* gene in strain AR-11. The G+C content of the *rmtA* gene was about 55%, and since the newly identified *rmtA* gene may well be mediated by some mobile genetic elements such as Tn*5041*, further dissemination of the *rmtA* gene could become an actual clinical problem in the near future.

Pseudomonas aeruginosa is an important opportunistic pathogen that is capable of causing chronic and severe invasive diseases in critically ill and immunocompromised patients. Aminoglycosides are clinically effective agents for treating infections caused by *P. aeruginosa* as well as other gram-negative bacilli. However, multidrug resistance is rapidly emerging in *P. aeruginosa*, whose spectrum of resistance often includes aminoglycosides as well as broad-spectrum β-lactams and fluoroquinolones (15). The most frequently encountered molecular mechanism for aminoglycoside resistance in *P. aeruginosa* is the production of aminoglycoside-modifying enzymes such as plasmid-dependent acetyltransferase (AAC), adenylyltransferase (AAD), and phosphotransferase (APH) (6, 17, 23). Among these, production of AAC(6′)-II and AAD(2″)-I is the most common mechanism for resistance to aminoglycosides in *P. aeruginosa* (1), although ribosomal mutations also play some part in aminoglycoside resistance (20). Arbekacin, one of the semisynthetic aminoglycosides belonging to the kanamycin group, is very efficacious for treatment of infections caused by both gram-positive and gram-negative bacteria, and since 1990 it has been approved, for chemotherapy of methicillin-resistant *Staphylococcus aureus* (MRSA) infections only, by the Japanese health insurance system. Unlike the other aminoglycosides, arbekacin is not inactivated by most of the modifying enzymes listed above. Only the bifunctional modifying enzyme composed of aminoglycoside-6′-*N*-acetyltransferase and 2″-*O*-phosphotransferase activity [AAC(6′)/APH(2″)] is able to inactivate arbekacin. However, such enzymes have not been found in gram-negative bacilli to date.

We recently reported a *P. aeruginosa* strain that was highly resistant to most aminoglycosides, including arbekacin. This strain harbors a novel aminoglycoside resistance gene named *rmtA*, which encodes a new 16S rRNA methylase (29). Production of 16S rRNA methylase had been reported among aminoglycoside-producing actinomycetes, including *Micromonospora* spp. and *Streptomyces* spp., but this novel aminoglycoside resistance mechanism had not been identified in clinical pathogens before, although a similar putative 16S rRNA methylase, ArmA, was found quite recently in *Klebsiella pneumoniae* in Europe (11). In the present study, we investigated the genetic environments of the *rmtA* genes harbored by two different *P. aeruginosa* strains isolated in separate Japanese hospitals.

(Some of the findings presented in this manuscript have been reported at the 102nd General Meeting of the American Society for Microbiology [abstr. A-28, 2002] by Y. Doi and at its 103rd General Meeting [abstr. A-105, 2003] by K. Yamane.)

MATERIALS AND METHODS

Screening of 16S rRNA methylase producers. In October 2001, a total of 903 nonrepetitive clinical strains of *P. aeruginosa* were collected from 278 medical institutions located in 22 prefectures across Japan. Potential producers of *rmtA* were first screened for a lack of susceptibility to gentamicin, amikacin, and arbekacin (MICs, ≥32 μg/ml). Our bacterial stock of 210 *P. aeruginosa* strains isolated clinically since 1997 was also subjected to a screening test for the *rmtA* gene. Strains that formed colonies on aminoglycoside-containing Mueller-Hinton agar plates were subjected to PCR analyses to check whether or not they harbored the *rmtA* gene. Primers used for amplification of the *rmtA* gene were RMTA-F (5′-CTA GCG TCC ATC CTT TCC TC-3′) and RMTA-R (5′-TTT GCT TCC ATG CCC TTG CC-3′), which amplify a 635-bp DNA fragment within the *rmtA* gene. Template DNAs used were prepared by boiling the bacterial suspension at 100°C for 10 min. Cycling parameters consisted of an initial cycle at 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 60°C for 30 s, and extension at 74°C for 2 min; and a final 5-min incubation at 74°C. Detection of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Characteristics	Source or reference
Strains		
P. aeruginosa AR-2, AR-3, AR-11, AR-15, AR-26, AR-101, AR-105, AR-112, and AR-118	Clinical isolates carrying the *rmtA* gene	This study
E. coli XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 lac* [F^- *proAB*$^+$ *lacI*^{q} ZΔM15::Tn*10*(Tet^r)]	Stratagene
Plasmids		
pBCSK+	Cloning vector; chloramphenicol resistant	Stratagene
pBCRMTH2	Recombinant plasmid carrying a 6.8-kb HindIII fragment containing the *rmtA* gene of *P. aeruginosa* strain AR-2	This study
pBCRMTE2	Recombinant plasmid carrying a 10.3-kb EcoRI fragment containing the *rmtA* gene of *P. aeruginosa* strain AR-2	This study
pBCRMTE11	Recombinant plasmid carrying a 15.8-kb EcoRI fragment containing the *rmtA* gene of *P. aeruginosa* strain AR-11	This study

AAC(6′)/APH(2″) was carried out as described by Ida et al. (13). Clinical isolates and plasmids used in this study are listed in Table 1.

Antibiotics and susceptibility testing. Antibiotics were obtained from the following sources: amikacin, Bristol Pharmaceuticals K. K., Tokyo, Japan; arbekacin, kanamycin, and streptomycin, Meiji Seika Kaisha Ltd., Tokyo, Japan; chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; gentamicin and sisomicin, Schering-Plough K. K., Osaka, Japan; hygromycin B, Sigma-Aldrich Japan K. K., Tokyo, 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., Ltd., Osaka, Japan. MICs were determined by the agar dilution method according to the protocol recommended by the National Committee for Clinical Laboratory Standards in document M7-A5 (19).

PFGE analysis. SpeI (New England Biolabs, Beverly, Mass.)-digested genomic DNAs of *P. aeruginosa* isolates were subjected to pulsed-field gel electrophoresis (PFGE) analysis by using a CHEF-DRII system (Bio-Rad Laboratories, Hercules, Calif.) under conditions described elsewhere (5). The pulses were increased linearly from 4 to 8 s for 10 h, after which the phase was 8 to 50 s for 12 h in this study. Banding patterns of the strains were compared visually; distinct patterns were defined by more than three fragment differences, in accordance with the criteria proposed by Tenover et al. (27).

Southern hybridization analysis of the *rmtA* gene. Total DNAs of all strains were digested with EcoRI (New England Biolabs), electrophoresed through 1.0% agarose gels, transferred to nylon membranes (Bio-Rad Laboratories) by the method of Southern (25), and then hybridized with digoxigenin-labeled *rmtA* gene fragments by use of the PCR DIG detection system (Roche Diagnostics, Tokyo, Japan).

Cloning of the *rmtA* gene. Basic recombinant-DNA techniques were carried out as described by Sambrook et al. (21). EcoRI and HindIII (New England Biolabs) were used for digestion of genomic DNA. The resultant fragments were ligated into the plasmid vector pBCSK+ (Stratagene, La Jolla, Calif.), and electrocompetent *Escherichia coli* XL1-Blue (Stratagene) was transformed with these recombinant plasmids. Transformants were selected on Luria-Bertani agar plates supplemented with 4 μg of arbekacin/ml and 30 μg of chloramphenicol/ml.

DNA sequencing. DNA sequences were determined as described by Sanger et al. (22) with BigDye Terminator Cycle Sequencing Ready Reaction kits and a model 3100 DNA sequence analyzer (Applied Biosystems, Foster City, Calif.). The sequences of the cloned fragments were determined with custom sequencing primers. Nucleotide sequence alignment was performed with GENETYX-MAC (version 10.1.1; Software Development Co., Ltd., Tokyo, Japan). The nucleotide sequence was analyzed by the FASTA service of the DNA Data Bank of Japan (DDBJ) homology search system.

Nucleotide sequence accession numbers. The nucleotide sequence data determined in this study will appear in the DDBJ database under nucleotide accession numbers AB083212 and AB120321.

RESULTS

Bacterial strains. Among 903 strains collected in October 2001, the MICs of arbekacin, gentamicin, and amikacin for 23 strains (2.5%) were greater than 32 μg/ml. Of these, four strains (AR-101, AR-105, AR-112, and AR-118), accounting for 0.4% of all isolates, were found to be positive for *rmtA* by PCR analysis. From our bacterial collection of 210 *P. aeruginosa* strains, 5 strains (AR-2, AR-3, AR-11, AR-15, and AR-26) were PCR positive for *rmtA*. AAC(6′)/APH(2″) was not detected in any of these nine strains by PCR analysis. Strains AR-2 and AR-3 were isolated from a hospital, as were strains AR-101 and AR-105. These nine *rmtA*-positive strains have been isolated from seven separate medical institutions in five prefectures in Eastern and Central Japan since 1997.

Susceptibility to antimicrobial agents. MICs of representative aminoglycosides for these nine strains carrying the *rmtA* gene are shown in Table 2. All the strains were highly resistant

TABLE 2. Results of antibiotic susceptibility testing

Aminoglycoside	MIC (μg/ml) for the following *P. aeruginosa* strain:								
	AR-2	AR-3	AR-11	AR-15	AR-26	AR-101	AR-105	AR-112	AR-118
Kanamycin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Amikacin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Tobramycin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Arbekacin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Gentamicin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Sisomicin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Isepacin	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
Neomycin	>1,024	>1,024	>1,024	128	>1,024	1,024	512	>1,024	1,024
Hygromycin B	>1,024	1,024	256	128	512	128	128	256	512
Streptomycin	128	128	128	>1,024	512	64	128	128	32

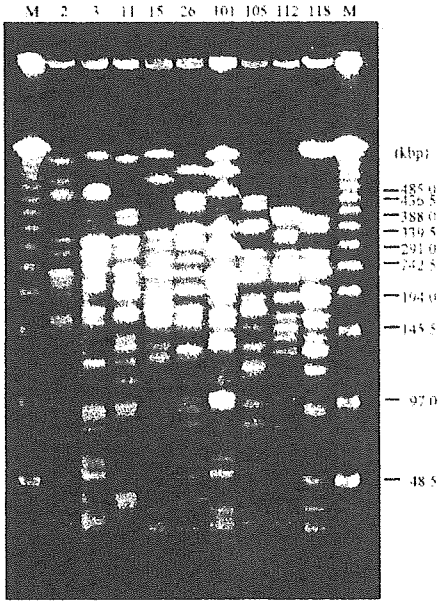

FIG. 1. PFGE fingerprinting of total DNAs from *P. aeruginosa* isolates digested with SpeI. M, PFGE molecular weight marker. The number above each lane indicates the AR strain number shown in Table 1.

to 4,6-disubstituted deoxystreptamines such as kanamycin, amikacin, tobramycin, and arbekacin, which belong to the kanamycin group, as well as to gentamicin, isepamicin, and sisomicin, belonging to the gentamicin group. In contrast, levels of resistance to neomycin, streptomycin, and hygromycin B varied. Strain AR-11 showed a multidrug-resistant profile to ceftazidime, imipenem, and ciprofloxacin as well as to most aminoglycosides.

PFGE profiles. The results of the PFGE analysis are shown in Fig. 1. The SpeI-digested patterns of the total DNAs of nine strains harboring the *rmtA* gene were apparently different from each other. This finding suggests not a clonal expansion of an *rmtA*-carrying strain but plasmid-mediated transmission of the *rmtA* gene among clinical strains with different genetic backgrounds by the help of some movable genetic elements such as a transposon and transferable plasmids.

Southern hybridization. DNA fragments digested with EcoRI showed two hybridization patterns. The *rmtA* probe hybridized with a 10.3-kbp EcoRI fragment for strains AR-2, AR-3, and AR-118 and with a 15.8-kbp fragment for strains AR-11, AR-15, AR-26, AR-101, AR-105, and AR-112 (Fig. 2).

Genetic environments harboring *rmtA* genes. A 6.8-kbp HindIII fragment and a 10.3-kbp EcoRI fragment containing the *rmtA* gene of AR-2 were cloned into the plasmid vector pBCSK+. The 6.8- and 10.3-kbp fragments were inserted into pBCRMTH2 and pBCRMTE2, respectively. The schematic structure of the 11.1-kbp sequenced region cloned from strain AR-2 is shown in Fig. 3. The *rmtA* gene was located within a 6.2-kbp genetic locus (the *rmtA* locus) flanked by a 262-bp sequence named the κγ element that was previously found in Tn*5041* and predicted to be a relic of mobile genetic elements (Fig. 3). The elements of the 6.2-kbp *rmtA* locus, comprising *rmtA*, *orfA*, and two additional specific sequences, were located in the following order: transposase gene-like sequence (sequence 1), probable tRNA ribosyltransferase gene (*orfA*), *rmtA*, and Na^+/H^+ antiporter gene-like sequence (sequence 2) (Fig. 3). The 5′ end of the HindIII fragment flanked *merR* of the *mer* operon found in Tn*5041*. However, the 3′ end of the EcoRI fragment was located within a 17-bp sequence which was completely identical to a part of the terminal inverted repeat of Tn*1721*. This 17-bp sequence was within *orfQ*, located upstream of *orfI* in Tn*5041*. The G+C content of the 6.2-kbp *rmtA* locus was about 55%. The 15.8-kbp EcoRI fragment of AR-11 containing the *rmtA* gene was also cloned into the plasmid vector pBCSK+, and the resultant recombinant plasmid was designated pBCRMTE11. In the 15.8-kbp EcoRI fragment, a 5′-truncated *orfA* (*orfA′*), *rmtA*, and sequence 2 were found between IS*6100* and a κγ element, and the sequence was completely identical to that of the corresponding region of the 6.2-kbp *rmtA* locus cloned from strain AR-2. The *orfQ* and *orfI* sequences of Tn*5041* were present both upstream of IS*6100* and downstream of a κγ element in the 15.8-kbp EcoRI fragment cloned from strain AR-11. In the sequenced areas, the fragments harboring the *rmtA* gene appeared to be inserted between the κγ sequences found in Tn*5041* (Fig. 3).

DISCUSSION

Aminoglycoside-producing actinomycetes such as *Micromonospora* spp. and *Streptomyces* spp. protect their 30S ribosome through methylation of its 16S rRNA at the aminoglycoside-binding A site (10, 30). For example, Kgm, which was isolated from *Micromonospora purpurea* (28), methylates G1405, and Kam, which was isolated from *Streptomyces tenjimariensis* (24), methylates A1408 (2). The 16S rRNA methylases had been thought to exist among aminoglycoside-producing environmental actinomycetes such as *Micromonospora* spp. or *Streptomyces* spp (7). However, we recently reported a novel 16S rRNA methylase, RmtA, that was identified in a *P. aeruginosa* clinical strain, AR-2 (29). This strain demonstrated an extraor-

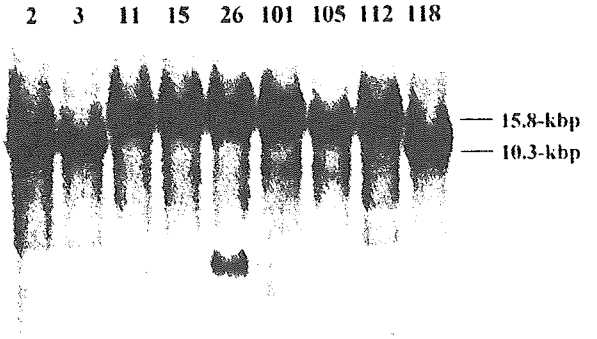

FIG. 2. Southern hybridization patterns of EcoRI-digested genomic DNAs. The number above each lane represents the AR strain number shown in Table 1. The nine strains tested appeared to be divided into two groups by the sizes of EcoRI-digested fragments (10.3 and 15.8 kbp, respectively).

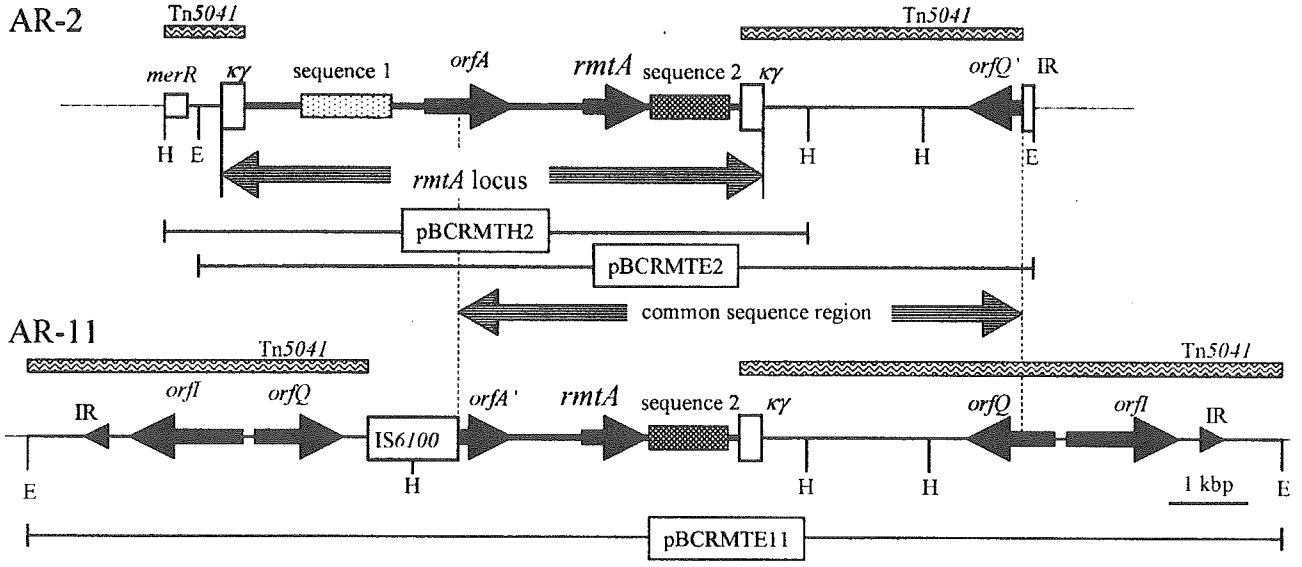

FIG. 3. Comparison of the genetic organizations of AR-2 and AR-11. Double-headed striped arrows indicate the position of the *rmtA* locus and that of the region common to both sequenced areas. Inserts of pBCRMTH2, pBCRMTE2, and pBCRMTE11 are indicated by horizontal lines. Rectangles filled with wavy lines, sequences similar to part of Tn*5041*. Solid arrowheads in the 15.8-kbp EcoRI fragment, terminal inverted repeats. *mer*, the mercury resistance operon, includes *merR*. Sequence 1, transposase gene-like sequence; sequence 2, Na^+/H^+ antiporter-like sequence; *orfA*, probable tRNA ribosyltransferase gene; *orfQ'*, part of *orfQ*; *orfA'*, part of *orfA*; IR, probable inverted repeat. Restriction sites: H, HindIII; E, EcoRI. Sequences 1 and 2 encode no complete proteins due to several frameshifts and deletions.

dinarily high level of aminoglycoside resistance to various 4,6-disubstituted deoxystreptamines, including semisynthetic arbekacin, as well as to gentamicin and kanamycin. In the present study, we investigated the genetic environments mediating the *rmtA* genes found in two different strains of *P. aeruginosa*. The G+C content of the *rmtA* gene was 55%, and those of 16S rRNA methylase genes found in aminoglycoside-producing actinomycetes were 64 to 72%. These observations suggested that the *rmtA* gene might have been acquired by *P. aeruginosa* from some environmental bacteria such as aminoglycoside-producing actinomycetes, although the *armA* gene, with a 30% G+C content, was speculated to have originated from unknown bacteria other than actinomycetes. At any rate, lateral gene transfer across bacterial genera would become much more important for acquisition of new antibiotic resistance profiles hereafter.

Although the PFGE patterns of the nine RmtA-producing strains in this study were highly divergent, Southern hybridization showed only two hybridization patterns when genomic DNAs were digested with EcoRI. This finding indicated that the *rmtA* gene might be mediated by some mobile genetic elements sharing similar genetic environments and spreading among genetically unrelated strains in geographically separate hospitals. This speculation would be supported by the finding that even strains AR-2 and AR-3, isolated at the same hospital, showed different PFGE patterns. Strains AR-101 and AR-105 also demonstrated quite different PFGE profiles despite being isolated at the same hospital. Furthermore, the arbakacin resistance profile of AR-2 was transferable to another *P. aeruginosa* strain by conjugation (29). This suggested that *rmtA* was mediated by some transferable plasmids in strain AR-2, but we failed to visualize the plasmid either by the method of Kado and Liu (14) or by cesium chloride-ethidium bromide density gradient ultracentrifugation (21). This is possibly due to the instability or the very low copy number of the plasmid which mediates the *rmtA* gene.

Tn*5041* was previously identified in a strain of a *Pseudomonas* species as a mercury resistance transposon (3, 16). Tn*5041* carries a 4-kbp insert of unknown origin between *orfQ* and the *mer* operon, and several nonfunctional pseudogenes and possible mobile elements such as the κγ element locate in this region. The 262-bp κγ element, containing 38 bp of imperfect inverted repeats starting with the sequence GGGG and terminating internally with the sequence TAAG, falls into the inverted repeats of Tn*3* family (4). Transposons belonging to the Tn*3* family usually contain transposase and resolvase genes and some additional genes encoding resistance to antimicrobial agents or heavy metals such as mercury between the terminal inverted repeats. The 6.2-kbp *rmtA* locus found in this study was flanked by an insertion element-like κγ element. Moreover, the *rmtA* locus had a transposase gene-like sequence (sequence 1) whose 5′ part showed 80.2% identity with part of the transposase gene derived from *Pseudomonas putida* (accession number AF109307); the 3′ part of sequence 1 had 67.2% identity with part of the transposase gene derived from *Pseudomonas pseudoalcaligenes* (accession number AF028594), but this sequence had no apparent initiation and stop codons. Thus, the 6.2-kb *rmtA* locus itself is unlikely to be an active transposon, although the nucleotide sequences outside of the two κγ elements were completely identical to the corresponding regions of Tn*5041*. The Na^+/H^+ antiporter gene-like sequences (sequence 2) found in strains AR-11 and AR-2 were completely identical, although they seemed nonfunctional. Multicopy expression of the intact transposase-like gene and the Na^+/H^+ antiporter-like gene might disturb systematic bacterial cell growth, so these genes might have been

inactivated during replication and translocation of the *rmtA* locus.

To examine whether strains other than AR-2 and AR-11 also carry part of the sequence found in Tn*5041*, Southern hybridization analysis was performed using a Tn*5041*-specific DNA probe containing a sequence between the right-hand κγ element and the *orfQ* gene, which is conserved in both strains AR-2 and AR-11. The DNA probes and the *rmtA* gene probe hybridized to the same fragments in all nine strains (data not shown). This finding strongly suggests the probable implication of some mobile genetic elements such as Tn*5041* in the dissemination of the *rmtA* gene among strains of *P. aeruginosa*.

The 5′ end of the *rmtA* locus was replaced by IS*6100* in strain AR-11. IS*6100* was originally discovered in *Mycobacterium fortuitum* (accession number X53635) (18) and was subsequently found in several gram-negative and -positive bacteria (9, 26). It has been reported that transposition of IS*6100* stimulates genetic rearrangement (12). Thus, it may be possible to speculate that the region containing *orfQ* and *orfI* found upstream of IS*6100* might be duplicated during IS*6100*-mediated recombination in strain AR-11. The outside sequences of both inverted repeats had no DNA homology to the genomic DNA of *P. aeruginosa* PAO-1. This finding suggests that the 15.8-kb EcoRI fragment of strain AR-11 might be carried by a much longer mobile genetic element, since the arbekacin-resistant profile of AR-11 was not transferred to another *P. aeruginosa* strain by conjugation, and no apparent plasmid was detected in this strain by the method of Kado and Liu (14). Additionally, *rmtA* gene probes hybridized to the position of chromosomal DNA (data not shown). These findings strongly suggested that the *rmtA* gene and its adjacent regions might be integrated into the chromosomal DNA in strain AR-11.

P. aeruginosa strains harboring the *rmtA* gene have already been found in several separate clinical settings in Japan, and a gene encoding the same kind of 16S rRNA methylase, called *armA*, has also been identified in members of the family *Enterobacteriaceae*, such as *Citrobacter freundii* (accession number NC004464) and *K. pneumoniae* (11) (accession number AY220558), in Europe. ArmA shares 29% identity with RmtA at the amino acid sequence level. Moreover, a new plasmid-mediated 16S rRNA methylase, RmtB, that shares 82% identity with RmtA at the amino acid sequence level, has also been identified in *Serratia marcescens* in Japan (8) (accession number AB103506). From our preliminary study on a bacterial stock, the presence of these genes was also suggested in several strains of *K. pneumoniae*, *E. coli*, and *Acinetobacter* species isolated in Japan. Thus, further dissemination of these genetic determinants to various pathogenic gram-negative bacilli could become a serious concern in the near future.

In Japanese clinical settings, various aminoglycosides have been used in the treatment of bacterial infections, since these agents still have very high efficacies against both gram-positive and gram-negative bacteria. Arbekacin is a semisynthetic aminoglycoside belonging to the kanamycin-group. It has been approved, for MRSA infection only, since 1990, and it is still very efficacious for MRSA infection. Under such clinical circumstances, arbekacin has been preferentially used in many clinical settings, although arbekacin-resistant strains which produce the bifunctional enzyme AAC(6′)/APH(2″) have emerged in MRSA. No such bifunctional enzymes, however, have been found in gram-negative bacilli to date. Thus, acquisition of 16S rRNA methylase would give gram-negative bacteria a great advantage in coping with clinical environments where huge amounts of semisynthetic aminoglycosides, including arbekacin, are consumed. Hence, one should recall again that bacteria can survive and proliferate in clinical environments, given their natural hereditary capacity to overcome the hazards of any environment.

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Nosocomial Spread of Ceftazidime-Resistant *Klebsiella pneumoniae* Strains Producing a Novel Class A β-Lactamase, GES-3, in a Neonatal Intensive Care Unit in Japan

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***Klebsiella pneumoniae* strain KG525, which showed high-level resistance to broad-spectrum cephalosporins, was isolated from the neonatal intensive care unit (NICU) of a Japanese hospital in March 2002. The ceftazidime resistance of strain KG525 was transferable to *Escherichia coli* CSH-2 by conjugation. Cloning and sequence analysis revealed that production of a novel extended-spectrum class A β-lactamase (pI 7.0), designated GES-3, which had two amino acid substitutions of M62T and E104K on the basis of the sequence of GES-1, was responsible for resistance in strain KG525 and its transconjugant. The bla_{GES-3} gene was located as the first gene cassette in a class 1 integron that also contained an *aacA1-orfG* fused gene cassette and one unique cassette that has not been described in other class 1 integrons and ended with a truncated 3′ conserved segment by insertion of IS*26*. Another five ceftazidime-resistant *K. pneumoniae* strains, strains KG914, KG1116, KG545, KG502, and KG827, which were isolated from different neonates during a 1-year period in the same NICU where strain KG525 had been isolated, were also positive for GES-type β-lactamase genes by PCR. Pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus-PCR analyses displayed genetic relatedness among the six *K. pneumoniae* strains. Southern hybridization analysis with a GES-type β-lactamase gene-specific probe showed that the locations of bla_{GES} were multiple and diverse among the six strains. These findings suggest that within the NICU setting genetically related *K. pneumoniae* strains carrying the bla_{GES} gene were ambushed with genetic rearrangements that caused the multiplication and translocation of the bla_{GES} gene.**

Resistance to β-lactam antibiotics mainly depends on the production of β-lactamases. To date, a large variety of β-lactamases which were classified by their amino acid sequences and functional substrate specificity profiles in various gram-negative bacilli such as *Pseudomonas* spp. and members of the family *Enterobacteriaceae* have been documented (6). Since the late 1980s, extended-spectrum β-lactamases (ESBLs) derived from TEM- and SHV-type penicillinases capable of hydrolyzing the oxymino-cephalosporins have been spreading globally, mainly in the *Enterobacteriaceae*, including *Klebsiella pneumoniae* and *Escherichia coli* (5, 23, 29). Moreover, various non-TEM-, non-SHV-type class A β-lactamases exhibiting extended-spectrum activities, including CTX-M-type (13, 31, 38, 39, 41), SFO-type (18), VEB-type (12, 20, 25), and GES-type (10, 11, 19, 24, 28, 37) β-lactamases, have also been reported in various gram-negative bacilli. Among the GES-type β-lactamases, GES-1, which was found to be produced by *K. pneumoniae* ORI-1, identified from a child transferred from French Guiana to France in 1998, was the first report of the GES-type class A β-lactamase (24); and GES-1-producing *K. pneumoniae* strains have caused nosocomial infections in Portugal (9). IBC-1 was identified in an *Enterobacter cloacae* clinical isolate from Greece in 1999 (11), and IBC-1-producing *E. cloacae* has also been reported to cause nosocomial infections in a neonatal intensive care unit (NICU) (17). GES-2, which displayed more extended-spectrum activity against imipenem compared with that of GES-1, was reported in *Pseudomonas aeruginosa* from South Africa (28) in 2000, and GES-2 producers also caused a nosocomial infection (27). All three genes, bla_{GES-1}, bla_{GES-2}, and bla_{IBC-1}, were found to be located as a gene cassette within similar class 1 integrons.

Recently, six clinical isolates of *K. pneumoniae* showing high-level resistance to various broad-spectrum cephalosporins, including ceftazidime, were identified from the NICU of a Japanese hospital, and conventional PCR analyses for TEM-derived ESBLs and CTX-M enzymes failed to specify their genetic determinants. In the present study, therefore, we characterized the molecular mechanism underlying the multiple-cephalosporin resistance among these six strains, as well as the organizations of their genetic environments.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Six *K. pneumoniae* clinical strains had been isolated from neonates over 1 year, from September 2001 to August 2002, and were stored in the clinical microbiology laboratory of the hospital until this study. Biochemical phenotypic identification of these strains was carried out by the analytical profile index procedure (API 20E system; bioMerieux, Marcy l'Etoile, France). A pre-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or Reference
K. pneumoniae KG914, KG1116, KG525, KG545, KG502, KG827	Clinical isolates from neonatal specimens	This study
E. coli		
CSH-2	*metB* F^- nalidixic acid^r rifampin^r	T. Sawai, Chiba University
XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17*(rK^- mK^+) *relA1 lac* [F^- *proAB*$^+$ *lacI*q*Z*ΔM15::Tn*10*(Tet^r)]	Stratagene
BL21(DE3)pLysS	F^- *ompT hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
Plasmids		
pKGC525	A natural plasmid carrying bla_{GES-3} of *K. pneumoniae* KG525	This study
pKGB525	A recombinant plasmid carrying a 6.7-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	This study
pKGM525	A recombinant plasmid carrying a 11.6-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	This study
pTAGES3	A recombinant plasmid carrying a PCR fragment with the entire bla_{GES-3} sequence and its promoter region cloned into the pCR2.1 vector	This study
pGES3	A recombinant plasmid carrying EcoR1 fragment from pTAGES3	This study
pIBC1	A recombinant plamid carrying bla_{IBC-1} constructed from pGES3	This study
pBCSK+	A cloning vector; chloramphenicol^r	Stratagene
pCR2.1	A cloning vector; ampicillin^r kanamycin^r	Invitrogen
pET29a(+)	An expression vector; kanamycin^r	Novagen
pET-GES3	A recombinant plasmid carrying PCR-amplified bla_{GES-3} gene ligated to pET29a(+)	This study

liminary double-disk synergy test was carried out with disks containing ceftazidime and amoxicillin-clavulanate. Bacteria were grown in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, unless specified otherwise.

Antibiotic susceptibility testing. The following antibiotics were obtained from the indicated sources: ampicillin, amoxicillin, and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; cefmetazole and chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime and cefpirome, Aventis Pharma, Ltd., Tokyo, Japan; ceftazidime and clavulanic acid, GlaxoSmithKline K. K., Tokyo, Japan; sulbactam, Pfizer Pharmaceutical Inc., Tokyo, Japan; tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan; cefepime, Bristol Pharmaceuticals K. K., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo Japan; and rifampin, Daiichi Pharamaceutical Co., Ltd., Tokyo, Japan. The MICs of the β-lactams were determined by the agar dilution method, according to the recommendations of National Committee for Clinical Laboratory Standards document M7-A5 (21). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains in the antimicrobial susceptibility testing.

PCR amplification. To amplify the broad-spectrum β-lactamase genes from the six clinical strains, PCR analyses were performed with sets of primers specific for various β-lactamase genes found in Japan—including the TEM-derived extended-spectrum β-lactamase (39); CMY-2-, MOX-1-, and DHA-1-type β-lactamases (8, 40, 41); and CTX-M-1-, CTX-M-2-, CTX-M-9-, IMP-1-, IMP-2-, and VIM-2-type β-lactamases (13, 26, 30, 31, 33, 39)—under the conditions described elsewhere (33). Detection of the SHV-type β-lactamase gene was not performed because most clinical *K. pneumoniae* strains carry the LEN-1 and/or SHV-1 β-lactamase gene on their chromosomes (1, 7). In order to detect the GES-type β-lactamase gene, an 827-bp internal fragment of the gene was amplified with primers GES-A (5′-CTT CAT TCA CGC ACT ATT AC-3′) and GES-B (5′-TAA CTT GAC CGA CAG AGG-3′) under the conditions described above.

Conjugal transfer of β-lactam resistance. Conjugal transfer of the ceftazidime resistance of *K. pneumoniae* KG525 to a recipient *E. coli* strain, strain CSH-2 (F^- *metB*, resistant to nalidixic acid and rifampin), was performed by the filter mating method. Transconjugants were selected on LB agar plates containing ceftazidime (2 μg/ml), rifampin (100 μg/ml), and nalidixic acid (50 μg/ml).

Cloning experiment and DNA sequencing. Basic recombinant DNA techniques were performed as described by Sambrook et al. (32). Total DNA of *K. pneumoniae* KG525 was extracted and digested with BamHI. The resultant fragments were ligated into cloning vector pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzyme. Transformants were selected on LB agar plates containing chloramphenicol (30 μg/ml) and ampicillin (50 μg/ml) or ceftazidime (2 μg/ml). The nucleotide sequence of the cloned fragment was determined with BigDye terminator cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.) by using custom sequencing primers.

Site-directed mutagenesis for comparison of GES-3 with IBC-1. PCR-based site-directed mutagenesis of the bla_{GES-3} gene was performed with the LA PCR In Vitro Mutagenesis kit (TAKARA Bio Inc., Ohtsu, Japan). In brief, the entire bla_{GES-3} gene and its promoter region were amplified by PCR and cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV, Leek, The Netherlands). One plasmid, pTAGES3, was selected after it was confirmed that it contained no amplification error and was then digested with EcoRI. The resultant fragment was recloned into pBCSK+. The resultant plasmid, pGES3, with an insert carrying the bla_{GES-3} gene and its promoter region was used to introduce a single nucleotide mutation (C to T) at nucleotide position 167, which leads to an amino acid substitution (T to M) at position 62 in GES-3, resulting in the conversion of the gene product from GES-3 to IBC-1 expressed under the same promoter.

Pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus (ERIC)-PCR analyses. Total DNA was prepared from six *K. pneumoniae* strains (34) and digested overnight with XbaI (New England Biolabs, Beverly, Mass.). The digested DNA was electrophoresed with a CHEF-DRII Drive Module (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: pulses ranging from 10 to 40 s at 6 V/cm for 20 h at 16°C. Six *K. pneumoniae* strains were also typed with the primer ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′). The PCR was carried out under the conditions described elsewhere (36).

Southern hybridization. Large plasmids were prepared from six *K. pneumoniae* strains by the procedure described by Kado and Liu (16). The chromosomal DNA was extracted from each isolate by the method of Stauffer et al. (35). Both plasmid and chromosomal DNA preparations were separately subjected to Southern hybridization experiments. The 827-bp DNA probes were amplified by a PCR with primers 5′-CTT CAT TCA CGC ACT ATT AC-3′ and 5′-TAA CTT GAC CGA CAG AGG-3′. The PCR amplicons were labeled with digoxigenin (DIG) by a random priming labeling method with the PCR DIG detection system, as recommended by the manufacturer (Roche Diagnostics, Tokyo, Japan). Southern hybridization was performed by the protocol of the manufacturer (Roche Diagnostics).

Purification of GES-3 β-lactamase. To overproduce GES-3 β-lactamase in *E. coli*, the bla_{GES-3} gene was amplified by using two primers, primer GES-F (5′-CAT ATG CGC TTC ATT CAC GCA CTA TTA CTG-3′), which was designed to add an NdeI linker (underlined), and primer GES-R (5′-GTC GAC

TABLE 2. MICs for six *K. pneumoniae* clinical isolates from a NICU

K. pneumoniae strain	Date of isolation (mo/day/yr)	Site of isolation	MIC (μg/ml)[a]													
			AMX + CLA	PIP	PIP + TZB	CAZ	CAZ + CLA	CTX	ATM	CMZ	FEP	IPM	GEN	AMK	LVX	CIP
KG914	9/14/01	Bronchial secretion	>128	>128	128	>1,024	512	64	64	16	32	0.13	1	32	<0.06	<0.06
KG1116	11/16/01	Bronchial secretion	>128	>128	128	>1,024	128	64	64	16	32	0.13	0.5	32	<0.06	<0.06
KG525	3/4/02	Stool	>128	128	128	>1,024	256	64	64	16	16	0.13	2	64	<0.06	<0.06
KG545	3/7/02	Nasal mucosa	>128	>128	>128	>1,024	1,024	128	128	128	64	0.5	2	64	0.25	0.25
KG502	5/2/02	Pus	>128	128	64	1,024	512	16	32	>128	8	8	2	32	0.13	<0.06
KG827	8/27/02	Bronchial secretion	>128	128	32	>1,024	256	16	64	>128	32	0.5	2	32	2	1

[a] Abbreviations: AMX, amoxicillin; CLA, clavulanic acid; PIP, piperacillin; TZB, tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; CMZ, cefmetazole; FEP, cefepime; IPM, imipenem; GEN, gentamicin; AMK, amikacin; LVX, levofloxacin; CIP, ciprofloxacin.

CTA TTT GTC CGT GCT CAG GAT GAG-3'), which was designed to add an SalI linker (underlined), and DNA polymerase (Expand High Fidelity PCR System; Roche Diagnostics), according to the instructions of the manufacturers. The resulting products were cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV) and subjected to confirmatory sequencing. One plasmid with no amplification error was selected and was partially double digested with NdeI and SalI and then subcloned into pET-29a(+) (Novagen, Madison, Wis.), which had been digested with the same enzymes. The expression vector constructed, named pET-GES3, was introduced into *E. coli* BL21(DE3) pLysS (Novagen). *E. coli* BL21(DE3) pLysS carrying plasmid pET-GES3 was cultured in 1 liter of LB broth containing kanamycin (50 μg/ml). Isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) was added when the culture reached an A_{600} of 0.6, and the culture was incubated for an additional 2 h. The cells were harvested by centrifugation and were suspended in 5 ml of 20 mM bis-Tris buffer (pH 6.5). The suspension was passed through a French pressure cell twice and was then centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used for subsequent chromatographic purification. Size-exclusion chromatography was performed on a HiLoad 16/60 Superdex 200 prep-grade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 20 mM bis-Tris buffer (pH 6.5). Fractions containing β-lactamase activity were collected and applied to an anion-exchange Hitrap Q HP column with the same buffer. β-Lactamase activity was recovered in the flowthrough and was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) overnight at 4°C. This partially purified enzyme was loaded onto a Hitrap Q HP column (Pharmacia Biotech) preequilibrated with the same buffer and eluted with a linear gradient of NaCl. Fractions presenting high levels of activity were pooled and dialyzed against 50 mM phosphate buffer (pH 7.0).

Isoelectric focusing (IEF). Fifty milliliters of the bacterial culture was centrifuged, and the cell pellet was suspended in 5 ml of distilled water. A crude periplasmic preparation containing β-lactamase was obtained by freezing-thawing the bacterial suspension three times, followed by ultracentrifugation (40,000 × *g*) for 1 h. The supernatant was condensed to 1/10 volume with an Ultrafree-15 Centrifugal Filter Device (Millipore Corporation, Bedford, Mass.). To determine the isoelectric point (pI), 5 μl of the condensed supernatant containing β-lactamase was loaded onto an Ampholine PAG plate (pH 3.5 to 9.5; Pharmacia Biotech) with a Multiphor II electrophoresis system (Pharmacia Biotech). The pI of the β-lactamase was measured by staining the gel with a 0.05% solution of nitrocefin. Purified GES-3 β-lactamase was also electrophoresed on the Ampholine PAG plate and stained with Coomassie blue.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession number AB113580.

RESULTS

Characteristics of six *K. pneumoniae* clinical isolates. The susceptibilities of the six isolates to β-lactams are presented in Table 2. All isolates were resistant to piperacillin, ceftazidime, and aztreonam. The MICs of cefotaxime, cefmetazole, cefepime, and imipenem for the isolates were variable. Despite the addition of clavulanic acid, these isolates kept their high-level resistance to ceftazidime (MICs, ≥128 μg/ml). This observation was consistent with the negative results of the double-disk synergy test with two disks containing ceftazidime and amoxicillin-clavulanate, respectively. Metallo-β-lactamase production was not detected by using a thiol compound (2). PCR analyses performed preliminarily to detect broad-spectrum β-lactamase genes including TEM derivatives, CTX-M-1, CTX-M-2, CTX-M-9, MOX-1 (CMY-9), CMY-2, DHA-1, IMP-1, IMP-2, and VIM-2, all of which had already been identified in Japan, failed to give positive results.

Transfer and cloning of β-lactamase genes. The ceftazidime resistance determinant of representative strain *K. pneumoniae* KG525 was successfully transferred to a recipient strain, *E. coli* CSH-2; and this finding indicated that the genetic determinant was located on a transferable plasmid. Two ceftazidime-resistant *E. coli* clones, each of which harbored a plasmid containing BamHI fragment inserts of approximately 6.7 and 11.6 kb, respectively, were obtained as a result of the cloning experiment. These two recombinant plasmids contained the same 864-bp open reading frame (ORF) encoding a putative β-lactamase which had conserved structural features of the active site of Ambler class A β-lactamases. The deduced amino acid sequence of the β-lactamase showed an amino acid substitution of M62T (a point mutation of T to C at nucleotide position 167) compared with the amino acid sequences of GES-1 (24), GES-2, and IBC-1, as well as an additional E104K substitution in comparison with the amino acid sequences of GES-1 and GES-2 (Fig. 1). Moreover, an N170G substitution was found in GES-3 compared with the amino acid sequence of GES-2, although the G residue at amino acid position 170 was conserved in IBC-1 and GES-1, as well as in GES-3. Therefore, we named this novel class A β-lactamase GES-3, although GES-1 is based on "Guiana extended spectrum" (24).

Antibiotic susceptibilities. The MICs of the β-lactams for parent strain *K. pneumoniae* KG525, transconjugant *E. coli* CSH-2(pKGC525), and transformant *E. coli* XL1-Blue(pKGB525) are listed in Table 3. Parental strain *K. pneumoniae* KG525 was resistant to most β-lactams except the cephamycins and carbapenems. The tranconjugant and transformant were resistant to ceftazidime, and the MICs of the other β-lactams were lower for the tranconjugant and the transformant than for the parent strain. The changes in the MICs of cefotaxime and ceftazidime for parent strain KG525 were apparently observed by the addition of β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, while decreases in the MICs of amoxicillin, ampicillin, and piperacillin, as well as cefotaxime and ceftazidime, were observed for the *E. coli* tranconjugant and transformant in the presence of the inhibitors.