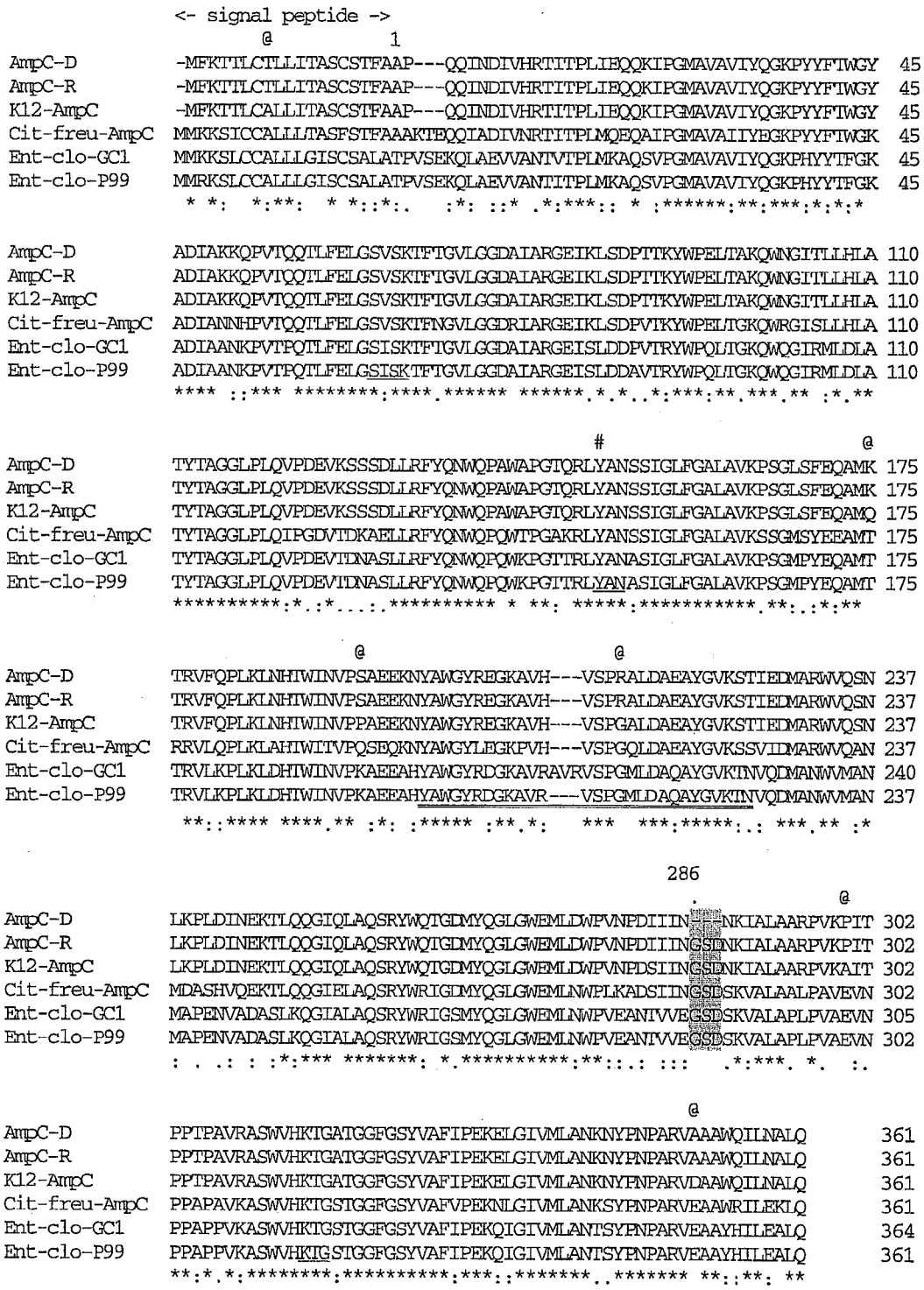


FIG. 1. Predicted amino acid sequence of the AmpC β-lactamase of *E. coli* HKY28 aligned with that of *E. coli* K-12 (10). The 3-amino-acid deletion in the HKY28 AmpC is shaded. Underlines, the β-lactamase active site SVSK, the conserved tripeptide KTG, and the class C motif YXN; #, position of Tyr150; @, positions of the amino acid substitutions observed between the AmpC^{D} 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.

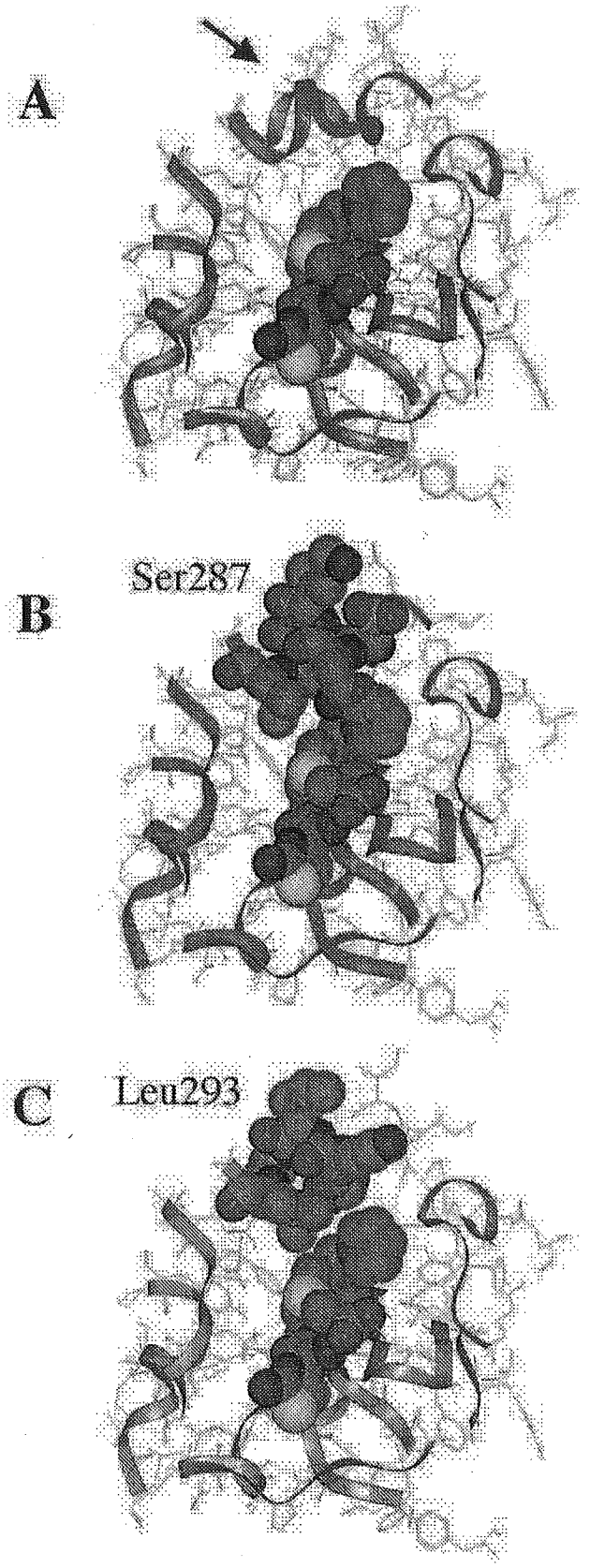
When the *ampC* gene was cloned and expressed in *E. coli* XL1-Blue, it conferred resistance to ceftazidime and cefotaxime, and the resistance could be reversed by any of the three commercially available β-lactamase inhibitors. Sulbactam and tazobactam were much more potent inhibitors in terms of lowering 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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Molecular Characterization of a Cephamycin-Hydrolyzing and Inhibitor-Resistant Class A β-Lactamase, GES-4, Possessing a Single G170S Substitution in the Ω-Loop

Jun-ichi Wachino,[1,2] Yohei Doi,[1] Kunikazu Yamane,[1] Naohiro Shibata,[1] Tetsuya Yagi,[1] Takako Kubota,[3] and Yoshichika Arakawa[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] Central Clinical Laboratory, Kagoshima Municipal Hospital, Kagoshima,[3] and Program in Radiological and Medical Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya,[2] Japan

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The nosocomial spread of six genetically related *Klebsiella pneumoniae* strains producing GES-type β-lactamases was found in a neonatal intensive care unit, and we previously reported that one of the six strains, strain KG525, produced a new β-lactamase, GES-3. In the present study, the molecular mechanism of cephamycin resistance observed in strain KG502, one of the six strains described above, was investigated. This strain was found to produce a variant of GES-3, namely, GES-4, which was responsible for resistance to both cephamycins (cefoxitin MIC, >128 μg/ml) and β-lactamase inhibitors (50% inhibitory concentration of clavulanic acid, 15.2 ± 1.7 μM). The GES-4 enzyme had a single G170S substitution in the Ω-loop region compared with the GES-3 sequence. This single amino acid substitution was closely involved with the augmented hydrolysis of cephamycins and carbapenems and the decreased affinities of β-lactamase inhibitors to GES-4. A cloning experiment and sequencing analysis revealed that strain KG502 possesses duplicate bla_{GES-4} genes mediated by two distinct class 1 integrons with similar gene cassette configurations. Moreover, the genetic environments of the bla_{GES-4} genes found in strain KG502 were almost identical to that of bla_{GES-3} in strain KG525. From these findings, these two phenotypically different strains were suggested to belong to a clonal lineage. The bla_{GES-4} gene found in strain KG502 might well emerge from a point mutation in the bla_{GES-3} gene harbored by its ancestor strains, such as strain KG525, under heavy antibiotic stress in order to acquire extended properties of resistance to cephamycins and carbapenems.

Over the past decade, a number of new plasmid-mediated β-lactamases with wide substrate specificities have appeared mainly in gram-negative bacilli belonging to the family *Enterobacteriaceae* (11, 19, 20, 27). In particular, the emergence of bacteria producing TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) has made chemotherapy for bacterial infections more complex than ever (13). Furthermore, non-TEM- and non-SHV-type ESBLs, such as the CTX-M-type (23, 31), GES-type (8, 21, 25, 28, 29), and VEB-type (3, 22) β-lactamases, have also been identified in these gram-negative bacilli. Generally, the β-lactamases described above are often plasmid encoded and can hydrolyze oximino-cephalosporins and monobactams as well as penicillins but not 7-α-methoxy-cephalosporins, the so-called cephamycins. Carbapenems are also very stable against these enzymes. Therefore, at present cephamycins and carbapenems are potent agents for the treatment of infections caused by the gram-negative bacilli that produce these new class A β-lactamases with wide substrate specificities. Among the various β-lactamase genes described above, the genes encoding the GES-type β-lactamases as well as the VEB-type β-lactamases are often located in integrons as gene cassettes (8, 17, 21, 22, 25, 29). Integrons have been described to play a sophisticated role in the accumulation and expression of genes responsible for antibiotic resistance as well as their dissemination among gram-negative bacilli (9, 10).

Among the GES-type β-lactamases, GES-1 was first reported from a *Klebsiella pneumoniae* clinical isolate in France in 1998 (21); and then two other GES-type β-lactamases, IBC-1 and GES-2, were found in *Enterobacter cloacae* and *Pseudomonas aeruginosa*, respectively (8, 25). GES-2 has an amino acid substitution (glycine to asparagine at position 170) compared to the sequence of GES-1 and shows a higher imipenem-hydrolyzing activity than GES-1.

We found that the high-level ceftazidime resistance of six genetically related *K. pneumoniae* clinical strains, which had been isolated from a neonatal intensive care unit (NICU) over a 1-year period, depended on the production of GES-type β-lactamases, and one of the six isolates was found to produce the GES-3 β-lactamase (30). The bla_{GES-3} gene encoding GES-3 was located as a gene cassette in a class 1 integron, as has been observed for the GES-type β-lactamase genes found in Europe. GES-3 production does not affect the level of cephamycin resistance in the *Escherichia coli* host, as has been reported for the other Ambler class A β-lactamases, including ESBLs. However, the levels of resistance to cephamycins varied widely among the six GES-type β-lactamase-producing strains. Among these, the highest MICs of the carbapenems as well as the cephamycins were seen for strain KG502 (30), which also showed an inhibitor resistance phenotype. The goal of this study was to elucidate the molecular mechanism responsible for resistance to cephamycins and carbapenems in strain KG502, as well as its inhibitor-resistant nature.

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
K. pneumoniae KG502	Clinical isolate, resistant to cephamycins, carrying bla_{GES-4}	This study
K. pneumoniae KG525	Clinical isolate, susceptible to cephamycins, carrying bla_{GES-3}	30
E. coli CSH-2	*metB* F$^-$ nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 lac* [F$^-$ *proAB*$^+$ *lacIqZ*ΔM15::Tn*10* (Tet^r)]	Stratagene
E. coli BL21(DE3)pLysS	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
Plasmids		
pKGL502	Recombinant plasmid carrying a 6.6-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGS502	Recombinant plasmid carrying a 6.0-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGB525	Recombinant plasmid carrying a 6.7-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	30
pET-GES4	Recombinant plasmid containing PCR-amplified bla_{GES-4} ligated to pET29a(+)	This study
pET-GES3	Recombinant plasmid containing PCR-amplified bla_{GES-3} ligated to pET29a(+)	This study
pBCSK+	Cloning vector, chloramphenicol^r	Stratagene
pCR2.1	Cloning vector, ampicillin^r kanamycin^r	Invitrogen
pET29a(+)	Expression vector; kanamycin^r	Novagen

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* strain KG502 was isolated in May 2002 from the pus of a neonate under treatment in the NICU of a general hospital in Japan. This strain was resistant to oximino-cephalosporins and cephamycins. GES-3-producing strain KG525 was isolated in the same NICU where strain KG502 was isolated.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton agar (Becton Dickinson, Cockeysville, Md.), according to the guidelines in National Committee for Clinical Laboratory Standards document M7-A5 (18). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains for MIC determinations. The double-disk synergy test for the detection of ESBL production and an inhibitory test with thiol compounds for the detection of metallo-β-lactamase producers were carried out by the methods described elsewhere (1, 6, 12), with the modification that 2-mercaptopropionic acid was replaced with sodium mercaptoacetic acid.

Transfer of β-lactam resistance genes. Conjugation experiments were performed by the filter mating method with rifampin- and nalidixic acid-resistant *E. coli* CSH-2 as the recipient. Transconjugants were detected on Luria-Bertani (LB) agar supplemented with rifampin (100 μg/ml), nalidixic acid (100 μg/ml), and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Transformation of *E. coli* XL1-Blue with the large plasmids of the parental strain *K. pneumoniae* KG502 was performed by electroporation. Transformants were selected on LB agar containing ceftazidime (4 μg/ml) or cefminox (2 μg/ml).

PCR amplification, cloning, and sequencing of β-lactamase gene. To amplify the bla_{GES} gene, PCR was performed with the primers under the conditions described elsewhere (30). The cloning experiment was carried out as follows: total DNA prepared from strain *K. pneumoniae* KG502 was digested with BamHI, and the resultant fragments were ligated to vector pBCSK+ (Stratagene, La Jolla, Calif.), which had been digested with the same enzyme. Transformants carrying recombinant plasmids were selected on LB agar plates containing chloramphenicol (30 μg/ml) and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Both strands of the DNA fragments inserted into the recombinant plasmids (pKGL502 and pKGS502) were sequenced.

Purification of β-lactamase. β-Lactamases were purified by exactly the same protocol described elsewhere (30). In brief, β-lactamases were overproduced with the pET system, extracted by use of a French press, and cleared by ultracentrifugation. After ultracentrifugation the supernatant was loaded onto a HiLoad 16/60 Superdex 200 and anion-exchange Hitrap Q HP column (Pharmacia Biotech, Uppsala, Sweden). The purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

β-Lactamase assay. Substrate hydrolyses by GES-4 and GES-3 were assayed at 30°C in phosphate buffer (50 mM; pH 7.0) by use of an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The molar extinction coefficients (Δε values) used were as follows: for benzylpenicillin (232 nm), 1.077 mM^{-1} cm^{-1}; for ampicillin (235 nm), 1.121 mM^{-1} cm^{-1}; for cephaloridine (300 nm), 0.384 mM^{-1} cm^{-1}; for cefotaxime (264 nm), 5.725 mM^{-1} cm^{-1}; for ceftazidime (274 nm), 6.123 mM^{-1} cm^{-1}; for cefpirome (290 nm), 4.057 mM^{-1} cm^{-1}; for cefoxitin (293 nm), 0.325 mM^{-1} cm^{-1}; for cefminox (298 nm), 1.878 mM^{-1} cm^{-1}; for imipenem (297 nm), 8.061 mM^{-1} cm^{-1}; and for aztreonam (315 nm), 0.68 mM^{-1} cm^{-1}. Fifty percent inhibitory concentrations (IC_{50}s) were determined with benzylpenicillin as the substrate and the inhibitors clavulanic acid, sulbactam, tazobactam, and imipenem. Purified enzyme and various concentrations of these inhibitors were preincubated in 50 mM phosphate buffer (pH 7.0) at 30°C for 5 min. Purified GES-4 and GES-3 β-lactamases and nonpurified extracts of 50-ml cultures of strain KG502 were subjected to isoelectric focusing (IEF) analysis with an Immmobiline Drystrip (pH 3 to 10; Pharmacia Biotech) and an IPGphor electrophoresis system (Pharmacia Biotech).

Nucleotide sequence accession numbers. The nucleotide sequences described in this work appear in the GenBank nucleotide database under accession numbers AB116260 and AB116723.

RESULTS

Properties of *K. pneumoniae* isolate KG502. *K. pneumoniae* KG502 was isolated from the pus of a neonate in May 2002. This strain was one of the six GES-type β-lactamase-producing strains that we reported previously (30). Strain KG502 exhibited resistance to oximino-cephalosporins and the cephamycins and intermediate susceptibility to carbapenems. No synergy between an amoxicillin-clavulanic acid disk and a ceftazidime and/or a cefotaxime disk was detectable against this strain. The lack of production of metallo-β-lactamases was also suggested by the results of inhibition testing with sodium mercaptoacetic acid. Preliminary PCR detection of some class A β-lactamase and metallo-β-lactamase genes was performed as we reported in our previous study (30), and all PCRs gave negative results.

Transfer of β-lactam resistance by conjugation and transformation. Our previous Southern hybridization experiment with a digoxigenin-labeled bla_{GES}-specific probe demonstrated that the GES-type β-lactamase genes of strain KG502 are located on two distinct plasmids. Therefore, we performed conjugation by filter mating in an attempt to transfer these plasmids to *E. coli* CSH-2, as well as electroporation to introduce them directly into *E. coli* XL1-Blue. However, the transfer of these plasmids into *E. coli* was unsuccessful, despite repeated attempts.

Cloning and sequencing of the β-lactamase gene. Sequencing of the DNAs of the PCR products obtained with the primers specific for the GES-type β-lactamase gene revealed the presence of a variant of the bla_{GES-3} gene in strain KG502. Cloning was performed by standard procedures to determine the entire nucleotide sequences of this new gene. Two distinct recombinant plasmids, one of which carried a 6.6-kb BamHI

```
                    20        30        40        50        62
GES-4  MRFIHALLLAGIAHSAYASEKLTFKTDLEKLEREKAAQIGVAIVDPQGEIVAGHRTAQRF
GES-3  ----------------------------------------------------T-----
GES-2  ----------------------------------------------------M-----
GES-1  ----------------------------------------------------M-----

          70        80        90       100 104   110       120
GES-4  AMCSTFKFPLAALVFERIDSGTERGDRKLSYGPDMIVKWSPATERFLASGHMTVLEAAQA
GES-3  ------------------------------------K-----------------------
GES-2  ------------------------------------E-----------------------
GES-1  ------------------------------------E-----------------------

         130       140       150       160 164   170       179
GES-4  AVQLSDNGATNLLLREIGGPAAMTQYFRKIGDSVSRLDRKEPEMSDNTPGDLRDTTTPIA
GES-3  ----------------------------------------G-------------------
GES-2  ----------------------------------------N-------------------
GES-1  ----------------------------------------G-------------------

         190       200       210       220       230       240
GES-4  MARTVAKVLYGGALTSTSTHTIERWLIGNQTGDATLRAGFPKDWVVGEKTGTCANGGRND
GES-3  ------------------------------------------------------------
GES-2  ------------------------------------------------------------
GES-1  ------------------------------------------------------------

         250       260       270       280       290
GES-4  IGFFKAQERDYAVAVYTTAPKLSAVERDELVASVGQVITQLILSTDK
GES-3  ---------------------------------------------
GES-2  ---------------------------------------------
GES-1  ---------------------------------------------
```

FIG. 1. Amino acid alignments of GES-4, GES-3, GES-2, and GES-1 β-lactamases. Hyphens indicate identical amino acids, and the Ω-loop region of β-lactamase is underlined.

fragment and the other of which carried a 6.0-kb BamHI fragment, were obtained independently. The nucleotide sequences of both genetic determinants for β-lactam resistance were the same and differed by a glycine (G)-to-alanine (A) mutation at position 509 compared with the sequence of bla_{GES-3}, so they were designated bla_{GES-4}. The deduced amino acid sequence of GES-4 had an amino acid substitution of G to serine (S) at position 170 (G170S) within the Ω-loop region of the Ambler class A β-lactamase compared with the sequence of GES-3 (Fig. 1). Among the GES-type β-lactamases, a similar amino acid substitution was reported at position 170, G to asparagine (N), leading to the conversion from GES-1 to GES-2 (25).

Sequencing of bla_{GES-4} flanking region. The inserts in recombinant plasmids pKGL502 and pKGS502 were sequenced, which revealed that both fragments commonly contained bla_{GES-4} gene in the class 1 integron separately, followed by an *aacA1-orfG* fused gene, as the first and second gene cassettes, respectively. The integron in pKGL502 differed from that in pKGS502, in that a third gene cassette, *orfA*, was present (Fig. 2). The product encoded by *orfA* had no significant homology with any other known protein at the amino acid sequence level, so the function of the product could not be presumed. Moreover, the backbone genetic structure surrounding the integron containing the bla_{GES-4} gene from strain KG502 was otherwise identical to that surrounding the integron containing the bla_{GES-3} gene from strain KG525, except that it lacked the outer 128-bp nucleotide sequences, including the 25-bp terminal repeat (IRt) at the left end of IS*6100*, as shown in Fig. 2.

Susceptibilities to various β-lactams. The MICs of β-lactams for *K. pneumoniae* strain KG502 and *E. coli* XL1-Blue harboring recombinant plasmid pKGL502 are listed in Table 2. GES-4 β-lactamase-producing strain KG502 exhibited resistance to cefminox, moxalactam, and cefmetazole and intermediate susceptibility to imipenem and meropenem, whereas GES-3-producing strain KG525 was susceptible to all these agents. These resistance trends were also observed in each of the *E. coli* clones harboring pKGL502 or pKGB525, but the overall resistance levels of the clones were lower than those of the parent strains.

Two notable differences were detected between the MICs for a GES-4-producing *E. coli* clone (pKGL502) and those for a GES-3-producing *E. coli* clone (pKGB525), expressed under the same promoters located within *intI1*. One was a difference in the levels of resistance to cephamycins. The MICs of cephamycins, such as cefminox, cefoxitin, moxalactam, and cefmetazole, were much higher for the GES-4-producing clone than the GES-3-producing clone. In addition, the meropenem MIC for the GES-4 producer was 16-fold higher than that for the GES-3 producer.

The other major differences were the inhibition profiles obtained when the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam were added. The MICs of ampicillin, amoxicillin, piperacillin, ceftazidime, and cefotaxime for the GES-4-producing *E. coli* clone were decreased a maximum of only 8-fold in the presence of β-lactamase inhibitors, whereas those for the GES-3-producing *E. coli* clone decreased at least 32- to 512-fold.

IEF analysis. IEF analysis of the crude extract of parent strain KG502 revealed the presence of two major bands with β-lactamase activities corresponding to pIs of 6.9 and 7.6, respectively. The band with a pI of 7.6 was likely the chromosomally encoded

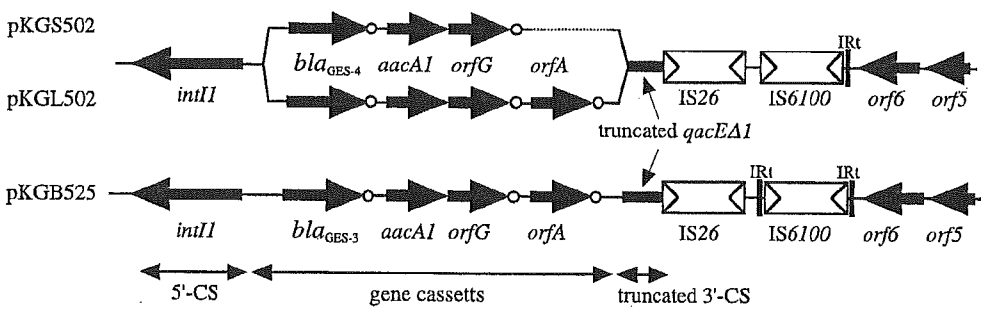

FIG. 2. Schematic comparison of the genetic environments of three class 1 integrons mediating bla_{GES-4} on pKGL502 and pKGS502 and bla_{GES-3} on pKGB525. Open circles represent the positions of the 59-base element. CS, conserved segment.

TABLE 2. MICs of β-lactams

β-Lactam[a]	MIC (μg/ml)				
	K. pneumoniae KG502(GES-4)	*K. pneumoniae* KG525(GES-3)	*E. coli* XL1-Blue pKGL502(GES-4)	*E. coli* XL1-Blue pKGB525(GES-3)	*E. coli* XL1-Blue pBCSK+
Ampicillin	>128	>128	>128	>128	4
Ampicillin + sulbactam	>128	>128	>128	2	2
Amoxicillin	>128	>128	>128	>128	4
Amoxicillin + clavulanate	>128	>128	>128	32	4
Piperacillin	128	128	64	16	1
Piperacillin + tazobactam	64	128	16	0.5	1
Cefotaxime	16	64	1	2	0.13
Cefotaxime + clavulanate	16	8	0.25	0.06	0.06
Cefotaxime + sulbactam	16	32	1	0.06	0.06
Cefotaxime + tazobactam	16	64	1	0.06	0.06
Ceftazidime	1,024	>1,024	64	128	0.13
Ceftazidime + clavulanate	512	256	8	4	0.06
Ceftazidime + sulbactam	>128	>128	32	0.25	0.13
Ceftazidime + tazobactam	>128	>128	32	0.5	0.13
Cephaloridine	>128	>128	64	16	2
Cefminox	>128	8	16	1	0.5
Cefoxitin	>128	128	>128	8	4
Moxalactam	128	4	16	0.5	0.13
Cefmetazole	>128	16	128	2	0.5
Cefpirome	128	>128	1	2	0.06
Cefepime	8	16	0.25	0.25	0.06
Aztreonam	32	64	2	4	0.06
Imipenem	8	0.25	0.25	0.13	0.13
Meropenem	8	0.5	0.25	0.015	0.015

[a] Clavulanate, tazobactam, and sulbactam were each used at a fixed concentration of 4 μg/ml.

SHV-type β-lactamase of *K. pneumoniae*. The estimated pI of 6.9 was identical to those of the purified GES-3 and GES-4 enzymes.

Kinetic studies. The kinetic parameters of the GES-4 and GES-3 β-lactamases for representative β-lactams are given in Table 3. The hydrolyzing efficiencies (k_{cat}/K_m) of GES-4 for the penicillins were about twice as high as those of GES-3, although GES-2 showed less efficient hydrolysis but lower K_m values for cephaloridine and penicillin. On the other hand, GES-3 hydrolyzed ceftazidime and cefotaxime more efficiently than GES-4 did. GES-4 measurably hydrolyzed cefoxitin, cefminox, and imipenem, which accounted for the increases in the MICs of these agents for the clone harboring pKGL502, but no measurable hydrolysis of these agents as substrates by GES-3 was observed under the same experimental conditions used in the present study. No measurable hydrolysis was observed for aztreonam as the substrate with each type of β-lactamase. The IC_{50}s measured with benzylpenicillin as the substrate are listed in Table 4. GES-2 was reported to be inhibited by lower concentrations of clavulanic acid and tazobactam; but GES-4 was inhibited 10-fold less by clavulanic acid, 16-fold less by sulbactam, 8-fold less by tazobactam, and 21-fold less by imipenem than GES-3 was. These results corroborate the inhibitor-resistant nature of GES-4.

DISCUSSION

GES-4 had a single G170S substitution within the Ω-loop region of class A β-lactamases compared with the sequence of

TABLE 3. Kinetic parameters of GES-4, GES-3, and GES-2

Substrate	GES-4			GES-3			GES-2[e]		
	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})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Benzylpenicillin	160 ± 10	130 ± 10	7.8×10^5	33 ± 7	15 ± 1	4.5×10^5	4	0.4	9.6×10^4
Ampicillin	62 ± 4	19 ± 11	3.1×10^5	120 ± 30	23 ± 4	1.9×10^5	NP[e]	NP	NP
Cephaloridine	2,200 ± 400	490 ± 30	2.3×10^5	2,300 ± 100	270 ± 10	1.2×10^5	7.7	0.5	6.5×10^4
Cefotaxime	700 ± 200	17 ± 6	2.4×10^4	1,100 ± 100	120 ± 10	1.1×10^5	890	2.2	2.5×10^3
Ceftazidime	1,500 ± 400	2.5 ± 0.5	1.7×10^3	990 ± 130	23 ± 2	2.3×10^4	>3,000	ND[d]	ND
Cefpirome	340 ± 70	3.6 ± 0.4	1.1×10^4	550 ± 30	36 ± 2	6.6×10^4	NP	NP	NP
Cefoxitin	810 ± 70	85 ± 3	1.1×10^5	NH	NC	NC	—[f]	—	—
Cefminox	370 ± 70	7.7 ± 2.0	2.0×10^4	NH	NC	NC	NP	NP	NP
Imipenem	4.7 ± 0.7	0.38 ± 0.07	8.1×10^4	NH	NC	NC	0.45	0.004	9.0×10^3
Aztreonam	NH[a]	NC[b]	NC	NH	NC	NC	—	—	—

[a] NH, no measurable hydrolysis was detected after 2 h of incubation.
[b] NC, not calculated.
[c] Data for GES-2 were reported in reference 25.
[d] ND, not determined due to very high K_m values.
[e] NP, not provided in reference 25.
[f] —, not hydrolyzed (the initial rate of hydrolysis was less than 0.001 μM^{-1} s^{-1}).

TABLE 4. Inhibition profiles of GES-type β-lactamases

Inhibitor	IC_{50} (μM)				
	GES-4	GES-3	GES-2[a]	GES-1[a,b]	IBC-1[c]
Clavulanic acid	15 ± 1.7	1.5 ± 0.15	1.0 ± 0.5	5.0	1.1
Sulbactam	15 ± 0.9	0.91 ± 0.10	—[d]	—	—
Tazobactam	1.4 ± 0.31	0.19 ± 0.03	0.5 ± 0.2	2.5	0.12
Imipenem	2.1 ± 0.16	0.10 ± 0.01	8 ± 2	0.1	0.06

[a] Data were reported in reference 25.
[b] Data were reported in reference 21.
[c] Data were reported in reference 8.
[d] —, data not provided in the references.

GES-3. Replacement of the side chain (—H) of the glycine residue with that (—CH_2—OH) of the serine residue may indeed contribute to the acceleration of cephamycin hydrolysis as well as the inhibitor resistance profile. The GES-2 β-lactamase, identified as a variant of GES-1, had a substitution from glycine to asparagine at position 170, which is the same position leading to the conversion from GES-3 to GES-4. In comparison with GES-1, GES-2 showed an extended substrate specificity for imipenem and a lower affinity for β-lactamase inhibitors (25), as was seen with GES-4. However, the obvious increases in the MICs of cephamycins and meropenem seen for GES-4 were not detected for GES-2. These findings suggest that a single amino acid substitution at position 170, the center of the Ω-loop region, would play a key role in the expansion of the substrate specificities among GES-type β-lactamases. To elucidate the nature of GES-4, molecular modeling analysis as well as X-ray crystallographic analysis will be undertaken in the next study.

Although amino acid substitutions in the Ω-loop region, which influence hydrolyzing activities against oximino-cephalosporins and carbapenems, have also been observed in several class A β-lactamases, such as those of the TEM type (5, 16), SHV type (2, 15), CTX-M type (23), and GES type (25). Disruption of the salt bridge between R164 and D179 was suggested to be mainly involved in the expansion of substrate specificity for oxyimino-cephalosporins in these enzymes. However, substitutions resulting in increased cephamycin resistance have not been reported in class A β-lactamases so far. To our knowledge, this is the first report of a class A β-lactamase with cephamycin-hydrolyzing ability as a result of a single amino acid substitution in the center of the Ω-loop region. Poyart et al. (26) also reported a similar phenomenon in a TEM-type β-lactamase (TEM-52), in which significant decreases in vitro susceptibilities to some cephamycins were not due to an amino acid substitution in the Ω-loop region. The same investigators reported, however, that the combination of three amino acid substitutions E104K, M182Y, and G238S (on the basis of the sequence of TEM-1) in TEM-25 was responsible for the elevated MICs of moxalactam and cefotetan.

The G170S substitution found in the GES-4 β-lactamase affected not only cephamycin and carbapenem resistance but also inhibitor resistance. The IC_{50}s of clavulanic acid, sulbactam, tazobactam, and imipenem for GES-4 were considerably higher than those of GES-3. Since 1990 IRT β-lactamases derived from TEM-type β-lactamases have been reported to be inhibitor resistance class A β-lactamases. The IRT β-lactamases differ from parental enzyme TEM-1 or TEM-2 by several amino acid substitutions at different locations. The IC_{50}s of clavulanic acid and tazobactam for GES-4 (15.2 and 1.43 μM, respectively) were similar to those of some IRT β-lactamases, including IRT-7 (23 and 0.9 μM, respectively), IRT-8 (25 and 1 μM, respectively), and IRT-14 (22.5 and 1.48 μM, respectively), while the IC_{50} of sulbactam for GES-4 was much lower than those for IRTs (4). GES-4 seems to be a very characteristic enzyme, because it has a strong inhibitor-resistant nature like IRT enzymes, while it maintains the capacity to hydrolyze cephamycins and carbapenems.

As with the other bla_{GES} genes, the bla_{GES-4} gene was located in the class 1 integron as a gene cassette. Strain KG502 was unique, in that it possessed two distinct class 1 integrons which carried similar gene cassette configurations, including the bla_{GES-4} gene cassette. The coexistence of class 1 integrons with similar gene cassette arrays might result from the duplication of a region containing one original class 1 integron by mobile elements, such as transposons in strain KG502. It was speculated that in this strain a region containing one original class 1 integron with the bla_{GES-4}, *aacA1-orfG*, and *orfA* gene cassettes was first duplicated in the bacterium. Next, one of the class 1 integrons might have excised the *orfA* gene cassette by a site-specific recombination mechanism catalyzed by some recombinases, including integrases or transposases. Consequently, strain KG502 might have come to have two class 1 integrons with very similar backbone structures.

The entire genetic structure of the flanking region containing bla_{GES-4} on pKGL502 was almost identical to that containing bla_{GES-3} on pKGB525. Taken together with the facts that both the bla_{GES-3} and the bla_{GES-4} genes were found in genetically related *K. pneumoniae* strains and that the genetic environments of these two genes are almost the same, it is probable that the bla_{GES-4} gene emerged from the point mutation in the bla_{GES-3} gene under conditions of antibiotic stress in order to acquire resistance to additional groups of drugs, i.e., the cephamycins and carbapenems.

We characterized here for the first time a novel class A β-lactamase, GES-4, which acquired extended substrate specificity for the cephamycins through a single amino acid substitution within the Ω-loop region. This finding indicates that β-lactamases which are capable of hydrolyzing cephamycins are not limited to the Ambler class B and class C β-lactamases. The emergence of a cephamycin-hydrolyzing class A β-lactamase might complicate treatment in clinical settings, because cephamycins have generally been considered stable to class A β-lactamases and to retain good efficacies for the treatment of infectious diseases caused by organisms producing class A β-lactamases. Moreover, the inhibitor resistance of the GES-4 β-lactamase may introduce confusion during the routine laboratory detection of class A β-lactamase-producing strains, including ESBL producers. The much higher IC_{50} of clavulanic acid for GES-4 hampered the detection of GES-4-producing clinical isolates by conventional double-disk synergy testing.

Since GES-type β-lactamase-producing gram-negative bacteria have been identified worldwide and nosocomial outbreaks caused by these microorganisms have been reported worldwide (7, 14, 24), due consideration must be given to the possible emergence of variants of GES-type β-lactamases like GES-4 which have acquired several amino acid substitutions to

expand their substrate specificities to cope with the extensive use of broad-spectrum β-lactams in clinical settings.

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Nosocomial Transmission of CTX-M-2 β-Lactamase-Producing *Acinetobacter baumannii* in a Neurosurgery Ward

Noriyuki Nagano,[1]* Yukiko Nagano,[1] Christophe Cordevant,[2] Naohiro Shibata,[3] and Yoshichika Arakawa[3]

Medical Microbiology Laboratory, Funabashi Medical Center, Chiba,[1] and Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[3] Japan, and Molecular Typing Center, Institut Pasteur de Lille, Lille, France[2]

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Three strains of cefotaxime (CTX)-resistant *Acinetobacter baumannii*, FM0209680, FM0300106, and FM0301433, were isolated from transtracheal aspirate cultures of three patients with probable nosocomial infections in a neurosurgery ward in Japan. The CTX MICs for these isolates were greater than 128 μg/ml but were drastically reduced in the presence of 4 μg of clavulanic acid per ml. These strains were also resistant to ceftriaxone, cefpodoxime, and aztreonam but were susceptible to ceftazidime and imipenem. The profile of resistance to various broad-spectrum β-lactams was transferred by conjugation. Strain FM0209680 was not eradicated from case patient 1 by administration of imipenem, ceftazidime, and levofloxacin, even after a 6-month hospitalization period. Strains FM0300106 and FM0301433 were isolated from case patients 2 and 3 during the sixth week following admission, respectively, and then each patient was colonized for 3 weeks. Eradication of FM0300106 was successfully obtained from case patient 2 by imipenem treatment, while administration of imipenem was continued to prevent pneumonia. Prophylactic antimicrobial therapy was discontinued in case patient 3 because of the lack of pneumonic symptoms, and FM0301433 disappeared after the discontinuation of antimicrobial chemotherapy. All three strains carried the $bla_{CTX-M-2}$ gene, and the appearance of colonies in the growth-inhibitory zones around disks of CTX and aztreonam in double-disk synergy tests suggested inducible β-lactamase production in these *A. baumannii* strains. The ribotyping investigation suggested that all these strains belong to the same clonal lineage. The plasmids harbored by *A. baumannii* had the same restriction profile as those harbored by *Proteus mirabilis* strains previously isolated in a urology ward of the Funabashi Medical Center.

Acinetobacter species, including *Acinetobacter baumannii*, had been regarded as one of the important groups of opportunistic pathogens implicated in various infections such as pneumonia, urinary tract infection, endocarditis, surgical site infection, meningitis, and septicemia, particularly in immunocompromised patients (4). *A. baumannii* has recently been rerecognized as an important causative pathogen of nosocomial infections (2). Patients admitted to intensive care units tend to become the main victims of this nosocomial pathogen, which occurs worldwide (12, 22, 29, 54). Increasing therapeutic difficulties due to the acquisition of a profile of multidrug resistance to major groups of antimicrobial agents by various bacterial species have been becoming a serious clinical concern (1, 6, 14, 21, 23, 31, 51). A wide variety of molecular mechanisms for resistance to broad-spectrum β-lactams have been elucidated, i.e., β-lactamase production, mutations of penicillin-binding proteins, and alterations to membrane permeability as well as augmented functioning of the active efflux system (4, 6, 20, 27). *Acinetobacter* species are renowned for their characteristic nature of readily accepting foreign DNA in order to survive in hazardous environments (15, 36). Various β-lactamases demonstrating broad-spectrum substrate specificities that allow *A. baumannii* to cope with broad-spectrum β-lactams, such as OXA-type class D β-lactamases (19, 39, 53), metallo-β-lactamases belonging to class B β-lactamases (45, 51, 57), and AmpC-type class C β-lactamases (7), have been found in *A. baumannii* so far. Moreover, PER-1 and VIB-1 class A β-lactamases demonstrating broad-spectrum substrate specificities have also recently been detected among nosocomially isolated *A. baumannii* strains in Turkey (52) and were then detected in France (40, 41) and Korea (26).

Since June 2002, the medical microbiology laboratory of the Funabashi Medical Center, Chiba, Japan, isolated three cefotaxime-resistant *A. baumannii* strains from inpatients in a neurosurgery ward. In the present study, we characterized the molecular mechanism of cefotaxime resistance in *A. baumannii* strains associated with a nosocomial infection episode.

MATERIALS AND METHODS

Bacterial strains. Three cefotaxime-resistant *A. baumannii* strains, FM0209680, FM0300106, and FM0301433, were isolated from cultures of transtracheal aspirates from three different inpatients in the neurosurgery ward of the Funabashi Medical Center since June 2002. Biochemical identification of the isolates was performed with the API 20NE system (bioMérieux, Marcy l'Etoile, France) combined with a complementary test for the ability to grow at 44°C. Alternatively, sequencing of the 16S rRNA gene was performed by the method described by Sasaki et al. (46). β-Lactamase testing was performed on the basis of acidometry by using a commercial product (P/Case test; Nissui Pharmaceutical, Tokyo, Japan). The bacterial strains were stored in Casitone medium (Eiken Chemical, Tokyo, Japan) at room temperature until they were used.

MIC determinations. MICs were determined by a microdilution broth method with a WalkAway-96 SI system (NEG Combo 5J and NEG MIC 5J panels; Dade Behring, Sacramento, Calif.) with an inoculum of 10^4 CFU per well. Suscepti-

* Corresponding author. Mailing address: Medical Microbiology Laboratory, Funabashi Medical Center, 1-21-1 Kanasugi, Funabashi, Chiba 273-8588, Japan. Phone: 81-47-438-3321. Fax: 81-47-438-7323. E-mail: naganoyn@d3.dion.ne.jp.

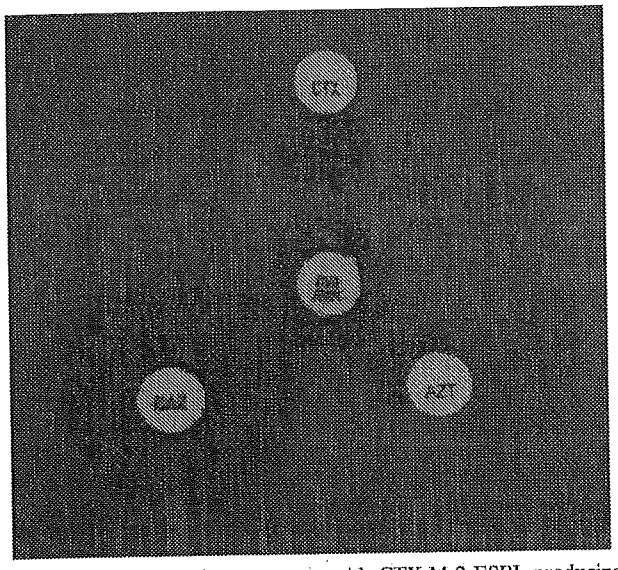

FIG. 1. Double-disk synergy test with CTX-M-2 ESBL-producing *A. baumannii* FM0209680. Disks: CTX, cefotaxime at 30 μg; CAZ, ceftazidime at 30 μg; AZT, aztreonam at 30 μg; CVA · AMPC, clavulanic acid at 10 μg and amoxicillin at 20 μg.

bility categories were determined according to the criteria of the National Committee for Clinical Laboratory Standards (35).

The ESBL plus panel (Dade Behring) with an inoculum of 10^4 CFU per well was used as complementary test for MIC measurements. The panel was incubated for 18 h at 35°C, and then the results were assessed visually.

β-Lactamase study. The double-disk synergy test was used to screen for extended-spectrum β-lactamase (ESBL)-producing strains. Kirby-Bauer disks containing cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and amoxicillin-clavulanic acid (20 μg-10 μg) for tests on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) were obtained from Nissui Pharmaceuticals. The distance between the disks was adjusted so that synergy could be detected correctly (48), as shown in Fig. 1.

The inducibility of AmpC β-lactamase production was tested by disk antagonism tests (30). Disks containing an inducing agent, cefoxitin at 30 μg or ceftazidime at 30 μg, were placed on Mueller-Hinton agar plates (BBL Microbiology Systems). The distance between the disks was adjusted so that the blunting phenomenon of the ceftazidime zone could be detected correctly.

PCR amplification and *bla* gene sequencing. The oligonucleotides used as primers for amplification and sequencing are shown in Table 1. A search for the bla_{TEM} (49), bla_{SHV} (41), $bla_{CTX-M-1}$ (3), $bla_{CTX-M-2}$ (24), and $bla_{CTX-M-9}$ (32) genes in the clinical isolates was performed by PCR amplification, as described previously (34). Detection of the *ampC* gene was performed as described by Bou and Martínez-Beltrán (7).

A resistance plasmid carrying the $bla_{CTX-M-2}$ gene was prepared from the *Escherichia coli* CSH2 (F^- *metB*, with resistance to both nalidixic acid and rifampin [18]) transconjugant. The 902-bp fragments corresponding to the main frame of the $bla_{CTX-M-2}$ gene were amplified by PCR with a set of primers (43) and were used for sequencing. The PCR products were labeled with fluorescent materials by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). Both strands of the DNA sequences were analyzed with an ABI PRISM 377 XL sequencer analyzer (Applied Biosystems). The nucleotide and deduced amino acid sequences were analyzed and compared with sequences in a database by using the FASTA analysis programs available at the National Institute of Genetics (Mishima, Japan) website (http://www.nig.ac.jp/section/service.html).

Plasmid preparation and restriction endonuclease analysis. The resident plasmids of three *A. baumannii* strains and previously isolated *Proteus mirabilis* strains (34) were prepared by a conventional protocol (38). The plasmids were digested with EcoRI, and the restriction fragment profiles were compared by agarose gel electrophoresis.

Automated ribotyping and analysis. Because pulsed-field gel electrophoresis analysis failed to give clear results due to the persistent degradation of DNA, we used automated ribotyping for analysis of the genetic relatedness of the three clinical isolates. Ribotyping was performed with the restriction enzyme EcoRI and a RiboPrinter (Qualicon Inc., Wilmington, Del.), as described previously (13, 33). Briefly, a single colony from a 5% sheep blood agar plate was suspended in a sample buffer and heat treated at 80°C for 15 min. After addition of lysis buffer to release the DNA, the sample was loaded into the RiboPrinter system. Further processing including EcoRI digestion, agarose gel separation, transfer to a nylon membrane, and hybridization with a chemiluminescence-labeled DNA probe containing the rRNA operon from *E. coli*, which was carried out by automated instruments in 8 h. Output ribotype patterns with similarity coefficients of >0.93 were considered a single ribogroup and were automatically given a code number. Computerized ribotypes were finally exported for analysis in text files and imported into BioNumerics software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium) by using the Qualicon macro. Clustering analysis was performed by the unweighted pair group with arithmetic averages method (48), based on the Dice coefficient (16) for band matching, with a position

TABLE 1. Nucleotide sequences of the oligonucleotides used for PCR amplification and DNA sequencing

Procedure and gene	Primer	Expected size of PCR amplicon (bp)	Reference
Amplification			
bla_{TEM-1}	5′-CCG TGT CGC CCT TAT TCC-3′ 5′-AGG CAC CTA TCT CAG CGA-3′	824	50
bla_{SHV-1}	5′-ATT TGT CGC TTC TTT ACT CGC-3′ 5′-TTT ATG GCG TTA CCT TTG ACC-3′	1,051	42
$bla_{CTX-M-1}$	5′-CGG TGC TGA AGA AAA GTG-3′ 5′-TAC CCA GCG TCA GAT TAC-3′	354	3
$bla_{CTX-M-2}$	5′-ACG CTA CCC CTG CTA TTT-3′ 5′-GCT TTC CGC CTT CTG CTC-3′	780	24
$bla_{CTX-M-9}$	5′-GCA GAT AAT ACG CAG GTG-3′ 5′-CGG CGT GGT GGT GTC TCT-3′	393	32
bla_{AmpC}	5′-ACT TAC TTC AAC TCG CGA CG-3′ 5′-TAA ACA CCA CAT ATG TTC CG-3′	663	7
Sequencing, $bla_{CTX-M-2}$	5′-TTA ATG ATG ACT CAG AGC ATT C-3′ 5′-GAT ACC TCG CTC CAT TTA TTG-3′	902	43

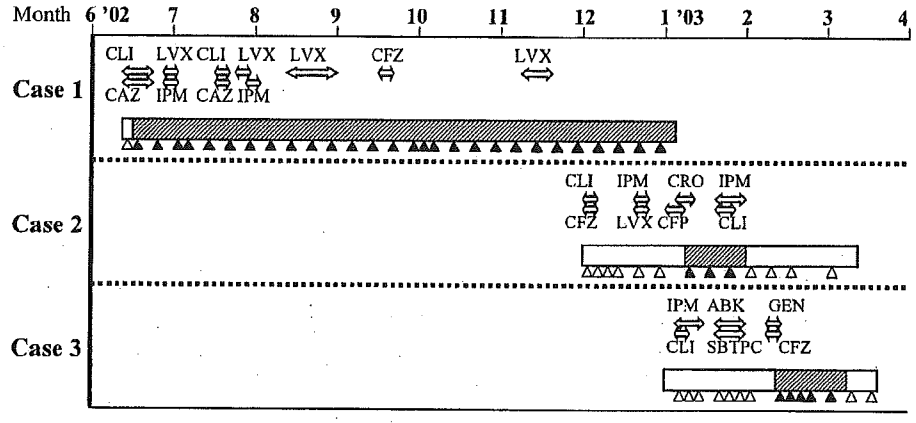

FIG. 2. Sequence of isolation of cefotaxime-resistant *A. baumannii* strains as well as the antimicrobial agents prescribed for three patients. Rectangles, periods of hospitalization; black triangles, times of isolation of cefotaxime-resistant *A. baumannii* from transtracheal aspirate cultures; open triangles, times that cefotaxime-resistant *A. baumannii* strains from transtracheal aspirate cultures were negative; hatched rectangles, times of probable colonization or infection with CTX-M-2 producers; double-headed arrows, prescription periods during which each antimicrobial agent was prescribed; CLI, clindamycin; LVX, levofloxacin; CFZ, cefazolin; CAZ, ceftazidime; IPM, imipenem-cilastatin; CRO, ceftriaxone; CFP, cefoperazone-sulbactam; ABK, arbekacin; GEN, gentamicin; SBTPC, sultamicillin.

tolerance setting of 1.0% (default values are 1% position tolerance and 0.5% optimization). Bands for analysis with the Dice coefficient were assigned manually, according to densitometric curves and the accompanying hard-copy photograph.

RESULTS

Identification of *A. baumannii* isolates and their clinical associations. Strain FM0209680 was derived from the culture of a transtracheal aspirate from a 24-year-old male patient (case patient 1) from whom the first cefotaxime-resistant *A. baumannii* strain was isolated on 19 June 2002. This patient was admitted to the neurosurgery ward on 14 June with a pneumonia-related condition and had a history as a patient in the same ward in October 1999 due to hemorrhaging of the brain caused by underlying cerebral arteriovenous malformation. Until his hospital discharge, on 6 January 2003, cefotaxime-resistant *A. baumannii* isolates were recurrently and predominantly detected in cultures of transtracheal aspirates from the patient. These data point out the great difficulty in eradicating the organisms. Strain FM0300106 was then recovered from the culture of a transtracheal aspirate from a 62-year-old female patient (case patient 2) who had been hospitalized in the neurosurgery ward due to brainstem infarction on 24 November 2002. The cefotaxime-resistant *A. baumannii* strain was first and predominantly detected in the specimen for culture taken on 6 January 2003, although it had not been found in a total of six samples for culture taken up to that time. Strain FM0301433 was recovered from the culture of a transtracheal aspirate from a 65-year-old male patient (case patient 3) who had been hospitalized in the neurosurgery ward due to cerebral hemorrhage on 27 December 2002. As was noted for case patient 2, the cefotaxime-resistant *A. baumannii* strain was first detected predominantly in the specimen for culture taken on 12 February 2003, although it had not been found in a total of seven specimens for culture obtained up to that time. In both case patients 2 and 3, the eradication of the cefotaxime-resistant *A. baumannii* strains was achieved within 3 weeks after the strains were first detected. Imipenem therapy was used for case patient 2 because a complication of pneumonia was a serious concern and the organisms disappeared. The cefotaxime-resistant *A. baumannii* strain was isolated from case patient 3 during prophylactic antimicrobial therapy with gentamicin and cefazolin. Administration of cefazolin may have induced colonization with this organism in case patient 3, in view of the fact that strain FM0301433 was resistant to cefazolin. The prophylactic therapy was then discontinued because no pneumonic clinical findings were noted, and, fortunately, the organism disappeared after 3 weeks.

The history of antimicrobial therapy before isolation of cefotaxime-resistant *A. baumannii* was untraceable for case patient 1 because this patient was transferred from another hospital to the Funabashi Medical Center. Sultamicillin, cefazolin, ceftriaxone, imipenem-cilastatin, cefoperazone-sulbactam, levofloxacin, clindamycin, gentamicin, and arbekacin were used prior to the isolation of *A. baumannii* in case patients 2 and 3. The sequence of isolation of the three cefotaxime-resistant *A. baumannii* strains, as well as the antimicrobial agents prescribed for the three case patients, are summarized in Fig. 2.

Three isolates, FM0209680, FM0300106, and FM0301433, which shared the same biochemical profile code, 0041073, by use of the API NE20 system, combined with a positive result for the ability to grow at 44°C as a complementary test, were eventually identified as *A. baumannii*. The 16S rRNA nucleotide sequences of these microorganisms shared 99% identity with that of *A. baumannii* type strain ATCC 19606 deposited in the EMBL database (accession number Z93435).

Antibiotic susceptibilities. The antibiotic susceptibility profiles of all three *A. baumannii* strains are shown in Table 2. These strains were broadly resistant to penicillins, cephalosporins, cephamycins, and monobactam but were highly susceptible to carbapenems. For all strains, addition of 4 μg of clavulanic acid per ml to the medium for antimicrobial susceptibility testing significantly decreased the MICs of cefotaxime from

TABLE 2. Antibiotic susceptibilities of *A. baumannii* clinical isolates

Antibiotic[a]	MIC (μg/ml)[b]		
	FM0209680	FM0300106	FM0301433
Ampicillin	>16	>16	>16
Amoxicillin-CLA	8/4	8/4	16/8
Piperacillin	>64	>64	>64
Cefazolin	>16	>16	>16
Cefotiam	>16	>16	>16
Cefoperazon-SUL	≤16/8	≤16/8	32/16
Cefotaxime	>128	>128	>128
Cefotaxime-CLA	2	8	4
Ceftazidime	8	8	8
Ceftazidime-CLA	1	4	2
Ceftriaxone	>64	>64	>64
Cefpirome	>16	>16	>16
Cefepime	>32	>32	>32
Cefozopran	>16	>16	>16
Cefaclor	>16	>16	>16
Cefpodoxime	>64	>64	>64
Cefoxitin	>32	>32	>32
Cefmetazole	32	32	>32
Cefotetan	32	32	16
Flomoxef	16	16	16
Imipenem	≤0.5	≤0.5	≤0.5
Meropenem	1	1	1
Aztreonam	>64	>64	>64
Gentamicin	>8	>8	2
Amikacin	32	>32	16
Minocycline	4	4	4
Levofloxacin	4	4	2
Fosfomycin	>16	>16	>16

[a] CLA, clavulanic acid at a fixed concentration of 4 μg/ml; SUL, sulbactam.
[b] NEG Combo 5J and NEG MIC 5J panels and ESBL plus panel were used for MICs determinations.

>128 μg/ml to 2 to 8 μg/ml, while the ceftazidime MICs decreased modestly from 8 μg/ml to 1 to 4 μg/ml.

β-Lactamase production. The production of β-lactamase was initially judged by the P/Case test (37), which can discriminate between penicillinase (with 1.5 mg of benzylpenicillin as the substrate) and cephalosporinase (with 1.5 mg of cephaloridine and 75 μg of clavulanic acid as the substrate). According to the instructions of the manufacturer, the bacterial colonies are rubbed onto two indicator disks containing benzylpenicillin and cephaloridine with clavulanic acid, respectively. When the strain produces any class A β-lactamase, such as TEM- or SHV-derived β-lactamases or CTX-M-type enzymes, a disk containing benzylpenicillin turns yellow, while the other disk containing cephaloridine with clavulanic acid remains purple, because hydrolysis of cephaloridine by the class A β-lactamases is blocked in the presence of clavulanic acid. If the strain produces class C β-lactamases, the disk containing cephaloridine with clavulanic acid turns yellow, because these enzymes are not blocked by clavulanic acid. The results of the test suggested that all three *A. baumannii* strains produce both class A and class C β-lactamases.

The isolates suggested to produce class A β-lactamases were investigated further to determine whether they produce ESBLs by the double-disk synergy test. The presence of an ESBL was suggested by the synergy between amoxicillin-clavulanic acid and cefotaxime, ceftazidime, and aztreonam against all three strains. However, growth of scattered colonies was also noted within the expanded growth-inhibitory zones around the cefotaxime and aztreonam disks (Fig. 1). Enhanced production of β-lactamases as well as alterations in membrane protein composition might be implicated in this phenomenon. A significant decrease in the MICs of cefotaxime from >128 μg/ml to 2 to 8 μg/ml in the presence of 4 μg of clavulanic acid per ml (Table 2) suggested the production of some CTX-M-type class A β-lactamases by these isolates.

As described above, the P/Case test suggested that these isolates produce an AmpC cephalosporinase, and the *ampC* gene (7) was actually detected as a 662-bp PCR amplicon in all three strains, although no obvious inducibility of AmpC β-lactamases was detected in any of them by cefoxitin-ceftazidime disk antagonism tests (data not shown).

No amplicons for the bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}9}$ genes were detected in any of the strains except strain FM0301433, which produced an 824-bp amplicon for the bla_{TEM} gene.

Sequencing of $bla_{CTX\text{-}M\text{-}2}$ gene and plasmid analysis. In a preliminary search by PCR, 780-bp amplification products specific for the $bla_{CTX\text{-}M\text{-}2}$ genes were observed in all three *A. baumannii* strains. The nucleotide sequence of the 902-bp amplicon encoding a full-length *bla* gene, which was amplified with a set of PCR primers (43), was exactly the same as that of the $bla_{CTX\text{-}M\text{-}2}$ gene (GenBank accession number X92507), suggesting that all these strains certainly harbor the $bla_{CTX\text{-}M\text{-}2}$ gene. The nucleotide sequences of the $bla_{CTX\text{-}M\text{-}2}$ gene harbored by the *A. baumannii* strains were exactly the same as that of a *P. mirabilis* strain previously isolated in the urology ward of the Funabashi Medical Center (34). Moreover, the $bla_{CTX\text{-}M\text{-}2}$-bearing plasmids prepared from three *A. baumannii* strains were digested into four different EcoRI fragments of approximately 6.6, 6.4, 4.1, and 3.8 kb (data not shown). The restriction fragment profiles of the plasmids were exactly the same as those of plasmids previously isolated from *P. mirabilis* strains (data not shown).

Ribotyping. In order to investigate the genetic relationship among FM0209680, FM0300106, and FM0301433, the isolates were analyzed by automated ribotyping. Figure 3 shows the results of a clustering analysis performed with the ribotype patterns of the three isolates and some collection strains of the genus *Acinetobacter*, including strains with patterns related to those of European outbreak clones I and II (11, 17) and a newly described clone, clone III (S. Brisse, personal communication). The three *A. baumannii* strains shared the same fingerprint pattern, indicating a clonal lineage. Moreover, the patterns of the three strains showed a high degree of similarity (92%) with those of a clone II strain. Nevertheless, the patterns of all three strains were different from those of the European clones, which, it has been suggested (11), are probably widespread worldwide.

DISCUSSION

This report describes the probable nosocomial transmission of CTX-M-2 β-lactamase-producing *A. baumannii* strains in a neurosurgery ward, where the majority of inpatients are represented by immunocompromised hosts who have undergone respiratory manipulations, such as a tracheotomy or mechanical ventilation, and where careful and strict monitoring is

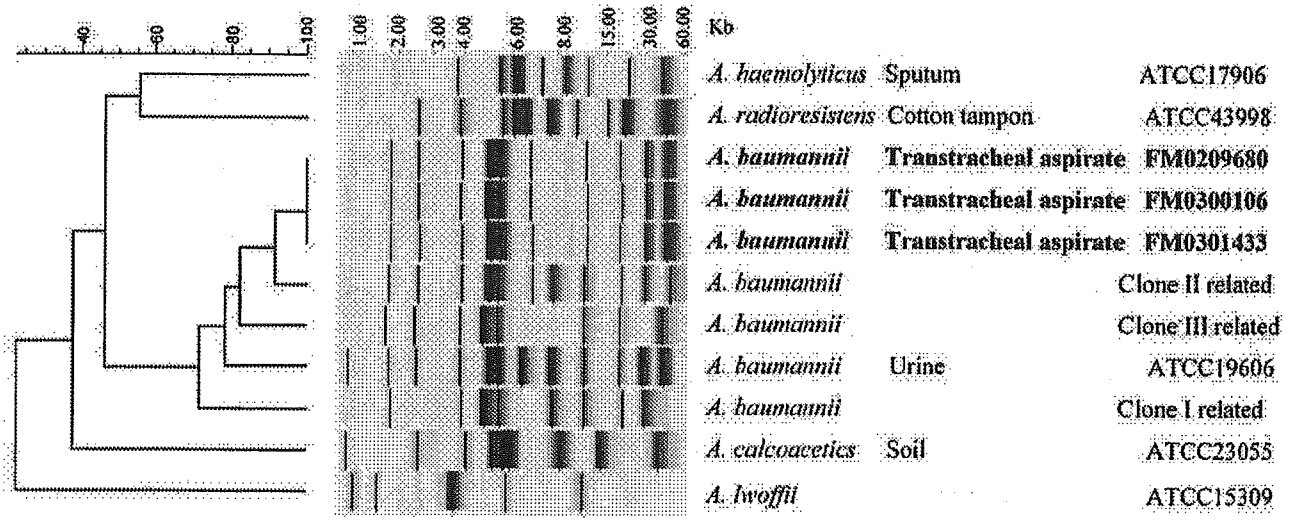

FIG. 3. EcoRI ribotypes of the three cefotaxime-resistant *A. baumannii* clinical isolates. A comparative analysis of these isolates with some collection strains of the genus *Acinetobacter*, including European outbreak clones I, II, and III of *A. baumannii*, is shown. Clustering was performed by the unweighted pair group method with arithmetic averages, and similarity analysis was based on the use of the Dice coefficient.

provided. For case patient 1, a CTX-M-2-producing *A. baumannii* strain was first isolated on day 5 after admission. During the 6-month hospitalization of this patient, cefotaxime-resistant *A. baumannii* strains were detected continuously, despite the administration of imipenem, ceftazidime, and levofloxacin, which consequently failed to eradicate the microorganism. For case patients 2 and 3, CTX-M-2-producing *A. baumannii* strains were detected at the sixth week after admission, and the patients were then colonized for 3 weeks. Imipenem treatment was used to eradicate *A. baumannii* from case patient 2 because a high risk of pneumonia was a serious concern in this patient. On the other hand, prophylactic therapy was discontinued in case patient 3 because no pneumonic clinical findings appeared and the microorganism consequently disappeared. As shown in Fig. 2, it seems very likely that an *A. baumannii* strain harboring the $bla_{CTX\text{-}M\text{-}2}$ gene was persistently transmitted from case patient 1 to case patient 3 via case patient 2 over a period of 9 months in the same neurosurgery ward.

The three *A. baumannii* strains, strains FM0209680, FM0300106, and FM0301433, shared the same ribotype pattern, suggesting a clonal lineage. For all three patients, multiple bacterial species other than *A. baumannii* were frequently coisolated throughout the periods of hospitalization, but fortunately, none was a CTX-M-2 producer. The genes for CTX-M-type β-lactamases are often encoded on plasmids and are easily transmitted among gram-negative bacilli, including members of the family *Enterobacteriaceae* (5, 38), and glucose nonfermenters, such as *Pseudomonas aeruginosa* (8). Since no CTX-M-2 ESBL-producing bacterial isolates, including *A. baumannii*, were previously detected in the neurosurgery ward, it is likely that case patient 1 already carried a CTX-M-2-producing *A. baumannii* isolate before his rehospitalization. However, in the Funabashi Medical Center, a previous outbreak of nosocomial infections in the urology ward, located on a different floor from the neurosurgery ward, involved 19 inpatients and was related to CTX-M-2-producing *P. mirabilis* (34). Agarose gel electrophoresis demonstrated that the $bla_{CTX\text{-}M\text{-}2}$-bearing plasmids found in both bacterial species had the same EcoRI digestion pattern. Therefore, this finding strongly suggests the possibility of the lateral transfer of the plasmids carrying the $bla_{CTX\text{-}M\text{-}2}$ gene between *P. mirabilis* and *A. baumannii* in the clinical setting described here.

A. baumannii strains which produce plasmid-mediated CTX-M-type β-lactamases or TEM- and SHV-derived ESBLs are still very rare. The class A β-lactamases PER-1 and VEB-1, which are genetically distant from the predominant TEM- and SHV-derived ESBLs and CTX-M-type enzymes, were first reported among nosocomial *A. baumannii* isolates in Turkish and French hospitals (41, 52). Similar strains have subsequently been isolated in France (40) and Korea (26). Among the CTX-M-type β-lactamases, the CTX-M-5 gene has been found in *A. baumannii* (GenBank accession number AF462635); however, no details have been published to date. *A. baumannii* usually produces a chromosomally encoded AmpC cephalosporinase, but this kind of enzyme generally cannot hydrolyze oxyiminocephalosporins, cephamycins, or carbapenems. Therefore, acquisition of plasmid-mediated enzymes with broad- and extended-spectrum substrate specificities could well allow this bacterial species to survive in present clinical environments. *A. baumannii* has become one of the major groups of bacteria that causes respiratory infections, especially among patients in intensive care units. Thus, the emergence of CTX-M-2-producing *A. baumannii* strains could become a serious clinical problem in Japan, because CTX-M-2-producing as well as CTX-M-1-producing microorganisms have already been frequently found among clinical isolates from humans (5, 25, 55) and cattle (47).

Early outbreaks due to β-lactamase-producing strains resulted in epidemics caused by isolates with only a single β-lactamase. However, more complex situations involving multiple-β-lactamase-producing microorganisms have been documented in recent nosocomial outbreaks (7, 9, 10, 56). The three *A. baumannii* strains reported in the present study pro-

duced two β-lactamases, the CTX-M-2 enzyme as well as the AmpC β-lactamase. Moreover, the third strain also produced a TEM-type penicillinase. In the present study, the induction of β-lactamase production in *A. baumannii* strains was suggested by the growth of colonies within the growth-inhibitory zones around cefotaxime and aztreonam disks. Such inducible class C β-lactamase production by *A. baumannii* was not detected by previously reported disk antagonism tests with cefoxitin and ceftazidime disks (30), although the presence of the *ampC* gene was confirmed by PCR. Thus, it is likely that some of the colonies appearing in the growth-inhibitory zone around the cefotaxime and aztreonam disks might be mainly constitutive CTX-M-2 producers. It is also possible that these colonies corresponded to AmpC-hyperproducing mutants in which the regulatory system of AmpC production was disrupted through mutations in the *ampC* promoter region or in genes implicated in the regulation of ApmC production, such as *ampR*, *ampG*, and *ampD* (28). These mutations might be easily induced in *A. baumannii* in the presence of cefotaxime, aztreonam, and clavulanic acid. Moreover, all three *A. baumannii* strains characterized in this study demonstrated resistance to cephamycins, such as cefmetazole, cefoxitin, and cefotetan. This may depend on a continuous and moderate level of production of chromosomal AmpC cephalosporinase as well as alterations in bacterial membrane permeability (44). Functional analysis of the *Acinetobacter* AmpC cephalosporinase and further characterization of the nature of the bacterial cells appearing around cefotaxime and aztreonam disks will be continued.

A. baumannii tends to accept foreign DNA as a means to adapt to environments hazardous for bacterial growth (15, 36) and has a high survival capacity in any environment with damp conditions and low temperatures. This inherent ability of *A. baumannii* may facilitate the development of a multidrug resistance profile with the widespread use of antimicrobial agents through the selection of strains with ever accumulating antibiotic resistance profiles in hospital environments. Thus, the emergence of CTX-M-2-producing *A. baumannii* strains not only may confer the potential for epidemics but also could serve as reservoirs for the plasmid-dependent CTX-M-2 enzyme for dispersal of the $bla_{CTX\text{-}M\text{-}2}$ gene among different gram-negative bacterial species. Indeed, although it might be difficult to detect the coproduction of class A β-lactamases like CTX-M-type enzymes in intrinsically cephalosporin-resistant *A. baumannii*, knowledge of the presence of these burdensome strains would seem to be very important in achieving suitable infection control measures in hospitals on a daily basis.

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Metallo-β-Lactamase-Producing Gram-Negative Bacilli: Laboratory-Based Surveillance in Cooperation with 13 Clinical Laboratories in the Kinki Region of Japan

Hisaaki Nishio,[1]* Masaru Komatsu,[2] Naohiro Shibata,[3] Kouichi Shimakawa,[2] Noriyuki Sueyoshi,[4] Toshiro Ura,[5] Kaori Satoh,[6] Masahiro Toyokawa,[7] Tatsuya Nakamura,[8] Yasunao Wada,[9] Tamaki Orita,[10] Tomomi Kofuku,[11] Katsutoshi Yamasaki,[12,13] Masako Sakamoto,[14] Shohiro Kinoshita,[15] Masanori Aihara,[2] and Yoshichika Arakawa[3]

Clinical Laboratory, Shiga Medical Center for Adults,[1] and Clinical Laboratory, Social Insurance Shiga Hospital,[4] Shiga, Department of Clinical Pathology, Tenri Hospital, Nara,[2] Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[3] Department of Clinical and Laboratory Medicine, National Cardiovascular Center,[5] Department of Medical Technology, Kinki University School of Medicine,[6] Laboratory for Clinical Investigation, Osaka University Hospital,[7] Clinical Central Laboratory, Kansai Medical University Hospital,[8] Division of Biomedical Informatics, Course of Health Science, Graduate School of Medicine, Osaka University,[13] and Clinical Laboratory, The Research Foundation for Microbial Diseases of Osaka University,[14] Osaka, Clinical Laboratory, Hyogo Medical University Hospital,[9] Clinical Laboratory, Takarazuka Municipal Hospital,[10] Clinical Laboratory, Hyogo Prefectural Amagasaki Hospital,[11] and Clinical Laboratory, Kobe University Hospital,[15] Hyogo, and Clinical Laboratory, Wakayama Rosai Hospital, Wakayama,[12] Japan

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A total of 19,753 strains of gram-negative rods collected during two 6-month periods (October 2000 to March 2001 and November 2001 to April 2002) from 13 clinical laboratories in the Kinki region of Japan were investigated for the production of metallo-β-lactamases (MBLs). MBLs were detected in 96 (0.5%) of the 19,753 isolates by the broth microdilution method, the 2-mercaptopropionic acid inhibition test, and PCR and DNA sequencing analyses. MBL-positive isolates were detected in 9 of 13 laboratories, with the rate of detection ranging between 0 and 2.6% for each laboratory. Forty-four of 1,429 (3.1%) *Serratia marcescens*, 22 of 6,198 (0.4%) *Pseudomonas aeruginosa*, 21 of 1,108 (1.9%) *Acinetobacter* spp., 4 of 544 (0.7%) *Citrobacter freundii*, 3 of 127 (2.4%) *Providencia rettgeri*, 1 of 434 (0.2%) *Morganella morganii*, and 1 of 1,483 (0.1%) *Enterobacter cloacae* isolates were positive for MBLs. Of these 96 MBL-positive strains, 87 (90.6%), 7 (7.3%), and 2 (2.1%) isolates carried the genes for IMP-1-group MBLs, IMP-2-group MBLs, and VIM-2-group MBLs, respectively. The class 1 integrase gene, *intI1*, was detected in all MBL-positive strains, and the *aac* (*6'*)-*Ib* gene was detected in 37 (38.5%) isolates. Strains with identical PCR fingerprint profiles in a random amplified polymorphic DNA pattern analysis were isolated successively from five separate hospitals, suggesting the nosocomial spread of the organism in each hospital. In conclusion, many species of MBL-positive gram-negative rods are distributed widely in different hospitals in the Kinki region of Japan. The present findings should be considered during the development of policies and strategies to prevent the emergence and further spread of MBL-producing bacteria.

Metallo-β-lactamases (MBLs) are enzymes belonging to Ambler's class B that can hydrolyze a wide variety of β-lactams, including penicillins, cephems, and carbapenems (14, 30, 42). The acquisition by gram-negative rods of MBLs, which are often encoded by mobile genetic elements such as cassettes inserted into integrons, confers a multidrug resistance profile against many clinically important β-lactams as well as other antimicrobial agents (1). This fact raises a significant problem with respect to antimicrobial chemotherapy (38). Plasmid-mediated MBLs are categorized into three major molecular types: they are IMP-type, VIM-type, and SPM-type enzymes (14, 21, 30, 32, 39, 42). Among them, IMP-1-type MBLs have been identified in various gram-negative bacilli belonging to the family *Enterobacteriaceae* and in several non-glucose-fermenters, such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. (13, 18, 19, 20, 36–38, 43). Furthermore, in Japan, several variants of the IMP-1 type, including IMP-3 from *Shigella flexneri* (15), IMP-6 from *Serratia marcescens* (47), IMP-10 from *P. aeruginosa* and *Alcaligenes xylosoxidans* (16), and IMP-11 (EMBL/GenBank accession no. AB074437) from *P. aeruginosa* and *Acinetobacter baumannii*, have been characterized recently. VIM-type MBLs, including VIM-1 and VIM-2 from *P. aeruginosa* isolates in Italy and France, respectively (21, 32), were first described in 1999. Outbreaks of VIM-type MBL-positive strains have also been reported in Italy and Greece (8, 40). SPM-1, a member of the third group of plasmid-mediated MBLs, was recently detected in *P. aeruginosa* isolates in Brazil, and SPM-1 producers appear to be widely disseminated in Brazil (11).

* Corresponding author. Mailing address: Clinical Laboratory, Shiga Medical Center for Adults, 5-4-30 Moriyama, Moriyama, Shiga 524-8524, Japan. Phone: 81-775-82-5031 (ext. 3241). Fax: 81-775-82-5426. E-mail: qq6w62t9@chive.ocn.ne.jp.

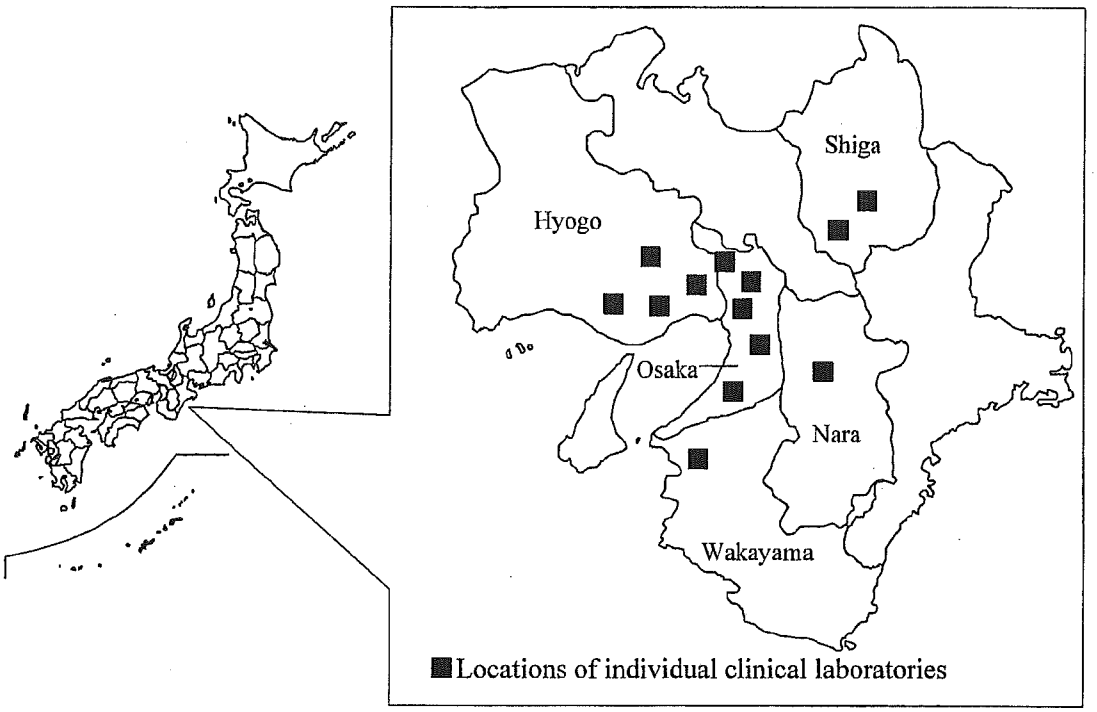

FIG. 1. Locations of the 13 institutions involved in this study of the Kinki region of Japan.

Previously reported survey data from the Kinki region of Japan revealed that 0.7% of isolates produced IMP-1-group MBLs (44). The prevalence of IMP-1-group MBLs among gram-negative rods has also been investigated (19, 36); however, the prevalence of the new plasmid-mediated MBLs, such as the IMP-2 group (33) and the VIM-2 type (32), in Japan remains unclear.

For the present study, to assess the prevalence and types of MBL-positive bacteria in the Kinki region of Japan, we investigated almost 20,000 isolates collected from 12 general hospitals and one commercial laboratory.

MATERIALS AND METHODS

Bacterial isolates. This laboratory-based surveillance study was conducted with the cooperation of 13 institutions (12 hospital clinical laboratories and one commercial laboratory) in the Kinki region, which is located in western Japan (Fig. 1), with the assistance of the National Institute of Infectious Diseases of Japan. Between October 2000 and March 2001 (first study period) and November 2001 and April 2002 (second study period), a total of 19,753 isolates of gram-negative bacilli, including *P. aeruginosa* (6,198 isolates), *Acinetobacter* spp. (1,108 isolates), *Escherichia coli* (4,347 isolates), *Klebsiella pneumoniae* (2,354 isolates), *S. marcescens* (1,429 isolates), *Enterobacter cloacae* (1,483 isolates), *Citrobacter freundii* (544 isolates), *Klebsiella oxytoca* (627 isolates), *Enterobacter aerogenes* (454 isolates), *Proteus mirabilis* (470 isolates), *Morganella morganii* (434 isolates), *Proteus vulgaris* (178 isolates), and *Providencia rettgeri* (127 isolates), were isolated from various clinical specimens and then tested. A single isolate was selected from each patient and identified by use of a MicroScan Neg Combo 5J panel (Dade Behring, Tokyo, Japan). Moreover, *Acinetobacter* isolates were identified by use of an ID TEST NF-18 panel (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). For *Acinetobacter* spp. other than *A. baumannii*, PCR amplification of the 16S rRNA gene was performed, with genomic DNA as the template, according to a previously published protocol (34), and the amplicons were sequenced. The sequence data were submitted to the DNA Data Bank of Japan (DDBJ) database to check the identity or similarity of each sequence against the database by use of the FASTA program (http://www.ddbj.nig.ac.jp/search/Welcome-e.html).

First screening for MBL production. MIC criteria for the first screening of MBL producers were >16 μg of ceftazidime/ml for *Acinetobacter* spp. and >16 μg of both ceftazidime and cefoperazone-sulbactam/ml for gram-negative organisms other than *Acinetobacter* spp. The production of MBLs was assessed with a 2-mercaptopropionic acid inhibition (2-MPA) test as described previously (2, 37). Test strains were cultured, adjusted to a 0.5 McFarland standard, diluted with 0.85% saline, and inoculated onto Mueller-Hinton agar plates according to the protocol recommended by the NCCLS (27). Two Senci-Disks (Becton Dickinson Co., Ltd., Tokyo, Japan) containing 30 μg of ceftazidime, 10 μg of imipenem, and 30 μg of cefepime were placed at a distance of 50 mm from each other on the plate, and one blank disk was placed near one of the Senci-Disks at a distance of 20 mm. Two to 3 μl of 2-MPA was added to the blank disk. After an overnight incubation at 35°C, if an expansion of the growth inhibition zone around either the ceftazidime, imipenem, or cefepime disk was observed, the strain was interpreted as being positive for MBL.

Susceptibility testing for antimicrobial agents. The MICs of antimicrobial agents for isolates that tested positive in the 2-MPA test were subjected to antimicrobial susceptibility testing by the broth microdilution method with dry plates (Eiken Chemical Co., Ltd., Tokyo, Japan), which conformed to NCCLS guidelines (26, 28). The following antimicrobial agents and concentrations were used: piperacillin (2 to 128 μg/ml), piperacillin-tazobactam (1-4 to 128-4 μg/ml), ceftazidime (1 to 128 μg/ml), cefepime (1 to 128 μg/ml), cefoperazone-sulbactam (1-0.5 to 128-64 μg/ml), aztreonam (1 to 128 μg/ml), cefmetazole (1 to 128 μg/ml), moxalactam (1 to 128 μg/ml), meropenem (0.25 to 32 μg/ml), imipenem (0.25 to 32 μg/ml), gentamicin (1 to 8 μg/ml), amikacin (4 to 32 μg/ml), minocycline (4 to 8 μg/ml), levofloxacin (2 to 4 μg/ml), trimethoprim-sulfamethoxazole (9.5-0.5 to 38-2 μg/ml), and chloramphenicol (8 to 16 μg/ml). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as reference strains for quality control of the tests (26).

PCR amplification and DNA sequencing. Isolates that tested positive in the 2-MPA test were then assessed for their MBL type by PCR and DNA sequencing. PCRs were performed as described previously (35). PCR primers for the amplification of each MBL gene were constructed as described in previous reports for bla_{IMP-1} (35), bla_{IMP-2} (33), and bla_{VIM-2} (32). Primers for the amplification of integrase genes (*intI1*, *intI2*, and *intI3*) (31, 37) and the aminogly-

TABLE 1. Primers for PCR and sequencing of MBL genes

Target	Use	Primer name	Primer sequence (5′ to 3′)	Position[a]	Product length (bp)	Reference
IMP-1	**Amplification**	**IMP1L**	**CTACCGCAGCAGAGTCTTTG**	**47–66**	**587**	**35**
		IMP2R	AACCAGTTTTGCCTTACCAT	633–614		
	Sequencing	IMP1-SQ-F	ACCGCAGCAGAGTCTTTGCC	49–68	587	37
		IMP1-SQ-R	ACAACCAGTTTTGCCTTACC	635–616		
IMP-2	**Amplification**	**IMP2L**	**GTGTATGCTTCCTTTGTAGC**	**23–42**	**174**	**33**
		IMP2R	CAATCAGATAGGCGTCAGTGT	196–176		
	Sequencing	IMP2-SQ-F	GTTTTATGTGTATGCTTCC	16–34	678	37
		IMP2-SQ-R	AGCCTGTTCCCATGTAC	693–677		
VIM	**Amplification**	**VIMB**	**ATGGTGTTTGGTCGCATATC**	**152–171**	**510**	**32**
		VIMF	TGGGCCATTCAGCCAGATC	661–643		
	Sequencing	VIM2-SQ-F	ATGTTCAAACTTTTGAGTAAG	1–21	801	37
		VIM2-SQ-R	CTACTCAACGACTGAGCG	801–784		

[a] Position number 1 for every MBL gene corresponds to the first base of the start codon.

coside resistance gene [*aac (6′)-Ib*] (35) were described previously. The PCR and DNA sequencing primers used are listed in Table 1.

PCR products were sequenced by the dideoxynucleotide chain termination method (45) in an automated DNA sequencer (ABI 3100; Perkin-Elmer Applied Biosystems, Foster City, Calif.). Similarity searches against sequence databases were performed with an updated version of the FASTA program available from the Center for Information Biology and DNA Data of Japan for Biotechnology Information server of the National Institute of Genetics of Japan (http://www.ddbj.nig.ac.jp/).

RAPD pattern analysis. The isolates that were confirmed to be positive for the MBL gene by PCR and chromosomal DNA typing were analyzed by random **amplified polymorphic DNA (RAPD) analysis to generate a DNA fingerprint** (29). The RAPD primers were ERIC2 (5′-AAGTAAGTGACTGGGGTGAGC G-3′) for *Enterobacteriaceae* other than *S. marcescens* (41), HLWL-74 (5′-CGT CTATGCA-3′) and 1254 (5′-AACCCACGCC-3′) for *S. marcescens* (12), 272 (5′-AGCGGGCCAA-3′) for *P. aeruginosa* (6), and A5 (5′-GCCGGGGCCT-3′) for *Acinetobacter* spp. (31).

RESULTS

Prevalence of MBL-positive isolates. The prevalence of isolates that produced MBLs is shown in Table 2. Seven hundred **fifty-seven isolates (3.8%) fulfilled the MIC criteria for the** production of MBLs. Of these 757 isolates, 96 (12.7%) were positive in the 2-MPA test. Of these 96 positive isolates, only 1 *E. cloacae* isolate appeared to have a weak and ambiguous growth inhibition zone (data not shown) in the 2-MPA test. All 96 isolates that tested positive in the 2-MPA test were positive for at least one MBL gene by PCR and DNA sequencing. The numbers of MBL-positive isolates with an MBL gene were 44 (3.1%) for *S. marcescens*, 22 (0.4%) for *P. aeruginosa*, 21 (1.9%) for *Acinetobacter* spp., 4 (0.7%) for *C. freundii*, 3 (2.4%) for *Providencia rettgeri*, 1 (0.2%) for *M. morganii*, and 1 (0.1%) for *E. cloacae*. Of 21 isolates of *Acinetobacter* spp., 18 were **identified as** *A. baumannii*, and the remaining three strains were *A. johnsonii*, *A. junii*, and *A. calcoaceticus* according to 16S rRNA sequencing analysis and their biochemical properties.

The results of the MBL assessments in each laboratory are shown in Table 3. These 13 laboratories included 5 laboratories in university hospitals, 7 laboratories in general hospitals, and 1 commercial laboratory. MBL-positive isolates were detected in 9 of 13 laboratories; the overall rate of detection was 0.5% and ranged from 0 to 2.6% in each laboratory.

Genetic characterization of MBL-producing isolates. Some characteristics and selected clinical associations of MBL-producing isolates are shown in Table 4. All MBL-producing iso-

TABLE 2. Prevalence of metallo-β-lactamase-producing isolates

Organism	No. of isolates collected			**No. of isolates fulfilling** MIC criteria			No. (%) of MBL-producing isolates[c]		
	2000[a]	2001[b]	Total	2000[a]	2001[b]	Total	2000[a]	2001[b]	Total
Pseudomonas aeruginosa	2,645	3,553	6,198	141	141	282	8 (0.3)	14 (0.4)	22 (0.4)
Escherichia coli	1,334	3,013	4,347	3	8	11	0 (0)	0 (0)	0 (0)
Klebsiella pneumoniae	867	1,487	2,354	1	2	3	0 (0)	0 (0)	0 (0)
Serratia marcescens	615	814	1,429	101	55	156	26 (4.2)	18 (2.2)	44 (3.1)
Enterobacter cloacae	565	918	1,483	81	90	171	0 (0)	1 (0.1)	1 (0.1)
Acinetobacter spp.	388	720	1,108	20	14	34	13 (3.4)	8 (1.1)	21 (1.9)
Citrobacter freundii	234	310	544	23	37	60	0 (0)	4 (1.3)	4 (0.7)
Klebsiella oxytoca	227	400	627	0	0	0	0 (0)	0 (0)	0 (0)
Enterobacter aerogenes	194	260	454	11	12	23	0 (0)	0 (0)	0 (0)
Proteus mirabilis	176	294	470	1	4	5	0 (0)	0 (0)	0 (0)
Morganella morganii	166	268	434	4	2	6	1 (0.6)	0 (0)	1 (0.2)
Proteus vulgaris	97	81	178	0	0	0	0 (0)	0 (0)	0 (0)
Providencia rettgeri	45	82	127	5	1	6	3 (6.7)	0 (0)	3 (2.4)
Total	7,553	12,200	19,753	391	366	757	51 (0.7)	45 (0.4)	96 (0.5)

[a] First study period, October 2000 to March 2001.
[b] Second study period, November 2001 to April 2002.
[c] Percentages were calculated as follows: no. of MBL-producing isolates/no. of isolates collected × 100%.