10- and CMY-11-producing isolates of the family *Enterobacteriaceae* was also reported in Korea (18, 19). In the present study, CMY-type β-lactamase-producing *K. pneumoniae* and *E. coli* clinical isolates from a Japanese general hospital were investigated. Through PFGE analysis, it was found that the five *K. pneumoniae* isolates had little genetic relatedness to each other, while the nine *E. coli* isolates belonged to the same clonal lineage. Interestingly, plasmid analysis showed that all 14 isolates harbored a very similar conjugal plasmid that encodes a CMY-type β-lactamase which was either CMY-9 or CMY-19, a variant that differs from CMY-9 by a single amino acid substitution (I292S). Since the flanking structures of the bla_{CMY} genes were identical in all plasmids, it is probable that one conjugal plasmid carrying bla_{CMY-9} was horizontally transferred to *E. coli*, *K. pneumoniae*, and then an *E. coli* clone and various *K. pneumoniae* strains harboring the bla_{CMY} gene-bearing plasmids might have spread in the hospital.

β-Lactamases can modify their substrate specificity through a single amino acid substitution (30). CMY-19 had a single amino acid substitution, I292S, near the H-10 helix domain, compared with the sequence of CMY-9 (Fig. 1). Indeed, a serine residue at the same amino acid position was found in all the FOX-type enzymes (15, 26), including CAV-1 (14), and also in CMY-11 (21), as shown Fig. 1; but no peculiar behavior against cefepime was documented with those enzymes. Through the I292S substitution, CMY-19 would have developed extended substrate specificity against cefepime and cefpirome, as well as ampicillin, piperacillin, cephaloridine, and ceftazidime, compared with that of CMY-9, although the hydrolyzing activities against ceftizoxime, cefotaxime, and cephamycins were impaired. The expansion of hydrolyzing activity against cefepime found in CMY-19 was a most remarkable property because cefepime is generally stable against AmpC β-lactamases (11). Similar developments of extended hydrolyzing activity against cefepime through amino acid substitutions or deletions adjacent to the H-10 helix have been observed in several chromosomally encoded AmpC β-lactamases, such as the AmpC of *Serratia marcescens* that lacks four amino acids at positions 293 to 296 (22), an AmpC of *E. coli* that lacks three amino acids at positions 286 to 288 (13), an AmpC of *Enterobacter cloacae* that lacks six amino acids at positions 289 to 294 (6), and an AmpC of *Enteroacter aerogenes* that acquired an L293P substitution (5). Furthermore, Barlow and Hall reported on the in vitro selection of CMY-2 β-lactamase variants with several amino acid substitutions, including replacements at positions 292, 293, 294, 296, and 298, which demonstrated increased resistance to cefepime (4). From our previous molecular modeling analyses (13), it was found that the expansion of an open space in the vicinity of the R-2 side chain of ceftazidime or cefepime through the deletion of tripeptides in the H-10 helix of *E. coli* AmpC (Fig. 1) played a crucial role in the acquisition of the greater hydrolyzing activity against those agents possessing a bulky R side chain. Although the actual mechanism for the higher cefepime-hydrolyzing activity in CMY-19 compared to that in CMY-9 has not been elucidated, a similar molecular distortion at the active center of the enzyme might well have occurred in CMY-19. This speculation would be substantiated by molecular modeling and X-ray crystallographic analyses.

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A Nosocomial Outbreak Due to Novel CTX-M-2-Producing Strains of *Citrobacter koseri* in a Hematological Ward

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Citrobacter koseri is a member of the family *Enterobacteriaceae*. Urinary tract infections caused by *C. koseri* have been observed in as many as 12% of all isolates in adults (1). In compromised hosts, *Citrobacter* spp. could cause pneumonitis, empyema (2), biliary infection (3), and bacteremia (4). *Citrobacter* spp. were formerly susceptible to oximinocephalosporins including cefotaxime (3), but recently, *C. koseri* has been reported to have developed resistance to some cephalosporins and cephamycins through the production of an inducible chromosomally-encoded cephalosporinase that can inactivate these agents (5). Most clinically isolated *C. koseri* are susceptible to oximinocephalosporins and carbapenems. Recently, oximinocephalosporin resistance among Gram-negative bacteria has been developed due to the hydrolysis of beta-lactams by beta-lactamases including extended-spectrum beta-lactamases (ESBLs). ESBLs show variable levels of resistance to cefotaxime, ceftazidime, and other broad-spectrum cephalosporins and monobactams. Nosocomial outbreaks due to SHV-4-type ESBL-producing strains and TEM-type ESBL-producing strains of *C. koseri* have already been reported (6,7). We have identified a novel CTX-M-2-type of ESBL among nosocomially isolated *C. koseri* strains, causing a probable outbreak in the hematological ward.

Sixty-eight strains of *C. koseri* were isolated from the blood, urine, feces, sputum, ascites, and pharynx of 31 patients with a hematological malignancy that had lasted over 18 months (Figure 1). *C. koseri* not only colonized but also caused bacteremia, urinary tract infection, enteritis, and peritonitis. These strains showed similar antibiotic susceptibility profiles (Table 1). We collected 5 strains of *C. koseri* from 4 patients (Table 2) and used the double-disk synergy test and plasmid profiling to screen for ESBL-producing strains as reported previously (8,9). All of the 5 strains harbored a plasmid mediating the CTX-M-2 type beta-lactamase gene. Epidemiological study using pulsed-field gel electrophoresis (PFGE) of total DNA prepared from the 5 strains revealed patterns that were indistinguishable from each other (Figure 2). The results suggested that the 5 strains characterized belong to a single epidemic strain.

In general, multiple factors may help to decrease the immunity of patients with hematological malignancies, including impairment of phagocytosis, impaired cellular immunity, and defective production of antibodies. Moreover, intensive chemotherapies usually induce severe granulocytopenia. Thus, bacterial infections are a major cause of complications and death in patients with hematological malignancies. Recently, two studies (10,11) revealed the efficacy of the prophylactic use of quinolon by neutropenic patients. As for febrile neutropenia, empirical antibiotic therapy using cefepime or cefotaxime has been emphasized (12,13). All 31 patients in this study had hematological malignancy and underwent intensive chemoradiotherapy. After that, most of the patients in our ward were administered prophylactic and therapeutic systemic antibiotics such as quinolon, cefepime, and cefotaxime, which might well be associated with the selection of antibiotic-resistant microorganisms. Unlike other members of the family *Enterobateriaceae*, CTX-M-2-producing *C. koseri* might survive in a patient's bowel flora, because of its resist-

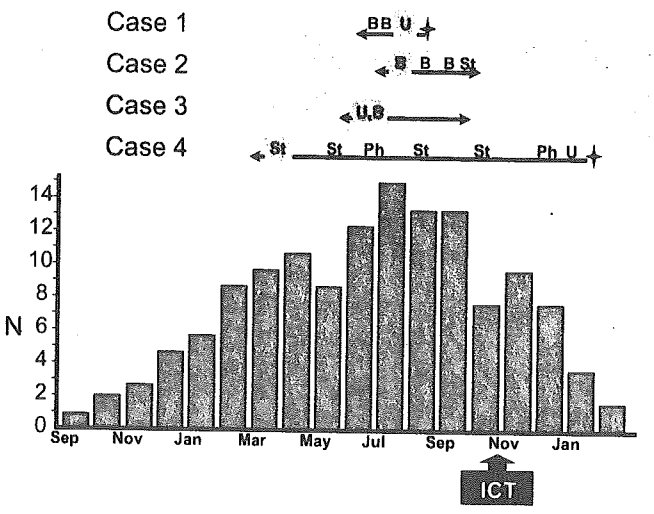

Fig. 1. The number of patients infected and/or colonized with *C. koseri*. Bars indicate the number of patients infected and/or colonized per month with *C. koseri*, the antibiotic susceptibility of which showed the same pattern. Case numbers are identical to those in Table 1. Arrows indicate the duration of each patient's hospitalization in the ward. The bald signs on each arrow indicate the samples, from which *C. koseri* was isolated. The network-breaking characters indicate the samples, from which genetically identical strains were isolated in our study. An infection control team (ICT) intervened in the ward to resolve the outbreak (see article). B, blood; U, urine; St, stool; Ph, pharynx.

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Table 1. Antibiotics susceptibility profile of *C. koseri* isolated in this outbreak

ampicillin	>16
ampicillin/ clavulanate	16
piperacillin	>64
cefazolin	>16
cefotaxime	>16
cefotiam	>32
ceftazidime	>16
cefpirome	>16
cefpodoxime proxetil	>4
cefcapene pivoxil	>1
cefmetazole	>32
flomoxef	32
sulbactam/cefoperazone	>32
aztoreonam	>16
imipenem	<1
gentamicin	<1
amikacin	<1
minomicin	2
levofloxacin	>4

MICs were determined by microdilution method recommended by NCCLS (currently CLSI) guideline with Muller-Hinton broth (Difco, Detroit, Mich., USA) using MicroScan-kit (Dade Behring, West Sacramento, Calif., USA).

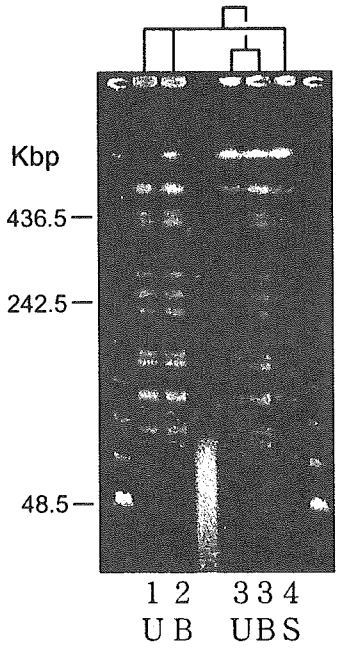

Fig. 2. PFGE analysis of *C. koseri* strains. Bacterial DNA was extracted, digested and subjected to PFGE, as previously described (8,9). Lanes 1, 2, 3, and 4, were sampled from patients Nos. 1, 2, 3, and 4, respectively. U, B, and S indicate urine, blood, and stool, respectively.

Table 2. Profiles of cases involved in the outbreak

No.	Age/Sex	Underlying disease	Therapy/Outcome	Infection	Sources of *C. koseri*
1	71/M	malignant lymphoma	chemotherapy/refractory	sepsis	Urine
2	61/M	adult T-cell leukemia	chemotherapy/partial response	sepsis	Blood
3	25/F	acute lymphoblastic leukemia	bone marrow transplantation	sepsis	Urine, Blood
4	63/F	acute lymphoblastic leukemia	chemotherapy/complete remission	enteritis	Stool

ance to quinolon, cefepime, and cefotaxime. In addition, urinary tract infections tended to be easily associated with urinary catheterization in our cases. We speculated that the situation was as follows. Once *C. koseri* colonizes in the bladder or intestine, it will then disseminate into the blood stream causing severe bacteremia during intensive chemotherapy. The symptoms of sepsis caused by *C. koseri* were often very serious, and could only be cured by appropriate and immediate administration of carbapenem. However, the use of carbapenem in high amounts and at high frequency in our ward could create a grave epidemiological problem.

The number of *C. koseri* infections increased significantly, and standard infection control measures were not effective to stop this outbreak. Therefore, we began to enforce the following precautions. We introduced barrier precautions against not only infected patients but also colonized patients, using disposable gloves and drapes. Mandatory hand washing was done immediately before and after any manipulation involved in the nursing care. Hand hygiene using commercial alcoholic disinfectant (Welpas; Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) was promoted not only for medical workers but also for patients. As for the environment, the water taps were converted to the hands-free types, and all doorknobs and bars for drip injection were sterilized using 70% alcohol twice daily. We also tried to restrict the prophylactic use of quinolon for high-risk patients with neutropenia decreasing under 100/μL which was keeping for more than 1 week. After these procedures, the incidence of *C. koseri* isolation decreased, but this type of infection has not yet been eradicated, as shown in Figure 1. We continue to make an effort to prevent nosocomial transmission of *C. koseri*.

In this report, we emphasize the appearance of *C. koseri* and its new type of drug resistance. We also warn that it is quite difficult to control the outbreak of such antimicrobial-resistant microorganisms in a hematological ward. In the future, we must pay close attention to the nosocomial spread of this type of *C. koseri*, which has demonstrated resistance to a broad spectrum of cephalosporins, cephamycins, and carbapenems.

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Novel Plasmid-Mediated 16S rRNA Methylase, RmtC, Found in a *Proteus mirabilis* Isolate Demonstrating Extraordinary High-Level Resistance against Various Aminoglycosides

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***Proteus mirabilis* ARS68, which demonstrated a very high level of resistance to various aminoglycosides, was isolated in 2003 from an inpatient in Japan. The aminoglycoside resistance of this strain could not be transferred to recipient strains *Escherichia coli* CSH-2 and *E. coli* HB101 by a general conjugation experiment, but *E. coli* DH5α was successfully transformed by electroporation with the plasmid of the parent strain, ARS68, and acquired an unusually high degree of resistance against aminoglycosides. Cloning and sequencing analyses revealed that the presence of a novel 16S rRNA methylase gene, designated *rmtC*, was responsible for resistance in strain ARS68 and its transformant. The G+C content of *rmtC* was 41.1%, and the deduced amino acid sequences of the newly identified 16S rRNA methylase, RmtC, shared a relatively low level of identity (≤29%) to other plasmid-mediated 16S rRNA methylases, RmtA, RmtB, and ArmA, which have also been identified in pathogenic gram-negative bacilli. Also, RmtC shared a low level of identity (≤28%) with the other 16S rRNA methylases found in aminoglycoside-producing actinomycetes. The purified histidine-tagged RmtC clearly showed methyltransferase activity against *E. coli* 16S rRNA in vitro. *rmtC* was located downstream of an IS*Ecp1*-like element containing *tnpA*. Several plasmid-mediated 16S rRNA methylases have been identified in pathogenic gram-negative bacilli belonging to the family *Enterobacteriaceae*, and some of them are dispersing worldwide. The acceleration of aminoglycoside resistance among gram-negative bacilli by producing plasmid-mediated 16S rRNA methylases, such as RmtC, RmtB, and RmtA, may indeed become an actual clinical hazard in the near future.**

Aminoglycosides have been widely used for the treatment of a variety of bacterial infections (9). These agents bind to the A site of the 16S rRNA of prokaryotic 30S ribosomal subunits and subsequently block bacterial growth through interference with protein synthesis (17). On the other hand, bacteria have acquired resistance to aminoglycosides by producing aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases, aminoglycoside nucleotidyltransferases, and aminoglycoside phosphotransferases (17, 24). Moreover, reduction of affinity for the target site within 16S rRNA by nucleic acid point mutations, the excretion of aminoglycosides by the augmented function of efflux systems, and altered membrane permeability, which leads to the reduced penetration of these agents, also contribute to the intrinsic clinical resistance of bacteria (3, 17).

Recently, as a new mechanism of resistance against aminoglycosides among clinically important pathogenic bacteria, plasmid-mediated 16S rRNA methylase (RmtA) was first characterized in a clinically isolated *Pseudomonas aeruginosa* strain, strain AR-2. This strain was isolated in 1997 in a Japanese hospital and demonstrated consistent resistance to various clinically important aminoglycosides (29). The total sequence of a large plasmid carrying genes for both CTX-M-3 and 16S rRNA methylase was then submitted to the EMBL/GenBank database (accession no. AF550415) on 18 October 2002 by M. Golebiewski et al., although they did not seem to be aware of the presence of the *armA* gene in the sequence deposited in the database. In 2003, the *armA* gene, found in a clinically isolated *Klebsiella pneumoniae* strain, was reported from France (7). RmtB, which was encoded on a nonconjugative plasmid of a clinically isolated *Serratia marcescens* strain, was also reported from Japan in 2004 (6). At present, the three types of plasmid-mediated 16S rRNA methylases described above have been found in pathogenic gram-negative rods. More recently, nosocomial outbreaks caused by 16S rRNA methylase-producing gram-negative bacteria was reported from Taiwan (28). The further global dissemination of 16S rRNA methylase genes among pathogenic bacilli will be a cause of great concern in the near future, because these genes were mediated by some bacterial site-specific recombination and translocation systems such as a transposon (6, 7, 26).

A *Proteus mirabilis* strain, strain ARS68, which displayed a very high level of resistance to various aminoglycosides, was isolated in 2003 from an inpatient in Japan. A preliminary PCR analysis, however, failed to detect any of the known three plasmid-mediated 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, in this strain. Therefore, it was strongly suspected that the *P. mirabilis* ARS68 strain would have a novel 16S rRNA methylase gene. In the present study, the molecular mecha-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source
Strains		
P. mirabilis ARS68	Clinical isolate resistant to various aminoglycosides	This study
E. coli DH5α	*supE44 ΔlacU169* (*ϕ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB*$^+$	TOYOBO
E. coli CSH-2	*metB* F$^-$ nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli HB101	*thi-1 hsdS20*(r_B^- m_B^+) *supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rps20* (Str^r) *xyl-5 mtl-1*	A. Ohta, Tokyo University
E. coli BL21(DE3)pLysS	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3) pLysS	Novagen
Plasmids		
pARS68	A natural plasmid carrying *rmtC* of *P. mirabilis* ARS68	This study
pBC-E1	A recombinant plasmid carrying a 7.7-kb EcoRI fragment containing *rmtC*	This study
pBC-KB1	A recombinant plasmid carrying a PCR-amplified fragment containing *rmtC* and its promoter	This study
pBC-Sa1	A recombinant plasmid carrying *aph(3′)*	This study
pGEM-rmtC	A recombinant plasmid carrying PCR-amplified *rmtC* ligated to the pGEM-T vector	This study
pET-His-rmtC	A recombinant plasmid carrying *rmtC* ligated to pET29a(+)	This study
pBCSK+	A cloning vector, chloramphenicol^r	Stratagene
pGEM-T	A cloning vector, ampicillin^r	Promega
pET29a(+)	An expression vector, kanamycin^r	Novagen

[a] r, resistant to the indicated antimicrobial agent.

nism underlying a very high level of resistance against various aminoglycosides found in strain ARS68 was elucidated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. mirabilis* strain ARS68 was isolated in August 2003 from a throat swab of an inpatient admitted to a general hospital in Japan. Biochemical phenotypic identification of this strain was performed with a commercially supplied API 20E system (bioMerieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing. The MICs of antimicrobial agents were determined by the agar dilution method with Mueller-Hinton agar plates, according to the protocol recommended by CLSI (formerly the National Committee for Clinical Laboratory Standards) (18). The following antibiotics were obtained from the indicated sources: amikacin, Bristol Pharmaceuticals K. K., Tokyo, Japan; arbekacin, kanamycin, and streptomycin, Meiji Seika Kaisha, 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; and tobramycin, Shionogi & Co. Ltd., Osaka, Japan.

PCR amplification. The sets of PCR primers and amplification conditions used to detect the three 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, are referred to in our recent study (27).

Transfer of aminoglycoside resistance. Conjugal transfer was performed by using *E. coli* CSH-2 (F$^-$ *metB*, resistant to both nalidixic acid and rifampin) or *E. coli* HB101 (resistant to streptomycin) as a recipient by a filter-mating method. Transconjugants were selected on Luria-Bertani (LB) agar plates containing rifampin (100 μg/ml) and kanamycin (30 μg/ml) or arbekacin (10 μg/ml) when *E. coli* CSH-2 was used as the recipient. Two kinds of streptomycin-containing (50 μg/ml) LB agar plates supplemented with kanamycin (30 μg/ml) or arbekacin (10 μg/ml) were also prepared when *E. coli* HB101 was used as the recipient. The plasmid DNA of *P. mirabilis* ARS68 was prepared by the method of Kado and Liu (14). *E. coli* DH5α was transformed with the plasmids of *P. mirabilis* ARS68

TABLE 2. Results of antibiotic susceptibility testing

Aminoglycoside	MIC (μg/ml)				
	P. mirabilis ARS68(pARS68)	*E. coli* DH5α(pARS68)	*E. coli* DH5α(pBC-E1)	*E. coli* DH5α(pBC-KB1)	*E. coli* DH5α(pBCSK+)
4,6-Substituted deoxystreptamine antimicrobials					
Kanamycin group					
Arbekacin	>1,024	512	512	>1,024	0.25
Amikacin	>1,024	1,024	512	>1,024	0.5
Kanamycin	>1,024	>1,024	>1,024	>1,024	1
Tobramycin	1,024	256	128	512	0.25
Gentamicin group					
Gentamicin	>1,024	256	512	>1,024	0.13
Sisomicin	>1,024	512	256	>1,024	0.13
Isepamicin	>1,024	>1,024	1,024	>1,024	0.13
4,5-Substituted deoxystreptamine antimicrobials					
Neomycin	512	16	0.5	1	0.5
Another aminoglycoside					
Streptomycin	4	2	2	2	2

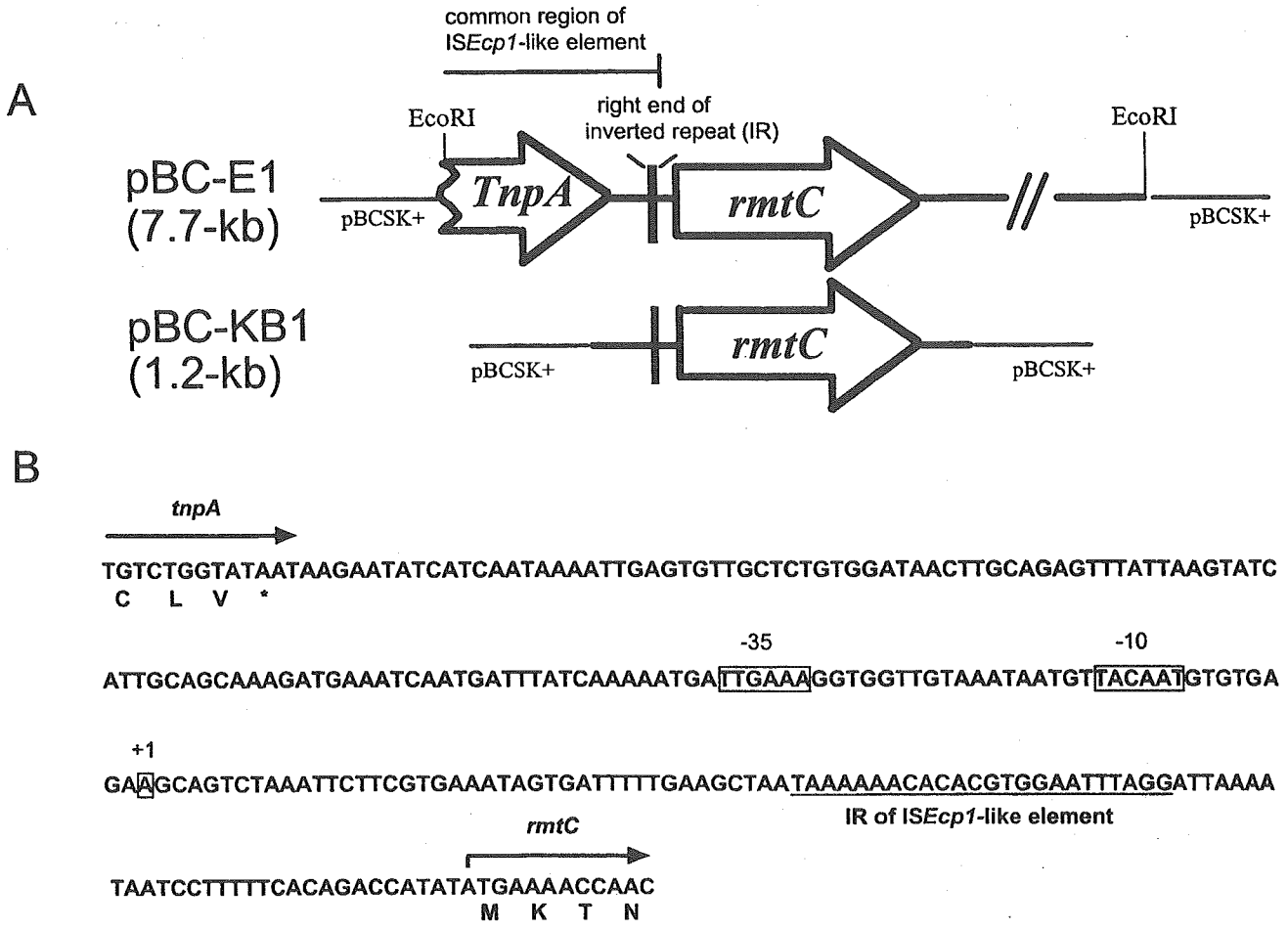

FIG. 1. (A) Schematic presentation of the 7.7-kb EcoRI fragment on pBC-E1 and the 1.2-kb PCR fragment on pBC-KB1. (B) Part of the nucleotide sequences encoding the 3′ end of an IS*Ecp1*-like element and the start region of *rmtC*. The predicted −35 and −10 promoter sequences and the +1 position of the putative transcriptional start of *rmtC* are boxed. These positions were cited elsewhere (4). Arrows indicate the transcription orientation. The deduced amino acid sequences are designated in single-letter code. The right inverted repeat (IR) of an IS*Ecp1*-like element is underlined.

by electroporation techniques. Transformants were selected on LB agar plates supplemented with arbekacin (4 μg/ml) or kanamycin (10 μg/ml).

Cloning and sequencing of aminoglycoside resistance determinants. Both total DNA and plasmid DNA were prepared from the bacterial strains as described previously (23) and restricted with endonucleases according to the recommendations of the supplier. The digested fragments were ligated to restriction enzyme-cleaved pBCSK+ (Stratagene, La Jolla, Calif.), and *E. coli* competent cells were transformed by electroporation with the mixture of recombinant plasmids. Transformants were selected on LB agar plates containing chloramphenicol (30 μg/ml) and arbekacin (4 μg/ml) or kanamycin (10 μg/ml). Both strands of the nucleotide sequences of the cloned fragment encoding the gene responsible for aminoglycoside resistance were determined with BigDye Terminator cycle sequencing ready reaction kits and an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, Calif.) by using several custom sequencing primers.

PCR cloning of aminoglycoside resistance gene. The DNA fragment carrying the aminoglycoside resistance gene and its promoter region was amplified by PCR with the primers rmtC-F (5′-CGC GGA TCC AGT GTA TGA AAA ATG TCT GG-3′) and rmtC-R (5′-CGG GGT ACC GGT GTG TTA GAA TTT GCC TT-3′) (where the underlining indicates the restriction site of BamHI or KpnI). The resultant fragments were digested with BamHI and KpnI and ligated to pBCSK+ (Stratagene).

Expression and purification of histidine-tagged enzyme. The gene responsible for aminoglycoside resistance was amplified from plasmid pBC-E1 by using primers that introduced NdeI and XhoI sites at the ends of the amplified fragments. This fragment was ligated to the pGEM-T vector (Promega, Madison, Wis.), and one plasmid with no amplification error (pGEM-rmtC) was selected. A single nucleotide mutation which leads to the silent mutation (T to C) at position 171 was introduced to destroy the NdeI site within the fragment inserted on pGEM-rmtC by using an LA PCR in vitro mutagenesis kit (Takara Bio Inc., Ohtsu, Japan). A resultant plasmid was digested with NdeI and XhoI and ligated into the pET-29a(+) vector (Novagen, Madison, Wis.) restricted with the same enzymes. The newly constructed expression vector, pET-His-rmtC, was introduced into *E. coli* (DE3)pLysS (Novagen) and cultured in 1 liter of LB broth containing both kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was added when the culture reached an A_{600} of 0.55, and the culture was incubated for an additional 3 h. The bacterial pellet harvested by centrifugation was washed with 50 mM phosphate buffer (pH 7.0) and suspended in 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. The suspension was passed through a French pressure cell (Ohtake Works Co., Ltd., Tokyo, Japan) at 120 MPa and then centrifuged at 100,000 × *g* for 1 h. The supernatant containing the fusion protein was loaded onto a HisTrap HP column and purified according to the manufacturer's instructions (Amersham Biosciences, K. K., Tokyo, Japan). The eluted fusion protein was dialyzed against 20 mM Tris-HCl buffer (pH 7.5), applied to an anion-exchange HiTrap Q HP column (Amersham Biosciences), and eluted with a linear gradient of NaCl. Finally, size-exclusion chromatography was performed with a Superdex 200 HR10/30 column (Amersham Biosciences). The purified protein was dialyzed against HRS buffer (10 mM HEPES-KOH, pH 7.5; 10 mM $MgCl_2$; 50 mM NH_4Cl; 3 mM 2-mercaptoethanol). The purity was checked by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The protein con-

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RmtC   MKTNDNYIEEVTAKVLTSGKYSTLYPPTVRRVTERLFDRYP-PKQLEKEVRKKLHQAYGA
RmtA   -----MSFDDALASILSSKKYRSLCPDTVRRILDQEWGRHKSPKLAVEATRTRLHGICGA
RmtB   -----MNINDALTSILASKKYRALCPDTVRRILTEEWGRHKSPKQTVEAARTRLHGICGA
ArmA   -----MDKNDVVKKILESKKYENLDSDIVEKVVSISEKKYK-LKEVENYSKKKLHQIWGS
                ::.  .:* * **  * .  *.::      ::   *   :  :.:**   *:

RmtC   YIGGIDGKRLEKKIEKIIHEIPNPTTDEATRTEWEKEICLKILNLHTSTNERTVAYDELY
RmtA   YVT--------------------PESLKAAAAALSVGDVQKALSLHASTKERLAELDCLY
RmtB   YVT--------------------PESLKAAAAALSAGDVKKALSLHASTKERLAELDTLY
ArmA   YYSAYP-----------------NWDKLLKKYNQGQLSIEDLLKIHSSTNERVATLNDFY
       *                                          . *.:*:**:** .  : :*

RmtC   QKIFEVTGVPTSITDAGCALNPFSFPFFTEAGMLGQYIGFDLDKGMIEAIEHSLRTLNAP
RmtA   DFIFSG-GVPHRVLDIACGLNPLALFIRDITSVWACDIHQGLGDVITPFAHH-----QGL
RmtB   DFIFSA-ETPRRVLDIACGLNPLALYERGIASVWGCDIHQGLGDVITPFARE-----KDW
ArmA   TYVFGNIKHVSSILDFGCGFNPLALYQWNENEKIIYHAYDIDRAEIAFLSSIIGKLKTTI
         :*        : * .*.:**:::                    :

RmtC   EGIVVKQGDILSDPSGESDLLLMFKLYTLLDRQEEASGLKILQEWKYKNAVISFPIKTIS
RmtA   DFTFALQDVMCTPPTETGDLALVFKLLPLLEREQAGAAMALLQALATPRIAVSFPTRSLG
RmtB   DFTFALQDVLCAPPAEAGDLALIFKLLPLLEREQAGSAMALLQSLNTPRMAVSFPTRSLG
ArmA   KYRFLNKESDVYKG--TYDVVFLLKMLPVLK-QQDVNILDFLQLFHTQNFVISFPIKSLS
       .  .  :           *: :::*: .:*. ::    : :**     . .:*** :::.

RmtC   GRDVGMEENYTVKFENDLVGSDLRIMQKLKLGNEMYFIVSRL--
RmtA   GRGKGMEANYSAWFEGALP-DEFEIEDTKTIGIELVYMIKRNK-
RmtB   GRGKGMEANYAAWFEGGLP-AEFEIEDKKTIGTELIYLIKKNG-
ArmA   GKEKGMEENYQLWFESFTK-GWIKILDSKVIGNELVYITSGFQK
       *:  *** **   **.      :.* :.  :* *: :: .
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FIG. 2. Alignment of the deduced amino acid sequence of RmtC with those of RmtA, RmtB, and ArmA. Asterisks indicate the conserved residues among the above four 16S rRNA methylases.

centration was estimated by use of the Coomassie Plus protein assay reagent and bovine serum albumin as a standard (Pierce Biotechnology, Rockford, Ill.). The N-terminal sequence of the purified protein was obtained by Edman degradation in a Shimadzu model PPSQ-23 automated protein sequencer.

Preparation of 30S ribosomal subunits. The 30S ribosomal subunits of *E. coli* DH5α were prepared as described by Skeggs et al. (25). After ultracentrifugation with sucrose density gradients, fractions of the 30S ribosomal subunits were collected and concentrated by centrifugation with an Ultrafree-15 centrifugal filter device (Millipore Corporation, Bedford, Mass.). The purity of the 30S ribosomal subunit was checked by denatured agarose gel electrophoresis of the 16S rRNA derived from the material, and the 30S ribosomal subunit was stored at −80°C in aliquots until use.

Methylation assay of 30S ribosomal subunits. The methylation assay of the 30S ribosomal subunits was carried out as described by Doi et al. (6), with some modifications, as follows. The reaction mixture contained 20 pmol 30S ribosomal subunits from *E. coli* DH5α, 20 pmol histidine-tagged RmtC, and 5 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]SAM); and this mixture was adjusted to 200 μl with methylation buffer (50 mM HEPES-KOH, pH 7.5; 7.5 mM $MgCl_2$; 37.5 mM NH_4Cl; 3 mM 2-mercaptoethanol). In control experiments, histidine-tagged RmtC was replaced by an equal volume of heat-inactivated histidine-tagged RmtC, bovine serum albumin, and HRS buffer. Samples (35 μl) were taken at 0, 5, 15, 30, and 60 min and purified with an RNeasy Mini kit (QIAGEN K.K., Tokyo, Japan), according to the instructions provided by the manufacturer. Two micrograms of eluted 16S rRNA was spotted onto a DEAE filter mat for MicroBeta (Perkin-Elmer Life Sciences Japan Co., Ltd., Tokyo, Japan). The filter mat was then covered with MeltiLex for MicroBeta filters (Perkin-Elmer) on a hot plate. Finally, it was applied to a 1450 MicroBeta TRILUX (Perkin-Elmer), and the radioactivity of each spot was counted.

Nucleotide sequence accession number. The open reading frame of *rmtC* was deposited in the EMBL and GenBank databases through the DDBJ database and has been assigned accession number AB194779.

RESULTS

Characteristics of *P. mirabilis* strain ARS68. Clinically isolated *P. mirabilis* strain ARS68 showed an extraordinary high level of resistance (MIC, ≥1,024 μg/ml) to the various clinically important aminoglycosides except streptomycin and neomycin, as shown in Table 2. PCR analyses were performed preliminarily to detect three 16S rRNA methylase genes, *rmtA*, *rmtB*, and *armA*, which were previously found in pathogenic gram-negative bacilli; but none of them was detected in this strain.

Transfer of aminoglycoside resistance. The aminoglycoside resistance of *P. mirabilis* strain ARS68 could not be transferred to the recipients *E. coli* CSH-2 and *E. coli* HB101 by conjugation under the experimental conditions used in this study. However, *E. coli* DH5α was successfully transformed by electroporation with the plasmid, pARS68, prepared from *P. mirabilis* ARS68. The size of plasmid pARS68 was estimated to be ca. >100 kb by summation of the SacI-digested DNA fragment sizes observed by agarose gel electrophoresis (data not shown).

TABLE 3. Amino acid identities among various 16S rRNA methylases

16S rRNA methylase	G+C content (%)	Identity (%) of amino acid residues							
		Plasmid-mediated 16S rRNA methylases among pathogenic gram-negative bacilli			Chromosomally encoded 16S rRNA methylases among aminoglycoside-producing actinomycetes				
		RmtA	RmtB	ArmA	GrmA	KgmB	GrmO	FmrO	Kmr
RmtC	41.1	27.7	29.5	27.8	26.5	23.1	25.4	23.0	22.0
RmtA	55.4		82.0	29.2	31.7	29.5	28.1	27.3	28.7
RmtB	55.6			28.9	31.7	26.4	28.9	28.5	26.3
ArmA	30.4				26.3	26.6	20.6	28.0	24.4

E. coli DH5α(pARS68) demonstrated a very high degree of resistance to various aminoglycosides, as was observed in the parent strain (Table 2).

Cloning of aminoglycoside resistance determinant. A cloning experiment was performed to confirm the genetic aminoglycoside resistance determinant of *P. mirabilis* ARS68 and its transformant, *E. coli* DH5α(pARS68). As a result, one recombinant plasmid (pBC-E1) with a 7.7-kb EcoRI insert derived from pARS68 was obtained by selection with arbekacin and chloramphenicol, and the insert was then sequenced. A part of the cloned fragment sequenced is shown in Fig. 1A. The first 0.5 kb of the insert contained the 3′ end of the *tnpA* gene with a terminal inverted repeat (IR). This region containing the IR had a high degree of similarity at the nucleotide level with the IS*Ecp1* element, which was often identified upstream of several genes encoding CTX-M-type β-lactamases and CMY-type cephalosporinases (2, 4, 10, 20, 21). One open reading frame, which encoded 281 amino acids, was located downstream of *tnpA*. A BLAST analysis of the deduced amino acid sequence revealed that the gene product exhibited low-level identities to the 16S rRNA methylases, RmtA, RmtB, and ArmA (28%, 29%, and 28%, respectively), found in pathogenic gram-negative bacilli. The predicted enzyme was designated RmtC, and a comparison of the deduced amino acid sequences of RmtA, RmtB, and ArmA is shown in Fig. 2. RmtC also has a low degree of similarity (≤28%) to other 16S rRNA methylases found in aminoglycoside-producing *Streptomyces* and *Micromonospora* species. The amino acid similarities among 16S rRNA methylases are summarized in Table 3. The putative promoter region of *rmtC* appeared to be located within the IS*Ecp1*-like element, just upstream of the IR generally found among several CTX-M-type and CMY-type β-lactamase genes (Fig. 1B) (4, 10, 20, 22). One Sau3AI fragment carrying the aminoglycoside phosphotransferase gene, *aph(3′)*, was also cloned from *P. mirabilis* strain ARS68 when kanamycin was used as a selection marker.

Antibiotic susceptibilities. The MICs of the aminoglycosides for parental strain *P. mirabilis* ARS68, *E. coli* DH5α(pARS68), *E. coli* DH5α(pBC-E1), and *E. coli* DH5α(pBC-KB1) are shown in Table 2. *E. coli* DH5α(pARS68) demonstrated resistance to all the various aminoglycosides except streptomycin and neomycin. RmtC-producing strains *E. coli* DH5α(pBC-E1) and *E. coli*(pBC-KB1) showed high levels of resistance to 4,6-disubstituted deoxystreptamine antimicrobials belonging to the kanamycin and gentamicin groups but were susceptible to the 4,5-disubstituted deoxystreptamine antimicrobial neomycin and another aminoglycoside, streptomycin. *E. coli* DH5α(pBC-Sa1), which carried the aminoglycoside phosphotransferase gene, *aph(3′)*, showed resis-

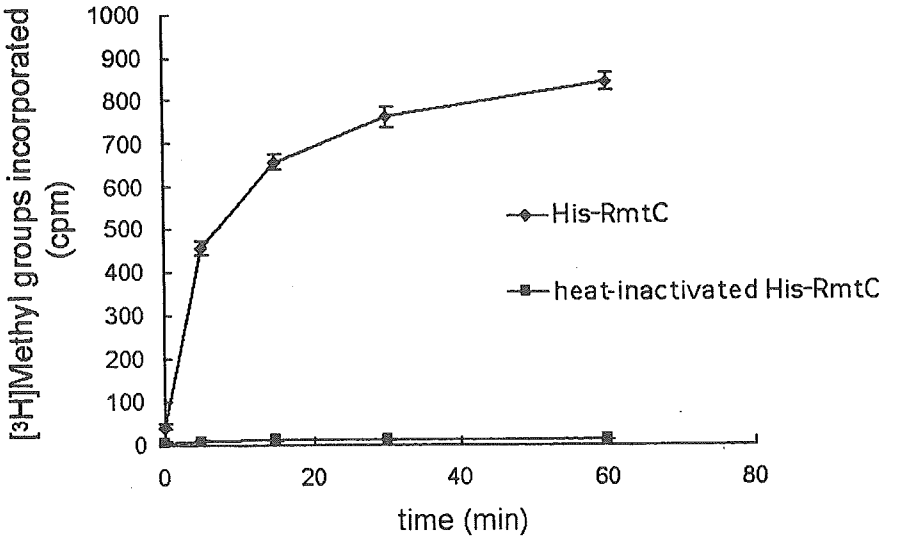

FIG. 3. Methylation of 16S rRNA. The 16S rRNA from *E. coli* DH5α was incubated with purified histidine-tagged RmtC (His-RmtC) by using [*methyl*-^{3}H]SAM as a cofactor. The value of each point was calculated with three datum points. Error bars indicate standard deviations.

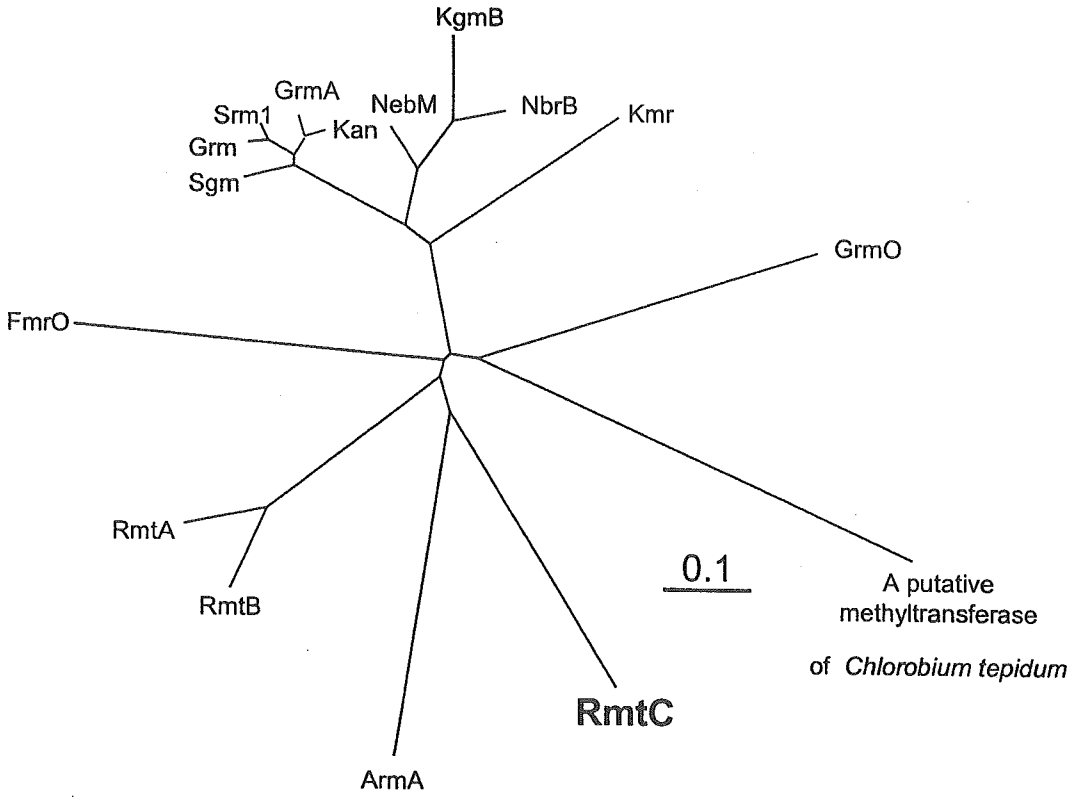

FIG. 4. Dendrogram of 16S rRNA methylases. Sequences are from *P. mirabilis* (RmtC; GenBank accession number AB194779), *P. aeruginosa* (RmtA; GenBank accession number AB083212) (29), *S. marcescens* (RmtB; GenBank accession number AB103506) (6), *K. pneumoniae* (ArmA; GenBank accession number AY220558) (7), *Micromonospora zionensis* (Sgm; GenBank accession number A45282) (16), *Micromonospora rosea* (Grm; GenBank accession number M55521) (15), *Micromonospora inyoensis* (Srm1; GenBank accession number AY661430), *Micromonospora echinospora* (GrmA; GenBank accession number AY524043), *Streptomyces* sp. (Kan; GenBank accession number AJ414669), *Streptomyces tenebrarius* (NebM; GenBank accession number AJ550991), *S. tenebrarius* (KgmB; GenBank accession number S60108) (13), *Streptoalloteichus hindustanus* (NbrB; GenBank accession number AF038408), *Streptoalloteichus kanamyceticus* (Kmr; GenBank accession number AJ582817) (5), *Micromonospora olivasterospora* (FmrO; GenBank accession number D13171) (19), *M. echinospora* (GrmO; GenBank accession number AY524043), and *Chlorobium tepidum* TLS (putative methytransferase; GenBank accession number AAM72273). The "0.1" scale represents a genetic unit reflecting 10% of the amino acid substitutions calculated with the ClustalW program (http://www.ddbj.nig.ac.jp/search/Welcome-e.html) provided by the DDBJ (http://www.ddbj.nig.ac.jp/Welcome-e.html).

tance to both neomycin (MIC, 1,024 μg/ml) and kanamycin (MIC, >1,024 μg/ml). The resistance to neomycin found in strain ARS68 seemed to be attributable to the presence of *aph(3')*.

Identification of RmtC as a 16S rRNA methyltransferase. Histidine-tagged RmtC-producing *E. coli* BL21(DE3)pLysS showed resistance to arbekacin, while *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS, which carried the pET29a(+) vector, were susceptible to arbekacin. This finding indicated that the production of histidine-tagged RmtC was responsible for the aminoglycoside resistance in *E. coli* BL21(DE3)pLysS. The N-terminal sequence of the purified protein was determined to be MKTND. The result of the methylation assay is shown in Fig. 3. Purified histidine-tagged RmtC readily methylated 30S ribosomal subunits prepared from *E. coli* DH5α in the presence of the methyl group donor [*methyl*-^{3}H]SAM as a cosubstrate in a time-dependent manner. On the other hand, incubation with heat-inactivated histidine-tagged RmtC did not increase the counts of radioactivity. When an equal volume of bovine serum albumin or HRS buffer was used in place of purified histidine-tagged RmtC, no increase in the radioactivity counts was observed (data not shown).

DISCUSSION

In the present study, we found a new 16S rRNA methylase gene, *rmtC*, in a clinical *P. mirabilis* isolate and characterized it precisely. The production of RmtC conferred a high degree of resistance mainly against 4,6-disubstituted deoxystreptamines but not against non-4,6-disubstituted deoxystreptamines, such as streptomycin and neomycin, as did RmtA, RmtB, and ArmA. Although the methylation site in the 16S rRNA has not been clarified yet, it was speculated that G1405 within the A site of 16S rRNA would be methylated by these plasmid-mediated 16S rRNA methylases, since the methylation of G1405 by some 16S rRNA methylases produced by actinomycetes was reported to confer resistance against 4,6-disubstituted deoxystreptamines but not against 4,5-disubstituted deoxystreptamines, such as neomycin (1). RmtC as well as RmtA, RmtB, and ArmA might well confer resistance against 4,6-disubstituted deoxystreptamines through a manner similar to that in aminolycoside-producing actinomycetes. The methylation site in the 16S rRNA introduced by these enzymes will be elucidated in a forthcoming study.

Interestingly, all the plasmid-mediated 16S rRNA methylase

genes found so far were associated with some genes implicated in gene recombination systems. For example, the *rmtA* gene was flanked by a 262-bp sequence called the κγ element that was initially found in Tn*5041* and that was predicted to be a relic of mobile genetic elements (26). The *rmtB* gene was located just downstream of the 3′ end of the insertion sequence of Tn*3* (6). As for the two genes described above, the mode of actual translocation of the fragments containing the 16S rRNA methylase genes has not been elucidated in detail. On the other hand, it was reported that the *armA* gene was mediated by a composite transposon Tn*1548* and was successfully transposed in vitro (8). Although the *rmtC* gene was also associated with a *tnpA* gene encoding a probable transposase, the actual mode of translocation of the regions carrying the *rmtC* gene is unclear. However, it is speculated that the presence of an IS*Ecp1*-like element located upstream of *rmtC* would be responsible for the actual translocation process, because several CTX-M-type β-lactamase genes located downstream of *tnpA* within the IS*Ecp1* element were able to be transposed in vitro (4, 21). Characterization of the genetic environment mediating the *rmtC* gene and the mode of translocation will be undertaken in another study.

As was observed in the phylogenic tree (Fig. 4), a cluster of plasmid-mediated 16S rRNA methylases is antithetical to that of the 16S rRNA methylases from actinomycetes. Although no progenitor of plasmid-mediated 16S rRNA methylases, including RmtA, RmtB, RmtC, and ArmA, has been found to date, these genes might have been derived from unknown environmental aminoglycoside-producing bacteria.

In conclusion, we identified a novel plasmid-mediated 16S rRNA methylase, RmtC, in a clinical *P. mirabilis* isolate that demonstrated an extraordinarily high level of aminoglycoside resistance like actinomycetes. The nosocomial transmission of pathogens that produce plasmid-dependent 16S rRNA methylases has recently been reported from Taiwan (28), and an ArmA-producing *E. coli* isolate was isolated from the feces of a diarrheic pig in Spain in 2002 (11, 12). Special caution should be taken because of the emergence and spread of pathogenic bacteria that have acquired various new antimicrobial resistance genes, including *rmtC*, *rmtB*, and *rmtA*, especially in both clinical and livestock farming environments, where large amounts of antimicrobial agents have routinely been used.

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Practical Methods Using Boronic Acid Compounds for Identification of Class C β-Lactamase-Producing *Klebsiella pneumoniae* and *Escherichia coli*

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Detection of the resistance mediated by class C β-lactamases remains a challenging issue, considering that transferable plasmid-mediated class C β-lactamases are of worldwide concern. Methods for the identification of strains that produce extended-spectrum β-lactamases (ESBLs) or metallo-β-lactamases (MBLs) have been developed and applied for routine use in clinical microbiology laboratories, but no practical methods for identification of plasmid-mediated class C producers have been established to date. We therefore developed three simple methods for clinical microbiology laboratories that allow identification of plasmid-mediated class C β-lactamase-producing bacteria using a boronic acid derivative, 3-aminophenylboronic acid (APB), one of the specific inhibitors of class C β-lactamases. Detection by the disk potentiation test was based on the enlargement of the growth-inhibitory zone diameter (by greater than or equal to 5 mm) around a Kirby-Bauer disk containing a ceftazidime (CAZ) or a cefotaxime (CTX) disk in combination with APB. In a double-disk synergy test, the discernible expansion of the growth-inhibitory zone around the CAZ or the CTX disk toward a disk containing APB was indicative of class C β-lactamase production. A greater than or equal to eightfold decrease in the MIC of CAZ or CTX in the presence of APB was the criterion for detection in the microdilution test. By using these methods, *Escherichia coli* and *Klebsiella pneumoniae* isolates producing plasmid-mediated class C β-lactamases, ACT-1, CMY-2, CMY-9, FOX-5, LAT-1, and MOX-1, were successfully distinguished from those producing other classes of β-lactamases, such as ESBLs and MBLs. These methods will provide useful information needed for targeted antimicrobial therapy and better infection control.

The production of β-lactamases is the major mechanism of resistance to β-lactams, which are most frequently used for the treatment of various infectious diseases. Class C β-lactamases, which belong to group 1 according to the classification of Bush et al. (7), are cephalosporinases, which are poorly inhibited by β-lactamase inhibitors, such as clavulanic acid (CLA) and sulbactam. Class C β-lactamases are clinically important because they usually confer resistance to a variety of β-lactams, including oxyiminocephalosporins and some cephamycins, as well as penicillins and monobactam, when they are produced in large amounts (14, 21, 32). They are usually chromosomally encoded AmpC enzymes in several bacterial species belonging to the family *Enterobacteriaceae*, including *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens*, and *Escherichia coli*, which are frequently encountered as nosocomial pathogens. Moreover, since the first report of transferable plasmid-mediated class C β-lactamases, such as MIR-1, in the late 1980s (3, 30), their increasing presence worldwide is becoming of great concern (9, 13, 22, 24). In Japan, MOX-1 (15), CMY-9 (10), and CMY-2 and DHA-1 (unpublished data) have been identified so far. Although simple methods for the identification of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) have been established and are already in laboratory use (1, 29), detection of the resistance mediated by class C β-lactamases still remains a challenging issue. Several methods that use the Kirby-Bauer (KB) disk potentiation method (20, 21, 34, 35, 45) with some β-lactamase inhibitors (2, 5) or the three-dimensional method (9, 22, 39) have been developed; and a cefoxitin agar medium-based assay that uses preparations of bacterial cell extracts has been reported (26). However, these methods are technically intricate, and interpretation of their results is not sufficiently simple for routine use in clinical microbiology laboratories. PCR or multiplex PCR analyses are able to provide satisfactory results in the identification and classification of genes for β-lactamases (25, 31, 38, 44), but equipment availability is limited to medical institutions, such as university hospitals. They are also costly and require time-consuming techniques. An enzyme-linked immunosorbent assay has also been developed and has known sensitivity and specificity for the detection of certain class C β-lactamases. This technique is less costly than genetic methods, but it is not sensitive for the detection of class C β-lactamases that possess less than 70% homology to CMY-2 (16). Thus, practical and simple methods for detection of the resistance mediated by plasmid-mediated class C β-lactamases are urgently needed for enhanced infection control.

In 1982, boronic acids were reported as reversible inhibitors of AmpC enzymes belonging to the class C β-lactamases (4). Serial studies revealed the structure-based mechanism of inhibition of AmpC β-lactamases by boronic acids (34, 37, 41), and novel compounds that inhibit AmpC β-lactamases with nano-

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TABLE 1. Bacterial strains used in this study and MICs of CAZ and CTX with or without β-lactamase inhibitors

Strain	β-Lactamase	MIC (μg/ml)								Reference
		CAZ	CAZ + CLA	CAZ + SMA	CAZ + APB	CTX	CTX + CLA	CTX + SMA	CTX + APB	
E. coli NS12	CMY-2	128	64	128	4	64	32	64	2	This study
E. coli HKY515	CMY-2	256	128	256	4	64	32	64	4	This study
E. coli HKY701	CMY-2	128	128	128	2	32	32	64	4	This study
E. coli MRY041197	CMY-2	>256	256	>256	8	128	128	128	8	This study
E. coli HKY581	CMY-2	256	128	256	8	128	128	256	4	This study
E. coli C502	CMY-2	256	256	256	16	16	32	32	2	This study
E. coli KG2	CMY-2	128	128	64	4	64	32	64	8	This study
E. coli MRY041243	CMY-8	256	256	>256	4	64	32	64	0.5	This study
E. coli M68	CMY-9	256	256	256	1	>256	>256	>256	16	10
E. coli Coral Gables 66040	FOX-5	>128	>128	>128	4	64	64	64	1	G. A. Jacoby
E. coli Coral J53 (a transformant)	ACT-1	8	4	4	≤0.25	2	2	2	≤0.25	G. A. Jacoby
E. coli HKY28	Mutant AmpC	64	32	64	4	16	4	8	1	11
K. pneumoniae NU2936	MOX-1	64	32	32	0.5	256	256	256	8	15
K. pneumoniae HKY-L1	MOX-1	32	32	32	1	128	128	128	4	This study
K. pneumoniae KPW142	CMY-8	32	32	64	1	128	128	128	4	This study
K. pneumoniae HKY209	CMY-9	>128	>128	>128	2	>128	>128	>128	2	This study
K. pneumoniae HKY327	CMY-19	>256	>256	>256	16	64	64	64	1	This study
K. pneumoniae 5064	FOX-5	64	64	64	2	8	16	16	0.5	36
K. pneumoniae Bronx Lebanon 18	ACT-1	64	64	128	64	8	16	8	0.5	G. A. Jacoby
K. pneumoniae P20	LAT-1	64	64	64	2	32	32	32	1	40
Hafnia alvei EE47[a]	AmpC	64	128	64	2	32	64	32	2	This study
E. coli NCB03522[b]	CMY-2 + CTX-M-9	64	16	64	16	256	8	256	256	This study
K. pneumoniae NCB02189[a]	DHA-1 + CTX-M-9	16	128	16	1	32	8	32	2	This study
E. coli AYW-1	TEM-26	>128	0.5	>128	>128	2	<0.06	2	2	44
E. coli HKY322	TEM-91	128	0.5	128	>128	1	<0.06	1	1	18
E. coli MRY041435	TEM-132	64	1	64	64	8	≤0.25	4	4	This study
E. coli HKY453	SHV-24	>128	2	>128	>128	2	0.13	2	2	17
E. coli NCB03515	CTX-M-3	32	≤0.25	16	16	>256	≤0.25	256	256	This study
E. coli MRY04718	CTX-M-3	64	1	128	32	>256	≤0.25	>256	>256	This study
E. coli AYW-2	CTX-M-2	8	0.13	8	4	>128	<0.06	>128	>128	This study
E. coli NCB03490	CTX-M-2	4	≤0.25	4	1	128	≤0.25	256	64	This study
E. coli NCB03520	CTX-M-14	2	0.25	4	1	128	0.13	>128	128	This study
E. coli AYW-3	CTX-M-9	0.5	<0.06	0.5	0.25	32	<0.06	64	16	This study
K. pneumoniae HKY402	SHV-12	>128	1	>128	>128	32	<0.06	32	32	44
K. pneumoniae MRY041410	TEM-132	64	1	64	64	4	≤0.25	4	8	This study
K. pneumoniae K108	CTX-M-1	2	0.25	1	2	64	<0.06	64	64	This study
K. pneumoniae MRY04332	CTX-M-3	16	1	8	8	128	≤0.25	128	128	This study
K. pneumoniae HKY495	CTX-M-2	16	1	16	16	128	0.13	>128	>128	This study
K. pneumoniae MRY04504	CTX-M-2	2	≤0.25	2	4	64	≤0.25	128	64	This study
K. pneumoniae NCB03502	CTX-M-9	0.5	0.06	1	1	32	<0.06	64	32	This study
K. pneumoniae NCB03081	CTX-M-9	4	≤0.25	4	4	32	≤0.25	32	32	This study
K. pneumoniae KG525	GES-3J	>128	>128	>128	>128	64	8	64	32	42
K. pneumoniae KG502	GES-4J	>128	>128	>128	>128	32	16	16	16	43
E. coli NCB03426	IMP-1	64	64	≤0.25	64	16	16	≤0.25	16	This study
E. coli NCB02465	IMP-1	128	128	≤0.25	128	32	64	≤0.25	64	This study
K. pneumoniae KP115	IMP-1	>128	128	1	>128	64	64	0.25	64	This study
K. pneumoniae NCB03034	IMP-1	64	64	≤0.25	64	128	128	≤0.25	128	This study
E. coli EE61	OXA-30	2	2	2	2	4	4	4	4	This study

[a] Production of AmpC or DHA-1 might be augmented in the presence of clavulanic acid.
[b] *E. coli* strain NCB03522 also produces the TEM-1 penicillinase.

molar K_i values were prepared by stereoselective organic synthesis (23). However, there are only a few reports of studies that applied boronic acids to the identification of class C β-lactamase-producing bacteria (19, 34). In the present study we used one of the boronic acids, 3-aminophenylboronic acid (APB), and here we propose simple and practical methods for the identification of class C β-lactamase-producing bacteria showing resistance to broad-spectrum β-lactams, including cephamycins. The methods constructed in the present study promise to be very helpful for the screening of plasmid-mediated class C β-lactamase-producing bacteria in clinical microbiology laboratories.

MATERIALS AND METHODS

Bacterial strains, chemicals, and antibiotics. The bacterial strains used in this study and the β-lactamases that they produce are shown in Table 1. The types of β-lactamase genes were previously confirmed by PCR analyses, cloning and sequencing experiments, as well as isoelectric focusing, as described elsewhere (6, 10, 15, 25, 36, 40, 42, 43, 44). APB, 3-nitrophenylboronic acid (NPB), and 2-thiopheneboronic acid (TPB) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mueller-Hinton (MH) agar and MH broth were obtained from Becton Dickinson and Company (Paramus, N.J.). KB disks were commercially supplied by Eiken Chemical Co., Ltd. (Tokyo, Japan).

Susceptibility test. The MICs of ceftazidime (CAZ) and cefotaxime (CTX) with or without β-lactamase inhibitors were determined by the agar dilution method with MH agar, according to the recommendations of CLSI (formerly the National Committee for Clinical Laboratory Standards) in document M2-A8 (28). Clavulanic acid (GlaxoSmithKline K.K., Tokyo, Japan) was added at a concentration of 4 μg/ml, and both sodium mercaptoacetic acid (SMA) and APB were added at a concentration of 300 μg/ml. The MIC of APB was generally above 2,400 μg/ml, so the concentration of APB employed in this study did not show any detectable effect on bacterial growth or susceptibilities to antimicrobial agents.

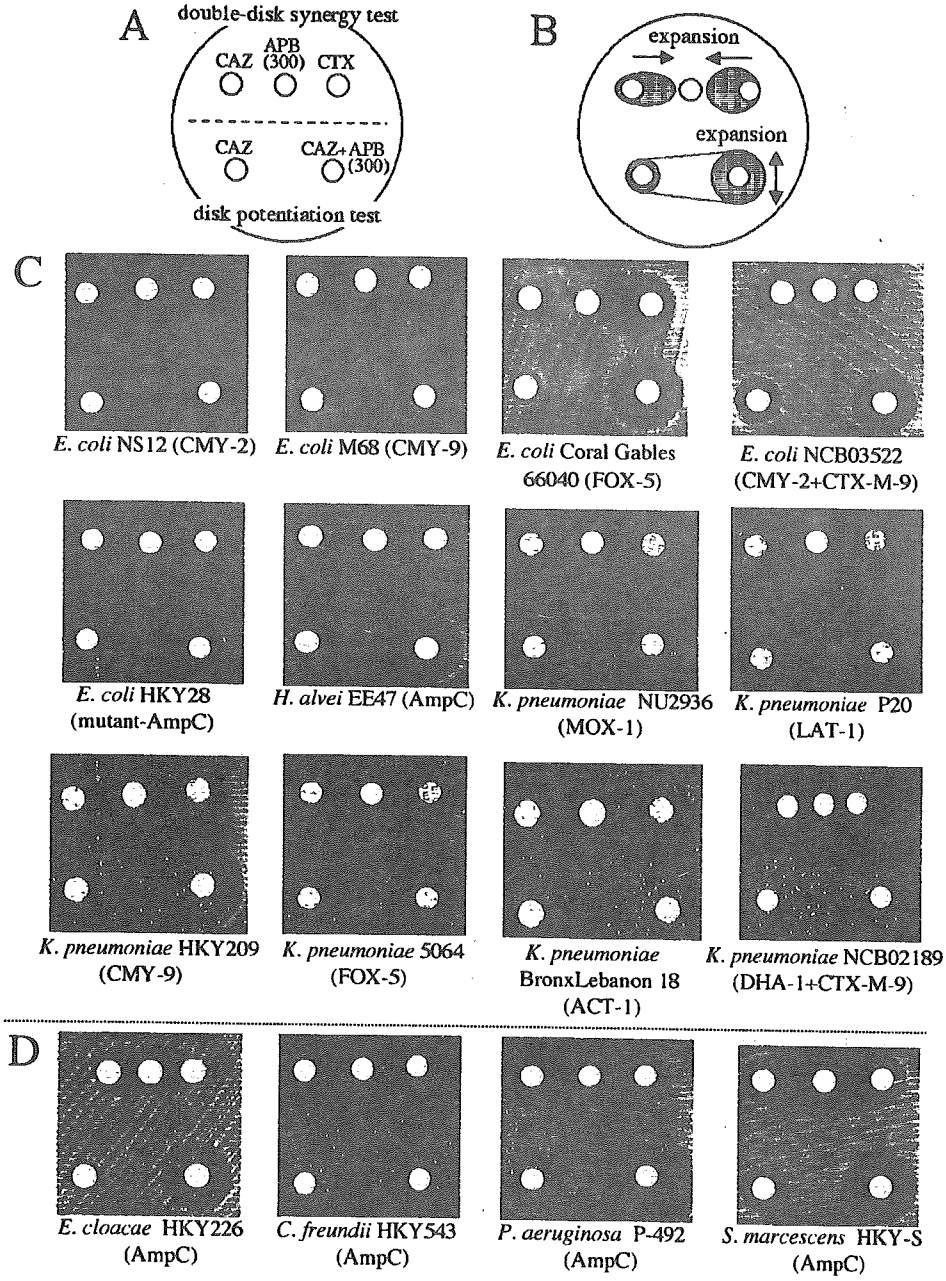

FIG. 1. DDST and disk potentiation test for class C β-lactamase producers. (A) Scheme of disk arrangement for the two tests. The upper three disks are for DDST, and the lower two are for the disk potentiation test. The amount of APB added to the disk was 300 μg. (B) Typical observations of the growth-inhibitory zones among class C β-lactamase producers. The growth-inhibitory zones are expanded toward the APB disk in DDST. In the disk potentiating test, the diameter of the growth-inhibitory zone is expanded around the disk containing both CAZ and APB compared with that around the disk containing solely CAZ. (C) Practical changes in the morphologies or the diameters of the growth-inhibitory zones among the class C β-lactamase-producing strains. Expansion of the growth-inhibitory zone toward the APB disk is observed around the disks containing CAZ or CTX in DDST (upper) among the class C β-lactamase producers. In the disk potentiation test (lower), enlargement of the diameter of the growth-inhibitory zone of greater than or equal to 5 mm is seen among all class C β-lactamase producers except *K. pneumoniae* BronxLebanon 18. (D) DDST and disk potentiation test against chromosomal AmpC producers. Similar findings are observed among gram-negative rods that produce chromosomally encoded inducible AmpC type β-lactamases, suggesting that the new identification method described in the present study can also be applied to chromosomal AmpC producers, as well as plasmid-mediated class C β-lactamase producers.

Detection of class C β-lactamase production. Class C β-lactamase production was determined by the following three methods. Clinical isolates of *Klebsiella pneumoniae* or *E. coli* producing the following plasmid-mediated class C β-lactamases were used as positive controls: ACT-1 (6), CMY-2 and CMY-9 (10), FOX-5 (36), LAT-1 (40), and MOX-1 (15). Because of the similarity of its chromosomal enzyme to one of the plasmid-mediated β-lactamases, ACC-1, an isolate of *Hafnia alvei* was added to the positive controls (24). As negative controls, we used clinical isolates of *K. pneumoniae* or *E. coli* producing other plasmid-mediated β-lactamases: TEM-26 (44); TEM-91 (17); SHV-12 (44); SHV-24 (18); CTX-M-1, CTX-M-2, CTX-M-9, and GES-3 (42); GES-4 (43); and

IMP-1 (Table 1). The boronic acids APB, NPB, and TPB were dissolved in DMSO at a concentration of 100 mg/ml and used for the following tests.

Disk potentiation test. A colony of a test strain which was suspected of being a class C β-lactamase producer was suspended in and diluted with MH broth medium to 10^8 CFU/ml and spread on an MH agar plate with a cotton swab, according to the protocol recommended by CLSI in document M2-A8 (28). Three hundred micrograms of one of the boronic acids, APB, NPB, or TPB, was added to a commercially available KB disk containing 30 μg of CAZ or CTX. These disks were placed on the MH agar plate described above in pairs with a KB disk containing 30 μg of CAZ or CTX with a center-to-center distance of 30 mm (Fig. 1A). The agar plates were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around a CAZ disk with APB was compared with that around a CAZ disk without APB for the detection of class C β-lactamase production.

Double-disk synergy test (DDST). Three hundred micrograms of APB was added to a disk that contained no antibiotics and that was the same size as the KB disk. This APB-containing disk was placed on an MH agar plate on which the bacterial suspension to be examined had been spread. Two other KB disks containing 30 μg of CAZ and CTX were also placed on the MH agar plate, with a center-to-center distance to the boronic acid-containing disk of 18 mm (Fig. 1A). The plate was incubated at 37°C overnight, and the change in the shape of the growth-inhibitory zone around the CAZ or the CTX disk through the interaction with the boronic acid-containing disk was observed for the detection of class C β-lactamase production (Fig. 1B).

Microdilution test. MH broth containing serial dilutions of CAZ or CTX at concentrations ranging from 0.125 to 256 μg/ml and containing 300 μg (1.9 mM) of APB with the same serial dilution of CAZ or CTX was prepared and placed in a 96-well plate. A bacterial suspension was inoculated into each well, according to the recommendation of CLSI in document M7-A6 (27). The plate was incubated at 37°C overnight. The decrease in the MIC of CAZ or CTX in combination with APB was used for the identification of a class C β-lactamase producer.

RESULTS AND DISCUSSION

Plasmid-mediated class C β-lactamases have been identified worldwide since the late 1980s, and they are emerging threats to antibiotic therapy for various infectious diseases because they confer to pathogenic bacteria, especially *E. coli* and *K. pneumoniae*, resistance to broad-spectrum β-lactams (9, 10, 13, 15, 22, 24, 32). Boronic acids have been recognized as specific inhibitors of AmpC β-lactamases since 1982 (4, 8, 34, 37, 41). Using three commercially available boronic acids, APB, NPB, and TPB, in the present study, we evaluated three different methods for the identification of bacteria producing class C β-lactamases which would be simple enough for routine use in a clinical microbiology laboratory.

First, we developed the disk potentiation test, which is similar to the confirmation test for ESBL production recommended by CLSI in document M100-S14 (29). We selected three commercially available boronic acids, APB, NPB, and TPB, as the specific inhibitors of class C β-lactamases and observed the enlargement of the growth-inhibitory zone diameter around the disk containing CAZ in combination with these inhibitors. The boronic acids were dissolved in DMSO and added to the KB disk containing 30 μg of CAZ. DMSO itself had no apparent effect on the growth of the isolates tested when it was added to the disk at a volume up to 10 μl (data not shown). Both NPB and TPB were found to have antibacterial activity by themselves at concentrations of about 300 μg/ml, leading to a misinterpretation of the changes in the diameter of the growth-inhibitory zone (data not shown). Therefore, we chose APB as the most practical candidate among the specific inhibitors of class C β-lactamases for further examination. Among the four drugs that we tested, CAZ, CTX, cefmetazole, and moxalactam, CAZ showed the best performance in combination with APB. When a cutoff value of a 5-mm enlargement of the growth-inhibitory zone diameter or greater was set, all *K. pneumoniae* and *E. coli* isolates producing the plasmid-mediated class C β-lactamases, except for ACT-1-producing *K. pneumoniae* BronxLebanon 18, could be detected (Fig. 1C); and the specificity of the test was nearly 100% for the negative controls of producers of other classes of β-lactamases (Fig. 2). The exception, *K. pneumoniae* BronxLebanon 18, was less inhibited by APB when CAZ was used. However, a successful test result was obtained with the combination of CTX and APB (data not shown). This strain was supposed to produce another ESBL or to have an alteration in the permeability of the outer membrane, and the test reported by Pitout et al. (33) might be useful for this kind of strain. *H. alvei* was also found to be positive as an AmpC β-lactamase producer. Also, this method could detect *E. coli* HKY28, a mutant AmpC producer which was moderately susceptible to β-lactamase inhibitors such as tazobactam and sulbactam (11). Two well-characterized isolates, *E. coli* NCB03522 and *K. pneumoniae* NCB02189, which produce plasmid-mediated CMY-2 and DHA-1, respectively, together with CTX-M-9, were examined with this disk potentiation test. Using the drug-inhibitor combinations of CAZ plus APB and CTX plus clavulanic acid, we could detect class C β-lactamases and CTX-M-9 separately, with no apparent interaction of these two different classes of β-lactamases.

Second, we applied DDST to the identification of class C β-lactamase producers. Powers et al. (34) first described the potentiation effect of a boronic acid, benzo(*b*)thiophene-2-

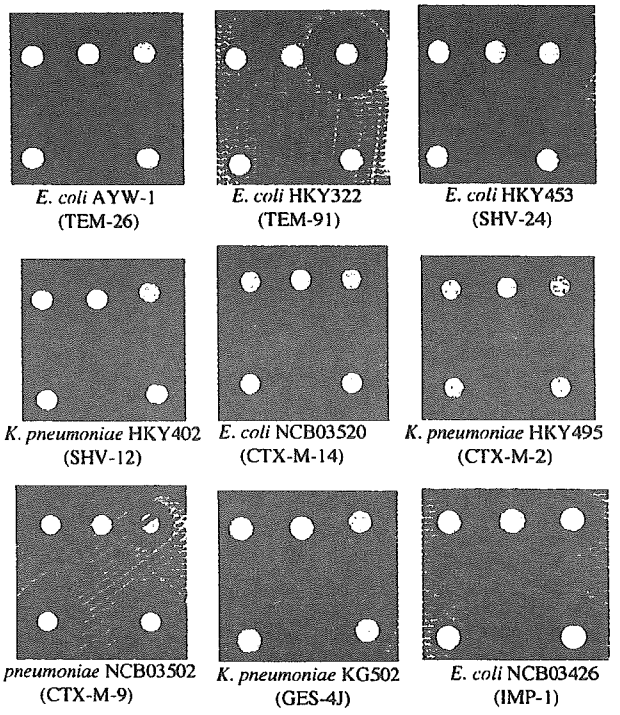

FIG. 2. DDST (upper) and disk potentiation test (lower) for non-class C β-lactamase producers. No apparent changes in the shapes or the diameters of the growth-inhibitory zones around the disks containing CAZ or CTX are observed in the presence of APB (300 μg per disk). The arrangement of the disks was as described for Fig. 1.

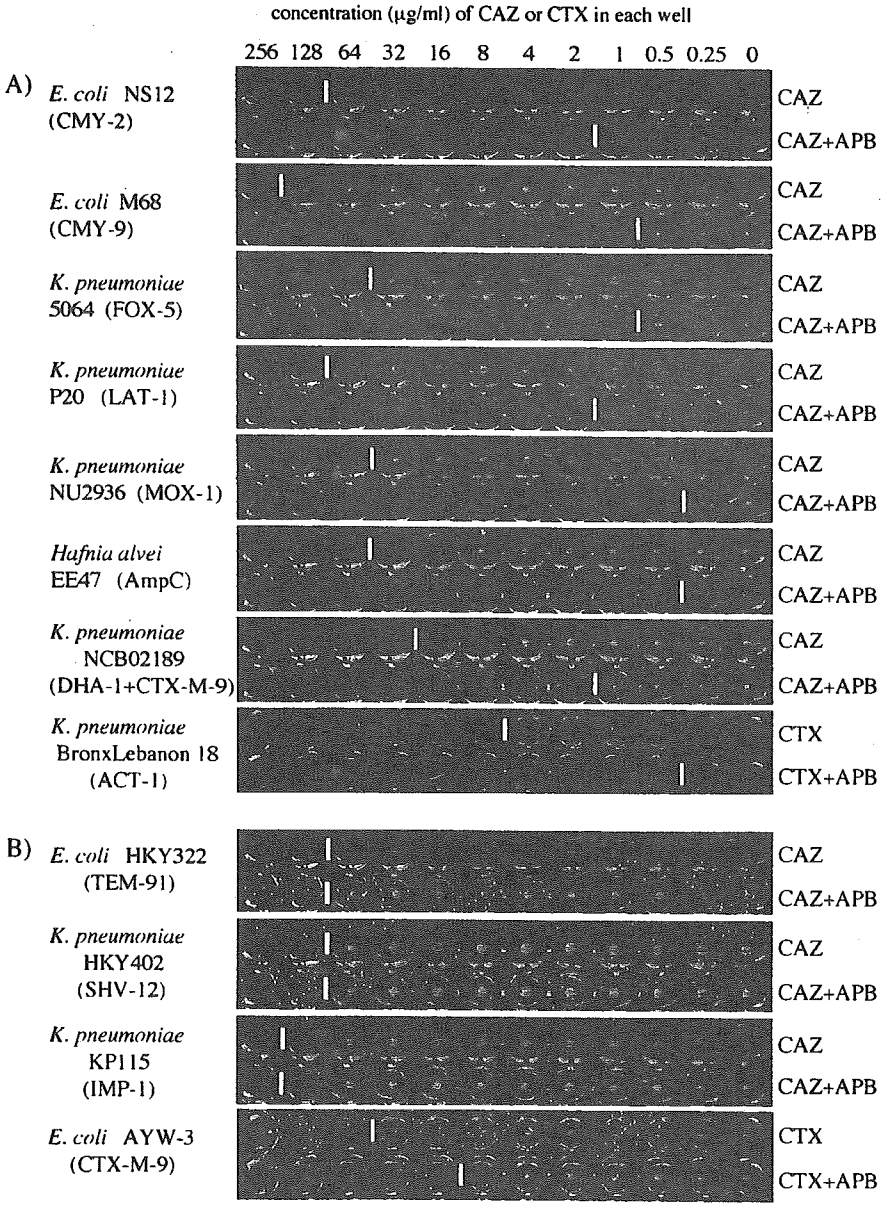

FIG. 3. Microdilution test with APB for detection of class C β-lactamases. APB was added to serial dilutions of CAZ or CTX, and the concentration of ABP in each well is 300 μg/ml. (A) Detection of plasmid-mediated class C β-lactamases in representative *E. coli* and *K. pneumoniae* isolates and chromosomal AmpC β-lactamase in *H. alvei* EE47. An eightfold or greater decrease in the MIC of CAZ or CTX with the addition of APB is indicative of the production of class C β-lactamases. (B) Negative results of microdilution test by using APB for *E. coli* and *K. pneumoniae* isolates producing class A ESBLs or a class B MBL, IMP-1. Among the strains tested, the level of resistance to cefotaxime was reduced in the presence of APB in a few strains, such as CTX-M-9-producing *E. coli* AYW-3; and the coproduction of chromosomal AmpC was suspected in this strain. It may even be possible to distinguish strains that chiefly produce a class A or a class B enzyme, together with a small amount of a class C enzyme, from those that mainly produce class C enzymes when the breakpoint was set at a decrease in the MIC of greater than or equal to eightfold (three tubes) in the presence of APB. White vertical bars between the wells indicate the upper limit of bacterial growth in each line.

boronic acid, to the antimicrobial activity of CAZ; and Liebana et al. (19) used this synergism test for confirmation of the presence of an AmpC-like enzyme. This method, similar to the simple test which we described earlier (1) for the detection of metallo-β-lactamases by the use of thiol compounds, was based on the interpretation of the change in morphology in the growth-inhibitory zone in order to detect class C β-lactamases. An APB-containing disk and a disk containing a test drug, CAZ or CTX, were placed on an MH agar plate which had been inoculated with a test isolate, with the center-to-center distance of 18 mm. After overnight incubation, expansion of the growth-inhibitory zone toward the APB-containing disk was interpreted to be a positive result for class C β-lactamase production. With the combination of APB and CAZ or CTX,

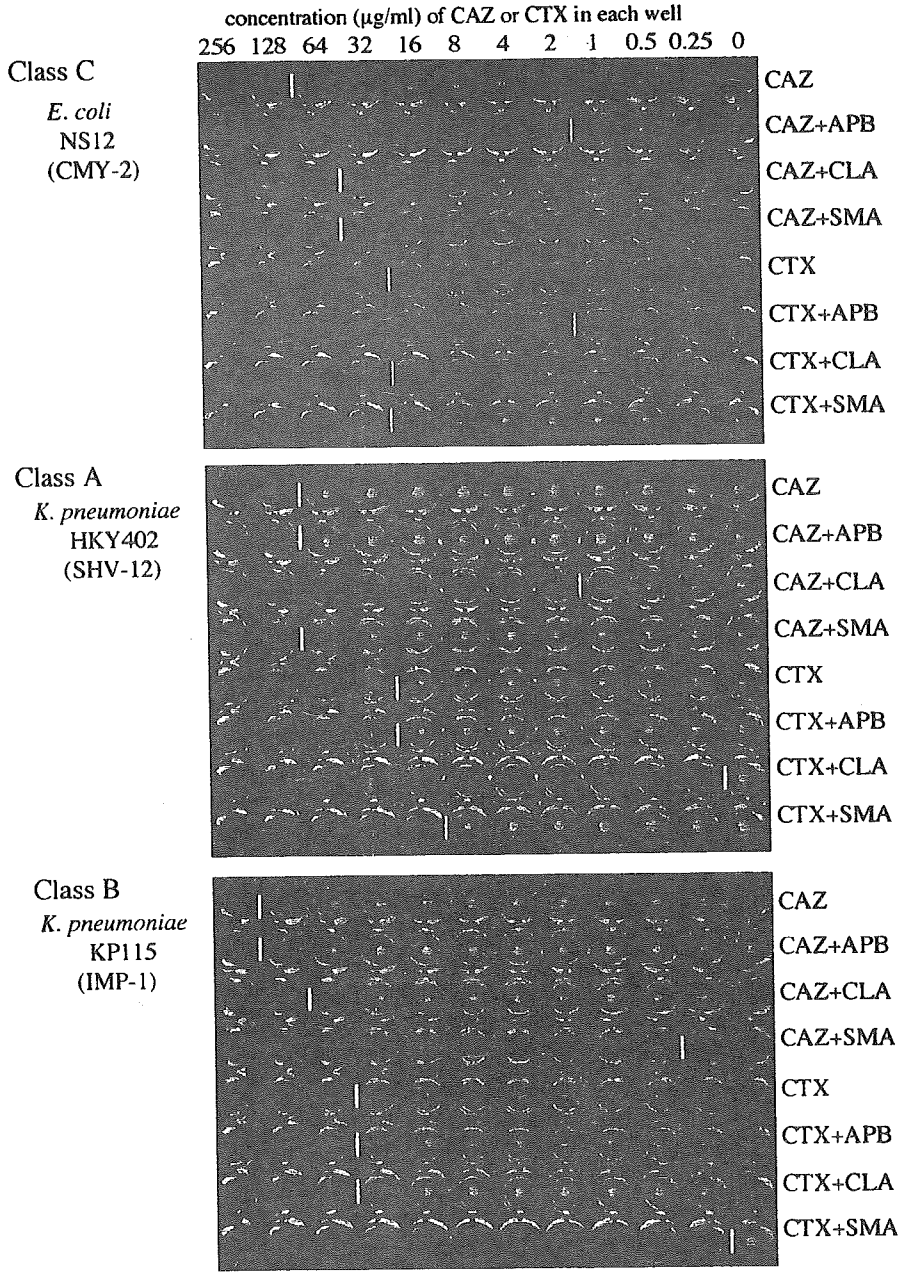

FIG. 4. Microdilution test using three inhibitors for detection of presumptive β-lactamase types. Three inhibitors, APB (300 μg/ml), CLA (4 μg/ml), and SMA (300 μg/ml), were added in each line of the wells. The inhibition patterns of each inhibitor for strains producing class A, class B, and class C β-lactamases are demonstrated by using cefotaxime and ceftazidime as indicators. White vertical bars between the wells indicate the upper limit of bacterial growth in each line.

all plasmid-mediated class C β-lactamases of the positive controls were detected (Fig. 1C), and no apparent changes in the morphology of the growth-inhibitory zone were observed for the negative controls producing other classes of β-lactamases (Fig. 2). For *E. coli* NCB03522 and *K. pneumoniae* NCB02189, which produce plasmid-mediated CMY-2 or DHA-1 together with CTX-M-9, the center-to-center distance between the CAZ and the APB disks should be shortened to 12 mm in order to detect a more discernible expansion of the growth-inhibitory zone around the CAZ disk toward the APB disk.

The microdilution method is one of the most familiar methods for the determination of MICs in clinical laboratories due to the recent introduction of rapid automated bacterial identification and antimicrobial susceptibility test systems. Three hundred micrograms of APB was added to the serial dilution of CAZ, and the MICs of CAZ determined with and without APB were compared according to the methods recommended by CLSI (27). The MICs appeared to be similar to those shown in Table 1, which were determined by the agar dilution method, according to the recommendations of CLSI (28). More

than or equal to an eightfold decrease in the MIC of CAZ in combination with APB was indicative of the production of plasmid-mediated class C β-lactamases in *E. coli* and *K. pneumoniae*. Most of the isolates showed more than or equal to an eightfold reduction in the MIC of CAZ in the presence of APB, while only a fourfold reduction of MIC was observed in *E. coli* NCB03522 (Fig. 3). For ACT-1-producing *K. pneumoniae* BronxLebanon 18, this test was positive, with a 16-fold reduction in the MIC of CTX in combination with APB (Fig. 3). As shown in Fig. 4, the classes of β-lactamases produced by clinical isolates can be easily distinguished from each other by using three kinds of inhibitors, especially when a strain chiefly produces a single type of β-lactamase.

Moreover, we applied the former two methods to several CAZ-resistant clinical isolates of *E. cloacae*, *C. freundii*, *S. marcescens*, and *Pseudomonas aeruginosa* for the detection of their chromosomal AmpC β-lactamases. Most of these isolates showed positive results, suggesting that they are probably hyperproducers of chromosomal AmpC β-lactamases. The results of both tests for the representative strains, *E. cloacae* HKY226, *C. freundii* HKY543, *S. marscecens* HKY-S, and *P. aeruginosa* P-492, are shown in Fig. 1D. For the *E. cloacae* isolates, successful detection was achieved by shortening the center-to-center distance of the two disks containing CAZ and APB from 18 mm to 12 mm in DDST. A few isolates of *S. marcescens* and *P. aeruginosa* were less inhibited by APB, so they could not be detected by either method (data not shown). They might produce additional unknown β-lactamases other than the AmpC type or overexpress their multidrug efflux systems (12).

According to these results, all three tests, the disk potentiation test, the double-disk synergy test, and the microdilution test with APB, were very simple, highly sensitive, and specific for the identification of bacteria producing class C β-lactamases. Thus, they are fully applicable for routine use in clinical microbiology laboratories. Although the results for the production of class C enzymes obtained by these methods is sometimes ambiguous when the strains also coproduce a large amount of ESBLs or MBLs, the methods provide useful information on the mechanism of drug resistance mediated by class C β-lactamases for enhanced infection control and effective antimicrobial therapy.

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