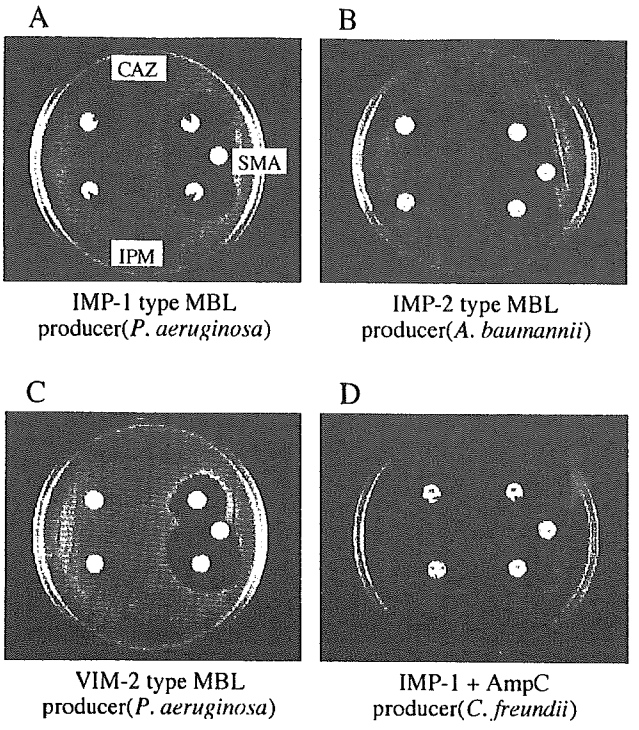

FIG. 1. Inhibition test for detection of MBL producers by use of SMA. Sample results of inhibition tests using SMA for detection of strains producing IMP-1, IMP-2, or VIM-2 type MBLs are shown. For MBL-producing strains, a clear expansion of the growth inhibition zone appears between the disks containing ceftazidime and SMA, respectively (A to C). In strains producing MBL as well as ESBLs or AmpC or CMY type cephalosporinases, the expansion of the growth inhibition zone is usually unclear. For such strains, the use of disks containing imipenem or meropenem instead of ceftazidime is recommended. By this substitution of disks, a clear growth inhibition zone appears between the disks containing imipenem and SMA, respectively (D). The MIC of imipenem or meropenem for MBL producers is sometimes lower than 32 μg/ml, but that of ceftazidime is usually higher than 64 μg/ml. Thus, disks containing 30 μg of ceftazidime are suitable for the first testing to detect MBL producers. IPM, imipenem; CAZ, ceftazidime.

Chromosomal DNAs prepared from four strains of *P. putida* were embedded in agarose gel plugs (InCert; Bio-Whittaker Molecular Applications, Rockland, Maine). Plugs were mounted in wells of agarose and electrophoresed after treatment with reaction mixtures according to the method described previously (20).

RESULTS

Screening of MBL production by using an SMA disk. Of 587 strains tested, 431 (73.4%) appeared to produce MBL, as determined by the screening tests using disks containing a thiol compound (2), SMA. *P. aeruginosa* (n = 116), *Alcaligenes xylosoxidans* (n = 53), *P. putida/fluorescens* (n = 51), *S. marcescens* (n = 47), *A. baumannii* (n = 30), *K. pneumoniae* (n = 23), and *E. coli* (n = 17) were the main species of SMA test-positive strains (Table 2). One hundred fifty-six strains were SMA negative even when a combination of disks containing imipenem and SMA, respectively, were used together. Since many of these strains were susceptible to meropenem, they were speculated to be either producers of class A enzymes

TABLE 2. Results of screening tests using SMA disks

Bacterial species	No. of strains testing:		Total
	Positive	Negative	
Pseudomonas aeruginosa	180	48	228
Pseudomonas putida/fluorescens	55	1	56
Alcaligenes xylosoxidans	53	0	53
Serratia marcescens	47	9	56
Acinetobacter baumannii	35	13	48
Klebsiella pneumoniae	23	16	39
Escherichia coli	17	40	57
Enterobacter cloacae	5	8	13
Burkholderia cepacia	5	2	7
Citrobacter freundii	3	8	11
Klebsiella oxytoca	2	4	6
Providencia rettgeri	2	0	2
Alcaligenes faecalis	1	0	1
Morganella morganii	1	2	3
Acinetobacter lwoffii	1	1	2
Enterobacter aerogenes	1	3	4
Proteus spp.	0	1	1
Total	431	156	587

such as ESBLs, hyperproducers of chromosomal class C enzymes, or producers of plasmid-mediated AmpC and CMY type cephamycinases; some of them may also acquire altered membrane permeability to β-lactams. Further characterization of these strains will be performed in the next study.

PCR typing of MBL genes. Of 431 SMA-positive strains, 357 appeared to carry bla_{IMP-1} or one of its mutants such as bla_{IMP-3} or bla_{IMP-6}, which are indistinguishable from bla_{IMP-1} with the PCR primers and conditions used in this study. Of these 357 strains, 116, 53, 51, 47, 30, and 23 were identified as *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, *S. marcescens*, *A. baumannii*, and *K. pneumoniae*, respectively (Table 3). Sixty-seven and seven strains appeared to carry bla_{VIM-2} and bla_{IMP-2}, respectively, and all these strains harbored the

TABLE 3. Number of strains of each MBL type among SMA test-positive strains

Bacterial species	No. of strains of type:			Total no. of strains
	IMP-1	IMP-2	VIM-2	
Pseudomonas aeruginosa	116	1	63	180
Pseudomonas putida/fluorescens	51	0	4[a]	55
Alcaligenes xylosoxidans	53	0	0	53
Serratia marcescens	47	0	0	47
Acinetobacter baumannii	30	5	0	35
Klebsiella pneumoniae	23	0	0	23
Escherichia coli	17	0	0	17
Enterobacter cloacae	5	0	0	5
Burkholderia cepacia	5	0	0	5
Citrobacter freundii	3	0	0	3
Klebsiella oxytoca	2	0	0	2
Providencia rettgeri	2	0	0	2
Alcaligenes faecalis	1	0	0	1
Morganella morganii	1	0	0	1
Acinetobacter lwoffii	0	1	0	1
Enterobacter aerogenes	1	0	0	1
Total	357	7	67	431

[a] These strains were reidentified as *P. putida*.

TABLE 4. Combination of MBL and *intI* genes among all SMA test-positive strains

Type of MBL	No. of strains with the following type of integrase gene:		Total no. of strains
	intI1	*intI1* + *intI3*	
IMP-1	353	4[a]	357
IMP-2	7	0	7
VIM-2	67	0	67
Total	427	4	431

[a] Reidentified as *P. putida*.

intI1 gene (Tables 3 and 4). No isolate carrying two or more different types of MBL genes together was found among the strains tested. Of 67 bla_{VIM-2}-positive strains, 63 were *P. aeruginosa* and the remaining 4 were reidentified as *P. putida* (Table 3) by 16S rRNA sequencing. As for seven bla_{IMP-2}-positive strains, one was *P. aeruginosa* and five were *A. baumannii* (Table 3). All SMA-positive strains were found to carry one of the MBL genes tested in this study, although no strain harboring bla_{VIM-1} or bla_{SPM-1} was detected among them.

PCR typing of integrase genes. Of 431 SMA-positive strains, 427 appeared to carry the *intI1* gene. Among these 427 strains, 180, 53, 51, 47, 35, and 23 were identified, respectively, as *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, *S. marcescens*, *A. baumannii*, and *K. pneumoniae*. Only four strains appeared to carry both the *intI1* and *intI3* genes, and these were reidentified as *P. putida* (Table 4) by sequencing of 16S rRNA as well as by the conventional identification protocol depending on the characteristic biochemical properties of each bacterial species. Of 51 *P. putida/fluorescens* strains harboring only the *intI1* gene, 47 and 4 appeared to carry bla_{IMP-1} and bla_{VIM-2}, respectively (Table 5). All four *P. putida* strains positive for both the *intI1* and *intI3* genes carried the IMP-1 type MBL gene. The four *intI1*-positive strains carrying the VIM-2 type MBL gene were also reidentified as *P. putida* (Table 5).

Sequencing analyses of *bla* and *intI* genes. The nucleotide sequences of 15 amplicons, consisting of 5 bla_{IMP-1}, 5 bla_{IMP-2}, and 5 bla_{VIM-2} amplicons, were consistent with the types predicted by the preceding PCR analyses, at least within the sequenced areas. Similarly, the nucleotide sequences of nine amplicons, consisting of five *intI1* and four *intI3* genes, also coincided with the results predicted by the PCR analyses.

Relationships of the *intI3* and bla_{IMP-1} genes. According to the result of a PCR analysis using a set of primers for amplification of a 609-bp fragment in the *intI3*–bla_{IMP-1} region (Table 1), the *intI3* gene was suggested to be located adjacent to the bla_{IMP-1} gene in the configuration reported previously (1) in all four *P. putida* strains carrying both the *intI3* and bla_{IMP-1} genes.

TABLE 5. Combination of MBL and *intI* genes in *P. putida/fluorescens*

Type of MBL	No. of strains with the following type of integrase gene:		Total no. of strains
	intI1	*intI1* + *intI3*	
IMP-1	47	4[a]	51
VIM-2	4[a]	0	4
Total	51	4	55

[a] Reidentified as *P. putida*.

***P. putida* strains carrying both the *intI1* and *intI3* genes.** Of four *P. putida* strains carrying both the *intI1* and *intI3* genes, three were isolated at hospital A and one was isolated at hospital B; both these hospitals are located in Mie Prefecture. Table 6 shows the clinical associations of these four strains. The *Spe*I-digested genomic DNA patterns of the four clinical isolates were classified into two types (Fig. 2). The three strains isolated in hospital A, NCB 01-121, NCB 02-182, and NCB 02-204, demonstrated very similar PFGE patterns, suggesting a clonal lineage.

DISCUSSION

Since 1988, transferable carbapenem resistance has been found in several *P. aeruginosa* strains isolated in Toyama Prefecture, Japan (12, 25). In 1991, an IMP-1 type MBL, initially characterized in a strain of *S. marcescens*, gave high-level resistance to various broad-spectrum β-lactams including imipenem (6, 14). This strain was isolated in a hospital in Aichi Prefecture and had the *intI1* gene just upstream of the bla_{IMP-1} gene cassette on the chromosome. In 1993, several *S. marcescens* strains harboring a plasmid-mediated bla_{IMP-1} gene were also identified in Aichi Prefecture (1, 6). Several of these strains were found to carry a novel integron-like element that was classified as a class 3 integron (5). The newly identified class 3 integrase, IntI3, has about 60.9% identity to the previously identified IntI1 at the amino acid sequence level (1). The genomic organization of a class 1 integron was characterized in a bla_{IMP-1}-carrying strain (9) which was isolated in Japan. The emergence and development of carbapenem resistance through the acquisition of genes for MBLs has since become a matter of general concern, especially in gram-negative bacteria (13, 21).

At least three genetically different clusters of MBLs have been found to date: IMP-1 to IMP-12 (4), VIM-1 to VIM-6 (EMBL accession no. AY165025), and SPM-1 (23) have been published or registered with the EMBL/GenBank database. Almost all of their genetic determinants are associated with class 1 integrons, and only a few strains have been found to carry class 3 integrons to date. In the present study, almost all MBL genes were found to correlate with class 1 integrons. To our knowledge, only *S. marcescens* strain AK9373 and a small number of strains isolated in Aichi Prefecture carry the *intI3* gene (N. Shibata, Y. Arakawa, H. Kurokawa, Y. Doi, and K. Shibayama, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. C524, 2001). Recently however, a class 3 integron mediating a gene for a GES-1 type class A β-lactamase, a kind of ESBL, was submitted from Portugal to EMBL/GenBank (accession no. AY219651). Because *intI* genes are usually accompanied by genes for aminoglycoside-modifying enzymes such as *aacA4* or *aadA1*, these gene cassettes in integrons may be derived from some nonpathogenic bacteria such as aminoglycoside-producing actinomycetes, which are widely distributed in the natural environment. Therefore, the two strains possessing class 3 integrons found in Japan (1) and Portugal may have

TABLE 6. Clinical associations of *P. putida* isolates carrying both the *intI1* and *intI3* genes[a]

Strain no.	Age of patient (yr)	Sex of patient	Disease	Specimen type	Hospital	Date of isolation (mo/yr)
NCB 01-121	77	Male	Prostatic cancer	Urine	A	6/2001
NCB 02-182	79	Male	Cerebral infarction	Urine	A	5/2002
NCB 02-190	66	Female	Cerebral infarction	Sputum	B	5/2002
NCB 02-204	76	Female	Gallbladder cancer	Biliary tract drainage tube	A	6/2002

[a] All these strains were isolated in Mie Prefecture.

been generated independently of each other by the integration of separate β-lactamase gene cassettes in clinical environments, where the strong influence of antimicrobial agents has continued.

In the present study, we confirmed that class 1 is the most abundant type of integron in Japan, although four *P. putida* strains carrying both class 1 and class 3 integrons have been newly identified. Although the reason why these four strains carry two types of integron is not well understood at present, analyses of the organization and function of class 1 and class 3 integrons in these strains may elucidate their biological advantage in the future. In any case, since *intI3* has already been identified in at least three gram-negative bacterial species (*S. marcescens*, *K. pneumoniae*, and *P. putida*) isolated from geographically different areas, the emergence and proliferation of class 3 integrons that carry various gene cassettes responsible for multiple antimicrobial resistance may become not so much a local problem as a global issue.

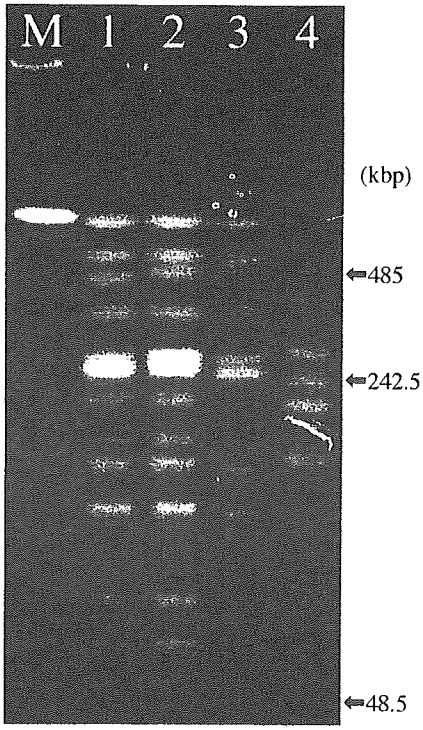

FIG. 2. PFGE of four *P. putida* strains carrying both the *intI1* and *intI3* genes. Lanes: M, size marker for PFGE; 1, *P. putida* strain NCB 01-121; 2, strain NCB 02-182; 3, strain NCB 02-204; 4, strain NCB 02-190. Clinical associations of the strains subjected to this PFGE analysis are listed in Table 6.

It was confirmed that the IMP-1 type of MBL is the most common MBL in Japan at present, although IMP-3 (7) and IMP-6 (27) have also been identified in Japan. Since very few amino acid substitutions exist among IMP-1, IMP-3, and IMP-6, the latter two MBLs are fundamentally variants of IMP-1. It is difficult to confirm whether all the bla_{IMP-1}-positive strains found in this study carry genuine bla_{IMP-1}, since the primer sets used in this study cannot distinguish bla_{IMP-3} and bla_{IMP-6} from bla_{IMP-1}. Strains producing IMP-3 or IMP-6 usually demonstrate susceptibility or low-level resistance to imipenem (MICs, 1 to 16 μg/ml) (7, 27). However, almost all bla_{IMP-1}-positive strains identified in this study, especially in *P. aeruginosa* and *S. marcescens*, demonstrated high-level resistance to imipenem (MICs, >32 μg/ml), as was reported previously (6, 18, 20). In any event, the bla_{IMP-1}-positive strains detected in this study appeared to produce MBLs belonging to the IMP-1 complex, consisting mainly of IMP-1 and probably small amounts of its variants, IMP-3 and IMP-6. Detailed typing of the MBLs produced by the 357 bla_{IMP-1}-positive strains will be continued in the next study.

IMP-2 was initially found in *A. baumannii* in Italy (18), and its variant IMP-8 was also identified in *K. pneumoniae* in Taiwan (26). In the present study, one strain of *P. aeruginosa* and five strains of *A. baumannii* were found to carry the bla_{IMP-2} gene in Japan. IMP-2 is not a variant of IMP-1, since many amino acid substitutions are found between them (18). One has to wonder why IMP-2 has been found in such widely separated geographical regions as Europe and the Far East. The increased frequency of international travel and transportation may well be involved in the transmission of IMP-2 producers, although they may have emerged independently in each area. Precise characterization of the genetic organization of each integron is necessary to determine their origins and relationships.

The present study suggests that VIM-2 producers are also increasing in Japan, especially in *Pseudomonas* spp. VIM-2 was initially identified in France in 1996 (17). VIM-2 producers have occasionally caused outbreaks in many countries recently, including Korea (11), where VIM-2 producers are reportedly increasing (28). It is not well understood why VIM-2 producers are becoming predominant in Korea, a country adjacent to Japan, where IMP-1 producers are very common. A similar observation was made in the isolation of ESBL producers. Strains producing TEM- or SHV-derived ESBLs have been increasing in Korea, while such ESBL producers still remain rare in Japan, though producers of CTX-M type enzymes, including Toho-1, are becoming common in Japan. These discrepancies may depend on differences in the administration of antimicrobial agents.

In the present study, 16 gram-negative bacterial species were found to carry genes for at least three types of MBLs. Indeed, although *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, and *S. marcescens* are the most frequent carriers of MBL genes among these 16 bacterial species, bla_{IMP-1} genes have already been transmitted into various bacterial species belonging to the family *Enterobacteriaceae*, such as *E. coli*, *E. cloacae*, *E. aerogenes*, *C. freundii*, *K. pneumoniae*, *Providencia rettgeri*, and *Morganella morganii*, through the mediation of class 1 integrons, which are usually carried on transferable plasmids. Isolation of MBL producers belonging to these bacterial species is still very rare even in Japan, which may be due to the instability of plasmids carrying MBL and integrase genes in these strains. However, the frequency of isolation of MBL producers belonging to these species is also likely to increase in the future, as it already has in *P. aeruginosa* and *S. marcescens*, if the gene cassettes or gene clusters locating in integron structures are transposed to stable resident plasmids or chromosomes in each bacterial species.

In hospital A, three strains of *P. putida* carrying *intI1*, *intI3*, and bla_{IMP-1} have been isolated from different patients for more than 12 months. Since these strains demonstrated very similar PFGE patterns, it is suggested that they are a clonal lineage and have been existing in the hospital for nearly 1 year. Since *P. putida* can grow at temperatures below 10°C and tends to be isolated from damp materials and environments such as plant surfaces, mops, sponges, and sinks, these strains might have survived in such nosocomial environments. Indeed, *P. putida* sometimes causes opportunistic infections such as respiratory and urinary tract infections, but its virulence is not as strong as that of *P. aeruginosa*. However, it can fully work as a reservoir of integrons and MBL genes. Thus, we should pay continuous attention even to such low-virulence bacterial species when they carry special genetic determinants such as MBL genes.

The bla_{IMP-1} genes move together with class 1 or class 3 integrons in many nosocomial gram-negative bacilli. Furthermore, genes for bla_{IMP-2} and bla_{VIM-2} have also been dispersing into various non-glucose-fermenting bacteria such as *P. putida* and *A. baumannii*. Hence, we should take special precautions against the further global dissemination of integron-associated MBL genes among a variety of nosocomial gram-negative bacterial species.

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Plasmid-Mediated 16S rRNA Methylase in *Serratia marcescens* Conferring High-Level Resistance to Aminoglycosides

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***Serratia marcescens* S-95, which displayed an unusually high degree of resistance to aminoglycosides, including kanamycins and gentamicins, was isolated in 2002 from a patient in Japan. The resistance was mediated by a large plasmid which was nonconjugative but transferable to an *Escherichia coli* recipient by transformation. The gene responsible for the aminoglycoside resistance was cloned and sequenced. The deduced amino acid sequence of the resistance gene shared 82% identity with RmtA, which was recently identified as 16S rRNA methylase conferring high-level aminoglycoside resistance in *Pseudomonas aeruginosa*. Histidine-tagged recombinant protein showed methylation activity against *E. coli* 16S rRNA. The novel aminoglycoside resistance gene was therefore designated *rmtB*. The genetic environment of *rmtB* was further investigated. The sequence immediately upstream of *rmtB* contained the right end of transposon Tn*3*, including bla_{TEM}, while an open reading frame possibly encoding a transposase was identified downstream of the gene. This is the first report describing 16S rRNA methylase production in *S. marcescens*. The aminoglycoside resistance mechanism mediated by production of 16S rRNA methylase and subsequent ribosomal protection used to be confined to aminoglycoside-producing actinomycetes. However, it is now identified among pathogenic bacteria, including *Enterobacteriaceae* and *P. aeruginosa* in Japan. This is a cause for concern since other treatment options are often limited in patients requiring highly potent aminoglycosides such as amikacin and tobramycin.**

Aminoglycoside antibiotics are widely used in clinical settings, especially for treatment of life-threatening infections caused by gram-negative bacteria. They bind to the highly conserved A-site of the 16S rRNA of the prokaryotic 30S ribosomal subunits, interfering with the protein synthesis with subsequent bacterial death (16). The most frequently encountered mechanism of resistance to aminoglycosides is their structural modification by specific enzymes produced by resistant organisms. The three classes of such enzymes are aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT or AAD), and aminoglycoside phosphotransferases (APH) (20). Other mechanisms of resistance include ribosomal alterations, efflux of the agents by extrusion pump, or altered permeability leading to reduced uptake (3). While ribosomal protection by methylation of 16S rRNA in aminoglycoside-producing actinomycetes gives high-level resistance to intrinsic aminoglycosides, no clinical isolate with this mechanism has been found among nosocomial bacteria (5, 6).

However, a novel plasmid-mediated 16S rRNA methylase which conferred an extraordinarily high level of resistance to aminoglycosides was identified quite recently in a *Pseudomonas aeruginosa* clinical strain in Japan (23) and submitted to the DNA Data Bank of Japan in April 2002 (DDBJ accession no. AB083212). It was the first report of aminoglycoside resistance mediated by 16S rRNA methylase in gram-negative bacteria. Acquisition of such an efficacious resistance mechanism by *P. aeruginosa* was alarming, and there was concern about possible further dissemination of 16S rRNA methylase genes among *P. aeruginosa* and also into other gram-negative bacterial species (23).

Serratia marcescens S-95 isolated from an inpatient in Japan displayed a very high degree of resistance to many aminoglycosides, including arbekacin. This was an unusual observation, because arbekacin is generally stable under enzymatic modification, and only the bifunctional enzyme AAC(6′)-APH(2″) is known to confer low-level resistance to arbekacin (15). Quite recently, another putative 16S rRNA methylase, ArmA, conferring high-level resistance to aminoglycosides, was found in a *Klebsiella pneumoniae* clinical isolate in France (10). The ArmA showed a moderate degree of similarity in amino acid sequence with some 16S rRNA methylases previously reported from actinomycetes. In the present study, we aimed to elucidate the mechanism of the high-level resistance to various aminoglycosides, including arbekacin, found in a clinically isolated *S. marcescens* strain.

MATERIALS AND METHODS

Clinical background. *S. marcescens* S-95 was isolated in March 2002 from sputum of a 76-year-old male patient at a 500-bed general hospital in Japan. He was originally admitted due to cerebral hemorrhage, but the course had been complicated with subdural hematoma, bronchial asthma, pneumonia, and urinary tract infection. The patient had received panipenem, minocycline, vancomycin, and levofloxacin in the month before isolation of the strain.

Bacterial strains and plasmids. The strains and plasmids used in the study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or agar plates (BD Diagnostic Systems, Sparks, Md.) supplemented with appropriate antibiotics.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Plasmid	Characteristic(s)
S. marcescens S-95	pKRC	Clinical isolate from sputum, Kochi, Japan
E. coli CSH2		Resistant to rifampin and nalidixic acid
E. coli XL1-Blue		*supE44 recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 lac* [F′ *proAB*$^+$ *lacI*^{q}ZΔM15::Tn*10*(Tet^r)]
E. coli XL1-Blue	pKRC	Transformant
E. coli XL1-Blue	pBCSK+	Chloramphenicol-resistant cloning vector
E. coli XL1-Blue	pS95B2	Transformant containing a 4.6-kb *Bam*HI fragment with *rmtB* ligated to pBCSK+
E. coli XL1-Blue	pS95S8	Transformant containing a 1.2-kb *Sau3*AI fragment with *rmtB* ligated to pBCSK+
E. coli XL1-Blue	pET29a(+)	Kanamycin-resistant cloning-expression vector
E. coli BL21(DE3)pLysS		F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *dcm gal*, λ(DE3) pLysS Cm^r
E. coli BL21(DE3)pLysS	pS95H5	Transformant containing a PCR-amplified *rmtB* ligated to pET29a(+)

Antibiotics and susceptibility testing. The following antibiotics were obtained from the indicated sources: amikacin, Bristol Pharmaceuticals Y. K., Tokyo, Japan; arbekacin, kanamycin, and streptomycin, Meiji Seika Kaisha Ltd., Tokyo, Japan; chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; gentamicin and sisomicin, Schering-Plough K. K., Osaka, Japan; hygromycin B, Sigma Aldrich Japan K. K., Tokyo, Japan; isepamicin, Asahi Kasei Corporation, Tokyo, Japan; neomycin, Nippon Kayaku Co., Ltd., Tokyo, Japan; rifampin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; and tobramycin, Shionogi Pharmaceutical Co., Osaka, Japan.

MICs were determined by the agar dilution method using Mueller-Hinton agar (BD Diagnostic Systems) and according to the protocol recommended by the National Committee for Clinical Laboratory Standards (17).

Transfer of aminoglycoside resistance genes. Conjugation experiments were conducted using *Escherichia coli* CSH2 as the recipient by broth mating and filter mating methods (7, 9). Transconjugants were selected on LB agar supplemented with rifampin (50 μg/ml), nalidixic acid (50 μg/ml), and kanamycin (25 μg/ml). Plasmid DNA of *S. marcescens* S-95 was purified by the method of Kado et al. (13). Transformation of *E. coli* XL1-Blue with the plasmid DNA of *S. marcescens* S-95 was performed using standard electroporation techniques. Transformants were selected on LB agar containing kanamycin (25 μg/ml).

Cloning and sequencing of aminoglycoside resistance genes. The basic recombinant DNA techniques were carried out as described by Sambrook et al. (19). The plasmid DNA of *S. marcescens* S-95 was digested with *Bam*HI, and the resultant fragments were ligated in plasmid vector pBCSK$^+$ (Stratagene, La Jolla, Calif.). Electrocompetent *E. coli* XL1-Blue was then transformed with these recombinant plasmids. Transformants were selected by resistance to chloramphenicol (30 μg/ml) and kanamycin (25 μg/ml). The enzymes used for gene manipulation were purchased from New England Biolabs, Inc. (Beverly, Mass.) or Takara Bio Inc. (Otsu, Japan). The DNA sequences were determined on both strands using BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). The alignments of nucleotide and amino acid sequences were performed with GENETYX-MAC (version 10.1.1; Software Development Co., Ltd., Tokyo, Japan).

Preparation of 30S ribosomal subunits. 30S ribosomal subunits of *E. coli* XL1-Blue were prepared as described by Skeggs et al. (21). After ultracentrifugation with sucrose density gradients, fractions corresponding to 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 subunits was checked by denatured agarose gel electrophoresis of 16S rRNA derived from the material, and the 30S ribosomal subunits were stored at −80°C in aliquots until use.

Expression and purification of histidine-tagged RmtB. For use in methylation assays, RmtB was purified using a histidine-tag purification system. The entire coding region of *rmtB* was amplified by PCR with primers MBH-F (5′-GGAA TTCCATATGAACATCAACGATGCCCT-3′) and MBH-R (5′-CCGCTCGA GTCCATTCTTTTTTATCAAGTA-3′). The product was partially double digested with *Nde*I and *Xho*I, and ligated to pET29a(+) (Novagen, Madison, Wis.) double digested with the same enzymes. Electrocompetent *E. coli* XL1-Blue was transformed with the recombinant plasmids, and transformants were selected on LB agar containing arbekacin (20 μg/ml). Several colonies obtained were found to harbor plasmids with inserts encoding RmtB tagged with six histidine residues at the C-terminal end. *E. coli* BL21(DE3)pLysS (Novagen) was transformed with one such plasmid, pS95H5. The transformants were cultured in 1 liter of LB broth supplemented with kanamycin (25 μg/ml) to an optical density (A_{620}) of approximately 0.7. IPTG (isopropyl-D-thiogalactopyranoside) (0.5 mM) was then added to the culture, and a further 2-h incubation was conducted before harvesting. The pellet was washed once with 50 mM phosphate buffer (pH 7.0) and suspended in 20 mM phosphate buffer (pH 7.4) containing 10 mM imidazole. The suspension was passed through a French pressure cell (Ohtake Works Co., Ltd., Tokyo, Japan) at 120 MPa twice and then centrifuged at 30,000 × *g* for 30 min. Histidine-tagged RmtB contained in the supernatant was purified using HiTrap Chelating HP included in the HisTrap kit (Amersham Biosciences, K. K., Tokyo, Japan) according to the manufacturer's instructions. It was eluted at an imidazole concentration of 300 mM, and found to be over 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Finally, the enzyme was dialyzed at 4°C against 2 × 200 volumes of HRS buffer (10 mM HEPES-KOH, pH 7.5; 10 mM $MgCl_2$; 50 mM NH_4Cl; 3 mM 2-mercaptoethanol) and stored at −80°C in aliquots until use.

Methylation assay of 16S rRNA. The reaction mixtures for methylation experiments contained 10 pmol of 30S ribosomal subunits from *E. coli* XL1-Blue, 10 pmol of histidine-tagged RmtB, and 2.5 μCi of [methyl-^{3}H]*S*-adenosyl methionine (SAM) and were adjusted to 100 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 RmtB was replaced by an equal volume of HRS buffer. The reactions were carried out at 35°C, and 30-μl aliquots of reaction mixtures were sampled at 0, 10, and 30 min, respectively. Each sample was purified immediately using an RNeasy Mini kit (QIAGEN K. K., Tokyo, Japan) according to the instructions provided by the manufacturer. The eluate (50 μl) containing purified 16S rRNA was spotted on DEAE Filtermat 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 1450 MicroBeta TRILUX (Perkin-Elmer), and the radioactivity of each spot was determined.

Nucleotide sequence accession number. The entire nucleotide sequence containing *rmtB* and determined in this study appears in the EMBL/GenBank/DDBJ databases under accession number AB103506.

RESULTS

Aminoglycoside resistance of *S. marcescens* S-95. The MICs of aminoglycosides for *S. marcescens* S-95 are listed in Table 2. S-95 showed a high level of resistance to kanamycin, tobramycin, amikacin, arbekacin, gentamicin, sisomicin, isepamicin, streptomycin (MIC, >1,024 μg/ml), and hygromycin B (MIC, 128 μg/ml), but not neomycin.

Transfer of aminoglycoside resistance. The aminoglycoside resistance of *S. marcescens* S-95 could not be transferred to the recipient *E. coli* strain CSH2 by conjugation despite repeated attempts. However, the aminoglycoside resistance could be transferred to *E. coli* XL1-Blue by electroporation, and the resultant transformants harbored a large nonconjugative plasmid of the parental strain, which was designated pKRC.

Cloning of aminoglycoside resistance gene. Competent cells of *E. coli* XL1-Blue were electrotransformed with recombinant plasmids of pBCSK$^+$ carrying a *Bam*HI-digested fragment of total DNA from *S. marcescens* S-95. Transformants obtained with selection by kanamycin and chloramphenicol were found

TABLE 2. Results of antibiotic susceptibility testing

Aminoglycoside	MIC (μg/ml)				
	S. marcescens S-95	*E. coli* XL1-Blue(pKRC)	*E. coli* XL1-Blue(pS95B2)	*E. coli* XL1-Blue(pS95S8)	*E. coli* XL1-Blue(pBCSK+)
Kanamycin	>1,024	>1,024	>1,024	>1,024	0.5
Tobramycin	>1,024	1,024	64	128	0.25
Amikacin	>1,024	1,024	1,024	>1,024	0.5
Arbekacin	>1,024	256	256	1,024	0.13
Gentamicin	>1,024	>1,024	1,024	1,024	0.13
Sisomicin	>1,024	>1,024	128	512	0.13
Isepamicin	>1,024	>1,024	1,024	1,024	0.25
Neomycin	2	0.5	0.5	0.5	0.5
Hygromycin B	128	16	8	16	16
Streptomycin	1,024	128	0.5	0.5	2

to possess recombinant plasmids with a 4.6-kb *Bam*HI insert. One such plasmid (pS95B2) was selected for further study. The 1.2-kb *Sau3*AI fragment was recloned with *Bam*HI-cleaved pBCSK+, and the resultant recombinant plasmid was assigned pS95S8. The MICs of aminoglycosides for *E. coli* XL1-Blue (pKRC), XL1-Blue(pS95B2), and XL1-Blue(pS95S8) are listed in Table 2. The spectrum of resistance of XL1-Blue(pS95B2) included aminoglycosides belonging to the kanamycin and gentamicin groups, while the degree of resistance was generally lower than that of the parental strain. MICs of aminoglycosides for XL1-Blue(pS95S8) were generally higher than those for XL1-Blue(pS95B2), and this might due to probable multicopy effect of small plasmid. Both transformants were susceptible to streptomycin and neomycin. XL1-Blue(pKRC) carrying the large plasmid from S-95 was resistant to streptomycin as well. This streptomycin resistance was attributed to the presence of the integron-borne streptomycin resistance gene *aadA2* on pKRC (data not shown).

DNA sequencing of pS95B2. The entire 4.6-kb insert of pS95B2 was sequenced in the search for a kanamycin-gentamicin resistance determinant. The overall structure of the sequenced region is depicted in Fig. 1. The first 1.4 kb comprised the right end of Tn*3* and included part of *tnpR* and bla_{TEM}, ending with the right-hand inverted repeat (11). An open reading frame encoding 251 amino acids was located immediately downstream of the inverted repeat. It showed 82% amino acid identity with *rmtA*, which was recently reported as an aminoglycoside resistance gene encoding 16S rRNA methylase in a *P. aeruginosa* clinical isolate (23), and therefore was designated *rmtB*. A comparison of deduced amino acid sequences of RmtA and RmtB is shown in Fig. 2. The identity of amino acid residues between RmtB and ArmA was 29%. Identities with other 16S rRNA methylases produced by *Streptomyces* and *Micromonospora* species were generally lower. Amino acid identities of RmtB were 33 and 32% with GrmB and Sgm methylases of sisomicin-producing *Micromonospora rosea* and *Micromonospora zionensis*, respectively (14); 32% with GrmA methylase of gentamicin-producing *Micromonospora purpurea* (14); 31% with Kmr methylase of kanamycin-producing *Streptomyces kanamyceticus* (8); 30% with FmrO methylase of fortimicin-producing *Micromonospora olivasterospora* (18); and 27% with KgmB of nebramycin-producing *Streptomyces tenebrarius* (12). The putative promoter region of *rmtB* appeared to be located within the right-hand end of Tn*3*, just upstream of the inverted repeat (Fig. 1b). The nucleotide sequence upstream of *rmtB* shared no significant similarity with that of *rmtA*. On the other hand, the sequences downstream of the two genes showed 78% identity for approximately 0.8 kb and then diverged. The only other open reading frame identified was truncated at the end of the cloned insert. The available sequence indicated that it encoded at least 358 amino acids, which shared 99% identity with Orf2, a transposase-like protein of *Salmonella enterica* serovar Typhimurium (2), and 56% identity with Orf513, a putative transposase known to be associated with *sul1*-type complex integrons (9).

Methylation activity of RmtB. Histidine-tagged RmtB-producing *E. coli* XL1-Blue demonstrated a high-level resistance to arbekacin (MIC, >128 μg/ml), as well as to the other aminoglycosides (data not shown). Therefore, this recombinant protein was purified and used as the enzyme in the methylation assay. The result is depicted in Fig. 3. The vigorous incorporation of radiolabeled methyl groups into 16S rRNA of 30S ribosomal subunits from *E. coli* XL1-Blue in the presence of purified RmtB confirmed that RmtB was in fact a functional 16S rRNA methylase.

DISCUSSION

Ribosomal protection by methylation of 16S rRNA has been known as a principal mechanism of aminoglycoside resistance among some aminoglycoside-producing organisms such as *Streptomyces* spp. and *Micromonospora* spp. Although production of such 16S rRNA methylases confers a very high level of aminoglycoside resistance to the producers, it had been thought that this mechanism was confined to environmental bacterial species without clinical relevance (5, 6).

This picture changed when a *P. aeruginosa* clinical strain AR-2 was found to produce 16S rRNA methylase, which conferred an extremely high level of resistance (MIC, >1,024 μg/ml) to a wide spectrum of aminoglycosides (23). The responsible gene, *rmtA*, was located on a self-transmissible plasmid, and therefore further dissemination of the gene among *P. aeruginosa* and other gram-negative bacteria was anticipated (23).

In fact, the present study identified the emergence of an *S. marcescens* clinical strain producing 16S rRNA methylase. This novel enzyme RmtB conferred high-level resistance to various aminoglycosides. The spectrum included 4,6-disubstituted deoxytreptamine aminoglycosides such as kanamycin, tobramycin, amikacin, arbekacin, gentamicin, sisomicin, and isepami-

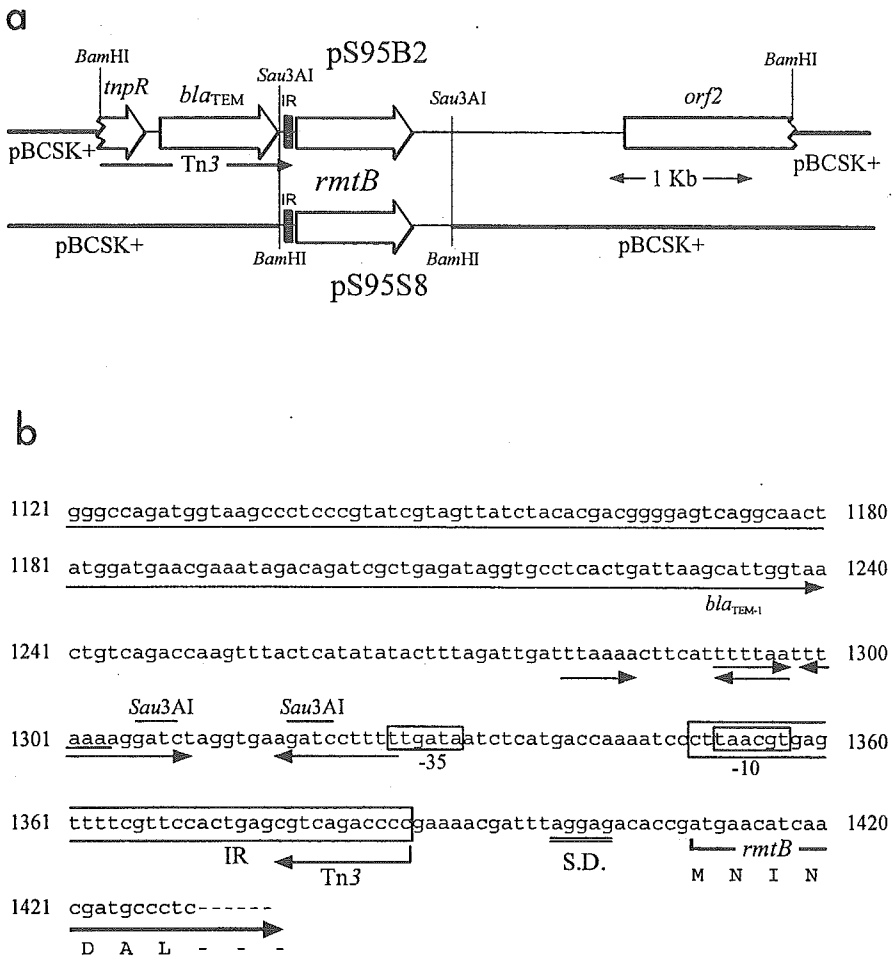

FIG. 1. (a) Schematic presentation of the 4.6-kb *Bam*HI fragment of pS95B2 and 1.2-kb *Sau3*AI fragment of pS95S8. Shaded boxes indicate terminal inverted repeats (IR) of Tn*3*. The 1.2-kb insert of pS95S8 carries only IR and the *rmtB* gene as well as its possible promoter. (b) Region upstream of the *rmtB* gene. The nucleotide sequence containing the inverted repeat (IR) of the transposon *3* (Tn*3*) and region upstream of the *rmtB* gene are shown. The open reading frame of bla_{TEM} is terminated at ^{1,238}TAA. Several dyad symmetries are indicated with horizontal arrows. Possible −35 and −10 regions are boxed. IR sequence of Tn*3* is enclosed with an open oblong box. A Shine-Dalgarno-like sequence (S.D.) (^{1,399}AGGAG) is located just upstream of the initiation codon (^{1410}ATG) of the *rmtB* gene.

cin. However, RmtB did not confer resistance to neomycin, streptomycin, and hygromycin B, all of which have different aminocyclitol components. This resistance pattern is consistent with that conferred by RmtA and includes most of the parenteral bactericidal aminoglycosides administered for serious infections caused by gram-negative bacteria (23).

RmtB shared 82% identity with RmtA of *P. aeruginosa*, while its similarity with the 16S rRNA methylases of the genera *Streptomyces* and *Micromonospora* was relatively low (up to 33%). As to the origin of the cluster of enzymes including RmtB and RmtA, at this stage we assume that the responsible genes have been mobilized to *S. marcescens* and *P. aeruginosa* independently from some yet unidentified aminoglycoside-producing bacterial species which are likely related to one another.

In previous studies, crude extracts of the 16S rRNA methylase-producing organisms were used as the enzyme for methylation assays (21, 22, 23). The incorporation rate of SAM was approximately twofold compared with controls in these reports. For improved specificity, we constructed histidine-tagged RmtB, which rendered its producer resistant to kanamycins and gentamicins. The protein was readily purified and subsequently used for methylation assay in place of crude enzyme. As a result, vigorous methylation of 16S rRNA could be observed, resulting in more than a 20-fold difference in the rate of incorporation of SAM between the RmtB-containing reaction mixtures and controls (Fig. 3).

16S rRNA methylases produced by aminoglycoside-producing actinomycetes are known to confer either a kanamycin-gentamicin resistance pattern or a kanamycin-apramycin resistance pattern (5). The MICs shown in Table 2 indicate that RmtB belongs to the former group of enzymes. GrmB produced by *M. purpurea*, which belongs to the kanamycin-gentamicin group, was previously shown to methylate G1405 within the A-site of 16S rRNA, resulting in resistance of the producer to kanamycin and gentamicin but not neomycin or apramycin (1). This methylation is known to prevent the formation of hydrogen bonds with ring III of gentamicin C1a, a

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RmtB  1 MNINDALTSILASKKYRALCPDTVRRILTEEWGRHKSPKQTVEAARTRLHGICGAYVTPE  60
RmtA    MSFDDALASILSSKKYRSLCPDTVRRILDQEWGRHKSPKLAVEATRTRLHGICGAYVTPE
ArmA    MDKNDVVKKILESKKYENLDSDIVEKVVSISEKKYKLKEVENYSKK-KLHQIWGSYYSAY
        *. :*.: .** ****. * .* *.::: . ::* : : : .** * *:* :

RmtB    -SLKAAAAALSAG--DVKKALSLHASTKERLAELDTLYDFIFS-AETPARVLDIACGLNP 116
RmtA    -SLKAAAAALSVG--DVQKALSLHASTKERLAELDCLYDFIFS-GGVPHRVLDIACGLNP
ArmA    PNWDKLLKKYNQGQLSIEDLLKIHSSTNERVATLNDFYTYVFGNIKHVSSILDFGCGFNP
         . .      . *  .::. *.:*:**:**:* *: :* ::*.       :**:.**:**

RmtB    LALYERGIAS---VWGCDIHQGLGDVITPFAREKDWDFTFALQDVLCAPPAEAGDLALIF 173
RmtA    LALFIRDITS---VWACDIHQGLGDVITPFAHHQGLDFTFALQDVMCTPPTETGDLALVF
ArmA    LALYQWNENEKIIYHAYDIDRAEIAFLSSIIGKLKTTIKYAFLNKESDVYKGTYDVVFLL
        ***:  . .     . **.:.  .::.: .    :.: : :  .    : *:.:::

RmtB    KLLPLLEREQAGSAMALLQSLNTPRMAVSFPTRSLGGRGKGMEANYAAWFEGGLPAEFEI 233
RmtA    KLLPLLEREQAGAAMALLQALATPRIAVSFPTRSLGGRGKGMEANYSAWFEGALPDEFEI
ArmA    KMLPVLK-QQDVNILDFLQLFHTQNFVISFPIKSLSGKEKGMEENYQLWFESFTKGWIKI
        *:**:*: :*    : :** : * .:.:*** :**.*: **** **  ***.     ::*

RmtB    EDKKTIGTELIYLIKKNG-                                         251
RmtA    EDTKTIGIELVYMIKRNK-
ArmA    LDSKVIGNELVYITSGFQK
         *.*.** **:*: .
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FIG. 2. Alignment of the deduced amino acid sequence of RmtB with those of RmtA and ArmA. Asterisks indicate amino acid identities, and dots denote conserved replacements. Residues conserved among representative 16S rRNA methylases (GenBank accession no. AB083212, AB103506, AY220558, M55520, M55521, M87057, S60108, and Y15838) are underlined.

major component of gentamicin which usually interacts with G1405 · C1496 and U1406 · U1495 base pairs (24). We may therefore speculate that RmtB methylates a nucleotide within the A-site in a similar fashion.

rmtB was carried on a large plasmid, pKRC, which was nonconjugative but transferable to *E. coli* by electroporation. The neighboring sequence of *rmtB* was interrupted only 22 bp upstream of the initiation codon in the presence of Tn*3*. However, the 0.8-kb region downstream of *rmtB* shared significant identity with the corresponding region of *rmtA*, thus reenforcing the idea that the two genes may have come from similar bacterial species. The mode of acquisition of *rmtB* by pKRC was not clear from the sequence information obtained in this study alone. This large plasmid also possessed an integron-mediated streptomycin resistance gene, *aadA2*, so the aminoglycoside-resistant phenotype seen in S-95 could be accounted for solely by the presence of pKRC. The integron is a well-recognized DNA recombination system that mediates the integration of antibiotic resistance genes through site-specific recombination (4). Future acquisition of additional antibiotic resistance genes by pKRC may well be possible.

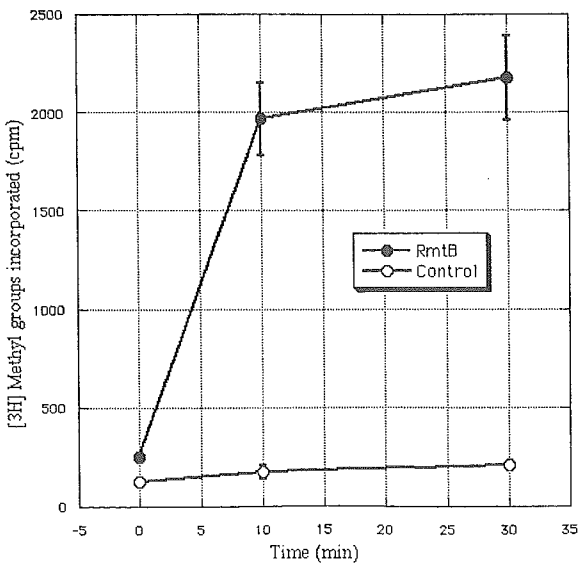

FIG. 3. Methylation of 16S rRNA. The 16S rRNA from *E. coli* XL1-Blue was incubated with purified RmtB using [methyl-^{3}H]SAM as a cofactor. The value of each point was calculated with three data points. Error bars, standard deviations.

Nosocomial bacteria producing RmtB or RmtA have been uniformly pan-resistant to 4,6-disubstituted deoxytreptamines, which cannot be accounted for by production of a single aminoglycoside-modifying enzyme except for the bifunctional enzyme AAC(6′)-APH(2″) produced by some methicillin-resistant *Staphylococcus aureus* strains. When a gram-negative pathogen with high-level resistance (MIC, >128 μl/ml) to both gentamicin and amikacin or tobramycin is detected in the clinical laboratory, additional susceptibility testing using arbekacin may prove useful. If the MIC of arbekacin exceeds 128 μl/ml, it strongly suggests that the strain produces 16S rRNA methylase.

In conclusion, we have described the emergence of high-level aminoglycoside resistance mediated by production of a

novel 16S rRNA methylase in an *S. marcescens* clinical isolate. Dissemination of *rmtB* to other enterobacterial species as well as among *S. marcescens* is of concern.

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A New TEM-Derived Extended-Spectrum β-Lactamase (TEM-91) with an R164C Substitution at the Ω-Loop Confers Ceftazidime Resistance

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A new plasmid-mediated TEM-derived extended-spectrum β-lactamase, TEM-91, was identified in a ceftazidime-resistant (MIC, >128 μg per ml) *Escherichia coli* strain isolated in 1996 in Japan. TEM-91 has three amino acid substitutions, R164C, M184T, and E240K, compared with TEM-1 penicillinase. The isoelectric point (pI), K_m, and k_{cat} of TEM-91 for ceftazidime were 5.7, 179 μM, and 29.0 s^{-1}, respectively. The K_i of clavulanic acid for ceftazidime hydrolysis was 30.3 nM.

Since broad-spectrum β-lactams, including expanded-spectrum cephalosporins, cephamycins, and carbapenems, are efficacious agents for the control of infectious diseases caused by gram-negative bacilli, the emergence and proliferation of such microorganisms that have acquired consistent resistance to the above-mentioned antimicrobial agents are becoming actual impediments in clinical settings (2, 3, 11). For instance, the worldwide proliferation of extended-spectrum-β-lactamase (ESBL)-producing clinical isolates belonging to the family *Enterobacteriaceae*, such as *Escherichia coli* and *Klebsiella pneumoniae*, is creating real problems in the provision of high-grade medical treatment, including organ transplantations, surgical operations, and chemotherapy for patients with malignancies (6, 7). More than 119 TEM-related β-lactamases, including inhibitor-resistant enzymes and TEM-derived ESBLs that hydrolyze oxyimino-cephalosporins and monobactams, have been recorded to date (12; Jacoby, G. A., and K. Bush, Amino acid sequences from TEM, SHV, and OXA extended-spectrum and inhibitor resistant β-lactamases [http://www.lahey.org/studies/webt.htm]). According to the report by the National Nosocomial Infections Surveillance system conducted by the Centers for Disease Control and Prevention in the United States, *K. pneumoniae* resistant to oxyimino-cephalosporins increased to >10% in intensive care units in 2001 (5), and most of these strains are speculated to be ESBL producers. In Japan, however, only a few TEM-derived ESBLs have been reported, although several *E. coli* and *K. pneumoniae* strains producing CTX-M-type enzymes, including Toho-1, or SHV-derived ESBLs, such as SHV-12, have been isolated in geographically separate medical institutions (9, 23, 24). In this study, a new TEM-derived ESBL, TEM-91, possessing three amino acid substitutions, R164C, M182T, and E240K, was characterized.

The ceftazidime (CAZ)-resistant *E. coli* strain HKY322 was isolated from a urine sample of a patient in 1996, and the MIC of CAZ for this strain was >128 μg per ml. The strain, however, was susceptible to other oxyimino-β-lactams, such as cefotaxime, as shown in Table 1. The CAZ resistance was transferred to *E. coli* CSH2 (F^- *metB* Na^r Rif^r) by conjugation analysis (10) concurrently with the transmission of a resident large plasmid. The inhibition tests with clavulanic acid and PCR analyses suggested that this strain produced a TEM-type enzyme. An *Eco*RI-digested DNA fragment carrying the gene for CAZ resistance in strain HKY322 was ligated with a cloning vector, pBCSK$^+$, and CAZ resistance was expressed in *E. coli* XL1-Blue cells by a transformation with the recombinant plasmids. The antibiotic susceptibility profiles of the parental strain HKY322 and the clone *E. coli* XL1-Blue(pBCTEM91) are shown in Table 1. Strain HKY322 showed resistance to CAZ, as well as to ampicillin and piperacillin, but did not show resistance to other oxyimino-cephalosporins, such as cefotaxime (MIC, 4 μg/ml) and ceftriaxone (MIC, 1 μg/ml) (Table 1).

Nucleotide sequencing analyses of both strands of the cloned DNA fragment were performed with an ABI Prism 377 DNA sequencer using the M13 universal primer and truncated mutants made with a deletion kit (Takara Co. Ltd., Kyoto, Japan). From cloning and sequencing analyses of the genetic determinant for CAZ resistance, strain HKY322 was found to produce a new TEM-derived ESBL, TEM-91. Four point mutations were found in the coding region of the bla_{TEM-91} gene compared with the bla_{TEM-1A} gene (EMBL accession number X54604), and these mutations caused three amino acid substitutions, R164C, M182T, and E240K, numbered according to the residue numbering scheme for class A β-lactamases by Ambler et al. (1) and Leflon-Guibout et al. (16) (Table 2).

The purification of TEM-91 from bacterial culture of *E. coli* XL1-Blue(pBCTEM91) was performed according to the methods described previously (14) with a HiLoad 16/60 Superdex 200 prepgrade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 50 mM Tris-HCl buffer (pH 8.0). Anionic-exchange chromatography was performed on a HiTrap Q HP column (Pharmacia Biotech) preequilibrated with the same buffer by using a high-performance liquid chromatography system (Pharmacia Biotech). Proteins were eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer. Fractions with activity were pooled and concentrated with an Ultra-

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TABLE 1. Susceptibilities of *E. coli* HKY322 producing TEM-91 and transconjugant to β-lactams and β-lactam–β-lactamase inhibitor combinations

E. coli strain	MIC (μg/ml)[a]										
	AMP	CER	CAZ	CAZ-CLA 4	CTX	CTX-CLA 4	CPM	MOX	CMNX	ATM	IPM
HKY322	>128	128	>128	0.5	4	<0.5	16	2	<0.5	32	<0.5
XL1-Blue-(pBCTEM91)	128	64	64	<0.5	1	<0.5	2	1	1	8	<0.5
XL1-Blue	4	2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Abbreviations: AMP, ampicillin; CER, cephaloridine; CAZ, ceftazidime; CLA, clavulanic acid; CTX, cefotaxime; CPM, cefpirome; MOX, moxalactam; CMNX, cefminox; ATM, aztreonam; IPM, imipenem. The number after "CLA" indicates concentration (micrograms per milliliter).

free-15 centrifugal filter device (Millipore Corporation, Bedford, Mass.). This elution process was repeated four times. Fractions with activity were then passed through the size exclusion and anion-exchange columns once again. The purified enzymes were used for subsequent β-lactamase assays. To determine the isoelectric point (pI), 10 μl of purified enzyme solution was loaded onto an Immobiline DryStrip (pHs 3 to 10; Pharmacia Biotech) with an IPGphor electrophoresis system (Pharmacia Biotech). The pI of TEM-91 was 5.7.

TEM-91 was assayed against various β-lactam substrates at 30°C in 50 mM phosphate buffer (pH 7.0) with an autospectrophotometer (model V-550; Nihon Bunko Ltd., Tokyo, Japan). The absorption maxima of the substrates used were as follows: for ampicillin, 235 nm; for aztreonam, 315 nm; for cefotaxime, 264 nm; for CAZ, 272 nm; and for cephaloridine, 295 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (20). K_m and k_{cat} values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. Inhibition studies were done at 30°C in 50 mM phosphate buffer (pH 7.0) with ampicillin or CAZ as substrates and clavulanate and sulbactam as inhibitors. Kinetic parameters of TEM-91 are shown in Table 3. CAZ was expected to have a relatively low affinity to the TEM-91 compared to those of cephaloridine and cefotaxime, but the relatively high hydrolytic velocity of TEM-91 for CAZ resulted in high hydrolysis of CAZ. However, TEM-91 hardly hydrolyzes cefotaxime, in contrast to TEM-72 (18), which has both the M182T and E240K substitutions. Moreover, TEM-91 showed low hydrolytic capacity (k_{cat}/K_m = 1.32×10^2) for aztreonam compared to that for CAZ, an infrequent occurrence in ESBLs, where aztreonam and CAZ hydrolysis are often closely associated. This enzyme was blocked effectively by clavulanic acid (K_i for CAZ, 30.3 nM).

R164H and R164S substitutions have been reported for several TEM-enzymes, such as TEM-5 and TEM-46. However, the substitution R164C found in TEM-91 is not common (http://www.lahey.org/studies/webt.htm). Only TEM-87 was reported to possess the R164C substitution, and this enzyme also hydrolyzes CAZ (17). Class A β-lactamases have a conserved structural domain Ω-loop consisting of amino acid residues R164 through D179, and this portion is fastened by a salt bridge across the side chains of R164 and D179 (12, 15, 21, 22, 23). It has also been suggested that steric hindrance of the tight Ω-loop structure toward the bulky 7β side chains of oxyiminocephalosporins results in the poor hydrolytic activity of TEM-1 penicillinase against these substrates (reference 23 and http://www.lahey.org/studies/webt.htm). From this perspective, it is reasonable to speculate that the hydrolytic activity of TEM-91 for CAZ may well depend on the enlargement of the active-site cavity through distortion of the Ω-loop structure as a result of the R164C substitution, which disrupts the salt bridge between R164 and D179 as was suggested for SHV-24 (14). The E240K substitution just near the active-site pocket of the enzyme would also reduce steric hindrance for bulky 7β functionality of CAZ as well as stabilization of the enzyme (19), and this might well result in the acceleration of the CAZ hydrolysis cycle of TEM-91. Actually, a high level of CAZ-hydrolyzing activity has been reported to occur in TEM-5 (CAZ-1), TEM-24 (CAZ-6), TEM-46 (CAZ-9), and TEM-61, which have E240K as well as the R164S or R164H substitution (http://www.lahey.org/studies/webt.htm).

The substitutions found at R164 are usually R164S and R164H and are caused by point mutations at nucleotide position 692 (CGT→AGT) and at nucleotide position 693 (CGT→CAT), respectively. The R164C substitution found in bla_{TEM-91}, however, is caused by a point mutation at nucleotide position 692 (CGT→TGT). Theoretically, the probability of occurrence of each point mutation should be the same among these three mutation types. The codon usage of UGU is not rare in *E. coli*, and molecular sizes of simple side chains of Ser (—CH_2 OH) and Cys (—CH_2 SH) are similar, while a long side chain [—$(CH_2)_3$ $C(NH_2)$—N^+H_2] of arginine is projected into the active-site pocket in wild-type class A β-lactamases. One might wonder, therefore, why only two enzymes, TEM-87 and TEM-91, have an R164C substitution among the TEM-derived enzymes reported, despite the fact that R164S and

TABLE 2. Silent mutations and amino acid substitutions found in TEM-91 compared with TEM-1A and TEM-1B

β-Lactamase	Codon (amino acid substitution, position) containing a mutation at position[a]:						
	209	226	436	604	692	747	917
TEM-91	ATG (M, 3)	TTC (F, 8)	GGT (G, 78)	GCG (A, 134)	TGT (C, 164)	ACG (T, 182)	AAG (K, 240)
TEM-1A	ATG (M, 3)	TTC (F, 8)	GGC (G, 78)	GCG (A, 134)	CGT (R, 164)	ATG (M, 182)	GAG (E, 240)
TEM-1B	ATG (M, 3)	TTT (F, 8)	GGT (G, 78)	GCT (A, 134)	CGT (R, 164)	ATG (M, 182)	GAG (E, 240)

[a] Standard numbering schemes for class A β-lactamases by Ambler et al. (1) and Leflon-Guibout et al. (16). Boldface nucleic acids are the mutation.

TABLE 3. Kinetic parameters for TEM-91 β-lactamase[a]

Agent	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_i (nM)[b]	
				CLA	SUL
Ampicillin	15.5	94.1	6.07×10^6	34.6	33.8
Cephaloridine	31.6	36.3	1.15×10^6	ND	ND
Ceftazidime	179	29.0	1.62×10^5	30.3	57.9
Cefotaxime	32.1	2.96	9.22×10^4	ND	ND
Aztreonam	94.8	0.0125	1.32×10^2	ND	ND

[a] Data are mean values from three measurements; each standard deviation was lower than 10%.
[b] Abbreviations: CLA, clavulanic acid; SUL, sulbactam; ND, not done.

R164H substitutions have thus far been found in at least 14 and 8 TEM-derived enzymes, respectively (http://www.lahey.org/studies/webt.htm). The R164C substitution observed in TEM-91 may thus have negative effects on the retention of the tertiary structure or function of this enzyme due to its susceptibility to oxidation or alkylating substances. The R164C substitution may also offset the folding and stability defects that occur with the M182T substitution (8), since substitutions at R164 are often associated with the M182T substitution, as has been observed for TEM-43, TEM-63, TEM-87, and TEM-107 (http://www.lahey.org/studies/webt.htm).

Strains producing TEM-derived ESBLs are still rare in Japan compared with their presence in Western countries, although some CTX-M-related class A β-lactamases, including Toho-1 and CAZ-hydrolyzing SHV-12 (9, 25), and several cephamycin-hydrolyzing class C β-lactamases, such as MOX-1 and CMY-9 (4), have been identified in Japan, as well as carbapenem-hydrolyzing metallo-β-lactamases such as IMP-1 (13). Thus, there is a need for more prudent use of broad-spectrum β-lactams and intensive surveillance of gram-negative bacilli that have acquired consistent resistance to oxyimino-β-lactams.

Nucleotide sequence accession number. The nucleotide sequence of bla_{TEM-91} and the deduced amino acid sequence of TEM-91 have been deposited in the EMBL and GenBank nucleotide sequence data banks through the DNA Data Bank of Japan with the assigned accession number AB049569.

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Nosocomial Outbreak of Infections by *Proteus mirabilis* That Produces Extended-Spectrum CTX-M-2 Type β-Lactamase

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Nineteen multidrug-resistant *Proteus mirabilis* strains were isolated from 19 patients suffering from infections probably caused by *P. mirabilis*. These strains were recovered from urine or other urogenital specimens of 16 inpatients and three outpatients with a hospitalization history in a urology ward of Funabashi Medical Center, from July 2001 to August 2002. These strains demonstrated resistance to cefotaxime, ceftriaxone, cefpodoxime, and aztreonam, while they were highly susceptible to ceftazidime (MIC, ≤0.5 μg/ml). The resistance level of these strains to cefotaxime was decreased by the presence of clavulanic acid. Therefore, the strains were speculated to produce extended-spectrum class A β-lactamases. These strains were later found to carry $bla_{CTX\text{-}M\text{-}2}$ genes by both PCR and sequencing analyses. The profiles of *Sma*I-digested genomic DNA of 19 isolates were distinguished into five different clusters by biased sinusoidal field gel electrophoresis. Four of them, consisting of 18 isolates, were suggested to be a clonal expansion. These findings suggested that a nosocomial outbreak of infections by CTX-M-2-producing *P. mirabilis* had occurred in our medical center. Most patients suffered from urogenital malignancies with long-term catheterization. Cefazolin, cefoperazone-sulbactam, and/or levofloxacin were mostly administered to the patients, but these agents seemed ineffective for eradication of CTX-M-2 producers. Early recognition and rapid identification of colonizing antimicrobial-resistant bacteria, including CTX-M-2-producing *P. mirabilis*, would be the most effective measures to cope with further spread of this kind of hazardous microorganism in clinical environments.

The increasing prevalence of plasmid-mediated extended-spectrum β-lactamases (ESBLs) in members of the family *Enterobacteriaceae* has become a serious clinical problem on a worldwide scale (8). ESBLs of Ambler's molecular class A (1) belonging to Bush's functional group 2be (10) are capable of hydrolyzing a wide range of β-lactams, including oxyimino-β-lactams and monobactam, but usually remain ineffective against cephamycins such as cefoxitin, cefmetazole, and cefotetan as well as carbapenems. These class A β-lactamases tend to be blocked by β-lactamase inhibitors such as clavulanic acid (10). The majority of ESBLs are derivatives of TEM-1, TEM-2, or SHV-1 enzymes, resulting from a few amino acid substitutions (10). In contrast to these TEM- and SHV-derived ESBLs, CTX-M type β-lactamases, which constitute a new family of class A enzymes, are exclusively active against cefotaxime compared to other oxyimino-cephalosporins, including ceftazidime (39).

More than 30 CTX-M-type β-lactamases have so far been described in various species of *Enterobacteriaceae* but mostly in *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Klebsiella pneumoniae* since the initial reports of Toho-1-producing *E. coli* in Japan (18) and CTX-M-1/MEN-1 in 1989 in Germany and France (2, 3). Strains producing other CTX-M enzymes have been isolated in separate geographic areas, including Europe (9, 15, 16, 35, 40), South America (4, 7, 30), and the Middle and Far East (4, 18, 23, 41). CTX-M-type β-lactamases can be classified into four clusters according to their amino acid sequences: CTX-M-1-group, with CTX-M-1 (4), -M-3 (16), -M-10 (27), -M-11 (GenBank accession no. AY005110), -M-12 (21), -M-15 (20), -M-22 (GenBank accession no. AY080894), and -M-23 (GenBank accession no. AF488377); CTX-M-2-group, with CTX-M-2 (4), -M-4 (15), -M-5 (9), -M-6 (14), -M-7 (14), -M-20 (36), -M-24 (GenBank accession no. AY143430), and Toho-1 (18); CTX-M-8 group (7); and CTX-M-9 group, with CTX-M-9 (35), -M-13 (12), -M-14/18 (29), -M-16 (6), -M-19 (32), -M-21 (36), and Toho-2 (23).

In this paper, we report a nosocomial outbreak of infections caused by CTX-M-2 β-lactamase-producing *Proteus mirabilis* in a urology ward. *P. mirabilis* is one of the most common causes of urinary tract infections. Because of the difficulty in eradicating *P. mirabilis* species from immunocompromised hosts (13), this bacterial species is usually considered an important cause of nosocomial infections (34). Although the most predominant plasmid-mediated β-lactamases found in clinical isolates of *P. mirabilis* are TEM-derived ESBLs (5, 11, 22, 24, 28, 33), the emergence of CTX-M-type enzymes with extended substrate specificity has been a serious concern (7, 36). In the present study, we investigated the CTX-M-2-producing *P. mirabilis* strains that caused a nosocomial outbreak in a urology ward in our medical center.

MATERIALS AND METHODS

Patients and bacterial strains. From July 2001 to August 2002, 19 nonduplicated multiresistant *P. mirabilis* clinical strains were isolated from 19 patients suffering from infections probably caused by *P. mirabilis*. These strains were recovered from urine or other urogenital specimens of 16 inpatients and three outpatients with a hospitalization history in a urology ward of Funabashi Medical Center. This hospital has 426 beds and serves as an acute-care municipal hospital

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TABLE 1. Origins of CTX-M-2-producing *P. mirabilis* isolates and medical records of patients

Patient no.	Strain no.[a]	Date of isolation (day/mo/yr)	Source	Age (yr)/sex	Underlying disease	Antibiotic used within 30 days before detection[b]
1	1	9/7/01	Operative wound	77/M	Bladder cancer	KAN, CFZ, CFP-SUL
2	2	24/7/01	Indwelling catheter urine	83/F	Bladder stone	CDR, CFZ, CFP-SUL
3	3	15/8/01	Indwelling catheter urine	66/M	Prostatic cancer	LVX, CFZ, CFP-SUL
4	4	30/8/01	Midstream urine	62/M	Retroperitoneal fibrosis, renal failure	LVX, CDR, CFZ, IPM/CS, CTM-HE
5	5	18/10/01	Indwelling catheter urine	83/M	Bladder cancer	LVX, CFZ, CFP-SUL, IPM/CS
6	6	22/10/01	Indwelling catheter urine	59/M	Bladder cancer	LVX, CFZ, CFP-SUL
7	7	29/10/01	Indwelling catheter urine	79/F	Postrenal failure, hydronephrosis	LVX, CFZ
8	8	8/11/01	Midstream urine	77/M	Bladder cancer	LVX, CFZ, CFP-SUL, IPM/CS
9	9	12/11/01	Midstream urine	73/M	Prostatic cancer	LVX, CFZ, CFP-SUL, CDR
10	10	12/11/01	Catheter urine	70/M	Renal failure, diabetes mellitus	IPM/CS, GEN, LVX, MIN
11	11	19/11/01	Midstream urine	72/M	Bladder cancer	CFZ, CFP-SUL
12	12	29/11/01	Midstream urine	62/M	Bladder cancer	KAN, LVX, CDR, CFZ
13	13	30/11/01	Indwelling catheter urine	44/M	Stomach cancer, hydronephrosis, pyelonephritis	CFP-SUL, ISP
14	14	6/12/01	Indwelling catheter urine	83/M	Prostatic cancer	CFZ, AMP, CAZ
15	15	30/4/02	Sputum	56/M	Rectal cancer, bladder cancer	FMOX, CAZ
16	16	19/6/01	Catheter urine	80/M	Bladder stone	LVX
17	17	13/12/01	Indwelling catheter urine	80/F	Bladder cancer	CFZ, CDR, IPM/CS, CFP-SUL
18	18	2/8/02	Midstream urine	65/M	Prostatic cancer	CFZ, CFP-SUL
19	19	29/8/02	Indwelling catheter urine	59/M	Bladder cancer	LVX, CFZ, CFP-SUL

[a] Strain no. 16, 17, and 18 were derived from outpatients with a hospitalization history in a urology ward.
[b] KAN, kanamycin; CFZ, cefazolin; CFP-SUL, cefoperazone-sulbactam; CDR, cefdinir; LVX, levofloxacin; IPM/CS, imipenem/cilastatin; CTM-HE, cefotiam-hexetil; GEN, gentamicin; MIN, minocycline; ISP, isepamicin; AMP, ampicillin; CAZ, ceftazidime; FMOX, flomoxef.

for a population of 560,000 in Funabashi City, Chiba, Japan. Tables 1 and 2 show the clinical background of patients for each isolate and their respective treatment outcomes. All 19 isolates were suggested to produce inhibitor-susceptible class A β-lactamase based on the double-disk synergy test results. Biochemical identification of isolates was performed with an NEG Combo 5J panel and WalkAway-96 SI System (Dade Behring, Sacramento, Calif.) according to the manufacturer's instructions. β-Lactamase testing was performed based on microacidimetry with a commercial product (P/Case Test; Nissui Pharmaceutical, Tokyo, Japan). Bacterial strains were stored before use in Casitone medium (Eiken Chemical, Tokyo, Japan) at room temperature.

Antimicrobial susceptibility testing. MICs were determined by a microdilution broth method with a WalkAway-96 SI System (NEG Combo 5J and NEG MIC 5J panels; Dade Behring) with an inoculum of 10^4 CFU per well. Susceptibility categories were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria (26).

ESBL plus Panel (Dade Behring) with an inoculum of 10^4 CFU per well was used complementarily for MIC measurements, with incubation for 18 h at 35°C, and then assessed visually.

Double-disk synergy test. For screening ESBL-producing strains, the double-disk synergy test was used. Antimicrobial disks for Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) tests, cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and amoxicillin-clavulanic acid (20 μg and 10 μg) were obtained from Nissui Pharmaceutical. The distance between disks was adjusted so that synergy could be detected accurately (38).

PCR analysis. A search for bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ genes in clinical isolates was performed by PCR amplification with the following sets of primers: 5′-CCGTGTCGCCCTTATTCC-3′ and 5′-AGG CACCTATCTCAGCGA-3′ for bla_{TEM}, 5′-ATTTGTCGCTTCTTTACTCG C-3′ and 5′-TTTATGGCGTTACCTTTGACC-3′ for bla_{SHV}, 5′-CGGTGCTG AAGAAAAGTG-3′ and 5′-TACCCAGCGTCAGATTAC-3′ for $bla_{CTX-M-1}$, 5′-ACGCTACCCCTGCTATTT-3′ and 5′-CCTTTCCGCCTTCTGCTC-3′ and for $bla_{CTX-M-2}$, and 5′-GCAGATAATACGCAGGTG-3′ and 5′-CGCCGTGG TGGTGTCTCT-3′ for $bla_{CTX-M-9}$. Freshly isolated colonies were suspended in distilled water and adjusted to a 0.5 MacFarland, which was boiled for 10 min. Supernatant obtained after centrifugation at 13,000 rpm for 5 min was used as template DNA.

PCRs were carried out in 50-μl volumes containing 5 μl of DNA, 0.5 μM each primer, 200 μM deoxynucleoside triphosphates, 1.25 U of TaKaRa Ex *Taq* (Takara), and PCR buffer (Takara) with the following parameters: initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, repeated for 30 cycles; and a final extension at 72°C for 5 min.

CTX-M-2-specific PCR and DNA sequencing. Amplification of the $bla_{CTX-M-2}$ gene and flanking regions was carried out with the oligonucleotide primers M-2-F (5′-TTCGCCGCTCAATGTTA-3′) and M-2-R (5′-GCATCAGAAACC GTGGG-3′), corresponding to nucleotides 22 to 38 and 852 to 868, respectively, of the structural gene. Plasmid DNA was prepared from each isolate by the Kado and Liu method (19) and used as templates for PCR analyses. PCRs were performed as described above. Cycling conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, repeated for 30 cycles. PCR-generated amplicons were purified with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Calif.), and sequenced directly on both strands with a BigDye terminator cycle sequencing ready reaction kit and ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.).

Genomic typing. Chromosomal DNAs from clinical isolates embedded in agarose gel plugs (InCert; Bio-Whittaker Molecular Applications, Rockland, Maine) were subjected to treatments with lysozyme and sodium dodecyl sulfate containing proteinase K, then incubated overnight at 30°C with 12.5 U of *Sma*I (Takara Shuzo Co., Kyoto, Japan). Plugs were mounted into the wells of a 1% SeaKem GTG Agarose (Bio-Whittaker) in 50 mM Tris-borate-EDTA buffer (pH 8.4). The biased sinusoidal field gel electrophoresis system (Atto Corp., Tokyo, Japan) (25), a modified pulsed-field gel electrophoresis technique utilizing a biased sinusoidal electric field for separation of large DNA molecules, was employed at 12°C with the field parameters Eb = 1.2 V/cm and Es = 7.3 V/cm. Lambda DNA ladders (48.5 kb to 1 Mb; Takara) were used as molecular size markers.

RESULTS

Bacterial strains and clinical features. Multiresistant *P. mirabilis* isolates were obtained from 16 inpatients in a urology ward and three outpatients with a hospitalization history in

TABLE 2. Treatment outcome for patients with respect to detection of CTX-M-2-producing *P. mirabilis* isolates

Patient no.	Antibiotic used after isolation of bacteria[a]		Treatment outcome[b]	Coisolates[c]
	During persistent detection	Upon eradication		
1	CFP-SUL, FMOX, CFZ		Failure	*S. aureus*, *Streptococcus agalactiae*
2	CDR		Failure	*K. pneumoniae*, *E. faecalis*
3	CFP-SUL, LVX		Indeterminate	ND
4	LVX, CDR, CFP-SUL		Failure	*P. aeruginosa*
5	LVX, CFZ, CFP-SUL, IPM/CS, ISP, CDR		Failure[d]	MRSA, *E. faecalis*, *P. aeruginosa*
6	CFP-SUL, FMOX, IPM/CS		Indeterminate[d]	*E. faecalis*
7	LVX	TMP, FMOX	Eradication	ND
8	LVX, TMP, CFPN-PI, GAT		Failure	ND
9	CFP-SUL, CDR		Failure	ND
10	LVX		Failure	MRSA, *S. agalactiae*
11	IPM/CS, MIN		Indeterminate[d]	ND
12		FMOX, LVX	Eradication[d]	*Tatumella ptyseos*
13	ISP, CFP-SUL	CFP-SUL, CAZ	Eradication[d]	ND
14	CAZ		Failure[d]	*E. faecalis*, *S. haemolyticus*, *E. cloacae*, MRSA
15	FMOX		Indeterminate[d]	*P. aeruginosa*, *S. aureus*, *K. oxytoca*
16	LVX		Indeterminate	*E. faecalis*
17			Indeterminate	MRSA, *Morganella morganii*
18	LVX		Indeterminate	ND
19		KAN, MTZ, LVX	Eradication	ND

[a] CFP-SUL, cefoperazone-sulbactam; FMOX, flomoxef; CFZ, cefazolin; CDR, cefdinir; LVX, levofloxacin; IPM/CS, imipenem/cilastatin; ISP, isepamicin; TMP, trimethoprim-sulfamethoxazole; CFPN-PI, cefcapene-pivoxil; GAT, gatifloxacin; MIN, minocycline; CAZ, ceftazidime; KAN, kanamycin; MTZ, metronidazole.
[b] Indeterminate, lack of necessary information.
[c] ND, not detected; MRSA, methicillin-resistant *S. aureus*.
[d] Died due to underlying disease.

that ward, 44 to 83 years old, 16 males and 3 females, from July 2001 to August 2002 (Table 1). Of 19 nonduplicated isolates, 9 isolates (strains 2, 3, 5, 6, 7, 13, 14, 17, and 19) were recovered from indwelling catheter urine samples, six isolates (strains 4, 8, 9, 11, 12, and 18) were from midstream urine samples, two isolates (strains 10 and 16) from catheter urine samples, one (strain 1) from an operative wound, and one (strain 15) from sputum. The common underlying disease was urogenital malignancies, including bladder cancer in nine patients, prostatic cancer in four patients, followed in order by renal failure in three, bladder stone in two, and stomach cancer as well as pyelonephritis in one patient. Cefazolin and cefoperazone-sulbactam had been administered most frequently in 12 patients, and levofloxacin had been prescribed to 11 patients within 30 days before isolation of the multiresistant *P. mirabilis*.

Table 2 shows the outcomes of antibiotic therapy for 19 patients after detection of the isolates, in which 12 patients were traceable for evaluation. Eradication was achieved in four patients with sulfamethoxazole-trimethoprim and flomoxef for patient 7, flomoxef and levofloxacin for patient 12, cefoperazone-sulbactam and ceftazidime for patient 13, and kanamycin, metronidazole, and levofloxacin for patient 19. In eight patients, *P. mirabilis* isolates were persistently detected despite the therapy with cefoperazone-sulbactam (patients 1, 4, 5, and 9), flomoxef (patient 1), imipenem/cilastatin (patient 5), and ceftazidime (patient 14). Treatment outcome could not be evaluated in seven patients due to lack of bacteriological follow-up in four and death from underlying disease in three patients. In 11 of 19 patients, other bacterial species were isolated besides *P. mirabilis*; *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were predominant. Moreover, no ESBL producers were detected among eight bacterial strains belonging to the family *Enterobacteriaceae* or *P. aeruginosa* isolated from 19 patients.

Determination of antibiotic susceptibility. MICs of antimicrobial agents for 19 clinical isolates are shown in Table 3. These isolates showed very similar susceptibility profiles, characterized by elevated MICs of cefotaxime (MICs, >128 μg/ml), ceftriaxone (MICs, >64 μg/ml), cefpodoxime (MICs, >64 μg/ml), aztreonam (MICs, 8 to >16 μg/ml), while they were susceptible to ceftazidime (MICs, ≤0.5 μg/ml). For all isolates, the MICs of cefotaxime were decreased drastically to ≤0.12 μg/ml in the presence of 4 μg of clavulanic acid per ml, whereas the MICs of ceftazidime for these strains were not obviously influenced by the presence of clavulanic acid. The MICs of cefoperazone-sulbactam were 8 to 32 μg/ml. MICs of other β-lactams, imipenem and melopenem, for the same strains were 2 μg/ml and ≤0.5 μg/ml, respectively. There was a trend towards resistance to gentamicin (MICs, 2 to >8 μg/ml), minocycline (MICs, >8 μg/ml), and levofloxacin (MICs, 2 to >4 μg/ml) among the isolates.

β-Lactamase study. The production of β-lactamase was detected by the P/Case test, which can distinguish between penicillinase (benzylpenicillin as the substrate) and cephalosporinase (cephaloridine and clavulanic acid as the substrate). Penicillinase production was detected in all 19 strains tested. With the double-disk synergy test, expanded growth-inhibitory zones indicative of class A β-lactamase production were observed with cefotaxime, ceftazidime, and aztreonam disks among all 19 strains (data not shown). MICs of cefotaxime (>128 μg/ml) decreased dramatically to ≤0.12 μg/ml in the

TABLE 3. Antibiotic susceptibilities of clinical *P. mirabilis* isolates presented in Table 1

Antibiotic(s)	MIC (μg/ml)[b] distribution (no. of isolates tested)
Ampicillin	>16 (19)
Amoxicillin/CLA	4/2 (19)
Piperacillin	>64 (19)
Cefazolin	>16 (19)
Cefotiam	>16 (19)
Cefoperazone/SUL	8/4 (2), 16/8 (14), 32/16 (3)
Cefotaxime	>128 (19)
Cefotaxime/CLA	≤0.12/4 (19)
Ceftazidime	≤0.5 (19)
Ceftazidime/CLA	≤0.12/4 (19)
Ceftriaxone	>64 (19)
Cefpirome	>16 (19)
Cefepime	>32 (19)
Cefozopran	>16 (19)
Cefaclor	>16 (19)
Cefpodoxime	>64 (19)
Cefoxitin	4 (10), 8 (8), 32 (1)
Cefmetazole	≤4 (18), 32 (1)
Cefotetan	≤0.5 (19)
Flomoxef	≤1 (17), 2 (2)
Imipenem	2 (19)
Meropenem	≤0.5 (19)
Aztreonam	8 (2), >16 (17)
Gentamicin	2 (4), 8 (1), >8 (14)
Amikacin	4 (5), 8 (14)
Minocycline	>8 (19)
Levofloxacin	2 (1), >4 (18)
Fosfomycin	>16 (19)

[a] NEG Combo 5J and NEG MIC 5J panels, and ESBL plus Panal were used for MIC determination.

presence of 4 μg of clavulanic acid per ml, suggesting the production of a CTX-M type class A β-lactamase (Table 2).

PCR and sequencing of $bla_{CTX-M-2}$ gene. The preliminary PCR search revealed that all 19 *P. mirabilis* isolates showed 780-bp amplification products for $bla_{CTX-M-2}$ genes, whereas no amplicons were observed for bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, or $bla_{CTX-M-9}$. The entire coding sequences of the $bla_{CTX-M-2}$ gene and flanking regions were subsequently amplified with more specific primers for $bla_{CTX-M-2}$, M-2-F and M-2-R, and sequenced on both strands. The BLAST analysis of the nucleotide sequences and the deduced protein sequences showed that *P. mirabilis* isolates produced CTX-M-2 group β-lactamase (4).

***Sma*I-digested genomic DNA profiles.** The *Sma*I-digested genomic DNAs of 19 clinical isolates were classified into five different clusters (Fig. 1). Thirteen isolates of strains 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 17 had the same restriction profiles (pattern I), while the patterns of strains 15, 18, and 19 differed from pattern I in only one band (pattern II). The patterns of strains 4 (pattern III) and 16 (pattern IV) were different from each other in three bands and differed in two bands from pattern I. Therefore, these 18 isolates were clonally related. The pattern of strain 2 (pattern V) was completely distinguishable from those of 18 isolates (Fig. 1).

DISCUSSION

ESBL production in *P. mirabilis* was first documented in 1993 (17), and the increase in clinical prevalence of ESBL-producing strains has recently been noted in survey studies in separate geographic areas, including the United States, Europe, and Asia. The proportion of ESBL-positive isolates has increased from 0.8% of *P. mirabilis* isolates in 1991 (17) to 6.9% in 1998 (13) in France. Surveillance studies conducted in the United States and Italy showed 9.5% and 8.8% ESBL prevalence among *P. mirabilis* isolates, respectively (22, 37). TEM-derived ESBLs showing a wide diversity were the most predominant among ESBLs (5, 11, 22, 24, 28, 33), but other enzymes belonging to group 2be (7, 36) have also been observed in *P. mirabilis*. Because of the production of such diverse class A β-lactamases in *P. mirabilis*, as well as its predilection for the urinary tract, the emergence and proliferation of multidrug resistant *P. mirabilis* could pose a threat, especially in catheterized patients with malignancy as a cause of subsequent nosocomial infections.

To our knowledge, this is the first report of a nosocomial outbreak of infections caused by CTX-M-2-producing *P. mirabilis* strains in a Japanese medical institution. Nineteen isolates found in its urology ward were initially speculated to produce CTX-M-type class A β-lactamase, since consistent high MICs

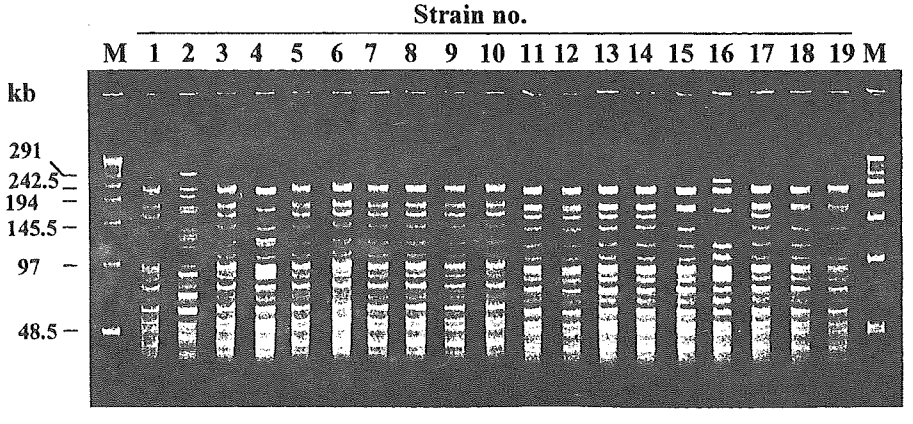

FIG. 1. Profiles of the genomic DNAs of 19 *P. mirabilis* isolates producing CTX-M-2 β-lactamase after digestion with *Sma*I. See Table 1 for the origins and backgrounds of the isolates. Lane M, lambda DNA ladder as molecular size markers.

of cefotaxime, ceftriaxone, cefpodoxime, and aztreonam were observed for these strains. The drastic reduction in the MICs of cefotaxime in the presence of clavulanic acid supported this speculation. Guidelines for screening and confirmatory tests for ESBL producers have been established by the National Committee for Clinical Laboratory Standards (26). These guidelines apply specifically to *K. pneumoniae*, *E. coli*, and *Klebsiella oxytoca*. However, the incidence of ESBLs producers has also been increasing in many other genera belonging to the family *Enterobacteriaceae* such as *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, and other gram-negative bacilli (8). This is also the case in our medical center, where nosocomial infection due to ESBL-producing *Acinetobacter baumannii* has recently been identified (unpublished data).

In our experience, the NCCLS guidelines could be applicable for detection of CTX-M-2 producers among *P. mirabilis* that produce no intrinsic AmpC cephalosporinases, although some modification in the NCCLS criteria might be needed. This would be of critical importance to be able to detect ESBL and CTX-M-type β-lactamase producers for effective clinical management of patients with infections by reliable and appropriate therapeutic options. Thus, accurate monitoring of ESBL prevalence would be mandatory to promote hospital infection control procedures.

The production of ESBL is alternatively confirmed by the double-disk synergy test, by which a synergistic effect on growth inhibition is observed with the coexistence of clavulanic acid and broad-spectrum cephalosporins, including cefotaxime, ceftazidime, or aztreonam. This test was also useful to detect CTX-M-2 production in *P. mirabilis* strains. The NCCLS recommends, however, reporting that *Klebsiella* spp. and *E. coli* strains producing ESBLs may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent in vitro susceptibility to some of these agents. This recommendation might cause confusion, implying that ceftazidime may be ineffective for treatment of infections caused by cefotaxime-resistant strains that produce CTX-M-type β-lactamase despite the fact that strains producing only CTX-M-type β-lactamase seem highly susceptible to ceftazidime.

As a practical matter, eradication of CTX-M-2-producing *P. mirabilis* isolates was successfully achieved by therapy with ceftazidime in patient 13, although combination therapy with cefoperazone-sulbactam was employed in this case. However, ceftazidime therapy alone failed to eradicate the organism in patient 14 (Table 2). There was a difference in treatment regimens between these two cases. Continuous administration of ceftazidime (1 g/day intravenously) for 8 days was used for patient 13, while two courses of 3-day-repeated administration of the same dosage at a 19-day interval was administered to patient 14. Furthermore, while patient 13 was infected only with *P. mirabilis*, patient 14 had polymicrobial infection with *E. faecalis*, *Staphylococcus haemolyticus*, *Enterobacter cloacae*, and methicillin-resistant *S. aureus* in addition to CTX-M-2-producing *P. mirabilis*. This, together with the incomplete antibiotic therapy, might explain the poor therapeutic response to ceftazidime in patient 14. To our knowledge, no clinical evidence obtained by double-blind clinical trials supporting the ineffectiveness of ceftazidime for infections with CTX-M-type producers has been reported. Clinical studies to address this issue should be conducted immediately to either corroborate or call into question the NCCLS recommendation.

Most patients from whom CTX-M-2-producing *P. mirabilis* strains were isolated had urological malignancies and long-term catheterization. All patients had received antibiotic therapy for 30 days before isolation of *P. mirabilis*, in which cefazolin, cefoperazone-sulbactam, and/or levofloxacin had been most frequently administered. Among the antibiotics used after detection of the organisms, cefoperazone-sulbactam, imipenem/cilastatin, and flomoxef showed low MICs. However, eradication with cefoperazone-sulbactam was noted in only one (patient 13) of five patients, and it was ineffective in combination with isepamicin. Since CTX-M-type enzymes can hydrolyze cefoperazone and tend to be hardly blocked by sulbactam, CTX-M producers usually demonstrate insusceptibility or resistance to the combination of cefoperazone-sulbactam (41). Thus, random or uniform prescription of cefoperazone-sulbactam may induce nosocomial spread of gram-negative bacilli which produce CTX-M-type enzymes.

Actually, 12 of 19 patients were prescribed cefoperazone-sulbactam prior to the isolation of CTX-M-2-producing *P. mirabilis*. The imipenem/cilastatin used in one patient (patient 5) proved in ineffective. Flomoxef was used in three patients (patients 1, 7, and 12), and eradication was achieved in the latter two cases. Levofloxacin, which tends to be preferentially used in urinary tract infections in Japan, showed high MICs for *P. mirabilis* isolates and seemed ineffective for most of the eight patients for whom it was used. These findings suggest difficulties in eradication of CTX-M-2-producing *P. mirabilis* strains even with antibiotics with low MICs. The biofilm-forming ability of bacteria, including *P. mirabilis*, on urinary catheters may be one reason for the failure to eradicate the organisms.

The restriction profiles of genomic DNAs from 19 *P. mirabilis* isolates shared concordant patterns (pattern I) among 13 isolates (strains 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 17), suggesting a clonal expansion of CTX-M-2 producers. Moreover, a clonal relatedness among five isolates (strains 15, 18, 19, 4, and 16 corresponding to patterns II, III, and IV) was also indicated. These five strains presumably relate to the previous 13 strains with pattern I. However, the pattern of strain number 2 (pattern V) was apparently distinct from those of the other 18 isolates classified as patterns I, II, III, and IV. These findings suggested that a nosocomial outbreak of 18 clonally related isolates and one isolate subjected to a different genetic lineage had occurred in a certain ward carrying the same CTX-M-2 β-lactamase determinant.

The Dienes test, which visualizes a unique feature of the swarming ability of *P. mirabilis*, has been utilized for an epidemiologic typing method (31), which we adopted in the present study for initial epidemiologic characterization of isolates. The 16 isolates demonstrating pattern I or II were indistinguishable and formed one Dienes type, whereas one isolate with patterns III and one isolate with pattern V formed independent Dienes type. Furthermore, one isolate subjected to pattern IV showed less than detectable swarming ability (data not shown). While the Dienes test was indeed partially applicable for typing of *P. mirabilis*, the biased sinusoidal field gel electrophoresis employed in this study was much more useful

for the epidemiological analyses, although it requires skill and involves somewhat complicated procedures.

Infection control at the initial stage of the outbreak was difficult due to frequent patient transfers within the urology ward. Eventually, the outbreak was successfully brought under control by intensive surveillance, improvement of facilities including disinfection equipment, prudent use of antibiotics, and due precautions to prevent contact transmission of microorganisms. The most effective measures to prevent the further spread of CTX-M-2 β-lactamase-producing *P. mirabilis* were rapid identification of colonization status of such bacteria among all immunocompromised patients with severe urological disorders by periodic urine culturing at admission and once-per-week follow-up testing with informed consent.

In our medical center, first- and third-generation cephalosporins have been preferentially used as first-line drugs. Carbapenems and penicillins as well as first- and third-generation cephalosporins have accounted for the great majority. These antibiotics might well provide selective pressure for proliferation of the CTX-M-2 producers. Early recognition of bacterial strains possessing antimicrobial resistance would contribute to an appropriate antibiotic treatment regimen that would be essential for prevention of nosocomial infections.

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