Micromonospora rosea (35%; SWISS-PROT accession number P24619) and *M zionensis* (34%; EMBL accession number JG0018), respectively. RmtA also showed similarities to the NbrB methylase of the nebramycin-complex-producing *Streptoalloteichus hindustanus* (33%; EMBL accession number AF038408), to the Kmr methylase from the kanamycin-producing *Streptomyces kanamyceticus* (30%; EMBL accession number CAA75800), and to the KgmB methylase from the nebramycin complex-producing *Streptomyces tenebrarius* (31%; EMBL accession number AAB20100). The dendrogram in figure 4 suggests the evolutionary relation between the 16S rRNA methylases and RmtA, implying a potential intergeneric transfer of the gene from some aminoglycoside-producing actinomycetes to *P aeruginosa*. The dendrogram was calculated with the CLUSTAL W program.[14]

Incorporation of a radiolabelled methyl group into the 30S ribosomal subunits prepared from *P aeruginosa* PAO1 (pTO001) was seen (figure 5).

Of 1113 clinically isolated *P aeruginosa* strains that have been isolated from Japanese hospitals and stocked in our laboratory since 1997, nine strains were shown to carry the *rmtA* gene by PCR. These strains were isolated from seven separate hospitals in five prefectures in Japan.

Discussion

We have reported a completely new mechanism for multiple aminoglycoside resistance—that is, enzymatic methylation of the 16S rRNA found in gram-negative bacteria.

Although intergeneric lateral gene transfer has been regarded as a method of acquisition of new phenotypes for bacteria to survive in hazardous environments,[18,19] its rate and background are not well known. The *rmtA* gene product, RmtA, showed considerable similarity to 16S rRNA methylases that protect 16S rRNA in aminoglycoside-producing actinomycetes such as *Streptomyces* spp and *Micromonospora* spp. In fact, a cell-free cytosolic fraction of *P aeruginosa* PAO1 (pTORmtA) containing RmtA accelerated uptake of the ^{3}H-labelled methyl group into the 30S ribosome of *P aeruginosa* PAO1. Moreover, the *rmtA* gene was suggested to be carried by the transposon Tn5041, which mediates mercury resistance. These results suggest that traces of the 16S rRNA methylase gene have moved by intergeneric lateral gene transfer from some aminoglycoside-producing bacteria into *P aeruginosa* because of the increasingly heavy clinical use of arbekacin, which is rarely inactivated by ordinary aminoglycoside-modifying enzymes generally found in gram-negative bacteria.

Since arbekacin resistance of strain AR-2 can be easily transferred to *P aeruginosa* strain 105 by conjugation (10^{-4}–10^{-5}), the *rmtA* gene could be contained on a self-transmissible large plasmid, though more precise characterisation is now underway. This finding indicates that further widespread dissemination of the *rmtA* gene in gram-negative bacteria is possible as an important ecological result of heavy antibiotic use in clinical settings.[20] In this study, nine *P aeruginosa* strains that carry the *rmtA* gene were isolated from seven separate hospitals located in five prefectures across Japan. This finding indicates that in Japanese clinical settings there has been stealthy multifocal proliferation of *P aeruginosa* strains that have acquired consistent and very high-level resistance to various clinically important aminoglycosides through production of the newly identified 16S rRNA methylase. Since resistance to fluoroquinolones and carbapenems has already developed in gram-negative bacteria including *P aeruginosa*,[3,21] emergence of multidrug resistant superbug strains through further acquisition of the *rmtA* gene threatens to become a serious clinical problem. Further global transmission of the *rmtA* gene in gram-negative bacteria could become a matter of grave concern in the future. Like vancomycin-resistant *S aureus* strains[22] and plasmid-mediated quinolone-resistant *Klebsiella pneumoniae*,[23] bacteria readily cope with hazardous environments by accepting any genes, even those from hereditarily distant microorganisms.[24,25]

Contributors

K Yokoyama cloned and characterised the *rmtA* gene and the product, RmtA. H Kurokawa obtained clinical isolates and initially isolated *P aeruginosa* AR-2. Y Doi, K Yamane, N Shibata, T Yagi, K Shibayama, and H Kato contributed to the characterisation of RmtA. Y Arakawa contributed to coordination of the study and writing and editing of the report.

Conflict of interest statement

None declared.

Acknowledgments

This work was supported by grants H12-Shinko-19 and H12-Shinko-20 from the Ministry of Health, Labor, and Welfare of Japan. *P aeruginosa* PAO1 and pTO001 were kindly provided by N Gotoh (Kyoto Pharmaceutical University, Kyoto, Japan).

References

1 Tacconelli E, Tumbarello M, Bertagnolio S, et al. Multidrug-resistant *Pseudomonas aeruginosa* bloodstream infections: analysis of trends in prevalence and epidemiology. *Emerg Infect Dis* 2002; **8**: 220–21.

2 Arakawa Y, Ike Y, Nagasawa M, et al. Trends in antimicrobial-drug resistance in Japan. *Emerg Infect Dis* 2000; **6**: 572–75.

3 Jones AM, Govan JR, Doherty CJ, et al. Spread of a multiresistant strain of *Pseudomonas aeruginosa* in an adult cystic fibrosis clinic. *Lancet* 2001; **358**: 557–58.

4 Thornsberry C, Barry AL, Jones RN, Baker CN, Badal RE, Packer RR. Comparison of in vitro activity of Sch 21420, a gentamicin B derivative, with those of amikacin, gentamicin, netilmicin, sisomicin, and tobramycin. *Antimicrob Agents Chemother* 1980; **18**: 338–45.

5 Umezawa H. Studies on aminoglycoside antibiotics: enzymic mechanism of resistance and genetics. *Jpn J Antibiot* 1979; **32** (Suppl): S1–14.

6 Westbrock-Wadman S, Sherman DR, et al. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrob Agents Chemother* 1999; **43**: 2975–83.

7 Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 1993; **57**: 138–63.

8 Tanaka N, Matsunaga K, Hirata A, Matsuhisa Y, Nishimura T. Mechanism of action of habekacin, a novel amino acid-containing aminoglycoside antibiotic. *Antimicrob Agents Chemother* 1983; **24**: 797–802.

9 Price KE. The potential for discovery and development of improved aminoglycosides. *Am J Med* 1986; **80**: 182–89.

10 Watanabe T, Ohashi K, Matsui K, Kubota T. Comparative studies of the bactericidal, morphological and post-antibiotic effects of arbekacin and vancomycin against methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 1997; **39**: 471–76.

11 Inoue M, Nonoyama M, Okamoto R, Ida T. Antimicrobial activity of arbekacin, a new aminoglycoside antibiotic, against methicillin-resistant *Staphylococcus aureus*. *Drugs Exp Clin Res* 1994; **20**: 233–39.

12 Kondo S, Hotta K. Semisynthetic aminoglycoside antibiotics: development and enzymatic modifications. *J Infect Chemother* 1999; **5**: 1–9.

13 National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing, 12th informational supplement, NCCLS document M100-S12. Wayne: National Committee for Clinical Laboratory Standards, 2002.

14 DNA data bank of Japan. DDBJ homology search system. http://www.ddbj.nig.ac.jp/E-mail/homology.html (accessed Aug 28, 2003).

15 Skeggs PA, Thompson J, Cundliffe E. Methylation of 16S ribosomal

RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. *Mol Gen Genet* 1985; **200**: 415–21.
16 Kelemen GH, Cundliffe E, Financsek I. Cloning and characterization of gentamicin-resistance genes from *Micromonospora purpurea* and *Micromonospora rosea*. *Gene* 1991; **98**: 53–60.
17 Thompson J, Skeggs PA, Cundliffe E. Methylation of 16S ribosomal RNA and resistance to the aminoglycoside antibiotics gentamicin and kanamycin determined by DNA from the gentamicin-producer, *Micromonospora purpurea*. *Mol Gen Genet* 1985; **201**: 168–73.
18 Nelson KE, Clayton RA, Gill SR, et al. Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature* 1999; **399**: 323–29.
19 Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. *Nature* 2000; **405**: 299–304.
20 Lipsitch M, Samore MH. Antimicrobial use and antimicrobial resistance: a population perspective. *Emerg Infect Dis* 2002; **8**: 347–54.
21 Kurokawa H, Yagi T, Shibata N, Shibayama K, Arakawa Y. Worldwide proliferation of carbapenem-resistant gram-negative bacteria. *Lancet* 1999; **354**: 955.
22 Gonzalez-Zorn B, Courvalin P. VanA-mediated high level glycopeptide resistance in MRSA. *Lancet Infect Dis* 2003; **3**: 67–68.
23 Martinez-Martinez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. *Lancet* 1998; **351**: 797–99.
24 Bootsma, HJ, van Dijk H, Vauterin P, Verhoef J, Mooi FR. Genesis of BRO β-lactamase-producing *Moraxella catarrhalis*: evidence for transformation-mediated horizontal transfer. *Mol Microbiol* 2000; **36**: 93–104.
25 Gomis-Ruth FX, Moncalian G, Perez-Luque R, et al. The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase. *Nature* 2001; **409**: 637–41.

Clinical picture

Peritoneal dialysis and an inguinal hernia

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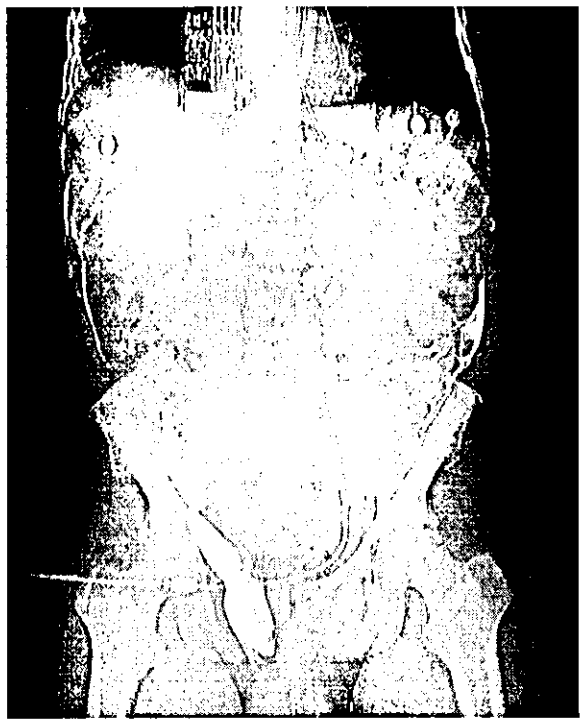
A 59-year-old man with diabetes started chronic ambulatory peritoneal dialysis (CAPD) in January, 2002, with 1500 mL exchanges four times daily. After 3 weeks, he developed massive scrotal oedema. We found a left inguino-scrotal hernia, which was surgically repaired, and the patient was switched to haemodialysis. CAPD was resumed after 4 weeks. Massive scrotal oedema recurred 10 days later. Abdominal multislice helical CT with intraperitoneal injection of 100 mL contrast medium showed a dialysate diffusion through a right inguinal hernia (figure, scout view), but absence of contralateral leakage, indicating an efficient surgical repair. Surgical repair was then done on the right side. 4 weeks later, we resumed CAPD without any further complications. Clinical examination had failed to detect any hernias before starting CAPD. The increased intraperitoneal pressure due to the dialysate was the probable cause.



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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2004, p. 491–496 Vol. 48, No. 2
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.2.491–496.2004

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

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Received 28 July 2003/Returned for modification 30 September 2003/Accepted 13 October 2003

***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 *Sau*3AI 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-

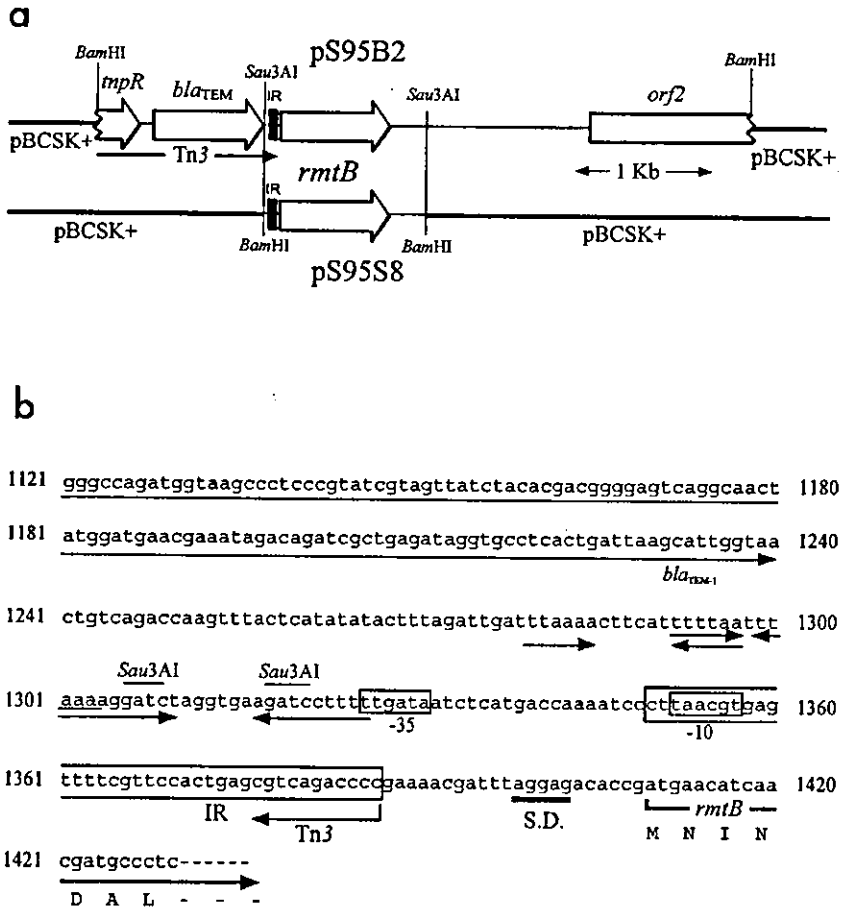

FIG. 1. (a) Schematic presentation of the 4.6-kb *Bam*HI fragment of pS95B2 and 1.2-kb *Sau*3AI fragment of pS95S8. Shaded boxes indicate terminal inverted repeats (IR) of Tn*3*. The 1.2-kb insert of pS95S8 carries only IR and the *rmtB* gene as well as its possible promoter. (b) Region upstream of the *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

```
RmtB  1 MNINDRLTSILASKKYRRLCPDTVRRILTEEWGRHKSPKQTVERRRTRLHGICGRYVTPE  60
RmtA    MSFDDRLRSILSSKKYRSLCPDTVRRILDQEWGRHKSPKLAVERTRTRLHGICGRYVTPE
ArmA    MDKNDVVKKILESKKYENLDSDIVEKVVSISEKKYKLKEVENYSKK-KLHQIWGSYYSRY
        *. :*.: .** ****. * .* *.::: . ::* : : : .** * *:* :

RmtB    -SLKRRRRRLSRG--DVKKRLSLHRSTKERLRELDTLYDFIFS-RETPRRVLDIRCGLNP 116
RmtA    -SLKRRRRRLSVG--DVQKRLSLHRSTKERLRELDCLYDFIFS-GGVPHRVLDIRCGLNP
ArmA    PNWDKLLKKYNQGQLSIEDLLKIHSSTNERVRTLNDFYTYVFGNIKHVSSILDFGCGFNP
         . .      . *  .::. *.:*:**:**:* *: :* ::*.       :**:.**:**

RmtB    LALYERGIRS---VHGCDIHQGLGDVITPFRREKDWDFTFRLQDVLCRPPRERGDLRLIF 173
RmtA    LRLFIRDITS---VHRCDIHQGLGDVITPFRHHQGLDFTFRLQDVMCTPPTETGDLRLVF
ArmA    LRLYQWNENEKIIYHRYDIDRAEIRFLSSIIGKLKTTIKYRFLNKESDVYKGTYDVVFLL
        ***: . .    . **.:.  .::.: .   :.: : : .    : *:.:::

RmtB    KLLPLLEREQRGSRMRLLQSLNTPRMAVSFPTRSLGGRGKGMEENYRRHFEGGLPREFEI 233
RmtA    KLLPLLEREQRGRRMRLLQRLRTPRIAVSFPTRSLGGRGKGMEENYSRHFEGRLPDEFEI
ArmA    KMLPVLK-QQDVNILDFLQLFHTQNFVISFPIKSLSGKEKGMEENYQLHFESFTKGHIKI
        *:**:*: :*    : :** : * .:.:*** :**.*: **** **  ***.     ::*

RmtB    EDKKTIGTELIYLIKKNG-                                          251
RmtA    EDTKTIGIELVYMIKRNK-
ArmA    LDSKVIGNELVYITSGFQK
         *.*.** **:*: .
```

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.

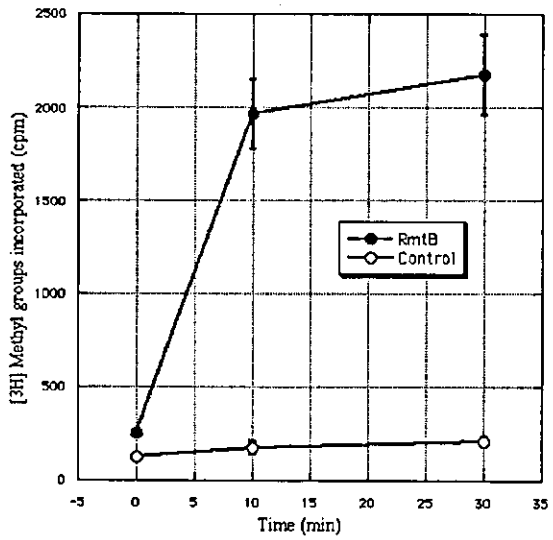

FIG. 3. Methylation of 16S rRNA. The 16S rRNA from *E. coli* XL1-Blue was incubated with purified RmtB using [methyl-^{3}H]SAM as a cofactor. The value of each point was calculated with three data points. Error bars, standard deviations.

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.

ACKNOWLEDGMENTS

We thank Kumiko Kai for expert technical assistance. We also thank Yoshiichi Yamaji for providing *S. marcescens* S-95 and Hiroya Tada for providing relevant clinical information.

This work was supported by grants H12-Shinkou-19 and H12-Shinkou-20 from the Ministry of Health, Labor, and Welfare of Japan. Part of the genetic analyses was supported by Grant-in-Aid for Young Scientists (B) 14771358 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

1. **Beauclerk, A. A., and E. Cundliffe.** 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J. Mol. Biol. 193:661–671.
2. **Boyd, D., G. A. Peters, A. Cloeckaert, K. S. Boumedine, E. Chaslus-Dancla, H. Imberechts, and M. R. Mulvey.** 2001. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. J. Bacteriol. 183:5725–5732.
3. **Bryan, L. E.** 1988. General mechanisms of resistance to antibiotics. J. Antimicrob. Chemother. 22(Suppl. A):1–15.
4. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. 174:1574–1585.
5. **Cundliffe, E.** 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. 43:207–233.
6. **Davies, J., and G. D. Wright.** 1997. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol. 5:234–240.
7. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. Methods Enzymol. 235:386–405.
8. **Demydchuk, J., Z. Oliynyk, and V. Fedorenko.** 1998. Analysis of a kanamycin resistance gene (*kmr*) from *Streptomyces kanamyceticus* and a mutant with increased aminoglycoside resistance. J. Basic Microbiol. 38:231–239.
9. **Doi, Y., N. Shibata, K. Shibayama, K. Kamachi, H. Kurokawa, K. Yokoyama, T. Yagi, and Y. Arakawa.** 2002. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. 46:2427–2434.
10. **Galimand, M., P. Courvalin, and T. Lambert.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. 47:2565–2571.
11. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in transposition of Tn*3*. Cell 18:1153–1163.
12. **Holmes, D. J., and E. Cundliffe.** 1991. Analysis of a ribosomal RNA methylase gene from *Streptomyces tenebrarius* which confers resistance to gentamicin. Mol. Gen. Genet. 229:229–237.
13. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.
14. **Kelemen, G. H., E. Cundliffe, and I. Financsek.** 1991. Cloning and characterization of gentamicin-resistance genes from *Micromonospora purpurea* and *Micromonospora rosea*. Gene 98:53–60.
15. **Kondo, S., and K. Hotta.** 1999. Semisynthetic aminoglycoside antibiotics: development and enzymatic modifications. J. Infect. Chemother. 5:1–9.
16. **Kotra, L. P., J. Haddad, and S. Mobashery.** 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob. Agents Chemother. 44:3249–3256.
17. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial susceptibility testing; 12th informational supplement. M100–S12. National Committee for Clinical Laboratory Standards, Wayne, Pa.
18. **Ohta, T., and M. Hasegawa.** 1993. Analysis of the self-defense gene (*fmrO*) of a fortimicin A (astromicin) producer, Micromonospora olivasterospora: comparison with other aminoglycoside-resistance-encoding genes. Gene 127: 63–69.
19. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. 57:138–163.
21. **Skeggs, P. A., J. Thompson, and E. Cundliffe.** 1985. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. Mol. Gen. Genet. 200:415–421.
22. **Thompson, J., P. A. Skeggs, and E. Cundliffe.** 1985. Methylation of 16S ribosomal RNA and resistance to the aminoglycoside antibiotics gentamicin and kanamycin determined by DNA from the gentamicin-producer, *Micromonospora purpurea*. Mol. Gen. Genet. 201:168–173.
23. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet 362:1888–1893.
24. **Yoshizawa, S., D. Fourmy, and J. D. Puglisi.** 1998. Structural origins of gentamicin antibiotic action. EMBO J. 17:6437–6448.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2004, p. 2069–2074 Vol. 48, No. 6
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.6.2069–2074.2004

Genetic Environments of the *rmtA* Gene in *Pseudomonas aeruginosa* Clinical Isolates

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Received 24 August 2003/Returned for modification 24 December 2003/Accepted 22 February 2004

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

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

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

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

MATERIALS AND METHODS

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

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TABLE 1. Bacterial strains and plasmids used in this study

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

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

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

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

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

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

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

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

RESULTS

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

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

TABLE 2. Results of antibiotic susceptibility testing

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

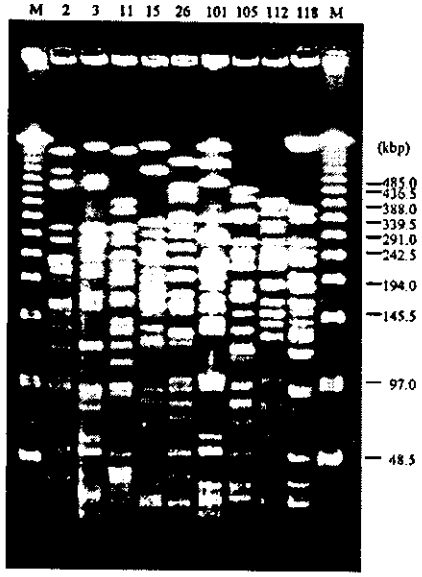

FIG. 1. PFGE fingerprinting of total DNAs from *P. aeruginosa* isolates digested with SpeI. M, PFGE molecular weight marker. The number above each lane indicates the AR strain number shown in Table 1.

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

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

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

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

DISCUSSION

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

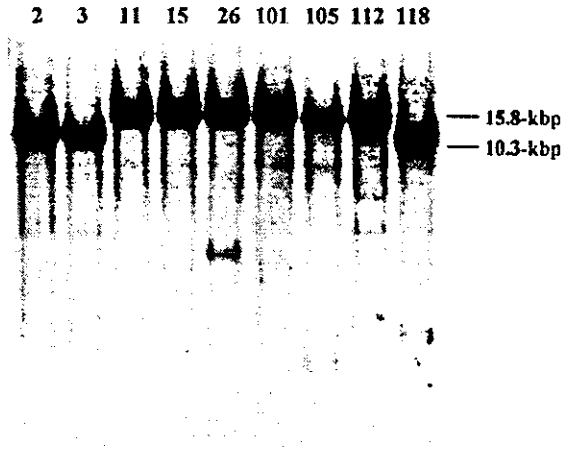

FIG. 2. Southern hybridization patterns of EcoRI-digested genomic DNAs. The number above each lane represents the AR strain number shown in Table 1. The nine strains tested appeared to be divided into two groups by the sizes of EcoRI-digested fragments (10.3 and 15.8 kbp, respectively).

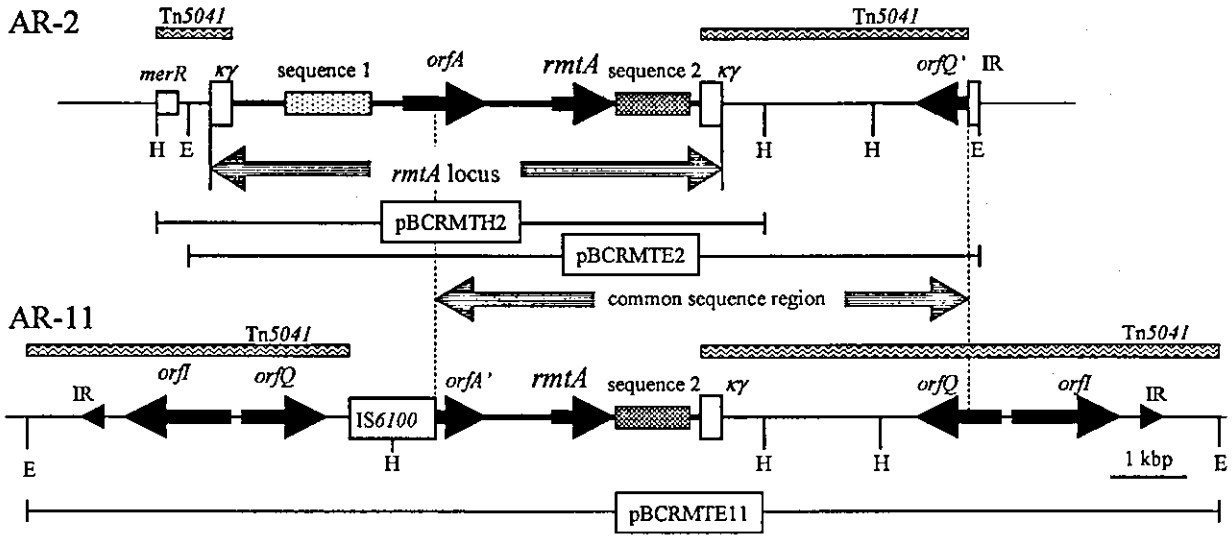

FIG. 3. Comparison of the genetic organizations of AR-2 and AR-11. Double-headed striped arrows indicate the position of the *rmtA* locus and that of the region common to both sequenced areas. Inserts of pBCRMTH2, pBCRMTE2, and pBCRMTE11 are indicated by horizontal lines. Rectangles filled with wavy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

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

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

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

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

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

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

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

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

ACKNOWLEDGMENTS

This work was supported by grants H12-Shinkou-19, H12-Shinkou-20, H15-Shinkou-9, and H15-Shinkou-10 from the Ministry of Health, Labor, and Welfare of Japan. The travel costs for presenting part of the data reported in this paper at the 103rd General Meeting of the American Society for Microbiology was supported by a grant from The Japan Antibiotics Research Association, sponsored by the Pfizer Infectious Diseases Research Fund 2003.

REFERENCES

1. **Aminoglycoside Resistance Study Groups.** 1994. Resistance to aminoglycosides in *Pseudomonas*. Trends Microbiol. 2:347–353.
2. **Beauclerk, A. A. D., and E. Cundliffe.** 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J. Mol. Biol. 193:661–671.
3. **Bogdanova, E. S., S. Z. Mindlin, E. S. Kalyaeva, and V. G. Nikiforov.** 1988. The diversity of mercury reductases among mercury-resistant bacteria. FEBS Lett. 234:280–282.
4. **Craig, N. L., R. Craigie, M. Gellert, and A. M. Lambowitz.** 2002. Mobile DNA II. ASM Press, Washington, D.C.
5. **D'Agata, E., L. Venkataraman, P. DeGirolami, and M. Samore.** 1997. Molecular epidemiology of acquisition of ceftazidime-resistant gram-negative baciili in a nonoutbreak setting. J. Clin. Microbiol. 35:2602–2605.
6. **Davies, J., and G. D. Wright.** 1997. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol. 5:234–240.
7. **Davies, J. D.** 1983. Resistance to aminoglycosides: mechanisms and frequency. Rev. Infect. Dis. 5:S261–S267.
8. **Doi, Y., K. Yokoyama, K. Yamane, J., Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa,.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. 48:491–496.
9. **Dubois, V., C. Arpin, P. Noury, and C. Quentin.** 2002. Clinical strain of *Pseudomons aeruginosa* carrying a bla_{TEM-21} gene located on a chromosomal interrupted Tn*4* type transposon. Antimicrob. Agents Chemother. 46:3624–3626.
10. **Fouemy, D., M. I. Recht, S. C. Blanchard, and J. D. Puglisi.** 1996. Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 274:1347–1371.
11. **Galimand, M., P. Courvalin, and T. Lambert.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. 47:2565–2571.
12. **Gunos, G., B. Smith, and P. Dyson.** 1999. Genetic instability associated with insertion of IS*6100* into one end of the *Streptomyces lividans* chromosome. Microbiology 145:2203–2208.
13. **Ida, T., R. Okamoto, C. Shimauchi, T. Okubo, A. Kuga, and M. Inoue.** 2001. Identification of aminoglycoside-modifying enzymes by susceptibility testing: epidemiology of methicillin-resistant *Staphylococcus aureus* in Japan. J. Clin. Microbiol. 39:3115–3121.
14. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.
15. **Karlowsky, J. A., D. C. Draghi, M. E. Jones, C. Thornsberry, I. R. Friedland, and D. F. Sahm.** 2003. Surveillance for antimicrobial susceptibility among clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2001. Antimicrob. Agents Chemother. 47:1681–1688.
16. **Kholodii, G. Y., O. V. Yurieva, Z. M. Gorlenko, S. Z. Mindlin, I. A. Bass, O. L. Lomovskaya, A. V. Kopteva, and V. G. Nikiforov.** 1997. Tn*5041*: a chimeric mercury resistance transposon closely related to the toluene degradative transposon Tn*4651*. Microbiology 143:2549–2556.
17. **Kotra, L. P., J. Haddad, and S. Mobashery.** 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob. Agents Chemother. 44:3249–3256.
18. **Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel.** 1990. Transposition of an antibiotic resistance element in mycobacteria. Nature 345:739–743.
19. **National Committee for Clinical Laboratory Standards.** 1988. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Document M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.

20. **Recht, M. I., and J. D. Puglisi.** 2001. Aminoglycoside resistance with homogeneous and heterogeneous populations of antibiotic-resistant ribosomes. Antimicrob. Agents Chemother. **45**:2414–2419.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
23. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzames. Microbiol. Rev. **57**:138–163.
24. **Skeggs, P. A., J. Thompson, and E. Cundliffe.** 1985. Methylation of 16S ribosomal RNA and resistance to the aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. Mol. Gen. Genet. **200**:415–421.
25. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
26. **Tauch, A., S. Gotker, A. Puhler, J. Kalinowski, and G. Thierbach.** 2002. The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenyltransferase gene cassette *aadA9* and the regulated tetracycline efflux system Tet 33 flanked by active copies of the widespread insertion sequence IS*6100*. Plasmid **48**:117–129.
27. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233–2239.
28. **Thompson, J., P. A. Skeggs, and E. Cundliffe.** 1985. Methylation of 16S ribosomal RNA and resistance to the aminoglycoside antibiotics gentamicin and kanamycin determined by DNA from the gentamicin-producer, *Micromonospora purpurea*. Mol. Gen. Genet. **201**:168–173.
29. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa,.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362**:1888–1893.
30. **Yoshizawa, S., D. Fourmy, and J. D. Puglisi.** 1998. Structural origins of gentamicin antibiotic action. EMBO J. **17**:6437–6448.

病院長様

平成15年6月 28日

拝啓

青葉若葉のみぎり、ますますご清栄のこととお喜び申し上げます。

さてこの度「アミノグリコシド高度耐性を付与する 16S rRNA メチレースの保有状況に関する調査」を実施する事となりました。

近年緑膿菌、セラチア等を中心としたグラム陰性桿菌の多剤耐性化が進行し、これらの耐性菌による院内感染の事例報告も増加しております。今回調査の対象となるアミノグリコシドに対して高度耐性を付与する 16S rRNA メチレースはグラム陰性桿菌では 2003 年に初めて報告された新しい耐性機序で臨床で用いられるほとんどのアミノグリコシドに対して高度耐性を示す特徴を有します。現在までに緑膿菌、セラチア、大腸菌などから分離されておりますが臨床現場にどの程度広がっているかわかっておりません。アミノグリコシドは重症のグラム陰性桿菌感染症の治療に対して広く使用され、このような耐性機序を保有するグラム陰性桿菌が臨床現場に蔓延することは非常に大きな問題となります。

そこで、臨床分離株を広く収集しこの耐性機序を有するグラム陰性桿菌の日本における割合を算出することを計画いたしました（別紙 1)。貴施設の御協力を賜る事ができましたら幸甚に存じます。

別添えの「承諾書（凡例)」を 7 月 15 日までに国立感染症研究所の荒川宜親宛に御返送いただければ幸いに存じ上げます。

敬具

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別紙 1

「アミノグリコシド高度耐性を付与する 16S rRNA メチレースの保有状況に関する調査」

調査の概要

期間：平成 16 年 8 月 1 日から 9 月 30 日までの 2 ヶ月間

対象菌株：
検査室において検査した全てアミノグリコシドに耐性を示すグラム陰性桿菌とする。アミノグリコシドの種類は問わない。1 患者について同一部位から複数回分離された場合は 1 菌種につき 1 株を収集する。

実施方法：
参加協力施設には移送用培地（25 本）を研究班から提供する。
菌株は 10 月 10 日までに国立感染症研究所　細菌第二部に送付する（着払い）。
試験・検査は国立感染症研究所　細菌第二部で実施する。

試験・検査の内容：
PCR 法による 16S rRNA methylase の検出
16S rRNA methylase 陽性株について NCCLS 法による MIC の測定
各菌種について日本における 16S rRNA メチレース保有割合の算出

プライバシーの保護に関して：
患者情報は報告しない。
万一メチラーゼ産生菌が分離された場合、施設名が第三者に特定されないように配慮する。

詳細につきましては参加協力いただける施設に別途御案内させていただきます。
ご協力の程、どうぞよろしくお願い致します。

検査部（科・室）長様
検査技師長様
細菌検査主任様

平成15年7月 27日

拝啓　時下ますますご健勝のほどお喜び申し上げます。

さて、この度は「アミノグリコシド高度耐性を付与する16S rRNAメチレースの保有状況に関する調査」にご参加頂き誠にありがとうございます。

多数の御施設からご参加をいただきました結果、輸送用培地の発送が 7 月中に間に合わなくなってしまいました。そこで、誠に勝手ではございますが、調査期間を 9 月 1 日から 10 月 31 日の 2 ヶ月間に変更させて頂きたいと考えております。そこで、参加協力施設各位には大変ご迷惑をおかけ致しますがどうぞよろしくお願い申し上げます。

なお調査方法の詳細や輸送用培地、輸送容器などは後日こちらから発送させていただきます。ご不明な点がございましたら下記までご連絡頂きますよう何卒よろしくお願い申し上げます。

敬具

平成16年7月 27日

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アミノグリコシド高度耐性グラム陰性桿菌が保有する 16S rRNA メチラーゼ分離状況の調査研究

1. 研究背景と目的

近年、グラム陰性桿菌の多剤耐性化が我が国の臨床現場で進行しており深刻な問題となっている。本研究はアミノグリコシド高度耐性を付与する 16S rRNA メチラーゼの我が国における分布状況を調査することを目的とする。

2. 実施方法

2.1 菌株の収集

協力施設において**2004 年 9 月 1 日から 10 月 31 日**の 2 ヶ月間に日常的な検査業務の中で検体として提出された臨床材料から下記の菌種が分離された場合、収集、保存し、感染研細菌第二部に提供する。

2.2 対象菌株

調査期間中に検査室において分離した菌株のうち、各施設で検査項目に入れているアミノグリコシド全てに耐性を示す腸内細菌と *Pseudomonas* 属、*Acinetobacter* 属とする。アミノグリコシドの種類は問わない。1 患者について複数回、同じ薬剤感受性パターンを示す株が分離された場合は、1 菌種につき 1 株を収集する。(検体の種類は問わない)

2.3 収集方法

参加施設にはあらかじめ輸送用培地を提供する。

各施設で調査期間内に日常の細菌検査業務によって分離・培養・同定された指定グラム陰性桿菌を保存する。保存された菌株はなるべく発送日に近い時期に輸送用培地に接種し、輸送用培地に菌株番号を油性マジックで記入する。

2.4 データ保存方法

同梱されたフロッピーディスク内の Excel ワークシートに保存菌株の情報を入力する。1 ページ目に「施設名」「病床数」「担当者名」等、2 ページ目に「依頼検体総数」、「分離菌体総数」、「グラム陰性菌分離総数」、「菌種別分離総数」を入力する。検体情報は菌株情報ファイル記入例を参考にして期間内に提出されたそれぞれの検体総数を入力する。3 ページ目については以下にデータ入力手順を示す。

a) A 列に「菌株同定結果」を入力する。(例：*Pseudomonas aeruginosa*)
b) B 列に「分離検体番号」を入力する。(各施設の検体番号)
c) C 列は空欄（感染研で記入)。
d) D 列に「検体採取日」を入力する。(例：2004.6.15)
e) E 列に「臨床検体名」を菌株情報ファイル記入例を参考に入力する。その他の検体は具体的に検体名を入力する。
f) F 列以降に薬剤感受性を入力する。検査していない薬剤欄は空欄。

記入例がフロッピーディスク内に入れてありますのでご参照下さい。

3. 菌株の送付

保存菌株は第一回目 10 月 7 日頃までに、第二回目は 11 月 7 日までに下記に送付する。菌株情報はフロッピーディスクに入力し二回目の送付時に菌株と一緒に送付する。

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荒川　宜親宛

4. 送付方法

1. 菌を接種した輸送用培地を栄研化学株式会社の 50 本入りの箱に入れる
2. ビニール袋を二重にして密閉する。
3. その上に「弱毒性菌株、ヒト腸管・環境常在菌、煮沸または 70%エタノールで 10 分間処理により死滅」と表記したタックシールをはる。
4. 保存菌株を往路に用いた発泡スチロール性の箱に入れる。
5. データを入力したフロッピーディスクと施設での同定感受性試験報告書のコピーを返信用ゆうパックの袋に入れる。
6. 送り伝票に「研究用試料」と明記して郵便（ゆうパック/室温）で国立感染症研究所に郵送する。

(ア) 解析内容

病床数ごとに、どの程度検体が細菌検査に提出されているか確認する。
集められた菌株はアルベカシン入り培地で再度スクリーニングを行うことにより対象を絞り、PCR 法で 16S rRNA メチラーゼ遺伝子の確認試験を行う。

5. 予定している集計解析結果

菌株毎に我が国における 16S rRNA メチラーゼ陽性菌株の分離率を算出する。
全体的な集計結果については、各参加施設に御報告させて頂く予定です。

6. 研究組織

研究代表者：国立感染症研究所　細菌第二部　荒川　宜親

7. 結果の発表

研究結果について研究報告書を作成し参加施設に送付する。さらに全体的な集計結果については、感染研の担当者が関連学会での発表や専門学術雑誌などへ発表することを予定している。
尚、16S rRNA メチラーゼ産生株が検出された各施設の検査担当者の方は、必要に応じて、微生物関連の学会または感染症関連の学会等で御発表頂く事は可能です。

8. 連絡先

不明な点がありましたら下記までご連絡下さい。

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