Acquisition of the newly identified 16S rRNA methylases, RmtA, RmtB and ArmA, in pathogenic Gram-negative bacilli has been a growing concern. The ArmA, which has so far been identified exclusively in Europe, was also found in several Gram-negative pathogenic bacilli isolated in Japan, suggesting global dissemination of hazardous multiple-aminoglycoside-resistance genes.

Multidrug-resistant Gram-negative super bugs have been emerging worldwide. Since carbapenems and fluoroquinolones are the last resort against infections caused by Gram-negative bacilli (1,2), the proliferation and dissemination of such clinical isolates that produce metallo-β-lactamases and acquire mutations in *gyrA* and *parC* genes have become an actual global clinical threat (3,4). Aminoglycosides such as amikacin and tobramycin are still potent agents to cope with the above-described resistant bacilli. The most common resistance mechanisms against aminoglycosides have been elucidated to be the production of aminoglycoside modifying enzymes, such as aminoglycoside acetyltransferases (AAC), aminoglycoside phosphorylases (APH) and aminoglycoside adenyltransferases (AAD) (5), which are mainly mediated by transferable large plasmids. Recently, however, a series of special methylases which through methylation protect bacterial 16S rRNA, the main target of aminoglycosides, was identified in several nosocomial pathogens including *Pseudomonas aeruginosa* (6), *Serratia marcescens* (7) and *Klebsiella pneumoniae* (8). The newly identified 16S rRNA methylases, RmtA and RmtB, were recently found in Japan (6,7). The gene for ArmA was initially sequenced in *Citrobacter freundii* isolated in Poland (EMBL/GenBank accession No. AF550415) and later characterized in *K. pneumoniae* isolated in France (8). Quite recently, the nosocomial spread of ArmA or RmtB producing *Escherichia coli* and *K.pneumoniae* was also reported from Taiwan (9). These enzymes are capable of conferring an extraordinary high-level resistance (MIC, >512 mg/L) against most clinically important aminoglycosides of the type observed

among aminoglycoside-producing actinomycetes, suggesting their probable phylogenic relation with the intrinsic 16S rRNA methylases of actinomycetes (Figure 1). RmtA shared 82% amino acid identity with RmtB, but the amino acid sequence similarities between 16S rRNA methylases isolated from pathogenic Gram-negative bacteria and those from aminoglycoside-producing actinomycetes were relatively low (up to 33%). From analyses of the genetic environments of genes encoding 16S rRNA methylases, the *rmtA* gene is probably associated with the mercury-resistant transposon-like Tn*5041* (10), and the *rmtB* gene was found in the flanking region of Tn*3*-like structure (7). The *armA* gene was associated with type 1 integron (8) that mediates various gene cassettes responsible for multiple antimicrobial resistance. The structure of these genetic environments implied that the genes for these 16S rRNA methylases are mediated by some mobile gene elements carried by transferable large plasmids (7, 8, 10). In fact, the *rmtA* gene was transferred from *P. aeruginosa* strain AR-2 to a wild strain of *P. aeruginoss*formation (7). The *armA* gene was located on a composite transposon Tn*1548* (Lambert et al. Abstract C1-1496, 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, 2004). Thus, the growing concern was that these newly identified aminoglycoside resistance genes could easily spread and be further disseminated among the glucose nonfermentative Gram-negative bacilli including *P. aeruginosa* and *Acinetobacter* spp., as well as the genus belonging to the family *Enterobacteriaceae*.

Therefore, we conducted a preliminary screening of the 16S rRNA methylase-producing bacilli on our Gram-negative bacterial stock of 2,877 strains isolated from

Japanese hospitals within the past several years. Arbekacin, a semisynthetic aminoglycoside belonging to the kanamycin group, requires two modifications at (6') amino-group and (2") hydroxyl group for inactivation, so this agent is hardly inactivated by the plasmid-mediated known aminoglycoside-modifying enzymes. Therefore, high level arbekacin-resistance (MIC, >512 mg/L) was used as a marker for screening the 16S rRNA methylase-producing strains. All arbekacin-highly-resistant strains were subjected to a PCR analysis to detect *rmtA*, *rmtB* or *armA*, and all strains were PCR-positive, except for a strain of *Acinetobacter* spp. demonstrating a very high level of resistance to arbekacin (MIC, 1,024 mg/L). This strain was later found to produce both aminoglycoside 6'-acetyltransferase and 2"-adenyltransferase (11), so arbekacin was inactivated in this strain by both 6'-acethylation and 2"-adenylation. Each PCR-primer set used for detection of *rmtA* and *rmtB* genes was shown in our previous reports (6,7). The PCR primers for amplification of *armA* was newly designed (forward: 5'-AGG TTG TTT CCA TTT CTG AG-3', reverse: 5'-TCT CTT CCA TTC CCT TCT CC-3') and the predicted size of the amplicon is 590 bp. So far as to our experience, these three sets of PCR primers were very reliable in detection of *rmtA*, *rmtB* and *armA* genes, respectively. Each PCR amplicon was then subjected to sequencing analyses on both strands to confirm its nucleotide sequences for detecting mutations in the methylase gene.

As reported in our previous study, *rmtA* and *rmtB* genes had been found in at least 9 clinically isolated *P. aeruginosa* isolates (6,10) and a strain of *S. marcescens* (7), respectively. As shown in Table 1, 5 *P. aeruginosa* strains isolated after our previous

report (6) were newly identified as *rmtA*-positive. The *rmtB* was additionally identified in 4 *K. pneumoniae*, 2 *E. coli*, and 1 *Klebsiella oxytoca* in Japan. To our surprise, the *armA* thus far found in various Gram-negative bacterial species belonging to the family Enterobacteriaceae exclusively in Europe as reported by Galimand et al. (abstract C2-59, 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, 2003) was also identified in Japan in 1 *E. coli*, 1 *S. marcescens*, and 1 *Acinetobacter* sp. Notably, the *armA* and *rmtB* genes were also identified quite recently in *K. pneumoniae* and *E. coli* in Taiwan (9). Furthermore, the genetic environments around the *armA* gene found in the *C. freundii* isolated in Poland was quite similar to that of *K. pneumoniae* isolated in France. The genetic environments around the *armA* gene found in the Japanese 3 bacterial species, *E. coli*, *S. marcescens*, and *Acinetobacter* sp. were quite similar to those found in Europe as well, as submitted to the EMBL/GenBank databases, accession Nos. AB116388, and AB117519. These findings strongly suggest that the ArmA-producing Gram-negative nosocomial bacteria that harbor a very similar genetic environment carrying *armA* gene have already spread globally.

As described previously, arbekacin still shows a very wide antibacterial spectrum from Gram-positive to Gram-negative nosocomial bacteria at present, and has been approved solely for treatment of MRSA infections in Japan since 1990, in order to assure the prudent use of this precious agent. The emergence and presence of the 16S rRNA methylase-producing Gram-negative bacilli, however, has not been well recognized in Japan to date, because arbekacin has not been listed among the antimicrobial agents for daily antimicrobial susceptibility testing of Gram-negative

bacteria.

The use of semisynthetic aminiglycosides including arbekacin in Japanese clinical settings for more than 10 years may have promoted the emergence and dissemination of the 16S rRNA methylases-producing Gram-negative bacteria in Japan. The huge amount of various aminoglycosides used in livestock farming environments could have also been a selective pressure for the emergence and spread of the pathogenic bacteria that harbor genetic determinants for the newly identified 16S rRNA methylases, as exemplified by recent isolation of ArmA producing *E. coli* from swine in Spain (EMBL/GenBank database accession no. AY522431).

Since acquisition of multidrug resistance against clinically important antimicrobial agents such as carbapenems and fluoroquinolones has already been rapidly developing worldwide, the acceleration of even greater aminoglycoside resistance among Gram-negative bacilli promises to become an actual clinical concern in the near future, just as vancomycin-resistant enterococci did in the 90s (12). The emergence of Gram-positive cocci including MRSA and VRE that acquire the 16S rRNA methylase could also be a grave clinical matter, although fortunately no such hazardous bacteria have been identified to date. Thus, steps must be taken to block further proliferation of these multidrug-resistant Gram-negative super bugs including *P. aeruginosa* and *Acinetobacter* spp., as well as multiple drug-resistant cocci such as MRSA and VRE, that have acquired an extraordinally high level resistance to various clinically important aminoglycosides through production of 16S rRNA methylases especially in clinical environments.

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Dr. Yamane is a medical doctor with a career working in intensive care units and emergency medical divisions. His current interest is molecular mechanisms of antimicrobial resistance in nosocomial bacteria.

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Address for correspondence: Yoshichika Arakawa, Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan; fax +81-42-461-7173; email: yarakawa@nih.go.jp

Figure Legend

Figure 1. Phylogenic relation among the 16S rRNA methylases. Each amino acid sequence datum subjected to the analysis referred to the following source: FmrO, accession No. JN0651; Kmr, accession No. AB164642; Grm, accession No. M55521; GrmA, accession No. AY524043; Kan, accession No. AJ414669; Sgm, accession No. A45282; KgmB, accession No. S60108; NbrB, accession No. AF038408; FMRO, Q08325; RmtA, (6); RmtB, (7); ArmA, (8); predicted enoyl-CoA hydratase/carnithine racemase of uncultured marine gamma proteobacterium EBAC20E09, accession No. AAS73112; putative methylase of *Chlorobium tepidum*, accession No. AAM72273; hypothetical protein of *Nanoarchaeum equitans*, accession No. AAR39385. The ClustalW program provided by the DNA Data Bank of Japan (DDBJ) <http://www.ddbj.nig.ac.jp/search/clustalw-e.html> was employed in this study. Dendrogram was illustrated by the TreeView program Version 1.6.5 for Macintosh. The "0.1" scale represents a genetic unit reflecting the 10% of aminoacid substitutions calculated with the CLUSTAL W program of the DDBJ.

Table 1. 16S rRNA methylase-producing strains identified after previous study (6)

Species & strain	Type	Isolation	Hospital	Prefecture
Pseudomonas aeruginosa P122	RmtA	2002	A	Aichi
P. aeruginosa P340	RmtA	2002	B	Gifu
P. aeruginosa 02-386	RmtA	2002	C	Saitama
P. aeruginosa 03-29	RmtA	2003	D	Aichi
P. aeruginosa 03-230	RmtA	2003	E	Shizuoka
E. coli 01-139	RmtB	2001	H	Yamanashi
Klebsiella pneumoniae 01-140	RmtB	2001	H	Yamanashi
Klebsiella oxytocae 01-141	RmtB	2001	H	Yamanashi
K. pneumoniae 01-142	RmtB	2001	H	Yamanashi
Escherichia coli C316	RmtB	2002	F	Hyogo
Serratia marcescens S95	RmtB	2002	G	Kohchi
K. pneumoniae 03-252	RmtB	2003	H	Yamanashi
K. pneumoniae 03-518	RmtB	2003	H	Yamanashi
E. coli C316-2	ArmA	2003	F	Hyogo
S. marcescens ARS8	ArmA	2003	I	Tochigi
Acinetobacter sp. ARS6	ArmA	2003	J	Kanagawa

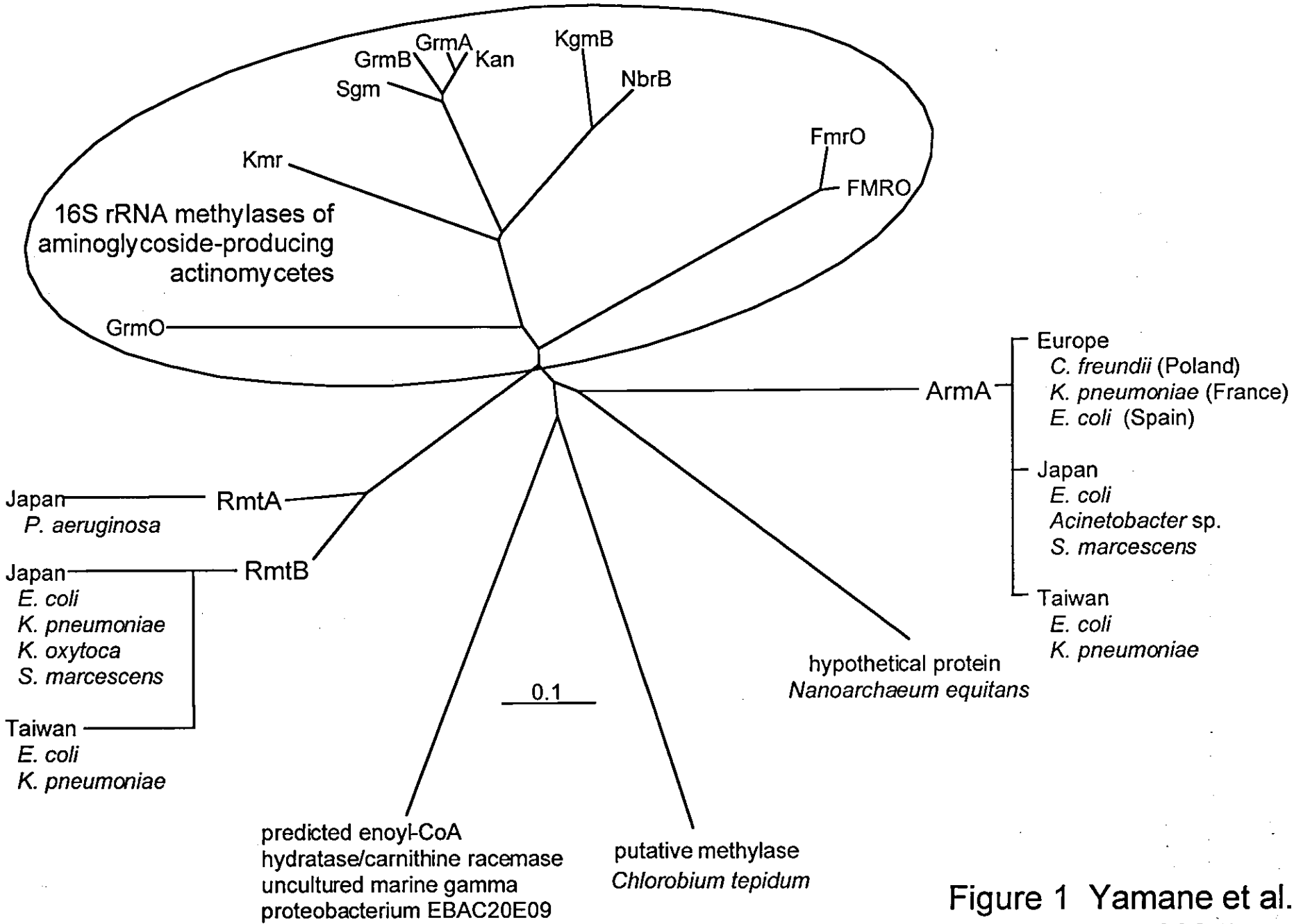

Figure 1 Yamane et al.
(EID Ms. #04-0924)

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Laboratory and Epidemiology Communications

Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens* in a Long-Term Care Facility for Patients with Severe Motor and Intellectual Disabilities

Teruo Kirikae[1], Osamu Tokunaga, Yasuhiro Inoue, Tomoko Fujino[1], Katsutoshi Saruta[1], Hiroshi Yoshikura[2], Tadatoshi Kuratsuji[1] and Takeshi Miyanomae*

Department of Pediatrics, Minami-Kyoto Hospital, National Hospital Organization, Kyoto 610-0113,
[1]Department of Infectious Diseases and Tropical Medicine,
International Medical Center of Japan, Tokyo 162-8655 and
[2]National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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Assessing the risk of nosocomial infection is necessary for optimizing the quality of patient care and the practice of infection control in long-term care facilities for patients with severe motor and intellectual disabilities (SMID). We conducted a molecular epidemiological study of pathogens in December 2002 and August 2003 in two wards of such a facility having three wards. Among the 39 inpatients in the wards, 20 had tracheotomy or were cared for with mechanical ventilators. The isolates were tested for chromosomal DNA typing by using a contour-clamped homogeneous electric field system (CHEF Mapper™: Bio-Rad Laboratories, Hercules, Calif., USA).

In December 2002, 14 of 20 patients carried at least one methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, or *Serratia marcescens* strain (Table 1). MRSA was isolated from 11 specimens from 9 patients, including eight patients' sputa, one patient's abscess, and one patient's eye mucus. Among these, two were obtained on different days from an abscess of patient P5 and two others from different sites of patient P7. *P. aeruginosa* was obtained from nine patients' sputa and *S. marcescens* from five patients' sputa. Three patients, P1, P4, and P6, carried MRSA, *P. aeruginosa*, and *S. marcescens* in the same specimen, and the other three patients, P3, P7, and P5, carried MRSA and *P. aeruginosa*.

The survey was repeated in August 2003. Eighteen patients carried at least one MRSA, *P. aeruginosa*, or *S. marcescens* strain (Table 1). MRSA strains were isolated from six patients, including four patients' sputa and two patients' urine. *P. aeruginosa* was isolated from 13 patients' sputa, and *S. marcescens* from three patients' sputa. No patient simultaneously carried MRSA, *P. aeruginosa*, and *S. marcescens* strains. Only one patient, P15, had both MRSA and *P. aeruginosa*, and two patients, P1 and P11, had *P. aeruginosa* and *S. marcescens*. Nine patients, P1, P2, P3, P4, P7, P8, P11, P13, and P14, carried MRSA, and either *P. aeruginosa* or *S. marcescens* both in December 2002 and in August 2003.

The PFGE patterns of these MRSA isolates are shown in Fig. 1A. From a total of 17 isolates, 12 different PFGE patterns were detected. Band-based cluster analysis of these patterns (Molecular Analyst™: Bio-Rad) revealed a cluster consisting of patterns A1, A3, and A16 (Fig. 1B) (patterns

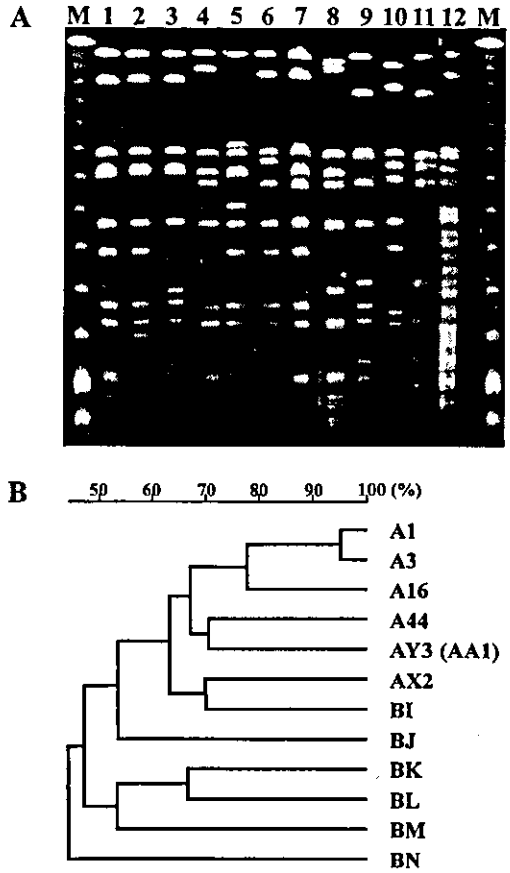

Fig. 1. Molecular analysis of MRSA isolate. A: pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA from MRSA isolates. M: low range PFG Marker. Lanes 1 to 12 corresponding to the following PFGE pattern; 1: A1, 2: A3, 3: A16, 4: A44, 6: AY3, 7: BJ, 8: BI, 9: BK, 10: BL, 11: BM, 12: BN. B: cluster analysis of MRSA isolates based on PFGE patterns of *Sma* I-digested genomic DNA.

*Corresponding author: Mailing address: Department of Pediatrics, Minami-Kyoto Hospital, National Hospital Organization, Naka-ashihara 11, Johyoh-shi, Kyoto 610-0113, Japan. Fax: +81-774-55-2765, E-mail: miyanomt@skyoto.hosp.go.jp

Table 1. Clinical characteristics of patients with MRSA, *P. aeruginosa* and *S. marcescens*, and PFGE patterns of these isolates

Isolate date	Patient No.	Disease	Tracheotomy	Respirator	Specimen	PFGE pattern		
						MRSA	*P. aeruginosa*	*S. marcescens*
Dec. 2002	P1	Hypoxic encephalopathy	+	+	Sputum	B1	P.D	S.A1
	P2	Mental retardation	+	–	Sputum	AY3(AA1)	–	–
	P3	Hypoxic encephalopathy	+	+	Sputum	A3	P.G1	–
	P4	Cerebral palsy	+	–	Sputum	A3	P.C1	S.A1
	P5	Anoxic encephalopathy	+	+	Abscess	A16/BM	–	–
	P5	Anoxic encephalopathy	+	+	Sputum	–	P.B	–
	P6	Cerebral palsy	+	–	Sputum	A3	P.I1	S.A2
	P7	Cerebral palsy	+	–	Sputum	BM	P.A1	–
	P7	Cerebral palsy	+	–	Eye mucus	BM	–	–
	P8	Developmental disability	+	+	Sputum	A44	–	–
	P9	MELAS[1)]	+	+	Sputum	BK	–	–
	P10	Hypoxic encephalopathy	+	+	Sputum	–	P.E1	–
	P11	Sequelae of encephalitis	+	+	Sputum	–	P.G2	–
	P12	Cerebral palsy	+	–	Sputum	–	P.E2	–
	P13	Hypoxic encephalopathy	+	+	Sputum	–	–	S.B
	P14	Cerebral palsy	+	–	Sputum	–	–	S.A1
Aug. 2003	P1	Hypoxic encephalopathy	+	+	Sputum	–	P.F	S.A1
	P2	Mental retardation	+	–	Sputum	BJ	–	–
	P3	Hypoxic encephalopathy	+	+	Sputum	–	P.H	-
	P4	Cerebral palsy	+	–	Sputum	A3	–	S.A1
	P7	Cerebral palsy	+	–	Sputum	–	P.A1	–
	P8	Cerebral palsy	+	+	Sputum	–	P.K	–
	P11	Sequelae of encephalitis	+	+	Sputum	–	P.G2	S.A1
	P13	Hypoxic encephalopathy	+	+	Sputum	BN	–	–
	P14	Cerebral palsy	+	–	Sputum	–	P.A2	–
	P15	Hypoxic encephalopathy	+	–	Sputum	A1	P.J2	–
	P16	Developmental disability	–	–	Urine	AX2	–	–
	P17	Viral encephalitis[2)]	–	–	Urine	BL	–	–
	P18	Herpatic encephalitis	+	–	Sputum	–	P.G3	–
	P19	Hypoxic encephalopathy	+	+	Sputum	–	P.J1	–
	P20	Cerebral palsy	+	+	Sputum	–	P.I2	–
	P21	Cerebral palsy	+	–	Sputum	–	P.C2	–
	P22	Cerebral palsy	+	–	Sputum	–	P.J1	–
	P23	Herpatic encephalitis	+	+	Sputum	–	P.A3	–

1): MELAS, nutochondrial myopathy and lactic acidosis.
2): caused by measles virus.

sharing a similarity of 70% or higher were grouped into a cluster). No other clustering was observed.

Among 11 MRSA isolates found in December 2002, there were two clusters, one consisting of three isolates of PFGE pattern A3 and the other of three isolates of pattern BM. In contrast, in six isolates found in August 2003, clustering was not detected (Table 1). The PFGE patterns obtained from this study were compared with those identified in previous studies conducted in 2000-2003 in Tokyo (1-4), in 2002-2003 in Kumamoto (5-7), and in 2003 in Sendai (8). Among the patterns detected in the present study, pattern A1 was detected in 2000-2003 both in Tokyo and Kumamoto; pattern A3 in 2000-2003 in Tokyo and in 2003 in Sendai; and pattern A16 in 2001 and 2002 in Tokyo. The other nine patterns we identified were not detected in the previous studies.

The PFGE patterns of *P. aeruginosa* isolates are shown in Fig. 2A. From a total of 22 isolates, 19 different PFGE patterns were detected. Band-based cluster analysis of these patterns revealed six clusters, A, C, E, G, I, and J (Fig. 2B). The isolates from patients P19 and P22 in August 2003 were of the same pattern, P.J1. The isolates in December 2002 and August 2003 from patient P7 were of the same pattern P.A1, and those from patients P11 in the two surveys were also of the same pattern P.G2.

A total eight *S. marcescens* isolates were obtained. These represented three different PFGE patterns (Fig. 3A), two of which were similar to each other (Fig. 3A, 3B). Three of five isolates found in December and all of the three isolates found in August were of pattern S.A1.

Comparison of the August 2003 data with December 2002 data clearly shows reduction of MRSA carriers and disappearance of genetically related MRSA clusters in the second survey. Probably interventions taken after the first survey reduced MRSA transmission among the inpatients. The interventions taken were i) an educational program for the ward staff that dealt with infection control practice, ii) promotion of compliance with hand washing, and iii) replacement of the multi-use catheter with the sterile single-use catheter for suction of respiratory tract secretions. The data also suggested that the above interventions were not as successful for control of *P. aeruginosa* and *S. marcescens* that were present in the environment of the facility.

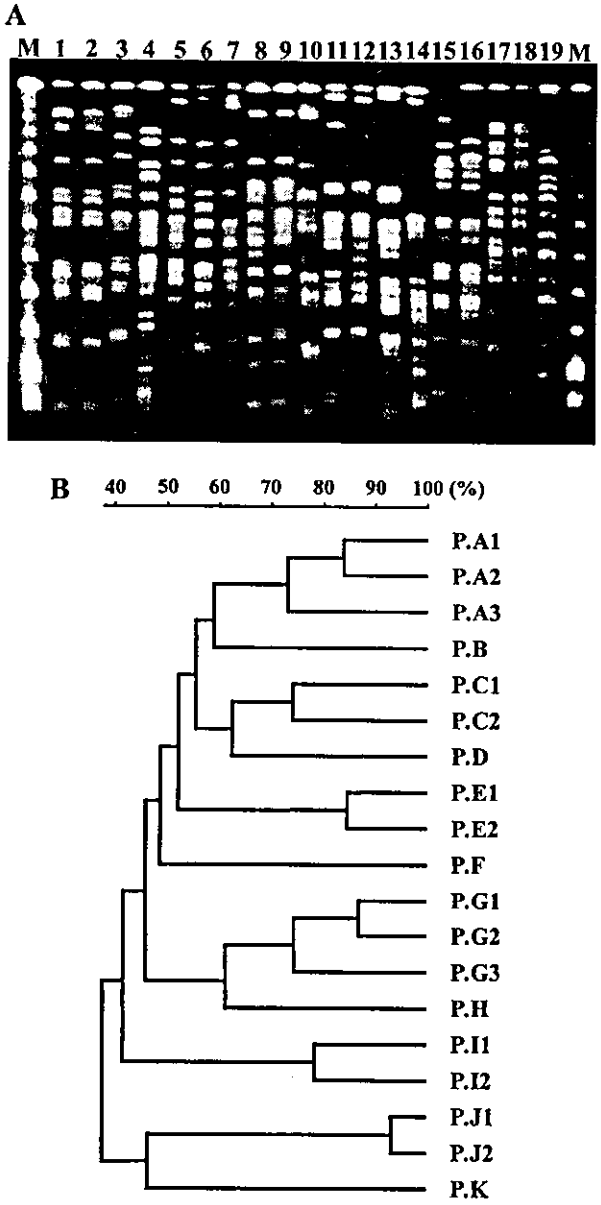

Fig. 2. Molecular analysis of *P. aeruginosa* isolate. A: pulsed-field gel electrophoresis of *Spe*I-digested genomic DNA from *P. aeruginosa* isolates. M: low range PFG Marker. Lanes 1 to 19 corresponding to the following PFGE pattern; 1: P.A1, 2: P.A2, 3: P.A3, 4: P.B, 5: P.C1, 6: P.C2, 7: P.D, 8: P.E1, 9: P.E2, 10: P.F, 11: P.G1, 12: P.G2, 13: P.G3, 14: P.H, 15: P.I1, 16: P.I2, 17: P.J1, 18: P.J2, 19: P.K. B: Cluster analysis of *P. aeruginosa* isolates based on PFGE patterns of *Spe*I-digested genomic DNA.

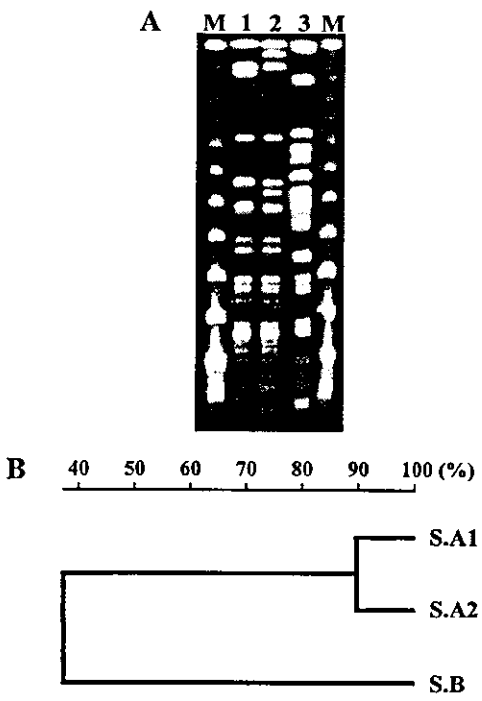

Fig. 3. Molecular analysis of *S. marcescens* isolate. A: pulsed-field gel electrophoresis of *Spe*I-digested genomic DNA from *S. marcescens* isolates. M: low range PFG Marker. Lanes 1 to 3 corresponding to the following PFGE pattern; 1: S.A1, 2: S.A2, 3: S.B. B: cluster analysis of *S. marcescens* isolates based on PFGE patterns of *Spe*I-digested genomic DNA.

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参考資料

Mechanisms of disease

Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*

Keiko Yokoyama, Yohei Doi, Kunikazu Yamane, Hiroshi Kurokawa, Naohiro Shibata, Keigo Shibayama, Tetsuya Yagi, Haru Kato, Yoshichika Arakawa

Summary

Background Bacteria develop resistance to aminoglycosides by producing aminoglycoside-modifying enzymes such as acetyltransferase, phosphorylase, and adenyltransferase. These enzymes, however, cannot confer consistent resistance to various aminoglycosides because of their substrate specificity. Notwithstanding, a *Pseudomonas aeruginosa* strain AR-2 showing high-level resistance (minimum inhibitory concentration >1024 mg/L) to various aminoglycosides was isolated clinically. We aimed to clone and characterise the genetic determinant of this resistance.

Methods We used conventional methods for DNA manipulation, susceptibility testing, and gene analyses to clone and characterise the genetic determinant of the resistance seen. PCR detection of the gene was also done on a stock of *P aeruginosa* strains that were isolated clinically since 1997.

Findings An aminoglycoside-resistance gene, designated *rmtA*, was identified in *P aeruginosa* AR-2. The *Escherichia coli* transformant and transconjugant harbouring the *rmtA* gene showed very high-level resistance to various aminoglycosides, including amikacin, tobramycin, isepamicin, arbekacin, kanamycin, and gentamicin. The 756-bp nucleotide *rmtA* gene encoded a protein, RmtA. This protein showed considerable similarity to the 16S rRNA methylases of aminoglycoside-producing actinomycetes, which protect bacterial 16S rRNA from intrinsic aminoglycosides by methylation. Incorporation of radiolabelled methyl groups into the 30S ribosome was detected in the presence of RmtA. Of 1113 clinically isolated *P aeruginosa* strains, nine carried the *rmtA* gene, as shown by PCR analyses.

Interpretation Our findings strongly suggest intergeneric lateral gene transfer of 16S rRNA methylase gene from some aminoglycoside-producing microorganisms to *P aeruginosa*. Further dissemination of the *rmtA* gene in nosocomial bacteria could be a matter of concern in the future.

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Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan (K Yokoyama PhD, Y Doi MD, K Yamane MD, H Kurokawa, N Shibata MD, K Shibayama MD, T Yagi MD, H Kato MD, Y Arakawa MD)

Correspondence to: Dr Yoshichika Arakawa (e-mail: yarakawa@nih.go.jp)

Introduction

Acquisition of multidrug resistance in nosocomial pathogens such as *Pseudomonas aeruginosa* has become a global concern.[1] For the treatment of infectious diseases caused by *P aeruginosa*, fluoroquinolones, broad-spectrum β lactams including carbapenems, and aminoglycosides such as the anti-pseudomonal drug amikacin, are the drugs of last resort. In Japan, however, about 20% of clinically isolated *P aeruginosa* have acquired resistance to imipenem or ciprofloxacin, while about 5% of clinical isolates also show resistance to amikacin.[2] Therefore, continuing amplification of resistance rates and levels, and expansion of resistance profiles to aminoglycosides in *P aeruginosa*, is becoming a general and genuine threat in clinical settings.[3]

Various aminoglycosides—such as gentamicin, kanamycin, amikacin, tobramycin, and isepamicin—have been developed and used for chemotherapy since the 1950s.[4] These drugs have high affinities for 16S rRNA of the bacterial 30S ribosome, and they block protein synthesis.[5] Over the past few decades, results of many studies on the mechanisms of resistance to aminoglycosides have shown self-modification of drugs to be the most typical mechanism; impermeability caused by upregulation of the active multidrug efflux system MexXY-OprM also confers broad but low-level resistance to aminoglycosides.[6] Several aminoglycoside-modifying enzymes—such as acetyltransferase, phosphorylase, and adenyltransferase—that catalyse covalent modification of specific amino or hydroxyl groups have been identified.[7] These enzymes have been noted in various nosocomial bacteria and are generally associated with transposable elements mediated by transferable R-plasmids. To overcome these modifying enzymes, a novel semisynthetic aminoglycoside, arbekacin, a derivative of kanamycin, was developed in Japan; this drug shows strong activity against various bacterial species and is rarely inactivated by single 6′ acetylation or 2″-phosphorylation.[8] Arbekacin showed effective antibacterial activity against various gram-positive and gram-negative bacteria by inhibition of 16S rRNA in bacterial 30S ribosome.[9,10]

Arbekacin has been used in Japan since 1990,[11] although this drug was approved only for control of infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) for prudent antibiotic use. However, several arbekacin-resistant MRSA strains have emerged in Japan, which produce the bifunctional enzyme, aminoglycoside-6′-*N*-acetyltransferase-2″-*O*-phosphotransferase, which mediates both 6′-acetylation and 2″-phosphorylation; this type of modification, however, confers only low-level drug resistance (minimum inhibitory concentration [MIC] between 4 and 32 mg/L).[12]

GLOSSARY

16S rRNA METHYLASES
Enzymes essential for folding and stabilisation of rRNA by methylation in bacterial ribosomes. Aminoglycoside-producing actimomycetes produce enzymes that mediate methylation of ribonucleotide residues at the aminoglycoside-binding site of 16S rRNA to protect their own 16S rRNAs from intrinsic aminoglycosides.

ACTINOMYCETES
A group of morphologically diverse gram-positive bacteria (order Actinomycetales) that produce various bioactive agents including antibiotics, enzymes, and vitamins. *Streptomyces* spp and *Micromonospora* spp belong to this bacterial order.

CONJUGATION
Transmission of bacterial plasmids through direct contact between bacterial cells.

SHINE-DALGARNO SEQUENCE
A specific nucleotide sequence essential for initiation of bacterial protein synthesis in bacterial ribosome, according to information encoded by mRNA. The 3'-terminal region of 16S rRNA in bacterial 30S ribosomal subunit recognises and attaches to this sequence. The ATG codon locating just downstream of the Shine-Dalgarno sequence generally functions as the initiation codon for formyl-methionine, which is usually the forefront aminoacid residue at the N-terminal of peptides.

TRANSCONJUGANTS
Bacterial cells that accept foreign plasmid by conjugation.

In this study, we aimed to characterise the genetic determinant for multiple and high-level aminoglycoside resistance in a clinically isolated *P aeruginosa* strain showing consistent and very high-level resistance to all clinically useful aminoglycosides, including amikacin and arbekacin. We also aimed to characterise the prevalence of the molecular mechanism of very high-level resistance to arbekacin found in *P aeruginosa* strain AR-2 among clinically isolated *P aeruginosa* strains.

Methods

Procedures

DNA manipulation, susceptibility testing, and gene analyses

We isolated *P aeruginosa* strain AR-2 from a clinical sample (sputum) taken in 1997. We used *E coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) as the transformation host and for propagation of plasmids. We used *P aeruginosa* strain 105 (ciprofloxacin-resistant, arbekacin-sensitive, amikacin-sensitive) as recipient in a CONJUGATION experiment. The plasmid pBC-SK+ (Stratagene) was used as the cloning vector, and pTO001—an *E coli-P aeruginosa* shuttle-cloning vector—was also used. *P aeruginosa* PAO1 served as the host for subcloning experiments. Unless noted otherwise, we grew cultures at 37°C in Luria-Bertani broth. We established MICs of aminoglycosides by an agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA), according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines M7-A5.[13]

Primers used

RMTA-forward
5'-CTAGCGTCCATCCTTTCCTC-3'
RMTA-reverse
5'-TTTGCTTCCATGCCCTTGCC-3'

DNA prepared from *P aeruginosa* AR-2 was digested with HindIII and ligated into the HindIII site of pBC-SK+ with T4 DNA ligase (Nippon Gene, Tokyo, Japan); the resulting recombinant plasmid was named pBCH9, and the deleted plasmid was named pBCH9-13 (figure 1). We selected *E coli* strain XL1-Blue transformants carrying a roughly 8-kb insert on Luria-Bertani agar plates containing both arbekacin (2 mg/L) and chloramphenicol (30 mg/L). We assayed MICs on both the parent strain and transformants, according to the guidelines of the NCCLS. We established the nucleotide sequence by the dideoxy-chain termination method with a model 3100 DNA sequencer (Applied Biosystems Japan, Tokyo, Japan). We did nucleotide and aminoacid sequence homology searches with the internet program FASTA (National Institute of Genetics, Mishima, Japan).[14] We aligned nucleotide and aminoacid sequences with GENETYX-MAC software, version 10.1.1 (Software Development, Tokyo, Japan).

To ascertain the transferability of the *rmtA* gene for arbekacin resistance, we did conjugation experiments with *P aeruginosa* strain 105 as a recipient. TRANSCONJUGANTS were selected on Mueller-Hinton agar

	P aeruginosa AR-2	*E coli* XL1-blue			*P aeruginosa* PAO1		*P aeruginosa*	
		pBCH9	pBCH9-13	pBC-SK+	pTORmtA	pTO001	Transconjugant	105*
4,6-substituted deoxystreptamine antimicrobials								
Kanamycin groups								
Arbekacin	>1024	>1024	>1024	0·5	>1024	1	>1024	4
Amikacin	>1024	>1024	>1024	1	>1024	8	>1024	4
Kanamycin	>1024	>1024	>1024	2	>1024	128	>1024	>1024
Tobramycin	>1024	>1024	512	1	>1024	1	>1024	256
Gentamicin groups								
Gentamicin	>1024	>1024	1024	0·5	>1024	256	>1024	>1024
Sisomicin	>1024	512	>1024	0·5	>1024	256	>1024	>1024
Isepamicin	>1024	>1024	>1024	0·5	>1024	4	>1024	8
4,5-substituted deoxystreptamine antimicrobials								
Neomycin	>1024	4	>1024	4	512	16	>1024	>1024
Other aminoglycosides								
Streptomycin	128	4	2	4	32	32	>1024	>1024
Hygromycin B	1024	64	2	32	512	512	1024	512
Others								
Ceftazidime	2	0·5	0·25	0·25	ND	ND	128	32
Imipenem	1	0·25	0·25	0·125	ND	ND	16	16
Ciprofloxacin	0·25	0·125	0·125	0·125	ND	ND	64	64

ND=not determined. pBCH9, pBCH9-13, pBC-SK+, pTORmtA, and pTO001 are the plasmids harboured by each transformant. pBC-SK+, pTO00=cloning vector, expression vector. pBCH9=pBC-SK+ + 8 kb insert fragment. pBCH9-13=pBC-SK+ + 1–2 kb insert fragment. pTORmtA=pTO001+rmtA. *105 was recipient for the conjugation study.

MICs (mg/L) for parental strain, transformants, and transconjugant

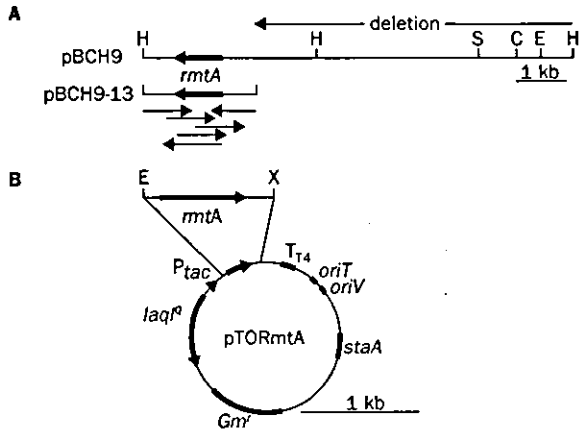

Figure 1: **Sequencing strategy (A) and restriction map (B) of the DNA insert**

Black bar represents the coding region of the *rmtA* gene and the arrow the direction of transcription. Horizontal thin arrows show the sequencing strategy. H=HindIII; C=ClaI; S=SacI; E=EcoRI; X=XbaI.

containing arbekacin (64 mg/L) and ciprofloxacin (5 mg/L). We did genomic DNA analysis of the parent, recipient, and transconjugants with pulsed-field gel electrophoresis. SpeI-digested genomic DNA fragments were separated for 22 h at 6 V/cm and 14°C with a CHEF-DR system (BioRad Laboratories, Tokyo, Japan). We did electrophoresis in two ramps as follows: pulse times were linearly increased from 4 s to 8 s for 12 h during the first ramp and from 8 s to 50 s for 10 h during the second ramp.

Assay of gene product

For thin layer chromatography analyses, we harvested bacterial cells grown in Luria-Bertani broth at the middle of the logarithmic phase. Cells were washed and resuspended with 0·1 mol/L phosphate buffer (pH 7·0). We disrupted the cell suspension by a French press (Ohtake, Tokyo, Japan), and then centrifuged it at 7700 *g* for 10 min at 4°C. The supernatant was ultracentrifuged at 100 000 *g* for 3 h at 4°C with a 65 Ti rotor (Beckman Instruments, Fullerton, CA, USA), and we stored the resulting cell-free extract at –20°C before use. We did acetylation or phosphorylation under the following conditions: 0·5 mmol/L arbekacin, 0·1 mol/L phosphate buffer (pH 7·0), 10% (volume/volume) cell-free extract, and 4 mmol/L acetylCoA or ATP in a 50 μL reaction mixture. After incubation for 3 h at 37°C, we monitored every reaction mixture by thin layer chromatography, which we did with a silica gel plate (Merck silica gel 60 F254; Merck, Darmstadt, Germany) developed with 5% KH_2PO_4 and stained with ninhydrin reagent.

Preparation of ribosomes, ribosomal subunits, and post-ribosomal supernatant containing material removed from 70S ribosomes by high salt washing (S100) was done as described by Skeggs and others.[15] For assay of methylase activity, the extract (S100) from a *P aeruginosa* clone that harbours a recombinant plasmid, pTORmtA, carrying the *rmtA* genes was used as a source of methylase together with S-adenosyl-L-methionine as cofactor. Radiolabelled methyl groups were incorporated into 16S rRNA at 35°C in reaction mixtures (100 μL total volume) made up in a buffer containing 50 mmol/L Hepes-KOH (pH 7·5 at 20°C), 7·5 mmol/L $MgCl_2$, 37·5 mmol/L NH_4Cl, and 3 mmol/L 2-mercaptoethanol. Such mixtures contained 20 pmol of *P aeruginosa* PAO1 (pTO001) 30S ribosomal subunits (substrates for methylation) together with 50 μL S100 from the clone of *P aeruginosa* (controls received S100 from *P aeruginosa* PAO1 [pTO001]) plus $9{\cdot}25\times10^4$ Bq S-adenosyl-[methyl-^{3}H]-L-methionine (18·5 GBq/mmol). We removed samples (10 μL) at intervals (0, 10, 30, and 50 min) and allowed them to permeate a DEAE (diethylaminoethyl) filtermat (glass fibre filter, with DEAE active groups; Wallac, Turku, Finland), and the filtermat was washed three times with ice-cold 50 mmol/L glycine hydrochloride (pH 4·5) and a further two times with ice-cold ethanol. The filtermat was dried and soaked in a scintillator, MeltiLexTM (Wallac), and then radioreactivity was counted by MicroBeta plus (Wallac).

PCR screening of rmtA *gene harbouring strains*

We screened a bacterial stock of 1113 clinically isolated *P aeruginosa* strains for the *rmtA* gene. PCR analyses with the primers shown in the panel, which amplify a 635-bp fragment within the *rmtA* gene, were done on strains showing a degree of resistance to gentamicin, amikacin, and arbekacin (MICs ≥32 mg/L) .

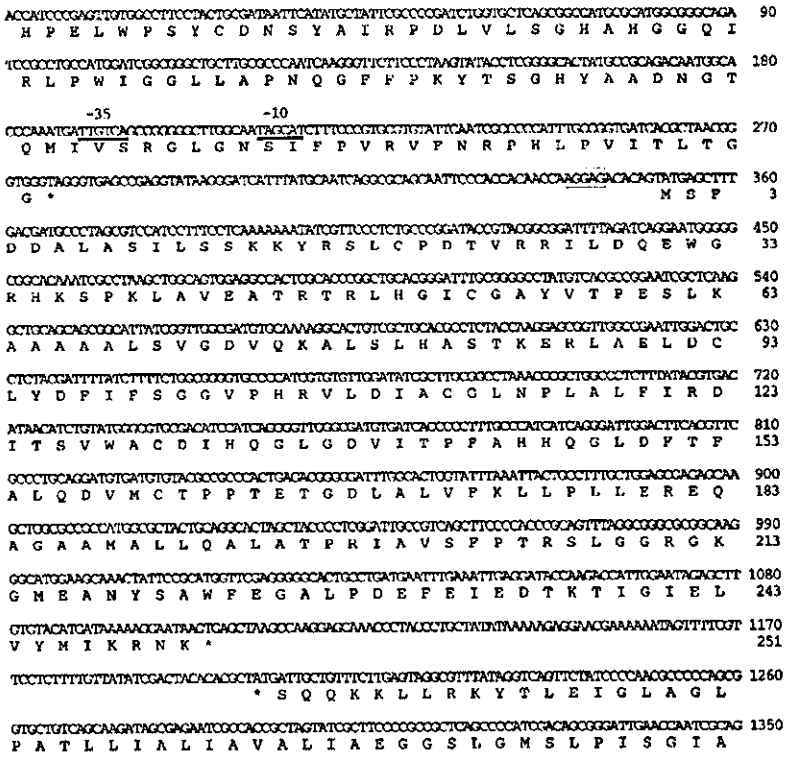

Figure 2: **Nucleotide and deduced aminoacid sequences of *rmtA* gene and RmtA protein**

The 1350 bp sequence of the 1662 bp area determined is shown. Stop codons are indicated by asterisks. A putative SHINE-DALGARNO SEQUENCE is boxed. The putative promoter –35 region is marked by a bold line at positions 190–195, and the possible –10 region is indicated by a bold line below the nucleotide sequence at positions 212–217.

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P. aer. AR-2/RmtA  ----------MSFIDALASILSSKKVRSLCPDIVRRILQDWGRHGSPKLAVEATR  46
M. ros. /Grm       ---MT-TSTGD--D-RIDQLQQATKSRRYQTVAPATVRRLARAALVASRGDVPDAV-KR  52
M. zio. /Sgm       ---MT-APAAD--D-RIDETERATTKSRRYQTVAPATVRRLARAALVAARGDVPDAV-KR  52
S. hin. /NbrB      MPHPA-PGPADAEDPRLAEVMAAVRSSRRYQSVAPETVRRLAANALVASRGDLAEAV-KR  58
S. kan. /Kmr       ----MSQSASDE-DPRLTRVVEAVRGGRRYRSVTDQAVRRLARAALAVEGGDVTRAT-KR  54
S. ten. /KgmB      MPHPA-PGPGDPEDPRLAEVVDAVRSSRRYQSVAPETVRRLATSALVASRGDLAEAV-KR  58
                                . . .   .  ...*..... .***..  .... ...   .. .*

P. aer. AR-2/RmtA  TR--LHGICGAYV--TPES---L-KAAAAALSVGDVQ--KA-LSLHA-----STKERLAE  90
M. ros. /Grm       TKRGLHETYGAFLPPSAPNYTALLRHLDSAVEAGDDEAVVRAD-RRAMSVHMSTRERVPH  111
M. zio. /Sgm       TKRGLHETYGAFLPPSPPNYAALLRHLDSAVDAGDDEAV-RAALLRAMSVHLSTRERLPH  111
S. hin. /NbrB      TKRSLHEVFGAYLPSPP-KYDALLRQLRDAVDAGDDEAVRAVLHRAMSTHAST-RERLPI  116
S. kan. /Kmr       TKRGLHEVFGAFMHMTPK-YEALLRDVPEALELDDPEATRTAL-KPALGAHSSTRERLPI  112
S. ten. /KgmB      TKRGLHEIPGAYLPSPP-KYDALLRQLRGAVDAATTRTCGHPAPRHVHARLHP-RA-LPI  115
                   *...**.. ** .. .  . .*.. .  *. ... ..      .  .......

P. aer. AR-2/RmtA  LDCLYDFIPSGGVPHRVL--DIACGLNPLAL-PIRDITS-V-W-ACDIHQGLGDVTTPFA  144
M. ros. /Grm       LDEFYREIPRHVPRNIL-RDLACGLNPLAVPWM-GLSDETVYVASDIDARLMDFVGAAL  169
M. zio. /Sgm       LDEFYRELPRHLPRNIL-RDLACGLNPLAAPWM-GLPAETVYTASDIDARLVGFVDEAL  169
S. hin. /NbrB      LEEFYREVFARLDAPTSV-RDLACGMNPLAAPWM-PGSDAFTYHASDIDTRLTEFLAAAL  174
S. kan. /Kmr       LTEMYAEVFDRLDTAPATVRDLACGMNPLTVPWM-PLPAGTTYLASDIDHRLMDFAGTVL  171
S. ten. /KgmB      LDEFYREVFARCADPASV-RDLACGMNPLAAPWM-PGSDAFTYHASDIDTRLMEFLDAAL  173
                   *...*.. *    .  .*.*** ***. ...      . *.**. .*  .  ..

P. aer. AR-2/RmtA  MHQGLDFTFALQDVMCTPPTEIGDLALVF-KLLPLLEREQVGANMALLQALATPRIAVSF  203
M. ros. /Grm       TRLGVAHRTSVVDLLEARLDEP-ADVTLLLKTLPCLETQQRGSGMEVDIVNSPIIVVTP  228
M. zio. /Sgm       TRLNVPHRTNVADLLEDRLDEP-ADVTLLLKTLPCLETQQRGSGMEVDIVNSPNIVVTP  228
S. hin. /NbrB      EILGVAHDVRVRDLMTGVG-EVATTDVTLIIKTLPCIEAQGRQQGMDLIDAIRSPVVVSF  233
S. kan. /Kmr       TALGVPNRVEVRDLLTDPDPEPAD-VISLLKAVKCLEAQCKCLGMKLLDQINSPVLVVSP  230
S. ten. /KgmB      EILGVAHDVRVRDLMTGVG-EVETDVTLLLKIVKCIEAQGRQQGMDLIDAIRSPLVVSP  232
                      ... .  . *.     *  ......*..*..* . .* .. ...   .*  .*.*

P. aer. AR-2/RmtA  PIRSLGGRGKGMEANYSAWFEGALPDE-FEIEDIKTTGIELVYMIKRNK  251
M. ros. /Grm       PIKSLGQRSKGMFQNYSQSFESQASERSCRIQRLEI-GNELIYVIIK--  274
M. zio. /Sgm       PIKSLGQRSKGMFQNYSQSFESQARERSCRIQRLEI-GNELIYVIQK--  274
S. hin. /NbrB      PIKSLGGRSKGMFNTYSANFGAWLENRPHIVEQVEF-RNELVYFVRKNA  281
S. kan. /Kmr       PIKTLGRRSRGMYQTHSAEFARRVAERPWKCIELEF-GDELVYVVIKG-  277
S. ten. /KgmB      PIKSLGQRSKGMFNTYSANFDAWLENRPHIVEQLEF-RNELVYFVRKNA  280
                   **..**.*..**.  .*. *     .     . ..**.*  .
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Figure 3: **Comparison of aminoacid sequences of known 16S rRNA methylases with *P aeruginosa* AR-2 RmtA**

Proteins in comparison: GrmB, *Micromonospora rosea*; Sgm, *M zionensis*; NbrB, *Streptoalloteichus hindustanus*; Kmr, *Streptomyces kanamyceticus*; and KgmB, *Streptomyces tenebrarius*. Identical aminoacid residues among all six enzymes are indicated by asterisks, and aminoacids with similar properties are indicated by dots. Dashes represent gaps introduced to improve alignment.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

P aeruginosa strain AR-2 showed very high-level resistance to various aminoglycosides (table). Arbekacin resistance was transferred from AR-2 to *P aeruginosa* PAO1 by conjugation, and the *E coli* clone (XL1-Blue) and transconjugant of *P aeruginosa* strain 105 showed similar resistance profiles to AR-2 against various aminoglycosides, as shown in the table. The pattern on pulsed-field gel electrophoresis of SpeI-digested genomic DNA fragments of the transconjugant was closely similar to that of the *P aeruginosa* recipient strain 105 (not shown). This finding suggested that the transconjugant was not a ciprofloxacin-resistant mutant of the donor strain *P aeruginosa* AR-2. By thin layer chromatography, however, no detectable conversion was noted in the rate of flow value of arbekacin after in-vitro acetylation or phosphorylation reactions (data not shown). Therefore, the mechanism underlying the wide range of resistance to various aminoglycosides is difficult to establish, since it is not merely production of known aminoglycoside-modifying enzymes. These findings suggest that in strain AR-2, novel molecular mechanisms determine multiple aminoglycoside resistance.

By sequencing of the plasmid carrying the *rmtA* gene, we determined a 1662-bp nucleotide sequence carrying arbekacin resistance (figure 1). An open reading frame of 756 bp was noted, with the initiation codon ATG at position 352 and the stop codon TGA at position 1105. The G+C content of the open reading frame was 55%. By part sequencing of the flanking region of the *rmtA* gene, the gene was suggested to be carried by Tn5041, which mediates Hg^{+}-resistance in *Pseudomonas* spp. The nucleotide sequence has been submitted to the EMBL, GenBank, and DDBJ databases and assigned accession number AB083212.

The open reading frame encoded a putative protein, RmtA, with 251 aminoacids (molecular weight 27 430; figure 2). The predicted aminoacid sequence of RmtA showed considerable similarity to the 16S rRNA METHYLASES produced by aminoglycoside-producing ACTINOMYCETES (figure 3).[16,17] The deduced 251 aminoacid sequence of RmtA was closely similar to GrmB and Sgm methylases found in sisomicin-producing

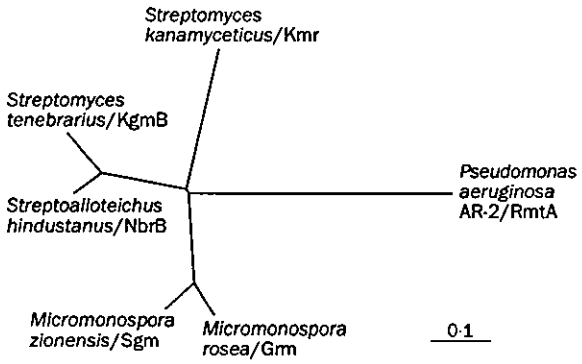

Figure 4: **Dendrogram of 16S rRNA methylases**

Units for bar are genetic units calculated with the CLUSTAL W program, which reflects the number of aminoacid exchanges.

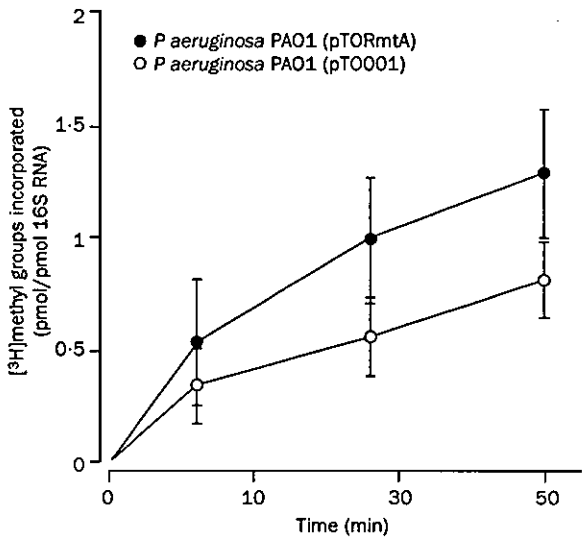

Figure 5: **Methylation of 30S ribosomal subunit by RmtA**

Error bars indicate SD.