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Novel Plasmid-Mediated 16S rRNA m[1]A1408 Methyltransferase, NpmA, Found in a Clinically Isolated *Escherichia coli* Strain Resistant to Structurally Diverse Aminoglycosides[▽]

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We have isolated a multiple-aminoglycoside-resistant *Escherichia coli* strain, strain ARS3, and have been the first to identify a novel plasmid-mediated 16S rRNA methyltransferase, NpmA. This new enzyme shared a relatively low level of identity (30%) to the chromosomally encoded 16S rRNA methyltransferase (KamA) of *Streptomyces tenjimariensis*, an actinomycete aminoglycoside producer. The introduction of a recombinant plasmid carrying *npmA* could confer on *E. coli* consistent resistance to both 4,6-disubstituted 2-deoxystreptamines, such as amikacin and gentamicin, and 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin. The histidine-tagged NpmA elucidated methyltransferase activity against 30S ribosomal subunits but not against 50S subunits and the naked 16S rRNA molecule in vitro. We further confirmed that NpmA is an adenine N-1 methyltransferase specific for the A1408 position at the A site of 16S rRNA. Drug footprinting data indicated that binding of aminoglycosides to the target site was apparently interrupted by methylation at the A1408 position. These observations demonstrate that NpmA is a novel plasmid-mediated 16S rRNA methyltransferase that provides a panaminoglycoside-resistant nature through interference with the binding of aminoglycosides toward the A site of 16S rRNA through N-1 methylation at position A1408.

Aminoglycosides such as kanamycin, gentamicin, and neomycin bind to the A site of the 16S rRNA of the bacterial 30S ribosomal subunit and subsequently block growth through interference with protein synthesis (25). These agents have been used for the treatment of a broad range of life-threatening infections due to both gram-positive and gram-negative bacteria in human and veterinary medicine (18, 37). However, bacteria have acquired various aminoglycoside resistance mechanisms, such as through the production of aminoglycoside-modifying enzymes (acetyltransferase, nucleotidyltransferase, and phosphotransferase), the reduction of antibiotic penetration on the outer membrane protein, the acquisition of reduced affinity by changing key nucleotides within the 16S rRNA, and augmented excretion by an efflux pump system (5, 25, 36, 42).

In 2003, a plasmid-mediated 16S rRNA methyltransferase, which confers a high level of resistance to various clinically important aminoglycosides, was reported to be involved as part of a novel aminoglycoside resistance mechanism in pathogenic gram-negative rods (16, 53). At present, five types of plasmid-mediated 16S rRNA methyltransferase genes, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *armA*, have been found worldwide in members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. (4, 7, 10, 11, 17, 20, 34, 48, 50–52). Also, these genes are mediated by bacterium-specific recombination systems, such as transposons, and are easily translocated to other DNA target sites (17, 19, 47, 49).

The 16S rRNA methyltransferases conferring aminoglycoside resistance are supposed to have evolved as a self-defense mechanism in aminoglycoside-producing actinomycetes, including *Streptomyces* spp. and *Micromonospora* spp (9). The methylation of 16S rRNA plays a crucial role in prevention of the adverse effects of intrinsic aminoglycosides that would block their own 16S rRNA. The 16S rRNA methyltransferase conferring aminoglycoside resistance consists of two different groups, one methylates the N-7 position of G1405 and confers panresistance to aminoglycosides belonging to both the kanamycin and the gentamicin groups (3, 44), and the other methylates the N-1 position of A1408 and provides resistance to kanamycin and apramycin (3, 22, 43). Recently, it was reported that the plasmid-mediated 16S rRNA methyltranferase ArmA methylates the N-7 position of G1405 within 16S rRNA (27). On the other hand, no plasmid-mediated 16S rRNA methyltransferase which modifies the N-1 position of A1408 has so far been found in any pathogenic bacteria isolated from clinical settings and natural environments. Therefore, we screened for a new plasmid-mediated methyltransferase that methylates A1408 among bacterial species belonging to the family *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. isolated in Japanese clinical settings. Apramycin resistance seemed to be a good indicator for the detection of an A1408 16S rRNA methyltransferase producer, since a previous study reported that the introduction of a recombinant plasmid encoding a gene for the A1408 16S rRNA methyltransferase derived from a *Streptomyces* sp. was also able to confer a high level of

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New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate[▽]

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Plasmid-mediated Qnr and AAC(6′)-Ib-cr have been recognized as new molecular mechanisms affecting fluoroquinolone (FQ) resistance. C316, an *Escherichia coli* strain demonstrating resistance to various FQs, was isolated in Japan. Resistance to FQs was augmented in an *E. coli* CSH2 transconjugant, but PCR failed to detect *qnr* genes, suggesting the presence of novel plasmid-mediated FQ resistance mechanisms. Susceptibility tests, DNA manipulation, and analyses of the gene and its product were performed to characterize the genetic determinant. A novel FQ-resistant gene, *qepA*, was identified in a plasmid, pHPA, of *E. coli* C316, and both *qepA* and *rmtB* genes were mediated by a probable transposable element flanked by two copies of IS*26*. Levels of resistance to norfloxacin, ciprofloxacin, and enrofloxacin were significantly elevated in *E. coli* transformants harboring *qepA* under AcrB-TolC-deficient conditions. QepA showed considerable similarities to transporters belonging to the 14-transmembrane-segment family of environmental actinomycetes. The effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on accumulation of norfloxacin was assayed in a *qepA*-harboring *E. coli* transformant. The intracellular accumulation of norfloxacin was decreased in a *qepA*-expressing *E. coli* transformant, but this phenomenon was canceled by CCCP. The augmented FQ resistance level acquired by the probable intergeneric transfer of a gene encoding a major facilitator superfamily-type efflux pump from some environmental microbes to *E. coli* was first identified. Surveillance of the *qepA*-harboring clinical isolates should be encouraged to minimize further dissemination of the kind of plasmid-dependent FQ resistance determinants among pathogenic microbes.

Fluoroquinolones (FQs) are synthetic chemical agents and among the most commonly prescribed antimicrobials because of their broad-spectrum antimicrobial activity. Extensive clinical and agricultural use of FQs has led to high rates of resistance to these agents among pathogenic microbes (6, 9). The most common mechanism for resistance to FQs among pathogenic microbes is the mutation of chromosomal genes encoding DNA gyrase and/or topoisomerase IV (12, 13). Changes in the expression of efflux pumps and porin proteins are also a common FQ resistance mechanism in bacteria (1), but no plasmid-mediated FQ efflux pump has been documented to date. Four chromosome-dependent efflux systems responsible for FQ resistance have so far been reported, e.g., the resistance nodulation division family, AcrAB-TolC in *Escherichia coli* (7, 28) and MexAB-OprM in *Pseudomonas aeruginosa* (24); the major facilitator superfamily (MFS), NorA of *Staphylococcus aureus* (34); the multidrug and toxic compound extrusion family, NorM of *Vibrio parahaemolyticus* (19); and the ATP-binding cassette family, VcaM of non-O1 *Vibrio cholerae* (14).

Two molecular groups have been identified as plasmid-mediated FQ resistance mechanisms to date. Qnr peptides, QnrA (30), QnrB (15), and QnrS (11), were identified from *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *E. coli*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter cloacae*, *Shigella flexneri*, and a *Salmonella* sp. (8, 16, 21, 31), and these peptides interfere with DNA gyrase by mimicking DNA (18). AAC(6′)-Ib-cr was also found to possess an N-acetylating activity of the piperazinyl substituent of ciprofloxacin and norfloxacin (26), and this enzyme has been reported to be geographically widespread (22, 25).

In the present study, we identified a novel FQ resistance mechanism, QepA, as a plasmid-mediated efflux pump found in an *E. coli* clinical isolate from Japan.

(These findings have been reported at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, 2006, San Francisco, CA [33].)

MATERIALS AND METHODS

Bacterial strains, susceptibility testing, and DNA manipulation. *E. coli* strain C316, which displayed a multiple-resistance profile to aminoglycosides, FQs, and broad-spectrum β-lactams, except for ceftazidime and imipenem, was isolated from the urine of an inpatient at a medical facility in Hyogo Prefecture, Japan, in March 2002. The strains and plasmids used in this study are listed in Table 1. The bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antimicrobial agents.

E. coli KAM32 (14), a highly susceptible antimicrobial laboratory strain, was transformed with *qepA*-carrying plasmids for a precise assay of the MICs of antimicrobials and chemical agents. Transformants were selected on LB agar plates containing chloramphenicol (20 μg/ml) and norfloxacin (0.025 μg/ml). The susceptibilities to antimicrobials, dyes, and other drugs were tested by the agar dilution method according to the procedure recommended by the NCCLS (presently CLSI) document M7-A6 (20).

Transconjugation analysis was performed with *E. coli* CSH2 as the recipient by the filter mating method. Transconjugants were selected on LB agar plates supplemented with rifampin (50 μg/ml) and kanamycin (25 μg/ml), because the R plasmid carried a kanamycin resistance determinant, *rmtB*. Plasmid DNA from a transconjugant was purified by the method of Kado and Liu (17). Transformation of *E. coli* DH10B with the plasmid DNA of the *E. coli* CSH2 transconjugant was performed by conventional electroporation techniques. Transformants were selected on LB agar containing kanamycin (25 μg/ml).

Basic recombinant DNA techniques described by Sambrook et al. (27) were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source or reference
E. coli strain		
ARS3	Clinical isolate	This study
CSH-2	*metB* F^-, nalidixic acid resistant, rifampin resistant	Laboratory strain
JM109	*endA1 recA1 gyrA96, thi-1 hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*]	Takara
BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* λ(DE3)pLysS (chloramphenicol resistant)	Novagen
Plasmid		
pARS3	115-kb conjugative plasmid carrying *npmA* of *E. coli* ARS3	This study
pMCL-H	Plasmid carrying 3,946 bp fragment containing *npmA* on pMCL210	This study
pMCL-BE	Plasmid carrying 980-bp fragment containing *npmA* on pMCL210	This study
pMCL-BH	Plasmid carrying *npmA* tagged with five histidine codons at its 3′ end and its promoter region on pMCL210	This study
pBCE	Plasmid carrying an EcoRI-digested fragment containing *npmA* on pBCSK+	This study
pBCSII	Plasmid carrying a SacII-digested fragment containing *npmA* on pBCSK+	This study
pCold-NpmA	Plasmid carrying *npmA* ligated to pCold-IV	This study
pMCL210	Cloning vector, chloramphenicol resistant	30
pBCSK+	Cloning vector, chloramphenicol resistant	Stratagene
pCold-IV	Protein expression vector, ampicillin resistant	Takara

resistance to apramycin (43). The use of this screening protocol on the basis of apramycin resistance allowed us to identify a panaminoglycoside-resistant *Escherichia coli* strain, strain ARS3, that produces a novel plasmid-mediated methyltransferase, newly assigned NpmA, that methylates A1408 at the A site of 16S rRNA. The aim of this study was to characterize the molecular mechanism underlying the panaminoglycoside resistance conferred by NpmA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain ARS3 was isolated in 2003 from the urine of an inpatient in a general hospital in Japan. The bacterial strains were grown in LB broth at 37°C with shaking, unless otherwise indicated. MICs were determined by the agar dilution method with Mueller-Hinton agar plates, according to the protocol recommended by the Clinical and Laboratory Standards Institute (8).

Conjugation. A conjugation experiment was performed as described elsewhere (48). *E. coli* strain CSH-2 was used as the recipient. Conjugants were selected on LB agar plates containing apramycin at 150 μg/ml and rifampin at 100 μg/ml.

Cloning of *npmA*. The transferable plasmid pARS3 was extracted from the *E. coli* conjugant and digested with restriction enzymes. The resultant fragments were ligated to cloning vectors and electroporated into *E. coli* strain JM109. The transformants were selected on LB agar plates supplemented with apramycin at 150 μg/ml and chloramphenicol at 30 μg/ml. The *npmA* gene was amplified with primer P1 (5′-CGG GAT CCA AGC ACT TTC ATA CTG ACG-3′) and primer P2 (5′-CGG AAT TCC AAT TTT GTT CTT ATT AGC-3′) (the underscored sequences indicate BamHI and EcoRI restriction sites, respectively) and cloned into the vector pMCL210.

N-terminal determination of NpmA. The DNA fragment carrying *npmA* and its promoter region was amplified by PCR with primers P1 and P3 (the sequence of primer P3 is 5′-CCC AAG CTT TTA atg atg atg atg atg ATG TTT TGA AAC ATG GCC-3′ [where the underscores indicate the Hind III restriction site and the sequence with lowercase letters represents the nucleotide sequence of C-terminal histidine tag]). Primer P3 was designed so that five histidine codons could be added to the 3′ end of *npmA*. The resultant fragments were ligated to pMCL210 and introduced into *E. coli* JM109. The cells were cultured in 1 liter of LB broth containing chloramphenicol at 30 μg/ml, disrupted with a French press, and centrifuged at 100,000 × *g* for 1 h. The supernatant containing the recombinant protein was loaded onto a HisTrap HP column (Amersham Biosciences) and purified according to the manufacturer's instructions. The N-terminal sequence of the purified protein was obtained by Edman degradation in a model Shimadzu PPSQ-23 automated protein sequencer.

Overexpression and purification of histidine-tagged NpmA. The *npmA* gene was amplified with primer P4 (5′-GGA ATT CCA TAT GTT AAT ACT CAA AGG AA-3′), which introduced an NdeI restriction site at the 5′ end, and primer P3, which introduced a HindIII restriction site and five histidine codons at the 3′ end. The amplified fragments were cloned into the pCold-IV vector (Takara) and introduced into *E. coli* BL21(DE3)pLysS. The purification of recombinant protein was performed as described above, with some modifications. After the step of nickel-nitrilotriacetic acid chromatography, the eluted protein was dialyzed against 50 mM sodium phosphate buffer (pH 6.4). Furthermore, the protein was applied to a cation-exchange HiTrap S HP column (Amersham Biosciences). Finally, the eluted protein was concentrated and the buffer was exchanged with 50 mM sodium phosphate buffer (pH 7.4).

Methylation assay. Both the 30S and the 50S subunits of *E. coli* JM109 were prepared as described previously (27). After ultracentrifugation with 10 to 30% sucrose density gradients, the 30S and 50S subunit fractions were collected. The purity of each subunit was checked by denatured agarose gel electrophoresis of the rRNA derived from the material. The methylation assay was carried out at 35°C, as follows. Thirty picomoles of substrate, 30 pmol of His$_5$-NpmA, and 7.5 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (76 Ci/mmol, 1 mCi/ml) were adjusted to 300 μl with methylation buffer (50 mM HEPES-KOH, pH 7.5; 10 mM $MgCl_2$; 100 mM NH_4Cl; 5 mM 2-mercaptoethanol). Aliquots were taken at 0, 5, 15, 30, and 45 min and purified with an RNeasy mini kit (Qiagen), according to the instructions provided by the manufacturer. The samples were counted with a scintillation counter.

RNase protection assay. One picomole of [^{3}H]methyl-labeled 16S rRNA was hybridized with 100 pmol of a deoxyoligonucleotide (positions 1421 to 1392 [5′-CAC TCC CAT GGT GTG ACG GGC GGT GTG TAC-3′] and positions 1507 to 1478 [5′-TAC CTT GTT ACG ACT TCA CCC CAG TCA TGA-3′]) in 50 μl of hybridization buffer (40 mM morpholineethanesulfonic acid, pH 6.4; 400 mM NaCl; 9 mM EDTA; 80% [vol/vol] formamide) The sample was incubated at 90°C for 10 min, cooled at room temperature for 15 min, and diluted with 450 μl of RNase buffer (10 mM Tris-HCl, pH 7.5; 300 mM NaCl; 5 mM EDTA) containing RNase T_1 (Roche). The digestion was performed at 37°C for 1 h. The reaction was stopped by adding 4.5 ml of 10% ice-cold trichloroacetic acid, and the reaction mixture was placed on ice for 10 min. The samples were passed through cellulose nitrate filters. The filters were dissolved in scintillation fluid, and the radioactivity was measured.

Primer extension. One microgram of 16S rRNA extracted from the 30S subunits methylated in vitro was hybridized with 50 pmol of a primer (5′-biotin-CCA ACC GCA GGT TCC CCT ACG G-3′) complementary to nucleotides 1530 to 1509 at 65°C for 10 min. The elongation was performed with Transcriptor reverse transcriptase (Roche) at 43°C for 1 h. The cDNA transcripts were analyzed on an 8% polyacrylamide gel containing 8 M urea.

HPLC assay of methylated adenine residue. 16S rRNA was extracted from the 30S subunits of *E. coli*. Sixty micrograms of extracted 16S rRNA was digested with nuclease P1 (3 U; Wako) and alkaline phosphatase (0.08 U; Takara) in 120 μl of a reaction mixture containing 25 mM HEPES-KOH (pH 7.5) at 37°C for 6 h. The resulting mixture was analyzed by high-performance liquid chromatog-

TABLE 2. Antimicrobial susceptibilities of parental strain, transconjugant, and transformant

Aminoglycoside	MIC (μg/ml)				
	E. coli ARS3(pARS3)	*E. coli* CSH-2(pARS3)	*E. coli* CSH-2	*E. coli* JM109(pMCL-BE)	*E. coli* JM109(pMCL210)
4,6-Disubstituted 2-deoxystreptamines					
Kanamycin group					
Arbekacin	64	4	0.13	4	0.25
Amikacin	256	16	0.13	32	0.5
Dibekacin	>256	128	0.13	128	0.25
Kanamycin	>256	>256	0.25	>256	0.5
Tobramycin	>256	128	≤0.06	128	0.13
Gentamicin group					
Gentamicin	>256	128	≤0.06	16	0.13
Isepamicin	>256	64	0.13	128	0.13
Netilmicin	>256	>256	≤0.06	>256	0.13
Sisomicin	>256	>256	≤0.06	256	0.13
4,5-Disubstituted 2-deoxystreptamines					
Lividomycin A	256	16	0.5	32	2
Neomycin	>256	64	0.13	64	0.25
Paromomycin	64	4	0.5	4	1
Ribostamycin	>256	>256	0.25	>256	0.5
Other aminoglycosides					
Apramycin	>256	>256	1	>256	2
Hygromycin B	128	16	16	32	32
Streptomycin	128	32	1	1	1
Spectinomycin	>256	>256	16	16	16

raphy (HPLC) with an HRC-ODS column (4.6 mm [inner diameter] by 250 mm; Shimadzu). The solvent system consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and 30% acetonitrile (solvent B) and was used as follows: 0% to 50% solvent B from 0 to 100 min, 50% to 99% solvent B from 100 to 110 min, and 99% solvent B from 110 to 130 min, with an effluent rate of 600 μl/min at 30°C.

Aminoglycoside binding to 30S subunit. Sixty picomoles of the wild-type or the modified 30S subunits was incubated in 100 μl of dimethylsulfate (DMS) buffer (80 mM sodium cacodylate, pH 7.2; 100 mM NH_4Cl; 20 mM $MgCl_2$; 1 mM dithiothreitol; 0.5 mM EDTA) at 42°C for 10 min. Addition of aminoglycosides (final concentration range, 1 μM to 1,000 μM) was followed by incubation at 37°C for 30 min and then on ice for 10 min. DMS (2 μl, 1:6 in ethanol) was added, and the mixture was incubated at 37°C for 10 min. The reaction was quenched by adding 25 μl of stop buffer (1.5 M sodium acetate, 1 M 2-mercaptoethanol). After ethanol precipitation, modified rRNA was obtained by extraction with phenol three times and chloroform twice. Reduction with sodium borohydride and aniline-induced strand scission were performed as described previously (27). A primer extension analysis was carried out as described above.

Nucleotide sequence accession number. The open reading frame of *npmA* was deposited in the EMBL and GenBank databases through the DDBJ database and has been assigned accession number AB261016.

RESULTS

Characteristics of *E. coli* strain ARS3. The MICs of various aminoglycosides for parent *E. coli* strain ARS3 are shown in Table 2. This strain demonstrated resistance to structurally diverse aminoglycosides. The panaminoglycoside-resistant phenotype of strain ARS3 was successfully transferred to the *E. coli* CSH-2 recipient strain at a frequency of 2×10^{-8} per donor by conjugation. The transconjugant acquired a transferable plasmid (pARS3), which was estimated to be about 115 kb in size by summation of the sizes of the EcoRI digestion products, and demonstrated resistance to various aminoglycosides (Table 2).

Genetic determinant of aminoglycoside resistance on transferable plasmid pARS3. A cloning experiment was performed to confirm the genetic aminoglycoside resistance determinant, which is mediated by pARS3. As a result, one recombinant plasmid (plasmid pMCL-H) was obtained by selection with apramycin and chloramphenicol, and both strands of the 3,946-bp HindIII insert were entirely sequenced. The schematic organization of probable genes found in the cloned fragment is shown in Fig. 1. To identify the gene responsible for apramycin resistance, Tn*5* (Tet^r) insertion mutants of clone pMCL-H were generated. A total of 12 insertion mutants were obtained (Fig. 1), and 3 of them that carried a Tn*5* insertion in *orf6* lost apramycin resistance. Recombinant plasmid pMCL-BE, which contained only *orf6* and its putative promoter region, showed apramycin resistance, as was seen in clone pMCL-H.

The deduced amino acid sequences of ORF6 exhibited low-level identities (<31%) to the chromosomally encoded 16S rRNA methyltransferases KamA, KamB, KamB2, KamB3, KamC, and Amr of actinomycetes that produce aminoglycosides. Several studies already revealed that some of these 16S rRNA methyltransferases of actinomycetes methylate the N-1 position of nucleotide A1408 in 16S rRNA and confer intrinsic aminoglycoside resistance to bacteria (3, 22, 43). Therefore, it is probable that the product of *orf6*, NpmA, has 16S rRNA methyltransferase activity and confers panresistance to aminoglycosides in a manner similar to that seen in aminoglycoside-producing actinomycetes. NpmA has a conserved residue (D) and the consensus GXGXG motif, which is considered the hallmark *S*-adenosylmethionine (SAM)-binding site of Ross-

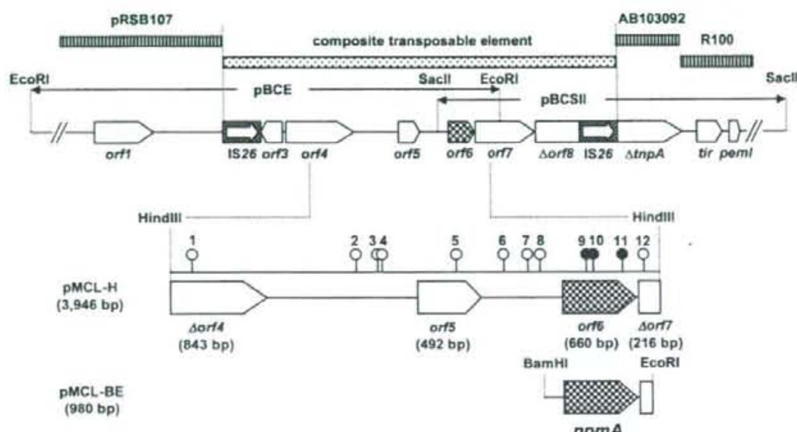

FIG. 1. Schematic presentation of open reading frames (ORFs) in the cloned fragment conferring aminoglycoside resistance. Open reading frames are shown as arrows indicating the transcription orientation. The positions where Tn*5* was inserted are indicated by open and closed circles. Mutants with Tn*5* insertions shown by open circles demonstrated aminoglycoside resistance, while those indicated by closed circles did not.

man fold SAM-dependent methyltransferases (Fig. 2) (26). SAM is often used as the source of the methyl group in methyltransferase reactions in various organisms (26).

Genetic environments of *npmA*. The structures of the flanking regions of *npmA* were determined (Fig. 1). The genes for *orf7* (which encodes a probable ABC transporter substrate binding protein) and *orf8* (which encodes a truncated mobilization protein) were located at the 3′-end region of *npmA*. Three open reading frames, *orf3* (which encodes a hypothetical protein), *orf4* (which encodes a possible replication protein), and *orf5* (which encodes a hypothetical protein), existed at the 5′-end region of *npmA*. The 9.1-kb region containing *orf3* to *orf8* was flanked by two IS*26* elements in direct orientation and composed a transposable element (12). The sequences around the 9.1-kb transposable element have significant sequence similarities to the sequences of a part of various multidrug resistance plasmids deposited in the EMBL/GenBank/DDBJ databases.

FIG. 2. Alignment of deduced amino acid sequences of NpmA with those of KamA and KamB. Chromosomal 16S rRNA methyltransferases (KamA and KamB) were found in aminoglycoside-producing actinomycetes (24, 32). Identical amino acids in all proteins are highlighted with a dark background. Physicochemically similar amino acids are highlighted with a gray background.

Antibiotic susceptibilities. The MICs of the aminoglycosides for the NpmA-producing *E. coli* transformant are shown in Table 2. The introduction of *npmA*-carrying plasmid pMCL-BE conferred resistance to both 4,6-disubstituted 2-deoxystreptamines, consisting of the kanamycin and gentamicin groups, and 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin. In addition, NpmA augmented the MIC of apramycin, whose structure is far different from those of the 4,6- and 4,5-disubstituted 2-deoxystreptamines. On the other hand, NpmA did not confer resistance to the non-A-site binders streptomycin and spectinomycin. On the whole, NpmA could confer resistance to various aminoglycosides which bind to the A site of the decoding region in 16S rRNA.

N-terminal sequence of NpmA. As shown in Fig. 2, the exact locations of the N termini of A1408 methyltransferases are still controversial. For example, the N-terminal position of the Kam family of proteins, including KamB and KamC, was previously reported to be position M61, shown in Fig. 2 (22). This fact, however, indicates the lack of a SAM-binding motif, which plays a crucial role in methyltransferase activity among the mature Kam family of enzymes. Most recently, Koscinski et al. reanalyzed the amended amino acid sequences of a Kam family protein and revealed that the SAM-binding motif is perfectly conserved in the missing N-terminal sequences of the Kam family of proteins (24). In this study, in order to determine the exact position of the N terminus in NpmA experimentally, the recombinant NpmA protein was purified from *E. coli* cells harboring pMCL-BH and was subjected to Edman protein sequencing. The N terminus of NpmA was exactly determined to be MLILK (Fig. 2), although TTG is uncommon as a bacterial initiation codon.

Methylation of 30S subunits by NpmA. *E. coli* BL21 (DE3)pLysS and the pCold-IV expression vector were used for

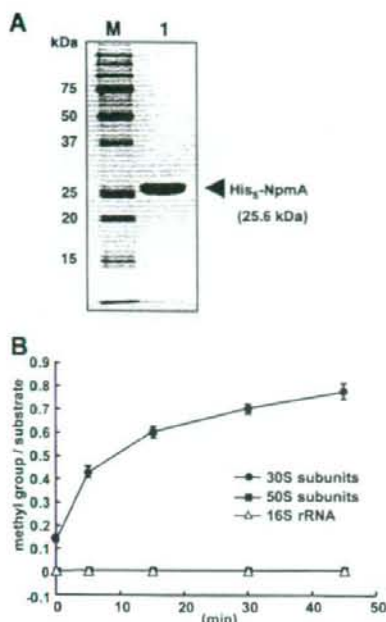

FIG. 3. Purification of NpmA and methylation assays. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified His_5-NpmA protein. Lanes: M, protein size marker; 1, purified His_5-NpmA. (B) Methyl acceptor activities of 30S subunits, 50S subunits, and naked 16S rRNA determined with [^{3}H]SAM and His_5-NpmA. The square symbols for the 50S subunits are hidden behind the triangle symbols for 16S rRNA.

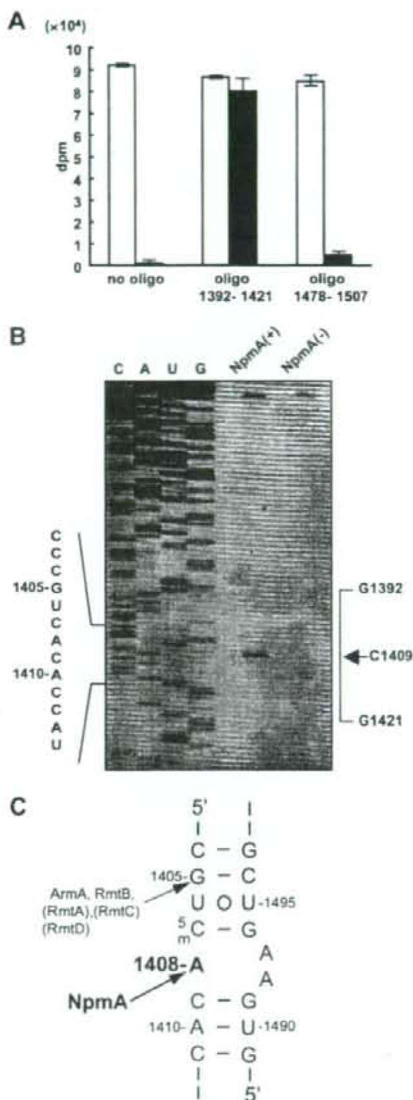

FIG. 4. Nuclease protection assay and primer extension analysis. (A) Nuclease protection assay with [^{3}H]-methyl-labeled 16S rRNA and DNA oligonucleotides (oligo) complementary to the regions from positions 1392 to 1421 or positions 1478 to 1507. The values are the averages of three measurements. Error bars indicate standard deviations. Open bars, undigested; solid bars, digested with RNase T_1. (B) Primer extension analysis of methylated 16S rRNA [NpmA(+)] and wild-type 16S rRNA [NpmA(−)]. Dideoxy sequencing lanes (C, A, U, and G) were generated with the amplified PCR products of *E. coli* 16S rRNA gene as the template. Primer extension termination at position C1409 is indicated by an arrow. (C) Methylation sites in the decoding region in 16S rRNA from *E. coli*. The exact methylation site by ArmA and RmtB was confirmed at G1405, but that by RmtA, RmtC, and RmtD has not yet been confirmed.

the overexpression and purification of NpmA. *E. coli* BL21 (DE3)pLysS carrying pCold-IV is susceptible to apramycin (MIC, 3.9 μg/ml), while *E. coli* BL21(DE3)pLysS carrying pCold-NpmA exhibited a very high level of resistance to apramycin (MIC, >1,000 μg/ml) in the microdilution susceptibility test. This result indicated that the histidine-tagged NpmA (His_5-NpmA) still has methylation activity and is responsible for apramycin resistance in *E. coli* BL21(DE3)pLysS. An optimized culture condition yielded 8 mg of purified protein per 1 liter of bacterial culture, and the purified enzyme gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining (Fig. 3A).

The methylation assay was performed with three different substrates, 50S subunits, 30S subunits, and 16S rRNA dissociated from the 30S subunits, to explore the activity of NpmA and its substrate specificity. NpmA was able to incorporate about 0.8 pmol of methyl groups into 1 pmol of 30S subunits after 45 min of incubation, whereas no significant incorporation of methyl groups into each 50S subunit or the dissociated 16S rRNA molecule was detected under the same experimental conditions (Fig. 3B). These results demonstrated that NpmA has optimal methyltransferase activity toward the properly assembled 30S subunit.

RNase protection assay. As described above, the in silico analysis indicated that NpmA exhibits amino acid sequence similarity to various chromosomally encoded A1408 16S rRNA

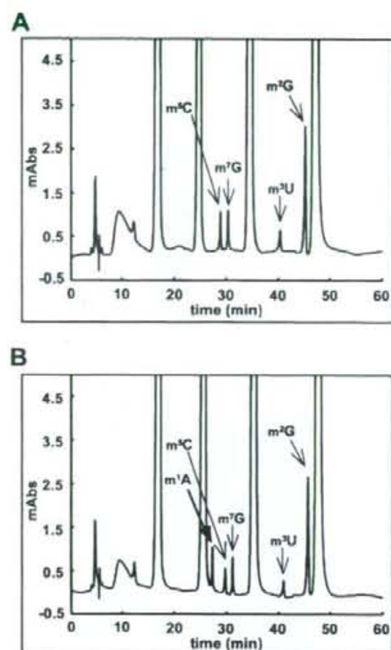

FIG. 5. HPLC analysis of methylated adenine residue. Purified 16S rRNA was completely digested with nuclease P1 and alkaline phosphatase and subjected to HPLC. (A) Wild-type 16S rRNA; (B) 16S rRNA methylated by NpmA. The m^1A peak indicates the formation of a methylated adenosine residue at 1408. mAb, milli-absorbance units.

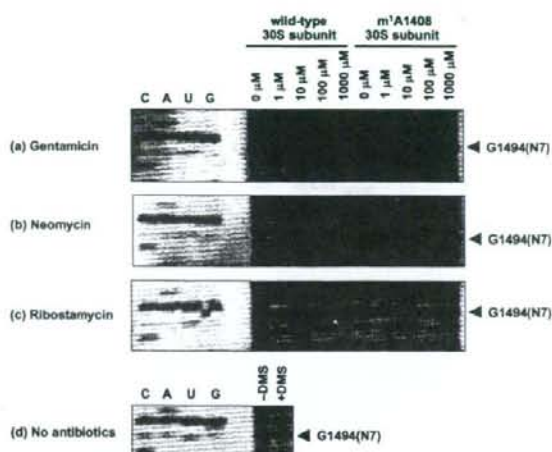

FIG. 6. Footprinting for confirmation of aminoglycoside binding to 30S subunits. The gentamicin, neomycin, and ribostamycin footprints are indicated at the position of G1494 (N-7) in the 16S rRNA. Dideoxy sequencing lanes (lanes C, A, U, and G) were generated with amplified PCR products on the *E. coli* 16S rRNA gene as the template. Each reaction was performed in the presence of 0, 1, 10, 100, and 1,000 µM aminoglycoside.

methyltransferases of aminoglycoside-producing actinomycetes. This suggested that NpmA would also modify the same position within 16S rRNA, as reported previously (3, 22, 43). To determine the exact position of methylated nucleotide, a hybridization protection study was first carried out with deoxynucleotides that were complementary to a part of the 16S rRNA sequence. Two oligomers from positions 1392 to 1421 and positions 1478 to 1507 were prepared to span the aminoglycoside-binding A-site region within the 16S rRNA. The hybridization with the oligomer from positions 1392 to 1421 served to keep the radioactivity of [^{3}H]methyl-labeled 16S rRNA after RNase T_1 digestion, while the oligomer from positions 1478 to 1507 was ineffective in protecting against RNase T_1 digestion (Fig. 4A). This finding indicated that the position of the methylated nucleotide is located within the region from residue 1392 to residue 1421 in the 16S rRNA.

Primer extension. Methylated 16S rRNA, prepared from 30S subunits which were incubated with His_5-NpmA in the presence of the methyl donor SAM, was used as the template RNA in reverse transcriptase extension. The extension terminated at position C1409, indicating that methylation surely occurs at position A1408 (Fig. 4B). In contrast, no termination signal was observed at the same position when unmethylated 16S rRNA was used for the reverse transcription experiment (Fig. 4B).

HPLC assay of methylated adenine residue. We determined the type of detailed modification by HPLC. When wild-type 16S rRNA was treated with nuclease P1 plus alkaline phosphatase, there was no peak corresponding to 1-methyladenosine (m^1A), due to the lack of an innate m^1A nucleoside in the 16S rRNA of a K-12-derived *E. coli* strain (Fig. 5A). On the other hand, the m^1A peak was clearly observed when the 16S rRNA methylated by NpmA was analyzed (Fig. 5B). These results clearly demonstrate that NpmA actually methylates the N-1 position of adenosine. Thus, NpmA is an adenine N-1 methyltransferase. Each peak corresponding to m^5C, m^7G, m^3U, and m^2G was detected with almost equal intensity between the wild-type and the methylated 16S rRNAs.

Binding of aminoglycosides to 30S subunits assayed by footprinting. The interaction between the 30S subunit and aminoglycosides was monitored by determining the changes in the chemical modification pattern of 16S rRNA by using DMS. The RNA footprints at G1494 were analyzed by primer extension with reverse transcriptase (Fig. 6). The apparent protection of G1494 was observed when wild-type 30S subunits were treated with gentamicin, neomycin, and ribostamycin at concentrations of 100 µM and 1,000 µM, indicating the certain binding of aminoglycosides to the 30S subunits. On the other hand, no decrease in the signal on G1494 was observed at 100 µM and 1,000 µM when m^1A1408-methylated 30S subunits were used under the same reaction conditions, indicating the interruption of aminoglycoside binding by methylation at A1408. Although a slight increase in the signal at position G1494 in the m^1A1408-methylated 30S subunits was observed in a gentamicin concentration-dependent manner, the precise reason for the phenomenon remains uncertain from the findings of the present study.

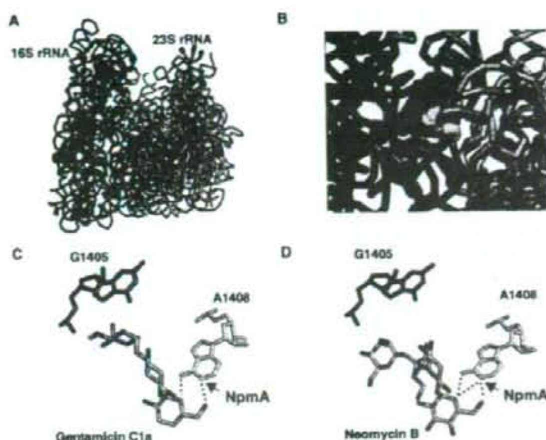

FIG. 7. Predicted interaction between aminoglycosides and 16S rRNA in the 30S ribosomal subunit. (A) Complex structure of 16S rRNA (black) and 23S rRNA (orange) in the 70S ribosome from *E. coli* (PDB codes 2AVY and 2AW4) (41). The positions of G1405 (magenta) and A1408 (cyan) are indicated with dots. (B) Enlargement of the region surrounding G1405 and A1408 at the same angle used for panel A. (C and D) Three-dimensional model from the crystal structure of complexes between aminoglycosides (C; gentamicin C1a, PDB code 2ET3) (D; neomycin B, PDB code 2ET4) and nucleotides G1405 and A1408 in the decoding A site (15). The dashed lines indicate probable hydrogen bonds. The figures were rendered with the PyMol program.

DISCUSSION

A careful review of the recent literature enabled us to understand the binding mode between aminoglycosides and the target site through the resolution structure investigated by X-ray crystallography and nuclear magnetic resonance imaging (6, 13–15, 29, 38, 39, 40, 54). Basically, aminoglycosides such as 4,5- and 4,6-disubstituted 2-deoxystreptamines form specific hydrogen bonds with the nucleotides in decoding region A-site within 16S rRNA (Fig. 4C and Fig. 7C and D). Thus, mutations and modifications in the key nucleotides that perturb the hydrogen bond result in the loss of binding between aminoglycosides and 16S rRNA and lead to resistance to aminoglycosides in bacteria. The production of methyltransferase, which converts guanosine to 7-methylguanosine (m^7G) at position 1405 in 16S rRNA, is one representative mechanism of aminoglycoside resistance in the manner described above. The genes encoding guanine N-7 methyltransferases, which methylate position G1405, have been found on the chromosomes of aminoglycoside-producing actinomycetes and on the plasmids of various pathogenic gram-negative pathogens isolated in both clinical and veterinary settings (7, 10, 11, 17, 20, 34, 48, 53). Furthermore, it is well known that adenine N-1 methyltransferases which modify position A1408 belong to another group of 16S rRNA methyltransferases that confer resistance to multiple aminoglycosides. However, this has so far been found exclusively on the chromosomes of aminoglycoside-producing actinomycetes. The present study is the first to describe the emergence of plasmid-mediated adenine N-1 methyltransferase, which confers panaminoglycoside resistance among pathogenic bacteria.

Position A1408 plays a crucial role in the binding of 4,6- and 4,5-disubstituted 2-deoxystreptamines, because the puckered sugar ring I of these agents is inserted into the A-site helix to form hydrogen bonds to Watson-Crick sites N-1 and N-6 of the universally conserved A1408 among bacteria (Fig. 7C and D) (6, 15, 46). Thus, alternation of A1408 to G leads to a repulsive interaction with the 6′-NH_3 group at ring I of 2-deoxystreptamines, while 2-deoxystreptamines carrying a 6′-OH group can still interact with 16S rRNA by accepting a hydrogen bond from the N-1 and N-2 positions of G1408 (35, 46). Likewise, methylation at the N-1 position of A1408 by NpmA will disturb the formation of the hydrogen bond toward the N-6′ or O-6′ of ring I of aminoglycosides, and this would, in turn, reduce the binding affinities of aminoglycosides to the target. In fact, the NpmA-producing strains demonstrate resistance to various aminoglycosides that combine the N-1 of A1408 through ring I. On the other hand, NpmA production did not confer resistance to non-A-site binders, such as streptomycin and spectinomycin. The results of susceptibility testing are well consistent with their footprinting patterns, with protection against DMS modification at G1494.

Additionally, it is speculated that m^1A1408 methylation will fundamentally affect the formation of the A1408 · A1493 base pair pocket, which is essential for aminoglycoside binding. However, a dynamic conformational change in RNA structure might impair a number of ribosomal innate functions, including decoding, aminoacyl transfer, and translocation. Actually, A1493 participates in codon-anticodon recognition during the tRNA selection step and involves a conformational change from a "tucked-in" form to a "flipped-out" form (28, 31). Although the effect of m^1A1408 methylation on the innate rRNA function remains uncertain, it seems unlikely that m^1A1408 methylation would be a serious disadvantage for bacterial proliferation, because there is no significant difference in the doubling times between NpmA-producing *E. coli* and wild-type *E. coli* strains under culture conditions with both rich and minimal medium compositions (data not shown). A growth competition assay may be required to elucidate the accurate biological cost induced by m^1A1408 methylation in bacteria.

The methylation reaction by an innate C1407 16S rRNA methyltransferase, YebU, of *E. coli* is specific for the 30S subunits and not for the naked 16S rRNA molecule (1). Docking of YebU onto the 30S subunit revealed several contacts between the methyltransferase domain of YebU and ribosomal protein S12 as well as 16S rRNA (21). Hallberg et al. concluded that interactions of YebU with ribosomal protein would explain the substrate specificity seen in YebU (21). Obviously, the accessibility of YebU to the 30S subunit would be supported by the fact that the C1407 position is exposed in the 30S subunit as well as in the 16S rRNA. As expected, the substrate specificity of NpmA is similar to that of YebU (Fig. 3B), and the explanation for this specificity might partially be the same reason suggested for YebU (Fig. 7A and B). A similar substrate specificity was also observed in a part of the aminoglycoside-resistant G1405 16S rRNA methyltransferase group (27). Methylation at an exposed position such as A1408 would occur in the late stage, during the assembly of the 30S subunit.

The G+C content of A1408 16S rRNA methyltransferase genes from aminoglycoside-producing actinomycetes is greater than 70%, whereas that of *npmA* is 34%. This discrepancy would make it unlikely that the origin of *npmA* is aminoglycoside producers with high G+C contents. A similar discrepancy was also observed in the case of the G1405 16S rRNA methyltransferases of actinomycetes and pathogenic bacteria. Liou et al. indicated that aminoglycoside producers with low G+C contents, such as *Bacillus circulans*, which naturally produces butirosin, might be the candidate sources of plasmid-mediated 16S rRNA methyltransferases (27). Although questions remain as to the presence of a 16S rRNA methyltransferase that confers aminoglycoside resistance in the genus *Bacillus*, the gene products of a putative ABC transporter substrate binding protein (*orf7*) and a mobilization protein (*orf8*) located at the 3' end of *npmA* certainly have relatively low levels of identity to those of *Bacillus* spp. The detailed characterization of 16S rRNA methyltransferases in aminoglycoside-producing *Bacillus* spp. demonstrating low G+C contents might provide clues to the identification of the origin of plasmid-mediated 16S rRNA methyltransferases, including *npmA*.

In conclusion, to our knowledge this is the first time that a novel plasmid-mediated m^1A1408 16S rRNA methyltransferase, NpmA, was identified in a panaminoglycoside-resistant *E. coli* clinical isolate. Indeed, methylation at A964 (pactamycin resistance) (2), G1405 (kanamycin-gentamicin resistance), and A1408 (kanamycin-apramycin resistance) and the loss of methylation at G527 (streptomycin resistance) (33), C1409 (capreomycin resistance) (23), and A1518-A1519 (kasugamycin resistance) (45) have been reported so far to be mechanisms of resistance to 30S subunit-targeting drugs in bacteria. However, these mechanisms have not been fully understood, especially in pathogenic bacteria that tend to be continuously or intermittently exposed to various aminoglycosides in both clinical and livestock farming environments. Further study is warranted to clarify the molecular mechanisms underlying the panaminoglycoside resistance that has been acquired by pathogenic bacteria.

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REFERENCES

1. **Andersen, N. M., and S. Douthwaite.** 2006. YebU is a m^5C methyltransferase specific for 16S rRNA nucleotide 1407. J. Mol. Biol. **359:**777–786.
2. **Ballesta, J. P., and E. Cundliffe.** 1991. Site-specific methylation of 16S rRNA caused by *pct*, a pactamycin resistance determinant from the producing organism, *Streptomyces pactum*. J. Bacteriol. **173:**7213–7218.
3. **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.
4. **Bogaerts, P., M. Galimand, C. Bauraing, A. Deplano, R. Vanhoof, R. De Mendonca, H. Rodriguez-Villalobos, M. Struelens, and Y. Glupczynski.** 2007. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. J. Antimicrob. Chemother. **59:**459–464.
5. **Bryan, L. E.** 1988. General mechanisms of resistance to antibiotics. J. Antimicrob. Chemother. **22**(Suppl. A):1–15.
6. **Carter, A. P., W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly, and V. Ramakrishnan.** 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature **407:**340–348.
7. **Chen, L., Z. L. Chen, J. H. Liu, Z. L. Zeng, J. Y. Ma, and H. X. Jiang.** 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. J. Antimicrob. Chemother. **59:**880–885.
8. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 7th ed. Document M7-A6. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Cundliffe, E.** 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. **43:**207–233.
10. **Doi, Y., D. de Oliveira Garcia, J. Adams, and D. L. Paterson.** 2007. Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrob. Agents Chemother. **51:**852–856.
11. **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.
12. **Doroshenko, V. G., and V. A. Livshits.** 2004. Structure and mode of transposition of Tn*2555* carrying sucrose utilization genes. FEMS Microbiol. Lett. **233:**353–359.
13. **Fourmy, D., M. I. Recht, and J. D. Puglisi.** 1998. Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16S rRNA. J. Mol. Biol. **277:**347–362.
14. **Fourmy, D., S. Yoshizawa, and J. D. Puglisi.** 1998. Paromomycin binding induces a local conformational change in the A-site of 16S rRNA. J. Mol. Biol. **277:**333–345.
15. **Francois, B., R. J. Russell, J. B. Murray, F. Aboul-ela, B. Masquida, Q. Vicens, and E. Westhof.** 2005. Crystal structures of complexes between aminoglycosides and decoding A site oligonucleotides: role of the number of rings and positive charges in the specific binding leading to miscoding. Nucleic Acids Res. **33:**5677–5690.
16. **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.
17. **Galimand, M., S. Sabtcheva, P. Courvalin, and T. Lambert.** 2005. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrob. Agents Chemother. **49:**2949–2953.
18. **Gilbert, D. N., R. C. Moellering, Jr., G. M. Eliopoulos, and M. A. Sande.** 2004. The Sanford guide to antimicrobial therapy, 34th ed. Antimicrobial Therapy, Inc., Hyde Park, VT.
19. **Gonzalez-Zorn, B., A. Catalan, J. A. Escudero, L. Dominguez, T. Teshager, C. Porrero, and M. A. Moreno.** 2005. Genetic basis for dissemination of *armA*. J. Antimicrob. Chemother. **56:**583–585.
20. **Gonzalez-Zorn, B., T. Teshager, M. Casas, M. C. Porrero, M. A. Moreno, P. Courvalin, and L. Dominguez.** 2005. *armA* and aminoglycoside resistance in *Escherichia coli*. Emerg. Infect. Dis. **11:**954–956.
21. **Hallberg, B. M., U. B. Ericsson, K. A. Johnson, N. M. Andersen, S. Douthwaite, P. Nordlund, A. E. Beuscher IV, and H. Erlandsen.** 2006. The structure of the RNA m^5C methyltransferase YebU from *Escherichia coli* reveals a C-terminal RNA-recruiting PUA domain. J. Mol. Biol. **360:**774–787.
22. **Holmes, D. J., D. Drocourt, G. Tiraby, and E. Cundliffe.** 1991. Cloning of an aminoglycoside-resistance-encoding gene, *kamC*, from *Saccharopolyspora hirsuta*: comparison with *kamB* from *Streptomyces tenebrarius*. Gene **102:**19–26.
23. **Johansen, S. K., C. E. Maus, B. B. Plikaytis, and S. Douthwaite.** 2006. Capreomycin binds across the ribosomal subunit interface using *tlyA*-encoded 2'-O-methylations in 16S and 23S rRNAs. Mol. Cell **23:**173–182.
24. **Koscinski, L., M. Feder, and J. M. Bujnicki.** 2007. Identification of a missing sequence and functionally important residues of 16S rRNA:m^1A1408 methyltransferase KamB that causes bacterial resistance to aminoglycoside antibiotics. Cell Cycle **6:**1268–1271.
25. **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.
26. **Kozbial, P. Z., and A. R. Mushegian.** 2005. Natural history of *S*-adenosylmethionine-binding proteins. BMC Struct. Biol. **5:**19.
27. **Liou, G. F., S. Yoshizawa, P. Courvalin, and M. Galimand.** 2006. Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. J. Mol. Biol. **359:**358–364.
28. **Meroueh, S. O., and S. Mobashery.** 2007. Conformational transition in the aminoacyl t-RNA site of the bacterial ribosome both in the presence and absence of an aminoglycoside antibiotic. Chem. Biol. Drug Des. **69:**291–297.
29. **Murray, J. B., S. O. Meroueh, R. J. Russell, G. Lentzen, J. Haddad, and S. Mobashery.** 2006. Interactions of designer antibiotics and the bacterial ribosomal aminoacyl-tRNA site. Chem. Biol. **13:**129–138.
30. **Nakano, Y., Y. Yoshida, Y. Yamashita, and T. Koga.** 1995. Construction of a series of pACYC-derived plasmid vectors. Gene **162:**157–158.
31. **Ogle, J. M., F. V. Murphy, M. J. Tarry, and V. Ramakrishnan.** 2002. Selection of tRNA by the ribosome requires a transition from an open to a closed form. Cell **111:**721–732.
32. **Ohta, T., and M. Hasegawa.** 1993. Analysis of the nucleotide sequence of

fmrT encoding the self-defense gene of the istamycin producer, *Streptomyces tenjimariensis* ATCC 31602; comparison with the sequences of *kamB* of *Streptomyces tenebrarius* NCIB 11028 and *kamC* of *Saccharopolyspora hirsuta* CL102. J. Antibiot. **46:**511–517.

33. **Okamoto, S., A. Tamaru, C. Nakajima, K. Nishimura, Y. Tanaka, S. Tokuyama, Y. Suzuki, and K. Ochi.** 2007. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol. Microbiol. **63:**1096–1106.
34. **Park, Y. J., S. Lee, J. K. Yu, G. J. Woo, K. Lee, and Y. Arakawa.** 2006. Co-production of 16S rRNA methylases and extended-spectrum β-lactamases in AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii* and *Serratia marcescens* in Korea. J. Antimicrob. Chemother. **58:**907–908.
35. **Pfister, P., S. Hobbie, Q. Vicens, E. C. Bottger, and E. Westhof.** 2003. The molecular basis for A-site mutations conferring aminoglycoside resistance: relationship between ribosomal susceptibility and X-ray crystal structures. Chembiochem **4:**1078–1088.
36. **Poole, K.** 2005. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**479–487.
37. **Prescott, J. F., J. D. Baggot, and R. D. Walkeer.** 2000. Antimicrobial therapy in veterinary medicine, 3rd ed. Iowa State University Press, Ames.
38. **Recht, M. I., S. Douthwaite, K. D. Dahlquist, and J. D. Puglisi.** 1999. Effect of mutations in the A site of 16S rRNA on aminoglycoside antibiotic-ribosome interaction. J. Mol. Biol. **286:**33–43.
39. **Recht, M. I., D. Fourmy, S. C. Blanchard, K. D. Dahlquist, and J. D. Puglisi.** 1996. RNA sequence determinants for aminoglycoside binding to an A-site rRNA model oligonucleotide. J. Mol. Biol. **262:**421–436.
40. **Russell, R. J., J. B. Murray, G. Lentzen, J. Haddad, and S. Mobashery.** 2003. The complex of a designer antibiotic with a model aminoacyl site of the 30S ribosomal subunit revealed by X-ray crystallography. J. Am. Chem. Soc. **125:**3410–3411.
41. **Schuwirth, B. S., M. A. Borovinskaya, C. W. Hau, W. Zhang, A. Vila-Sanjurjo, J. M. Holton, and J. H. Cate.** 2005. Structures of the bacterial ribosome at 3.5 Å resolution. Science **310:**827–834.
42. **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.
43. **Skeggs, P. A., D. J. Holmes, and E. Cundliffe.** 1987. Cloning of aminoglycoside-resistance determinants from *Streptomyces tenebrarius* and comparison with related genes from other actinomycetes. J. Gen. Microbiol. **133:**915–923.
44. **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.
45. **van Buul, C. P., and P. H. van Knippenberg.** 1985. Nucleotide sequence of the *ksgA* gene of *Escherichia coli*: comparison of methyltransferases effecting dimethylation of adenosine in ribosomal RNA. Gene **38:**65–72.
46. **Vicens, Q., and E. Westhof.** 2001. Crystal structure of paromomycin docked into the eubacterial ribosomal decoding A site. Structure **9:**647–658.
47. **Wachino, J., K. Yamane, K. Kimura, N. Shibata, S. Suzuki, Y. Ike, and Y. Arakawa.** 2006. Mode of transposition and expression of 16S rRNA methyltransferase gene *rmtC* accompanied by IS*Ecp1*. Antimicrob. Agents Chemother. **50:**3212–3215.
48. **Wachino, J., K. Yamane, K. Shibayama, H. Kurokawa, N. Shibata, S. Suzuki, Y. Doi, K. Kimura, Y. Ike, and Y. Arakawa.** 2006. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob. Agents Chemother. **50:**178–184.
49. **Yamane, K., Y. Doi, K. Yokoyama, T. Yagi, H. Kurokawa, N. Shibata, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Genetic environments of the *rmtA* gene in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **48:**2069–2074.
50. **Yamane, K., J. Wachino, Y. Doi, H. Kurokawa, and Y. Arakawa.** 2005. Global spread of multiple aminoglycoside resistance genes. Emerg. Infect. Dis. **11:**951–953.
51. **Yamane, K., J. Wachino, S. Suzuki, H. Kato, K. Shibayama, K. Kimura, K. Kumiko, I. Satoshi, Y. Ozawa, K. Toshifumi, and Y. Arakawa.** 2007. 16S rRNA methylase-producing, gram-negative pathogens, Japan. Emerg. Infect. Dis. **13:**642–646.
52. **Yan, J. J., J. J. Wu, W. C. Ko, S. H. Tsai, C. L. Chuang, H. M. Wu, Y. J. Lu, and J. D. Li.** 2004. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. J. Antimicrob. Chemother. **54:**1007–1012.
53. **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.
54. **Yoshizawa, S., D. Fourmy, and J. D. Puglisi.** 1998. Structural origins of gentamicin antibiotic action. EMBO J. **17:**6437–6448.

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New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate▽

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Plasmid-mediated Qnr and AAC(6′)-Ib-cr have been recognized as new molecular mechanisms affecting fluoroquinolone (FQ) resistance. C316, an *Escherichia coli* strain demonstrating resistance to various FQs, was isolated in Japan. Resistance to FQs was augmented in an *E. coli* CSH2 transconjugant, but PCR failed to detect *qnr* genes, suggesting the presence of novel plasmid-mediated FQ resistance mechanisms. Susceptibility tests, DNA manipulation, and analyses of the gene and its product were performed to characterize the genetic determinant. A novel FQ-resistant gene, *qepA*, was identified in a plasmid, pHPA, of *E. coli* C316, and both *qepA* and *rmtB* genes were mediated by a probable transposable element flanked by two copies of IS*26*. Levels of resistance to norfloxacin, ciprofloxacin, and enrofloxacin were significantly elevated in *E. coli* transformants harboring *qepA* under AcrB-TolC-deficient conditions. QepA showed considerable similarities to transporters belonging to the 14-transmembrane-segment family of environmental actinomycetes. The effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on accumulation of norfloxacin was assayed in a *qepA*-harboring *E. coli* transformant. The intracellular accumulation of norfloxacin was decreased in a *qepA*-expressing *E. coli* transformant, but this phenomenon was canceled by CCCP. The augmented FQ resistance level acquired by the probable intergeneric transfer of a gene encoding a major facilitator superfamily-type efflux pump from some environmental microbes to *E. coli* was first identified. Surveillance of the *qepA*-harboring clinical isolates should be encouraged to minimize further dissemination of the kind of plasmid-dependent FQ resistance determinants among pathogenic microbes.

Fluoroquinolones (FQs) are synthetic chemical agents and among the most commonly prescribed antimicrobials because of their broad-spectrum antimicrobial activity. Extensive clinical and agricultural use of FQs has led to high rates of resistance to these agents among pathogenic microbes (6, 9). The most common mechanism for resistance to FQs among pathogenic microbes is the mutation of chromosomal genes encoding DNA gyrase and/or topoisomerase IV (12, 13). Changes in the expression of efflux pumps and porin proteins are also a common FQ resistance mechanism in bacteria (1), but no plasmid-mediated FQ efflux pump has been documented to date. Four chromosome-dependent efflux systems responsible for FQ resistance have so far been reported, e.g., the resistance nodulation division family, AcrAB-TolC in *Escherichia coli* (7, 28) and MexAB-OprM in *Pseudomonas aeruginosa* (24); the major facilitator superfamily (MFS), NorA of *Staphylococcus aureus* (34); the multidrug and toxic compound extrusion family, NorM of *Vibrio parahaemolyticus* (19); and the ATP-binding cassette family, VcaM of non-O1 *Vibrio cholerae* (14).

Two molecular groups have been identified as plasmid-mediated FQ resistance mechanisms to date. Qnr peptides, QnrA (30), QnrB (15), and QnrS (11), were identified from *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *E. coli*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter cloacae*, *Shigella flexneri*, and a *Salmonella* sp. (8, 16, 21, 31), and these peptides interfere with DNA gyrase by mimicking DNA (18). AAC(6′)-Ib-cr was also found to possess an N-acetylating activity of the piperazinyl substituent of ciprofloxacin and norfloxacin (26), and this enzyme has been reported to be geographically widespread (22, 25).

In the present study, we identified a novel FQ resistance mechanism, QepA, as a plasmid-mediated efflux pump found in an *E. coli* clinical isolate from Japan.

(These findings have been reported at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, 2006, San Francisco, CA [33].)

MATERIALS AND METHODS

Bacterial strains, susceptibility testing, and DNA manipulation. *E. coli* strain C316, which displayed a multiple-resistance profile to aminoglycosides, FQs, and broad-spectrum β-lactams, except for ceftazidime and imipenem, was isolated from the urine of an inpatient at a medical facility in Hyogo Prefecture, Japan, in March 2002. The strains and plasmids used in this study are listed in Table 1. The bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antimicrobial agents.

E. coli KAM32 (14), a highly susceptible antimicrobial laboratory strain, was transformed with *qepA*-carrying plasmids for a precise assay of the MICs of antimicrobials and chemical agents. Transformants were selected on LB agar plates containing chloramphenicol (20 μg/ml) and norfloxacin (0.025 μg/ml). The susceptibilities to antimicrobials, dyes, and other drugs were tested by the agar dilution method according to the procedure recommended by the NCCLS (presently CLSI) document M7-A6 (20).

Transconjugation analysis was performed with *E. coli* CSH2 as the recipient by the filter mating method. Transconjugants were selected on LB agar plates supplemented with rifampin (50 μg/ml) and kanamycin (25 μg/ml), because the R plasmid carried a kanamycin resistance determinant, *rmtB*. Plasmid DNA from a transconjugant was purified by the method of Kado and Liu (17). Transformation of *E. coli* DH10B with the plasmid DNA of the *E. coli* CSH2 transconjugant was performed by conventional electroporation techniques. Transformants were selected on LB agar containing kanamycin (25 μg/ml).

Basic recombinant DNA techniques described by Sambrook et al. (27) were

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TABLE 1. Bacterial strains and plasmids used in this study

E. coli strain	Plasmid	Characteristic(s)
C316	pHPA	Multidrug-resistant clinical isolate harboring an R plasmid, pHPA, from urine (Hyogo, Japan); GyrA mutations at Leu83 and Asn87
CSH2	None	Host strain of conjugation; resistant to rifampin and nalidixic acid
DH10B	None	Δ(*ara-leu*)*7696 araD139 galE15 galK16* Δ(*lac*)*X74 rpsL nupG* [φ80Δ(*lacZ*)M15]
KAM32	None	Highly susceptible antimicrobial laboratory strain (Δ*acrB ydhE hsd*) derived from *E. coli* K-12[a]
	pHPA	Transformant harboring the R plasmid, pHPA, derived from a wild strain *E. coli*, C316
	pSTV28	Transformant harboring a chloramphenicol-resistant cloning vector, pSTV28
	pSTVqepA	Transformant harboring a recombinant plasmid that carries a 3.2-kbp SacI-SalI fragment containing a *qepA* gene ligated to pSTV28
	pSTVΔqepA	Transformant harboring a recombinant plasmid carrying a disrupted *qepA* gene by EZ-Tn5 <KAN-2> insertion

[a] See references 14 and 19.

employed in gene manipulations. The FQ resistance plasmid, pHPA, was prepared from *E. coli* DH10B and digested with SacI and SalI. The resultant fragments were ligated into a cloning vector, pSTV28 (Takara Bio, Inc., Otsu, Japan), that was restricted with the same enzymes, and the resultant recombinant plasmid that affects FQ resistance was named pSTVqepA. The nucleotide sequence of the insert of pSTVqepA was determined on both strands using BigDye Terminator cycle sequencing ready reaction kits and a DNA sequence analyzer, model 3100 (Applied Biosystems, Foster City, CA). The alignments of nucleotide and amino acid sequences were performed with Sequencher version 4.2.2 (Hitachi Software Engineering Co., Ltd., Yokohama, Japan) and GENETYX-MAC, version 12.2.3 (Software Development Co., Ltd., Tokyo, Japan).

Using the in vitro mutagenesis system of the EZ-Tn*5* <KAN-2> insertion kit (Epicenter, Madison, WI), a kanamycin-resistant transposon was inserted into the FQ resistance gene, *qepA*, for its inactivation, and the resultant plasmid was named pSTVΔqepA. Disruption of the *qepA* gene in pSTVΔqepA was checked by sequencing analysis using specific primers for the transposon ends.

Functional assay of gene product. The assay for norfloxacin accumulation in bacterial cells was performed according to Huda et al. (14) and Morita et al. (19). *E. coli* KAM32(pSTV28), *E. coli* KAM32(pSTVqepA), and *E. coli* KAM32 (pSTVΔqepA) cells were grown in the LB broth supplemented with 30 μg/ml of chloramphenicol. The cells were harvested at the late logarithmic phase and washed twice with 0.2 M MOPS (morpholinepropanesulfonic acid)-Tris buffer (pH 7.0), and the bacterial pellet was resuspended in the same buffer and adjusted to 50 μg of bacterial cells (wet weight) per ml for the assay. Norfloxacin (100 μM) was added to the assay mixture, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100 μM) was added to the same mixture 10 min later. The first sample containing no CCCP was obtained 10 min after the addition of norfloxacin, and the second sample containing CCCP was taken 30 min after the addition of norfloxacin. Samples were centrifuged at 10,000 × *g* for 1 min at 4°C. The pellet was suspended in 1 ml of 100 mM glycine-HCl (pH 3.0), and the suspension was shaken overnight at room temperature and centrifuged at 10,000 × *g* for 5 min at room temperature. The supernatant was diluted twofold with 100 mM glycine-HCl (pH 3.0), and fluorescence was measured with excitation at 370 nm and emission at 450 nm with a Hitachi F2000 fluorescence spectrophotometer (Hitachi High-Technologies Co., Ltd., Tokyo, Japan).

Statistical analyses. The statistical analyses were done with SPSS 14.0J for Windows (SPSS Japan, Inc., Tokyo, Japan). Student's *t* test was used for analyses

TABLE 2. Results of susceptibility tests

Antimicrobial agent or chemical	MIC (μg/ml) for:						Fold increase in MIC for *E. coli* KAM32(pSTVqepA) vs. *E. coli* KAM32(pSTV28)
	E. coli C316	*E. coli* KAM32	*E. coli* KAM32(pHPA)	*E. coli* KAM32(pSTV28)	*E. coli* KAM32(pSTVqepA)	*E. coli* KAM32(pSTVΔqepA)	
Ampicillin	>128	2	>128	2	2	2	1
Erythromycin	>128	2	>128	1	2	1	2
Kanamycin	>128	1	>128	1	1	>128	1
Tetracycline	>128	0.25	0.25	0.25	0.25	0.125	1
Nalidixic acid	>128	1	1	1	2	1	2
Norfloxacin	>128	0.016	0.25	0.016	1	0.016	64
Enrofloxacin	>128	0.008	0.25	0.008	0.25	0.008	32
Tosufloxacin	>128	0.002	0.032	0.002	0.032	0.002	16
Levofloxacin	32	0.008	0.016	0.004	0.016	0.004	4
Ciprofloxacin	>128	0.004	0.064	0.004	0.125	0.004	32
Lomefloxacin	>128	0.032	0.064	0.032	0.064	0.032	2
Pazufloxacin	16	0.004	0.016	0.004	0.016	0.004	4
Sparfloxacin	16	0.001	0.002	0.001	0.002	0.001	2
Moxifloxacin	32	0.002	0.016	0.002	0.016	0.002	8
Gatifloxacin	32	0.004	0.016	0.002	0.016	0.004	8
CCCP	16	16	16	16	16	16	1
Acriflavine	>128	2	4	2	4	2	2
Rhodamine 6G	>128	4	4	4	4	4	1
Crystal violet	16	2	2	2	2	2	1
Sodium dodecyl sulfate	>128	128	128	128	128	128	1
Ethidium bromide	>128	4	8	4	8	4	2
Deoxycholate	>1,024	1,024	512	512	512	512	1
Benzalkonium	64	4	4	4	4	4	1

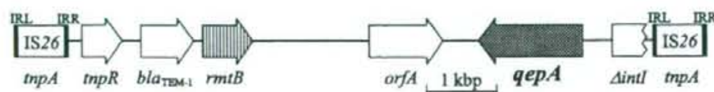

FIG. 1. Organization of the element mediating *qepA*. *qepA* is located in a 10-kb region flanked by two copies of IS*26*, and this probable transposable element also contains *rmtB*, together with *tnpR*, bla_{TEM-1}, and several probable open reading frames. IRL, 5′-GGCACTGTTGCAAA-3′; IRR, 5′-TTTGCAACAGTGCC-3′.

of norfloxacin accumulation in both *qepA*-expressing and -nonexpressing clones with or without CCCP.

Nucleotide sequence accession number. The nucleotide sequence of the *qepA* gene and its flanking regions appears in the EMBL/GenBank/DDBJ (E/G/D) databases under accession number AB263754.

RESULTS

Transfer of FQ resistance and antimicrobial susceptibility. FQ resistance was successfully transferred from *E. coli* C316 to *E. coli* CSH2 at a frequency of 10^{-5} to 10^{-6} cells per recipient cell by conjugation, and the MIC of norfloxacin for the CSH2 transconjugant was elevated above 0.125 μg/ml. Strain CSH2 shows resistance to nalidixic acid by *gyrA* mutation; therefore, the exact norfloxacin MIC was measured in *E. coli* DH10B. The norfloxacin MIC for DH10B was elevated from <0.008 μg/ml to 0.25 μg/ml by introduction of the plasmid pHPA, carrying *qepA*. For the *E. coli* KAM32 transformants that harbor pSTVqepA carrying a 3.2-kbp SacI-SalI fragment of pHPA, 32- to 64-fold-higher MICs of three FQs, norfloxacin, ciprofloxacin, and enrofloxacin, were observed and compared to those for both host strains of *E. coli* KAM32 with or without the vector plasmid (Table 2). The MICs of the other antimicrobial agents and various chemicals, including erythromycin, acriflavine, and ethidium bromide, were not changed when the 3.2-kbp SacI-SalI fragment of pHPA was introduced with pSTVqepA in *E. coli* KAM32. The *E. coli* KAM32 transcon-

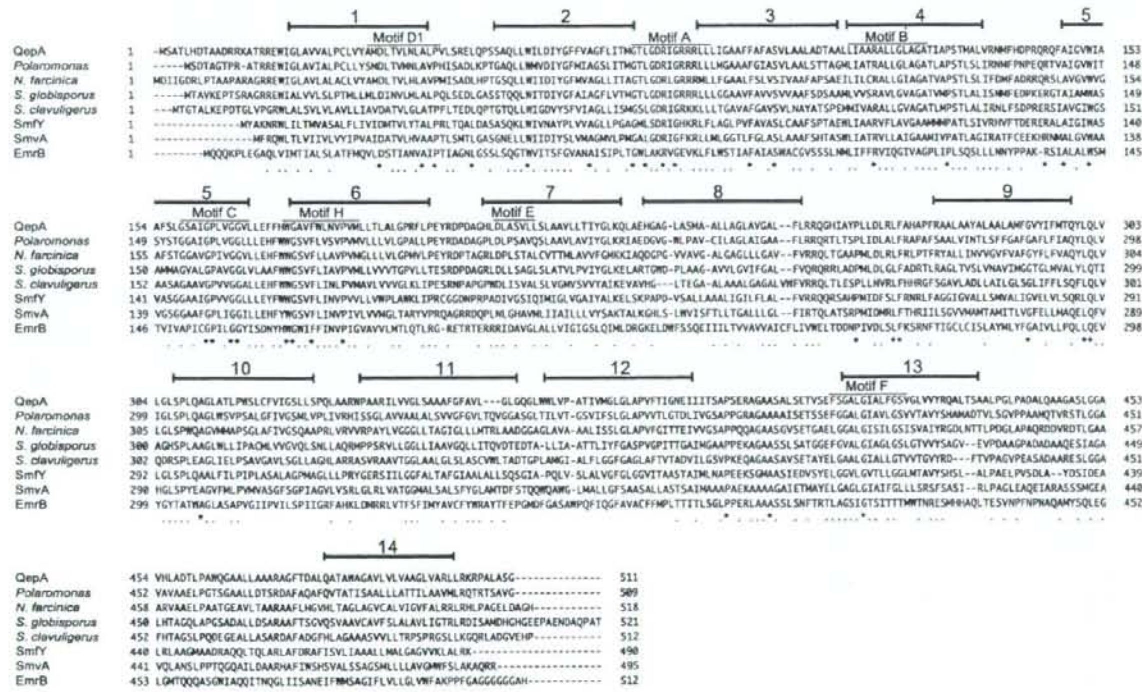

FIG. 2. Comparison of amino acid sequences of 14-TMS MFS-type efflux pumps with QepA. Fourteen portions of probable 14-TMS motifs and seven specific motifs, A, B, C, D1, E, F, and H, are shown by horizontal lines above the alignments. Asterisks and dots indicate identical and similar amino acid residues, respectively. Dashes represent gaps. *Polaromonas*, a putative efflux pump of the *Polaromonas* sp. strain JS666 chromosome (NCBI Protein Data Bank accession number ABE43318); *N. farcinica*, the putative arabinose efflux permease AraJ of the *Nocardia farcinica* chromosome (NCBI Protein Data Bank accession number BAD57397); *S. globisporus*, SgcB of *Streptomyces globisporus* (E/G/D accession number AY048670); *S. clavuligerus*, the putative efflux pump EpeA of the *Streptomyces clavuligerus* chromosome (E/G/D accession number AJ302083); SmfY, a *Serratia marcescens* chromosomal efflux pump protein (E/G/D accession number AB251607); SmvA, a *Salmonella enterica* chromosomal efflux pump protein (E/G/D accession number P37594); and EmrB, an *Escherichia coli* chromosomal efflux pump protein (E/G/D accession number AAC75733).

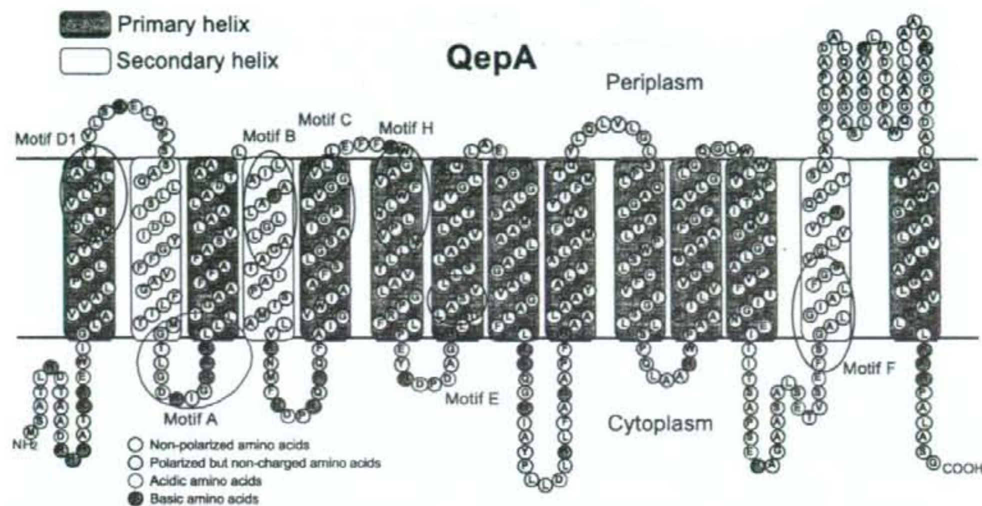

FIG. 3. Predicted secondary structure of QepA. The secondary transmembrane structure of QepA (511 amino acid residues) was calculated and illustrated by the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/). Fourteen probable α-helix TMS and seven specific motifs are indicated. Classes of amino acid residues are differentiated by four colors.

jugant harboring pHPA showed high-level resistance to ampicillin, erythromycin, and kanamycin (Table 2). These resistance profiles were later found to depend on carriage by pHPA of a CTX-M-type β-lactamase gene ($bla_{CTX-M-12}$), a macrolide phosphotransferase gene (*mphA*), and a 16S rRNA methylase gene (*rmtB*) (data not shown). No positive band was observed in the strain harboring pHPA by a PCR analysis for detecting plasmid-mediated FQ resistance *qnr* genes (data not shown). Therefore, pHPA was considered to harbor a new plasmid-mediated FQ resistance determinant. The nucleotide sequence in the *ori* region of pHPA was very similar to that of R100, suggesting that it belongs to the group of IncFII plasmids.

Characteristics of FQ resistance determinant and its product. Sequence analysis of the 3.2-kbp SacI-SalI pHPA fragment cloned into pSTVqepA revealed a 1,536-bp open reading frame with a high G+C content (72%) that was named *qepA*. Considerable similarities between the codon usage patterns of *qepA* and genes for the MFS-type efflux pumps of *Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus* were observed; however, no apparent similarities between the manners of codon usage of *qepA* and those of gram-negative bacteria, such as *rmrA* (E/G/D accession number AF233286), *smvA* (E/G/D accession number D26057), and *smfY* (E/G/D accession number AB251607) (data not shown) were seen. The *qepA*-harboring transferable plasmid, pHPA, also carried a 16S rRNA methylase gene, *rmtB* (5) (E/G/D accession number AB103506), which shows a considerable similarity to the 16S rRNA methylases essential for self-protection of actinomycetes from the hazardous effects of their own aminoglycosides (4, 29). The *qepA* gene is located in an ~10-kb region, flanked by two copies of IS*26* containing *tnpA*, and this region also contained several open reading frames, including *tnpR*, bla_{TEM-1}, and *rmtB*, as shown in Fig. 1.

The *qepA* gene encoded a putative protein, QepA, of 511 amino acids. The predicted amino acid sequence of QepA was subjected to a calculation with the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/) and was found to have probably 14 transmembrane segments (TMS) (Fig. 2 and 3).

Structural characteristics of QepA. The deduced amino acid sequence of QepA showed considerable similarity to probable membrane transporters of members of the order *Actinomycetales*, such as *Nocardia farcinica* (51%; NCBI protein database accession number BAD57397), *Streptomyces globisporus* (49%; E/G/D accession number AY048670), and *Streptomyces clavuligerus* (46%; E/G/D accession number AJ302083). A lower sequence homology (less than 38%) was seen with the major gram-negative bacterial 14-TMS family of MFS-type efflux pumps, such as EmrB (E/G/D accession number AAC75733), SmvA (E/G/D accession number P37594), and SmfY (E/G/D accession number AB251607), and with putative MFS-type efflux pumps of *Actinomycetales*. The phylogenetic tree in Fig. 4, which was calculated with the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-e.html), also suggested that QepA belongs to the 14-TMS family transporters of gram-positive *Actinomycetales* but not those of gram-negative bacteria.

Efflux of norfloxacin by QepA. The accumulation of norfloxacin in *qepA*-harboring *E. coli* KAM32(pSTVqepA) was significantly lower ($P < 0.01$) than that in *qepA*-nonharboring *E. coli* KAM32(pSTV28) or *qepA*-disrupted *E. coli* KAM32 (pSTVqepA). The accumulation of norfloxacin in *E. coli* KAM32(pSTVqepA) increased significantly after the addition of CCCP (Fig. 5), suggesting the proton potential-dependent efflux of norfloxacin by QepA.

DISCUSSION

We first characterized a new quinolone efflux pump protein (QepA) responsible for FQ resistance. QepA showed a con-

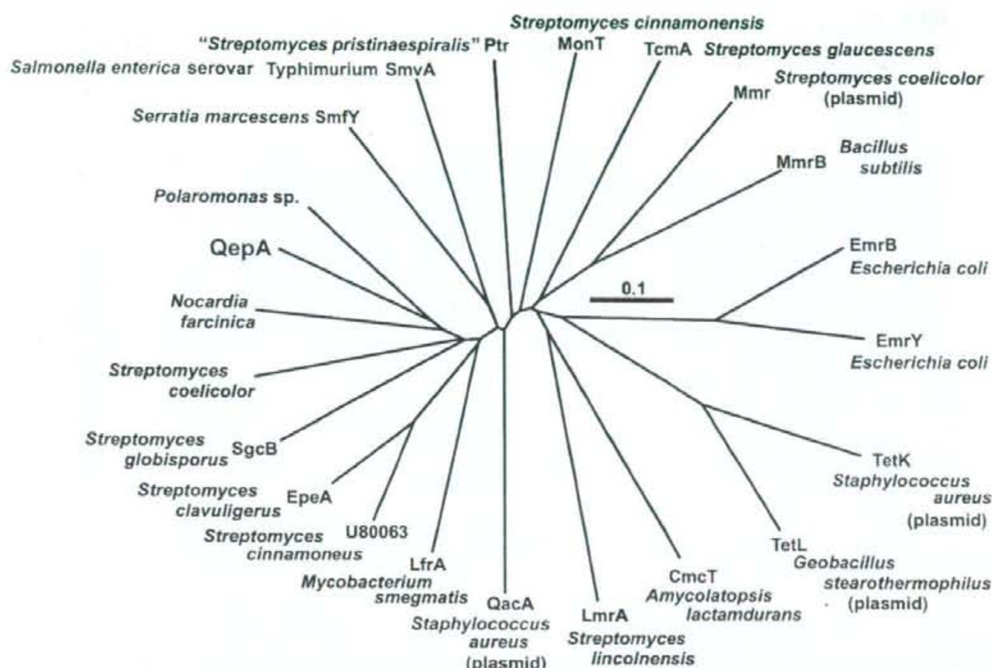

FIG. 4. Phylogenetic tree of 14-TMS efflux pumps belonging to MFS transporters. Each amino acid sequence was subjected to analysis with reference to the following sources: LfrA, a *Mycobacterium smegmatis* proton antiporter efflux pump (E/G/D accession number U40487); Ptr, a "*Streptomyces pristinaespiralis*" pristinamycin compound and rifampin resistance protein (E/G/D accession number X84072); TcmA, a *Streptomyces glaucescens* tetracenomycin C resistance and export protein (E/G/D accession number M80674); EmrB, an *E. coli* multidrug efflux system protein (E/G/D accession number AAC75733); EmrY, an *E. coli* multidrug resistance protein (E/G/D accession number D78168); TetK, a *Staphylococcus aureus* tetracycline efflux protein (E/G/D accession number M16217); TetL, a *Geobacillus stearothermophilus* tetracycline efflux protein (NCBI Protein Data Bank accession number P07561); Mmr, a *Streptomyces coelicolor* methylenomycin A resistance protein (E/G/D accession number M18263); MmrB, a *Bacillus subtilis* methylenomycin A resistance protein (E/G/D accession number X66121); CmcT, an "*Amycolatopsis lactamdurans*" cephamycin export protein (NCBI Protein Data Bank accession number Q04733); LmrA, a *Streptomyces lincolnensis* lincomycin resistance protein (E/G/D accession number X59926); QacA, an *S. aureus* antiseptic resistance protein (E/G/D accession number X56628); SmfY, a *Serratia marcescens* multidrug efflux pump (E/G/D accession number AB251607); SmvA, a *Salmonella enterica* serovar Typhimurium methyl-viologen resistance protein (NCBI Protein Data Bank accession number P37594); U80063, a *Streptomyces cinnamoneus* proton-dependent transport protein (E/G/D accession number U80063); EpeA, a *Streptomyces clavuligerus* DHA2 subfamily multidrug transporter (E/G/D accession number AJ302083); *Nocardia farcinica*, *N. farcinica* strain IFM 10152, probable arabinose efflux permease AraJ (NCBI Protein Data Bank accession number BAD57397); *Polaromonas* sp., *Polaromonas* sp. strain JS666, a probable MFS efflux pump (NCBI Protein Data Bank accession number ABE43318); QepA, an *E. coli* plasmid-mediated FQ efflux pump (E/G/D accession number AB263754); *Streptomyces coelicolor*, a probable membrane transporter of *S. coelicolor* (NCBI Protein Data Bank accession number CAC37879); SgcB, a *Streptomyces globisporus* transmembrane efflux protein (E/G/D accession number AAL06672); and MonT, a *Streptomyces cinnamonensis* putative monensin transporter (NCBI Protein Data Bank accession number AAO65793). Blue, actimomycetes; brown, gram-positive cocci; green, gram-negative rods; purple, *Mycobacterium smegmatis*. The horizontal bar (0.1) indicates a 10% change in the amino acid residues calculated by the CLUSTAL W program.

siderable similarity to the MFS-type efflux pumps belonging to the 14-TMS family of environmental microorganisms, including actinomycetes (Fig. 2). Norfloxacin accumulation in the *qepA*-expressing *E. coli* strain was significantly lower than that in the *qepA*-nonexpressing strains. Moreover, the addition of CCCP readily augmented the accumulation of norfloxacin even in the *qepA*-expressing strain (Fig. 5). These findings clearly revealed that the QepA protein is involved in the excretion of norfloxacin from the cytoplasm to the exterior of bacterial cells. As with FQs, the MICs of erythromycin, acriflavine, and ethidium bromide for the *qepA*-expressing clinical isolate and transconjugant were higher than those for the *qepA*-nonexpressing strains. However, the chemical substances tested, except FQs, showed at most twofold elevations in the MICs for a strain carrying only the *qepA* gene (Table 2). Therefore, it was strongly suggested that the QepA protein is involved mainly with the excretion of FQs as a proton antiporter efflux pump system.

Interestingly, the *qepA* gene had a high G+C content (72%), and the deduced amino acid sequence of QepA showed considerable homology to the probable efflux pumps belonging to the MFS-type membrane transporters found in *Polaromonas* spp. and members of the order *Actinomycetales* (Fig. 4). Moreover, considerable similarities between the codon usage patterns of *qepA* and genes for the MFS-type efflux pumps of *Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus* were observed, and both *qepA* and *rmtB* genes were mediated by a probable transposable element flanked by

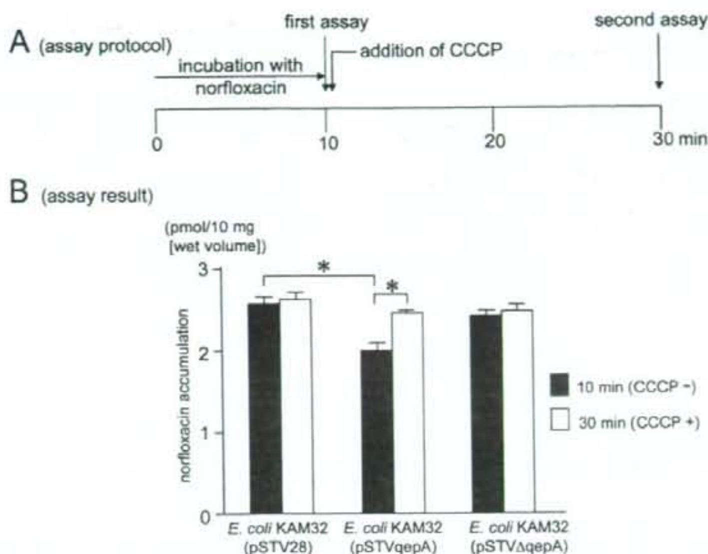

FIG. 5. Assay protocol and accumulation of norfloxacin in *E. coli* cells. The accumulations of norfloxacin in transformants that harbor a vacant plasmid (pSTV28), a *qepA*-carrying plasmid (pSTVqepA), and a disrupted *qepA*-carrying plasmid (pSTVΔqepA) are shown. *, statistical significance ($P < 0.01$) in the reduction of intracellular accumulation of norfloxacin that was seen in *E. coli* strains that produce QepA compared to QepA-nonproducing *E. coli* strains. The effect of CCCP on the accumulation of norfloxacin was also statistically significant in the QepA-producing strain. However, no statistical significance was seen in the norfloxacin levels of *E. coli* KAM32(pSTVqepA) cells between a sample taken at 10 min and a sample taken at 30 min in the absence of CCCP.

two copies of IS*26* on a transferable plasmid, pHPA. We therefore speculated that the chromosomal fragment of some actinomycetes with high G+C contents carrying both *qepA* and *rmtB* genes might be introduced into an IncFII plasmid by transposition and that pHPA could have been subsequently transferred to *E. coli*.

Antibiotic-producing actinomycetes have their own intrinsic genes for antibiotic efflux transporters for excretion of their metabolic products. FQs, however, are fully synthetic chemical agents, so no innate FQ transporter is expected to exist in the microbial world. It is possible, however, that some bacterial transporters that excrete antibiotics or bioactive agents possessing structural similarities to FQs might become transporters that can excrete FQs. Thus, we speculate that the QepA protein may well have originated from environmental microbes that produce bioactive metabolites, including antibiotics with structural similarity to FQs. A similar finding has been reported for OqxAB, a resistance nodulation division-type efflux pump that gives resistance to an artificial growth promoter, olaquindox (10).

Since resistance to broad-spectrum β-lactams has already developed among gram-negative bacteria, the emergence of multidrug-resistant gram-negative bacteria that harbor plasmids bearing *qepA* and/or *qnr*, as well as *rmtB* and genes for CTX-M-type extended-spectrum β-lactams and/or metallo-β-lactamases, could become a serious clinical concern. Although the MICs of FQs for QepA-producing strains are not high at present and the potential impact of *qepA*-harboring strains on FQ therapy has not been fully understood, the additive effect of QepA production on FQ resistance caused by mutations in genes for DNA gyrase and/or topoisomerase IV might promote further spread of FQ-resistant strains in clinical settings.

Quite recently, the *qepA* gene was also found in an RmtB-producing *E. coli* strain isolated in a Belgian hospital (23), suggesting a probable worldwide dissemination of *qepA* accompanied by *rmtB* that has already been identified in various gram-negative bacteria isolated in many countries of Asia (3, 5, 32) and Europe (2). QepA production confers resistance to enrofloxacin, a veterinary FQ, and *rmtB*-producing *E. coli* strains have frequently been isolated from pigs (3), for which aminoglycosides have tended to be used as a growth promoter in some countries or geographical areas. Thus, coproduction of QepA and RmtB may well give an advantage to bacteria to survive in livestock breeding environments as well as in human clinical settings. Hence, active surveillance of *qepA*-harboring gram-negative bacteria in animals might reveal a greater prevalence of such kinds of multidrug-resistant microbes.

In conclusion, we have newly identified a plasmid-mediated novel efflux pump, QepA, that is responsible for the elevation of levels of resistance to several clinically important FQs, such as ciprofloxacin, norfloxacin, and veterinary enrofloxacin.

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REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
2. **Bogaerts, P., M. Galimand, C. Bauraing, A. Deplano, R. Vanhoof, R. De Mendonca, H. Rodriguez-Villalobos, M. Struelens, and Y. Glupczynski.** 2007. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. J. Antimicrob. Chemother. **59:**459–464.
3. **Chen, L., Z. L. Chen, J. H. Liu, Z. L. Zeng, J. Y. Ma, and H. X. Jiang.** 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. J. Antimicrob. Chemother. **59:**880–885.
4. **Doi, Y., and Y. Arakawa.** 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. **45:**88–94.
5. **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.
6. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. Emerg. Infect. Dis. **7:**24–34.
7. **Fralick, J. A.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178:**5803–5805.
8. **Gay, K., A. Robicsek, J. Strahilevitz, C. H. Park, G. A. Jacoby, T. J. Barrett, F. Medalla, T. M. Chiller, and D. C. Hooper.** 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin. Infect. Dis. **43:**297–304.
9. **Ginsburg, A. S., J. H. Grosset, and W. R. Bishai.** 2003. Fluoroquinolones, tuberculosis, and resistance. Lancet Infect. Dis. **3:**432–442.
10. **Hansen, L. H., E. Johannesen, M. Burmølle, A. H. Sørensen, and S. J. Sørensen.** 2004. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in *Escherichia coli*. Antimicrob. Agents Chemother. **48:**3332–3337.
11. **Hata, M., M. Suzuki, M. Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae.** 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob. Agents Chemother. **49:** 801–803.
12. **Hooper, D. C.** 2001. Emerging mechanisms of fluoroquinolone resistance. Emerg. Infect. Dis. **7:**337–341.
13. **Hopkins, K. L., R. H. Davies, and E. J. Threlfall.** 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. Int. J. Antimicrob. Agents **25:**358–373.
14. **Huda, N., E.-W. Lee, J. Chen, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Molecular cloning and characterization of an ABC multidrug efflux pump, VcaM, in non-O1 *Vibrio cholerae*. Antimicrob. Agents Chemother. **47:**2413–2417.
15. **Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, H. Oh, A. Robicsek, and D. C. Hooper.** 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50:**1178–1182.
16. **Jonas, D., K. Biehler, D. Hartung, B. Spitzmüller, and F. D. Daschner.** 2005. Plasmid-mediated quinolone resistance in isolates obtained in German intensive care units. Antimicrob. Agents Chemother. **49:**773–775.
17. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
18. **Martínez-Martínez, L., A. Pascual, and G. A. Jacoby.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:**797–799.
19. **Morita, Y., K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1998. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob. Agents Chemother. **42:**1778–1782.
20. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. M7-A6. National Committee for Clinical Laboratory Standards, Wayne, PA.
21. **Nordmann, P., and L. Poirel.** 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. J. Antimicrob. Chemother. **56:** 463–469.
22. **Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahm, and D. C. Hooper.** 2006. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. **50:**3953–3955.
23. **Périchon, B., P. Courvalin, and M. Galimand.** 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob. Agents Chemother. **51:**2464–2469.
24. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
25. **Robicsek, A., G. A. Jacoby, and D. C. Hooper.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. **6:**629–640.
26. **Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, and D. C. Hooper.** 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat. Med. **12:**83–88.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B. DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer.** 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob. Agents Chemother. **45:**1126–1136.
29. **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.
30. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA **99:**5638–5642.
31. **Wang, M., J. H. Tran, G. A. Jacoby, Y. Zhang, F. Wang, and D. C. Hooper.** 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob. Agents Chemother. **47:**2242–2248.
32. **Yamane, K., J. Wachino, S. Suzuki, H. Kato, K. Shibayama, K. Kimura, K. Kumiko, I. Satoshi, Y. Ozawa, K. Toshifumi, and Y. Arakawa.** 2007. 16S rRNA methylase-producing, gram-negative pathogens, Japan. Emerg. Infect. Dis. **13:**642–646.
33. **Yamane, K., J. Wachino, K. Kimura, S. Suzuki, N. Shibata, and Y. Arakawa.** 2006. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-586.
34. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172:**6942–6949.

16S Ribosomal RNA Methylation: Emerging Resistance Mechanism against Aminoglycosides

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Methylation of 16S ribosomal RNA (rRNA) has recently emerged as a new mechanism of resistance against aminoglycosides among gram-negative pathogens belonging to the family *Enterobacteriaceae* and glucose-nonfermentative microbes, including *Pseudomonas aeruginosa* and *Acinetobacter* species. This event is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside-producing actinomycetes. Their presence confers a high level of resistance to all parenterally administered aminoglycosides that are currently in clinical use. The responsible genes are mostly located on transposons within transferable plasmids, which provides them with the potential to spread horizontally and may in part explain the already worldwide distribution of this novel resistance mechanism. Some of these organisms have been found to coproduce extended-spectrum β-lactamases or metallo-β-lactamases, contributing to their multidrug-resistant phenotypes. A 2-tiered approach, consisting of disk diffusion tests followed by confirmation with polymerase chain reaction, is recommended for detection of 16S rRNA methylase–mediated resistance.

Aminoglycosides continue to play an important role in the management of serious infections caused by gram-negative pathogens, often in combination with broad-spectrum β-lactams. They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis [1]. The most commonly encountered mechanism of resistance to aminoglycosides is enzymatic inactivation, which is mediated by 3 classes of enzymes: acetyltransferases, nucleotidyltransferases, and phosphotransferases [2]. They are further divided into subclasses that are based on the site of modification and the spectrum of resistance within the class of antimicrobials. Other known mechanisms of aminoglycoside resistance include defect of cellular permeability, active efflux, and, rarely, nucleotide substitution of the target molecule [1].

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Aminoglycosides are produced by species of actinomycetes, such as *Streptomyces* species and *Micromonospora* species. These actinomycetes are intrinsically resistant to the aminoglycosides that they produce [3]. In many cases, this resistance is caused by ribosomal protection through methylation of specific nucleotides within the A-site of 16S rRNA, which hampers binding of aminoglycosides to the 30S ribosomal subunits and serves as a means of self-protection. Until recently, this resistance mechanism was believed to be absent in clinically relevant species.

However, clinical strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* that produced 16S rRNA methylases were reported in 2003 [4, 5]. These enzymes were found to confer extraordinarily high levels of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin, and gentamicin. Since 2003, the literature on this newly recognized resistance mechanism has grown rapidly, documenting identification of new enzymes and their spread to different species in various parts of the world. In the present article, we will first review aminoglycoside resistance caused by 16S rRNA methylation in aminoglycoside-producing actinomycetes. We will then discuss the current knowledge of this emerging, plasmid-mediated resistance mechanism that is found among gram-negative path-

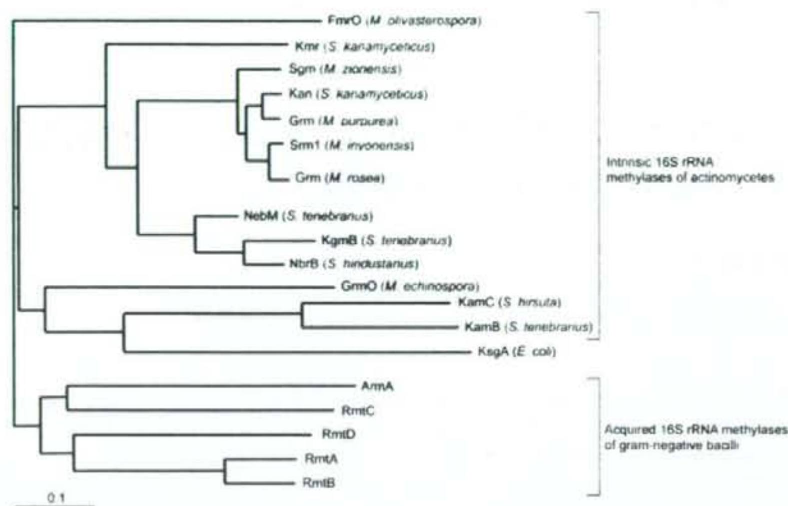

Figure 1. Dendrogram of 16S rRNA methylases of gram-negative bacteria and of representative actinomycetes. KsgA is a nonresistance 16S rRNA methylase intrinsic in *Escherichia coli*. The protein sequences were obtained from the databases of GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan and were aligned using ClustalW [11]. *M. echinospora, Micromonospora echinospora; M. inyonensis, Micromonospora inyonensis; M. olivasterospora, Micromonospora olivasterospora; M. purpurea, Micromonospora purpurea; M. rosea, Methylocystis rosea; M. zionensis, Micromonospora zionensis; S. hindustanus, Streptoalloteichus hindustanus; S. hirsuta, Saccharopolyspora hirsuta; S. kanamyceticus, Streptomyces kanamyceticus; S. tenebrarius, Streptomyces tenebrarius.*

ogens, with an emphasis on the diagnostic and therapeutic implications.

MODIFICATION OF rRNA IN BACTERIA

The ribosome is a large enzyme consisting of multiple proteins and RNA components [6]. In bacteria, it comprises 30S and 50S subunits, the former containing 16S rRNA and the latter containing 23S and 5S rRNAs. Posttranscriptional modification events of RNAs, such as methylation of nucleosides, take place following the generation of initial RNA transcripts. They are predominantly reported in tRNA, but they are also reported in rRNA. *Escherichia coli*, for example, is known to contain 10 methylated nucleosides in 16S rRNA and 14 methylated nucleotides in 23S rRNA [7]. The primary roles of rRNA methylation likely include modulation of rRNA maturation, stabilization of rRNA structures, and alteration of translation rates. For instance, mutants of *E. coli* that were deficient in the production of KsgA (RsmA) showed an increased leakiness of nonsense and frameshift mutants and alteration in decoding fidelity at both the A-site and peptidyl-tRNA site of 16S rRNA [8, 9]. In addition, some of the posttranscriptional methylation events are known to confer resistance to antimicrobials that target rRNA.

RESISTANCE IN ACTINOMYCETES MEDIATED BY 16S rRNA METHYLATION

A number of actinomycetes are known to be intrinsically resistant to aminoglycosides that they produce themselves. The mechanisms of resistance include inactivation of aminoglycosides by production of aminoglycoside-modifying enzymes and protection of 16S rRNA within the 30S ribosome subunit by production of 16S rRNA methylase. The latter mechanism results in high-level resistance to multiple aminoglycosides. It represents an efficient means to avoid inhibition of their own protein synthesis and is prevalent among aminoglycoside-producing actinomycetes (figure 1) [3].

Two sites of methylation within 16S rRNA that lead to different aminoglycoside-resistance phenotypes have been identified [10]. One group of 16S rRNA methylases, such as that produced by the istamycin producer *Streptomyces tenjimariensis*, methylates residue A1408 (figure 2). Another group of 16S rRNA methylases, exemplified by those produced by gentamicin-producer *Micromonospora purpurea*, methylates residue G1405. The former confers resistance to kanamycin and apramycin but not gentamicin, whereas the latter confers resistance to kanamycin and gentamicin but not apramycin. Both of these residues are located within the A-site–decoding region of 16S

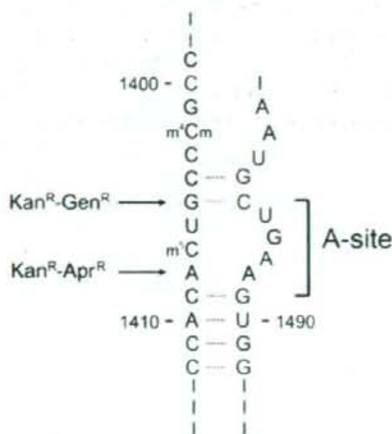

Figure 2. The positions of modifications in the aminoacyl site (A-site) decoding region in 16S rRNA (modified from Beauclerk et al. [10] with permission). ArmA is known to methylate G1405 [19]. Based on the common Kan^R-Gen^R phenotype, the other methylases in gram-negative organisms likely methylate the same residue. Methylases that modify A1408 have only been reported in actinomycetes. Apr, apramycin; Gen, gentamicin; Kan, kanamycin.

rRNA, where aminoglycosides are known to bind and interfere with accurate translation through blocking translocation of peptidyl-tRNA from the A-site to the peptidyl-tRNA site [12].

RESISTANCE IN GRAM-NEGATIVE PATHOGENS MEDIATED BY 16S rRNA METHYLATION

Because it became evident that many aminoglycoside-producing actinomycetes used ribosomal resistance afforded by methylation of 16S rRNA, the question was raised as to why the same resistance mechanism was not identified in clinically relevant species. It was speculated that such resistance mechanisms could exist but were possibly missed because of limited screening methods, because the resistance pattern could mimic that of organisms producing multiple aminoglycoside modifying enzymes. In 2002, a gene encoding 16S rRNA methylase, later designated ArmA, was deposited to the European Molecular Biology Laboratory and GenBank as part of a plasmid sequence from *Citrobacter freundii* in Poland (accession number AF550415). No additional findings have been published to date.

Then, in 2003, an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan was reported to produce 16S rRNA methylase [5]. The deduced amino acid sequence of this new enzyme, designated RmtA, shared modest (up to 35%) identity with 16S rRNA methylases of various actinomycetes. RmtA displayed methylation activity against 16S rRNA of 30S ribosomal subunits derived from a susceptible strain of *P. aeruginosa*. When *rmtA* was cloned and expressed in *E. coli* and *P. aeruginosa*, it was found to confer a high degree of resistance to all 4,6-disubstituted deoxystreptamines, which include gentamicin, tobramycin, and amikacin. As described above, a putative 16S rRNA methylase gene was initially found in a *C. freundii* clinical isolate from Poland. This gene was also identified in *K. pneumoniae* from France [4]. The gene product ArmA was also shown to confer high-level resistance to 4,6-disubstituted deoxystreptamines. The identity of the amino acid sequence of ArmA with those of RmtA and other 16S rRNA methylases from actinomycetes was only modest, ranging between 30% and 35%. The structural gene of RmtA was associated with a genetic element that resembled a mercury-resistance transposon Tn*5041* on a transferable plasmid [13]. The guanine cytosine content of *rmtA* was 55%, suggesting its origin from some guanine cytosine–rich microbe, including actinomycetes. The structural gene for ArmA was reported to be located on functional composite transposon Tn*1548* [14]. The guanine cytosine content of *armA* was 30%, suggesting that it was derived from some microbe with lower guanine cytosine content. These findings point to the possibility that these genes were acquired horizontally from diverse nonpathogenic environmental microbes, but their exact origins remain unknown.

Several other 16S rRNA methylases were subsequently discovered among gram-negative bacteria, and a total of 5 are known to date (figure 1). RmtB was identified in *Serratia marcescens* from Japan [15]. RmtB is closest to RmtA, sharing 82% identity at the amino acid level. The structural gene for RmtB was located adjacent to a Tn*3*-like transposon on a large transferable plasmid. RmtC was found in a *Proteus mirabilis* clinical strain from Japan that was rather distant in phylogeny from the 3 enzymes already reported [16]. The structural gene for RmtC is also located next to a transposon-mediated recombination system termed IS*Ecp1*, and the methylase gene was shown to be mobilizable from plasmid to plasmid [17]. The most recently identified 16S rRNA methylase is RmtD, which shares moderate identity (40%–42%) with RmtA and RmtB [18]. RmtD was found to be produced by a *P. aeruginosa* clinical strain from Brazil, which also produced metallo-β-lactamase SPM-1. This particular strain was, therefore, highly resistant to carbapenems as well as to aminoglycosides.

These newly identified 16S rRNA methylases in gram-negative bacilli all confer resistance to 4,6-disubstituted deoxystreptamines, including gentamicin, tobramycin, and amikacin, but not including 4,5-disubstituted deoxystreptamines, such as neomycin, 4-monosubstituted deoxystreptamines, such as paromomycin, or streptomycin, which lacks a deoxystreptamine ring. The level of resistance to tobramycin appears to be slightly lower than the level of resistance to other 4,6-disubstituted deoxystreptamines when the responsible genes are cloned and expressed in experimental strains of *E. coli*, such as XL1-Blue, DH5α, or INVaF′ (MIC, 64–256 μg/mL) (table 1). However,