

Discovery of a Gene Conferring Multiple-Aminoglycoside Resistance in *Escherichia coli*[∇]

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Bovine-origin *Escherichia coli* isolates were tested for resistance phenotypes using a disk diffusion assay and for resistance genotypes using a DNA microarray. An isolate with gentamicin and amikacin resistance but with no corresponding genes detected yielded a 1,056-bp DNA sequence with the closest homologues for its inferred protein sequence among a family of 16S rRNA methyltransferase enzymes. These enzymes confer high-level aminoglycoside resistance and have only recently been described in Gram-negative bacteria.

Aminoglycosides interfere with bacterial 16S rRNA function by binding at the site where codon-anticodon accuracy is assessed (the A site) (14). In Gram-negative pathogens, resistance to aminoglycosides is mediated primarily by enzymes that modify the drug by acetylation, adenylation, or phosphorylation and less commonly by other methods, including efflux mechanisms (27). Aminoglycoside-producing bacteria (*Streptomyces* and *Micromonospora* species) have intrinsic resistance to aminoglycosides through methylation of nucleotides within the A site of 16S rRNA, preventing disruption of translation by the aminoglycoside. The earliest reports of clinical Gram-negative isolates with plasmid-borne rRNA methylase aminoglycoside resistance genes were from Japan (33, 35) and France (14), followed by Taiwan (34), Spain (15, 16), South Korea (19), Belgium (4), and China (7). All were bacteria infecting human patients, except for reports from Spain (16) and China (7) of similar genes in *Escherichia coli* and *Enterobacter cloacae* isolates from swine.

As part of a larger study, 81 cattle origin commensal *E. coli* isolates were assayed for the presence of antibiotic resistance genes using a DNA oligonucleotide microarray (9a). The *E. coli* isolates used in this study were from different animals on the same farm or, if from the same animal, had different resistance phenotypes. The microarray includes 30 probes for detecting aminoglycoside resistance genes, five of which code for amikacin resistance and 10 for gentamicin resistance (Table 1). The results of the array hybridizations were compared to the resistance phenotypes as measured by a standard disk diffusion assay (2). The panel of antimicrobial disks included four aminoglycosides, amikacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), and streptomycin (10 µg). The resistance breakpoints were those recommended by the Clinical and Laboratory Standards Institute (9). Among the 81 *E. coli* isolates, 11 isolates were phenotypically resistant to amikacin, gentamicin, kanamycin, and streptomycin, but no amikacin resistance genes were detected by microarray assay. Nine of these isolates

were from separate animals, and two were from the same animal but had different resistance profiles (Table 2).

The initial attempts to transform sonicated plasmid DNA fragments from isolates with amikacin resistance but no corresponding gene, as described here, were successful for isolate 2517, which was therefore characterized further. The plasmid profile of *E. coli* isolate 2517 was performed as previously described (18); it contained two plasmids of approximately 60 and 95 kb. Plasmid DNA was extracted from isolate 2517 (Qiaprep spin miniprep kit; Qiagen, Valencia, CA) and electroporated into competent *E. coli* cells (GeneHogs; Invitrogen, Carlsbad, CA). Four resulting transformants that grew on gentamicin-supplemented medium (10 µg/ml) were all resistant to amikacin, gentamicin, kanamycin, and streptomycin as measured by disk diffusion assay, as was the donor isolate, 2517. The transformants each had a single plasmid of approximately 95 kb which probably corresponded to the 95-kb plasmid of the donor isolate. Plasmid DNA was extracted from the transformants and sonicated. After blunt-end repair and dephosphorylation, DNA fragments were ligated into a pCRII-Blunt-TOPO vector (Invitrogen) and chemically transformed into One Shot TOP10 cells (Invitrogen). They were then plated onto LB medium with gentamicin (10 µg/ml). The insert DNA was PCR amplified from the resulting transformants using flanking M13 primer binding sites. Two transformants yielded products that were 1,056 and 883 bp in length. Sequencing revealed an 819-bp open reading frame (ORF) (GenBank accession no. GU201947). Within the 1,056-bp fragment, the GC content of the ORF was 37.4%, and the GC content of its flanking sequences was 46.0%.

A BLASTx (NCBI) query of the deduced protein indicated that its amino acid sequence was most similar to that of 16S rRNA methyltransferases that confer high-level aminoglycoside resistance (11). The newly identified gene was amplified in each of the remaining 10 isolates identified with the same phenotype-genotype discordance (Table 2), using PCR primers GM1-Forward (5'-ATGAATATTGATGAAATGGTTGC) and GM1-Reverse (5'-TGATTGATTTCTCCGTTTTT). To confirm the aminoglycoside resistance phenotype in the 11 isolates that were PCR positive for the newly discovered gene, those isolates were tested for MICs using the Trek Diagnostics (Cleve-

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TABLE 1. Aminoglycoside resistance genes represented by probes on the resistance gene microarray

Gene	Accession no.	Target(s)	Enzyme encoded	Reference
<i>aac(3)-Ia</i>	DQ370505	Gentamicin	3-N-Acetyltransferase	29
<i>aac(3)-Ib</i>	L06157	Gentamicin	3-N-Acetyltransferase	13
<i>aac(3)-Id</i>	AY458224	Gentamicin	3-N-Acetyltransferase	13
<i>aac(3)-III</i>	X13542	Gentamicin	3-N-Acetyltransferase	1
<i>aac(3)-IVa</i>	X01385	Gentamicin	3-N-Acetyltransferase	8
<i>aac(3)-Vb</i>	M97172	Gentamicin	3-N-Acetyltransferase	24
<i>aac(6')-I30</i>	AY289608	Amikacin	6'-N-Acetyltransferase	22
<i>aac(6')-Ib</i>	AY103455	Amikacin, kanamycin	6'-N-Acetyltransferase	13
<i>aac(6')-IIa</i>	AY123251	Gentamicin	6'-N-Acetyltransferase	22
<i>aac(6')-Ia</i>	M18967	Amikacin, kanamycin	6'-N-Acetyltransferase	28
<i>aacC1</i>	U04610	Gentamicin	3-N-Acetyltransferase	13
<i>aacC2</i>	S68058	Gentamicin	3-N-Acetyltransferase	32
<i>aacCA5</i>	AY463797	Gentamicin, kanamycin, amikacin	3-N-Acetyltransferase	22
<i>aadA1</i>	EF422367	Streptomycin	3'-Adenylyltransferase	32
<i>aadA2</i>	AF071555	Streptomycin	3'-Adenylyltransferase	5
<i>aadA21</i>	AY171244	Streptomycin	3'-Adenylyltransferase	22
<i>aadA5</i>	AB126604	Streptomycin	3'-Adenylyltransferase	22
<i>aadA7</i>	AY458224	Streptomycin	3'-Adenylyltransferase	12
<i>aadB</i>	AY204504	Gentamicin, kanamycin	2'-Adenylyltransferase	13
<i>aadE</i>	AF516335	Streptomycin	6-Adenylyltransferase	13
<i>aph(3)-Ia</i>	V00359	Kanamycin	3'-Phosphotransferase	6
<i>aph(3)-IIa</i>	V00618	Kanamycin	3'-Phosphotransferase	6
<i>aph4</i>	V01499	Hygromycin B	Aminocyclitol phosphotransferase	20
<i>aphA-3</i>	AF516335	Kanamycin	3'-Phosphotransferase	13
<i>aphA7</i>	AY509004	Kanamycin	3'-Phosphotransferase	22
<i>aphD</i>	Y00459	Streptomycin	6-Phosphotransferase	10
<i>aphE</i>	X53527	Streptomycin	3'-Phosphotransferase	30
<i>aphIII</i>	V01547	Kanamycin	3'-Phosphotransferase	17
<i>strA</i>	AY055428	Streptomycin	6-Phosphotransferase	3
<i>strB</i>	NC_005014	Streptomycin	6-Phosphotransferase	3

land, OH) Sensititre plate COMEQ3F. The MICs for amikacin and gentamicin were greater than 32 µg/ml and 8 µg/ml, respectively.

To assess the phylogenetic relationships between the newly identified gene and other 16S rRNA methyltransferase genes, its inferred amino acid sequence and those of previously identified 16S rRNA methyltransferases were analyzed using CLUSTALW (<http://www.ddbj.nig.ac.jp/search/top-e.html>). The results were illustrated with the TreeViewX program, version 0.5.0 for Macintosh OS X (Fig. 1). It is clear from this analysis and from its low percent identity (33%) to the closest match in the NCBI protein database that the deduced product of the

aminoglycoside resistance gene identified in this study is distinct from other recognized homologues. This gene product can be designated RmtE (11). Its proximity on the tree to other enzymes for which the G1405 methylation site has been confirmed (ArmA [21] and RmtB [23]) suggests that the newly discovered RmtE probably also methylates that site (Fig. 1).

This is the first report of cattle-associated field isolates of *E. coli* bearing a plasmid-mediated aminoglycoside resistance gene of this type. The isolates in which the newly discovered RmtE gene was detected were collected from calves in a type of facility associated with intense antimicrobial use (25, 26, 31). While it seems biologically plausible that antibiotic selection

TABLE 2. Resistance gene microarray hybridization results for *E. coli* isolates having an aminoglycoside resistance phenotype not explained by any genes detected using the oligonucleotide microarray

Isolate	Unexplained resistance phenotype ^a	<i>aac(3)-III</i>	<i>aac(6')-IIa</i>	<i>aac(3)-IVa</i>	<i>aac(3)-IVa</i>	<i>aacC2</i>	<i>aadA1</i>	<i>aadA2</i>	<i>aadA21</i>	<i>aadA5</i>	<i>aph(3')-Ia</i>	<i>aph4</i>	<i>aphA7</i>	<i>strA</i>	<i>strB</i>
1090	Gentamicin, amikacin							+	+		+		+	+	+
2517	Gentamicin, amikacin										+		+	+	+
2521	Gentamicin, amikacin								+		+		+	+	+
2534	Amikacin				+		+	+			+		+	+	+
2538	Amikacin	+				+		+	+		+		+	+	+
2545	Amikacin				+		+	+	+		+	+	+	+	+
2550 ^b	Gentamicin, amikacin									+	+		+	+	+
2551 ^b	Amikacin	+	+				+	+	+		+		+	+	+
2577	Gentamicin, amikacin										+		+	+	+
2612	Amikacin	+	+			+				+	+	+	+	+	+
2614	Amikacin	+				+				+	+		+	+	+

^a Phenotypic resistance characteristic for which no explanatory gene was detected on the array. All isolates were resistant to the four aminoglycosides, amikacin, gentamicin, kanamycin, and streptomycin, as measured by a standard disk diffusion assay, and all isolates demonstrated a MIC of >32 µg/ml for amikacin and >8 µg/ml for gentamicin. All isolates were PCR positive for the new methyltransferase gene (GenBank accession no. GU201947) described herein.

^b Isolates 2550 and 2551 were from the same calf fecal sample.

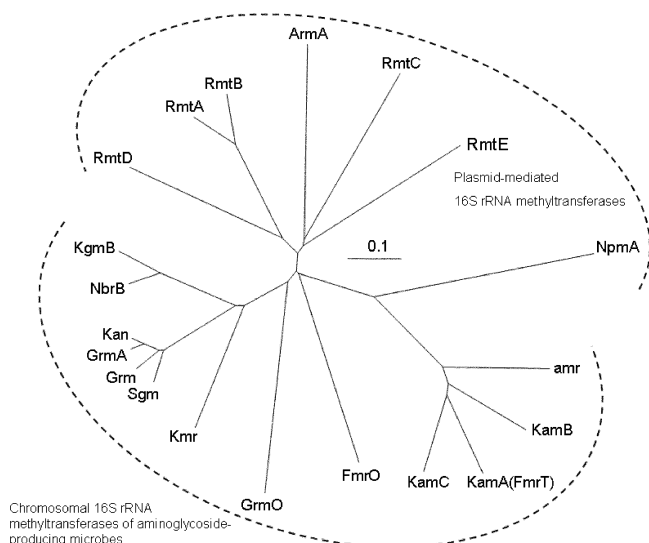


FIG. 1. Phylogenetic relationships among 16S rRNA methyltransferases. Twenty amino acid sequences of known 16S rRNA methyltransferases were compared in CLUSTALW, and the results were illustrated using the TreeViewX program, version 0.5.0 for Macintosh OS X. GenBank or EMBL accession numbers associated with each gene are as follows: RmtA, AB120321; RmtB, AB117036; ArmA, AF550415; RmtC, AB194779; RmtD, DQ914960; RmtE, GU201947; NpmA, AB261016; FmrO, D13171; GrmO, AY524043; Grm, M55521; Sgm, A45282; GrmA, AY524043; Kmr, AB164642; Kan, AJ414669; NbrB, AF038408; KgmB, AAB20100; KamA (FmrT), D13170; KamC, AAA26499; KamB, CAF33037; and Amr, AAB08.

pressure may promote the acquisition of novel resistance mechanisms by commensal enteric bacteria, we lack data on antimicrobial use associated with the specific animals from which these isolates originated. The difference between the GC content of the ORF itself and the GC content of the flanking regions is high, supporting the idea of a gene transfer event between species or genera of bacteria. As we have not characterized the plasmid or mobile elements associated with the gene, we cannot make further inferences about its origins. The array hybridization results for the isolates that were PCR positive for the novel gene indicate variations in the presence or absence of specific aminoglycoside resistance genes in this collection of isolates (Table 2). Because the *E. coli* isolates described here were from a single farm, these results cannot be generalized with regard to occurrence or prevalence on other premises.

Our purpose here is to report the finding of a previously unreported aminoglycoside resistance gene in the family of 16S rRNA methyltransferases in an *E. coli* isolate from an unusual setting and location. Further characterization, including protein expression, determination of the methylation site, and description of the plasmid and mobile elements associated with the new gene, is to follow.

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