

RecA-Mediated Gene Conversion and Aminoglycoside Resistance in Strains Heterozygous for rRNA

THERDSAK PRAMMANANAN, PETER SANDER, BURKHARD SPRINGER,
AND ERIK C. BÖTTGER*

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30623 Hannover, Germany

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Clinical resistance to aminoglycosides in general is due to enzymatic drug modification. Mutational alterations of the small ribosomal subunit rRNA have recently been found to mediate acquired resistance in bacterial pathogens in vivo. In this study we investigated the effect of 16S rRNA heterozygosity (wild-type [wt] and mutant [mut] operons at position 1408 [1408^{wt}/1408^{mut}]) on aminoglycoside resistance. Using an integrative vector, we introduced a single copy of a mutated rRNA operon (1408 A→G) into *Mycobacterium smegmatis*, which carries two chromosomal wild-type rRNA operons; the resultant transformants exhibited an aminoglycoside-sensitive phenotype. In contrast, introduction of the mutated rRNA operon into an *M. smegmatis* *rrnB* knockout strain carrying a single functional chromosomal wild-type rRNA operon resulted in aminoglycoside-resistant transformants. Subsequent analysis by DNA sequencing and RNase protection assays unexpectedly demonstrated a homozygous mutant genotype, rRNA^{mut}/rRNA^{mut}, in the resistant transformants. To investigate whether RecA-mediated gene conversion was responsible for the aminoglycoside-resistant phenotype in the rRNA^{wt}/rRNA^{mut} strains, *recA* mutant strains were generated by allelic exchange techniques. Transformation of the *recA rrnB* *M. smegmatis* mutant strains with an integrative vector expressing a mutated rRNA operon (*Escherichia coli* position 1408 A→G) resulted in transformants with an aminoglycoside-sensitive phenotype. Subsequent analysis showed stable heterozygosity at 16S rRNA position 1408 with a single wild-type allele and a single resistant allele. These results demonstrate that rRNA-mediated mutational resistance to aminoglycosides is recessive.

Aminoglycoside antibiotics comprise a large group of antimicrobial agents which have in common structures that contain cyclic alcohols (aminocyclitols) in glycosidic linkage with amino-substituted sugars; the most common aminocyclitol is deoxystreptamine. 2-Deoxystreptamine antibiotics bind to the ribosome and affect protein synthesis by inducing codon misreading and inhibiting translation (9). Three major mechanisms of resistance to aminoglycoside antibiotics have been described in both gram-positive and gram-negative bacteria: (i) enzymatic drug modification by either plasmid-encoded or chromosomally encoded aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases, aminoglycoside adenyltransferases, and aminoglycoside phosphoryltransferases (10, 14, 15, 19, 29, 30); (ii) reduced intracellular drug accumulation due to, for example, mutations that affect energy metabolism, such as electron transport, resulting in impaired aminoglycoside uptake or increased efflux (6, 7, 17, 20); and (iii) alterations of the antibiotic target structure.

Alterations of the antibiotic target involve a conserved region within the rRNA, i.e., methylation of an adenine at either 16S rRNA position 1405 or 16S rRNA position 1408, and have been found in aminoglycoside-producing organisms such as *Streptomyces* spp. and *Micromonospora* spp. This posttranscriptional modification has been shown to protect these organisms from their own metabolites by interfering with the binding of the aminoglycoside to the respective ribosomal target of the drug (4, 8, 13, 22, 31, 32, 36). Genetic studies with *Escherichia coli* have demonstrated that *cis* regulatory rRNA mutations that weaken base pairing between 16S rRNA positions 1409

and 1491 confer resistance to paromomycin and other aminoglycosides (11).

Aminoglycoside resistance mechanisms have been intensively studied in mycobacteria. Aminoglycoside acetyltransferases are universally present in both fast- and slow-growing mycobacteria (1, 2, 21, 37, 39). However, no correlation between the activities of these enzymes and the level of aminoglycoside resistance has been found. Recently, we have demonstrated that a single point mutation at 16S rRNA position 1408 (A→G; *E. coli* numbering) is associated with high-level resistance to 2-deoxystreptamine aminoglycosides in mycobacteria containing a single rRNA operon, such as *M. tuberculosis*, *M. abscessus*, and *M. chelonae* (24, 27).

The question of recessivity or dominance of aminoglycoside resistance mutations is a matter of debate. Apirion and Schlessinger (3) provided evidence that aminoglycoside sensitivity is dominant over resistance; De Stasio et al. (11) postulated that resistance to aminoglycosides is dominant at low drug concentrations (10 µg/ml) but recessive at high antibiotic concentrations in *E. coli*. Our own attempts to generate spontaneous aminoglycoside-resistant mutants of *M. smegmatis*, an organism containing two rRNA operons, were unsuccessful (mutation rate, <10⁻¹¹); resistant mutants could be obtained only in *M. smegmatis* *rrnA* or *rrnB* mutant strains, genetically engineered derivatives of *M. smegmatis* which carry a single functional rRNA operon (27). In contrast, Taniguchi et al. (35) were able to isolate kanamycin-resistant *M. smegmatis* mutants upon UV irradiation, suggesting the dominance of resistance.

To address the issue of dominance versus recessivity of aminoglycoside resistance mutations, we report here on our investigations of aminoglycoside resistance in heterozygous *M. smegmatis* rRNA^{wt}/rRNA^{mut} (wt represents wild type, and mut represents mutant) strains.

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, 30623 Hannover, Germany. Phone: (511) 5324348. Fax: (511) 5324366. E-mail: Boettger.Erik@Mh-Hannover.de.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Source or reference
Strains	
<i>E. coli</i> XL 1-Blue MRF	Stratagene
Mycobacteria	
<i>M. smegmatis</i> mc ² 155	Snapper et al. (33)
<i>M. smegmatis</i> mc ² 155 SMR5 <i>rrnB</i> mutant	Sander et al. (27)
<i>M. smegmatis</i> mc ² 155 SMR5 <i>rrnB recA</i> mutant	This work
Plasmids	
pT7-blue	Novagen
ptrpA-1-rpsL ⁺	Sander et al. (26)
precA(Ms)::hyg-rpsL ⁺	Frischkorn et al. (12)
pMV 261-rRNA 2058G ^a	Sander et al. (28)
pMV 361-H-rRNA 2058G ^b	Sander et al. (28)
pMV 261-rRNA 1408G/2058G ^a	This work
pMV 361-H-rRNA 1408G/2058G ^b	This work

^a Vector confers resistance to kanamycin and clarithromycin.

^b Vector confers resistance to kanamycin, hygromycin, and clarithromycin.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study are listed in Table 1. *E. coli* XL1-Blue MRF' (*mcrA*)183 (*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lac*Δ*ZΔM15*], Tn10(Tet^r) (Stratagene) was used for the cloning and propagation of the plasmids. Transformants were grown in Luria-Bertani (LB) medium containing either ampicillin (50 μg/ml) or kanamycin (50 μg/ml). Clarithromycin was kindly provided by Abbott GmbH, Wiesbaden, Germany. Selection of *M. smegmatis* transformants was performed by using LB medium containing either kanamycin (25 μg/ml), hygromycin B, or clarithromycin at a concentration of 50 μg/ml.

DNA techniques. Standard methods were used for restriction endonuclease digestion of DNA and other manipulations (25). Plasmid DNA was isolated either by the alkaline lysis method (5) or with the Qiagen preparation according to the manufacturer's instruction (Diagen).

Nucleic acid sequencing was done manually with ³²P-labelled dCTP and Sequenase (U.S. Biochemicals) or with fluorescence-labelled nucleotides and by *Taq* cycle sequencing (Applied Biosystems).

Analysis of 16S rRNA position 1408 was performed by amplification of genomic DNA with primer 285 and primer 261 (the primers used in this study are listed in Table 2); the amplified products were subsequently sequenced with primer 289. The peptidyltransferase region of the 23S rRNA was investigated with primer 18 and primer 92 in a PCR. Subsequent sequencing of the amplified product was performed manually with primer 99.

Construction of vectors for transformation. To introduce the 16S rRNA mutation 1408G into plasmids pMV261-rRNA 2058G and pMV361-H-rRNA 2058G, mutagenesis was performed by PCR. The mutated 16S rRNA (1408 A→G) was generated by PCR amplification of the *rrn* operon of *M. smegmatis* with primer 285 in combination with mutagenesis primer 90 and mutagenesis primer 89 in combination with primer 294. The two amplified fragments were gel purified and used as template in a fusion PCR with primer 285 and primer 294. The products were subcloned in vector pT7-blue (Novagen). Doubled-stranded sequencing confirmed that only the desired mutation was introduced. Subsequently, a *SacI-Eco47III* fragment of plasmids pMV261-rRNA 2058G and pMV361-H-rRNA 2058G was replaced by the corresponding fragments containing the 16S rRNA gene mutation 1408 A→G, resulting in plasmids pMV261-rRNA 1408G/2058G and pMV361-H-rRNA 1408G/2058G, respectively.

Generation of *M. smegmatis* *rrnB recA* mutant strains. Plasmid precA(Ms)::hyg-rpsL⁺, which is a derivative of plasmid ptrpA-1-rpsL⁺ (26) and which carries the wild-type *rpsL* gene and the hygromycin-inactivated *recA* gene, was used for inactivation of the chromosomal *recA* gene of the *M. smegmatis* *rrnB* mutant strain. Hygromycin and streptomycin were used as positive and negative selectable markers, respectively, for gene replacement, resulting in *recA* mutant transformants. Plasmid precA(Ms)::hyg-rpsL⁺ was transformed into the streptomycin-resistant *M. smegmatis* *rrnB* mutant (27), and transformants were selected by plating on LB medium containing hygromycin (50 μg/ml) and streptomycin (50 μg/ml). Subsequent analysis of the mutant *recA* phenotype was performed by determination of susceptibility to DNA-damaging agents, such as ethyl methanesulfonate (0.1% [vol/vol]); the genotypes of the *recA* mutant strains were confirmed by Southern blot analysis.

Isolation of genomic DNA. Mycobacteria grown on Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (Difco) were harvested. The cells were suspended in 500 μl of NaCl (0.9%), heat inactivated for 10 min at 80°C, and pelleted by centrifugation (6,000 × g for 5 min). The pellet was resuspended in 400 μl of Tris-EDTA-Tween-lysozyme (10 mM, 10 mM, 0.1% [wt/vol], and 2 mg/ml, respectively) and incubated for 2 h at 37°C with constant shaking. Sodium dodecyl sulfate and proteinase K were added to final concentrations of 1% and 0.1 mg/ml, respectively. Incubation was continued for 1 h. Proteins were extracted by the addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]). The aqueous solution was extracted once with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated by adding 2.5 volumes of ethanol and 0.1 volume of 5 M NaCl. After incubation at -70°C for 30 min, the DNA was pelleted by centrifugation. The pellet was washed once with 1 ml of 70% ethanol, dried, and resuspended in TE (10 mM Tris, 1 mM EDTA).

RNAse protection assay. To generate DNA fragments as templates for in vitro transcription, standard PCR amplification reactions were performed (23). Sp6 RNA polymerase promoters were introduced into the amplified DNA fragment by using primers containing the RNA polymerase Sp6 recognition site. To amplify the peptidyltransferase region of the 23S rRNA gene, primer 99-Sp6 was used in combination with primer 100 (reaction a) and primer 100-Sp6 was used in combination with primer 99 (reaction b). Primer 261-Sp6 was used in combination with primer 297 (reaction a) and primer 297-Sp6 was used in combination with primer 261 (reaction b) to generate PCR products of the 16S rRNA gene. Amplified DNA fragments of reactions a and b were used as templates for the generation of sense and antisense transcripts, respectively. For in vitro transcription, approximately 100 ng of the PCR fragment derived from reaction a and 100 ng of the PCR fragment derived from reaction b were mixed together in a volume

TABLE 2. Primers used in this work

Primer	Sequence ^a	Species	Gene	Position ^b
18	AGTCGGGACCTAAGGCGAG	<i>M. smegmatis</i>	<i>rrl</i>	1342–1360
89	CCGCCCCTCGCGTCATGAAAG	<i>M. smegmatis</i>	<i>rrs</i>	1399–1419
90	CTTTCATGACGCGACGGGCGG	<i>M. smegmatis</i>	<i>rrs</i>	1419–1399
92	CATCCCCTCGATATGGACTC	<i>M. smegmatis</i>	<i>rrl</i>	2710–2691
99	GGTATTTCAACAACGACTCCA	<i>M. smegmatis</i>	<i>rrl</i>	2174–2154
99-Sp6	<u>GGATTTAGGTGACACTATAGAA</u> GGTATTTCAACAACGACTCCA			
100	CCTGAGAGGTGATGCATAGC	<i>M. smegmatis</i>	<i>rrl</i>	1734–1753
100-Sp6	<u>GGATTTAGGTGACACTATAGAA</u> CCTGAGAGGTGATGCATAGC			
261	AAGGAGGTGATCCAGCCGCA	<i>M. smegmatis</i>	<i>rrs</i>	1539–1514
261-Sp6	<u>GATTTAGGTGACACTATAGAA</u> AAGGAGGTGATCCAGCCGCA			
285	GAGAGTTTGATCCTGGCTCAG	<i>M. smegmatis</i>	<i>rrs</i>	9–29
289	AAGTCGGAGTCGCTAGTAAT	<i>M. smegmatis</i>	<i>rrs</i>	1323–1342
294	GATGCTCGCAACCACTATCC	<i>M. smegmatis</i>	<i>ITS I</i> ^c	1746–1727
297	TTCCTTGTGGCCTGTGTGCA	<i>M. smegmatis</i>	<i>rrs</i>	1017–1036
297-Sp6	<u>GATTTAGGTGACACTATAGAA</u> TCCCTTGTGGCCTGTGTGCA			

^a Sp6 promoter sequences are underlined. Mutagenized nucleotides are boxed.

^b *E. coli* numbering.

^c ITS I, internal transcribed spacer between the 16S rRNA and 23S rRNA genes.

TABLE 3. MICs for *M. smegmatis* strains transformed with replicative and integrative vectors

Strain	Transformed plasmid ^a	Aminoglycoside ^b MIC (μg/ml)
mc ² 155	None	0.6
	pMV361-H-rRNA 2058G	0.6
	pMV 361-H-rRNA 1408G/2058G	0.6
	pMV 261-rRNA 1408G/2058G	0.6
mc ² 155 <i>rrnB</i> mutant	None	0.6
	pMV361-H-rRNA 2058G	0.6
	pMV 361-H-rRNA 1408G/2058G	>200.0
	pMV 261-rRNA 1408G/2058G	>200.0
mc ² 155 <i>rrnB recA</i> mutant	None	0.6
	pMV361-H-rRNA 2058G	0.6
	pMV 361-H-rRNA 1408G/2058G	0.6
	pMV 261-rRNA 1408G/2058G	0.6

^a Four independent transformants of each plasmid were investigated.

^b Amikacin, gentamicin, and tobramycin.

of 10 μl in 0.75× buffer in the presence of ribosomal nucleoside triphosphates and 20 U of Sp6 RNA polymerase according to the manufacturer's instructions (Ambion). The mixture was then incubated for 90 min at 37°C. Afterward, an equal amount of hybridization buffer was added and the reaction mixtures were heated to 95°C for 3 min before they were slowly cooled to room temperature. An aliquot of 4 μl of the mixture was incubated either with RNase I (100-fold dilution in RNase buffer) for transcripts of the 23S rRNA gene or with a mixture of RNase II plus RNase III (100-fold dilution in RNase buffer) for transcripts of the 16S rRNA gene in RNase digestion buffer (16 μl) for 45 min at 37°C. The fragments were separated on a 2% agarose gel.

Transformation of mycobacteria. The parental strain *M. smegmatis* mc²155 (33), a single rRNA allelic strain *M. smegmatis* mc²155 SMR5 *rrnB* mutant rRNA allele (27), and strain *M. smegmatis* mc²155 SMR5 *rrnB recA* mutants were used for the transformation experiments. All strains were clarithromycin sensitive. Bacteria were made electrocompetent by the method described by Jacobs et al. (16), with the exception that the bacteria were grown in brain heart infusion medium (Oxoid) with 0.05% Tween 80 as described previously (26). The transformants were primarily selected on LB agar plates containing either kanamycin (25 μg/ml), clarithromycin (50 μg/ml), or hygromycin (50 μg/ml). After 3 to 5 days of incubation, single colonies were picked and colony purified for further investigations.

RESULTS

Transformation of *M. smegmatis* with plasmids carrying mutated rRNA alleles. To investigate the possible gene dosage effects of the rRNA^{mut} and rRNA^{wt} genes, two different *M. smegmatis* strains, wild-type strain *M. smegmatis* mc²155 with two functional rRNA operons and genetically modified strain *M. smegmatis* mc²155 *rrnB* mutant and one functional rRNA operon, were transformed with plasmids carrying a mutated *rrn* operon. As vectors, derivatives of the replicative plasmid pMV261 and the integrative plasmid pMV361 carrying a mutated rRNA operon (16S rRNA position 1408 A→G [*E. coli* numbering] and 23S rRNA position 2058 A→G [*E. coli* numbering]) were used. Plasmid pMV261-rRNA 1408G/2058G is based on the autonomously replicating plasmid pMV261 (34), which is present in at three to five copies per genome; plasmid pMV361-H-rRNA 1408G/2058G is based on the mycobacteriophage L5 *att/int* and integrates in a single copy per genome at the bacterial *attB* site (34). The mutation in 23S rRNA (2058 A→G) confers resistance to macrolides, such as clarithromycin, and is dominant in heterozygous strains (28). This mutation was used as a *cis*-selectable marker for expression of the transfected *rrn* operon. Transformants were selected on LB agar plates containing clarithromycin. The MICs of aminoglycosides such as amikacin, gentamicin, and tobramycin (0.1 to

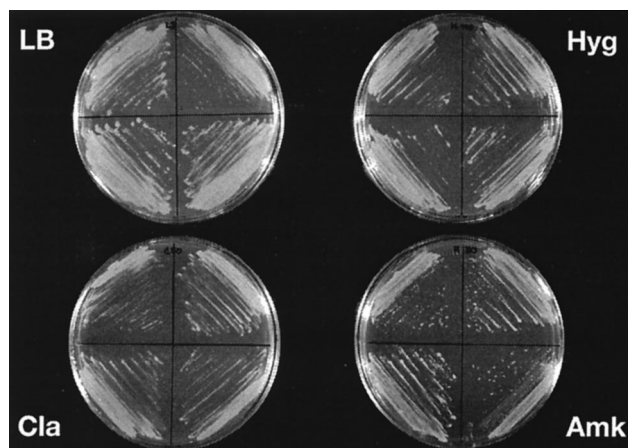


FIG. 1. Drug susceptibility patterns of four independent transformants of *M. smegmatis* *rrnB* mutants with integrative vector pMV361-H-rRNA 1408G/2058G; growth on control LB agar and on LB agar containing antibiotics. Clarithromycin (Cla) and hygromycin (Hyg) were used at 50 μg/ml each. Amikacin (Amk) was used at 200 μg/ml.

200 μg/ml) were determined to investigate the effects of the mutated rRNA alleles.

All strains obtained upon transformation of *M. smegmatis* mc²155 (four of four strains investigated) showed an aminoglycoside-sensitive phenotype (MIC, 0.6 μg/ml), regardless of whether the single-copy vector pMV361-H-rRNA 1408G/2058G or the multicopy vector pMV261-rRNA 1408G/2058G was used. In contrast, transformants of mutant strain *M. smegmatis* *rrnB* showed high-level aminoglycoside resistance even if vector pMV361-H-rRNA 1408G/2058G was used (Table 3 and Fig. 1). As illustrated in Fig. 1, growth of pMV361-H-rRNA 1408G/2058G transformants on amikacin was comparable to growth on LB, hygromycin (selectable marker on plasmid pMV361-H), or clarithromycin.

Characterization of *M. smegmatis* *rrnB* mutant transformants with an aminoglycoside-resistant phenotype. The genotype of the aminoglycoside-resistant transformants obtained by transformation of *M. smegmatis* mc²155 *rrnB* mutant with pMV361-H-rRNA 1408G/2058G was investigated by DNA sequencing and RNase protection assays. For this purpose, subcultures grown under selective conditions (on LB medium containing amikacin) and under nonselective conditions (on LB medium or LB medium containing clarithromycin) were used. Unexpectedly, the 16S rRNA genotypes of the transformants grown on medium containing aminoglycosides differed from the genotypes of the transformants grown on nonselective medium or on medium containing clarithromycin. Determination of the DNA sequences of transformants grown on LB medium revealed a heterozygous genotype with a wild-type (A) and a mutant (G) nucleotide at 16S rRNA position 1408, as demonstrated by the presence of a double band at the respective sequence position. In contrast, a homozygous genotype with a single mutant nucleotide (G) at this position was found in transformants grown in the presence of amikacin (Fig. 2A).

Analysis of the 23S rRNA genes of transformants grown on LB medium and LB medium containing clarithromycin was performed and revealed the simultaneous presence of a wild-type (A) and a mutant (G) nucleotide, indicating a heterozygous genotype of the 23S rRNA gene position 2058 for both subcultures (Fig. 2B).

To confirm the results obtained by DNA sequencing, RNase protection assays were performed for 16S rRNA region 1408

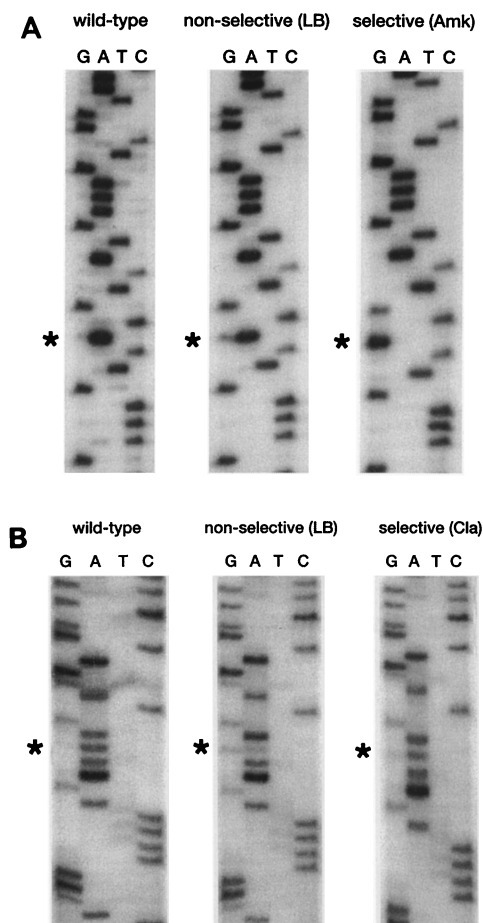


FIG. 2. (A) Nucleotide sequence of a short region of the PCR-amplified 16S rRNA gene of *M. smegmatis* *rrnB* mutants transformed with integrative vector pMV361-H-rRNA 1408G/2058G. The region shown corresponds to *E. coli* positions 1401 to 1416. Sequencing was performed with primer 289. The sequence of a nontransformed wild-type strain is shown along with those of the transformants grown on nonselective medium and selective medium (Amk, amikacin). The mutated base is indicated by an asterisk. (B) Nucleotide sequence of a short region of the PCR-amplified 23S rRNA gene of *M. smegmatis* *rrnB* mutants transformed with integrative vector pMV361-H-rRNA 1408G/2058G. The region shown corresponds to *E. coli* positions 2051 to 2066. Sequencing was performed with primer 99. The sequence of a nontransformed wild-type strain is shown along with those of the transformants grown on nonselective medium and selective medium (Cla, clarithromycin). The mutated base is indicated by an asterisk.

and 23S rRNA region 2058. In this assay, the RNA probes to be tested are hybridized to complementary antisense probes whose sequences are known (e.g., wild-type or mutant sequence). Mismatches in the RNA duplex are recognized and are cleaved by RNase, and the products that are obtained are separated by gel electrophoresis. DNA amplification of the corresponding regions was performed with a primer containing an Sp6 promoter, which allows in vitro transcription. Transcripts of the coding and the noncoding strands were synthesized by in vitro transcription with phage Sp6 RNA polymerase and the PCR product as the template. Sense transcripts were generated from PCR-amplified DNA fragments derived from a wild-type strain, from a cloned mutated (1408 A→G) *rrnB* operon, and from *M. smegmatis* *rrnB* transformants grown on nonselective medium and selective medium. Antisense transcripts were generated from PCR-amplified fragments derived from a cloned wild-type and a cloned mutated *rrnB* operon.

Hybridization of sense and antisense transcripts was followed by RNase digestion, and the cleavage products were separated by agarose gel electrophoresis. In this assay, homoduplexes withstand RNase cleavage and result in a RNA fragment with a molecular size of 521 bp for the 16S rRNA region. In contrast, heteroduplexes are cleaved by RNase. The cleaved products are indicated by the appearance of an RNA fragment with a smaller molecular size. A homozygous mutant genotype is present when a sense transcript of the strain to be investigated results in a noncleaved product upon hybridization to a mutated antisense probe, while a cleaved product is observed upon hybridization to a wild-type antisense probe. A heterozygous genotype is present when cleaved products are obtained upon RNase digestion when both a wild-type antisense probe and a mutated antisense probe are used.

The 16S rRNA gene (rDNA) transcripts from transformants grown on selective medium were cleaved by RNase when the transcripts were hybridized to wild-type antisense probes. No digestion products were observed when those transcripts were hybridized to mutated antisense probes. In contrast, 16S rDNA transcripts from transformants grown on nonselective medium were cleaved by RNase, irrespective of whether they were hybridized to wild-type or mutated antisense probes (Fig. 3A). Controls, i.e., homoduplexes of wild-type and mutated rRNAs, gave only a single noncleaved band after RNase digestion. These results confirm the conclusions drawn from DNA sequencing: a homozygous mutated 16S rRNA genotype was found in those transformants grown on selective medium, whereas a 16S rRNA heterozygous genotype was observed in transformants grown under nonselective conditions. As expected, cleaved products were obtained when 23S rDNA transcripts from transformants grown on selective or nonselective medium were hybridized to the corresponding wild-type and mutated antisense probes, indicating the heterozygous genotype of these transformants at the 23S rRNA allele (Fig. 3B).

Our results indicate that in a heterozygous strain and with growth under selective conditions, the wild-type 16S rRNA position 1408 acquired the respective drug resistance-conferring mutation. We determined the frequency of this event by plating serial dilutions of transformants on nonselective LB agar and on LB medium containing amikacin (50 µg/ml). By counting the numbers of CFU, we determined that the frequency of resistance in a heterozygous strain is approximately 10^{-4} . Apparently, mere streaking of strains on solid medium in order to investigate a drug-resistant phenotype grossly overestimates the proportion of resistant cells in a population (Fig. 1). The frequency that was determined (10^{-4}) is much higher than the frequency of spontaneous 16S rRNA mutations conferring aminoglycoside resistance in a strain with a single rRNA allele (10^{-8}) (27).

Characterization of *M. smegmatis* *recA* mutant transformants carrying resistant rRNA alleles. We hypothesized that RecA-mediated gene conversion is responsible for converting the wild-type allele into the resistant allele in our transformants. To prove this hypothesis, *recA* knockout derivatives from a strain *M. smegmatis* *rrnB* mutant were generated. Investigations with DNA-damaging agents indicated that mutant *M. smegmatis* *rrnB* *recA* strains were highly susceptible to DNA-damaging agents, a phenotype characteristic of mutant *recA* strains (12, 18, 38). The mutated *recA* genotype was confirmed by Southern blot analysis (data not shown). To investigate the effect of RecA on aminoglycoside resistance in a heterozygous rRNA^{wild}/rRNA^{mut} strain, an *M. smegmatis* *rrnB* and *recA* mutant was transformed with plasmids pMV261-rRNA 1408G/2058G and pMV361-H-rRNA 1408G/2058G, and transformants were selected by plating on clarithromycin.

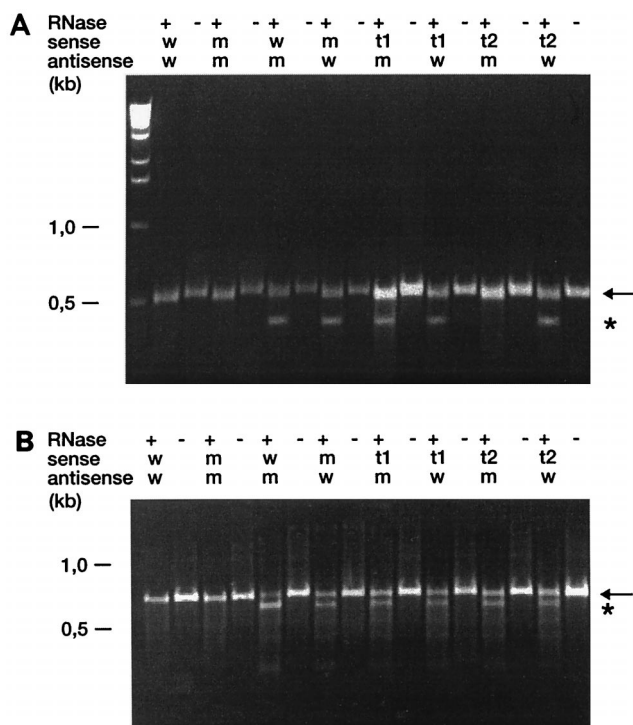


FIG. 3. (A) RNase protection assay of a 16S rRNA region encompassing *E. coli* nucleotide position 1408. A mutant *M. smegmatis* *rrnB* strain transformed with integrative vector pMV361-H-rRNA 1408G/2058G was analyzed. RNA hybrids were incubated in the presence (+) or absence (–) of RNase at 37°C for 45 min. Sense RNAs were generated with PCR products from a cloned wild-type rRNA operon (w), from a cloned mutated rRNA operon 1408 A→G (m), or from the transformants grown on nonselective medium (t1) or selective medium (t2). Antisense RNAs were derived from cloned wild-type (w) and mutated (m) rRNA operons. The cleaved and noncleaved products are indicated by an asterisk and an arrow, respectively. (B) RNase protection assay of a 23S rRNA region encompassing *E. coli* nucleotide position 2058. A mutant *M. smegmatis* *rrnB* strain transformed with integrative vector pMV361-H-rRNA 1408G/2058G was analyzed. RNA hybrids were incubated in the presence (+) or absence (–) of RNase at 37°C for 45 min. Sense RNAs were generated with PCR products from a cloned wild-type rRNA operon (w), from a cloned mutated rRNA operon 2058 A→G (m), or from the transformants grown on nonselective medium (t1) or selective medium (t2). Antisense RNAs were derived from cloned wild-type (w) and mutated (m) rRNA operons. The cleaved and noncleaved products are indicated by an asterisk and an arrow, respectively.

Subsequently, the MICs of different aminoglycosides were determined, and an aminoglycoside-sensitive phenotype was demonstrated for these transformants (Table 3 and Fig. 4). No evidence of a gene dosage effect was observed, because transformants with the replicative vector carrying approximately five copies of the mutated operon showed a sensitive phenotype. The drug susceptibilities of the transformants that were obtained were identical to that of the nontransformed parental strain (amikacin MICs, 0.6 µg/ml).

DNA sequencing of the *recA* mutant transformants demonstrated sequence ambiguities in terms of double bands that indicated a mixed genotype at 16S rRNA position 1408 (Fig. 5A). The mixed genotype was also confirmed by an RNase protection assay after PCR-mediated DNA amplification of the 16S rRNA gene (Fig. 6A). As a control, DNA sequencing and RNase protection assays of the 23S rRNA gene were performed and showed a mixed genotype at 23S rRNA position 2058 in these transformants (Fig. 5B and 6B).

These results indicate (i) that aminoglycoside sensitivity is dominant over resistance in a heterozygous strain and (ii) that

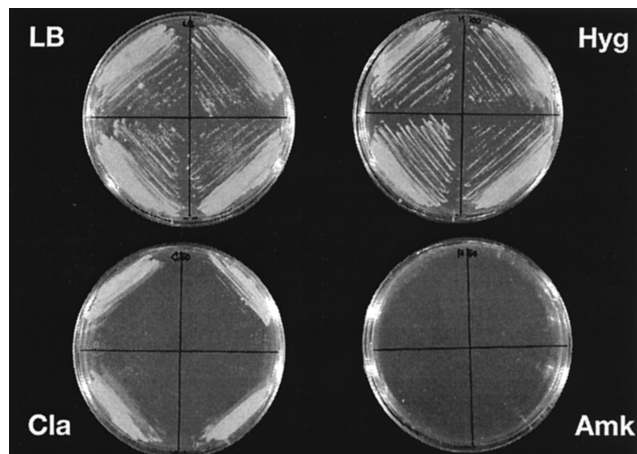


FIG. 4. Drug susceptibility patterns of four independent transformants of *M. smegmatis* *rrnB recA* mutants with integrative vector pMV361-H-rRNA 1408G/2058G; growth on control LB agar and on LB agar containing antibiotics. Clarithromycin (Cla) and hygromycin (Hyg) were used at 50 µg/ml each. Amikacin (Amk) was used at 200 µg/ml.

the resistant phenotype of a previously heterozygous rRNA^{wt}/rRNA^{mut} strain is due to RecA-dependent gene conversion.

DISCUSSION

Methylation of 16S rRNA positions 1405 and 1408 in aminoglycoside-producing organisms, such as *Streptomyces* spp. and *Micromonospora purpurea*, confers resistance to 2-deoxystreptamine aminoglycosides. Posttranscriptional modification of these nucleotides by methylation prevents binding of the antibiotics to their targets (4, 8, 22, 31, 32, 36). Our previous work has demonstrated that ribosomal proteins play no role in acquired resistance to 2-deoxystreptamine aminoglycosides but that mutational target alterations are limited to the rRNA of the small ribosomal subunit. Specifically, a mutation at 16S rRNA position 1408 (A→G) conferred resistance to 2-deoxystreptamine aminoglycosides (27). Spontaneous aminoglycoside-resistant mutants were found only for organisms which carry a single rRNA operon. Efforts to generate aminoglycoside-resistant mutants of organisms with two rRNA operons were unsuccessful (27), suggesting that the dominance of aminoglycoside sensitivity over resistance prevented the isolation of resistant mutants.

The question of whether recessivity or dominance of mutational target alterations causes aminoglycoside resistance has been a matter of debate (3, 11, 35). Mutations in the *E. coli* 16S rRNA-decoding region that confer resistance to aminoglycosides were demonstrated to be dominant in cells growing in the presence of low antibiotic concentrations but recessive in cells growing in the presence of high drug concentrations (11). Apirion and Schlessinger (3) demonstrated that kanamycin resistance in *E. coli* is recessive. Using a conjugation system, Taniguchi et al. (35) suggested that aminoglycoside susceptibility is dominant in heterogenomic strains of *M. smegmatis*. In contrast, upon UV irradiation the same investigators (35) obtained aminoglycoside-resistant mutants at a frequency of approximately 10⁻⁹ for organisms carrying two wild-type rRNA operons, indicating a dominance of aminoglycoside resistance (35).

Targeted inactivation of the *recA* gene in merodiploids carrying a wild-type rRNA operon and a drug-resistant mutated rRNA operon allowed us to establish that resistance due to

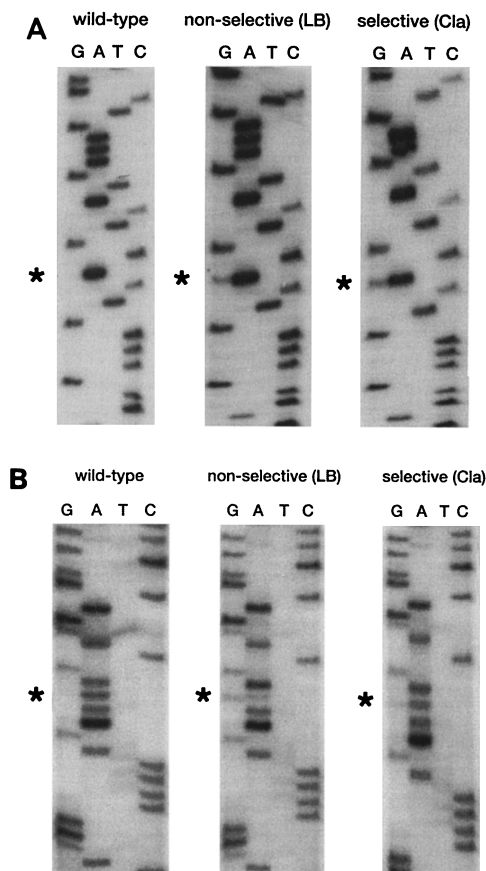


FIG. 5. (A) Nucleotide sequence of a short region of the PCR-amplified 16S rRNA gene of *M. smegmatis* *rmB recA* mutants transformed with integrative vector pMV361-H-rRNA 1408G/2058G. The region shown corresponds to *E. coli* positions 1401 to 1416. Sequencing was performed with primer 289. The sequence of a nontransformed wild-type strain is shown along with those of the transformants grown on nonselective medium and selective medium (Cla, clarithromycin). The mutated base is indicated by an asterisk. (B) Nucleotide sequence of a short region of the PCR-amplified 23S rRNA gene of *M. smegmatis* *rmB recA* mutants transformed with integrative vector pMV361-H-rRNA 1408G/2058G. The region shown corresponds to *E. coli* positions 2051 to 2066. Sequencing was performed with primer 99. The sequence of a nontransformed wild-type strain is shown along with those of the transformants grown on nonselective and selective medium (Cla, clarithromycin). The mutated base is indicated by an asterisk.

mutational target alteration is recessive. Interestingly, $rRNA^{wt}/rRNA^{mut}$ heterozygous strains with a *recA*⁺ genotype were genetically unstable under selective pressure. Transformants able to grow on aminoglycoside-containing medium demonstrated a homozygous mutant genotype at 16S rRNA position 1408. RecA-mediated gene conversion was shown to be responsible for conversion of the wild-type allele into the mutant allele in these transformants. In organisms with a limited number of rRNA operons and a heterozygous rRNA genotype, RecA-mediated gene conversion promotes the generation of homozygous strains at a high frequency. Cells obtained by transformation of the mutant *M. smegmatis* *rmB recA* strain containing one chromosomal wild-type and one plasmid-encoded mutated rRNA operon were genetically stable and highly sensitive to aminoglycoside antibiotics. From these results one must draw the conclusion that aminoglycoside resistance is recessive in heterozygous $rRNA^{wt}/rRNA^{mut}$ strains in the presence of high as well as low drug concentrations.

Our results and the conclusions based thereon explain why

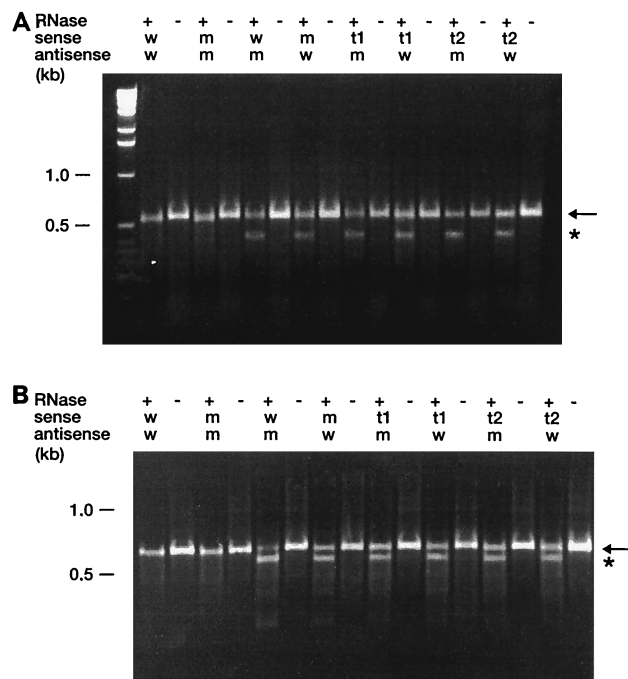


FIG. 6. (A) RNase protection assay of a 16S rRNA region encompassing *E. coli* nucleotide position 1408. A mutant strain of *M. smegmatis* *rmB recA* transformed with integrative vector pMV361-H-rRNA 1408G/2058G was analyzed. RNA hybrids were incubated in the presence (+) or absence (-) of RNase at 37°C for 45 min. Sense RNAs were generated with PCR products from a cloned wild-type rRNA operon (w), from a cloned mutated rRNA operon 1408 A→G (m), or from the transformants grown on nonselective medium (t1) or selective medium (t2). Antisense RNAs were derived from cloned wild-type (w) and mutated (m) rRNA operons. The cleaved and noncleaved products are indicated by an asterisk and an arrow, respectively. (B) RNase protection assay of a 23S rRNA region encompassing *E. coli* nucleotide position 2058. A mutant strain of *M. smegmatis* *rmB recA* transformed with integrative vector pMV361-H-rRNA 1408G/2058G was analyzed. RNA hybrids were incubated in the presence (+) or absence (-) of RNase at 37°C for 45 min. Sense RNAs were generated with PCR products from a cloned wild-type rRNA operon (w), from a cloned mutated rRNA operon 2058 A→G (m), or from the transformants grown on nonselective medium (t1) or selective medium (t2). Antisense RNAs were derived from cloned wild-type (w) and mutated (m) rRNA operons. The cleaved and noncleaved products are indicated by an asterisk and an arrow, respectively.

aminoglycoside resistance due to mutational rRNA alterations has not been found in organisms that carry more than one rRNA operon, such as members of the family *Enterobacteriaceae*, staphylococci, and streptococci. The data presented here provide the experimental evidence for the observed rarity of acquired aminoglycoside resistance during drug therapy in such organisms.

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