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Mechanisms of Streptomycin Resistance: Selection of Mutations in the 16S rRNA Gene Conferring Resistance

BURKHARD SPRINGER, YISHAK G. KIDAN, THERDSAK PRAMMANANAN,†
KERSTIN ELLROTT, ERIK C. BÖTTGER,‡ AND PETER SANDER*

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

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Chromosomally acquired streptomycin resistance is frequently due to mutations in the gene encoding the ribosomal protein S12, *rpsL*. The presence of several rRNA operons (*rrn*) and a single *rpsL* gene in most bacterial genomes prohibits the isolation of streptomycin-resistant mutants in which resistance is mediated by mutations in the 16S rRNA gene (*rrs*). Three strains were constructed in this investigation: *Mycobacterium smegmatis rrnB*, *M. smegmatis rpsL*$^{3+}$, and *M. smegmatis rrnB rpsL*$^{3+}$. *M. smegmatis rrnB* carries a single functional *rrn* operon, i.e., *rrnA* (comprised of 16S, 23S, and 5S rRNA genes) and a single *rpsL*$^{+}$ gene; *M. smegmatis rpsL*$^{3+}$ is characterized by the presence of two *rrn* operons (*rrnA* and *rrnB*) and three *rpsL*$^{+}$ genes; and *M. smegmatis rrnB rpsL*$^{3+}$ carries a single functional *rrn* operon (*rrnA*) and three *rpsL*$^{+}$ genes. By genetically altering the number of *rpsL* and *rrs* alleles in the bacterial genome, mutations in *rrs* conferring streptomycin resistance could be selected, as revealed by analysis of streptomycin-resistant derivatives of *M. smegmatis rrnB rpsL*$^{3+}$. Besides mutations well known to confer streptomycin resistance, novel streptomycin resistance conferring mutations were isolated. Most of the mutations were found to map to a functional pseudoknot structure within the 530 loop region of the 16S rRNA. One of the mutations observed, i.e., 524G→C, severely distorts the interaction between nucleotides 524G and 507C, a Watson-Crick interaction which has been thought to be essential for ribosome function. The use of the single rRNA allelic *M. smegmatis* strain should help to elucidate the principles of ribosome-drug interactions.

Many antibiotics inhibit the growth of bacteria by targeting protein biosynthesis (3, 7, 34). Streptomycin, an aminocyclitol aminoglycoside, has been shown to interact directly with the small ribosomal subunit (8, 24). The ribosome accuracy center is a highly conserved component of the translational apparatus (1), comprising an rRNA domain and several polypeptides of the small subunit, including the ribosomal protein S12 (8, 29). A number of mutations in the *rpsL* gene encoding the S12 polypeptide generate resistance to streptomycin (10, 36, 46, 47, 49).

Rather than being a mere scaffold for ribosomal proteins, the rRNA has important functions and is a main target for drugs interfering with bacterial protein synthesis (12, 26, 33, 42). Mutations within rRNA genes have been found to confer drug resistance; for some of these mutations experimental proof for a cause-effect relationship has been provided (9, 21, 40, 50). More recently, mutations in rRNA genes have been found to be associated with in vivo acquired drug resistance in bacterial pathogens, e.g., in *Mycobacterium tuberculosis* resistant to streptomycin (10); most of the mutations found mapped to the 530 region of 16S rRNA (15, 27). This unique mechanism of acquired resistance due to mutational rRNA alterations has been attributed to the presence of a single *rrn* operon in this pathogen (7).

The 530 loop region is one of the most highly conserved 16S rRNA regions both in sequence and in secondary structure (13). The 530 loop region is part of the aminoacyl-tRNA binding site (A-site) and is involved in the decoding process (8, 25). Several lines of evidence indicate that the universally conserved 530 loop of 16S rRNA—in particular a pseudoknot structure formed by residues 526-CCG-524 and 505-GGC-507—plays a crucial role in translation, with mild perturbations of this structure, e.g., creation of G-U wobble base pairs, generating resistance to and reducing affinity for streptomycin (31). Data from in vitro assembly studies suggest that the pseudoknot structure is stabilized by ribosomal protein S12, which protects these bases from attack by kethoxal and dimethyl sulfate (44). The specificity of these probes for N1 and N2 of guanine and N3 of cytosine provided direct evidence for a Watson-Crick pairing, rather than some alternative mode of base pair interaction.

During the past years tremendous progress has been made in the analysis of ribosomes and most recently their structure has been resolved at an atomic resolution (4, 28, 39, 48). The crystal structure of the 30S subunit complexed with streptomycin (8) identified the 16S rRNA nucleotides directly involved in drug binding. However, streptomycin resistance-associated mutations have not only been observed in nucleotides directly involved in drug binding (10, 15, 16, 27). In addition, translation is a highly dynamic process which requires alterations in base pairing resulting in conformational changes (20). Thus, besides crystallographic analyses, additional investigations including chemical, biochemical, and genetic methods will be necessary to elucidate the mechanisms of antibiotic action.

M. tuberculosis is a pathogenic microorganism which grows very slowly (generation time, 20 to 24 h) and is hardly amena-

* Corresponding author. Present address: Institut für Medizinische Mikrobiologie, Universität Zürich, Gloriastr. 30/32, CH-8028 Zurich, Switzerland. Phone: 41-1-634-2684. Fax: 41-1-634-4906. E-mail: sander.peter@gmx.de.

† Present address: Division of Mycology and Mycobacteriology, Faculty of Medicine, Mahidol University, Bangkok 10700, Thailand.

TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Source or reference	Description
Strains		
E. coli XL-1 Blue MRF′	Stratagene	
M. smegmatis mc^2 155	41	Two functional *rrn* operons, one *rpsL*
M. smegmatis rpsL^{3+}	This study	Two functional *rrn* operons, three *rpsL*
M. smegmatis rrnB	This study	One functional *rrn* operon, one *rpsL*
M. smegmatis rrnB rpsL^{3+}	This study	One functional *rrn* operon, three *rpsL*
Plasmids		
prRNA4-4K1	36	
pGEM-T vector	Promega	
pLO2	19	
pHRM3-Gm[a]	11	
pMV361-H-rRNA2058G	38	
prRNA::*aph-sacB*[b]	This study	
pMV361-H-rRNA523C/2058G[c]	This study	
pMV361-H-rRNA524C/2058G[c]	This study	
pMV361-H-rRNA526T/2058G[c]	This study	

[a] *pyrF* targeting vector.
[b] Targeting vector for inactivation of *rrnB*.
[c] Integrating vector with mutated *rrn* operon.

ble to genetic manipulations. The lack of a eubacterium suitable for genetic manipulations, which allows the isolation of in vivo selection-driven mutational *rrs* alterations conferring resistance to streptomycin, severely hampers our understanding of structure-function relationship within rRNAs. The presence of a limited number of rRNA operons, its apathogenic nature, the high growth rate, and the ability for genetic manipulations make *Mycobacterium smegmatis* an ideal host for the investigation of eubacterial rRNA structure-function relationships (32, 37, 38). More recently, functional inactivation of the *Escherichia coli rrn* operons has been used to generate strains with a single rRNA operon (2).

To learn more about (i) mechanisms of streptomycin resistance in general, (ii) structure-function relationships in rRNA, in particular 16S rRNA-mediated streptomycin resistance, and (iii) dominance-recessivity relationships of ribosomal resistance mechanisms, *M. smegmatis* strains with different copy numbers of genes encoding the targets of streptomycin, i.e., *rpsL* and *rrs,* were generated. Strains were constructed to allow selection of streptomycin resistance-conferring mutations to appear exclusively in *rrs*. Drug resistance mutations were mapped in *rrs*, and by allele exchange techniques a cause-effect relationship of the mutations observed was demonstrated. We made the surprising finding that a base pair interaction thought to be required for proper ribosome function is severely affected by one of the most frequently identified mutations.

MATERIALS AND METHODS

DNA manipulations. Standard methods were used for restriction endonuclease digestion of DNA, hybridization analysis, and other manipulations (35). Plasmid DNA was isolated by the alkaline lysis method (5) or by using the Qiagen plasmid DNA preparation kit according to the manufacturer's instructions. All initial cloning procedures were performed with *E. coli* XL-1 Blue MRF′ (Stratagene). Transformants were grown in Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Ampicillin, kanamycin, gentamicin, and hygromycin were obtained from Sigma; clarithromycin was a generous gift from Abbott GmbH (Wiesbaden).

DNA probes for Southern blot hybridization were labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim).

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Plasmid prRNA::*aph-sacB* was constructed by digestion of plasmid prRNA4-4K1 with *Sal*I and ligation to a 2-kbp *sacB* gene fragment (*Eco*RV/*Bam*HI) from plasmid pLO2 (19).

16S rRNA gene fragments carrying mutations *rrs*523A→C, *rrs*524G→C, and *rrs*526C→T were obtained by PCR amplification using DNA isolated from spontaneous streptomycin-resistant *M. smegmatis* strains carrying the corresponding mutation. PCR amplification was performed with primers #285 and #251 (Table 2). PCR products were subcloned into the pGEM-T vector (Promega). A 600-bp *Xho*I/*Eco*RV fragment containing the 530 loop region was isolated and used to replace the homologous gene fragment in plasmid pMV361-H-rRNA2058G, which previously was digested to completion with *Eco*RV and partially digested with *Xho*I. Plasmid pMV361-H-rRNA2058G expresses a functional *rrnB* operon under control of its own promoter. Sequencing with primers #242 and #259 confirmed that only the desired mutation was present. Resulting plasmids were pMV361-H-rRNA523C/2058G, pMV361-H-rRNA524C/2058G, and pMV361-H-rRNA526T/2058G, respectively.

Transformation of mycobacteria. Mycobacteria were made electrocompetent essentially as described previously (36). This method is a modification of the method described by Jacobs et al. (17). In short, *M. smegmatis* strains were grown until an optical density at 600 nm of 0.4 to 0.6 was achieved, and cells were incubated on ice for 1.5 h. The cells were then collected by centrifugation, washed several times with ice-cold glycerol (10%, vol/vol), and finally were resuspended in a 1/500 volume of glycerol (10%). Cells (100 μl) were mixed with 1 μg of plasmid DNA. Electroporation was performed in 0.2-cm cuvettes with a single pulse (2.5 kV, 25 μF, 1,000 Ω) in a Bio-Rad gene pulser. Cells were immediately resuspended in 1 ml of brain heart infusion medium and incubated for 2 h with vigorous shaking at 37°C. Afterwards serial dilutions were plated. When appropriate, antibiotics or sucrose was added at the following concentrations: kanamycin, hygromycin, or clarithromycin, 50 μg/ml; gentamicin, 15 μg/ml; sucrose, 7.5% (wt/vol).

TABLE 2. Primers used in the study

Primer	Sequence	Source	Gene or phage amplified	Position[a]
#11	GTCGAGGTCACGGCGTAC	*M. tuberculosis*	*rpsL*	181–198
#211	CCCACCATTCAGCAGCTGGT	*Mycobacteria*	*rpsL*	
#212	GTCGAGCGAACCGCGAATGA	*Mycobacteria*	*rpsL*	
#242	CTACGGGAGGCAGCAGTGGG	*M. smegmatis*	*rrs*	340–359
#251	GGCATCGCAGCCCTTTGTAC	*M. smegmatis*	*rrs*	1,256–1,237
#259	TTTCACGAACAACGCGACAA	*M. smegmatis*	*rrs*	599–580
#261	AAGGAGGTGATCCAGCCGCA	*M. smegmatis*	*rrs*	1,539–1,520
#264	TGCACACAGGCCACAAGGGA	*M. smegmatis*	*rrs*	1,072–1,052
#285	GAGAGTTTGATCCTGGCTCAG	*M. smegmatis*	*rrs*	9–29
#612	GTAATACGACTCACTATAGGGC	pHRM3-GM	T7	625–646
#651	AATTAACCCTCACTAAAGGG	pHRM3-GM	T3	2,890–2,871

[a] *E. coli* numbering. The positions of primers #211 and #212 can be found in reference 15.

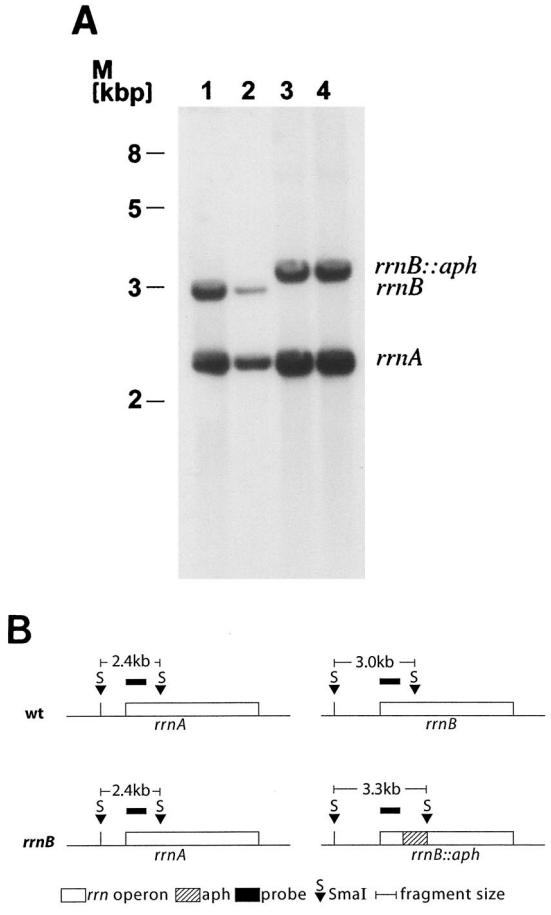

FIG. 1. Southern blot analysis (A) and schematic drawing (B) of the *rrn* loci. Lane 1, *M. smegmatis* mc^2 155; lane 2, *M. smegmatis* mc^2 155 *rpsL*$^{3+}$; lane 3, *M. smegmatis rrnB* (#1432); lane 4, *M. smegmatis rrnB* (#1434). Approximately 200 ng of genomic DNA was digested to completion with *Sma*I and hybridized to a 5′ probe of the *M. smegmatis* 16S rRNA gene (16S rRNA positions 5 to 788). Hybridization was performed under stringent conditions. The lower band corresponds to the *rrnA* operon, and the upper band corresponds to the *rrnB* operon. A shift of the upper band indicates functional inactivation of the *rrnB* operon. wt, wild type; M, molecular size standards.

Mapping of streptomycin resistance-conferring mutations. Spontaneous streptomycin-resistant mutants were generated by spreading 1×10^8 to 5×10^9 bacteria on brain heart infusion agar plates containing streptomycin at a concentration of 20 μg/ml; in parallel, dilutions of the bacterial suspension were plated on antibiotic-free medium. The frequency of resistance mutations was calculated by dividing the number of CFU on selective medium by the number of CFU on nonselective medium. An approximation of the mutational rate is given as the median of at least five independent experiments. MICs of streptomycin were determined by spreading mutants on agar plates containing 20, 100, and 200 μg of streptomycin/ml. Incubation time was between 3 and 5 days.

For nucleic acid extraction, a small loop of bacteria was dispersed in 100 μl of H_2O and heated for 10 min at 80°C. After addition of glass beads (100-μm diameter; Sigma) a tissue disintegrator (H. Mickle) was used to disrupt the cells. Following centrifugation the supernatant was transferred to a fresh microcentrifuge tube, and 5 μl was used as template for amplification PCRs.

Primers #211 and #212 (15) (see Table 2) were used to amplify *rpsL*, and primers #285 and #261 were used for amplification of *rrs*. Sequencing of *rpsL* was performed with #211; sequencing of *rrs* was performed with primers #242, #259, #261, #264, and #285. Primer #11 in combination with primer #612 or #651 was used to confirm transformation with plasmid pHRM3-Gm. An ABI 373 sequencer was used for sequence determination.

RecA-mediated gene conversion. To obtain strains which had undergone homologous recombination, clones obtained by transformation with vectors pMV361-H-rRNA523C/2058G, pMV361-H-rRNA524C/2058G, and pMV361-H-rRNA526T/2058G were grown in liquid broth, and serial dilutions were plated on LB agar containing streptomycin at a concentration of 20 μg/ml. The frequency of streptomycin-resistant recombinants was calculated by dividing the number of CFU obtained on agar plates containing streptomycin by the number of CFU obtained on nonselective medium.

Transformants were analyzed by manual DNA sequencing of the 16S ribosomal DNA (rDNA) PCR products (see above) using ^{32}P-labeled dCTP, sequenase (U.S. Biochemicals), and primer #259 to investigate their genotype (homogeneous or heterogeneous).

RESULTS

Generation of strains. The genome of *M. smegmatis* carries two *rrn* operons (*rrnA* and *rrnB*) and a single *rpsL* gene. Genetic engineering techniques were used to generate strains of *M. smegmatis* with different copy numbers of the main streptomycin target genes. The strains and plasmids used are shown in Table 1. prRNA::*aph-sacB* carries an inactivated *rrnB* operon from *M. smegmatis*, including the promoter region, the 5′ part of *rrs*, the 3′ part of *rrl*, and the entire *rrf*. An *aph* cassette cloned between the partially deleted *rrs* and *rrl* confers resistance to kanamycin; the inactivated *rrn* operon was cloned proximal to a *sacB* cassette. *sacB* facilitates the isolation of

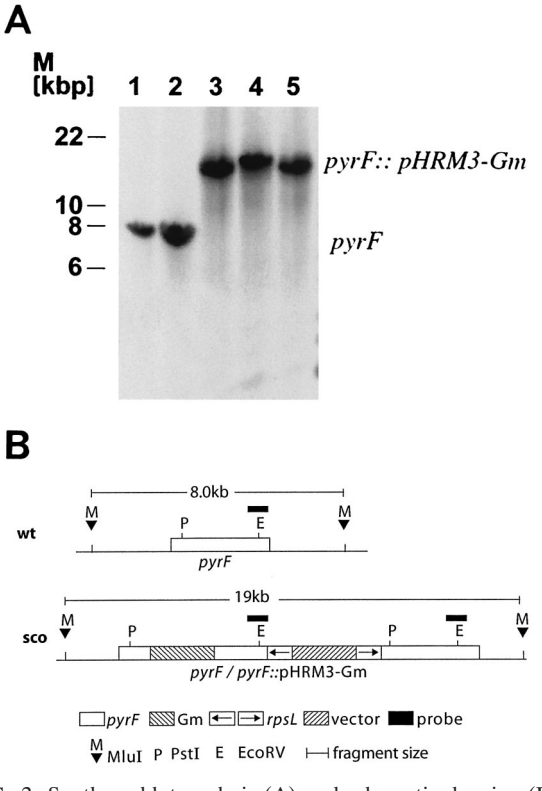

FIG. 2. Southern blot analysis (A) and schematic drawing (B) of the *pyrF* locus. Lane 1, *M. smegmatis* mc^2 155; lane 2, *M. smegmatis rrnB*; lane 3, *M. smegmatis rpsL*$^{3+}$; lane 4, *M. smegmatis rrnB rpsL*$^{3+}$; lane 5, streptomycin-resistant mutant (*rrs*524C) of *M. smegmatis rrnB rpsL*$^{3+}$. M, molecular size standard. Approximately 200 ng of genomic DNA was digested to completion with *Mlu*I and hybridized to a 1-kb *Sac*I fragment of the *pyrF* gene. Additional recognition sites are given for better orientation. Integration of vector pHRM3-Gm at the *pyrF* locus by a single crossover (sco) is indicated by a shift of the hybridizing fragment. wt, wild type.

TABLE 3. Frequency of streptomycin-resistant mutants of *M. smegmatis* strains carrying different numbers of functional *rrn* and *rpsL* genes

Strain	No. of *rrn* operons	No. of *rpsL* genes	Median mutation frequency[a]	Mutation frequency range
M. smegmatis mc^2 155 (wild type)	2	1	1.3×10^{-8}	6.6×10^{-8}–1.5×10^{-9}
M. smegmatis rpsL^{3+}	2	3	$<2 \times 10^{-12}$	
M. smegmatis rrnB	1	1	6.6×10^{-9}	2.1×10^{-8}–1.3×10^{-9}
M. smegmatis rrnB rpsL^{3+}	1	3	4.4×10^{-10}	1.3×10^{-8}–2.0×10^{-10}

[a] Median from at least five experiments.

allelic replacement mutants, as it confers sensitivity towards sucrose in mycobacteria (30).

Following transformation with prRNA::*aph-sacB*, clones resistant to kanamycin and sucrose were characterized genetically. Investigation by PCR and Southern blot analysis (Fig. 1) demonstrated inactivation of the *rrnB* operon in two of seven strains analyzed (*M. smegmatis rrnB* strains 1432 and 1434).

We next wanted to integrate additional *rpsL*$^+$ alleles into the genome of *M. smegmatis*. Previous investigations have revealed that the integrative vector pMV361 is unstable under negative selective pressure and is frequently lost from the genome (43). To ensure that the introduced *rpsL* alleles are stably maintained, they were integrated into a specific chromosomal position using a targeting vector. Strains *M. smegmatis rrnB* 1432 and 1434 as well as the parental strain *M. smegmatis* mc^2 155 (41) were transformed with plasmid pHRM3-Gm, a derivative of the suicide vector pHRM3, which targets *pyrF* (11). The coding region of *pyrF* is interrupted by a gentamicin resistance marker gene. Each side of the *pyrF* gene (5′ and 3′ regions) is flanked by a wild-type *rpsL* gene from *Mycobacterium bovis* BCG. Transformants were selected on uracil-free medium (to ensure a single crossover at the *pyrF*, thereby retaining a functional *pyrF* allele) in the presence of gentamicin. The presence of two additional *M. bovis* BCG *rpsL* wild-type genes in the transformants was confirmed by PCR analysis (data not shown). Southern blot analysis demonstrated that plasmid pHRM3-Gm had integrated at the genomic *pyrF* locus by homologous recombination (Fig. 2).

After transformation, strains with the following genotypes were generated: (i) *M. smegmatis rrnB* (strain with a single functional *rrn* operon and a single *rpsL*$^+$ gene), (ii) *M. smegmatis rpsL*$^{3+}$ (*rrn* wild-type strain with three *rpsL*$^+$ genes), and (iii) *M. smegmatis rrnB rpsL*$^{3+}$ (single *rrn* allelic strain with three *rpsL*$^+$ genes [Table 3]).

Isolation of streptomycin-resistant mutants. To select for streptomycin-resistant mutants, strains *M. smegmatis* mc^2 155, *M. smegmatis rrnB*, *M. smegmatis rpsL*$^{3+}$, and *M. smegmatis rrnB rpsL*$^{3+}$ were plated on agar containing streptomycin at a concentration of 20 μg/ml. Mutants were readily obtained for strains containing either a single *rpsL*$^+$ gene (*M. smegmatis* mc^2 155, *M. smegmatis rrnB*) or a single functional rRNA operon (*M. smegmatis rrnB rpsL*$^{3+}$). No streptomycin-resistant mutants could be isolated from *M. smegmatis* carrying two *rrn* operons and three *rpsL* genes (*M. smegmatis rpsL*$^{3+}$). The mutation frequencies determined are given in Table 3.

Characterization of spontaneous streptomycin-resistant strains. To locate the genetic alterations associated with streptomycin resistance, sequence analysis of *rpsL* and *rrs* genes was performed. Investigation of 20 streptomycin-resistant derivatives of *M. smegmatis* mc^2 155 identified *rpsL* mutations in each of these mutants. Mutations were restricted to codons 42 and 87. Changes in codon 42 resulted in the replacement of Lys by Arg, Thr, Asn, or Met; changes in codon 87 resulted in the replacement of Lys by Glu or Arg. The streptomycin-resistant derivatives of the single *rrn* allelic strain *M. smegmatis rrnB* also revealed an altered *rpsL* sequence (four of four investigated; see Table 4). Sequence analysis of the single functional *rrs* gene in streptomycin-resistant derivatives of *M. smegmatis rrnB rpsL*$^{3+}$ demonstrated alterations in the 530 loop region in all mutants (22 of 22 investigated; see Table 4) (Fig. 3). The frequency with which mutants appeared on the selection plate corresponds to the frequency of single point mutations in *M. smegmatis* rDNA (10^{-8} to 10^{-10} [37, 38]). The most frequent mutation (found in 12 out of 22 sequences) observed was a G→C transversion at position 524 (*E. coli* numbering). A mutation at this position previously has not been associated with resistance to streptomycin. The other prominent mutation was a 526 C→T transition (found in 7 out of 22 sequences). Two mutants exhibited a C→T transition at position 522, and a single resistant strain showed a 523A→C transversion. No mutations were found in *rrs* nucleotides directly interacting with streptomycin (e.g., G527, A913, and A914). However, the possibility that additional mutants, e.g., in the 912 region (15), might be isolated when screening a larger number of mutants cannot be excluded.

The MICs determined (Table 5) showed high levels of streptomycin resistance for *rpsL* mutant strains and for *rrs* mutation 524G→C (>200 μg/ml). *rrs* mutations 526C→T, 522 C→T, and 523 A→C conferred an intermediate level of resistance (100 μg/ml). Strains carrying the mutation *rrs*524G→C grew poorly in the absence of streptomycin; growth was restored in the presence of streptomycin, indicating that *rrs*524C confers a streptomycin-dependent phenotype.

Introduction of sensitive and resistant *rrs* alleles into wild-type and mutant strains. To demonstrate that the observed streptomycin-resistant phenotype in strains carrying *rrs* mutations maps to the small subunit rRNA and to exclude other unknown mutations, transformations with vector pMV361-H-rRNA2058G (38) were carried out in strains carrying mu-

TABLE 4. Streptomycin resistance-associated mutations

Mutated gene or nucleotide change	*M. smegmatis* strain investigated (no. of altered sequences/total no. investigated)		
	mc^2 155	*rrnB*	*rrnB rpsL*$^{3+}$
rpsL	20/20	4/4	0/22
rrs	0/20	0/4	22/22
522C→T			2/22
523A→C			1/22
524G→C			12/22
526C→T			7/22

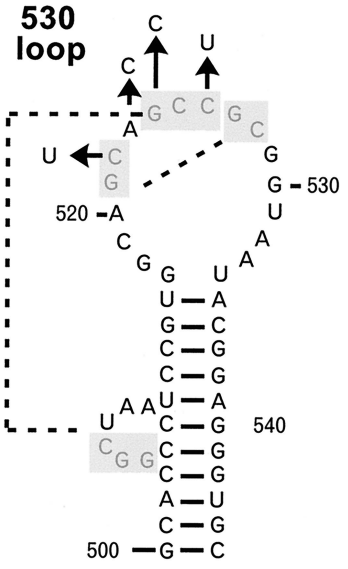

FIG. 3. Secondary structure of the 530 loop region. *rrs* mutations associated with streptomycin resistance and isolated in this study are indicated by an arrow. Nucleotides involved in tertiary structure interaction resulting in pseudoknots are shown in gray and are connected by dotted lines.

tations *rrs*523*C*, *rrs*524C, and *rrs*526T. pMV361 is a vector that integrates once into the mycobacterial genome at the *attB* site (45). pMV361-H-rRNA2058G contains a hygromycin resistance gene and the entire *rrnB* operon from *M. smegmatis*. An A→G transition at position 2058 in the 23S rRNA gene confers resistance to clarithromycin (resistant phenotype is dominant [38]), allowing phenotypic characterization of the plasmid-carried rRNA operon. Given that streptomycin resistance-conferring mutations are recessive in a merodiploid strain (18), we reasoned that transfection with a wild-type *rrs* allele should render the strains with a streptomycin resistance-conferring mutation in *rrs* sensitive to this drug. Following selection on hygromycin, the transformants were plated on streptomycin- or clarithromycin-containing agar. While transformants exhibited a clarithromycin-resistant phenotype, plating on streptomycin demonstrated that upon transformation with the *rrn*-2058G operon a streptomycin-sensitive phenotype was restored (see Table 5). These results indicate that the resistant phenotype maps to *rrn*.

RecA-mediated gene conversion (32) was used to experimentally verify that the 16S rRNA mutations observed confer a resistant phenotype and to exclude the possibility of compensatory mutations. Site-directed mutations were introduced into plasmid pMV361-H-rRNA2058G, resulting in plasmids pMV361-H-rRNA523C/2058G, pMV361-H-rRNA524C/2058G, and pMV361-H-rRNA526T/2058G. The plasmids were subsequently transformed into the single *rrn* allelic strain *M. smegmatis rrnB*. Following selection on hygromycin to ensure integration of the vector, the cells were plated on hygromycin plus streptomycin to select for RecA-mediated gene conversion of the mutant allele (32). Streptomycin-resistant transformants were obtained with a frequency of 10^{-5} to 10^{-6}. This frequency is several orders of magnitude higher than the frequency of spontaneous resistance (10^{-8} to 10^{-10}).

Using PCR-mediated sequence analysis, the 16S rRNA gene

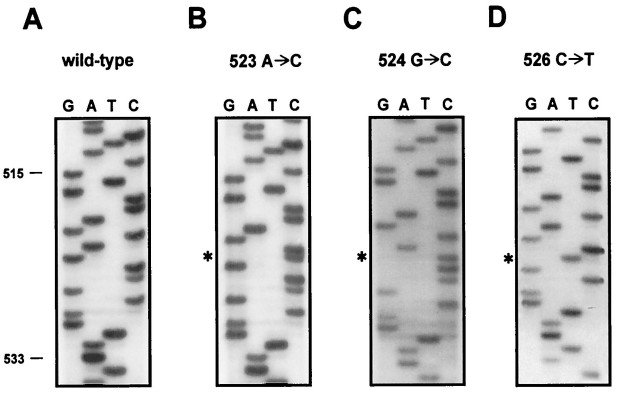

FIG. 4. 16S rDNA sequence of the 530 loop region. The wild-type sequence of a single rRNA allelic strain (A) is shown along with the same region of transformants obtained with plasmids pMV361-H-rRNA523C/2058G (B), pMV361-H-rRNA524C/2058G (C), and pMV361-H-rRNA526T/2058G (D) after selection on streptomycin. The mutated nucleotide is indicated by an asterisk.

of streptomycin-resistant mutants was analyzed. In transformants grown in the presence of streptomycin, a homogeneous mutant genotype was observed at the mutated 16S rRNA position introduced, i.e., positions 523, 524, and 526 (Fig. 4). These transformants originate from RecA-mediated homologous recombination between the vector-derived *rrn* operon and

TABLE 5. Streptomycin MICs for *rpsL* and *rrs* mutant strains

Strain	MIC (μg/ml)
Parental strains	
M. smegmatis mc^2 155	<20
M. smegmatis rpsL^{3+}	<20
M. smegmatis rrnB	<20
M. smegmatis rrnB rpsL^{3+}	<20
rpsL mutant strains	
*rpsL*42Lys→Arg	>200
*rpsL*42Lys→Asn	>200
*rpsL*42Lys→Thr	>200
*rpsL*42Lys→Met	>200
*rpsL*88Lys→Glu	200
*rpsL*88Lys→Arg	200
rrs mutant strains	
*rrs*524G→C	>200
*rrs*526C→T	20–100
*rrs*522C→T	20–100
*rrs*523A→C	20–100
rrs mutant strains transformed with *rrs* wild-type gene	
*rrs*524G→C::pMV361-H-rRNA2058G	<20
*rrs*526C→T::pMV361-H-rRNA2058G	<20
*rrs*523A→C::pMV361-H-rRNA2058G	<20
rrnB knockout strain transformed with different *rrs* alleles[a]	
pMV361-H-rRNA2058[b,c]	<20
pMV361-H-rRNA524C2058G[b]	>200
pMV361-H-rRNA526T2058G[b]	20–100
pMV361-H-rRNA523C2058G[c]	20–100

[a] Mutants were tested after selection on medium containing hygromycin (50 μg/ml) plus streptomycin (20 μg/ml).
[b] Strain *rrnB* was transformed.
[c] Strain *rrnB rpsL*$^{3+}$ was transformed.

the single functional chromosomal *rrn* operon resulting in gene conversion of the mutant allele.

Physiological investigations demonstrated that streptomycin MIC levels for the strains with RecA-mediated homogeneous mutant rRNA alleles were identical to those for the spontaneous resistant mutants with the corresponding mutation (see Table 5). In particular, introduction of mutation *rrs*524C resulted in a streptomycin-dependent phenotype.

DISCUSSION

Ribosomal protein S12 (RpsL) and the small subunit rRNA are the main targets of streptomycin-mediated translational inhibition. By altering the number of genes expressing sensitive alleles in *M. smegmatis*, we have generated strains of eubacteria which allow a detailed investigation of the mechanisms underlying streptomycin resistance. Our results seem to be somewhat in contrast to those of a previous report, where mutations in *rpsD* of *Salmonella enterica* serovar Typhimurium have been postulated to confer streptomycin resistance. However, it should be noted that these mutations were isolated by selecting for chromosomal alterations, which would compensate for streptomycin-dependent *rpsL* alleles, rather than by selecting for streptomycin resistance itself (6). Although we cannot exclude the theoretical possibility that some rare mutations outside *rrs* and *rpsL* may cause primary ribosomal resistance to streptomycin and were missed in our selection procedure, we consider this possibility highly unlikely, as (i) a low drug concentration (20 μg/ml) was used in the selection procedure, and (ii) no resistant mutants were isolated from *M. smegmatis* carrying two *rrs* genes and three *rpsL* genes. Our data are supported by recent structural analyses of the 30S ribosomal subunit demonstrating that the streptomycin binding site is composed of defined *rrs* nucleotides and stabilized by the RpsL protein (8).

These data suggest that strain *M. smegmatis rrnB rpsL*$^{3+}$ can only become streptomycin resistant by mutations in a rRNA gene. We have isolated resistant strains covering a total of four different mutations. All streptomycin-resistant mutants isolated in these experiments show a single point mutation in a small region of the 16S rRNA.

The frequency of appearance of resistant rRNA mutants (10^{-10}) falls within the mutation frequency of *M. smegmatis*, strongly suggesting that no second-site mutations (for example, in ribosomal protein genes) contributed to resistance. This conclusion was further corroborated by introducing the respective mutations into *rrnB* strains; the mutants were resistant to streptomycin. These experiments also effectively rule out the possibility of compensatory mutations.

Mutation *rrs*523A→C has been found previously to be associated with streptomycin resistance in *Chlamydomonas* chloroplasts (http://www.fandm.edu/departments/biology/databases/16SMDBexp.html); mutations *rrs*522C→T, 523A→C, and 526C→T have been observed to be associated with resistance to streptomycin in clinical isolates of *M. tuberculosis* (10, 22). However, with the exception of *rrs*523A→C (23), experimental proof for a cause-effect relationship was lacking. Structural analyses may offer a rationale for some resistance-conferring mutations (8), but prediction of resistance-conferring mutations is not exhaustive.

Previous investigations on the universally conserved pseudoknot structure within the 530 region and resistance-conferring 16S rRNA mutations were limited by a lack of selection-driven mutational resistance. With respect to the interaction between nucleotides 505-GGC-507 and 526-CCG-524, these studies demonstrated that (i) mutations that disrupt pairing between these bases are deleterious in *E. coli*, i.e., 505G-526A, 506A-525C, and 507C-524A; (ii) compensating changes restoring base pairing restored growth, i.e., 505U-526A, 506A-525U, and 507U-524A; and (iii) certain mild perturbations creating G/U wobble base pairs are compatible with 16S rRNA function and confer resistance to streptomycin, i.e., 506G-525U and 507U-524G (31). Although these landmark studies clearly established that mild perturbations within the pseudoknot structure can lead to streptomycin resistance, those mutations investigated, e.g., *rrs*507C→T and 525C→T, had to be chosen at will and bear little relationship to selection-derived mutations within this structure, i.e., 524G→C and 526C→T. The possibility of investigating selection-driven mutational alterations allows one to define and characterize those rRNA mutations which confer a resistant phenotype while simultaneously respecting the structural and functional confinements of the ribosome.

One of the most frequent mutations isolated in our work (*rrs*524G→C) so far has neither been investigated in genetically engineered mutants nor has it been observed in in vitro-selected mutants or in clinical isolates. The absence of *rrs*524C mutants in clinical isolates of *Mycobacterium tuberculosis* most likely is due to its streptomycin-dependent phenotype. As demonstrated by genetic exchange experiments, the 524C mutation confers high-level streptomycin resistance. The 524G→C alteration is particularly interesting, as it dramatically weakens the proposed pseudoknot structure between bases 505-GGC-507 and 526-CCG-524. As discussed above, perturbations of this pseudoknot structure have been shown to severely impair growth, which could be restored partially by compensatory mutations of nucleotides involved in base pairing (31). These effects have been investigated in mutants with a 525C→T or 507C→T alteration, as these mutations allow wobble base pairing between the respective nucleotides, i.e., 505-GGC-507–526-CUG-524 and 505-GGU-507–526-CCG-524, respectively. While our data are in accordance with the view that creation of a G-U wobble base pair within the pseudoknot structure is compatible with rRNA function and confers resistance to streptomycin (as exemplified by the mutation 526C→T), either the isolation of mutation 524G→C questions the postulate of a strict base pairing requirement within the pseudoknot structure for ribosome function (31) or streptomycin induces a distortion that compensates for the perturbation of the base pairing within the pseudoknot.

Streptomycin is an antibiotic which causes misreading of the genetic code by stabilizing the ribosomal ambiguity state, a conformation in which the aminoacyl-tRNA binding site of the ribosome has high affinity even to noncognate tRNAs. Streptomycin resistance mutations in *rpsL* often lead to hyperaccurate but slower ribosomes. While a weak hyperaccuracy results in streptomycin resistance, a strong hyperaccuracy causes streptomycin dependence. Mutations causing streptomycin dependence have been found to map in *rpsL* but also in *rrs* (16). In mutant *rrs*524C the ribosome most likely is trapped in the

restrictive state unless streptomycin suppresses this mutational effect (8).

The approach taken in this work, i.e., the generation of a nonpathogenic single rRNA allelic eubacterium carrying a multitude of stably integrated *rpsL* genes, allows selection of streptomycin resistance-conferring mutations in the 16S rRNA gene. *E. coli* is the model organism for eubacteria in general and for investigation of ribosome structure in particular. However, the establishment of additional model systems, such as the single rRNA allelic *M. smegmatis*, a gram-positive microorganism, will help to elucidate common principles in ribosome action and ribosome-drug interaction. In addition, gram-positive and gram-negative bacteria differ with respect to susceptibility to drugs targeting the bacterial ribosome, e.g., oxazolidinones (14). The availability of a suitable gram-positive microorganism will extend our possibilities to study structure-function relationships of rRNAs.

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