**Novel Mutation in 16S rRNA Associated with Streptomycin Dependence in *Mycobacterium tuberculosis***

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Received 30 September 1994/Returned for modification 29 November 1994/Accepted 3 January 1995

Molecular characterization of a streptomycin-dependent mutant of *Mycobacterium tuberculosis* revealed the presence of a novel mutation in the *rrs* gene encoding 16S rRNA. Insertion of an additional cytosine in the 530 loop of 16S rRNA, a region known to be involved in streptomycin susceptibility and resistance, was associated with streptomycin dependence.

Significant progress has been made toward understanding the molecular basis of resistance to streptomycin in the tubercle bacillus, *Mycobacterium tuberculosis*, and it is now clear that high-level resistance results from missense mutations in the genes encoding two components of the ribosome, the 16S rRNA and the S12 protein (2, 3, 8, 12, 14). The most frequently occurring mutation associated with streptomycin resistance is an A-to-G transition in codon 43 of the *rpsL* gene, which results in the substitution of Arg for Lys at position 43 (Lys-43Arg) in the ribosomal protein S12 (7). As the primary structure of this protein has been well conserved during evolution, this mutation is common in various streptomycin-resistant eubacteria and chloroplasts (4, 5, 11, 18, 19). Two other mutations associated with streptomycin resistance have also been found in the *rpsL* gene of *M. tuberculosis*, and these lead to the substitutions Lys-43Thr and Lys-88Arg. Mutation of Lys-88 to Gin has also been reported, but its significance is less clear, as the corresponding strain also harbored a second mutation in the *rrs* gene encoding the 16S rRNA (2, 3, 12).

In some streptomycin-resistant strains wild-type S12 proteins were found, and resistance was attributed to mutations in *rrs* affecting one of two highly conserved loops that are adjacent in the 16S rRNA (2, 3, 8, 12). In *Escherichia coli* these are the 530 loop and the region centered around nucleotide 912 (13), which may form part of a site involved in codon-anticodon interaction and proofreading.

Streptomycin dependence is another phenotype associated with the ribosomes from some resistant mutants, and in the case of *E. coli*, this has been shown to result from the missense mutations Arg-85Ser and Pro-90Leu (9, 18). The same mutations have been described for chloroplasts from streptomycin-dependent *Chlamydomonas reinhardtii* (11). As strains of *M. tuberculosis* showing streptomycin dependence have been described (6), it was of interest to determine the nature of the mechanism involved.

In 1955, Hashimoto isolated a streptomycin-dependent mutant, 18b, from the laboratory strain of *M. tuberculosis* H$_3$ (6). On retesting, nearly 40 years later, we found that strain 18b had retained its streptomycin dependence and that it was incapable of growth on Löwenstein-Jensen medium containing less than 50 µg of the antibiotic per ml. Interestingly, when streptomycin-free plates that had been inoculated with 18b were supplemented with the antibiotic after a period of 28 days, growth was observed, indicating that the strain does not lose viability.

To determine whether mutations in *rpsL* might be responsible for streptomycin dependence, the gene was amplified by the PCR and subjected to single-strand conformation polymorphism (SSCP) analysis (15, 16) using two different sets of oligonucleotide primers (8). In both cases, wild-type SSCP profiles were found (data not shown). To exclude the possibility that mutations escaped detection by PCR-SSCP analysis, the complete *rpsL* gene was sequenced by PCR-based solid-phase sequencing (8), and again, this revealed a wild-type *rpsL* sequence.

Subsequently, the second gene associated with streptomycin resistance, *rrs*, was examined and two positions known to be prone to mutations were screened. When the regions around nucleotide 904 and the 530 loop were subjected to PCR-SSCP analysis, the latter showed an abnormal profile suggesting the presence of a mutation. DNA sequence analysis revealed the presence of an additional cytosine residue in the gene (Fig. 1) at the position corresponding to nucleotides 512 and 513 of the 16S rRNA (10). No other deviations from the wild-type *rrs* sequence were found in the 400 bp sequenced.

The insertion associated with streptomycin dependence is situated close to four other nucleotides in the 530 loop which have been identified as being involved in the streptomycin susceptibility of *M. tuberculosis*. When cytosine-512, adenine-513, and cytosine-516 are substituted by other bases, high-level resistance results (2, 3, 8, 12). The insertion of an extra base after position 512 in the 16S rRNA may result in a bulge in the 530 loop and in the disruption of interactions between different components within the 30S ribosomal subunit, as the 530 loop is in contact with the S12 protein (17). In *E. coli*, and probably in *M. tuberculosis*, a higher-order structure, called the pseudoknot (3, 17), is formed by base pairing between the sequences GCC, at positions 524 to 526, and CGG, at positions 505 to 507 (Fig. 2). It is interesting to note that the mutation associated with streptomycin dependence results in a second copy of the triplet GCC adjacent to the motif involved in pseudoknot formation, suggesting possible interference. Presumably, the presence of streptomycin stabilizes these interactions in some way and leads to normal ribosome function in strains such as 18b.

The mutation in the 16S rRNA that confers both streptomycin resistance and dependence is a novel one. In other microorganisms, this phenotype results from amino acid substitutions involving Arg-85 or Pro-90 in the S12 protein (9, 11,
form the higher-order pseudoknot structure are indicated by arcs. Numbering is based on that found in streptomycin-dependent strain 18bis indicated by the large arrow. Those bases which give rise to resistance are indicated (CC). Numbering is based on that found in streptomycin-dependent strain 18bis indicated by the large arrow. Those bases which give rise to resistance are indicated (CC).

FIG. 1. Direct DNA sequence analysis of \textit{rrs} from the streptomycin-dependent strain 18b of \textit{M. tuberculosis} and the fully susceptible reference strain H37Rv. Lanes 1, strain 18b; lanes 2, strain H37Rv. The additional cytosine after position 912 is indicated (CC). Mutations to the 16S rRNA resulting in streptomycin resistance are very difficult to isolate, as most bacteria have multiple copies of the rRNA genes and the mutations are, therefore, recessive. As demonstrated here, and pointed out by Böttger recently (2), slowly growing mycobacteria are useful for studying resistance to drugs acting on ribosomal components, particularly the rRNAs, as they contain a single \textit{rn} operon (1).

We thank Beate Heym and Sei-ichi Katayama for helpful discussions and Catherine Schurra for technical assistance. This investigation received financial support from the National Institute of Allergy and Infectious Diseases (AI37015), the Association Française Raoul Follereau, the Institut National de la Santé et de la Recherche Médicale (CRE 910604), and the Institut Pasteur.

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