ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1994, p. 238–242
0066-4804/94/$04.00+0

Vol. 38, No. 2

Streptomycin Resistance in Mycobacteria

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Received 21 July 1993/Returned for modification 8 October 1993/Accepted 22 November 1993

Streptomycin, the first antibiotic used in tuberculosis control programs, perturbs protein synthesis at the ribosome level. It is shown here that streptomycin resistance in some clinical isolates of *Mycobacterium tuberculosis* is associated either with missense mutations in the *rpsL* gene, which encodes ribosomal protein S12, or with base substitutions at position 904 in the 16S rRNA. The primary structure of the S12 protein is well conserved among the mycobacteria, even those, such as *M. avium*, *M. gordonae*, and *M. szulgai*, that are naturally resistant to streptomycin. This suggests that permeability barriers may be responsible for the resistance to the antibiotic.

Streptomycin was the first antibiotic shown to be active against the etiologic agent of tuberculosis, *Mycobacterium tuberculosis*, and was used in control programs for many years (3). However, as a result of significant levels of drug resistance when streptomycin was used in monotherapy, some side effects, and, above all, the availability of better drugs, such as isoniazid and rifampin, streptomycin usage declined greatly in industrialized countries in the 1960s (3). Recently, the emergence in the United States (1, 3, 25) of strains of *M. tuberculosis* displaying resistance to some or all of the major antituberculosis drugs has led to renewed interest in streptomycin. It is conceivable that this aminoglycoside antibiotic, in association with other compounds, could again play a valuable role in controlling tuberculosis, especially the form of the disease caused by strains which are resistant to the components of the standard chemotherapy program (3, 13, 25), i.e., isoniazid, rifampin, ethambutol, and pyrazinamide. Clearly, if this were to be the case, it would be useful to know whether new clinical isolates of *M. tuberculosis* were already resistant to streptomycin.

Streptomycin acts on ribosomes and causes misreading of the genetic code, inhibition of initiation of translation of mRNA, and aberrant proofreading (18). Resistance to the antibiotic has been studied intensively in many bacteria and plants and has been shown to result from a limited number of missense mutations in the *rpsL* gene, which encodes the ribosomal protein S12 (7, 8, 15, 28, 30). Additional mutations conferring streptomycin resistance have also been found in the 16S rRNA gene, *rrs*, in some bacteria and in chloroplasts (7–9, 15, 18–20, 22, 28, 30), and these mutations affect the conserved regions around nucleotides 530 and 912 (22). In some bacteria streptomycin resistance is due to the production of aminoglycoside-modifying enzymes (2) whose genes are borne by plasmids or transposable elements. As neither transposons nor plasmids have been found in any strains of *M. tuberculosis*, this resistance mechanism seems less likely (16).

The nucleotide sequence of the *rpsL* gene from the leprosy bacillus, *Mycobacterium leprae*, was determined recently (10), and this enabled primers to be designed that were suitable for amplification of homologous genes from *M. tuberculosis* and other mycobacteria by PCR. The goals of the present study were to determine the molecular basis of streptomycin resistance in *M. tuberculosis* and to attempt to develop a simple diagnostic test for resistance.

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MATERIALS AND METHODS

Mycobacteria and drug susceptibility testing. The mycobacterial strains employed in this study and their sources are described in Table 1. When appropriate, drug susceptibility testing was performed by the agar dilution method, and organisms capable of growth at a drug concentration of ≥4 μg/ml were scored as streptomycin resistant. For some strains MICs were determined in Middlebrook medium (7H9 supplemented with OADC) containing different concentrations of streptomycin.

PCR procedures. DNA for PCR analysis was extracted by the following procedure. A loopful of organisms from a colony was suspended in 200 μl of H_2O, overlaid with 100 μl of mineral oil (Sigma Chemical Co.), and then subjected to the freeze-boiling technique as described previously (29). Aliquots (10 μl) were then used directly in PCR as described previously (29). Primers ML51 (CCCACCATTCAGCAGCTGGT) and ML52 (GTCGAGCGAACCGCGAATGA) were used to amplify most of *rpsL*, and in some experiments ML51 was used with TB59 (AGAACCTGTTCACCAACTGG) to amplify the complete gene plus some 65 bp of downstream sequences. Primers TB53 (GATGACGGCCTTCGGGTTGT) and TB54 (TCTAGTCTGCCCGTATCGCC) and primers TB55 (GTA GTCCACGCCGTAAACGG) and TB56 (AGGCCACAAG GGAACGCCTA) were employed for analysis of the 530 loop and the 912 region of the *rrs* gene (14), respectively. In all cases the following 35-cycle procedure was used with a PHC-1 thermal cycler (Techne): 1 min at 92°C, 2 min at 61°C, and 2 min at 72°C. To ensure complete synthesis of products, the final elongation step was performed for 10 min at 72°C.

When PCR products were destined for single-strand conformation polymorphism (SSCP) analysis (12, 23, 27) or for use as probes for Southern blots, the same PCR procedure was employed except for the inclusion of 5 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International Plc) in the reaction mixture. To detect mutations in the *rpsL* fragment, SSCP analysis using the conditions described recently (12) was employed. Essentially, 10 μl of the ^{32}P-labelled PCR fragment was mixed with 15 μl of H_2O and 25 μl of a solution containing sodium dodecyl sulfate (SDS) (0.1%) and EDTA (2 mM). Aliquots (5 μl) were mixed with an equal volume of sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol

TABLE 1. Properties of the bacterial strains used in this study[a]

Bacterium	Strain	Source	Streptomycin resistance	MIC (μg/ml)	Mutant allele (where known)	Resistance to other antituberculosis drugs
M. avium	ATCC 25291	V. Vincent	R	ND	None	NA
M. gordonae	CIPT0310002	V. Vincent	R	ND	None	NA
M. smegmatis	MC^2155	This laboratory	S	ND	None	NA
M. szulgai	CIPT0240007	V. Vincent	R	ND	None	NA
M. tuberculosis	H37Rv	This laboratory	S	<4	None	None
	9445	J. Grosset	S	<4	None	INH
	9363	J. Grosset	S	<4	None	INH
	9468	J. Grosset	S	<4	None	None
	9465	J. Grosset	R	40	None	INH
	9420	J. Grosset	R	ND	None	RIF
	9247	J. Grosset	R	>1,280	*rrnS1*	INH
	9428	J. Grosset	R	160	*rrnS2*	INH, RIF
	9181	J. Grosset	R	ND	*rpsL1*	INH
	9106	J. Grosset	R	>1,280	*rpsL1*	INH, RIF
E. coli	HB101	This laboratory	R	ND	*rpsL20*	NA

[a] Abbreviations: R, resistant; S, susceptible; NA, not applicable; ND, not determined; INH, isoniazid; RIF, rifampin.

blue, 0.05% xylene cyanol) and heated at 95°C for 10 min to denature the PCR fragment. Samples were then quickly chilled on ice to prevent renaturation and were loaded immediately on a 5% polyacrylamide gel (30% acrylamide, 0.5% bisacrylamide; 20 by 50 cm) containing TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was conducted for 2.5 h at constant power (65 W) with TBE buffer. The gel was dried and subjected to autoradiography.

General nucleic acid techniques. Southern blotting, DNA-DNA hybridization, and subcloning were performed by standard procedures (5, 24). DNA sequences were obtained directly from unlabelled PCR products by using the CircumVent cycled sequencing kit (New England Biolabs), primers which had been 5′ end labelled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International Plc), and T4 polynucleotide kinase, according to the protocol recommended by the manufacturer. Prior to sequencing, template DNA was subjected to phenol extraction, ethanol precipitation, and droplet dialysis. In some cases, when cycled sequences were ambiguous because of compressions, appropriate PCR fragments were end repaired and then cloned into M13mp18 and sequenced as described previously (11). The resultant sequences were always identical to those obtained by direct sequencing of PCR products. Sequences were compiled and analyzed with the Staden package (26).

RESULTS

Experimental rationale, primer design, and PCR analysis of various mycobacteria. Streptomycin resistance has been shown to result from a limited set of amino acid substitutions in the evolutionarily well-conserved ribosomal protein S12, encoded by the *rpsL* gene of *Escherichia coli*, and in certain chloroplasts (7, 8, 15, 26, 28, 30). The majority of the mutations affect amino acid residue 42, although others, affecting positions 41 to 47 or 90 to 92 and resulting in streptomycin resistance or streptomycin dependence, respectively, have been described (7, 8, 15, 26, 28, 30). Recently, the sequence of the *rpsL* gene from *M. leprae* was determined, and the primary structure of the S12 protein was deduced (10). Consequently, we were able to use this sequence information to design primers ML51 and ML52, which were suitable for amplifying the region of interest from other mycobacteria, in particular streptomycin-susceptible and -resistant strains of *M. tuberculosis*.

ML51 and ML52 directed the synthesis of a PCR product of 306 bp, when *M. leprae* DNA was used as the template, corresponding to amino acids 1 to 102 of the mature S12 protein. When this fragment was used as a hybridization probe, homologous sequences were detected in Southern blots of genomic DNA extracted from *M. tuberculosis* H37Rv, *Mycobacterium gordonae*, *Mycobacterium szulgai*, *Mycobacterium avium* (Fig. 1) and *Mycobacterium smegmatis* (not shown). Encouraged by these findings, we employed ML51 and ML52 in PCRs to amplify the corresponding segments of *rpsL* from all five mycobacteria, and in each case the expected 306-bp fragment was obtained.

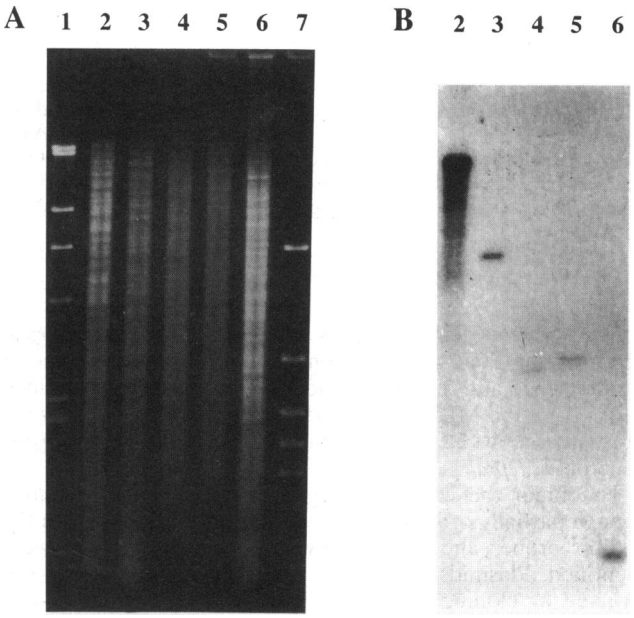

FIG. 1. Southern blot analysis of *rpsL* in various mycobacteria. After digestion of genomic DNA with *Rsr*II, samples were separated on an agarose gel (0.6%), visualized by staining with ethidium bromide (A), and then transferred to a nylon membrane and hybridized with the 306-bp ^{32}P-labelled probe made by PCR, using primers ML51 and ML52 with *M. leprae* DNA (B). Size markers are in lanes 1 (23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb) and 7 (6.0, 2.9, 2.1, 1.65, 1.32, and 0.75 kb). Lane 2, *M. leprae*; lane 3, *M. tuberculosis* H37Rv; lane 4, *M. gordonae*; lane 5, *M. szulgai*; lane 6, *M. avium*.

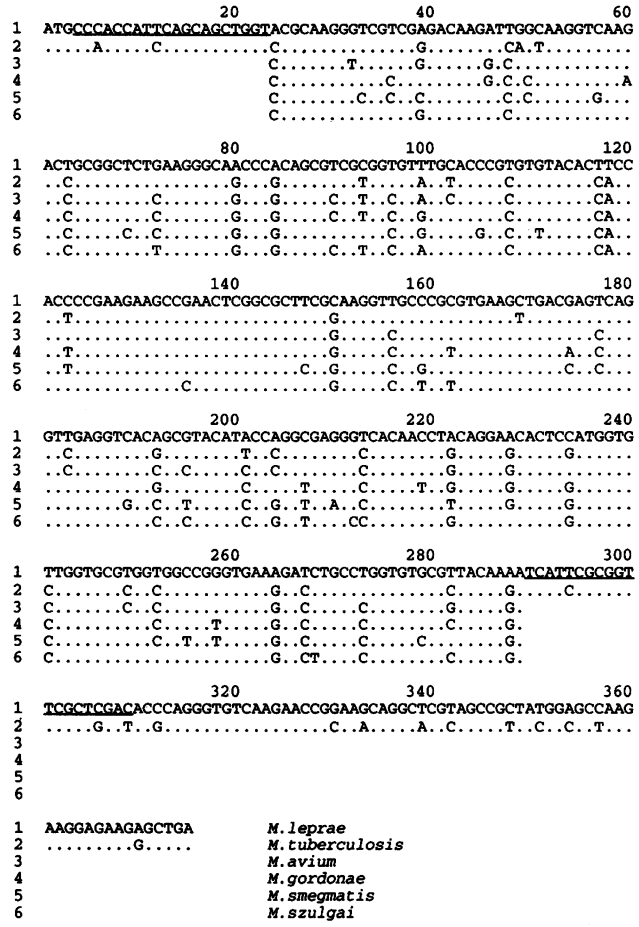

FIG. 2. Partial nucleotide sequences of mycobacterial *rpsL* genes. The sequences derived from the 306-bp PCR fragments of the *rpsL* genes from *M. avium*, *M. gordonae*, *M. smegmatis*, and *M. szulgai* are aligned with the complete gene sequences from *M. leprae* and *M. tuberculosis* H37Rv. The last two sequences start from the ATG initiation codon and end with the TGA translational stop codon. Only nucleotides which differ from those in the *M. leprae* sequence are shown. The positions corresponding to primers ML51 and ML52 are underlined.

Nucleotide sequence analysis of various mycobacterial *rpsL* genes. After PCR amplification, the various *rpsL* fragments were purified and their nucleotide sequences were determined with ML51 and ML52 as primers. The resultant sequences are presented in Fig. 2, where they are aligned with that of the *rpsL* gene of *M. leprae*.

To obtain the complete sequence of the *M. tuberculosis* gene, a partially ordered cosmid library was screened with the 306-bp probe, and two positively hybridizing clones were identified. Plasmids pNH55 (−) and pNH56 (+) were constructed by cloning an ~5-kb *Eco*RI fragment from cosmid T732, carrying the complete gene, into pUC19 in both orientations with respect to the *lac* promoter. Clone pNH56 was then used as a source of DNA to obtain the complete *rpsL* sequence.

When present in the streptomycin-resistant *E. coli* strain HB101, pNH56 was not capable of restoring susceptibility to the antibiotic. This apparent lack of complementation is most likely due to the production of insufficient levels of the *M. tuberculosis* S12 protein, as documented for other mycobacterial gene products (4), and not to its inability to interact efficiently with the other components of the *E. coli* ribosome for heterologous complementation, has been demonstrated with genes from other organisms (15).

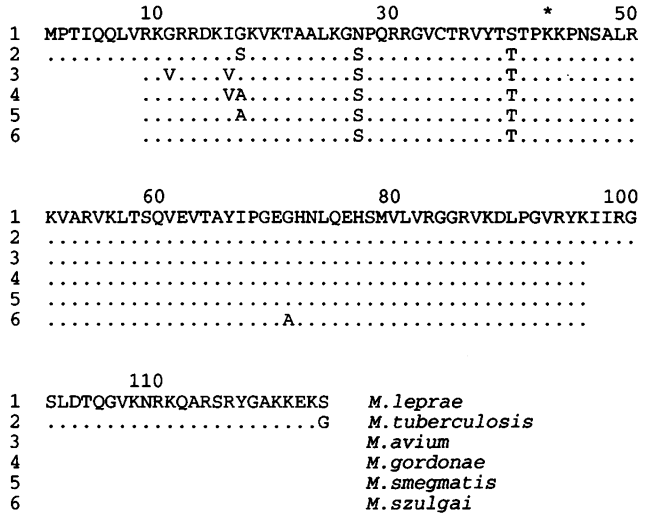

FIG. 3. Comparison of partial primary structures of S12 proteins from *M. avium*, *M. gordonae*, *M. smegmatis*, and *M. szulgai* with the complete S12 sequences from *M. leprae* and *M. tuberculosis* H37Rv. Only those amino acids which differ from those in the *M. leprae* sequence are shown. The position of the mutated Lys residue is indicated by an asterisk.

Deduced S12 protein sequences. The primary structures of the various mycobacterial S12 proteins deduced from the gene sequences are shown aligned in Fig. 3. There is perfect amino acid conservation in the regions found to be associated with streptomycin resistance in other organisms. Very few amino acid substitutions were seen among the various mycobacterial proteins, and the S12 proteins of *M. leprae* and *M. tuberculosis* show 96% identity with each other and have 69% identity with the *E. coli* S12 protein (data not shown).

Molecular and SSCP analyses of streptomycin-resistant *M. tuberculosis* isolates. To determine whether streptomycin resistance in *M. tuberculosis* was also due to mutations in *rpsL* and whether this was the sole mechanism responsible, a panel of nine clinical isolates, six of which were streptomycin resistant and three of which were streptomycin susceptible, was examined by PCR using the primers ML51 and ML52 and strain H37Rv as a positive control. In all cases the 306-bp fragment was obtained; to determine whether this harbored any point mutations, the strands were separated and subjected to SSCP analysis (27). As can be seen in Fig. 4, striking differences in the SSCP profiles were apparent in two of the six resistant

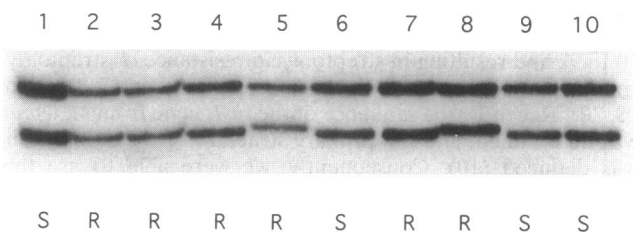

FIG. 4. SSCP analysis of streptomycin-susceptible (S) and -resistant (R) strains of *M. tuberculosis*. Lanes: 1, 9445; 2, 9247; 3, 9428; 4, 9420; 5, 9181; 6, 9363; 7, 9465; 8, 9106; 9, 9468; 10, H37Rv.

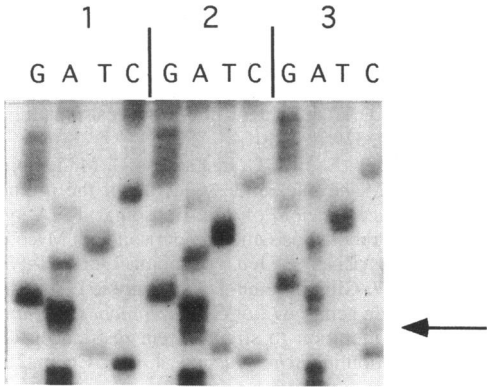

FIG. 5. Direct DNA sequence analysis of *rrs* from streptomycin-resistant and -susceptible clinical isolates of *M. tuberculosis*. 1, strain 9247; 2, strain 9248; 3, strain 9363. The mutated cytosine at position 904 is indicated by the arrow.

mutants (lanes 5 and 8), indicating that the *rpsL* genes probably had mutations. To confirm this, the nucleotide sequences of the PCR fragments from all nine strains were determined. As predicted by the SSCP analysis, point mutations were found in only two cases (strains 9106 and 9181), while the remaining strains displayed the wild-type sequence. In both mutants, the Lys codon, AAG at position 43 in *rpsL* (Fig. 3), had changed to an Arg codon, AGG. The MIC of streptomycin for strain 9106 was found to be >1,280 μg/ml (Table 1).

To exclude the possibility that streptomycin resistance stemmed from mutations at the 3′ end of *rpsL* that we were unable to detect by SSCP analysis using primers ML51 and ML52, another set of primers was employed. When ML51 was used together with TB59, a 457-bp PCR fragment was obtained but no differences in the SSCP profiles were seen when the mutants were compared with wild-type strains (data not shown).

As it was conceivable that mutations affecting the 16S rRNA were responsible for the streptomycin resistance in the remaining four resistant isolates, primers TB53 and TB54 were used for PCR to amplify a 238-bp fragment corresponding to the 530 loop, and TB55 and TB56 were used to produce a 238-bp fragment spanning the highly conserved 912–915 domain of the rRNA. Again, in all cases the sequences of the PCR products were determined and compared with that of the wild-type *rrs* gene (14). No deviations from the published sequence were found in the segment corresponding to the 530 loop, but in two cases, single-base substitutions were observed in nucleotide 904, which corresponds to position 912 in the 16S rRNA of *E. coli* (14, 22). As can be seen in Fig. 5, strains 9247 and 9428 have C-to-G and C-to-A transversions at position 904, respectively, and the MICs of streptomycin for both strains are high (1,280 and 160 μg/ml, respectively (Table 1).

In two of the six streptomycin-resistant strains of *M. tuberculosis* examined, 9465 and 9420 (Table 1), no mutations were found in the *rpsL* gene or in the selected regions of *rrs*. This suggests the existence of a third resistance mechanism, and this mechanism is likely to confer resistance to lower levels of streptomycin than the mutations described above, as the MIC of streptomycin for strain 9465 is 40 μg/ml (Table 1).

DISCUSSION

In the present study a PCR strategy has been used to investigate the molecular basis of streptomycin resistance in mycobacteria. A pair of primers (ML51 and ML52) that was capable of amplifying the critical region of the *rpsL* gene from the six mycobacteria examined was developed. Nucleotide and protein sequence comparisons showed these organisms to form a homogeneous group, and their S12 proteins have about 69% sequence identity with that of *E. coli*. When antibiotic-resistant clinical isolates of *M. tuberculosis* were tested by using this procedure in conjunction with SSCP analysis, it was found that strains harboring point mutations in *rpsL* could be readily identified. PCR-SSCP analysis (12, 23, 27) could thus represent a useful tool for rapidly monitoring for streptomycin susceptibility and could be of value to the clinician. However, as at least two loci are involved in streptomycin resistance, several PCR-SSCP assays will be required, in contrast to the system used for the detection of rifampin resistance in *M. tuberculosis*, in which a single set of primers detects all of the mutations in *rpoB* (12, 27).

One mechanism for streptomycin resistance can be attributed to missense mutations in *rpsL*, and the mutation found here, Lys-42 to Arg, has also been shown to be associated with resistance in enteric bacteria and in chloroplasts from *Nicotinia tabacum* and *Chlamydomonas reinhardtii* (7, 8, 17, 28, 30). Several other mutations affecting well-conserved regions of the S12 protein, such as residues 90 to 92, have been described previously but were not detected in this study with *M. tuberculosis*. However, since the submission of this paper, Finken et al. (6) have described a mutation affecting codon 88 in the *rpsL* gene of streptomycin-resistant isolates of *M. tuberculosis*.

It is also interesting that although mycobacteria such as *M. gordonae*, *M. szulgai*, and *M. avium* are considered to be naturally resistant to clinically significant levels of streptomycin, their S12 proteins appear to be of the antibiotic-susceptible type, as all of the conserved residues known to interact with streptomycin in other organisms are present. This suggests that a second factor, such as the cell envelope acting as a permeability barrier, might account for this phenotype.

Alterations in the 16S rRNA also appear to confer streptomycin resistance in *M. tuberculosis*, as two independent mutations affecting the highly conserved position 904, equivalent to cytosine 912 in *E. coli* (14, 22), have been found. Streptomycin is known to bind to the 16S rRNA at this position and to protect it from attack by alkylating agents and nucleases (18, 20). Base substitutions at the corresponding site are also responsible for streptomycin resistance in mutants of *E. coli* and *Euglena gracilis* (17–20) isolated in vitro, and the present work shows that similar mutants emerge in vivo during chemotherapy for tuberculosis. In contrast to the situation in *E. coli* and *E. gracilis*, in which the only substitutions found were C-912 to T, it is now clear that this cytosine residue can be replaced by any of the three possible nucleotides without any obvious detrimental effects. No mutations affecting the 530 loop, the site of interaction of the 16S rRNA with the S12 protein in the ribosome, were found in this study, although these have been shown to cause streptomycin resistance in *E. coli* and *Chlamydomonas* spp. (9, 17, 21) and, very recently (6), in some resistant strains of *M. tuberculosis*.

Another conclusion which can be drawn from the present study concerns the basis of multidrug resistance in *M. tuberculosis* (3, 13, 25), at least for the small number of strains examined. As exemplified by strains 9106 and 9428 (Table 1) the streptomycin resistance component appears to result from mutation of the antibiotic's target and not from a novel resistance mechanism.

Finally, it is intriguing that some of the *M. tuberculosis* isolates studied here appear to have normal 16S rRNA and S12 proteins yet display streptomycin resistance. This indicates the

existence of another means of resistance, and several possible mechanisms can be envisaged. These include modification of other components of the ribosome (18, 28, 30), although it seems more likely that changes in cell envelope permeability, like those believed to be operational in *M. avium*, may be involved. Indirect support for this interpretation is provided by our finding that for streptomycin-resistant strains which do not have mutations in *rpsL* or *rrs*, MICs of the antibiotic are lower (ca. 40 μg/ml) than they are for strains with altered ribosomal subunits (>160 μg/ml) (Table 1 and our unpublished data). Another possible explanation for this low-level resistance would be the acquisition of genes encoding aminoglycoside-modifying enzymes (2), and additional studies are required to address this.

ACKNOWLEDGMENTS

We thank Jacques Grosset for providing strains and encouragement, Véronique Vincent for a kind gift of mycobacterial DNA, and Beate Heym and Wolfgang Philipp for helpful discussions.

This investigation received financial support from the Association Française Raoul Follereau, the Institut National de la Santé et de la Recherche Médicale (CRE 910604), the Agence Nationale pour les Recherches sur le SIDA, and the Institut Pasteur.

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