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Sequence and Analysis of the *rpoB* Gene of *Mycobacterium smegmatis*

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The *rpoB* gene encodes the beta subunit of the DNA-dependent RNA polymerase of bacteria. Mutations in defined areas result in resistance to rifampin. *Mycobacterium smegmatis* is naturally resistant to rifampin, but analysis of the *rpoB* gene revealed no identifiable rifampin resistance mutations. Another mechanism of resistance may be present.

The *rpoB* gene of bacteria encodes the beta subunit of the DNA-dependent RNA polymerase (13). It is an important target for antimycobacterial drugs, such as rifampin, which exerts its effect by binding to the RNA polymerase and preventing transcription. Rifampin resistance in *Escherichia coli* has been shown to result from point mutations in four well-defined regions, or clusters, of *rpoB* (6, 14). Recently, strains of *Mycobacterium tuberculosis* resistant to rifampin have been shown to possess mutations in the largest of these rifampin resistance clusters. The types of mutations include single nucleotide changes and in-frame deletions and insertions. The *rpoB* gene of *M. tuberculosis* has recently been cloned and sequenced (11, 17).

Mycobacterium smegmatis is a species that rarely causes disease in humans, but it is an important tool for the genetic manipulation of mycobacterial DNA (6). For instance, certain strains are efficient hosts for the electroporation of DNA (16). Also, the low pathogenicity of this species, its ability to grow rapidly over days instead of weeks, and its 90% genetic homology to *M. tuberculosis* are reasons why *M. smegmatis* is an important model for genetic studies of mycobacterial species. Here we report the cloning and sequencing of the *rpoB* gene of *M. smegmatis* and identify characteristics that may shed additional light on the genetics of resistance to rifampin.

M. smegmatis (ATCC 14468) was grown in Middlebrook medium for 3 days at 37°C with shaking. The MIC of rifampin was determined to be 4 μg/ml by the E-Test (AB Biodisk, Solna, Sweden). The DNA was isolated by first disrupting cells with the use of glass beads in a Bead-Beater apparatus (Biospec Products, Bartlesville, Okla.) and phenol-chloroform extraction. DNA was amplified by PCR with the primers described by Telenti et al. (17). The resultant 432-bp product was sequenced by using the PCR primers, the fmol cycle sequencing kit (Promega, Madison, Wis.), and a 6% polyacrylamide gel (Sequagel-6; National Diagnostics, Atlanta, Ga.). We amplified sequences upstream and downstream from this core fragment by the method of rapid amplification of genomic DNA ends (12). By repeating the procedure in both upstream and downstream directions, we sequenced overlapping fragments totalling 4 kbp of *M. smegmatis* DNA that spanned the entire *rpoB* gene. All fragments were shown to belong to the *rpoB* gene by homology to the *rpoB* gene of *E. coli* (GenBank accession no. V00340).

We cloned the entire *rpoB* gene of *M. smegmatis* using the following strategy. We used PCR to prepare a 2-kbp, [^{32}P] dCTP-labelled probe (Prime-It II kit; Stratagene, La Jolla, Calif.). The probe was used to identify a 6-kbp fragment of *Sph*I-cut *M. smegmatis* DNA by Southern hybridization (2). A mini-library was prepared by ligation of DNA from the 6-kbp band to a *Sph*I-cut vector (pGem3Zf; Promega) and transformation into *E. coli* DH5α. Bacterial colonies of the resultant mini-library were screened directly by PCR with the PCR primers of Telenti et al. (17). One colony of 96 screened was positive. Plasmid DNA was recovered from a culture of this clone by using the Qiagen plasmid maxi kit (Qiagen, Chatsworth, Calif.). The clone was partially sequenced, including the start and stop codons, with the Sequenase kit (US Biochemical Corp., Cleveland, Ohio). Areas of compression were sequenced again by cycle sequencing. Both strands were sequenced at least twice. All sequence data were analyzed with the Wisconsin Package software (Genetics Computer Group, Madison, Wis.).

Of the total of 3,752 bases sequenced, there was 85% homology with the *rpoB* gene of *M. tuberculosis* and 82.6% homology with that of *Mycobacterium leprae*. The partial sequence of the *rpoB* clone contained an open reading frame of 3,507 bases. Like the *M. tuberculosis* counterpart, the *M. smegmatis rpoB* gene uses GTG as the probable start codon. The 1,169-amino-acid protein encoded by the *rpoB* gene was 90.1 and 88.8% homologous to the beta subunits of the RNA polymerases from *M. tuberculosis* and *M. leprae*, respectively (Fig. 1).

By analogy to the *E. coli rpoB* gene, four regions potentially associated with rifampin resistance were identified. The valine at position 167 corresponds to Val-146 of *E. coli*, where mutations have been rarely identified with rifampin resistance (10). Three clusters contain the majority of resistance mutations identified in *E. coli*. The 23 amino acids at positions 427 to 449 correspond to cluster I, those at positions 479 to 490 correspond to cluster II, and the single amino acid Arg-604 corresponds to cluster III (a misnomer, since there is but one location) (7, 15). Mutations in clinical *Mycobacterium* isolates resistant to rifampin have been localized only to cluster I (4, 7, 17). A small percentage of rifampin-resistant *M. tuberculosis* strains have no identifiable mutations in *rpoB* (7, 17).

In the *M. smegmatis rpoB* gene there were no mutations corresponding to any of the known rifampin resistance genotypes, despite the fact that the MIC for this isolate was 4 μg/ml, well above the level considered to indicate resistance. Guerrero et al. obtained similar results upon sequencing cluster I (Rif locus) of *Mycobacterium avium* and *Mycobacterium intracellulare* (3). In that study, all strains, regardless of rifampin susceptibility, had amino acid identity to the sequence found in

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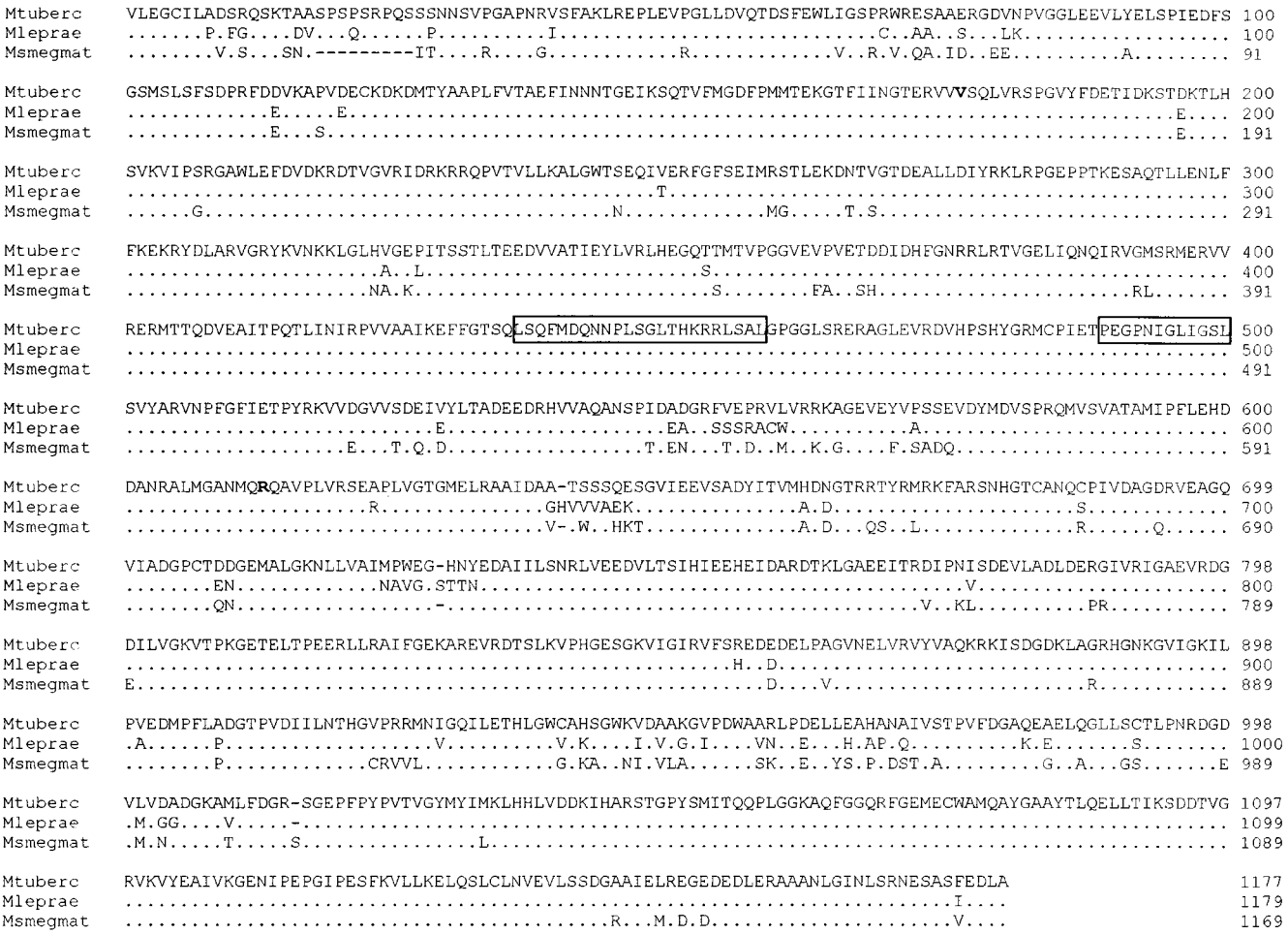

FIG. 1. Multiple amino acid sequence alignment of the *rpoB* gene products of *M. tuberculosis*, *M. leprae*, and *M. smegmatis*. For the latter two sequences, only differences from the *M. tuberculosis* sequence are shown. The dashes indicate gaps inserted to maintain optimal alignment. Val-167 and Arg-604 are shown in bold; the two boxes outline clusters I and II.

rifampin-susceptible *M. tuberculosis*. Thus, there must be factors other than the mutations in the identified *rpoB* clusters that cause the rifampin resistance of *M. smegmatis* and perhaps those isolates of *M. tuberculosis* that lack cluster I mutations. One possibility is that there are other areas of *rpoB* in which mutations may confer resistance. Alternatively, mutations in other subunits of the polymerase could contribute to rifampin resistance, perhaps by altering the conformation of the rifampin binding region of the beta subunit. In this regard, Levin and Hatfull raised a rifampin-resistant mutant of *M. smegmatis* (MIC $>$ 50 μg/ml) (9). They demonstrated in an in vitro transcription system that purified RNA polymerase from the mutant was less sensitive to rifampin than polymerase from the parent strain. Neither the genetic mechanism of the decreased sensitivity nor the subunit localization of the presumed mutation was defined. Another report identified a non-*rpoB* mutation causing rifampin resistance in *E. coli* (8). These results, however, do not exclude the intriguing possibility that another mechanism of drug resistance is present, such as diminished antibiotic entry into the bacteria, efflux, or enzymatic degradation or modification of rifampin. Recent reports hint that the latter mechanism may be present in *Nocardia* spp. (1, 18).

Nucleotide sequence accession number. The sequence of the *M. smegmatis rpoB* gene is in the GenBank database under accession no. U24494.

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REFERENCES

1. **Andersen, S. J., and E. R. Dabbs.** 1991. Cloning of nocardioform DNA conferring the ability to inactivate rifampicin. FEMS Microbiol. Lett. **63:** 247–250.
2. **Ausubel, F. M.** 1987. Current protocols in molecular biology. John Wiley and Sons, Inc., New York.
3. **Guerrero, C., L. Stockman, F. Marchesi, T. Bodmer, G. D. Roberts, and A. Telenti.** 1994. Evaluation of the *rpoB* gene in rifampicin-susceptible and -resistant *Mycobacterium avium* and *Mycobacterium intracellulare*. J. Antimicrob. Chemother. **33:**661–663.
4. **Honore, N., and S. T. Cole.** 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae*. Antimicrob. Agents Chemother. **37:**414–418.
5. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper, R. A. Udani, W. Jones, R. G. Barletta, and B. R. Bloom.** 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.
6. **Jin, D. J., and C. A. Gross.** 1988. Mapping and sequencing of mutations in the Escherichia coli *rpoB* gene that lead to rifampicin resistance. J. Mol. Biol. **202:**45–58.
7. **Kapur, V., L.-L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser.** 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J. Clin. Microbiol. **32:**1095–1098.

8. **Kollenda, M. C., D. Kamp, and G. R. Hartmann.** 1986. Mu-induced rifamycin-resistant mutations not located in the *rpoB* gene of Escherichia coli. Mol. Gen. Genet. **204:**192–194.
9. **Levin, M. E., and G. F. Hatfull.** 1993. *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance. Mol. Microbiol. **8:**277–285.
10. **Lisitsyn, N. A., E. D. Sverdlov, E. P. Moiseyeva, O. N. Danilevskaya, and V. G. Nikiforov.** 1984. Mutation to rifampicin resistance at the beginning of the RNA polymerase beta subunit gene in Escherichia coli. Mol. Gen. Genet. **196:**173–174.
11. **Miller, I. P., J. T. Crawford, and T. M. Shinnick.** 1994. The *rpoB* gene of *Mycobacterium tuberculosis.* Antimicrob. Agents Chemother. **38:**805–811.
12. **Mizobuchi, M., and L. A. Frohman.** 1993. Rapid amplification of genomic DNA ends. BioTechniques **15:**214–216.
13. **Ovchinnikov, Y. A., G. S. Monastyrskaya, V. V. Gubanov, S. O. Guryev, O. Y. Chertov, N. N. Modyanov, V. A. Grinkevich, I. A. Makarova, T. V. Marchenko, I. N. Polovnikova, V. M. Lipkin, and E. D. Sverdlov.** 1981. The primary structure of Escherichia coli RNA polymerase. Nucleotide sequence of the *rpoB* gene and amino-acid sequence of the beta-subunit. Eur. J. Biochem. **116:**621–629.
14. **Severinov, K., M. Soushko, A. Goldfarb, and V. Nikiforov.** 1993. Rifampicin region revisited. New rifampicin-resistant and streptolydigin-resistant mutants in the beta subunit of Escherichia coli RNA polymerase. J. Biol. Chem. **268:**14820–14825.
15. **Singer, M., D. J. Jin, W. A. Walter, and C. A. Gross.** 1993. Genetic evidence for the interaction between cluster I and cluster III rifampicin resistant mutations. J. Mol. Biol. **231:**1–5.
16. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. Mol. Microbiol. **4:**1911–1919.
17. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer.** 1993. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet **341:**647–650.
18. **Yazawa, K., Y. Mikami, A. Maeda, M. Akao, N. Morisaki, and S. Iwasaki.** 1993. Inactivation of rifampin by *Nocardia brasiliensis*. Antimicrob. Agents Chemother. **37:**1313–1317.