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The *rpoB* Gene of *Mycobacterium tuberculosis*

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A portion of the *Mycobacterium tuberculosis* gene encoding the β subunit of RNA polymerase (*rpoB*) was amplified by PCR using degenerate oligonucleotides and used as a hybridization probe to isolate plasmid clones carrying the entire *rpoB* gene of *M. tuberculosis* H37Rv, a virulent, rifampin-susceptible strain. Sequence analysis of a 5,084-bp *Sac*I genomic DNA fragment revealed a 3,534-bp open reading frame encoding an 1,178-amino-acid protein with 57% identity with the *Escherichia coli* β subunit. This *Sac*I fragment also carried a portion of the *rpoC* gene located 43 bp downstream from the 3′ end of the *rpoB* open reading frame; this organization is similar to that of the *rpoBC* operon of *E. coli*. The *M. tuberculosis rpoB* gene was cloned into the shuttle plasmid pMV261 and electroporated into the LR223 strain of *Mycobacterium smegmatis*, which is highly resistant to rifampin (MIC > 200 μg/ml). The resulting transformants were relatively rifampin susceptible (MIC = 50 μg/ml). Using PCR mutagenesis techniques, we introduced a specific *rpoB* point mutation (associated with clinical strains of rifampin-resistant *M. tuberculosis*) into the cloned *M. tuberculosis rpoB* gene and expressed this altered gene in the LR222 strain of *M. smegmatis*, which is susceptible to rifampin (MIC = 25 μg/ml). The resulting transformants were rifampin resistant (MIC = 200 μg/ml). The mutagenesis and expression strategy of the cloned *M. tuberculosis rpoB* gene that we have employed in this study will allow us to determine the *rpoB* mutations that are responsible for rifampin resistance in *M. tuberculosis*.

Rifampin, introduced in 1971, has proved to be an effective antituberculosis agent against susceptible strains as well as strains resistant to isoniazid or streptomycin (21, 30). Rifampin is rapidly bactericidal against *Mycobacterium tuberculosis*, and its use has greatly shortened the duration of chemotherapy necessary for the successful treatment of drug-susceptible tuberculosis (2, 4). Rifampin resistance heralds a more prolonged treatment for the patient and a poor outcome if the isolate is also resistant to isoniazid (21).

High-level resistance to rifampin occurs at a rate of 10^{-8} in *M. tuberculosis* in vitro and is thought to be a one-step mutational event (4, 29). Current assumptions about the mechanism of rifampin resistance in *M. tuberculosis* (35) are based on studies of the DNA-dependent RNA polymerase of *Escherichia coli* (3, 18), which is a complex oligomer containing four different subunits (α, β, β′, and σ) and assembled in two major forms: a core enzyme ($\alpha_2\beta\beta'$) and a holoenzyme ($\alpha_2\beta\beta'$ plus a σ subunit) (12). The core enzyme can perform RNA polymerization but requires a σ subunit to initiate site-specific transcription at promoter sites (12). The genes encoding subunits α, β, β′, and σ have been designated *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively (3).

Reconstitution experiments with subunits from rifampin-susceptible and rifampin-resistant strains of *E. coli* have demonstrated that RNA polymerase containing the α and β′ subunits from a resistant strain and the β subunit from a susceptible strain is sensitive to rifampin, while RNA polymerase containing the α and β′ subunits from a susceptible strain and the β subunit from a resistant strain is resistant to rifampin (33). In strains of *E. coli* that are rifampin susceptible, rifampin binds to the β subunit of RNA polymerase and leads to abortive initiation of transcription (15). Rifampin has a similar mechanism of action on RNA polymerase from *Mycobacterium smegmatis* (16). The majority of mutations causing rifampin resistance in *E. coli* have been mapped to three distinct loci near the center of the *rpoB* gene (14).

Taking advantage of the observation that portions of the amino acid sequences of RNA polymerase β subunits from different bacteria, plants, and some eukaryotes are highly conserved (7, 36), we used oligonucleotide primers corresponding to conserved domains of the β subunit in PCR (19) to amplify a portion of the *rpoB* gene from *M. tuberculosis* chromosomal DNA. This product was used as a hybridization probe to isolate plasmid clones carrying the entire *rpoB* gene.

MATERIALS AND METHODS

Strains, plasmids, and DNA. The bacterial strains and plasmids used in this study are described in Table 1. Chromosomal DNA from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *Mycobacterium bovis* BCG, *M. smegmatis*, and *Mycobacterium leprae* were isolated according to published methods (32, 34). Chromosomal DNA from *E. coli* K-12 was provided by A. Steigerwalt (Centers for Disease Control). Plasmid DNAs were isolated and purified by using Magic-Miniprep columns (Promega Corp., Madison, Wis.). Electrocompetent *E. coli* XL-1 Blue cells (Stratagene, La Jolla, Calif.) and *M. smegmatis* LR222 and LR223 were prepared as previously described (6, 13). *E. coli* XL-1 Blue was grown at 37°C in Luria-Bertani (LB) broth supplemented with 37.5 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) and 12.5 μg of tetracycline (Sigma) per ml or with 50 μg of kanamycin (Sigma) per ml when appropriate. *M. smegmatis* LR222 and LR223 were grown at 37°C in complete Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.)–10% (vol/vol) albumin-dextrose catalase enrichment (Difco)–0.05% (vol/vol) Tween 80, supplemented with 10 μg of kanamycin per ml when appropriate.

Oligonucleotide primers. All primers used in this study were synthesized by using the model 381A DNA synthesizer (Ap-

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TABLE 1. Bacterial strains and plasmids

Species and strain or plasmid	Relevant characteristic(s)	Source or reference[a]
M. smegmatis		
LR222	Rifampin-susceptible, electroporatable strain	M. Beggs-Altizer, VAMC, Little Rock, Ark.
LR223	Rifampin-resistant, electroporatable strain	C. Woodley, CDC, Atlanta, Ga.
E. coli XL-1 Blue	Tetracycline-resistant host strain	Stratagene
Plasmids		
pUC19	Ampicillin-resistant cloning vector	Pharmacia
pBluescript II SK$^+$	Ampicillin-resistant cloning vector	Stratagene
pMV261	Kanamycin-resistant *E. coli*-mycobacterial shuttle vector	27
p18	35 kb of *M. tuberculosis* H37Rv genomic DNA in cosmid pair pJC98-pJC100 containing portion of *rpoB*	This study
pLM49	4.9-kb *Pst*I genomic fragment of *M. tuberculosis* H37Rv containing portion of *rpoB* in pUC19	This study
pNK49	4.9-kb *Pst*I fragment of pLM49 in pBluescript II SK$^+$	This study
pLM51	5.1-kb *Sac*I genomic fragment of *M. tuberculosis* H37Rv containing entire *rpoB* gene in pUC19	This study
pNK51	5.1-kb *Sac*I fragment of pLM51 in pBluescript II SK$^+$	This study
pMV261::*lacZ*	*lacZ* gene in pMV261	27
pLN-2	5.1-kb *Bam*HI-*Eco*RI *rpoB* fragment of pLM51 in pMV261	This study
pMR-1	Mutant of pLN-2, with leucine (TTG) mutation at serine (TCG) codon 456 in the *rpoB* gene	This study

[a] VAMC, Veterans Affairs Medical Center; CDC, Centers for Disease Control.

plied Biosystems, Inc., Foster City, Calif.) in the National Center for Infectious Diseases Biotechnology Core Facility. The sequences and locations of these primers are listed in Table 2.

PCR. The DNA template (1 to 20 ng) was amplified in a 100-μl reaction volume containing each deoxynucleoside triphosphate at 200 μM, each primer at 1.0 μM, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin. The amplifications were carried out in either a model 480 or a model 4000 thermocycler (Perkin-Elmer Cetus). To amplify the 384-bp *rpoB* fragment by using primers POLB-A and POLB-E, 35 cycles of 2 min at 94°C, 2 min at 45°C, and 2 min at 72°C were used. To amplify the 619-bp *rpoB* fragment using primers POLB-5A and POLB-5B, 30 cycles of 1 min 30 s at 94°C, 1 min 45 s at 55°C, and 2 min 30 s at 72°C were used.

Recombinant DNA libraries. A cosmid library of *M. tuberculosis* H37Rv DNA (20) prepared by using the cosmid pair pJC98 and pJC100 was kindly provided by S. Mundayoor. A plasmid library of *M. tuberculosis* H37Rv genomic DNA *Sac*I fragments in dephosphorylated pUC19 DNA was prepared by previously described methods (25) and was electroporated into *E. coli* XL-1 Blue cells in a 0.2-cm electrode gap cuvette in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) set at 2,500 V, 25 μF, and 200 Ω according to the manufacturer's protocol (6). After overnight incubation at 37°C, the library was harvested in LB broth and stored in LB broth–15% (vol/vol) glycerol at −70°C. A library of *M. tuberculosis* H37Rv genomic DNA *Pst*I fragments in pUC19 was generated similarly.

Colony blotting. For the cosmid library, 100 individual recombinants were plated onto each 100-mm LB agar plate containing 25 μg of kanamycin per ml, and for the plasmid libraries, 2,000 to 4,000 individual recombinants were plated onto each 150-mm LB agar plate containing 37.5 μg of ampicillin and 12.5 μg of tetracycline per ml. Colony lifts and hybridizations were performed by using Hybond-N+ disc membranes (Amersham Corp., Arlington Heights, Ill.) and the ECL direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's recommendations.

DNA sequencing. Sequencing of the 200-bp *Sau*3AI fragment of the 384-bp PCR product was performed with a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's protocol. All subsequent sequencing was performed with a model 373 automated DNA sequencer

TABLE 2. Oligonucleotide primers used for PCR

Primer	Position[a]	Strand	Sequence (5'–3')[b]
Degenerate			
POLB-A	2892–2906	+	*Eco*RI N M Q R Q CCGAATTC AAC ATG CAG CGS CAG
POLB-E	3260–3243	−	*Eco*RI D E F N Y G CCGAATTC GTC CTC GAA GTT GTA ICC
M. tuberculosis H37Rv *rpoB*			
POLB-5A	2316–2335	+	ATCAACATCCGGCCGGTGGT
POLB-5B	2935–2916	−	GGGGCCTCGCTACGGACCAG

[a] Numbers refer to nucleotides in Fig. 2.
[b] S, C or G; I, inosine.

(Applied Biosystems). Plasmid subclones were sequenced with an AmpliTaq Dye Primer PRISM kit (Applied Biosystems) and a Catalyst Molecular Biology Lab Station (Applied Biosystems). PCR products were sequenced with an AmpliTaq Dye Terminator PRISM kit (Applied Biosystems) and a model 480 thermocycler (Perkin-Elmer Cetus).

Both strands of the *M. tuberculosis rpoB* gene were sequenced independently, and data were obtained an average of three to four times for each base. Sequence editing was performed with the use of the Sequence Editor software package (Applied Biosystems), and sequence analysis was performed with the Genetics Computer Group (University of Wisconsin) sequence analysis package (5).

Transformation of *M. smegmatis*. One microgram of purified plasmid DNA was electroporated into *M. smegmatis* cells as previously described (13). Following the addition of 1 ml of complete Middlebrook 7H9 broth and incubation at 37°C for 3 h, the transformation mixture was plated on 100-mm Trypticase soy agar (Difco) plates containing 10 μg of kanamycin per ml and was incubated for 72 h at 37°C.

Rifampin susceptibility tests. Individual *M. smegmatis* transformants were grown overnight in complete Middlebrook 7H9 broth containing 10 μg of kanamycin per ml at 37°C. Following determination of optical density at 600 nm, each culture was diluted to ~5,000 cells per ml and 0.1-ml portions were plated on complete Middlebrook 7H10 agar plates containing 10 μg of kanamycin per ml and graded concentrations of rifampin (Sigma) (0 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, or 200 μg/ml). The plates were incubated for 72 h at 37°C and then scored for visible growth. The rifampin MIC was defined as the lowest concentration of rifampin that prevented macroscopic growth after 72 h.

Nucleotide sequence accession number. The sequence of the *Sac*I fragment containing the *M. tuberculosis rpoB* gene was assigned GenBank accession number L27989.

RESULTS

Cloning of the *M. tuberculosis rpoB* gene. Primers POLB-A and POLB-E are degenerate oligonucleotides corresponding to conserved domains of the *rpoB* gene flanking amino acids 684 to 814 of the *E. coli* gene product, but with the codon bias typical of mycobacteria (7, 26). These primers amplified a product of the anticipated size (384 bp) from genomic DNA of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. smegmatis*, but not from *E. coli* (data not shown). To confirm that the 384-bp amplicon contained *rpoB* sequences, a 200-bp *Sau*3AI fragment of the 384-bp product from *M. tuberculosis* H37Rv was subcloned into *Bam*HI-cleaved pUC19 and a partial sequence was determined. The deduced amino acid sequence of one open reading frame (ORF) on this fragment displayed 47.1% identity with the corresponding region of the *E. coli* β subunit, and the nucleotide sequence had a G + C content (66.7%) which is typical of *M. tuberculosis* DNA (data not shown).

A clone carrying an ~5,100-bp *Sac*I fragment was isolated from a library of *M. tuberculosis* H37Rv *Sac*I fragments in pUC19 by colony hybridization using a 619-bp fragment of the *M. tuberculosis* H37Rv *rpoB* gene as a probe. The 619-bp fragment is located 5′ to the 200-bp sequenced fragment in the *rpoB* gene; it is the amplified product of oligonucleotide primers POLB-5A and POLB-5B. The sequence information used to design these primers was obtained by partially sequencing a clone, designated p18, from a cosmid library of *M. tuberculosis* H37Rv DNA which contained a partial copy of the *rpoB* gene (data not shown). Using the 619-bp probe, we also

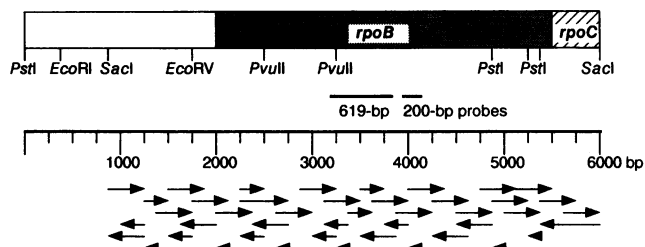

FIG. 1. Restriction map and sequencing strategy. The bar on top represents the restriction map of the cloned *M. tuberculosis* H37Rv genomic DNA containing the *rpoB* gene and a portion of the *rpoC* gene. The two lines below the map indicate the locations of the 619-bp and 200-bp hybridization probes. The 4.9-kb *Pst*I and 5.1-kb *Sac*I fragments were used as templates to generate two sets of nested deletion subclones using exonuclease III and the Erase-a-Base System (Promega). Overlapping sequences are indicated by the arrows.

isolated a clone from a library of *M. tuberculosis* H37Rv *Pst*I fragments in pUC19. Preliminary sequence analyses of the ends of the *Pst*I fragment revealed that this fragment contained the majority of the *rpoB* gene but lacked the 3′ end of the gene. Restriction mapping of the cloned *Pst*I and *Sac*I fragments indicated that they were overlapping fragments (data not shown) and that the *Sac*I fragment should contain the entire *rpoB* gene.

Sequence determination of the *rpoB* gene. The sequencing strategy is shown schematically in Fig. 1, and the sequence of the *Sac*I fragment is shown in Fig. 2. The sequence contains a 3,534-bp ORF which has an overall G + C composition of 64.2% and a G + C content of 86.4% for the bases that occupy the third position of the codons, as is typical for *M. tuberculosis* (26, 31). The first GTG codon of this ORF is at position 1065 to 1067, while the first ATG is at position 1371 to 1373. Assuming that the first GTG codon is the initiator codon and the TAA at position 4596 to 4598 is the stop codon, this ORF could encode a protein of 1,178 amino acids. Several partial matches with the consensus sequence for an *E. coli* ribosome binding site are present within the first 12 bases upstream from the GTG codon.

The deduced amino acid sequence of the 1,178-residue ORF has 93.3% and 57% identity with the β subunits of *M. leprae* and *E. coli*, respectively, and contains matches with each of the six previously described highly conserved regions (36). The *M. tuberculosis* β subunit displays excellent alignment with the sequences of the β subunits from *M. leprae* and *E. coli* and from the tobacco plant chloroplast (Fig. 3), although several gaps and insertions are necessary to align the 1,178-amino-acid sequence of the *M. tuberculosis* β subunit with the 1,342-amino-acid sequence of the β subunit of *E. coli*.

The sequence also contains a second, partial ORF beginning 43 bp downstream of the TAA codon. The putative GTG initiation codon of this ORF is located at position 4641 to 4643, and matches (AGGAAAGG) with the consensus sequence for an *E. coli* ribosome binding site are present 8 to 15 bases upstream of this codon. The deduced amino acid sequence of this partial ORF has 60% identity with the corresponding portion of the *E. coli rpoC* gene product. No other ORFs of greater than 100 amino acids and typical *M. tuberculosis* codon usage are present in the sequence.

A four-of-six match with the −10 consensus sequence for *E. coli* promoters (TATAAT) is located 61 to 66 bp upstream from the presumed start codon of *M. tuberculosis rpoB*, and a three-of-six match with the −35 consensus sequence (TTGA

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   1 GAGCTCGCCAAGCGCTCGGCCGACCCGCGCCTCACCGATGAACTCAACGACTTGGCCGGTCATACCCTCGACGACCTGTGACCGAGGGCGAGGTCGGGGTAGGCCTGCTAGATACGTCGG
 121 TCTTCATTGCGCGCGAGAGCGCGGTGCAATCGCGGACCTGCCTGAACGCGTGGCGCTTTCGGTTATGACGATCGGTGAGCTGCAACTCGGTCTGCTCAATGCTGGCGATTCGGCGACCCG
 241 ATCACGACGCGCCGACACCCTCGCGCTAGCGCGCACGGCCGATCAGATCCCTGTCAGTGAAGCGGTGATGATTTCGTTGGCTCGACTCGTCGCGGACTGCCGAGCCGCGGGCGTGCGGCG
 361 GTCGGTGAAGCTGACCGACGCTCTCATTGCGGCAACCGCGGAGATCAAGGTGTGACACCGAGGACTGATGAAGGTGCCGCTGCACCCTGCCTGATGCCTGACGTCACGATGCCCGTGAAG
 481 CGTGGTGATGCCCGGGGAGCTTTGGGTGTGGGTCCAGCTTTGTTCGTGGTGAGCGTGAGCAGCTCGCTGGTGAGGGCCAGGAGCTGTCGTTGCACGGCGGATTGATCGATTCGACCGCAT
 601 CCATCTGGAGCTACTGCCCCAGACCGGACTCGCAGCCTTGGCAAGCCGCTACGCGGGCATTCTCACCTGAGGCAACGAAGGGCGCTATGCGCGCATTGTGGGTGAGTCAACGCGAGGACT
 721 TGACGGCAGACGCTAAACGGGTCAATCTGTTGGGCAGCATGCGCCGCATGTGGCCAAAGGAAGTCGAGATCGCCAGCTAGCGCCGATATCCGGGGATGGTTATTGCCGGGTATTTGAGGA
 841 ATGCGCCGTCCTGCGCTATTGTTGGACGTTGCGCTGGCTACTTCCTGCCCACCTCACCCGCCACTTGACACCGTGGTCTTAGTCTGAGCCCAGTTTGCGGCTCAGCGGTTTAGTTGCGTG
                                                                                                RBS           V  L  E  G  C  I
 961 CGTGAGATCCGGACAGATCGTTCGCCGGCCGAAACCGACAAAATTATCGCGGCGAACGGGCCCGTGGGCACCGCTCCTCTAAGGGCTCTCGTTGGTCGCATGAAGTGCTGGAAGGATGCA
       L  A  D  S  R  Q  S  K  T  A  A  S  P  S  P  S  R  P  Q  S  S  S  N  N  S  V  P  G  A  P  N  R  V  S  F  A  K  L  R  E
1081 TCTTGGCAGATTCCCGCCAGAGCAAAACAGCCGCTAGTCCTAGTCCGAGTCGCCCGCAAAGTTCCTCGAATAACTCCGTACCCGGAGCGCCAAACCGGGTCTCCTTCGCTAAGCTGCGCG
       P  L  E  V  P  G  L  L  D  V  Q  T  D  S  F  E  W  L  I  G  S  P  R  W  R  E  S  A  A  E  R  G  D  V  N  P  V  G  G  L
1201 AACCACTTGAGGTTCCGGGACTCCTTGACGTCCAGACCGATTCGTTCGAGTGGCTGATCGGTTCGCCGCGCTGGCGCGAATCCGCCGCCGAGCGGGGTGATGTCAACCCAGTGGGTGGCC
       E  E  V  L  Y  E  L  S  P  I  E  D  F  S  G  S  M  S  L  S  F  S  D  P  R  F  D  D  V  K  A  P  V  D  E  C  K  D  K  D
1321 TGGAAGAGGTGCTCTACGAGCTGTCTCCGATCGAGGACTTCTCCGGGTCGATGTCGTTGTCGTTCTCTGACCCTCGTTTCGACGATGTCAAGGCACCCGTCGACGAGTGCAAAGACAAGG
       M  T  Y  A  A  P  L  F  V  T  A  E  F  I  N  N  N  T  G  E  I  K  S  Q  T  V  F  M  G  D  F  P  M  M  T  E  K  G  T  F
1441 ACATGACGTACGCGGCTCCACTGTTCGTCACCGCCGAGTTCATCAACAACAACACCGGTGAGATCAAGAGTCAGACGGTGTTCATGGGTGACTTCCCGATGATGACCGAGAAGGGCACGT
       I  I  N  G  T  E  R  V  V  V  S  Q  L  V  R  S  P  G  V  Y  F  D  E  T  I  D  K  S  T  D  K  T  L  H  S  V  K  V  I  P
1561 TCATCATCAACGGGACCGAGCGTGTGGTGGTCAGCCAGCTGGTGCGGTCGCCCGGGGTGTACTTCGACGAGACCATTGACAAGTCCACCGACAAGACGCTGCACAGCGTCAAGGTGATCCC
       S  R  G  A  W  L  E  F  D  V  D  K  R  D  T  V  G  V  R  I  D  R  K  R  R  Q  P  V  T  V  L  L  K  A  L  G  W  T  S  E
1681 CGAGCCGCGGCGCGTGGCTCGAGTTTGACGTCGACAAGCGCGACACCGTCGGCGTGCGCATCGACCGCAAACGCCGGCAACCGGTCACCGTGCTGCTCAAGGCGCTGGGCTGGACCAGCG
       Q  I  V  E  R  F  G  F  S  E  I  M  R  S  T  L  E  K  D  N  T  V  G  T  D  E  A  L  L  D  I  Y  R  K  L  R  P  G  E  P
1801 AGCAGATTGTCGAGCGGTTCGGGTTCTCCGAGATCATGCGATCGACGCTGGAGAAGGACAACACCGTCGGCACCGACGAGGCGCTGTTGGACATCTACCGCAAGCTGCGTCCGGGCGAGC
       P  T  K  E  S  A  Q  T  L  L  E  N  L  F  F  K  E  K  R  Y  D  L  A  R  V  G  R  Y  K  V  N  K  K  L  G  L  H  V  G  E
1921 CCCCGACCAAAGAGTCAGCGCAGACGCTGTTGGAAAACTTGTTCTTCAAGGAGAAGCGCTACGACCTGGCCCGCGTCGGTCGCTATAAGGTCAACAAGAAGCTCGGGCTGCATGTCGGCG
       P  I  T  S  S  T  L  T  E  E  D  V  V  A  T  I  E  Y  L  V  R  L  H  E  G  Q  T  T  M  T  V  P  G  G  V  E  V  P  V  E
2041 AGCCCATCACGTCGTCGACGCTGACCGAAGAAGACGTCGTGGCCACCATCGAATATCTGGTCCGCTTGCACGAGGGTCAGACCACGATGACCGTTCCGGGCGGCGTCGAGGTGCCGGTGG
       T  D  D  I  D  H  F  G  N  R  R  L  R  T  V  G  E  L  I  Q  N  Q  I  R  V  G  M  S  R  M  E  R  V  V  R  E  R  M  T  T
2161 AAACCGACGACATCGACCACTTCGGCAACCGCCGCCTGCGTACGGTCGGCGAGCTGATCCAAAACCAGATCCGGGTCGGCATGTCGCGGATGGAGCGGGTGGTCCGGGAGCGGATGACCA
       Q  D  V  E  A  I  T  P  Q  T  L  I  N  I  R  P  V  V  A  A  I  K  E  F  F  G  T  S  Q  L  S  Q  F  M  D  Q  N  N  P  L
2281 CCCAGGACGTGGAGGCGATCACACCGCAGACGTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAAGGAGTTCTTCGGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGC
       S  G  L  T  H  K  R  R  L  S  A  L  G  P  G  G  L  S  R  E  R  A  G  L  E  V  R  D  V  H  P  S  H  Y  G  R  M  C  P  I
2401 TGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGTGAGCGTGCCGGGCTGGAGGTCCGCGACGTGCACCCGTCGCACTACGGCCGGATGTGCCCGA
       E  T  P  E  G  P  N  I  G  L  I  G  S  L  S  V  Y  A  R  V  N  P  F  G  F  I  E  T  P  Y  R  K  V  V  D  G  V  V  S  D
2521 TCGAAACCCCTGAGGGGCCCAACATCGGTCTGATCGGCTCGCTGTCGGTGTACGCGCGGGTCAACCCGTTCGGGTTCATCGAAACGCCGTACCGCAAGGTGGTCGACGGCGTGGTTAGCG
       E  I  V  Y  L  T  A  D  E  E  D  R  H  V  V  A  Q  A  N  S  P  I  D  A  D  G  R  F  V  E  P  R  V  L  V  R  R  K  A  G
2641 ACGAGATCGTGTACCTGACCGCCGACGAGGAGGACCGCCACGTGGTGGCACAGGCCAATTCGCCGATCGATGCGGACGGTCGCTTCGTCGAGCCGCGCGTGCTGGTCCGCCGCAAGGCGG
       E  V  E  Y  V  P  S  S  E  V  D  Y  M  D  V  S  P  R  Q  M  V  S  V  A  T  A  M  I  P  F  L  E  H  D  D  A  N  R  A  L
2761 GCGAGGTGGAGTACGTGCCCTCGTCTGAGGTGGACTACATGGACGTCTCGCCCCGCCAGATGGTGTCGGTGGCCACCGCGATGATTCCCTTCCTGGAGCACGACGACGCCAACCGTGCCC
       M  G  A  N  M  Q  R  Q  A  V  P  L  V  R  S  E  A  P  L  V  G  T  G  M  E  L  R  A  A  I  D  A  A  T  S  S  S  Q  E  S
2881 TCATGGGGGCAAACATGCAGCGCCAGGCGGTGCCGCTGGTCCGTAGCGAGGCCCCGCTGGTGGGCACCGGGATGGAGCTGCGCGCGGCGATCGACGCGGCGACGTCGTCGTCGCAAGAAA
      G  V  I  E  E  V  S  A  D  Y  I  T  V  M  H  D  N  G  T  R  R  T  Y  R  M  R  K  F  A  R  S  N  H  G  T  C  A  N  Q  C
3001 GCGGCGTCATCGAGGAGGTGTCGGCCGACTACATCACTGTGATGCACGACAACGGCACCCGGCGTACCTACCGGATGCGCAAGTTTGCCCGGTCCAACCACGGCACTTGCGCCAACCAGT
       P  I  V  D  A  G  D  R  V  E  A  G  Q  V  I  A  D  G  P  C  T  D  D  G  E  M  A  L  G  K  N  L  L  V  A  I  M  P  W  E
3121 GCCCCATCGTGGACGCGGGCGACCGAGTCGAGGCCGGTCAGGTGATCGCCGACGGTCCCTGTACTGACGACGGCGAGATGGCGCTGGGCAAGAACCTGCTGGTGGCCATCATGCCGTGGG
       G  H  N  Y  E  D  A  I  I  L  S  N  R  L  V  E  E  D  V  L  T  S  I  H  I  E  E  H  E  I  D  A  R  D  T  K  L  G  A  E
3241 AGGGCCACAACTACGAGGACGCGATCATCCTGTCCAACCGCCTGGTCGAAGAGGACGTGCTCACCTCGATCCACATCGAGGAGCATGAGATCGATGCTCGCGACACCAAGCTGGGTGCGG
       E  I  T  R  D  I  P  N  I  S  D  E  V  L  A  D  L  D  E  R  G  I  V  R  I  G  A  E  V  R  D  G  D  I  L  V  G  K  V  T
3361 AGGAGATCACCCGCGACATCCCGAACATCTCCGACGAGGTGCTCGCCGACCTGGATGAGCGGGGCATCGTGCGCATCGGTGCCGAGGTTCGCGACGGGGACATCCTGGTCGGCAAGGTCA
       P  K  G  E  T  E  L  T  P  E  E  R  L  L  R  A  I  F  G  E  K  A  R  E  V  R  D  T  S  L  K  V  P  H  G  E  S  G  K  V
3481 CCCCGAAGGGTGAGACCGAGCTGACGCCGGAGGAGCGGCTGCTGCGTGCCATCTTCGGTGAGAAGGCCCGCGAGGTGCGCGACACTTCGCTGAAGGTGCCGCACGGCGAATCCGGCAAGG
       I  G  I  R  V  F  S  R  E  D  E  D  E  L  P  A  G  V  N  E  L  V  R  V  Y  V  A  Q  K  R  K  I  S  D  G  D  K  L  A  G
3601 TGATCGGCATTCGGGTGTTTTCCCGCGAGGACGAGGACGAGTTGCCGGCCGGTGTCAACGAGCTGGTGCGTGTGTATGTGGCTCAGAAACGCAAGATCTCCGACGGTGACAAGCTGGCCG
       R  H  G  N  K  G  V  I  G  K  I  L  P  V  E  D  M  P  F  L  A  D  G  T  P  V  D  I  I  L  N  T  H  G  V  P  R  R  M  N
3721 GCCGGCACGGCAACAAGGGCGTGATCGGCAAGATCCTGCCGGTTGAGGACATGCCGTTCCTTGCCGACGGCACCCCGGTGGACATTATTTTGAACACCCACGGCGTGCCGCGACGGATGA
       I  G  Q  I  L  E  T  H  L  G  W  C  A  H  S  G  W  K  V  D  A  A  K  G  V  P  D  W  A  A  R  L  P  D  E  L  L  E  A  H
3841 ACATCGGCCAGATTTTGGAGACCCACCTGGGTTGGTGTGCCCACAGCGGCTGGAAGGTCGACGCCGCCAAGGGGGTTCCGGACTGGGCCGCCAGGCTGCCCGACGAACTGCTCGAGGCGC
       A  N  A  I  V  S  T  P  V  F  D  G  A  Q  E  A  E  L  Q  G  L  L  S  C  T  L  P  N  R  D  G  D  V  L  V  D  A  D  G  K
3961 ATGCGAACGCCATTGTGTCGACGCCGGTGTTCGACGGCGCCCAGGAGGCCGAGCTGCAGGGCCTGTTGTCGTGCACGCTGCCCAACCGCGACGGTGACGTGCTGGTCGACGCCGACGGCA
       A  M  L  F  D  G  R  S  G  E  P  F  P  Y  P  V  T  V  G  Y  M  Y  I  M  K  L  H  H  L  V  D  D  K  I  H  A  R  S  T  G
4081 AGGCCATGCTCTTCGACGGGCGCAGCGGCGAGCCGTTCCCGTACCCGGTCACGGTTGGCTACATGTACATCATGAAGCTGCACCACCTGGTGGACGACAAGATCCACGCCCGCTCCACCG
       P  Y  S  M  I  T  Q  Q  P  L  G  G  K  A  Q  F  G  G  Q  R  F  G  E  M  E  C  W  A  M  Q  A  Y  G  A  A  Y  T  L  Q  E
4201 GGCCGTACTCGATGATCACCCAGCAGCCGCTGGGCGGTAAGGCGCAGTTCGGTGGCCAGCGGTTCGGGGAGATGGAGTGCTGGGCCATGCAGGCCTACGGTGCTGCCTACACCCTGCAGG
       L  L  T  I  K  S  D  D  T  V  G  R  V  K  V  Y  E  A  I  V  K  G  E  N  I  P  E  P  G  I  P  E  S  F  K  V  L  L  K  E
4321 AGCTGTTGACCATCAAGTCCGATGACACCGTCGGCCGCGTCAAGGTGTACGAGGCGATCGTCAAGGGTGAGAACATCCCGGAGCCGGGCATCCCCGAGTCGTTCAAGGTGCTGCTCAAAG
       L  Q  S  L  C  L  N  V  E  V  L  S  S  D  G  A  A  I  E  L  R  E  G  E  D  E  D  L  E  R  A  A  A  N  L  G  I  N  L  S
4441 AACTGCAGTCGCTGTGCCTCAACGTCGAGGTGCTATCGAGTGACGGTGCGGCGATCGAACTGCGCGAAGGTGAGGACGAGGACCTGGAGCGGGCCGCGGCCAACCTGGGAATCAATCTGT
       R  N  E  S  A  S  F  E  D  L  A  *                                    RBS           V  L  D  V  N  F  F  D  E  L  R  I  G  L
4561 CCCGCAACGAATCCGCAAGTTTCGAGGATCTTGCGTAAAGCTGTCGCAAAATTACTAAACCCGTTAGGAAAGGGAGTTACGTGCTCGACGTCAACTTCTTCGATGAACTCCGCATCGGTC
       A  T  A  E  D  I  R  Q  W  S  Y  G  E  V  K  K  P  E  T  I  N  Y  R  T  L  K  P  E  K  D  G  L  F  C  E  K  I  F  G  P
4681 TTGCTACCGCGGAGGACATCAGGCAATGGTCCTATGGCGAGGTCAAAAAGCCGGAGACGATCAACTACCGCACGCTTAAGCCGGAGAAGGACGGCCTGTTCTGCGAGAAGATCTTCGGGC
       T  R  D  W  E  C  Y  C  G  K  Y  K  R  V  R  F  K  G  I  I  C  E  R  C  G  V  E  V  T  R  A  K  V  R  R  E  R  M  G  H
4801 CGACTCGCGACTGGGAATGCTACTGCGGCAAGTACAAGCGGGTGCGCTTCAAGGGCATCATCTGCGAGCGCTGCGGCGTCGAGGTGACCCGCGCCAAGGTGCGTCGTGAGCGGATGGGCC
       I  E  L  A  A  P  V  T  H  I  W  Y  F  K  G  V  P  S  R  L  G  Y  L  L  D  L  A  P  K  D  L  E  K  I  I  Y  F  A  A  Y
4921 ACATCGAGCTTGCCGCGCCCGTCACCCACATCTGGTACTTCAAGGGTGTGCCCTCGCGGCTGGGGTATCTGCTGGACCTGGCCCCGAAGGACCTGGAGAAGATCATCTACTTCGCTGCCT
       V  I  T  S  V  D  E  E  M  R  H  N  E  L
5041 ACGTGATCACCTCGGTCGACGAGGAGATGCGCCACAATGAGCTC
```

FIG. 2. Nucleotide sequence of the *M. tuberculosis* H37Rv *Sac*I fragment and the deduced amino acid sequence of the β subunit and the amino terminus portion of the β′ subunit. The *rpoB* gene product starts at position 1065 and ends at position 4598. The *rpoC* gene product starts at position 4641. RBS, potential ribosome binding sites.

CA) is located 79 to 84 bp upstream from the start codon (Fig. 2). A four-of-six match with the −10 consensus sequence is also located 29 to 34 bp upstream from the presumed GTG start codon of *rpoC*, and a three-of-six match with the −35 consensus sequence is located 45 to 50 bp upstream from the start codon and overlaps with the 3′ end of *rpoB* (Fig. 2).

Expression of the *M. tuberculosis* β subunit in *M. smegmatis*. A *Bam*HI-*Eco*RI fragment of pLM51 carrying the entire *M. tuberculosis* β subunit ORF was cloned into the *E. coli*-mycobacterial shuttle vector pMV261 (27) to create pLN-2, which was then electroporated into rifampin-susceptible *M. smegmatis* LR222 and rifampin-resistant *M. smegmatis* LR223. The MICs of rifampin for both LR222(pMV261::*lacZ*) and LR222(pLN-2) were 25 μg/ml. In contrast, LR223(pMV261::*lacZ*) was resistant to >200 μg of rifampin per ml, but LR223 (pLN-2) was relatively susceptible to rifampin (MIC = 50 μg/ml). At least six independent clones from each group of recombinants were tested in duplicate and yielded similar results (data not shown).

Expression of the mutant *M. tuberculosis* β subunit in *M.*

FIG. 3. Alignment of the deduced amino acid sequence of the *M. tuberculosis* H37Rv β subunit with the sequences of the β subunits from *M. leprae* (10), *E. coli* (23), and tobacco plant chloroplasts (22). An asterisk indicates identical amino acids in the three bacterial β subunits, and a colon indicates conservative replacements of amino acids in the three bacterial β subunits.

smegmatis. Using PCR mutagenesis techniques (8, 9), we introduced a single point mutation in the *rpoB* gene of pLN-2 (designated pMR-1), changing the serine (TCG) codon at position 2430 to 2432 to leucine (TTG), corresponding to residue 456 of the *M. tuberculosis* β subunit. This mutation has been noted to occur frequently in clinical strains of rifampin-resistant *M. tuberculosis* (28). The desired mutation in pMR-1 was confirmed by nucleotide sequencing, and pMR-1 was electroporated into rifampin-susceptible *M. smegmatis* LR222. The MICs of rifampin for both LR222(pMV261::*lacZ*) and LR222(pLN-2) were 25 μg/ml. In striking contrast, LR222 (pMR-1) was resistant to rifampin (MIC = 200 μg/ml). At least six independent clones from each group of recombinants were tested in duplicate and yielded similar results (data not shown).

DISCUSSION

In *E. coli*, the *rpoB* and *rpoC* genes are usually transcribed as part of the *rplJ-rplL-rpoB-rpoC* operon (1) by using the P1 promoter located approximately 1,600-bp upstream from *rpoB* (24). In contrast, a different *rpoB* gene organization has been noted in *M. leprae* (10). Honoré et al. described the *M. leprae rpoB* and *rpoC* genes in a tandem arrangement with the *rplL* gene located ~4,000 bp upstream from *rpoB*. These investigators described an additional ORF, designated *mkl* (homologous to the *E. coli proV* gene, which encodes a transport protein), which ends ~550 bp proximal to the *rpoB* gene (10). In *M. tuberculosis*, the *rpoB* and *rpoC* genes are in tandem with no homologs of *rplL* or *mkl* found within the ~1,000 bases upstream of these genes. The promoters actually used to

transcribe the *M. tuberculosis rpoB* and *rpoC* genes remain to be defined.

The β subunit of RNA polymerase is involved in chain initiation and elongation and rifampin resistance (15, 33). To characterize the amino acid changes in the β subunit responsible for rifampin resistance, Jin and Gross (14) isolated 42 spontaneous rifampin-resistant *E. coli* mutants and mapped the mutations within the *rpoB* gene. A total of 17 different mutations affecting 14 amino acid residues were found (507 to 511, 513, 516, 517, 522, 526, 529, 531, 532, 533, 563, 564, 572, and 687), including 14 point mutations, 2 deletions, and 1 insertion. Only one known mutation affecting amino acid 146 (17) was not included in their collection. To prove that the identified mutations were responsible for rifampin resistance, each was transferred by allelic exchange to a pBR322-derived plasmid expressing *rpoB* (14). The presence of the rifampin-resistant mutation on the plasmid was verified by showing that the plasmid conferred a rifampin-resistant phenotype to a rifampin-susceptible strain (14).

To characterize the changes associated with the *rpoB* gene in nine rifampin-resistant clinical strains of *M. leprae*, Honoré and Cole (11) PCR amplified and sequenced a 710-bp *M. leprae rpoB* gene product that corresponded to the *E. coli rpoB* gene region mapped by Jin and Gross (14). A total of nine mutations were found, including eight missense mutations involving serine codon 425, which corresponds to a mutated site identified by Jin and Gross in *E. coli* (11, 14), and one insertion mutation between codons 408 and 409. Using a similar approach, Telenti et al. (28) PCR amplified and sequenced a 411-bp product corresponding to residues 380 to 516 of the *M. tuberculosis* β subunit (residues 455 to 591 of the *E. coli* β subunit) from 66 rifampin-resistant and 56 rifampin-susceptible isolates of *M. tuberculosis*. Two of the rifampin-resistant strains and all 56 rifampin-susceptible strains had identical sequences for this 411-bp region. Fifteen different mutations, which altered eight codons, were identified in the remaining 64 rifampin-resistant strains. Each of the mutated sites in the *M. tuberculosis rpoB* gene corresponds to a mutated site identified by Jin and Gross in *E. coli* (14, 28). Although this correlation suggests that the mechanism of rifampin resistance in clinical isolates of *M. tuberculosis* may be the same as in *E. coli*, it had not been shown experimentally that any of the changes observed in the 411-bp amplicon are actually responsible for rifampin resistance.

An allelic exchange experiment is one method that can be used to determine the role of a mutation in rifampin resistance (14). Unfortunately, this method is not yet practical because gene replacement techniques have not been developed for *M. tuberculosis*. Another approach utilizes the observation that the rifampin-susceptible phenotype is dominant in *rpoB* merodiploids unless the rifampin-resistant genotype is present on a high-copy-number plasmid (14). We have shown that transformants of *M. smegmatis* that express both a plasmid-encoded *M. tuberculosis* rifampin-susceptible β subunit and a chromosomally encoded *M. smegmatis* rifampin-resistant β subunit display a relatively rifampin-susceptible phenotype. We have also shown that by introducing a specific *rpoB* mutation implicated in rifampin resistance into the *rpoB* gene of pLN-2 through in vitro mutagenesis techniques (8, 9) and then analyzing rifampin resistance in *M. smegmatis* transformants which carry the altered gene, the role of a specific mutation in rifampin resistance in *M. tuberculosis* can be determined. The rifampin susceptibility studies of LR222(pMR-1) indicate that by introducing a rifampin-resistant allele on pLN-2 we are able to overcome the chromosomal rifampin-susceptible allele of *M. smegmatis* LR222 and effect a rifampin-resistant phenotype. Further studies of different *M. tuberculosis rpoB* mutations associated with rifampin resistance are now in progress, and we plan to use the same approach to determine the *rpoB* mutations in *M. tuberculosis* that are responsible for resistance to other rifamycin compounds, especially rifabutin.

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