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Contribution of *rpoB* Mutations to Development of Rifamycin Cross-Resistance in *Mycobacterium tuberculosis*

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The contributions of 23 insertion, deletion, or missense mutations within an 81-bp fragment of *rpoB*, the gene encoding the β-subunit of the DNA-dependent RNA polymerase of *Mycobacterium tuberculosis*, to the development of resistance to rifamycins (rifampin, rifabutin, rifapentine, and KRM-1648) in 29 rifampin-resistant clinical isolates were defined. Specific mutant *rpoB* alleles led to the development of cross-resistance to all rifamycins tested, while a subset of mutations were associated with resistance to rifampin and rifapentine but not to KRM-1648 or rifabutin. To further study the impact of specific *rpoB* mutant alleles on the development of rifamycin resistance, mutations were incorporated into the *rpoB* gene of *M. tuberculosis* H37Rv, contained on a mycobacterial shuttle plasmid, by in vitro mutagenesis. Recombinant *M. tuberculosis* clones containing plasmids with specific mutations in either codon 531 or 526 of *rpoB* exhibited high-level resistance to all rifamycins tested, whereas clones containing a plasmid with a mutation in codon 516 exhibited high-level resistance to rifampin and rifapentine but were susceptible to both rifabutin and KRM-1648. These results provided additional proof of the association of specific *rpoB* mutations with the development of rifamycin resistance and corroborate previous reports of the usefulness of *rpoB* genotyping for predicting rifamycin-resistant phenotypes.

The emergence of rifampin-resistant strains of *Mycobacterium tuberculosis* has led to an increased interest in the antimycobacterial actions of rifamycin S analogs (rifamycins) (3–5, 8, 13, 14). Rifampin resistance has been shown to be associated with genetic alterations in an 81-bp region of the *rpoB* gene encoding the DNA-dependent RNA polymerase β-subunit (9, 16, 17). The association of *rpoB* mutations with the development of rifampin resistance has been further supported by the use of genetic complementation studies in which a mutant *rpoB* allele of *M. tuberculosis* H37Rv was transferred into *Mycobacterium smegmatis* LR222 on a mycobacterial shuttle vector (7). Resultant clones were resistant to high levels of rifampin. Similar studies with *M. tuberculosis*, using mutant *rpoB* alleles and rifampin or other rifamycins that demonstrate potent in vitro activity against *M. tuberculosis*, have not been conducted.

The antimycobacterial activities of several rifamycins against rifampin-resistant *M. tuberculosis* clinical isolates with known *rpoB* mutations have been analyzed to correlate the levels of rifamycin resistance with specific *rpoB* genotypes (1, 2, 8, 11, 18). These in vitro studies indicated that a subset of mutations appeared to be associated with the development of high-level rifamycin cross-resistance.

We have previously described mutations within an 81-bp fragment of the *rpoB* genes of a large collection of rifampin-resistant clinical isolates (n = 177) of *M. tuberculosis* from 11 countries and 12 laboratories within the United States (17–20). Resistance to rifampin was associated with altered *rpoB* alleles in 96% of these strains (Table 1). Twenty-three distinct missense, deletion, or insertion mutations were present in these strains, and mutations in either codon 531, 526, or 516 were present in 86% of all of the strains analyzed. No mutations were observed in 50 susceptible isolates.

Our goal in the present study was to correlate the level of rifamycin resistance with specific *rpoB* genotypes and to study the direct effect of mutant *rpoB* alleles on the development of rifamycin cross-resistance in *M. tuberculosis*. To accomplish this, a collection of 31 isolates, consisting of 29 rifampin-resistant strains with 23 unique *rpoB* alleles and 2 susceptible strains, was selected from our strain collection and tested against rifampin, rifabutin, rifapentine, and KRM-1648 to determine the MICs of each of these drugs. In addition, the three most frequently encountered *rpoB* mutations associated with the development of the rifampin-resistant phenotype were independently incorporated into the *M. tuberculosis rpoB* gene, contained on the pLN-2 plasmid (7), by PCR–site-directed mutagenesis. Resultant *M. tuberculosis* transformants were analyzed for susceptibility to the four rifamycins.

Rifampin was purchased from Sigma Chemical Co., St. Louis, Mo.; rifabutin was obtained from Pharmacia-Upjohn, Dublin, Ohio; rifapentine was obtained from Hoechst Marion Roussel, Kansas City, Mo.; and KRM-1648 was obtained from Pathogenesis Corp., Seattle, Wash. Stock solutions of each drug were prepared in dimethyl sulfoxide (DMSO), and serial twofold dilutions were prepared in DMSO.

Strains were obtained for susceptibility testing by streaking each clinical isolate on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) containing 1 μg of rifampin/ml. A single colony of each strain was added to Middlebrook 7H9 broth containing 1 μg of rifampin/ml and cultured for 8 to 12 days. Seed cultures of these strains were obtained by adding 100 μl of each culture to 4 ml of 7H12 broth (BACTEC 12B vials; Becton Dickinson, Towson, Md.) and incubating these

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TABLE 1. Mutant *rpoB* alleles in 177 rifampin-resistant clinical isolates of *M. tuberculosis*

Isolate phenotype	Amino acid(s) affected[a]	Amino acid change(s)	No. of isolates (frequency [%])	Origin of isolate(s) (no.)
Resistant[b]	509, 526	Ser→Thr, His→Asp	1 (0.6)	Canada (1)
	511	Leu→Pro	1 (0.6)	U.S.[c] (1)
	513	Gln→Leu	1 (0.6)	U.S. (1)
	513	Gln→Pro	2 (1.1)	Philippines (1), U.S. (1)
	514	Phe insertion	1 (0.6)	U.S. (1)
	516	Asp→Val	13 (7.4)	U.S. (9), Switzerland (1), Peru (1), Yemen (2)
	516	Asp→Gly	1 (0.6)	U.S. (1)
	517	Gln deletion	1 (0.6)	U.S. (1)
	518	Asn deletion	2 (1.1)	U.S. (2)
	519	Asn→Lys	1 (0.6)	U.S. (1)
	521	Leu→Met	1 (0.6)	U.S. (1)
	522	Ser→Leu	2 (1.1)	U.S. (1), Japan (1)
	522, 516	Ser→Leu, Asp→Val	1 (0.6)	Honduras (1)
	526	His→Pro	4 (2.3)	Japan (1), Peru (1), U.S. (2)
	526	His→Leu	5 (2.9)	Rwanda (1), U.S. (4)
	526	His→Asp	8 (4.6)	Switzerland (1), U.S. (7)
	526	His→Arg	9 (5.2)	Rwanda (2), Japan (4), U.S. (3)
	526	His→Tyr	35 (20.0)	U.S. (30), Yemen (1), Peru (1), Philippines (1), Honduras (2)
	527	Lys→Gln	1 (0.6)	U.S. (1)
	527, 526	Lys→Gln, His→Pro	1 (0.6)	U.S. (1)
	531	Ser→Leu	74 (42.0)	Belgium (1), Japan (5), Rwanda (9), Canada (2), Philippines (3), Vietnam (1), Yemen (4), U.S. (44), Peru (2), Honduras (3)
	531	Ser→Phe	1 (0.6)	U.S. (1)
	533	Leu→Pro	3 (1.7)	U.S. (2), Philippines (1)
	None	None	8 (4.6)	Rwanda (2), Berundi (1), U.S. (5)
Susceptible	None	None	50	U.S. (42), Honduras (8)

[a] Amino acid numbers correspond to the *E. coli* numbering system for the RNA polymerase β-subunit.
[b] Resistant to rifampin at 2 μg/ml by the BACTEC radiometric method or at 1 μg/ml by the proportion method.
[c] U.S., United States.

cultures at 37°C until a growth index (GI) reading of 800 was obtained in a BACTEC 460 instrument (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.).

The susceptibilities of these strains to rifamycins were determined by adding 100-μl aliquots of the seed cultures to BACTEC 12B vials containing DMSO alone or serial twofold dilutions of each rifamycin in DMSO, with concentrations ranging from 0.015 to 64 μg/ml for rifampin and rifabutin, from 0.031 to 64 μg/ml for rifapentine, and from 0.0156 to 10.24 μg/ml for KRM-1648. Drug-free controls consisted of a 1:100 dilution of the original inoculum added to BACTEC 12B vials (1:100 control). Cultures were incubated at 37°C, and GI readings were recorded daily with the BACTEC 460 instrument until the GI of the 1:100 control reached ≥30. The MIC for each rifamycin was defined as the lowest concentration that inhibited more than 99% of the bacterial population as assessed by GI readings in the BACTEC instrument.

Rifamycin MICs for rifampin-resistant clinical isolates of *M. tuberculosis* with unique *rpoB* mutant alleles are shown in Table 2. Eighteen of the *rpoB* mutations were associated with MICs of rifampin, rifabutin, rifapentine, and KRM-1648 above those obtained for susceptible strains. The remaining five *rpoB* mutations, in codons 511, 516, 519, and 522, were associated with resistance to rifampin and rifapentine but susceptibility to rifabutin and KRM-1648. These results confirmed earlier observations that strains with mutations in selected *rpoB* codons remain moderately susceptible to rifabutin or KRM-1648 or exhibit low-level resistance to these rifamycins (1, 8, 18). Strains with the same *rpoB* genotype but from different geographical locations showed similar patterns of resistance (Tables 1 and 2).

To further define the role of mutations in the development of rifamycin cross-resistance in *M. tuberculosis*, we employed a molecular genetic approach involving a PCR-based site-directed mutagenesis protocol. Mutagenesis was used to incorporate silent mutations (signature nucleotides) in codons 510 and 511 of the *rpoB* gene of pLN-2 (Fig. 1). These signature nucleotides would be used to identify the presence of the plasmid copy of *rpoB* and, therefore, eliminate the possibility that the rifampin-resistant phenotype of transformants resulted from the selec-

TABLE 2. Rifamycin MICs for clinical isolates of *M. tuberculosis* with mutant *rpoB* alleles

Clinical isolate	Affected amino acid(s) (change)[b]	MIC (μg/ml) of[a]: Rifampin	Rifabutin	Rifapentine	KRM-1648
Resistant[c]					
TB54	511 (Leu→Pro)	2	0.5	1	<0.01
TB022	513 (Gln→Pro)	32	8.0	32	0.32
TB872	513 (Gln→Pro)	32	4.0	32	0.32
TB713	513 (Gln→Leu)	16	2.0	16	0.16
TBU36	514 (Phe insertion)	32	<2.0	32	<0.16
TB3908	516 (Asp→Val)	32	<0.5	32	0.01
TBYE-67	516 (Asp→Val)	32	0.5	32	0.01
TBGro-1	516 (Asp→Tyr)	2	<0.5	8	0.04
TB33	517 (Gln deletion)	32	8.0	64	2.56
TB4183	518 (Asn deletion)	64	64	>32	5.12
TB053	518–519 (Asn deletion)	>64	>32	>32	>10.24
TBRos-1	519 (Asn→Lys)	32	0.5	32	0.01
TB3505	521 (Leu→Met)	32	8.0	32	2.56
TBNor-1	522 (Ser→Leu)	32	0.5	32	0.01
TB2230	522 (Ser→Leu), 516 (Asp→Val)	>32	>1.0	32	0.12
TBRR-1	526 (His→Arg)	64	64	<32	5.12
TB5095	526 (His→Leu)	16	8	16	0.56
TBRR-7	526 (His→Pro)	>64	32	>64	2.56
TB160	526 (His→Pro)	>64	>32	>64	5.12
TBYE-68	526 (His→Tyr)	>64	32	>64	>2.56
TB3081	526 (His→Tyr)	>64	>32	>64	5.12
TB052	526 (His→Asp)	>64	64	>32	>2.56
TB3140	526 (His→Asp)	>64	64	>32	5.12
TB91	526 (His→Asp), 509 (Ser→Leu)	>64	8	>64	0.64
TB023	527 (Lys→Gln)	>32	16	32	2.56
TBYE-14	531 (Ser→Leu)	>64	32	>64	10.24
TB3241	531 (Ser→Leu)	>64	32	>64	>10.24
TB019	531 (Ser→Phe)	64	32	>64	10.24
TB3126	533 (Leu→Pro)	64	8	64	2.56
Susceptible					
TBSmi-1	None	0.5	<0.5	<0.12	<0.01
TBH1-7	None	0.5	<0.5	0.12	<0.01

[a] MICs determined by the BACTEC radiometric method.
[b] Amino acid numbers correspond to the *E. coli* numbering system for the RNA polymerase β-subunit.
[c] Clinical isolates of *M. tuberculosis* resistant to rifampin at ≥2 μg/ml by the BACTEC radiometric method or at 1 μg/ml by conventional agar plate testing.

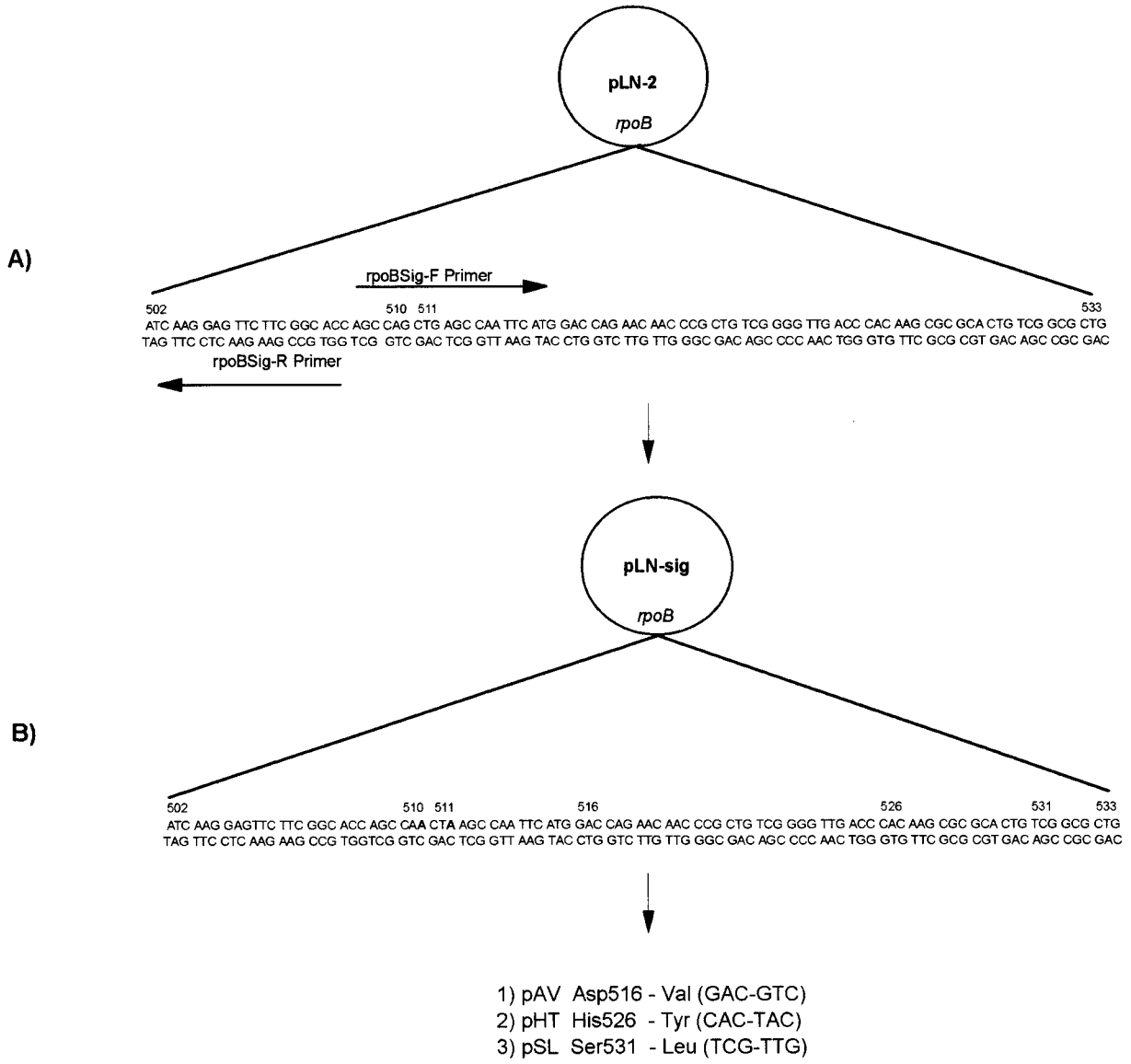

FIG. 1. Nucleotide substitutions were incorporated into the *rpoB* gene of *M. tuberculosis* H37Rv contained on pLN-2, using mutagenic primers (Table 3) and long-fragment PCR–site-directed mutagenesis. (A) Signature nucleotides were incorporated into pLN-2 at codons 510 (CAG→CAA) and 511 (CTG→CTA) to yield the pLN-sig plasmid. (B) Mutant *rpoB* alleles were incorporated into pLN-sig as follows: 1) a GAC→GTC nucleotide substitution in codon 516 of pLN-sig to yield plasmid pAV, 2) a CAC→TAC nucleotide substitution in codon 526 of pLN-sig to yield plasmid pHT, and 3) a TCG→TTG nucleotide substitution in codon 531 of pLN-sig to yield plasmid pSL.

tion of spontaneously rifampin-resistant mutants. Briefly, 250 ng of pLN-2 was combined with XL-PCR reagents (Perkin-Elmer, Norwalk, Conn.) and primers rpoBSig-F and rpoBSig-R (Table 3) and amplified, using the manufacturer's recommended protocol to incorporate nucleotide substitutions and produce linearized plasmid DNA. PCR products were treated with 5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) to polish the ends of the linear plasmid DNA and digested with 40 U of the restriction endonuclease *Dpn*I (Stratagene) to eliminate parental plasmid DNA. The preparation was treated with 4 U of T4 ligase (Gibco-BRL, Gaithersburg, Md.), and recircularized plasmids were cloned into *Escherichia coli* XL1-Blue (Stratagene). Kanamycin-resistant colonies were analyzed for the presence of *rpoB* containing signature nucleotides by PCR-direct DNA sequencing using primers and conditions previously described (19). Plasmid (pLN-sig) DNA was purified from a positive clone by using a QIAquick plasmid kit (Qiagen Inc., Chatsworth, Calif.).

Specific *rpoB* mutant alleles (531 Leu, 526 Tyr, and 516 Val), associated with the rifampin-resistant phenotype of *M. tuberculosis* (Table 1), were independently incorporated into pLN-sig by PCR–site-directed mutagenesis (Fig. 1) with the primers described in Table 3. All resultant plasmids were cloned into *E. coli* XL1-Blue, and recombinant clones with signature nucleotides and mutant *rpoB* alleles were identified by PCR-direct DNA sequencing.

Plasmid DNA of one clone for each mutant allele was purified by using a large-scale plasmid DNA preparation protocol (6). The presence of signature nucleotides and mutant *rpoB* alleles and the absence of spurious mutations in *rpoB* in these plasmids were confirmed by PCR-DNA sequencing with primers that span the entire insert containing *rpoB* (data not shown).

TABLE 3. Primers for PCR–site-directed mutagenesis of *M. tuberculosis rpoB* in plasmid pLN-2

Primer	Primer sequence[a]	Mutation[b]
rpoBSig-F	5′ CAGCCAACTAAGCCAATTC 3′	Signature nucleotides
rpoBSig-R	5′ GTGCCGAAGAACTCCTTGAT 3′	
rpoBVal516-F	5′ TCATGGTCCAGAACAACCCG 3′	Asp 516→Val (GAC→TAC)
rpoBVal516-R	5′ ATTGGCTTAGTTGGCTG 3′	
rpoBTyr526-F	5′ GTTGACCTACAAGCGCCGA 3′	His 526→Tyr (CAC→TAC)
rpoBTyr526-R	5′ CCCGACAGCGGGTTGTTCTGGTC 3′	
rpoBLeu531-F	5′ GACTGTTGGCGCTGG 3′	Ser 531→Leu (TCG→TTG)
rpoBLeu531-R	5′ GGCGCTTGTGGGTCAA 3′	

[a] All primers were phosphorylated on the 5′ end.
[b] Mutations in the *rpoB* of *M. tuberculosis* H37Rv contained on plasmid pLN-2 by use of PCR–site-directed mutagenesis.

Next, using a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) and a 0.2-cm-light-path cuvette, 1 to 2 μg of each plasmid was electroporated (at 2.5 kV, 25 μF, and 1,000 Ω) into electrocompetent (glycerol-treated) *M. tuberculosis* H37Rv (ATCC 27294) cells. Clones were selected on 7H11 agar containing 30 μg of kanamycin/ml.

Mutant β-subunits were expressed in these recombinants, and the susceptibilities of recombinant *M. tuberculosis* clones to rifamycins were determined radiometrically with the BACTEC instrument, using a liquid medium as indicated above. Rifampin-susceptible *M. tuberculosis* H37Rv (ATCC 27294) and rifampin-resistant *M. tuberculosis* H37Rv-Rif-r (ATCC 35838) were used as controls.

The results of these experiments indicated that *M. tuberculosis* recombinant clones containing plasmids with specific *rpoB* mutant alleles affecting either codon 531 or 526 showed high-level resistance to all rifamycins analyzed (Table 4). Clones containing a mutation within codon 516, resulting in the substitution of a valine for an aspartic acid residue in the β-subunit, showed a differential susceptibility to rifamycins, with KRM-1648 and rifabutin being the most active against this mutant. Clones containing either pLN-2 or pLN-sig (i.e., containing wild-type *rpoB* or *rpoB* with signature nucleotides, respectively) demonstrated rifamycin MICs consistent with the rifampin-susceptible phenotype of *M. tuberculosis* (Table 4). In addition, it was previously shown that the same vector with *lacZ* (not *rpoB*) as the insert (pMV261::lacZ) did not affect the rifamycin phenotype of either a rifampin-susceptible or a rifampin-resistant strain of *M. smegmatis* (7). Therefore, these results demonstrated that expression of the plasmid-encoded β-subunit was responsible for the alteration of the rifamycin susceptibility of recombinant clones.

The lower MICs of some of the recombinant clones compared to those of clinical isolates most likely resulted from the merodiploid state of *rpoB* in these cells (wild-type *rpoB* and *rpoB* containing mutant alleles). However, expression of plasmid-encoded β-subunits containing mutant *rpoB* alleles dramatically altered the rifampin phenotype in most of the recombinants. The most plausible explanation for this is as follows. pLN-2 is a multicopy vector, a derivative of pMV261, which has been shown to efficiently transform mycobacterial species and to maintain about five copies per genome equivalent in these mycobacterial cells (15). Therefore, the plasmid-encoded β-subunit is produced in abundance and thereby effectively outcompetes the single chromosomally encoded copy to make a mutant RNA polymerase holoenzyme. This altered enzyme results in the development of rifampin resistance.

Previously, we demonstrated that transformation of *M. smegmatis* LR222 with these plasmids resulted in the conversion of the rifampin-susceptible phenotype to the resistant phenotype (18). The present study extended these observations by including the performance of complementation studies in *M. tuberculosis* and analysis of three of the most frequently occurring *rpoB* mutant alleles of clinical isolates. In addition, the contribution of specific *rpoB* mutations in the development of rifamycin cross-resistance in *M. tuberculosis* was further defined by using this approach, with the results demonstrating that *rpoB* mutations present in rifampin-resistant clinical isolates are solely responsible for the development of the rifamycin-resistant phenotype.

These experiments define the role of specific *rpoB* mutant alleles that are involved in the development of selective susceptibility to rifampin analogs with specific chemical compositions. Rifamycin analogs that retain antituberculosis activity in the presence of specific mutant *rpoB* alleles have been identified. The ability of rifabutin and KRM-1648 to overcome rifampin resistance in vitro in a selected group of strains containing specific *rpoB* mutant alleles suggests that amino acid positions and specific substitutions in these regions of the β-subunit of the RNA polymerase are very important for the selective affinity and activity of specific rifamycin structural analogs for mutant β-subunits. Therefore, it appears that rifamycin structure can potentially be modified to circumvent rifampin resistance in some rifampin-resistant strains. However, this has not been proven in vivo in human clinical trials, in which issues such as solubility and attainable blood levels must be investigated.

The data obtained in the present study suggest that the detection of *rpoB* mutant alleles by several molecular genetic-based analyses, such as the line probe assay (2), PCR-heteroduplex formation (19, 20), PCR-SSCP (16), and PCR-direct DNA sequencing (10, 12), could be used to rapidly determine the susceptibility of a clinical strain to various rifamycins and provide information that may have an impact on treatment strategies and check further spread of drug-resistant mutants. In addition, this highly characterized collection of rifampin-resistant clinical isolates and genetically engineered *M. tuberculosis* clones expressing mutant β-subunits should be useful for studying specific structure-activity relationships between various structurally modified rifamycin analogs and wild-type and mutant β-subunits. Understanding how rifamycins kill *M. tu-*

TABLE 4. Rifamycin resistance of *M. tuberculosis* recombinant clones

Strain[a]	Mutated amino acid[b]	MIC (μg/ml) of[c]:			
		Rifampin	Rifabutin	Rifapentine	KRM-1648
H37Rv	None	0.25	≤0.015	0.031	≤0.015
H37Rv-pLN-2	None	0.25	≤0.015	0.031	≤0.015
H37Rv-pLN-sig	None	0.25	≤0.015	≤0.031	≤0.015
H37Rv-pAV	Val 516	32	≤0.015	16	≤0.015
H37Rv-pHT	Tyr 526	64	16	16	8
H37Rv-pSL	Leu 531	>64	16	>64	8
H37Rv-Rif-r	Leu 531	>64	16	>64	8

[a] H37Rv, *M. tuberculosis* H37Rv (ATCC 27294); H37Rv-pLN-2, *M. tuberculosis* H37Rv clone containing plasmid pLN-2 with wild-type *rpoB*; H37Rv-pLN-sig, *M. tuberculosis* H37Rv clone containing pLN-sig with signature nucleotides in *rpoB*; H37Rv-pAV, *M. tuberculosis* H37Rv clone containing plasmid pAV with Val 516 mutant allele in *rpoB*; H37Rv-pHT, *M. tuberculosis* H37Rv clone containing plasmid pHT with Tyr 526 mutant allele in *rpoB*; H37Rv-pSL, *M. tuberculosis* H37Rv clone containing plasmid pSL with Leu 531 mutant allele in *rpoB*; H37Rv-Rif-r, *M. tuberculosis* H37Rv (ATCC 35838).
[b] Amino acid numbering corresponds to that of the *E. coli* RNA polymerase β-subunit.
[c] MICs determined by the BACTEC radiometric method.

berculosis will potentially provide information for the rational design of a rifamycin analog(s) or to identify existing analogs which can be used to circumvent rifampin resistance.

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