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Characterization of Spontaneous, In Vitro-Selected, Rifampin-Resistant Mutants of *Mycobacterium tuberculosis* Strain H37Rv

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Resistance to rifampin in *Mycobacterium tuberculosis* results from mutations in the gene coding for the beta subunit of RNA polymerase (*rpoB*). At least 95% of rifampin-resistant isolates have mutations in *rpoB*, and the mutations are clustered in a small region. About 40 distinct point mutations and in-frame insertions and deletions in *rpoB* have been identified, but point mutations in two codons, those coding for Ser_{531} and His_{526}, are seen in about 70% of rifampin-resistant clinical isolates, with Ser_{531}-to-Leu (TCG-to-TGG) mutations being by far the most common. To explore this phenomenon, we isolated independent, spontaneous, rifampin-resistant mutant versions of well-characterized *M. tuberculosis* laboratory strain H37Rv by plating 100 separate cultures, derived from a single low-density inoculum, onto rifampin-containing medium. Rifampin-resistant mutants were obtained from 64 of these cultures. Although we anticipated that the various point mutations would occur with approximately equal frequencies, sequencing the *rpoB* gene from one colony per plate revealed that 39 (60.9%) were Ser_{531} to Leu. We conclude that, for unknown reasons, the associated *rpoB* mutation occurs at a substantially higher rate than other *rpoB* mutations. This higher mutation rate may contribute to the high percentage of this mutation seen in clinical isolates.

Rifampin and isoniazid remain the two most important drugs for the treatment of tuberculosis, and resistance to either drug represents a serious impediment to successful therapy. Resistance to rifampin is associated with mutations in the gene coding for the beta subunit of RNA polymerase (*rpoB*). Early studies examined the rate of rifampin resistance mutations in *Mycobacterium tuberculosis* (3). More recently, the sequencing of the *rpoB* gene in *M. tuberculosis* and the development of direct sequencing of PCR products have allowed determination of the actual mutations (11, 16), and there has been extensive analysis of mutations in the *rpoB* gene of rifampin-resistant patient isolates of *M. tuberculosis* (2, 4–7, 9–11, 13–17). Telenti et al. demonstrated that at least 95% of rifampin-resistant isolates have mutations in *rpoB* and that the mutations are clustered in an 81-bp region (16). Because RNA polymerase is an essential enzyme, there must be a limited number of possible mutations that confer rifampin resistance and retain polymerase activity, and this is reflected in the very low rate of mutation to resistance. About 40 distinct point mutations and in-frame insertions and deletions in *rpoB* have been identified in *M. tuberculosis* isolates. Point mutations in two codons, those encoding Ser_{531} and His_{526}, are seen in about 70% of rifampin-resistant clinical isolates, with Ser_{531}-to-Leu (TCG-to-TTG) mutations being by far the most common. We hypothesized that the various point mutations (single base changes) occur at about the same frequencies and that the finding of predominant mutations in clinical isolates is likely a result of specific mutations having a selective advantage, at the phenotypic level, for proliferation in humans following rifampin therapy. To explore this question, we isolated independent spontaneous rifampin-resistant mutant versions of well-characterized *M. tuberculosis* laboratory strain H37Rv by plating 100 separate cultures onto rifampin-containing medium. We then identified the *rpoB* mutations in the resulting 64 mutants. Contrary to our expectations, the distribution of mutations mirrored that seen in patient isolates.

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MATERIALS AND METHODS

Isolation of rifampin-resistant mutants. *M. tuberculosis* H37Rv from our laboratory stock was cultured in Middlebrook 7H9 broth at 37°C for 32 days to an optical density at 600 nm of 0.83, corresponding to approximately 10^8 CFU/ml. The culture was diluted and inoculated into 500 ml of 7H9 broth to yield a concentration of about 10^3 CFU/ml, and 5-ml aliquots were dispensed into 100 culture tubes. The tubes were incubated at 37°C for 32 days with daily shaking. Approximately 2 ml of culture containing most of the cell mass was pipetted from the bottom of each tube and transferred to a sterile screw-cap microcentrifuge tube. The tube was centrifuged for 30 s, the supernatant was aspirated, and the bacterial pellet was suspended in 1 ml of 0.5% Tween 80 in water. The tube was centrifuged, and the supernatant was aspirated, leaving a small amount of liquid. The pellet was suspended, and the entire volume was spread onto one quadrant of a plate containing Middlebrook 7H10 agar with 1 μg of rifampin/ml. The plates were incubated for 4 weeks at 37°C. Colonies from these plates were subcultured in 7H9 broth without Tween 80 containing 2 μg of rifampin/ml.

Amplification and sequencing of the *rpoB* gene. Genomic DNA was isolated using a mechanical cell disruption procedure. Briefly, 1 ml of a 7H9 broth culture was added to a 1.5-ml screw-cap microcentrifuge tube containing approximately 250 mg of siliconized zirconia and silica beads (0.1 mm in diameter), 200 μl of chloroform, and 300 μl of Tris-EDTA buffer. This mixture was vigorously agitated for 2 min using a Mickle cell disrupter (Brinkman Instruments, Inc., Westbury, N.Y.) and then centrifuged at 10,000 rpm in an Eppendorf model 5917R microcentrifuge for 5 min. The aqueous phase, containing genomic DNA, was collected and stored at 4°C.

The rifampin resistance-determining region of the *rpoB* gene was amplified by PCR using primers BC35 (5′-ATCAACATCCGGCCGGTGGT-3′) and BC41R (5′-TACACCGACAGCGAGCCGAT-3′). Each 25-μl PCR mixture contained 1.0 μl of template genomic DNA, 1.25 U of HotStart*Taq* DNA polymerase (Qiagen, Inc., Valencia, Calif.), deoxynucleotide triphosphates (200 μM each), 1.5 mM $MgCl_2$, and 300 nM (each) primer in 1× PCR buffer. Amplification was performed in a Gene-Amp PCR system 2400 thermal cycler (Perkin-Elmer, Inc., Foster City, Calif.). The amplification profile consisted of an initial 15-min denaturation and enzyme activation at 95°C followed by 35 cycles of 94°C denaturation for 30 s, 62°C annealing for 30 s, and 72°C elongation for 45 s and a final 8-min elongation.

Automated DNA sequencing was performed using dichlororhodamine BigDye Terminator chemistry according to the protocol supplied by the manufacturer (Perkin-Elmer, Inc.). The fluorescent elongation products were electrophoresed on a model 373XL DNA sequencer (Perkin-Elmer, Inc.). The 257-bp *rpoB* amplicons were sequenced with the same primers used for amplification. All postrun analysis was performed using Sequence Navigator, version 1.0.1, software (Perkin-Elmer, Inc.). Each sequencing run included rifampin-susceptible H37Rv as a wild-type control. Each sequence was compared both with the

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control strain sequence and with the published *rpoB* sequence (GenBank accession no. L27989). The codon numbers are based on the alignment with the *Escherichia coli rpoB* sequence and are not the actual codon numbers of the *M. tuberculosis* sequence.

RESULTS

The procedure for isolating a set of independent mutants was essentially the approach used in the classic fluctuation test of Luria and Delbruck (8). A dense culture of wild-type, rifampin-susceptible *M. tuberculosis* H37Rv was diluted to yield a 500-ml culture containing approximately 10^3 CFU/ml. Results of a previous study of the frequency of mutations to rifampin resistance and our own experience indicated that no rifampin-resistant mutants should be present in the total population of 5×10^5 CFU in this culture (3). The culture was aliquoted into 100 tubes, and these parallel cultures were incubated to allow growth to the high density needed to obtain rifampin-resistant mutants. Most of the cell mass from each of the 100 cultures was harvested and plated onto 7H10 agar containing rifampin. We expected this procedure to produce mutants derived from independent mutational events in each culture. Of the 100 cultures, 32 did not yield colonies. Colonies picked from four plates failed to grow on subculture in 7H9 broth containing rifampin. The average number of mutations per culture (m) can be calculated based on the number of cultures that yielded no mutants using the Poisson equation, $p_0 = e^{-m}$, where p_0 is the proportion of cultures yielding no mutants. This value is 1.13 for $p_0 = 0.32$ or 1.02 for $p_0 = 0.36$, depending on which value is used for plates with no mutants. It should be noted that we plated most, but not all, of the cells in each 5-ml culture, and the number of cultures with no mutants may have been slightly lower had we recovered all mutants. Regardless, this result indicates that in most cases all colonies derived from each culture are the progeny from a single mutational event. The remaining 64 cultures yielded rifampin-resistant mutants (Table 1). Of these, 62 plates had from 1 to 11 colonies (average, 2.32 colonies), with 29 plates having only a single colony. One colony was picked from each of the plates for subculture. The remaining two plates, plates 10 and 76, had 27 and 288 colonies, respectively, and 10 colonies were picked from each of these two plates.

All of the point mutations detected in this study have been reported previously (Table 1). All 10 colonies picked from plate 76 had the same mutation, Ser_{531} to Leu (TCG to TTG), consistent with the conclusion that the mutation occurred early in this culture and yielded many progeny. Similarly, 9 of the 10 colonies from plate 10 had the Ser_{531}-to-Leu mutation. The other colony gave a result indicating a mixture of mutants with mutations Ser_{531} to Leu and His_{526} to Tyr (CAC to TAC), i.e., peaks for both C and T were present at each position. For simplicity, we did not consider the latter mutant in the analysis but instead counted culture 10 as representing a Ser_{531}-to-Leu mutation.

Of the 64 mutants, 39 (60.9%) had the Ser_{531}-to-Leu mutation (Table 2). This is the most common mutation seen in patient isolates also. Two mutants had a different mutation in codon 531, resulting in a Ser_{531}-to-Trp change. Four mutations in codon 526 were identified in a total of 16 mutants. This is the second-most-frequent site for mutations seen in patient isolates. Four other mutations were identified: two point mutations, a three-base deletion, and a three-base insertion.

The sizes of the colonies on the rifampin plates were recorded as tiny, small, medium, and large. There was no correlation between colony size and mutation; in particular, we observed all colony sizes for Ser_{531}-to-Leu mutants.

TABLE 1. *rpoB* mutations detected in 100 independent cultures

Plate[a]	Colony count	Mutation[b]
2	1	H526R
3	1	S531L
4	1	S531L
6	2	S531L
7	1	S531L
9	2	S531L
10	27	S531L[c]
11	3	H526Y
12	11	S522L
13	1	S531W
14	4	S531L
18	1	S531L
20	6	H526Y
25	1	S531L
27	2	S531L
28	1	H526R
29	2	S531L
31	3	H526D
33	1	Deletion
34	1	H526R
35	1	S531L
37	1	S531L
39	3	S522L
41	1	S531L
42	1	H526D
44	1	S531L
45	1	S531L
47	4	H526P
49	4	H526R
50	2	S531L
53	3	S531L
55	3	S531L
57	1	D516V
59	2	H526Y
60	4	S531L
61	5	H526R
62	1	S531L
64	1	S531L
66	2	S531L
67	1	S531L
68	1	S531W
69	2	S531L
70	3	S522L
71	1	H526Y
72	1	Insertion
73	1	S531L
74	3	S531L
76	288	S531L[d]
77	5	H526D
78	1	S531L
79	3	S531L
80	2	S531L
82	1	S531L
83	2	H526D
84	2	S531L
85	1	H526Y
88	1	S531L
89	5	S531L
90	9	S531L
91	3	S531L
93	5	S522L
96	2	S531L
98	2	H526Y
100	1	S531L

[a] The other 36 plates yielded no colonies or colonies that failed to grow on subculture in broth.
[b] Corresponding codon changes are listed in Table 2.
[c] Ten colonies were picked for sequencing. The electropherogram for one colony showed a mixture of S531L and H526Y. The other nine colonies gave S531L.
[d] Ten colonies were picked for sequencing; all gave S531L.

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TABLE 2. *rpoB* mutations observed in rifampin-resistant mutants

Mutation	Codon change	No. (%) of mutations
S531L	TCG→ TTG	39 (60.9)
H526Y	CAC→ TAC	6 (9.4)
H526R	CAC→ CGC	5 (7.8)
H526D	CAC→ GAC	4 (6.2)
S522L	TCG→ TTG	4 (6.2)
S531W	TCG→ TGG	2 (3.1)
H526P	CAC→ CCC	1 (1.6)
D516V	GAC→ GTC	1 (1.6)
Deletion	GAC, codon 516[a]	1 (1.6)
Insertion	TTC, after codon 513 (Q)	1 (1.6)
Total		64

[a] Mutation not previously reported.

DISCUSSION

In this study we demonstrated that the *rpoB* mutations most commonly seen in rifampin-resistant clinical isolates of *M. tuberculosis*, especially the one resulting in the Ser_{531}-to-Leu change, also predominate in independent strain H37Rv mutants obtained by selection in vitro on 7H10 medium containing rifampin. This was not the result we anticipated; we expected that all point mutations would occur at about the same frequency. In patients with tuberculosis, rifampin resistance evolves following treatment when the rare spontaneously resistant mutants proliferate and replace the population of sensitive bacilli that have been killed by the drug. Although mutations imparting rifampin resistance occur at low frequency, the bacillary load present in cavitary tuberculosis is sufficient to allow for their selection. Expansion of the population of resistant mutants requires several weeks, and it is reasonable to assume that if several distinct mutants are present at the onset of therapy, the more-robust mutants, perhaps those with Ser_{531}-to-Leu mutations, will likely predominate and ultimately be isolated from the patient's sputum. The fitness of the mutant might also affect the probability of transmission, increasing the proportion of such mutants in patients with primary resistance. Although there have been significant studies of the evolution of rifampin resistance in patients, none have dealt with the emergence of specific mutations, which has been possible only in recent years.

The recent paper of Billington et al. also describes the isolation of rifampin-resistant H37Rv mutants (1). They determined that Ser_{531} to Leu is the most common mutation seen with in vitro-generated rifampin-resistant mutants and demonstrated that a mutant with this mutation shows the least physiological cost relative to the rifampin-susceptible parent strain and thus has a selective advantage. However, they sampled a smaller number of batch cultures than we did and, in contrast to our study, picked multiple colonies from the same cultures. Surprisingly, they detected only four mutations, Ser_{531} to Leu and three mutations in the His_{526} codon, among 156 colonies screened. Their results indicate either that these mutations occur at a higher frequency than other point mutations or that these mutants grow more rapidly and thus are more likely to be detected using their sampling procedure. The method used in the present study, essentially that of Luria and Delbruck (8), was intended to avoid the latter possibility. As in the classic fluctuation test, variation in the number of resistant colonies obtained from each separate culture reflects the time at which the mutation occurred. If the selection occurs at the appropriate time, most colonies derived from each culture represent progeny from a single event. In this case, the low frequency of rifampin-resistant mutants is advantageous, because few mutants are present even in dense cultures. The fact that one-third of the cultures did not yield mutants is consistent with single-mutation events in most of the cultures. If each culture contains only a single mutant there is no opportunity for competition between different mutants and enrichment of the faster-growing mutant prior to plating. Thus, the distribution of mutations with this procedure should reflect the relative rates of occurrence of the various mutations. We did not sequence all colonies from all plates, and it is possible that some selection bias occurred. Looking only at the cultures that yielded a single colony, in which there could be no bias in the selection of the colony for sequencing, 18 of 29 (62%) mutations were Ser_{531} to Leu.

Two possible mechanisms could explain the higher rate of occurrence of the Ser_{531}-to-Leu mutants. This could result from a higher rate of occurrence of the corresponding specific base substitution, i.e., an increased error rate at that site. Studies of DNA repair in *M. tuberculosis* have been reviewed recently (12), but we are not aware of any data that would explain a higher frequency of the Ser_{531}-to-Leu mutation. An alternative explanation is that other mutations occur at the same frequency but are less successful in making the transition to expression of the mutant beta subunit (as a result of genotypic and phenotypic lag), either in the absence of rifampin (in 7H9 broth) or upon initial exposure to rifampin on plates. To impact our results, this would require a lethal effect rather than merely a reduction in growth rate. Undoubtably many mutations in *rpoB* are lethal, but about 40 distinct mutations have been identified in patient isolates, indicating that all of the associated mutants are sufficiently fit to proliferate in the host, grow on laboratory media, and demonstrate rifampin resistance in standard susceptibility testing procedures, such as growth on 7H10 medium at the critical concentration of 1 μg of rifampin/ml (7a). Differences in MICs of rifampin for various *rpoB* mutants have been demonstrated. Because of this, we used the minimum concentration of rifampin (1 μg/ml) that reliably inhibits wild-type *M. tuberculosis* to avoid a selective advantage for certain mutants.

Whatever the mechanism, our results indicate that the Ser_{531}-to-Leu mutation and the multiple mutations in codon 526 occur at a significantly higher frequency than other point mutations. The precise rate of occurrence of these mutations cannot be determined from this limited study, but the overall trend is clear. Such higher occurrence rates may contribute to the high rate at which these mutants are isolated from patients with tuberculosis, although the emergence of rifampin resistance in humans is much more complex than that reflected in the simple in vitro experiments reported here.

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