Acquisition of rifabutin resistance by a rifampicin resistant mutant of *Mycobacterium tuberculosis* involves an unusual spectrum of mutations and elevated frequency

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Abstract

**Background:** Mutations in a small region of the *rpoB* gene are responsible for most rifamycin resistance in *Mycobacterium tuberculosis*. In this study we have sequentially generated resistant strains to first rifampicin and then rifabutin. Portions of the *rpoB* gene were sequenced from 131 randomly selected mutants. Second round selection resulted in a changed frequency of specific mutations.

**Methods:** *Mycobacterium tuberculosis* (strain Mtb72) rifamycin resistant mutants were selected in vitro with either rifampicin or rifabutin. One mutant R190 (*rpoB* S522L) selected with rifampicin had a rifampicin MIC of 32 µg/ml but remained sensitive to rifabutin (MIC<0.8 µg/ml). This mutant was subjected to a second round of selection with rifabutin.

**Results:** All 105 first round resistant mutants derived from the parent strain (Mtb72) screened acquired mutations within the 81 bp *rpoB* hotspot. When the rifampicin resistant but rifabutin sensitive S522L mutant was subjected to a second round of selection, single additional *rpoB* mutations were identified in 24 (92%) of 26 second round mutants studied, but 14 (54%) of these strains contained mutations outside the 81 bp hotspot (codons 144, 146, 148, 505). Additionally, spontaneous rifabutin resistant mutants were produced at >10 times the frequency by the S522L mutant than the parent strain.

**Conclusion:** First round selection of mutation S522L with rifampicin increased the frequency and changed the spectrum of mutations identified after selection with rifabutin.

Introduction

It has been estimated that one third of the World’s population is infected with *Mycobacterium tuberculosis* (MTB) resulting in 2 million deaths annually. In uncomplicated cases short course therapy (6 months) using a multiple-drug regimen is highly effective. An essential component of this regimen is rifampicin (RIF). In MTB resistance to antimicrobial agents appears to be solely due to
spontaneous mutation, as no horizontal transfer of genetic elements carrying a resistance genotype has been described. For this reason MTB strains or sub-populations with an unusual spectrum or rate of mutations are of considerable interest [1-3] as potentially they are more prone to develop resistance to antimicrobial drugs.

Mutations within an 81-bp locus of MTB rpoB have been seen in almost all (≥ 95%) rifamycin resistant isolates, whether they be clinical [4] or laboratory generated mutants [5]. These mutations are not only markers of resistance as the region of RpoB coded for by this locus has been shown to bind rifamycins and mutations in this region allow RpoB to function in the presence of rifamycins [6]. Although mutations associated with RIF resistance have been reported throughout this locus, mutations in two codons account for approximately 70% of all mutants identified (codon-526 approximately 20%, and codon-531 approximately 50%), from most collections of isolates studied.

Rifabutin (RFB) is a second therapeutically useful rifamycin, which is often used when M. avium / intracellulare (MAI) infection is suspected or confirmed. RIF and RFB resistances are not invariably cross-resistant although the most common mutations (codons 526 and 531) are present in almost all (> = 95%) rifamycin resistant isolates, whether they be clinical [4] or laboratory generated mutants. RIF and RFB resistances are not invariably cross-resistant although the most common mutations (codons 526 and 531) are reported to result in high-level resistance to both drugs.

Here we report the generation and characterisation (sequencing subgeneric fragments of the rpoB gene) of two sets of rifamycin resistant mutants selected from a single parent strain using either RIF or RFB. Mutations that confer resistance to RIF but not RFB cannot be selected for by RFB. Thus certain mutations are not possible when RFB is used for the selection of mutant cells and different selections and/or frequencies of mutations would be expected. Strains with mutations conferring resistance to RIF only can be subjected to a second round of selection with RFB and the type and frequency of resistant mutants compared to that of the sensitive parent strain [2]. In this study the single isolate identified with resistance to RIF only, was subjected to a second round of selection with RFB; after which rpoB gene fragments of 26 randomly selected colonies were re-sequenced in order to identify any additional mutations.

**Methods**

**Strain used**

*Mycobacterium tuberculosis* Mtbi72 (ATCC35801) belongs to the Haarlem genotype and was the parent strain of all isolates generated in this study.

**Generation and selection of mutants**

Bacteria were cultured in Middlebrook 7H9 (Difco) broth for 14 days in a shaking incubator at 37°C. Then 0.5 ml of broth was plated out onto Middlebrook 7H11 (Difco) media containing either RIF 8 μg/ml (Sigma) or RFB 0.8 μg/ml (Pharmacia Corporation, MI, USA) for the first round selection or 0.8 μg/ml and 8 μg/ml RFB for the second round selection. These plates were incubated at 37°C and colonies picked between 21 and 42 days culture. Selected colonies were streaked on drug containing media to confirm the resistant phenotype (RFB 0.8 μg/ml, RIF 8 μg/ml).

MICs were measured on Middlebrook 7H11 plates containing serial dilutions RIF (512 μg/ml to 4 μg/ml) or RFB (128 μg/ml to 1 μg/ml) for 24 isolates chosen so as to represent the full range of genotypes identified. Only strains showing growth of more than 1% of the inoculate were scored as resistant.

**PCR/ DNA sequencing**

A 271 bp fragment of the rpoB gene containing the 81 bp mutation hotspot (Cluster I) was amplified and sequenced from the parent and all 131 mutants studied. Crude DNA extracts were prepared for PCR by heating cell suspensions to 95°C for 20 min in TE buffer containing 1% Triton X-100. PCR of rpoB cluster I was carried out on all 132 isolates included in the study, in 20 μl volumes, containing 2 μl 10× PCR buffer (Bioline Ltd., London, UK); 0.5 unit Taq-DNA polymerase (Bioline); 0.5 μl 2 mM dNTP mixture (Bioline); 0.5 μl 20 μM primer mix (containing rpoBP1, 5’gtgcccagctgctggtgag and rpoB1420R, 5’gtaggtcgctcagctcgcagctc) 15.5 μl water and 1 μl of DNA extract. Thermal cycling was performed in a Thermocycler using the following programme 5 min at 95°C, 30 × (30 sec at 95°C, 30 sec at 65°C, 60 sec at 72°C), 5 min at 72°C. The presence of PCR products was confirmed by agarose gel electrophoresis. These products were diluted 1/100 in purified water and sequenced using CEQ Quick Start sequencing kits and a CEQ 8000 instrument (Beckman Coulter, High Wycombe, UK) according to the manufacturer’s instructions. The PCR products were sequenced in both directions using the amplification primers given above. All codon numbers are reported using the *E. coli* numbering system.

An additional 365 bp region of the rpoB gene (containing codon 176 [10]) from all 26 second round mutants and the two parent strains was also amplified and sequenced using primers rpoB7F (cttcctcgggtctggtggt) and rpoB7R (CGCGGCTTGTGCAGGCTAAACTC). PCR was carried out in 25 μl volumes, containing 2.5 μl 10× HotGoldstar PCR buffer (Eurogentec); 2 μl 25 mM MgCl2; 0.15 μl 5 units/μl HotGoldstar DNA polymerase (Eurogentec); 0.2 μl 25 mM dNTP mixture (Amersham Bioscience); 0.25 μl 10 μM M primer mix (containing rpoBP1, 5’gtgcccagctgctggtgag and rpoB1420R, 5’gtaggtcgctcagctcgcagctc) 15.5 μl water and 1 μl of DNA extract. Thermal cycling was performed in a Thermocycler using the following programme 5 min at 95°C, 30 × (30 sec at 95°C, 30 sec at 65°C, 60 sec at 72°C), 5 min at 72°C. The presence of PCR products was confirmed by agarose gel electrophoresis. These products were diluted 1/100 in purified water and sequenced using CEQ Quick Start sequencing kits and a CEQ 8000 instrument (Beckman Coulter, High Wycombe, UK) according to the manufacturer’s instructions. The PCR products were sequenced in both directions using the amplification primers given above. All codon numbers are reported using the *E. coli* numbering system.
Table 1: Distribution of rpoB spontaneous mutations identified after in vitro exposure to rifampicin (8 μg/ml) or rifabutin (0.8 μg/ml).

| Selective agent (Strain) | CAA>CTA | TCG>TGG | CAC>GAC | CAC>TAC | CAC>GGC | CAC>CCC | TCG>TGG | TCG>TGG | Others | No. tested |
|-------------------------|----------|----------|---------|---------|---------|---------|---------|---------|--------|------------|
| Rifabutin 0.8 μg/ml (Mtb72) | 1 | 0 | 5 | 27 | 6 | 0 | 6 | 1 | 1 | 47 |
| Rifampicin 8 μg/ml (Mtb72) | 0 | 2 | 6 | 26 | 4 | 2 | 35 | 0 | 0 | 58 |

* = 525 ACC>ACG + 526 CAC>CCC + 527 AAG>CAG

μM of each primer, 18.6 μl water and 1 μl of crude DNA extract. Thermal cycling was performed in a Thermocycler using the following programme: 10 min at 95°C, 35× (30 sec at 96°C, 30 sec at 60°C, 60 sec at 72°C). The presence of PCR products was confirmed by agarose gel electrophoresis. These products were diluted 1/10 in purified water and sequenced using the dideoxy chain termination method with the Big Dye Terminator cycle sequencing Kit (Applied Biosystems, CA, USA). PCR was carried out, using quarter reactions with either the forward or reverse primer. Sequence analysis was performed on a 310 Genetic Analyzer (Applied Biosystems).

Mutation frequency was measured by plating 1.5 ml of two entirely independent exponentially growing bacterial cultures in antibiotic free Middlebrook 7H9 broth onto a series of Middlebrook 7H11 plates, one set containing 0.8 μg/ml RFB and one set containing 8 μg/ml RFB. Decimal dilution series of each of these cultures were prepared and plated onto non-selective Middlebrook 7H11 plates, the CFU present on these plates was used to calculate the inoculum size. The mutation frequency, calculated separately for each drug concentration used, was the number of resistant colonies / number of CFU inoculated.

Results

Mutations in the 81-bp locus of the MTB rpoB gene were detected in cluster I (between codons 512 and 532) in all 105 first round resistant mutants that were sequenced (Table 1); no mutations were detected in the parent strain and two blinded wild type isolates included in this panel as sequencing controls.

Two regions of the rpoB gene from 26 randomly selected RFB second round mutants were sequenced (Table 2). Single mutations in addition to the original S522L mutation in the rpoB gene were identified in 24 of the 26 strains (92%). Twelve strains had mutations between codons 505 and 531, and 12 strains had mutations between codons 144 and 148, the final two strains did not have any additional mutations in the regions sequenced. No (additional) mutations were detected in the two parent strains (Mtb72 and R190) sequenced as controls.

The MICs of selected isolates representing the spectrum of mutations obtained are shown in table 3. The single strain identified as resistant to rifampicin (MIC 32 μg/ml) but sensitive to rifabutin (strain R190, MIC<0.8 μg/ml, mutation S522L) was subjected to a second round of selection using RFB, in parallel with the parent strain (Mtb72). The frequency of mutants produced after 28 days incubation at 37°C by R190 (S522L) on two different RFB concentrations was compared to the mutation frequency of the parent strain. After selection with 0.8 μg/ml RFB the parent strain generated RFB resistant mutants with a frequency of 1.25 × 10−7 colonies per CFU plated and the R190 (S522L) mutant generated RFB resistant mutants with a frequency of 1.93 × 10−6 per CFU plated. After selection with 8.0 μg/ml RFB the parent strain generated RFB resistant mutants with a frequency of 1.42 × 10−8 colonies per CFU plated and the R190 mutant generated RFB resistant mutants with a frequency of 1.7 × 10−7 per CFU plated. Thus, spontaneous RFB mutants were generated by R190 with 11.6 times the frequency of the parent strain with 8 μg/ml RFB and 15.4 times the frequency of the parent strain with 0.8 μg/ml RFB.

Discussion

In this study a single M. tuberculosis isolate (R190), with a S522L mutation, was detected that was resistant to RIF (MIC 32 μg/ml) but remained sensitive to RFB (MIC<0.8 μg/ml) (Table 3). Specific mutations in codon 522 have previously been shown to result in only low level RFB resistance from clinical isolates of MTB [7,9]. The distribution of second round RFB mutations from R190 was strikingly different from that obtained from the parent strain (Table 2) and the frequency of resistant mutants increased > 10 fold when measured on two separate occasions. Additional second round mutations in the rpoB gene were identified in 24 of 26 (92%) of the R190 second round
Table 2: Additional mutations acquired in 26 randomly selected colonies of rifampicin resistant isolate R190 (S522L) after a second round of selection with rifabutin.

| Strain | Conc. of RFB used for selection µg/ml | Additional mutations identified* codons 505–531 (Cluster I 507–533) | Additional mutations identified* codons 144–148 |
|--------|------------------------------------|-------------------------------------------------|------------------------------------------|
| 1      | 0.8                                | F505L TTC>TTA                                    | None                                     |
| 2      | 0.8                                | F505L TTC>TTG                                    | None                                     |
| 3      | 0.8                                | SS12G AGC>G GC                                  | None                                     |
| 4      | 8                                  | SS12G AGC>G GC                                  | None                                     |
| 5      | 8                                  | SS12G AGC>G GC                                  | None                                     |
| 6      | 8                                  | SS12G AGC>G GC                                  | None                                     |
| 7      | 0.8                                | HS26Y CAC>TAC                                    | None                                     |
| 8      | 8                                  | HS26Y CAC>TAC                                    | None                                     |
| 9      | 0.8                                | SS31L TCG>TTG                                   | None                                     |
| 10     | 0.8                                | SS31L TCG>TTG                                   | None                                     |
| 11     | 0.8                                | SS31L TCG>TTG                                   | None                                     |
| 12     | 0.8                                | SS31L TCG>TTG                                   | None                                     |
| 13     | 8                                  | None                                             | V144M GTG>ATG                            |
| 14     | 8                                  | None                                             | V144M GTG>ATG                            |
| 15     | 8                                  | None                                             | V144G GTG>GGG                            |
| 16     | 0.8                                | None                                             | V146F GTC>TTC                            |
| 17     | 0.8                                | None                                             | V146F GTC>TTC                            |
| 18     | 0.8                                | None                                             | V146F GTC>TTC                            |
| 19     | 0.8                                | None                                             | V146F GTC>TTC                            |
| 20     | 8                                  | None                                             | V146F GTC>TTC                            |
| 21     | 8                                  | None                                             | V146F GTC>TTC                            |
| 22     | 8                                  | None                                             | Q148H CAG>CAC                            |
| 23     | 0.8                                | None                                             | Q148H CAG>CAC                            |
| 24     | 0.8                                | None                                             | Q148H CAG>CAC                            |
| 25     | 0.8                                | None                                             | None                                     |
| 26     | 0.8                                | None                                             | None                                     |

*All isolates retained the original S522L TCG>TTG mutation from their R190 parent and had at most a single additional mutation identified. Codons are numbered according to the E. coli numbering system.

Table 3: Rifampicin and rifabutin MICs of selected first generation in vitro mutants.

| rpoB Mutation | Number of Isolates | RIF MIC µg/ml | Number of Isolates | RFB MIC µg/ml |
|---------------|--------------------|---------------|--------------------|---------------|
| Wild          | 2                  | <4            | 2                  | <0.8          |
| Q513L         | 1                  | 256           | 1                  | >128          |
| SS22L         | 1                  | 32            | 1                  | <0.8          |
| HS26Y         | 3                  | 256           | 1                  | >128          |
|               | 1                  | 128           | 2                  | 128           |
|               |                    |               | 1                  | 64            |
| HS26D         | 3                  | 256           | 1                  | >128          |
|               | 1                  | 128           | 3                  | 128           |
| HS26R         | 1                  | 512           | 5                  | 128           |
|               | 3                  | 256           |                    |               |
|               | 1                  | 128           |                    |               |
| HS26P         | 1                  | 256           | 1                  | >128          |
| SS31L         | 4                  | 256           | 1                  | >128          |
|               |                    |               | 2                  | 128           |
|               |                    |               | 1                  | 64            |
| SS31W         | 1                  | 256           | 1                  | 128           |
RFB resistant mutants (Table 2), only six of which (23%) were similar to those seen in the first round selection (either in codon 526 or 531). A single mutant with a change in codon 512 at the beginning of cluster I was also identified. Mutations in codon 512 have only been reported previously in association with additional mutations [10] as in this study (S512G + S522L). The remaining mutations were outside cluster 1, codons 144, 146, 148, and just before cluster I in codon 505 (Table 2).

This dramatic change in mutations identified indicates either, the range of viable SNPs resulting in high-level RFB resistance was significantly altered by the presence of the S522L mutation, or the range of spontaneous mutations occurring in this strain changed. Thus, it is possible, for example, that the mutations in codon 505 occurred in both cases but alone would not have resulted in resistance or in the absence of the S522L mutation may be lethal. Interestingly, these results could also be explained by a change in both the frequency and spectrum of spontaneous mutations after the first round of selection, i.e. the rate of spontaneous mutations in codons 505, 512, 144, 146, and 148, has increased after the first round of selection. As 9 different codon changes were identified among the 26 isolates tested (Table 2), from two independent experiments (drug concentrations), a single spontaneous mutant in an early generation of this culture (a "Jackpot" mutation) [11] cannot explain this result.

The distribution of in vitro first round spontaneous mutations in M. tuberculosis has previously been reported [5] and 7 of the 9 codon changes identified were also seen in this study. The most striking difference between this data and the first round selection data presented here is the higher frequency of the C>T H526Y mutation in our study with both RIF and RFB. Some variation between random selections of mutations would be expected and methodological differences, notably the use of a different bacterial strain [5,12], probably contributed to this effect.

Mutations in codons 526 and 531 predominate in most of the published studies but there is also some indication that certain strains may be prone to develop specific mutations [4,12] and marked differences in the distribution of mutations have been observed in different geographical locations [4,13]. Our data from the first round selection (Table 1) suggest that for the strain and conditions we used RFB may be more likely to select for C>T H526Y mutations than rifampicin.

All first round mutations identified in this study have been reported previously from clinical isolates [4,5,8-10,14] except the one triple mutant identified (Table 1). The second round mutations seen in codons 505, 512, 144, and 148 present in addition to the S522L mutation (Table 2), have to our knowledge not been reported previously from in vitro or clinical isolates. Although, it should be noted that these mutations lie outside the 81 bp hotspot region in a region of the rpoB gene that has been subjected to much less extensive investigation.

The possibility of an MTB strain with altered or raised mutation rate is important. Selection of a mutator phenotype is recognised as a consequence of antibiotic challenge in many bacterial species [11,15]. The selection of strains with increased mutation rates will result in a greater chance of acquiring resistance to other drugs but may also impact on the pathogenicity of the strain. It has been reported that many MDR-MTB strains have, at least initially, reduced pathogenicity [16] and mutations in three putative mutator genes as well as evidence for a reversion back to a more pathogenic non-mutator state after the acquisition of drug resistance has been reported in W-Beijing strains [3]. However, some rpoB mutations are associated with only a modest decrease in in vitro fitness [17]. Interestingly, induction of the proposed MTB error prone DNA repair enzyme was associated with survival of the bacteria in vivo [1], so the effect of a mutator phenotype on pathogenicity is difficult to predict [18].

In conclusion, the presence of a different spectrum of secondary rifabutin mutations implies that either, the mutation rate of individual mutations has changed, due to a defect in DNA repair or replication, or that additional spontaneous mutations are viable (and lead to resistance) in the presence of the S522L mutation [2]. A further consequence of this observation is that a proportion clinical isolates with mutations in cluster 1 of the rpoB gene associated with rifampicin resistance only may in fact be resistant to rifabutin as a consequence of addition mutations in other regions of this gene. We believe further study is warranted, of this and similar strains which should include generating mutants to other antimicrobials and measuring mutation rates [19,20], allowing the contribution of each of these possible explanations to be explored. The details of how resistance mutations arise would be valuable when formulating standard treatment regimens with the aim of minimising the emergence of resistance in treated populations [21].

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References
1. Boshoff HIM, Reed MB, Barry III CE, Mizrahi V: DnaE2 polymerase contributes to In Vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. Cell 2003, 113:183-93.
2. Mokrousov I: Multiple rpoB mutants of Mycobacterium tuberculosis and second-order selection. Emerg Infect Dis 2004, 10:1337-338.
9. Yuen LKW, David L, Coloe PJ: Mutations in putative mutator genes of Mycobacterium tuberculosis strains of the W-Beijing family. Emerg Infect Dis 2003, 9:838-45.
10. Heep M, Brandstatter B, Reiger U, Lehn N, Richter E, Rusch-Gerdes S, Niemann S: Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical Mycobacterium tuberculosis isolates. J Clin Microbiol 2001, 39:107-10.
11. Martinez JL, Baquero F: Mutations frequencies and antibiotic resistance. Antimicrob Agents Chemother 2000, 44:1771-7.
12. Hillemann D, Kubicz T, Rusch-Gerdes S, Niemann S: Disequilibrium in distribution of resistance mutations among Mycobacterium tuberculosis Beijing and non-beijing strains isolated from patients in Germany. Antimicrob Agents Chemother 2005, 49:1229-31.
13. Mokrousov I, Bhanu NV, Suffys PN, Kadival GV, Yap S-F, Cho S-N, Jordaan AM, Narvskaya O, Singh UB, Gomes HM, Lee H, Kulkarni SP, Lim KC, Khan BK, van Soolingen D, Victor TC, Schouls LM: Multi-center evaluation of reverse line blot assay for detection of drug resistance in Mycobacterium tuberculosis clinical isolates. J Microbiol Methods 2004, 57:323-35.
14. Herrera L, Jimenez S, Valverde A, Garcia-Aranda MA, Juan A, saez-Nieto : Molecular analysis of rifampicin-resistant Mycobacterium tuberculosis isolated in Spain (1996–2001). Description of new mutations in the rpoB gene and review of the literature. Int J Antimicrob Agents 2003, 21:403-8.
15. Matic I, Radman M, Todde F: Highly variable mutation rates in commensal and pathogenic Escherichia coli. Science 1997, 277:1833-4.
16. Gillespie SH: Evolution of drug resistance in Mycobacterium tuberculosis: Clinical and molecular perspective. Antimicrob Agents Chemother 2002, 46:267-74.
17. Billington OJ, McHugh TD, Gillespie SH: Physiological cost of rifampin resistance induced in vitro in Mycobacterium. Antimicrob Agents Chemother 1999, 43:1866-9.
18. Tanaka MM, Bergstrom CT, Levin BR: The evolution of mutator genes in bacterial populations: the roles of environmental change and timing. Genetics 2003, 164:843-54.
19. Luria SE, Delbruck M: Mutations of bacteria from virus sensitivity to virus resistance. Genetics 1943, 28:491-511.
20. Werngren J, Hoffner SE: Drug-susceptible Mycobacterium tuberculosis Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. J Clin Microbiol 2003, 41:1520-24.
21. Gumbo T, Louie A, Deziel ER, Parsons LM, Salfinger M, Drusano GL: Selection of a moxifloxacin dose that suppresses drug resistance in Mycobacterium tuberculosis, by use of an in vitro pharmacodynamic infection model and mathematical modeling. J Infect Dis 2004, 190:1642-51.