J Antimicrob Chemother 2013; **68**: 2493–2497
doi:10.1093/jac/dkt224 Advance Access publication 11 June 2013

Journal of Antimicrobial Chemotherapy

Genetic characterization of compensatory evolution in strains carrying *rpoB* Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates

Gerrit Brandis and Diarmaid Hughes*

Department of Medical Biochemistry and Microbiology, Box 582 Biomedical Center, Uppsala University, Uppsala, Sweden

*Corresponding author. Tel: +46-18-4714507; Fax: +46-18-4712000; E-mail: diarmaid.hughes@imbim.uu.se

Received 16 March 2013; returned 8 April 2013; revised 1 May 2013; accepted 10 May 2013

Objectives: The evolution of rifampicin resistance in *Mycobacterium tuberculosis* is a major threat to effective tuberculosis therapy. Much is known about the initial emergence of rifampicin resistance, but the further evolution of these resistant strains has only lately been subject to investigation. Although resistance can be caused by many different mutations in *rpoB*, among clinical *M. tuberculosis* isolates the mutation *rpoB* S531L is overwhelmingly the most frequently found. Clinical isolates with *rpoB* S531L frequently carry additional mutations in genes for RNA polymerase subunits, and it has been speculated that these are fitness-compensatory mutations, ameliorating the fitness cost of the primary resistance mutation. We tested this hypothesis using *Salmonella* as a model organism.

Methods: We created the *rpoB* S531L mutation in *Salmonella* and then evolved independent lineages with selection for mutants with increased relative fitness. Relative fitness associated with putative compensatory mutations was measured after genetic reconstruction in isogenic strains.

Results: Compensatory mutations were identified in genes coding for different subunits of RNA polymerase: *rpoA*, *rpoB* and *rpoC*. Genetic reconstructions demonstrated that each of these secondary mutations reduced the fitness cost of the *rpoB* S531L resistance mutation.

Conclusions: The compensatory mutations identified in *Salmonella* cluster in similar locations to the additional mutations found in *M. tuberculosis* isolates. These new data strongly support the idea that many of the previously identified *rpoA*, *rpoB* and *rpoC* mutations in rifampicin-resistant *M. tuberculosis* (*rpoB* S531L) are indeed fitness-compensatory mutations.

Keywords: antibiotic resistance, S531L, *Mycobacterium tuberculosis*

Introduction

The antibiotic rifampicin is an essential part of short-course anti-tuberculosis treatment.[1] Rifampicin targets the β-subunit of the RNA polymerase (RNAP), where it inhibits RNA transcription shortly after initiation.[2] Studies of *in vitro*-selected rifampicin-resistant mutants,[3–5] supported by analysis of clinical isolates of *Mycobacterium tuberculosis*,[6] show that rifampicin resistance (Rif^R) is nearly always caused by mutations affecting a small part of the β-subunit close to the catalytic centre of RNAP.[2,7,8]

The Rif^R mutation most frequently found in clinical isolates of *M. tuberculosis* is *rpoB* S531L. In *M. tuberculosis* from countries around the world, *rpoB* S531L was identified in 40%–93% of all Rif^R isolates.[9–13] One explanation for this high frequency might be low fitness cost. Characterization of paired strains carrying *rpoB* S531L showed that clinical isolates had lower fitness costs compared with laboratory-constructed strains,[14] suggesting that fitness-compensatory mutations had been acquired in clinical isolates in the time between developing Rif^R and the taking of the clinical sample.[14]

Model organisms, such as *Escherichia coli* and *Salmonella enterica,* are frequently used to study antibiotic resistance fitness costs and the genetics of fitness-compensatory evolution.[15] We have previously used *Salmonella enterica* serovar Typhimurium as a model organism to study compensatory evolution using the high-cost Rif^R mutation *rpoB* R529C as a starting point.[16] We showed that compensatory mutations were selected in the genes *rpoA*, *rpoB* and *rpoC*, which encode the α, β and β′ subunits, respectively, of RNAP.[16] Studies on *M. tuberculosis* revealed that mutations in *rpoA*, *rpoB* and *rpoC* are also common among clinical isolates with the *rpoB* S531L mutation,[17,18] and that secondary mutations might play a role in facilitating transmission of tuberculosis.[19]

Previous genetic studies in *E. coli* or *S. enterica* have not included *rpoB* S531L, the most frequently found *rpoB* mutation in Rif^R *M. tuberculosis*. Therefore, it has not been demonstrated experimentally that the secondary mutations found in *M. tuberculosis* isolates carrying *rpoB* S531L are compensatory mutations. Furthermore, studies on compensation in Rif^R tuberculosis isolates have focused on the part of *rpoC* encoding the $\alpha-\beta'$ subunit interaction surface,[17,19] whereas compensatory mutations for the Rif^R mutation *rpoB* R529C are also found in other parts of the *rpoC* gene.[16]

Because we have previously studied Rif^R fitness cost and compensatory evolution in *Salmonella*,[16] we constructed an *S. enterica* strain with the Rif^R mutation *rpoB* S531L and evolved it with selection for increased relative fitness. This mutation is infrequent in *S. enterica,* probably because the serine to leucine codon change requires two nucleotide substitutions in *S. enterica* (TCC to TTG), whereas in *M. tuberculosis* only one nucleotide substitution is required (TCG to TTG). Our aims were: (i) to identify and test putative fitness-compensatory mutations in the genes *rpoA*, *rpoB* and *rpoC*; and (ii) to identify hotspots for compensatory mutations in these genes to help determine whether the secondary mutations in *M. tuberculosis* are likely to be compensatory mutations.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains are listed in Table S1 (available as Supplementary data at *JAC* Online). *S. enterica* serovar Typhimurium 14028s[20] was used in evolution and fitness experiments. The *rpoB* S531L mutation was made by oligonucleotide recombineering[21] (Table S2, available as Supplementary data at *JAC* Online). Genetic markers were moved between strains by P22 transduction.[16] Bacteria were grown at 37°C in Luria broth (LB) or on solid LA medium (LB solidified with 1.5% Oxoid agar) with 0.2% glucose and 3 mM $CaCl_2$. Rifampicin was added as required at 100 mg/L. Growth rates were measured using a Bioscreen C machine (Oy Growth Curves Ab Ltd).[16] Rifampicin MIC values were determined using broth dilution.[16]

Evolution by serial passage

Independent lineages of TH8380 were grown in 15 mL tubes with shaking at 37°C overnight in LB and LB+rifampicin, and serially passaged after each cycle of growth by transferring 2 μL of culture into 2 mL of fresh medium to initiate the next cycle. Each lineage was evolved for 28 cycles (280 generations), after which dilutions were plated on LA and incubated overnight at 37°C. Two colonies per lineage of larger size than the ancestral strain were picked for analysis.

PCR amplification and DNA sequencing

Preparation of DNA was performed as previously described.[16] Primers for amplification and sequencing of *rpoA*, *rpoB* and *rpoC* are described in Table S3 (available as Supplementary data at *JAC* Online). DNA sequencing was carried out at Macrogen (Seoul, Korea) using purified PCR product.

Results

Characterization of rpoB S531L

The constructed strain harbouring *rpoB* S531L (TH8380), an infrequent mutation in *S. enterica*, showed Rif^R (MIC: wild-type 12 mg/L; mutant 3000 mg/L) and a fitness cost of the mutation in LB of about 27% (LB generation time: wild-type 19.55 min; mutant 26.84 min).

Selection of compensatory mutations

Twenty independent lineages of TH8380 (*rpoB* S531L) were evolved by serial passage in rich medium (10 in LB and 10 in LB+rifampicin). After 280 generations, diluted cultures were plated on LA agar. Two colonies of larger size than the ancestral strain were isolated from each of the 20 lineages. The genes *rpoA*, *rpoB* and *rpoC* were sequenced for each of these 40 strains. The initial *rpoB* S531L mutation was still present in each of the 40 isolates. A secondary mutation in one of the three sequenced genes was carried by 45% (9 of 20) of the isolates evolved in LB and 95% (19 of 20) of the isolates evolved in LB+rifampicin. In total, 18 different putative compensatory mutations were identified (Table 1).

The secondary mutations are necessary and sufficient for growth compensation

The growth rates of the strains containing each one of the secondary mutations in the RNAP genes were determined, and all showed an increase compared with the ancestral Rif^R strain (Table 1). Isogenic strains were constructed, and the growth rates of the reconstructed mutant strains were indistinguishable from the growth rates of the respective parental strains (Table S4, available as Supplementary data at *JAC* Online), showing that the individual secondary mutations found in *rpoA*, *rpoB* and *rpoC* are sufficient to generate the growth-compensated phenotypes. In 12 of the 40 evolved strains no secondary mutation was found in the three RNAP genes, although they grew faster than the ancestral strain (Table S5, available as Supplementary data at *JAC* Online), confirming that compensatory evolution had taken place. Since the focus of this study was on the overlap of compensatory mutations within the *rpoA*, *rpoB* and *rpoC* genes with secondary mutations found in clinical isolates, these strains were excluded from further analysis.

Compensatory mutations overlap with secondary mutations identified in RNAP genes in clinical isolates

Two recent studies based on genome sequencing of *M. tuberculosis* isolates identified a set of mutations in RNAP genes as putative compensatory mutations for the Rif^R mutation *rpoB* S531L.[17,18] Together with the 18 compensatory mutations found in this study, a total of 55 secondary mutations associated with *rpoB* S531L have been identified in *rpoA* ($n=9$), *rpoB* ($n=10$) and *rpoC* ($n=36$). The 37 different secondary mutations identified in *M. tuberculosis* carrying *rpoB* S531L are clustered at a few locations within the primary sequences of *rpoA* (codons 189–199), *rpoB* (codons 516–561 and 848) and *rpoC* (codons 257, 358–452 and 606–674) (Figure 1). Compensatory mutations identified in this study map within, or very close to, clusters carrying 36 of the 37 *M. tuberculosis* secondary mutations. Thus, there is a very good overlap between the locations of secondary mutations identified in clinical isolates of *M. tuberculosis*[17,18] and the locations of fitness-compensatory mutations selected in this study. In addition, we selected several novel compensatory mutations at

Table 1. Compensatory mutations found in *rpoA*, *rpoB* and *rpoC*

Mutations in *rpoA*, *rpoB* or *rpoC*					Selection[c]	
Rif^R	compensatory	Rifampicin MIC (mg/L)	Dt (min) ± SD[a]	*n*[b]	LB	LB + rifampicin (100 mg/L)
wild-type	wild-type	12	19.45 ± 0.82	—	−	−
rpoB S531L	wild-type	3000	26.84 ± 1.13	—	−	−
rpoB S531L	*rpoA* A189E	3000	23.46 ± 0.29	1	−	+
rpoB S531L	*rpoA* T196S	3000	22.64 ± 0.70	1	+	−
rpoB S531L	*rpoB* N339H	3000	23.37 ± 0.41	1	−	+
rpoB S531L	*rpoB* D340Y	3000	23.23 ± 0.88	2	−	+
rpoB S531L	*rpoB* E546K	3000	20.30 ± 0.91	1	+	−
rpoB S531L	*rpoB* G809A	3000	24.10 ± 0.62	1	+	−
rpoB S531L	*rpoB* Q1264R	3000	22.11 ± 0.62	1	−	+
rpoB S531L	*rpoC* H419P	3000	22.16 ± 0.72	1	+	−
rpoB S531L	*rpoC* A446V	3000	21.75 ± 0.32	1	−	+
rpoB S531L	*rpoC* D622E	3000	22.41 ± 1.16	1	−	+
rpoB S531L	*rpoC* R634P	3000	24.04 ± 0.79	1	−	+
rpoB S531L	*rpoC* T674I	3000	22.58 ± 0.50	1	−	+
rpoB S531L	*rpoC* I937T	3000	21.95 ± 0.86	1	−	+
rpoB S531L	*rpoC* S942L	3000	21.08 ± 0.77	1	+	−
rpoB S531L	*rpoC* R943H	3000	20.69 ± 0.40	1	−	+
rpoB S531L	*rpoC* R1075C	3000	23.64 ± 1.04	1	+	−
rpoB S531L	*rpoC* R1075L	3000	22.53 ± 0.73	1	−	+
rpoB S531L	*rpoC* G1136A	3000	23.04 ± 1.14	1	−	+

[a]Dt is the doubling time (generation time) in LB at 37°C, based on nine independent measurements for each strain.
[b]Number of independent isolates (from independent lineages) of each mutant.
[c]Growth medium in which growth-compensatory mutants were selected.

other locations within *rpoB* and *rpoC* (Figure 1). These additional mutations could be specific for *S. enterica* or, alternatively, could be mutations not yet found in *M. tuberculosis* (see the Discussion section).

Discussion

We evolved a rifampicin-resistant *S. enterica* strain carrying the Rif^R mutation *rpoB* S531L (the most frequently found *rpoB* mutation in Rif^R *M. tuberculosis*), with selection for increased growth fitness. We identified 18 different secondary mutations within the genes *rpoA*, *rpoB* and *rpoC*. Genetic reconstructions showed that each of these secondary mutations is individually sufficient to ameliorate the fitness cost caused by the *rpoB* S531L Rif^R mutation.

In the absence of rifampicin, some strains evolved increased relative fitness without acquiring a secondary mutation within the three RNAP genes. However, strains that were compensated by secondary mutations in *rpoA*, *rpoB* or *rpoC* benefited from a greater increase in growth rate in the presence of rifampicin than strains that acquired compensatory mutations in other loci (Figure S1, available as Supplementary data at *JAC* Online), plausibly explaining their overwhelming predominance under those selective conditions. This suggests that there may be a close connection between exposure to rifampicin and selection of compensatory mutations affecting the RNAP genes.

Finally, we compared the locations of compensatory mutations selected in this study with the locations of secondary mutations identified in clinical isolates of *M. tuberculosis* carrying *rpoB* S531L.[17,18] All of the secondary mutations within *rpoA* cluster in a section of the protein 11 amino acids long, where we also identified compensatory mutations (Figure 1). Only a few secondary mutations were identified in *rpoB*, of which three are known to be Rif^R mutations (D516V, D516Y, S522L) and we confirmed compensatory mutations at these and other locations within *rpoB* (Figure 1). Within *rpoC*, most secondary mutations are found in two separate regions, and compensatory mutations were also selected in this study in each of these regions of the gene. The majority (26 of 37) of the RNAP secondary mutations identified in clinical isolates are found in two clusters (Figure 1), in regions of *rpoA* and *rpoC*, which encode the α–β′ interaction region of RNAP.[2] However, because most (21 of 25) of the secondary mutations in *rpoC* were found in a study that sequenced only the α–β′ interaction region, it cannot be excluded that some clinical isolates carried secondary mutations in other parts of the *rpoC* gene.[17]

In conclusion, our findings genetically support the empirical data that secondary mutations within the genes *rpoA*, *rpoB* and *rpoC*, found in clinical *M. tuberculosis* isolates, do indeed compensate for the growth defect caused by the Rif^R mutation *rpoB* S531L. Furthermore, we have identified several distinct regions in the genes *rpoA*, *rpoB*, and *rpoC* that are potentially important

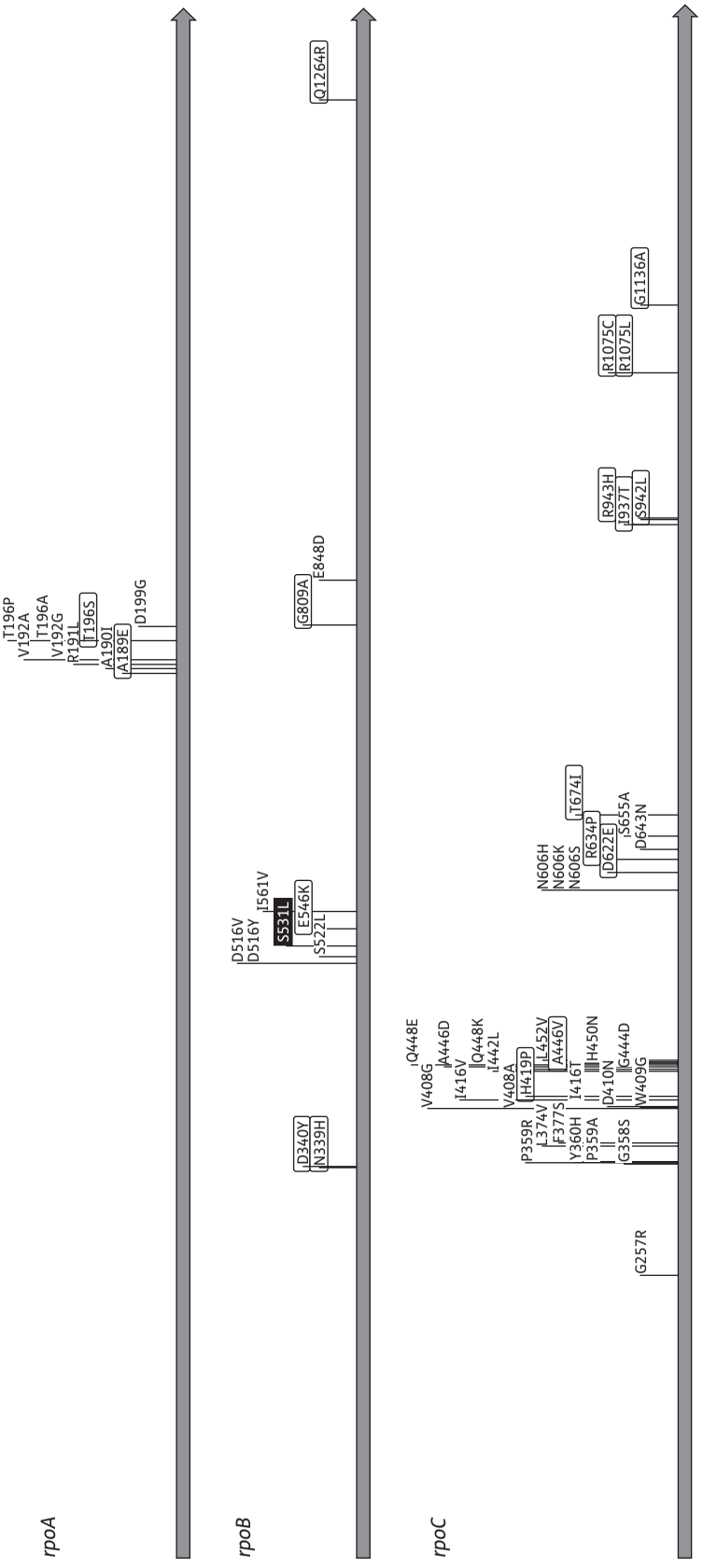

Figure 1. Distribution of secondary mutations in RNAP genes. Distribution of compensatory mutations identified in this study (in boxes) and putative compensatory mutations previously identified in clinical isolates of *M. tuberculosis* in the genes *rpoA*, *rpoB* and *rpoC*. The primary Rif^R mutation *rpoB* S531L is shown in reversed colour.

regions in the compensatory evolution of *rpoB* S531L, the most frequently found *rpoB* mutation in Rif^R *M. tuberculosis*.

Acknowledgements

We thank Rahel Alemayehu, who worked on parts of this project as her research training course.

Funding

This work was supported by grants from the Swedish Research Council (Vetenskapsrådet), the Knut and Alice Wallenberg Foundation (RiboCore Project), the EU 7th Framework Programme (project PAR), the Swedish Strategic Research Foundation (SSF) and the Swedish Governmental Agency for Innovation Systems (Vinnova).

Transparency declarations

None to declare.

Supplementary data

Tables S1 to S5 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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