Novel insertion and deletion mutants of RpoB that render *Mycobacterium smegmatis* RNA polymerase resistant to rifampicin-mediated inhibition of transcription

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The startling increase in the occurrence of rifampicin (Rif) resistance in the clinical isolates of *Mycobacterium tuberculosis* worldwide is posing a serious concern to tuberculosis management. The majority of Rif resistance in bacteria arises from mutations in the RpoB subunit of the RNA polymerase. We isolated *M. smegmatis* strains harbouring either an insertion (6 aa) or a deletion (10 aa) in their RpoB proteins. Although these strains showed a compromised fitness for growth in 7H9 Middlebrook medium, their resistance to Rif was remarkably high. The attenuated growth of the strains correlated with decreased specific activities of the RNA polymerases from the mutants. While the RNA polymerases from the parent or a mutant strain (harbouring a frequently occurring mutation, H442Y, in RpoB) were susceptible to Rif-mediated inhibition of transcription from calf thymus DNA, those from the insertion and deletion mutants were essentially refractory to such inhibition. Three-dimensional structure modelling revealed that the RpoB amino acids that interact with Rif are either deleted or unable to interact with Rif due to their unsuitable spatial positioning in these mutants. We discuss possible uses of the RpoB mutants in studying transcriptional regulation in mycobacteria and as potential targets for drug design.

Received 7 December 2009
Revised 2 February 2010
Accepted 5 February 2010

INTRODUCTION

Rifamycin was isolated from *Amycolatopsis rifamycinica* in 1957. Rifampicin (Rif), a semi-synthetic molecule with the activity of rifamycin was synthesized in 1959 and introduced as an important ingredient of the combinatorial drug therapy (together with isoniazid, ethambutol and streptomycin) in 1967 for the treatment of tuberculosis. Since then, Rif has continued as a frontline drug against not only *Mycobacterium tuberculosis* but also many other pathogenic bacteria such as *Staphylococcus aureus* (methicillin-resistant *S. aureus*), *Neisseria meningitidis*, *Haemophilus influenzae*, etc. Rif binds to the β subunit (RpoB) of the DNA-dependent RNA polymerase and inhibits its activity in transcription (Hinkle *et al.*, 1972; McClure & Cech, 1978). The crystal structure of *Thermus aquaticus* RpoB bound to Rif shows that the antibiotic makes contact with amino acids that participate in catalysis (Campbell *et al.*, 2001). However, the emergence of

Rif resistance in *M. tuberculosis* is a major clinical concern, and a better understanding of the mechanism of development of Rif resistance in mycobacteria is crucial for the continued use of Rif or its derivatives in the treatment of tuberculosis.

A substantial body of work has revealed that the majority of the mutations that confer Rif resistance to mycobacteria map to the *rpoB* gene (Cole, 1996). Within *rpoB*, most of the mutations are found in a short 81 nt stretch referred to as the rifampicin resistance-determining region (RRDR) (Jin & Gross, 1988). There is a wealth of data available for the clinical samples of *M. tuberculosis* or other bacteria which show that a wide spectrum of point mutations, and in some instances small insertions or deletions in RRDR, are responsible for Rif resistance (Herrera *et al.*, 2003). The most common among these are the substitution mutations at His-442 and Ser-447 of *Mycobacterium smegmatis* RpoB (corresponding to 526 and 531, respectively, according to *Escherichia coli* numbering and 445 and 450, respectively according to *M. tuberculosis* numbering) (Cole, 1996). Spontaneously arising Rif-resistant mutants in the

Abbreviations: LBT, Luria–Bertani broth containing 0.2% Tween 80; PEI, polyethyleneimine; Rif, rifampicin; RRDR, rifampicin resistance-determining region.

laboratory strains of mycobacteria (*M. tuberculosis*, *M. smegmatis*) also show identical amino acid changes in RRDR (Boshoff *et al.*, 2003; Huitric *et al.*, 2006) and studies have shown that the mutants display varying levels of tolerance to the antibiotic (Huitric *et al.*, 2006; Ohno *et al.*, 1996; Taniguchi *et al.*, 1996). Interestingly, the property of spontaneously arising Rif-resistance mutations has also been widely used to determine the mutation rates in bacteria, including mycobacteria, and the nature of the changes at specific positions has been exploited to understand the mechanism of action of the various DNA repair enzymes.

As a part of our studies on DNA repair mechanisms in mycobacteria, we have sequenced a large number of RRDR amplicons from the Rif-resistant isolates to correlate the altered mutation spectrum to the loss of a particular DNA-repair process (Garibyan *et al.*, 2003; Jain *et al.*, 2007). During the course of these studies, we not only observed the most commonly occurring point mutations in RRDR but also isolated Rif-resistant *M. smegmatis* strains that carry either an insertion of 18 nt or a deletion of 30 nt in RRDR. Although the insertion and deletion mutations in RRDR have been reported earlier (Herrera *et al.*, 2003), their sizes were smaller than the ones we observed and the mechanisms of how such mutations in *rpoB* confer Rif resistance have not been investigated. In this study, we have purified RNA polymerases from Rif-resistant *M. smegmatis*

strains and carried out transcription assays. Together with the 3D structure modelling, these analyses have allowed us to infer the molecular basis of the Rif resistance of the strains due to the insertion and deletion mutations in RRDR and to propose that these mutants could offer a valuable target for drug design against Rif-resistant mycobacteria strains.

METHODS

Oligodeoxyribonucleotides, strains and culture conditions. The bacterial strains were cultured in 7H9 Middlebrook medium (Difco) supplemented with 0.2 % (v/v) glycerol and 0.2 % (v/v) Tween 80 or Luria–Bertani (Difco) broth containing 0.2 % (v/v) Tween 80 (LBT). For culturing bacteria on a solid surface, 1.5 % agar (Difco) was added to LBT. When needed, Rif was added to the medium at a concentration of 50–300 µg ml⁻¹. A list of strains derived from *M. smegmatis* mc²155 (referred to as wild-type or the parent strain) and their relevant details, and various plasmids and DNA oligomers are provided in Table 1.

Rif sensitivity assay. Isolated colonies of *M. smegmatis* strains from LBT agar Rif (50 µg ml⁻¹) plates were inoculated in LBT medium and grown to mid-exponential phase (OD₆₀₀ ~0.6). The cultures of *M. smegmatis* were serially diluted 1:5 in LBT in a 96-well plate. Samples from the third dilution onwards were spotted on LBT agar plates containing varying concentrations of Rif, with a 48-pronged spotter (Sigma) and incubated at 37 °C for 3–5 days.

Table 1. List of bacterial strains, DNA oligomers and plasmids

Relevant details/sequence		Reference
Strain		
<i>M. smegmatis</i> mc ² 155	High-efficiency transformation strain.	Snapper <i>et al.</i> (1990)
<i>rpoB</i> (H442Y)	<i>M. smegmatis</i> mc ² 155 <i>rpoB</i> has a C–T substitution mutation at codon 442 in the RRDR leading to H442Y amino acid change in RpoB.	This study
<i>rpoBA</i> 10	<i>M. smegmatis</i> mc ² 155 strain that has a deletion of 30 bp corresponding to aa 429–438 in the RRDR of the encoded RpoB.	This study
<i>rpoB</i> ::6	<i>M. smegmatis</i> mc ² 155 <i>udgB</i> :: <i>hyg</i> strain that contains an insertion of 18 bp corresponding to a sequence between aa 434 and 435 within RRDR of the encoded RpoB.	This study
<i>rpoB</i> ::6 pMV261 <i>udgB</i>	<i>M. smegmatis</i> <i>rpoB</i> ::6 mutant harbouring pMV261 <i>udgB</i> .	This study
<i>rpoB</i> (WT)	Designation used in this study for mc ² 155 harbouring pDK20 (<i>kan</i> ^R) at L5 <i>attB</i> site in the chromosome.	Venkatesh <i>et al.</i> (2003)
Oligomer*		
MsmUdgBPFp	GACACCGGTACCGGCGATCAGTG	This study
MsmUdgBRp	CAGAAGCTTTGGTATCCGAGCCG	This study
Msm- <i>rpoB</i> -seq-Fp	GTCTGCGCACCGTCCGGTG	Jain <i>et al.</i> (2007)
306- <i>rpoB</i> -Fp	CGACCACTTCGGCAACCG	Jain <i>et al.</i> (2007)
306- <i>rpoB</i> -Rp	CGATCAGACCGATGTTGG	Jain <i>et al.</i> (2007)
Plasmid		
pJET1.2	CloneJET PCR cloning kit.	MBI Fermentas
pMV261 (<i>kan</i> ^R)	A replicative vector with the <i>E. coli</i> origin of replication and mycobacterial origin of replication pAL5000 and <i>kan</i> ^R marker.	Stover <i>et al.</i> (1991)
pMV261 <i>udgB</i>	A replicative vector pMV261 <i>kan</i> ^R containing <i>M. smegmatis</i> MSMEG_5031 ORF.	This study

*Oligomers are given in 5'–3' orientation.

Analysis of RRDR. The amplicons containing RRDR segments were analysed by DNA sequencing as reported previously (Jain *et al.*, 2007).

Growth kinetics of rifampicin-resistant *M. smegmatis*. Isolated colonies of *M. smegmatis* strains were grown in triplicate in 7H9 Middlebrook medium with appropriate antibiotics to saturation (55–60 h). Cultures were serially diluted 1:100 in LBT with 0.5% (w/v) BSA, seeded in the wells of honeycomb plates and incubated in a Bioscreen C kinetic growth reader at 37 °C with constant shaking at maximum amplitude. Growth was monitored by measuring OD₆₀₀ at 3 h intervals.

Genomic blot analysis. Genomic DNA (~2.5 µg) from the parent and the Rif-resistant strains of *M. smegmatis* were digested with an excess of restriction enzymes, *SacI* or *HindIII* and *ScaI* (20 U), separated on a 0.7% agarose gel using TBE buffer, transferred to a nylon membrane (Amersham Hybond XL; GE Healthcare) and hybridized (Reed & Mann, 1985; Vasanthakrishna *et al.*, 1997) with a body-labelled probe generated by PCR using [α -³²P]dATP and primers MsmRpoBFp and MsmRpoBRp (Sambrook *et al.*, 1989).

Generation of *udgB* complemented strain. *M. smegmatis* UdgB (MSMEG_5031) was amplified by PCR and subcloned into pMV261 to generate pMV261*udgB*, which was then introduced into an *M. smegmatis* *rpoB*::6 mutant by electroporation to generate a complemented strain (Malshetty *et al.*, 2010).

Purification of RNA polymerases. RNA polymerases were purified by a two-column chromatography method involving Superdex S-200 gel filtration and heparin Sepharose (GE Healthcare). Briefly, 2 l of 7H9 Middlebrook medium was inoculated with the desired *M. smegmatis* strain and grown at 37 °C for 22 h (OD₆₀₀ ~1). The culture was harvested by centrifugation at 8000 g for 10 min, and the pellet was resuspended in the lysis buffer (50 mM Tris/HCl, pH 8.0, 2 mM Na₂EDTA, 5% glycerol, 233 mM NaCl, 5% glycerol, 0.1 mM DTT, 1 mM β -mercaptoethanol, 130 µg lysozyme ml⁻¹ and 23 µg PMSF ml⁻¹). Cells were subjected to lysis using a French press and centrifuged at 30 000 g for 1 h. The supernatant was subjected to 0.35% polyethyleneimine (PEI) precipitation and the precipitate was recovered by centrifugation and washed with TGED (10 mM Tris/HCl, pH 7.9, 5% glycerol, 0.5 mM Na₂EDTA and 100 µM DTT) containing 400 mM NaCl. Proteins were extracted from the pellet of PEI-precipitated material using TGED containing 1 M NaCl. The extracted proteins were subjected to a 0–50% ammonium sulfate precipitation, the precipitate was collected by centrifugation and resuspended in TGED containing 1 M NaCl, and fractionated on a Superdex S-200 gel filtration column with a flow rate of 0.5 ml min⁻¹. The fractions enriched for the transcription activity were pooled, dialysed against TGED with 150 mM NaCl and further purified by heparin Sepharose chromatography using a linear gradient of 150–1000 mM NaCl in 50 ml volume. The fractions were analysed by SDS-PAGE and those enriched for RNA polymerase were pooled and dialysed against the storage buffer (10 mM Tris/HCl, pH 8.0, 50% glycerol, 0.1 mM Na₂EDTA, 0.1 mM DTT and 150 mM KCl).

Determination of specific activity of RNA polymerase and inhibition by Rif. The specific activities of RNA polymerases were determined by promoter non-specific transcription assays using calf thymus DNA as template. The reaction buffer contained 40 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM Na₂EDTA, 14 mM β -mercaptoethanol, 200 µM NTPs, 20 µCi (740 kBq) [³H]UTP ml⁻¹ and 15 µg calf thymus DNA ml⁻¹. Reactions were carried out at 37 °C for 30 min and stopped by spotting on Na₂EDTA-soaked DE81 filter papers. Filters were washed once with 5% Na₂HPO₄ followed by two washes with deionized water. The filter paper was soaked in ethanol, dried and the [³H]UMP incorporation was measured by liquid scintillation counting. Specific activities of the RNA polymerases were

expressed as nmoles [³H]UMP incorporated per mg protein in 30 min. The specific activities were also measured in the presence of varying concentrations of Rif in reactions.

Three-dimensional structural modelling of RpoB. Three-dimensional structural modelling of *M. smegmatis* RpoB/RRDR sequences was carried out using the comparative modelling approach COMPOSER (Srinivasan & Blundell, 1993) available in the SYBYL (Tripos) suite of software. Structural modelling was based on the crystal structure of *T. aquaticus* RpoB bound to Rif (Protein Data Bank code 1YNN; Campbell *et al.*, 2005), which shows 50% sequence identity with *M. smegmatis* RpoB. During the course of comparative modelling, the side chains in the wild-type and mutant RpoB proteins were positioned on the basis of the side-chain rotamer rules (Sutcliffe *et al.*, 1987). While modelling the insertion and deletion regions in such mutants, the loop-modelling protocol that employs structural environment-dependent amino acid substitution table was used (Topham *et al.*, 1993). Structural models of the wild-type and mutants were subjected to energy minimization using AMBER force field (Weiner *et al.*, 1984) available in SYBYL. Structural models were analysed in the interactive graphics using Setor (Evans, 1993).

RESULTS

Identification of insertion and deletion mutants of RpoB

During the analysis of RRDR loci from *M. smegmatis* strains (wild-type, or deficient in certain parts of DNA repair), we noted that while a vast majority of RRDR from Rif-resistant strains possessed the frequently occurring point mutations, a small minority showed an unusual occurrence of either a hexapeptide (KFMDQN) insertion between residues N434 and N435 (*rpoB*::6) or a decapeptide (QFMDQNNPLS) deletion from residues Q429 to S438 (*rpoBA*10) in RRDR (Fig. 1a). The positions of these amino acids corresponding to *M. tuberculosis* (*Mtu*) and *E. coli* (*Eco*) RpoB are also shown in Fig. 1(a) for comparison.

Genomic blot of *rpoB* mutants and steady-state accumulation of RpoB in the Rif-resistant strains

To rule out the possibility of *rpoB* gene amplification in the Rif-resistant isolates, we carried out genomic blot analysis and probed for *rpoB* gene organization. A schematic representation of the *rpoB* locus is depicted in Fig. 1(b). For the genomic blot analysis, we chose restriction endonucleases that cleave within the *rpoB* gene (*SacI*, *BamHI*) and in the flanking regions of the gene (*SacI*, *ScaI*). Moreover, in this and the analyses detailed below, we also included a strain that harboured a most frequently occurring mutation in RRDR (H442Y). As shown in Fig. 1(c), the *SacI* digests revealed the presence of single bands of ~3.7 kb in the wild-type (lane 1) and the Rif-resistant isolates (lanes 2, 3 and 4). Similarly, the *BamHI*/*ScaI* double digests also resulted in detection of ~4.0 kb bands (lanes 5–8) in the strains. The presence of single bands of the expected sizes and of similar intensities suggested that the *rpoB* gene in the Rif-resistant isolates was not amplified. Furthermore, by carrying out immuno-

Fig. 1. (a) Multiple sequence alignment of *M. smegmatis* RpoB (RRDR, amino acids 421–474) sequence of the wild-type (*rpoB*), hexapeptide insertion mutant (*rpoB*::6) and a decapeptide deletion mutant (*rpoB*Δ10) strains in the region. The gaps (–) indicate deletion and the inverted triangle indicates insertion of the amino acids in the RRDR. The corresponding sequences of *M. tuberculosis* (*Mtu*) and *E. coli* (*Eco*) RpoB are also shown. The numbering of amino acids is as per the codon numbers in the ORFs of the *rpoB* genes. (b, c) Genomic blot analysis. (b) A schematic representation of the expected sizes of the fragments in genomic blot from the *rpoB*. The location of the *rpoB* gene-specific probe of 383 bp used in the genomic blot is indicated by a dotted line above the *rpoB* gene. (c) Digital image of the genomic blot of the *M. smegmatis* strains. Lanes 1–4 correspond to *SacI* digests, and 5–8 correspond to *BamHI/SacI* digests of the genomic DNA. Lanes: 1 and 5, *rpoB* (wild-type); 2 and 6, *rpoB*(H442Y); 3 and 7, *rpoB*Δ10; 4 and 8, *rpoB*::6. Sizes of DNA bands (in kb) are as indicated.

blot analysis of total cellular extracts using anti-RpoB rabbit antibodies (data not shown), we observed that in all the strains, RpoB accumulated to a similar extent and that the mutations within RpoB had little, if any, significant impact on their stabilities.

Growth fitness of *rpoB* mutants

Resistance to antibiotics is often at the cost of fitness. There are reports on mycobacterial strains (Billington *et al.*, 1999; Mariam *et al.*, 2004) showing that drug-resistant mutants have varying fitness costs. As the isolated mutants had insertion and deletion mutations within an essential gene, we compared the growth properties of the strains in 7H9 Middlebrook medium. As shown in Fig. 2, compared with the growth of the parent (*M. smegmatis* mc²155), the growth of the mutants was compromised. Of these, growth of the deletion mutant was the most compromised. It may be noted that after about 36 h growth, the OD₆₀₀ values of the *rpoB*Δ10 and *rpoB*(H442Y) strains were observed to decline because of an early clumping in these cultures.

Analysis of Rif sensitivity

It has been reported that mutations in RpoB result in different levels of Rif resistance (Huitric *et al.*, 2006; Ohno *et al.*, 1996). As shown in Fig. 3(a), in the absence of any Rif, the growth of all dilutions of the strains following spotting onto the agar medium were comparable. As expected, while the parent strain was susceptible to the presence of 50 µg Rif ml^{−1}, the remainder of the strains grew even in the presence of 100 µg Rif ml^{−1} in the medium. When the concentration of Rif was increased to 200 and 300 µg ml^{−1}, only the deletion mutant (*rpoB*Δ10) showed growth, suggesting that the decapeptide deletion in RRDR conferred the highest resistance. However, it should be mentioned that while the H442Y mutation and the *rpoB*Δ10 mutation in *rpoB* occurred in the wild-type parent strain background, the *rpoB*::6 mutation was obtained in an *udgB*-deficient background. Although, we have shown that UdgB deficiency does not result in any growth defects (Malshetty *et al.*, 2010), to ensure that *udgB* and the *rpoB*::6 mutation together did not result in any synthetic effects we complemented the *rpoB*::6 strain with a plasmid

Fig. 2. Saturated cultures of various *M. smegmatis* strains [*rpoB* (WT), ○; *rpoB* (H442Y), □; *rpoB*::6, ▽; and *rpoB*Δ10, △] were diluted 100-fold in LBT containing 0.5% (w/v) BSA. Cultures were grown at 37 °C in a Bioscreen C kinetic growth reader using honeycomb plates. The culture growth was monitored by measuring OD₆₀₀ at regular intervals. Growth curves were prepared from three independent colonies for each strain, and the mean values (±SD) are shown.

copy of the *udgB* gene to generate *rpoB*::6pMV261*udgB* strain (Table 1). As shown in Fig. 3(b), resistance of this strain to Rif was the same as in Fig. 3(a), suggesting that the Rif sensitivity of the strain was not affected by the *UdgB* deficiency.

In vitro transcription analysis of the RNA polymerases

To understand the mechanism of the varying levels of Rif resistance of the strains (Fig. 3), we purified RNA polymerases from the wild-type (*rpoB*), *rpoB*(H442Y),

rpoB::6 and the *rpoB*Δ10 strains. Analysis of 1 and 2 µg preparations of RNA polymerases revealed similar stoichiometries of the different subunits (Fig. 4a), indicating that the mutations in RRDR had no impact on the subunit composition of the mutant RNA polymerases. We then analysed template (calf thymus DNA)-dependent NTP polymerization (incorporation) by the RNA polymerases from the parent and the mutant strains. As shown in Fig. 4(b), while the specific activity of NTP incorporation by the H442Y mutant was similar to that of the wild-type enzyme, the insertion mutant was compromised about threefold and the activity of the deletion mutant was severely compromised (~15-fold).

It has been reported that RNA polymerase from wild-type *M. smegmatis* is sensitive to the action of rifampicin *in vitro*. However, the RNA polymerases from the Rif-resistant strains are less sensitive to the action of Rif (Levin & Hatfull, 1993). Hence, to further our understanding of the Rif resistance of the isolates, we analysed the effect of Rif on the transcription activities of the respective RNA polymerases. As shown in Fig. 5(a), the activity of the polymerase from the parent strain (*rpoB*) was highly sensitive to Rif. Its activity was nearly totally inhibited by Rif at as low as 0.25 µg ml⁻¹. The activity of the *rpoB*(H442Y)-containing polymerase showed a greater resistance to Rif (Fig. 5b), but it was strongly inhibited by the presence of ~100 µg Rif ml⁻¹. However, the activities of the RNA polymerases from the insertion (*rpoB*::6, Fig. 5c) and deletion (*rpoB*Δ10, Fig. 5d) strains remained essentially unchanged even in the presence of 1 mg Rif ml⁻¹.

Structural modelling of RpoB mutants

As the sequence identity between the *M. smegmatis* RpoB and its *T. aquaticus* homologue is quite high (50%), the

Fig. 3. (a) Rif sensitivity assay on *M. smegmatis* [*rpoB* (WT); *rpoB*(H442Y); *rpoB*Δ10 and *rpoB*::6] strains. Dilutions (as indicated) of early exponential phase cultures were spotted onto LBT agar containing Rif (concentrations indicated above each plate). The plates were then incubated at 37 °C for 4 days and photographed. (b) Rif sensitivity assay on *M. smegmatis* strains [*rpoB*::6 and *rpoB*::6:pMV261*udgB*] were performed as in (a).

Fig. 4. (a) RNA polymerases from the various strains were purified (Methods) and analysed (1 μg and 2 μg of each preparation, as indicated) by using SDS-PAGE (8 %) and silver staining. (b) Determination of the specific activity. The activities were determined by promoter-non-specific transcription initiation assay using calf thymus DNA as template. Error bars indicate SD.

accuracy of the structural model for *M. smegmatis* RpoB generated on the basis of the crystal structure of *T. aquaticus* homologue was expected to be good. Moreover, one of the crystal structures of *T. aquaticus* RNA polymerase (PDB code 1YNN) is available in complex with Rif. Hence, to further our understanding of the Rif binding to *M. smegmatis* RpoB proteins and to obtain structural insights for the Rif resistance of the RpoB proteins (Fig. 5), we modelled these proteins by making use of the available crystal structure of *T. aquaticus* RNA polymerase (Campbell *et al.*, 2005).

The crystal structure of the *T. aquaticus* RpoB complexed with Rif showed that the Q393, F394, D396, H406, R409 and S411 (residue numbering as followed in the crystal structure) either directly interact with Rif or are located proximal to the binding site, making them potentially capable of influencing the Rif binding to RpoB. Interestingly, all these residues are also conserved in *M. tuberculosis* and *M. smegmatis* (Campbell *et al.*, 2001; Hetherington *et al.*, 1995). Fig. 6 shows the location of these conserved residues (Q429, F430, D432, H442, R445 and S447 as per the *M. smegmatis* RpoB) in the

Fig. 5. Transcription assays carried out in the presence of increasing concentrations of Rif with 1 μg RNA polymerases. RpoB (wild-type) in the presence of 0–1 μg Rif ml⁻¹ (a), RpoB(H442Y) (b), RpoB::6 (c) and RpoBΔ10 (d) in the presence of between 1 μg and 1 mg Rif ml⁻¹. The specific activity of transcription at each Rif concentration is normalized with the transcription activity in absence of the drug (taken as 100 %) for each protein. Error bars indicate SD.

Fig. 6. Three-dimensional structural analysis. (a) Superposition of the crystal structure of *T. aquaticus* RpoB (pink) with the model of *M. smegmatis* homologue (green). Side chains of Rif-binding residues are shown with numbering corresponding to the *M. smegmatis* homologue. Rif is shown in white. (b–d) Superposition of the model of *M. smegmatis* RpoB (wild-type, green) and the models of various mutants (yellow). Rif is shown in white. Rif-binding residues in the wild-type protein are shown in blue and the corresponding residues in the mutants [indicated by prime (') numbers] are shown in brown. (b) RpoB(H442Y), (c) RpoB::6 and (d) RpoBΔ10. Representations of protein structures were generated using SETOR (Evans, 1993).

superimposed crystal structure of *T. aquatics* and comparative model of *M. smegmatis*.

As shown in Fig. 6(a) and (b), H442 is one of the Rif-binding residues. In fact, the side chain of the equivalent H406 in the *T. aquatics* crystal structure forms a hydrogen bond with Rif and this feature is also noted with H442 in the structural model of the *M. smegmatis* homologue (Fig. 6b). In the RpoB(H442Y) mutant, although the Tyr side chain presents a hydrogen-bonding group, due to its bigger size, it is expected to disturb the position of Rif from its canonical binding site noted in the wild-type RpoB. This could affect the hydrogen bonding between Rif and the side chain. Also, a possibility of hydrogen bonding between Rif and the OH group in the side chain of Tyr is likely to weaken other interactions of Rif to RpoB(H442Y) due to disturbance of the canonical binding mode of Rif.

In the *M. smegmatis* homologue (Fig. 6a), the region 429–453 harbours six of the amino acids that constitute the Rif-binding site. In RpoB::6, there is an insertion of 6 aa between N434 and N435 (Fig. 1a). Many of the Rif-binding residues such as F430, D432, H448 (corresponding to H442 in the wild-type) and R451 (corresponding to R445 in the

wild-type) are immediately flanking the inserted sequence. Accommodation of six residues between positions 434 and 435 will necessarily require major conformational changes in the flanking regions, repositioning the Rif-binding residues. Indeed, a model of this mutant (RpoB::6) showed profound structural differences and altered positioning of Rif-binding residues compared with the wild-type RpoB (Fig. 6c), agreeing with the observation that RpoB::6 was essentially recalcitrant to Rif inhibition (Fig. 5c). Likewise, the RpoBΔ10 homologue involved deletion of a 10 residue region Q429–S438 (Fig. 1a). In the wild-type allele, three of the six Rif-binding residues, namely Q429, F430 and D432, are located within this region. Clearly, deletion of a region with Rif-binding residues can seriously hamper the ability of the mutant to bind to Rif. Moreover, accommodation of the deletion region in the structural scaffold will mean major structural alterations in the regions flanking the deletion (Fig. 6d). All these structural features in the model of the deletion mutant are consistent with the observations in Fig. 5(d).

DISCUSSION

The antibiotic Rif is widely used to control bacterial infection. In fact, it is a frontline drug to combat *M. tuberculosis* infection. Rif affects the vital process of transcription by binding to RpoB (β subunit) of the bacterial RNA polymerases. The crystal structure of *T. aquaticus* RpoB bound to Rif showed that the antibiotic makes contact with amino acids that take part in catalysis, and provides a basis for inhibition of the process of productive transcription initiation (Campbell *et al.*, 2001). As shown in the modelled structures of *M. smegmatis* RpoB (Fig. 6), mutations that confer Rif resistance destabilize the Rif–RpoB interaction. This region of RpoB is highly conserved among the various prokaryotes and more so between *M. tuberculosis* and *M. smegmatis* (91 % similarity and 85 % identity) (Hetherington *et al.*, 1995). Importantly, therefore, the Rif-resistant mutations that arise under laboratory conditions in the *M. smegmatis* model (Jain *et al.*, 2007) have direct relevance to the clinical isolates of Rif-resistant *M. tuberculosis*.

The insertion and deletion mutants identified and characterized in this study were attenuated in growth (Fig. 2). Such fitness costs are often associated with the strains that gain resistance to the antibiotics (Billington *et al.*, 1999; Mariam *et al.*, 2004). In principle, this observation may suggest that these strains would fail to establish themselves in the field (Sander *et al.*, 2002). However, it has also been reported that in spite of such fitness costs, the strains may persist in the patients (Hershberg *et al.*, 2008), especially if the treatment regimens are not strictly adhered to; the existence of such strains may pose serious challenges for disease management. In addition, the drug-resistant bacterial pathogens with attenuated fitness may rapidly accumulate compensatory mutations and restore their fitness (Böttger *et al.*,

1998). Thus, the mutants of RpoB that we have characterized here could be significant to further our understanding of the drug resistance in the clinical isolates.

We noted that while the transcription activities of the RNA polymerases containing the insertion and deletion mutants of RpoB were essentially refractory to Rif inhibition (even at a concentration of 1 mg ml⁻¹) for their *in vitro* transcriptional activity, the *M. smegmatis* mutants from which these RNA polymerases were isolated showed growth inhibition at Rif concentrations as low as 200 µg ml⁻¹. The exact reasons for this seemingly conflicting observation have not been investigated. However, it is important to mention that the inhibitory effects of Rif, at least on *E. coli* RNA polymerase, have been reported to be promoter-specific, and the effectiveness of the Rif-mediated inhibition may also vary with the class of sigma factor present in the RNA polymerase (Hu *et al.*, 2000; Wegrzyn *et al.*, 1998). In this context, mycobacteria possess a larger number of sigma factors (~13 in *M. tuberculosis* and ~26 in *M. smegmatis*; Rodrigue *et al.*, 2006; Waagmeester *et al.*, 2005), and the processes of Rif-mediated inhibition of transcription from specific promoters with RNA polymerases containing different sigma factors have not been studied. It may be quite likely that *in vivo* transcription of some of the housekeeping/vital genes becomes susceptible to high concentrations of Rif, resulting in growth inhibition of even the strains that harbour the insertion and deletion mutants of RpoB (Fig. 3). In addition, studies with *M. tuberculosis* have shown that the presence of high concentrations of Rif renders them uncultivable, even though the RNA polymerase remains transcriptionally competent (Hu *et al.*, 2000).

Finally, as shown in Fig. 4, among the RNA polymerases characterized in this study, while the substitution of the wild-type RpoB with RpoB(H442Y) in the enzyme retains full activity, substitution with RpoB::6 retains about 30% activity. However, the RNA polymerase containing RpoBΔ10 is severely compromised for its transcriptional activity *in vitro* (Fig. 4). In *E. coli*, it is known that the mutations in RpoB that confer Rif resistance also affect the processes of transcriptional elongation, termination and anti-termination (Jin *et al.*, 1988; Yanofsky & Horn, 1981). Impacts of such mutations on the various steps of transcription are unknown in mycobacteria. It should also be said that various aspects of transcription in mycobacteria (and other Gram-positive bacteria) are notably different from *E. coli* (Henkin, 2000; Smith *et al.*, 2005; Unniraman *et al.*, 2002). Thus, the *M. smegmatis* RNA polymerases characterized in this study may provide us with valuable tools to investigate the impact of the mutations (that result in Rif resistance) on various regulatory mechanism of transcription in mycobacteria. Also, while our modelling studies (Fig. 6) were designed to provide us with the basis for the development of the Rif resistance in the mutants we have isolated, in principle, similar computational analysis could also afford the design of newer derivatives of the drug, wherein introduction of

alternate chemical groups may compensate for the impact of the lost interactions. Clearly, such studies constitute a crucial aspect of developing newer compounds as drugs.

ACKNOWLEDGEMENTS

We thank our laboratory colleagues for their suggestions on the manuscript. This work was supported by funding from the Department of Biotechnology, and the Department of Science and Technology, New Delhi.

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Edited by: G. R. Stewart