Role of an RNA polymerase interacting protein, MsRbpA, from *Mycobacterium smegmatis* in phenotypic tolerance to rifampicin

Abhinav Dey, Amit Kumar Verma and Dipankar Chatterji

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

Correspondence

Dipankar Chatterji
dipankar@mbu.iisc.ernet.in

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Rifampicin and its derivatives are at the forefront of the current standard chemotherapeutic regimen for active tuberculosis; they act by inhibiting the transcription activity of prokaryotic RNA polymerase. Rifampicin is believed to interact with the β subunit of RNA polymerase. However, it has been observed that protein–protein interactions with RNA polymerase core enzyme lead to its reduced susceptibility to rifampicin. This mechanism became more diversified with the discovery of RbpA, a novel RNA polymerase-binding protein, in *Streptomyces coelicolor* that could mitigate the effect of rifampicin on RNA polymerase activity. MsRbpA is a homologue of RbpA in *Mycobacterium smegmatis*. On deciphering the role of MsRbpA in *M. smegmatis* we found that it interacts with RNA polymerase and increases the rifampicin tolerance levels, both *in vitro* and *in vivo*. It interacts with the β subunit of RNA polymerase. However, it was found to be incapable of rescuing rifampicin-resistant RNA polymerases in the presence of rifampicin at the respective IC_{50} .

INTRODUCTION

Tuberculosis (TB) is responsible for the loss of more human lives every week than any other infectious disease. Chemotherapy for TB has resulted from the success of a handful of natural products and synthetic molecules that were tested as inhibitors for *Mycobacterium tuberculosis*. However, the chemotherapeutic options have been limited to a very small number of compounds, introduced 40–50 years ago, viz. rifampicin, isoniazid, pyrazinamide and ethambutol, which form the armoury of the first-line chemotherapy against TB (Nguyen & Thompson, 2006). Although a plethora of small-molecule inhibitors have been reported over the years targeting bacterial DNA-dependent RNA polymerase, the only drug approved for clinical administration for treating TB, along with other infectious diseases, is rifampicin. Rifampicin belongs to the family of ansamycins, which were discovered in a strain of *Nocardia mediterranei* (Villain-Guillot *et al.*, 2007). Notwithstanding the efficacy of rifampicin in TB treatment, resistant mutants arise spontaneously, especially due to improper drug administration. Rifampicin resistance in all eubacterial species has been attributed to structural changes in RNA polymerase resulting from missense mutations in the *rpoB* gene (Jin & Gross, 1988; Miller *et al.*, 1994). Apart from the classical mechanism of action of rifampicin on the β

subunit, another mechanism has also been proposed (Artsimovitch *et al.*, 2005). Rifampicin seems to be nearer to region 3 of the σ -factor than expected from the crystal structure of the rifampicin–core RNA polymerase complex. This proximity could provide an explanation for the σ -dependence of the inhibition of transcription by rifampicin; for example, the inhibition is stronger when the polymerase uses σ^{70} compared with σ^{32} (Węgrzyn *et al.*, 1998).

M. tuberculosis exhibits rifampicin tolerance – a reversible physiological state involving lowered sensitivity to rifampicin – which limits bactericidal kinetics. In other words, rifampicin cannot quickly kill persistent populations of *M. tuberculosis* which are found in clinical disease. The long period of chemotherapy which is needed leads to poor patient compliance, and in turn, difficulties in disease control. Rifampicin-tolerant *M. tuberculosis* cells exhibit low but detectable levels of RNA polymerase activity in the presence of rifampicin (Hu *et al.*, 2000). This behaviour was thought to be due to alternative forms of RNA polymerase holoenzymes associated with various factors, leading to lowered affinity of RNA polymerase to rifampicin. Rapid purification of RNA polymerase from biomass cultured to different stages of growth (Mukherjee & Chatterji, 2008) showed that the IC_{50} of rifampicin for stationary-phase RNA polymerase was double that for exponential-phase RNA polymerase. The composition of the transcription complex at the stationary phase was studied; this revealed the association of several proteins

Abbreviations: AB, azidobenzoate; NHS, *N*-hydroxysuccinimide; sulfo-HSAB, *N*-hydroxysulfosuccinimidyl-4-azidobenzoate; TB, tuberculosis.

Supplementary material is available with the online version of this paper.

with RNA polymerase throughout the purification process, prominent among them being GroEL1, DNA polymerase 1, transcription repair coupling factor and preprotein translocase. Thus, it was speculated that in the stationary phase, RNA polymerase could be insulated from rifampicin by such associated proteins. The role of a DNA replication machinery protein, DnaA, has recently been reported (Flåtten *et al.*, 2009) with respect to its *in vitro* interaction with RNA polymerase in *Escherichia coli*, and the partial protection that RNA polymerase received from rifampicin as a result of this interaction.

In an independent line of study, a new protein, RbpA, was discovered in *Streptomyces coelicolor* (Newell *et al.*, 2006). This protein can interact with RNA polymerase, causes rifampicin tolerance of RNA polymerase activity *in vitro* and leads to basal levels of rifampicin resistance *in vivo*. RbpA was earlier observed as a protein that copurifies with RNA polymerase preparations in *S. coelicolor* (Paget *et al.*, 2001). This protein was named as RbpA (RNA polymerase-binding protein). Moreover, RbpA has sequence homologues exclusively in the actinomycete family, with nearest neighbours in mycobacteria. Paget *et al.* (2001) discovered RbpA as a part of the σ^R regulon in *S. coelicolor*. The comparison of the upstream sequence of *rbpA* between *S. coelicolor* and *M. tuberculosis* revealed a strong conservation of the -10 and -35 regions, making σ^H a possible candidate for regulating *rbpA_{MTB}* (Rv2050). Interestingly, when *rbpA* null mutants of *S. coelicolor* were transformed with the *M. tuberculosis rbpA* gene, the resistance to rifampicin increased from 0.75 $\mu\text{g ml}^{-1}$ to 2 $\mu\text{g ml}^{-1}$ *in vivo*, which pointed towards an analogous role of *rbpA_{MTB}*. Saturation mutagenesis studies carried out on *M. tuberculosis* have placed *rbpA_{MTB}* (Rv2050) on the list of genes needed for optimal growth (Sasseti *et al.*, 2003). The similarities and the speculations over mycobacterial RbpA made a compelling case for deciphering its role in mycobacteria, especially in the context of rifampicin tolerance. Bioinformatic analysis identified a homologue of *rbpA_{MTB}* (Rv2050) in *M. smegmatis* in the form of the MSMEG_3858 product, which is 92 % identical and 100 % similar to *rbpA_{MTB}*. Here we report the role of

MSMEG_3858 (MsRbpA) with respect to rifampicin tolerance in mycobacteria. To the best of our knowledge, this is the first study on RbpA after that of Newell *et al.* (2006).

METHODS

Bacterial strains, plasmids and growth conditions.

Mycobacterium smegmatis mc²155 is the wild-type strain. SM07 is a recombinant strain derived from mc²155, harbouring a chromosomal hexahistidine-encoding tag on the *rpoC* gene (Mukherjee & Chatterji, 2008). Jmc²155 and JRmc²155 are plasmid-transformed versions of mc²155, carrying the plasmids pJAM2 (Triccas *et al.*, 1998) and pJAM2MsRbpA, respectively. SM0747, SM0734 and SM0748 are rifampicin-resistant variants of SM07. All *M. smegmatis* strains were grown on MB7H9 medium (Difco) (supplemented with Bacto-agar when required) along with 2 % (w/v) glucose and 0.05 % (v/v) Tween 80 (in the case of liquid cultures).

SM07, SM0747, SM0734 and SM0748 were grown with 50 $\mu\text{g ml}^{-1}$ hygromycin. Jmc²155 and JRmc²155 were grown with 25 $\mu\text{g ml}^{-1}$ kanamycin. Rifampicin concentrations varied from 2.5 $\mu\text{g ml}^{-1}$ to 400 $\mu\text{g ml}^{-1}$.

Escherichia coli strain DH5 α was used for cloning experiments. The protein expression and purification experiments were carried out using *E. coli* strain BL21(DE3) in Luria-Bertani medium.

All plasmids used in this study are listed in Table 1.

Strain constructions

Jmc²155. pJAM2 encodes an acetamide-inducible expression system used for the purification of recombinant proteins in *M. smegmatis* (Triccas *et al.*, 1998). pJAM2 was electroporated into *M. smegmatis* mc²155 competent cells and the transformants were selected on MB7H9 medium with 2 % (w/v) glucose and kanamycin.

JRmc²155. pJAM2MsRbpA contains MSMEG_3858 cloned in pJAM2. *M. smegmatis* mc²155 competent cells were electroporated with pJAM2MsRbpA and the colonies were screened on MB7H9 medium supplemented with 2 % (w/v) glucose and kanamycin.

SM0747, SM0734 and SM0748. Construction of these strains is described in the Supplementary Methods available with the online version of this paper.

Table 1. Plasmids used in this study

Plasmid	Description	Reference
pET21b	Cloning and expression vector	Novagen
pJAM2	Shuttle vector for overexpression of genes in <i>M. smegmatis</i>	Triccas <i>et al.</i> (1998)
pETMsRbpA	MSMEG_3858 cloned in pET21b in <i>NdeI</i> - <i>XhoI</i> site	This work
pJAM2MsRbpA	MSMEG_3858 cloned in pJAM2 in <i>Bam</i> HI- <i>Xba</i> I site	This work
pFPVH	Vector carrying <i>M. tuberculosis rrn</i> promoter	Gift from Professor Jaya Tyagi, AIIMS
pVJP16	Vector carrying <i>M. tuberculosis rel</i> promoter cloned in pSD5B	Jain <i>et al.</i> (2005)
pAZI0612A	<i>M. tuberculosis</i> RNA polymerase β' subunit cloned in pET3a	Gift from AstraZeneca, Bangalore
pAZI0611	<i>M. tuberculosis</i> RNA polymerase β subunit cloned in pET3a	Gift from AstraZeneca, Bangalore
pETRNP α Ms	<i>M. smegmatis</i> RNA polymerase α subunit cloned in pET21b	Kumar & Chatterji (2008)
pETOsm	<i>M. smegmatis</i> RNA polymerase ω subunit cloned in pET21b	Mathew <i>et al.</i> (2005)
pARC8171	<i>M. tuberculosis</i> SigA-overexpressing plasmid	Gift from AstraZeneca, Bangalore

Plasmid constructions

pETMsRbpA. MSMEG_3858 was amplified from *M. smegmatis* genomic DNA using PCR and primers MsRbpXNdF and MsRbpXNdR (see Table 2). The 367 bp amplicon was cloned in the *XhoI/NdeI*-digested pET21b vector (Novagen) to create pETMsRbpA. The clone was confirmed by DNA sequencing and MALDI-TOF MS from a sample obtained from in-gel trypsin digestion of the band corresponding to 14 kDa in 15 % SDS-PAGE.

pJAM2MsRbpA. Primers MsRbpJAM2f and MsRbpJAM2r (see Table 2) were used to amplify MSMEG_3858 from *M. smegmatis* genomic DNA, which was later cloned in pJAM2, pre-digested with *XbaI/BamHI*. The clone was confirmed by PCR using internal primers pRbpintf and pRbpintr (see Table 2), specific for MSMEG_3858, as well as Western blotting using anti-MsRbpA antibody on acetamide-induced cultures.

Purification of His-tagged MsRbpA. pETMsRbpA was transformed into *E. coli* BL21(DE3) and after 3 h of induction with 1 mM IPTG, cells were harvested. The pellet was resuspended in 20 ml ice-cold lysis buffer [50 mM Tris/HCl, 5 % (v/v) glycerol, 0.1 mM EDTA, 1 M NaCl, 1 mM DTT, 6 mM β -mercaptoethanol, 1 mM imidazole, 23 μ g PMSF ml⁻¹, 200 μ g hen egg white lysozyme ml⁻¹, pH 7.8]. The remaining steps of the protocol were similar to the method followed by Kumar & Chatterji (2008).

Pull-down assay for wild-type lysate of *M. smegmatis* over an immobilized (His)₆MsRbpA-Ni-NTA matrix. Cells from a 24 h culture of *M. smegmatis* mc²155 were resuspended in lysis buffer [50 mM Tris/HCl, 5 % (v/v) glycerol, 0.1 mM EDTA, 1 M NaCl,

1 mM DTT, 6 mM β -mercaptoethanol, 23 μ g PMSF ml⁻¹, 200 μ g hen egg white lysozyme ml⁻¹, pH 7.8] and incubated in ice for 30 min with 0.2 % (w/v) sodium deoxycholate, with occasional shaking. After incubation, the suspension was subjected to sonication and French press treatment. The lysate was centrifuged and the supernatant was treated with 0.6 % (v/v) polyethylenimine P and centrifuged. The resulting pellet was extracted with 1 M NaCl in TGE buffer [10 mM Tris/HCl, 5 % (v/v) glycerol, 0.1 mM EDTA, 6 mM β -mercaptoethanol] followed by 50 % ammonium sulfate precipitation. The pellet was resuspended in TGE buffer containing 1 M NaCl and 1 mM imidazole and kept for binding with a Ni-NTA matrix, pre-equilibrated with purified (His)₆MsRbpA. After 6 h incubation on a rotary shaker at 4 °C, the Ni-NTA matrix was washed with 100 column volumes of TGE buffer containing 0.6 M NaCl and 10 mM imidazole. (His)₆MsRbpA and associated proteins were eluted in TGE buffer with 400 mM NaCl and 250 mM imidazole.

Reconstitution of a heterologous mycobacterial core RNA polymerase. The various subunits of core RNA polymerase were purified as described in the Supplementary Methods. The reconstitution was carried out by dialysing 2 mg purified *M. smegmatis* RNA polymerase α subunit (50 nmol), 8 mg each of purified *M. tuberculosis* RNA polymerase β' subunit and *M. tuberculosis* RNA polymerase β subunit (100 nmol each), and *M. smegmatis* RNA polymerase ω subunit (50 nmol), against storage buffer [100 mM Tris/HCl pH 7.9, 50 % (v/v) glycerol, 0.1 mM EDTA, 0.15 M KCl and 0.1 mM DTT] overnight. The overall concentration of all the proteins was kept below 0.5 mg ml⁻¹. This fraction was subsequently mixed with Ni-NTA agarose (equilibrated in storage buffer) and kept for binding at 4 °C for 6 h. The column was then washed with five column volumes of TGE buffer [50 mM Tris/HCl pH 7.9, 5 % (v/v) glycerol, 6 mM

Table 2. Primers used in this study

Primer	Sequence (5'–3')	Purpose	Vector
MsRbpXNdF	GGAGTGATGCATATGGCTGATCG	Forward primer for cloning of MSMEG_3858	pET21b
MsRbpXNdR	GTCAGCTCGAGTGTGCTTCCGG	Reverse primer for cloning of MSMEG_3858	pET21b
MsRbpJAM2f	GCGTGAGGATCCATGGATCGTGTC	Forward primer for cloning of MSMEG_3858	pJAM2
MsRbpJAM2r	CCTCAGCTCTCTAGAGCTTCCGGT	Reverse primer for cloning of MSMEG_3858	pJAM2
pRbpintf	TCGGAGCCGTGAGCTACGAGAC	Internal forward primer to screen for cloned MSMEG_3858 inside plasmids	pETMsRbpA and pJAM2MsRbpA
pRbpintr	GCTTGGCCTTGATCAGGTCGAGAC	Internal reverse primer to screen for cloned MSMEG_3858 inside plasmids	pETMsRbpA and pJAM2MsRbpA
PrelF	GTGTCGTGATCATTGACGACG	Forward primer for amplification of <i>M. tuberculosis rel</i> promoter for <i>in vitro</i> transcription assays	pVJP16
PrelR	CTGGTCTTAAGAGTCTCGACCG	Reverse primer for amplification of <i>M. tuberculosis rel</i> promoter for <i>in vitro</i> transcription assays	pVJP16
RNCF	GGCTCGATGTCACTGTCCTTCTCGGA	Forward primer to screen for mutations in N-terminal Rif ^R cluster of <i>M. smegmatis rpoB</i> gene	
RNCR	ACCTTGACGCTGTGCAGCGTCTTC	Reverse primer to screen for mutations in N-terminal Rif ^R cluster of <i>M. smegmatis rpoB</i> gene	
R3CF	CGTCGGTGAGCTGATCCAGAACCAGATCC	Forward primer to screen for mutations in Rif ^R clusters I, II and III of <i>M. smegmatis rpoB</i> gene	
R3CR	CCATGACCGTGATGTAGTCGGCCGAGA3	Reverse primer to screen for mutations in Rif ^R clusters I, II and III of <i>M. smegmatis rpoB</i> gene	

β -mercaptoethanol, 0.1 mM EDTA]. The bound proteins were eluted with TGEB + 0.4 M NaCl + 250 mM imidazole. The eluted fractions were dialysed against TGED + 0.4 M NaCl for 12 h in two batches to remove imidazole. This fraction was subjected to another dialysis in TGED + 0.15 M NaCl. The final dialysed fraction was loaded onto a heparin-Sepharose column (pre-equilibrated with TGED + 0.15 M NaCl) and kept for binding for 6 h. The column was washed with five column volumes of TGED + 0.15 M NaCl buffer. The proteins were eluted with TGED + 0.6 M NaCl buffer. The eluted fractions were then analysed for their purity by 10% SDS-PAGE and subsequently assayed for RNA synthesis activity.

Promoter-specific single-round gel-based transcription assay.

The *M. tuberculosis* *rel* promoter was amplified from pVJP16 (Jain *et al.*, 2005) using primers PreIF and PreIR (Table 2). The amplicon thus obtained was used as a template for the single-round promoter-specific transcription assay. Purified σ^A (200 nM) from *M. tuberculosis* was incubated with reconstituted core RNA polymerase (100 nM) in molar ratio 2:1 in transcription buffer [40 mM Tris/HCl, 10 mM MgCl₂, 75 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 14 mM β -mercaptoethanol, 0.025 mg ml⁻¹ BSA (nuclease free)] at 37 °C for 10 min. The DNA template *rel* amplicon (10 nM) was then added at a 0.1 molar ratio to RNA polymerase and a further incubation of 37 °C for 5 min was carried out. Finally, the NTP mix comprising 0.15 mM ATP, 0.15 mM GTP, 0.15 mM CTP, 0.05 mM UTP, 2 μ Ci (74 kBq) [³²P]UTP and 200 μ g heparin ml⁻¹ was added, followed by incubation for 30 min at 37 °C. The reaction was stopped by addition of stop solution (5 mM EDTA and 100 μ g *E. coli* tRNA ml⁻¹). The transcription products were subjected to 10% denaturing PAGE in the presence of 6 M urea and analysed by phosphorimager (FLA2000; Fujifilm) (Chowdhury *et al.*, 2007).

In the transcription reactions involving the addition of rifampicin, the core RNA polymerase, σ^A and rifampicin were incubated together in transcription assay buffer at 37 °C for 10 min before the addition of DNA, NTPs and heparin. In the transcription reactions involving the presence of MsRbpA, the core RNA polymerase, σ^A and rifampicin were incubated together in transcription assay buffer at 37 °C for 10 min and then MsRbpA was added to the mix followed by incubation at 37 °C for 5 min. This was followed by addition of DNA, NTP and heparin in order to complete the transcription reaction.

Cross-linking of MsRbpA with RNA polymerase using the heterobifunctional cross-linker sulfo-HSAB. With the objective of determining the binding partner of MsRbpA on RNA polymerase, a chemical cross-linking reaction was carried out between MsRbpA and *M. smegmatis* RNA polymerase. Sulfo-HSAB (*N*-hydroxysulfosuccinimideyl-4-azidobenzoate; Pierce) was used as a cross-linker. *M. smegmatis* core RNA polymerase was purified from a 30 h culture and dialysed in PBS (pH 7.8). (His)₆MsRbpA labelled with [³⁵S]methionine was also

purified and dialysed against PBS (pH 7.8). ^{35}S -labelled (His) $_6$ MsRbPA (70 μM) was mixed with 50-fold molar excess of sulfo-HSAB dissolved in PBS (pH 7.8). After incubation at 37 $^\circ\text{C}$ in the dark for 30 min, the mix was loaded on a Sephadex-G25 column to separate unreacted sulfo-HSAB from the protein. The labelling of MsRbPA with sulfo-HSAB was followed by monitoring the increase in A_{260} as a measure of *N*-hydroxysuccinimide (NHS) ester hydrolysis caused as a result of labelling. The protein was detected in the void volume by scintillation counting. The labelled MsRbPA was incubated with fivefold molar excess of RNA polymerase at 37 $^\circ\text{C}$ for 30 min followed by exposure to 254 nm UV light for 8 min. The reaction mix was immediately subjected to 12 % SDS-PAGE, and the complexes formed as a result of chemical cross-linking were detected with anti-MsRbPA antibodies, and antibodies against the *M. tuberculosis* β' , *M. tuberculosis* β , *M. smegmatis* α and *M. smegmatis* ω subunits of RNA polymerase (subsequently referred to as anti-*M. tuberculosis* β' , anti-*M. tuberculosis* β , anti-*M. smegmatis* α and anti-*M. smegmatis* ω antibodies).

RESULTS

MsRbpA is highly conserved in pathogenic and non-pathogenic mycobacterial species

RbpA was discovered as a protein co-eluting with RNA polymerase during purification from *S. coelicolor* (Paget *et al.*, 2001). The characterization of this protein for its role in rifampicin resistance in *S. coelicolor* was reported later by the same group (Newell *et al.*, 2006). Bioinformatic analysis identified an RbpA homologue in *M. smegmatis* with accession number MSMEG_3858, which was 92 % identical to the *M. tuberculosis* relative Rv2050 (Fig. 1). Further analysis revealed that MsRbpA homologues are highly conserved in mycobacterial species (Fig. 2). Another conspicuous feature is the occurrence of a homologue of RbpA in *Nocardia farcinica* (Supplementary Fig. S1), the only sequenced genome from the genus *Nocardia*, which includes the rifampicin producer, *N. rifamylicina*. This could be a possible defence mechanism that *Nocardia* might use against its own metabolite rifampicin.

MsRbpA can interact with RNA polymerase

MsRbpA was tagged with a stretch of six histidines at the C-terminus by cloning MSMEG_3858 in pET21b (pre-

Fig. 1. Bioinformatic search for homologues of *S. coelicolor* RbpA (SCO1421) and *M. tuberculosis* RbpA (Rv2050) in the genome of *M. smegmatis* mc²155. The BLAST search revealed the existence of a homologue MSMEG_3858 showing 50% amino acid sequence identity with SCO1421 and 92% identity with Rv2050.

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MT_2110_M.tuberculosis CDC155      MADRVLRGSRGAVSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
NTL01MB2075_M.bovis subsp. bo      MADRVLRGSRGAVSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
NTL02MT02045_M.tuberculosis H      MADRVLRGSRGAVSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
NTL03MA1798_M.avium paratuber      MADRVLRGSRGAVSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
MAV_2447_M.avium 104                -----MSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
NTL01ML1440_M.leprae TN            MADRVLRGSRGAVSYETDRNHD LAPRQIARYRTDNGEEFEVFPFADDAEI
MSMEG_3858                          MADRVLRGSRGAVSYETDRNHD LAPRQVARYRTDNGEEFDVFPFADDAEI
MSMEG_3858_M.smegmatis MC2          MADRVLRGSRGAVSYETDRNHD LAPRQVARYRTDNGEEFDVFPFADDAEI
                                     :*****:*****:*****:*****:
                                     :*****:*****:*****:*****:

MT_2110_M.tuberculosis CDC155      PGTWLCRNGMEGT LIEGDLPEPKVKPPRTHWDM LLERSIEELEELLKE
NTL01MB2075_M.bovis subsp. bo      PGTWLCRNGMEGT LIEGDLPEPKVKPPRTHWDM LLERSIEELEELLKE
NTL02MT02045_M.tuberculosis H      PGTWLCRNGMEGT LIEGDLPEPKVKPPRTHWDM LLERSIEELEELLKE
NTL03MA1798_M.avium paratuber      PGTWLCRNGMEGT LIEGDLPEPKVKPPRTHWDM LLERSIEELEELLKE
MAV_2447_M.avium 104                PGTWLCRNGMEGT LIEGDLPEPKVKPPRTHWDM LLERSIEELEELLKE
NTL01ML1440_M.leprae TN            PGTWLCRNGMEGT LIEGDLPEPKMKVPPRTHWDM LLERSIEELEELLKE
MSMEG_3858                          PGTWLCRNGLEGT LIEGDPVPEPKVKPPRTHWDM LLERSV EEELEELLKE
MSMEG_3858_M.smegmatis MC2          PGTWLCRNGLEGT LIEGDPVPEPKVKPPRTHWDM LLERSV EEELEELLKE
                                     *****:*****:*** *****:*****:*****:

MT_2110_M.tuberculosis CDC155      RLELIRSRRRG---
NTL01MB2075_M.bovis subsp. bo      RLELIRSRRRG---
NTL02MT02045_M.tuberculosis H      RLELIRSRRRG---
NTL03MA1798_M.avium paratuber      RLEIIRSRRRG---
MAV_2447_M.avium 104                RLEIIRSRRRG---
NTL01ML1440_M.leprae TN            RLDLIRSRRRG---
MSMEG_3858                          RLDLIKAKRRGTGS
MSMEG_3858_M.smegmatis MC2          RLDLIKAKRRGTGS
                                     **:***:***

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Fig. 2. Multiple sequence alignment of MSMEG_3858 with other mycobacterial homologues in pathogenic mycobacterial species using CLUSTAL W 2.

digested with *NdeI* and *XhoI*). The protein purified as MsRbpA was confirmed by DNA sequencing and MALDI-TOF MS (Supplementary Fig. S2, Supplementary Table S1). The (His)₆MsRbpA was immobilized on a Ni-NTA matrix (Qiagen); wild-type *M. smegmatis* lysate fractionated with 50% ammonium sulfate was loaded on the matrix, and left for binding overnight at 4 °C. The eluates obtained showed the presence of RNA polymerase (Fig. 3a). The eluates were screened for RNA polymerase subunits using anti-*M. tuberculosis* β' and anti-*M. tuberculosis* β antibody (Fig. 3b). However, a pull-down assay on crude cell lysate is not the best way to establish protein-protein interaction (Mackay *et al.*, 2007). Therefore, we repeated the pull-down assay using a pure RNA polymerase from wild-type *M. smegmatis* (Fig. 3c, Supplementary Fig. S3). The pull-down assay using purified RNA polymerase reconfirmed the interaction between MsRbpA and RNA polymerase and also indicated that this interaction was not mediated by other proteins present in the cell lysate.

MsRbpA rescues RNA polymerase from the inhibitory effect of rifampicin

In order to test the role of MsRbpA in rescuing RNA polymerase from the inhibitory effect of rifampicin, we reconstituted core RNA polymerase comprising pure β' , β ,

α and ω subunits from either *M. tuberculosis* or *M. smegmatis* (Supplementary Fig. S4). Due to the greater than 90% identity in different subunits of RNA polymerase across the species of mycobacteria, the reconstituted enzyme is the same from either of the species. The purpose of reconstitution was to rule out any possibility of other proteins which might co-purify with RNA polymerase and influence the results. We tested those concentrations of rifampicin where the transcription activity is completely inhibited and challenged the same conditions with the addition of MsRbpA (Fig. 4a). We found that MsRbpA could rescue the *in vitro* activity of RNA polymerase in a promoter-specific single-round transcription assay, carried out from the *rel* promoter of *M. tuberculosis* (Jain *et al.*, 2005). Transcription occurred even at 100 μ M rifampicin, when the molar ratio of RNA polymerase to MsRbpA was kept at 1:2. When we increased the molar ratio of RNA polymerase to MsRbpA from 1:0.5 to 1:3 at constant rifampicin concentration of 40 μ M (Fig. 4b), we observed that the use of threefold molar excess of MsRbpA restored the *in vitro* activity of RNA polymerase to the normal level.

Overexpression of MsRbpA in *M. smegmatis* increases rifampicin tolerance

We cloned MsRbpA under the acetamidase promoter in pJAM2 (Triccas *et al.*, 1998) and electroporated the

Fig. 3. Interaction between MsRbpA and *M. smegmatis* RNA polymerase. (a) $(\text{His})_6\text{MsRbpA}$ was immobilized over a Ni-NTA agarose column. *M. smegmatis* wild-type culture was grown and cells were lysed as described in Methods. The supernatant of the lysate after 50 % ammonium sulfate precipitation was treated with the affinity matrix and subsequently eluted with 250 mM imidazole. Lane M contains molecular mass markers (Perfect Protein Marker, Novagen); lane C, the elution of $(\text{His})_6\text{MsRbpA}$ without any treatment with lysate, served as control. Lanes E1 to E6 show different elution fractions, all containing RNA polymerase subunits, as proved by Western analysis. (b) Western analysis for RNA polymerase subunits β' (upper panel) and β (lower panel), using polyclonal antibodies developed against *M. tuberculosis* β' and β subunits, for detection of RNA polymerase co-eluting in the Ni-NTA agarose pull-down assay carried out using immobilized $(\text{His})_6\text{MsRbpA}$. Ref, RNA polymerase; L, load of processed cell-free lysate; FT, flowthrough; W, wash; E1 to E5, elutions 1 to 5. (c) Ni-NTA agarose pull-down assay carried out using immobilized $(\text{His})_6\text{MsRbpA}$ on purified RNA polymerase from wild-type *M. smegmatis*. A 10 μmol sample of RNA polymerase was mixed with 70 μmol $(\text{His})_6\text{MsRbpA}$ and loaded on an Ni-NTA agarose column; elutions were carried out at 250 mM imidazole. (Ref, reference core RNA polymerase; M, markers; W, wash; E1 to E5, elutions 1 to 5).

5b). We also carried out growth-dependent Western blot analysis using anti-MsRbpA antibody on wild-type *M. smegmatis* lysates to determine the levels of MsRbpA (Fig. 5c). The level of MsRbpA increased in stationary-phase cultures of *M. smegmatis*. However, the level of expression was much less as compared to the RNA polymerase α subunit, which was taken as the loading control.

MsRbpA interacts with the β subunit of RNA polymerase

The chemical cross-linker HSAB had been previously used to locate the binding site of the ω subunit to the β' subunit on RNA polymerase (Gentry & Burgess, 1993). We used the same strategy to locate the binding of MsRbpA on *M. smegmatis* core RNA polymerase purified from 30 h cultures of *M. smegmatis* SM07. The hetero-bifunctional cross-linker sulfo-HSAB is modified by the attachment of a sulfo group compared to the free HSAB used by Gentry & Burgess (1993), rendering it water soluble. Cross-linking of MsRbpA to RNA polymerase involved two steps: derivatizing [^{35}S]MsRbpA (Supplementary Fig. S5a) with sulfo-HSAB, followed by incubating the derivatized MsRbpA with core RNA polymerase and exposing the mixture to UV light (Supplementary Fig. S5b). The first step, derivatization of [^{35}S]MsRbpA, added an azidobenzoate (AB) group to free amino groups on MsRbpA to make MsRbpA-AB. MsRbpA was made, in this case, from cells labelled with [^{35}S]methionine in order to create a tag distinct from the spectrophotometric identity. The product of the cross-linking, N-hydroxysuccinimide (NHS), absorbs at 260 nm. Seven residues can be modified in this way (MsRbpA has six lysines and the amino-terminus). This step was monitored

construct pJAM2MsRbpA into competent mc^2155 cells. As a control, pJAM2 vector was also electroporated into competent mc^2155 cells. The vector pJAM2 has a kanamycin-resistance marker. The resulting strains Jmc 2155 (carrying pJAM2) and JRmc 2155 (carrying pJAM2MsRbpA) were tested at different concentrations of rifampicin. The strain overexpressing MsRbpA in the inducing conditions of 2 % acetamide grew at rifampicin concentrations of 20, 40 and 80 $\mu\text{g ml}^{-1}$, while the strain carrying pJAM2 was incapable of growing at these concentrations (Fig. 5a). The MIC of rifampicin for Jmc 2155 was 20 $\mu\text{g ml}^{-1}$ while that for JRmc 2155 was 85 $\mu\text{g ml}^{-1}$. Thus overexpression of MsRbpA leads to an increase in the rifampicin tolerance levels in an otherwise rifampicin-sensitive strain. Colony morphology was compared between wild-type *M. smegmatis* transformed with pJAM2 (Jmc 2155) and pJAM2MsRbpA (JRmc 2155). The MsRbpA-overexpressing strain showed smaller colonies compared to the vector-carrying strain (Fig.

Fig. 4. MsRbpA rescues RNA polymerase activity in the presence of rifampicin. (a) Single-round promoter-specific gel-based transcription assay in the presence of different concentrations of rifampicin at constant concentration of MsRbpA (200 nM). The assays were carried out on the *M. tuberculosis* *rel* promoter fragment amplified from pVJP16 using primers PRelF and PRelR. Rifampicin was dissolved in DMSO. (b) Single-round promoter-specific transcription assay in the presence of different concentrations of MsRbpA and a fixed concentration of rifampicin (40 μ M).

by observing the increase in A_{260} due to release of NHS ester (Supplementary Fig. S5c). [35 S]MsRbpA eluted in the void volume of a Sephadex G25 column, and the released product of cross-linking, NHS, eluted in the included volume. The last step involved the actual cross-linking of MsRbpA to RNA polymerase. When the phenyl azide groups (attached to [35 S]MsRbpA-AB) were exposed to UV light (254 nm) there was formation of highly reactive phenylnitrene, which created covalent linkages with a variety of molecules. Such cross-linked products to RNA polymerase subunits were detected due to the formation of a covalent adduct on SDS gel which was identified by both anti-MsRbpA and anti-RNA polymerase subunit antibodies. The observed product was expected to have a size approximating the molecular mass of MsRbpA plus that of the specific subunit. The cross-linking reactions were carried out in the dark, according to the manufacturer's instructions. The products of cross-linking were probed with anti-MsRbpA, anti-*M. tuberculosis* β , anti-*M. tuberculosis* β' , anti-*M. smegmatis* α and anti-*M. smegmatis* ω antibodies. We observed a shift in the molecular mass of the β subunit after the photo-cross-linking reaction, in agreement with the shift in the molecular mass of the MsRbpA band (lanes 5 and 6 in Fig. 6a; lane 2 in Fig. 6b). The Western analysis with anti-*M. tuberculosis* β' , anti-*M. smegmatis* α and anti-*M. smegmatis* ω antibodies did not show any shift in band position (Fig. 6c, d).

MsRbpA does not restore the activity of Rif^R RNA polymerases in the presence of rifampicin at the IC₅₀

As both rifampicin and MsRbpA bind to the β subunit of RNA polymerase, we determined the effect of MsRbpA on

an RNA polymerase purified from a rifampicin-resistant (Rif^R) strain of *M. smegmatis*. We generated Rif^R mutants of *M. smegmatis* SM07 by serial culturing of the strain from lower (5 μ g ml⁻¹) to higher (50 μ g ml⁻¹) concentrations of rifampicin (Supplementary Fig. S6a). After scoring the Rif^R strains for resistance to 100, 200, 300 and 400 μ g rifampicin ml⁻¹ (Supplementary Fig. S6b), we checked whether the strains undergo reversion in the absence of antibiotic (Supplementary Fig. S6c, d). The selected strains were named as SM0747 (MIC of rifampicin ~200–300 μ g ml⁻¹), SM0734 (MIC of rifampicin ~300–400 μ g ml⁻¹) and SM0748 (MIC of rifampicin >400 μ g ml⁻¹). The Rif^R strains were also grown in MB7H9 broth and their growth curves were recorded at different concentrations of rifampicin (Supplementary Fig. S6e). They were characterized for the *rpoB* mutations by sequencing amplicons obtained using primers R3CF and R3CR (Table 2) on genomic DNA of *M. smegmatis* strains SM07, SM0747, SM0734 and SM0748. DNA sequencing was carried out by cycle sequencing using the BigDye Terminator Cycle Sequencing kit v3.0 (Applied Biosystems) and was analysed on an ABI3100 Genetic Analyser (Applied Biosystems). To detect the sequence variants in the Rif^R clusters of the *rpoB* gene, we compared the SM07 sequence with those of SM0747, SM0734 and SM0748 using CLUSTAL W 2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The mutations were scored as D432Y (in the case of SM0747) and S447L (in the case of SM0734 and SM0748) (Supplementary Fig. S6f). The results of the Rif^R mutations are in agreement with those obtained by Jin & Gross (1988) in *E. coli*. The IC₅₀ (concentration equivalent to the minimum concentration required for 50% inhibition of multiple-round transcription activity) of rifampicin for the

Fig. 5. *In vivo* studies on MsRbpA in *M. smegmatis*. (a) Overexpression of MsRbpA increases the rifampicin tolerance in *M. smegmatis*. Strains mc²155, Jmc²155 (pJAM2) and JRmc²155 (pJAM2MsRbpA) were plated on MB7H9 agar supplemented with 2% (w/v) glucose (plates A to F), 2% (w/v) acetamide (plates A to F) and 25 µg kanamycin ml⁻¹ (plates B to F), along with different concentrations of rifampicin: plate A, 0 µg ml⁻¹; plate B, 0 µg ml⁻¹; plate C, 10 µg ml⁻¹; plate D, 20 µg ml⁻¹; plate E, 40 µg ml⁻¹; plate F, 80 µg ml⁻¹. Strain JRmc²155 (pJAM2MsRbpA) showed growth up to 80 µg rifampicin ml⁻¹, while Jmc²155 (pJAM2) did not grow at 20, 40 or 80 µg rifampicin ml⁻¹. (b) Change in colony morphology upon overexpressing MsRbpA in *M. smegmatis*. Colonies of strain mc²155 transformed with pJAM2 (strain Jmc²155) and pJAM2MsRbpA (strain JRmc²155) are shown. The strains were grown for 7 days on MB7H9 agar supplemented with 2% (w/v) glucose, 2% (w/v) acetamide and 25 µg kanamycin ml⁻¹. (c) Growth-dependent Western analysis using anti-MsRbpA antibody on the cell-free lysates from 24 h, 48 h and 72 h *M. smegmatis* cultures (left panel); samples containing 100 µg protein were loaded in each lane. The control blot was probed using anti-*M. smegmatis* α antibody (right panel). The expression level of MsRbpA rises in stationary phase but the amount appears to be very low as compared to RNA polymerase, whose comparative expression was greater throughout the growth cycle as judged by the Western analysis. Left panel: lane 1, prestained molecular mass markers showing 20 kDa and 26 kDa bands; lane 2, control protein MsRbpA; lane 3, 24 h culture load; lane 4, 48 h culture load; lane 5, 72 h culture load. Right panel: lane 1, control protein *M. smegmatis* RNA polymerase α subunit; lane 2, prestained molecular mass markers showing 34 kDa and 47 kDa bands; lane 3, 24 h culture load; lane 4, 48 h culture load; lane 5, 72 h culture load.

Fig. 6. Cross-linking of MsRbpA with RNA polymerase. (a) Western blot analysis of MsRbpA–RNA polymerase cross-linked products separated by 12 % SDS-PAGE and probed using anti-MsRbpA antibody. The lanes represent the following reaction conditions: lane 1, MsRbpA; lane 2, MsRbpA + sulfo-HSAB; lane 3, MsRbpA + sulfo-HSAB + RNA polymerase; lane M, Prestained Marker SMO441 (Fermentas; sizes in kDa indicated); lane 4, MsRbpA + sulfo-HSAB + UV light (254 nm for 8 min); lane 5, MsRbpA + sulfo-HSAB + RNA polymerase + UV light (254 nm for 6 min); lane 6, MsRbpA + sulfo-HSAB + RNA polymerase + UV light (254 nm for 8 min) (R*, cross-linked adduct of MsRbpA–RNA polymerase; R, free MsRbpA). (b) Western blot analysis of MsRbpA–RNA polymerase cross-linked products separated by 6 % SDS-PAGE and probed using anti-*M. tuberculosis* β antibody. The lanes represent the following reaction conditions: lane 1, RNA polymerase + MsRbpA + sulfo-HSAB (no UV); lane 2, RNA polymerase + MsRbpA + sulfo-HSAB + UV light (254 nm for 8 min); M, molecular mass marker, 120 kDa; lane 3, reference *M. tuberculosis* β (B*, β –MsRbpA cross-linked adduct; B, free β subunit). (c) Western blot analysis of MsRbpA–RNA polymerase cross-linked products separated by 6 % SDS-PAGE and probed using anti-*M. tuberculosis* β' antibody. The lanes represent the following reaction conditions: lane 1, RNA polymerase + MsRbpA + sulfo-HSAB (no UV); lane 2, RNA polymerase + MsRbpA + sulfo-HSAB + UV light (254 nm for 8 min); lane 3, reference *M. tuberculosis* β' . (d) Western blot analysis of MsRbpA–RNA polymerase cross-linked products separated by 6 % SDS-PAGE and probed using anti-*M. smegmatis* α and anti-*M. smegmatis* ω antibodies. The lanes represent the following reaction conditions: lane 1, RNA polymerase + MsRbpA + sulfo-HSAB (no UV); lane 2, RNA polymerase + MsRbpA + sulfo-HSAB + UV light (254 nm for 8 min); lane 3, reference *M. smegmatis* RNA polymerase.

rifampicin-sensitive RNA polymerase from SM07 was $0.05 \mu\text{g ml}^{-1}$. The RNA polymerases purified from these strains (Supplementary Fig. S6g) had IC_{50} values of 4.4 mg ml^{-1} (SM0747), 6.4 mg ml^{-1} (SM0734) and 6.2 mg ml^{-1} (SM0748) (Supplementary Table S2). The rifampicin-sensitive RNA polymerase from SM07 had an IC_{50} value of $0.05 \mu\text{g ml}^{-1}$.

We tested for the rescue of rifampicin inhibition by MsRbpA of Rif^R RNA polymerases as against Rif^S RNA polymerase. In these cases, promoter-specific multiple-round transcription assays were carried out for the Rif^S and Rif^R RNA polymerases at their respective IC_{50} levels of rifampicin. Although the activity of Rif^S RNA polymerase was rescued by MsRbpA at the IC_{50} value of $0.05 \mu\text{g ml}^{-1}$, there was no recovery of transcription activity of Rif^R RNA polymerases from SM0747, SM0734 and SM0748, at their respective IC_{50} values of 4.4, 6.4 and $6.2 \text{ mg rifampicin ml}^{-1}$, even when assayed at 8 : 1 molar ratio of MsRbpA to RNA polymerase (Supplementary Fig. S6h).

DISCUSSION

The work on RbpA in *S. coelicolor* by Newell *et al.* (2006) laid the foundation for the novel mechanism of protection of RNA polymerase from the classical inhibitor rifampicin. Whether protein–protein interactions within the transcription machinery ultimately lead to the rifampicin tolerance is an important consideration. We have already mentioned that differential sensitivity to rifampicin of core RNA polymerase- σ^{70} or $-\sigma^{32}$ complex (Węgrzyn *et al.*, 1998) may arise from altered conformation of the transcription machinery, although it was not demonstrated clearly. Previous proteomic analyses by us indicated that in stationary phase, rifampicin binding to the enzyme is blocked by several proteins (Mukherjee & Chatterji, 2008). Similarly, activation of σ^B -dependent transcription in *Bacillus subtilis* by sublethal doses of rifampicin may arise due to the other proteins interacting with RNA polymerase (Bandow *et al.*, 2002). Proof of the transcriptional machinery of *M. tuberculosis* surviving the lethal rifampicin

treatment was obtained when mRNA transcripts were detected after treatment of this organism with rifampicin (Hu *et al.*, 2000), further raising the question of whether the stress-induced changes in the proteome lead to insulation of mycobacterial RNA polymerase against rifampicin.

In this study we have attempted to dissect the role of MsRbpA with respect to its ability to confer rifampicin tolerance to RNA polymerase in mycobacteria. Our findings are summarized below.

(a) The interaction between MsRbpA and reconstituted RNA polymerase mitigates the inhibition of transcription even at 100 μ M rifampicin, in an *M. tuberculosis* *rel* promoter-specific single-round transcription assay.

(b) MsRbpA increases the IC₅₀ of rifampicin for purified RNA polymerase from *M. smegmatis* SM07 in a multiple-round transcription assay on the *M. tuberculosis* *rel* promoter and *M. tuberculosis* *rrn* promoter (Supplementary Table S3), indicating that perhaps the effect of MsRbpA is not promoter specific.

(c) MsRbpA acts by binding to the β subunit of RNA polymerase. As the β subunit is the site of action of rifampicin, we checked the action of MsRbpA on Rif^R RNA polymerase. For that purpose we developed Rif^R mutants of *M. smegmatis* SM07 and mapped the mutation sites on the Rif^R clusters of the β subunit. However, we observed no change in the IC₅₀ values in these cases, even with an 8:1 molar ratio of MsRbpA:RNA polymerase.

At this point, we do not know where exactly MsRbpA binds the β subunit. However, our results indicate that the binding site may overlap with the rifampicin-binding domain. Such a proposition seems reasonable, as we know that there are four such domains along the length of the β subunit (Jin & Gross, 1988). The mode of action of MsRbpA in relieving RNA polymerase from rifampicin inhibition could be by shielding the polymerase against rifampicin or causing a conformational change in the binding site of rifampicin so that the interaction between RNA polymerase and rifampicin cannot take place. It should be mentioned here that we were unable to detect any interaction between MsRbpA and rifampicin *in vitro* (data not shown).

It was also intriguing why MsRbpA did not have a similar rifampicin rescue effect on a Rif^R RNA polymerase as it had on a Rif^S RNA polymerase. Such behaviour makes it tempting to speculate upon a kind of switching in the behaviour of the cell in response to rifampicin when RNA polymerase evolves from a Rif^S to a Rif^R form, in which case the role of MsRbpA in providing phenotypic tolerance to rifampicin becomes redundant. Thus, it appears that MsRbpA plays a role in protecting the Rif^S RNA polymerase when the cell attempts to evolve into a genetically Rif^R strain.

(d) The *in vivo* data regarding the *M. tuberculosis* homologue of MsRbpA, Rv2050, have been discussed

previously. The overexpression of Rv2050 in an *rbpA* null mutant of *S. coelicolor* increased the resistance level from 0.75 to 2 μ g rifampicin ml⁻¹. On similar lines, when we overexpressed MsRbpA in wild-type *M. smegmatis*, we found that the MIC values for rifampicin rose from 20 μ g ml⁻¹ to 85 μ g ml⁻¹. Thus, our *in vivo* data corroborated the *in vitro* data on MsRbpA obtained from the transcription assays.

(e) Microarray data of Murphy & Brown (2007) revealed that Rv2050 was upregulated under conditions of aerated starvation, starvation under 50 % dissolved oxygen, and *in vitro* hypoxia (Wayne model; Wayne & Hayes, 1996), as well as in mouse macrophages. Rv2050 was also upregulated under conditions of heat shock, diamide stress and dormant stage (AstraZeneca R&D, Bangalore, personal communication). Thus, it appears that Rv2050 is induced by multiple stress conditions. MsRbpA (MSMEG_3858) (which is 100 % similar to Rv2050) was also found to be upregulated in stationary-phase cultures of *M. smegmatis*. It will be interesting to find out whether MsRbpA has any effect on cells other than those described.

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