

MtrA, an essential response regulator of the MtrAB two-component system, regulates the transcription of resuscitation-promoting factor B of *Mycobacterium tuberculosis*

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The resuscitation-promoting factors of *Mycobacterium tuberculosis* are hydrolytic enzymes, which are required for resuscitation of dormant cells. RpfB, a peptidoglycan remodelling enzyme similar to the lytic transglycosylase of *Escherichia coli*, is required for reactivation of *M. tuberculosis* from chronic infection *in vivo*, underscoring the need to understand its transcriptional regulation. Here, we identified the transcriptional and translational start points of *rpfB*, and suggested from *rpf* promoter-driven GFP expression and *in vitro* transcription assays that its transcription possibly occurs in a SigB-dependent manner. We further demonstrated that *rpfB* transcription is regulated by MtrA – the response regulator of the essential two-component system MtrAB. Association of MtrA with the *rpfB* promoter region *in vivo* was confirmed by chromatin immunoprecipitation analysis. Electrophoretic mobility shift assays (EMSAs) revealed a loose direct repeat sequence associated with MtrA binding. Binding of MtrA was enhanced upon phosphorylation. MtrA could be pulled down from lysates of *M. tuberculosis* using a biotinylated DNA fragment encompassing the MtrA-binding site on the *rpfB* promoter, confirming that MtrA binds to the *rpfB* promoter. Enhanced GFP fluorescence driven by the *rpfB* promoter, upon deletion of the MtrA-binding site, and repression of *rpfB* expression, upon overexpression of MtrA, suggested that MtrA functions as a repressor of *rpfB* transcription. This was corroborated by EMSAs showing diminished association of RNA polymerase (RNAP) with the *rpfB* promoter in the presence of MtrA. *In vitro* transcription assays confirmed that MtrA inhibits RNAP-driven *rpfB* transcription.

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INTRODUCTION

Tuberculosis is second only to HIV/AIDS as the leading cause of death due to a single infectious agent (<http://www.who.int/mediacentre/factsheets/fs104/en/>). According to the World Health Organization, tuberculosis caused 1.3 million deaths in 2012. About a third of the world's population has latent tuberculosis, with no symptoms of the disease. This is associated with the ability of *Mycobacterium tuberculosis*, the

causative agent of tuberculosis, to lie dormant within its host over long periods of time, emerging from dormancy only when conditions are favourable. Both dormancy and reactivation represent processes which are likely to be good targets for therapeutic intervention.

M. tuberculosis has an efficient stress management machinery which enables it to survive in a dormant state under conditions of stress. It also must have a stress-responsive signalling system, together with machinery that allows it to reactivate and replicate when conditions are favourable. Dormancy and reactivation are regulated by differential expression and functional activation of distinct sets of genes. This involves: (i) the expression and activation of extracytoplasmic function sigma factors (Sachdeva *et al.*, 2010), (ii) Ser/Thr phosphorylation mediated by a family

Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IVT, *in vitro* transcription; qRT, quantitative real-time; RNAP, RNA polymerase; TCS, two-component system; TSP, transcriptional start point; TSS, translational start site.

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of Ser/Thr kinases (Cousin *et al.*, 2013), and (iii) signal sensing and gene expression regulated by two-component systems (TCSs) (Bretl *et al.*, 2011). Out of the 11 paired TCSs of *M. tuberculosis*, the response regulator MtrA alone is essential (Zahrt & Deretic, 2000). MtrB phosphorylates MtrA. Phosphorylated MtrA binds to the promoters of *oriA*, *fbpB*, *ripA* and *dnaA* (Fol *et al.*, 2006; Rajagopalan *et al.*, 2010). Merodiploid strains expressing *mtrA* with alterations in the signal-receiving domain show growth defects *in vitro* (Al Zayer *et al.*, 2011). In the fast-growing *Mycobacterium smegmatis*, the sensor kinase MtrB is not essential (Plocinska *et al.*, 2012). Recent studies in *M. smegmatis* have shown that MtrB associates with the septa in an FtsZ- and FtsI-dependent manner (Plocinska *et al.*, 2012). Interaction of MtrB with FtsI is required for expression of the *mtrA* regulon. MtrB interacts with Wag31 in a phosphorylation-independent manner to regulate the localization of Wag31 (Plocinska *et al.*, 2014). A lipoprotein modulates activity of the MtrAB system in mycobacteria to regulate drug resistance and cell wall homeostasis (Nguyen *et al.*, 2010). These reports suggest that the MtrAB TCS has a role in regulating cell wall synthesis, cell division and morphology in *M. smegmatis*. However, the *mtrA* regulon is poorly understood.

Hydrolytic enzymes are required at the site of cell division to facilitate peptidoglycan remodelling and division into two daughter cells. The hydrolases of *M. tuberculosis* include CwlM (Deng *et al.*, 2005), ChiZ (Vadrevu *et al.*, 2011) and the five resuscitation-promoting factors (RpfA–E) (Kana & Mizrahi, 2010), which are thought to correspond to peptidoglycan lytic transglycosylases in Gram-negative organisms. As with lytic transglycosylases, resuscitation-promoting factors are not essential for *in vitro* growth. Thus, deletion of all five Rpf homologues in *M. tuberculosis* has no effect on bacterial viability in broth culture, although delayed colony formation has been observed on solid agar (Downing *et al.*, 2004; Kana *et al.*, 2008). In contrast, the resuscitation-promoting factors are essential for growth and persistence in a mouse model of *M. tuberculosis* infection (Kana *et al.*, 2008). Cell wall growth and division can apparently proceed *in vitro* in the absence of resuscitation-promoting factors. However, the resuscitation-promoting factors are important for virulence and resuscitation from dormancy, and are expressed in *M. tuberculosis*-infected human tissue (Davies *et al.* 2008). A mutant of *M. tuberculosis* lacking *rpfB* shows delayed reactivation from chronic tuberculosis (Russell-Goldman *et al.*, 2008; Tufariello *et al.*, 2006). RpfB is capable of hydrolysing the 1,4- β -glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (Cohen-Gonsaud *et al.*, 2005), and is similar to the lytic transglycosylase Slt70 of *Escherichia coli* (Engel *et al.*, 1991; Hett & Rubin, 2008). It controls outer membrane integrity and is associated with β -lactam tolerance (Wivagg & Hung, 2012). RpfB interacts with the endopeptidase RipA to hydrolyse peptidoglycan (Hett *et al.*, 2007, 2008, 2010). The balance between cell wall synthesis and degradation is likely maintained by multiprotein complexes such as the PBP1–RpfB–RipA

complex. PBP1 inhibits peptidoglycan hydrolysis mediated by the RpfB–RipA complex (Hett *et al.*, 2010). RpfB is characterized by multiple domains as defined by the Pfam database. In addition to the catalytic domain (~ 75 residues), RpfB (362 residues) contains a G5 domain and three DUF348 domains. G5 domains are important molecular components of proteins that are involved in a variety of biological processes, such as cell wall degradation and biofilm formation. Considering the importance of the mycobacterial hydrolases in general, and RpfB in particular, in cell wall remodelling and in growth under stress or during reactivation from stress, it is of obvious importance to understand the transcriptional regulation of *rpfB*.

In our efforts to understand the transcriptional control of cell wall remodelling enzymes in *M. tuberculosis*, we initiated efforts to decipher the regulation of *rpfB* gene expression. Here, we partially characterized the *rpfB* promoter. We show that *rpfB* is regulated by SigB. In addition, chromatin immunoprecipitation (ChIP) analyses showed that MtrA associates with the *rpfB* promoter region. Electrophoretic mobility shift assays (EMSAs) and DNase I footprinting analyses with MtrA showed that MtrA binds in and around -35 regions of *rpfB*. EMSAs and *in vitro* transcription (IVT) suggested that MtrA acts as a transcriptional repressor by blocking the binding of RNA polymerase (RNAP) to the promoter region. Taking into account the fact that RpfB is required for reactivation of dormant cells, we suggest that by repressing *rpfB*, MtrA could possibly facilitate slowing down of bacterial multiplication, depending on the environmental cues sensed by sensors upstream of MtrA. This view is supported by independent observations that overexpression of MtrA prevents bacterial multiplication in macrophages (Nguyen *et al.*, 2010).

METHODS

Bacterial strains and growth conditions. *E. coli* TOP10 and DH5 α were used for cloning. *E. coli* C41BL21(DE3) or BL21(DE3) was used for protein expression. All *E. coli* strains were grown in Luria–Bertani (LB) broth and were supplemented with antibiotics [ampicillin (100 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$) or spectinomycin (50 $\mu\text{g ml}^{-1}$)] wherever required. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth containing 0.05% Tween 20 supplemented with albumin/dextrose/catalase (ADC) (Difco). For recombinant mycobacterial strains, appropriate antibiotics [kanamycin (20 $\mu\text{g ml}^{-1}$) or streptomycin (20 $\mu\text{g ml}^{-1}$)] were used. When needed, strains were plated on Middlebrook 7H10 supplemented with 10% (v/v) ADC and appropriate antibiotics. Growth was monitored by measuring OD₆₀₀.

Molecular biological procedures. Standard procedures were used for cloning and analysis of DNA, PCR, electroporation, and transformation. Primer sequences are available on request. Enzymes used to manipulate DNA were from Roche, Fermentas and New England Biolabs. DNA sequencing was performed using an automated DNA sequencer (BigDye Terminator v3.1; Applied Biosystems). All constructs made by PCR were sequenced to verify their integrity.

Cloning, expression and purification of MtrA and SigB. Full-length Rv3246c/*mtrA* of *M. tuberculosis* excluding the stop codon was amplified and cloned between the *Nde*I and *Hind*III sites of pET20b+ in-frame with a C-terminal His-tag to generate a construct

mtrA::pET20b+. The recombinant plasmid was transformed in *E. coli* C41BL21(DE3) and cells were grown in LB broth to OD₆₀₀ 0.6. IPTG was added to a final concentration of 500 μM and growth was continued at 16 °C for 20 h. Cells were harvested, resuspended and lysed in TGβ buffer (20 mM Tris/HCl, pH 8.0, 5% glycerol and 20 mM 2-mercaptoethanol) containing protease inhibitor cocktail (Roche), 2 mg lysozyme ml⁻¹, 0.2% (v/v) Triton X-100 and 5 μg DNase I ml⁻¹. Cell-free supernatant was obtained by centrifugation at 100000 g and recombinant His-tagged protein was purified using Ni²⁺-NTA agarose (Qiagen). Protein was concentrated using a protein concentrator (Pierce) and stored at -80 °C until required.

SigB_{MTB} (Rv2710) was expressed and purified according to Jacques *et al.* (2006). In brief, Rv2710 was amplified and cloned in pET30a+. The recombinant plasmid *sigB*::pET30a+ was used to transform *E. coli* BL21(DE3). Cells were grown to OD₆₀₀ 0.6 and SigB_{MTB} was induced by the addition of 500 μM IPTG at 16 °C for 20 h. Cells were lysed and SigB was purified as described above.

Identification of the transcriptional start point (TSP) of *rpfB*.

The TSP of *rpfB* was identified using a 5'/3'-RACE kit (2nd Generation; Roche), according to the manufacturer's protocol. Briefly, *M. tuberculosis* was grown to mid-exponential phase. Cells were lysed with 0.1 mm zirconia beads using a mini bead beater and RNA was purified using a RNeasy Mini kit (Qiagen). Purified RNA was treated with RNase-free Turbo DNase I (Ambion) for 30 min at 37 °C, followed by purification of RNA using a RNeasy Mini kit. First-strand cDNA was synthesized using the primer 5'-GTCCACACCTGCTTAGCGTC-3' followed by addition of a homopolymeric A-tail to the 3' end of first-strand cDNA. Finally, the dA-tailed cDNA was PCR amplified using FastStart *Taq* DNA polymerase (Roche), oligo(dT) anchor primer (supplied by the manufacturer) and the primer 5'-ACGGCTACGCCGACGACGA-3'. The PCR product was gel-purified and further amplified using PCR anchor primer (Roche) and 5'-AATGAATCCGTCGACTGAGAACCCGT-3'. Finally, the PCR product was purified, cloned and sequenced.

β-Galactosidase assays. For generation of translational fusions in pMYT131 (a generous gift from Dr Roberta Provvedi, University of Padova, Padova, Italy), a 352 bp DNA fragment starting 227 bp upstream and ending 122 bp downstream of the annotated translational start site (TSS) of *rpfB* was amplified using the primer pair 5'-ATTAAGCTTCCATGGTAGGGTGGATGCGCCAA-3' and 5'-ATTAAGCTTTTCATCGTGGTCACCCGCAT-3', and cloned into the *Hind*III site of pMYT131. The resulting plasmid contained a translational fusion with *lacZ*. Site-directed mutagenesis was carried out at the annotated TSS using appropriate mutagenic primer pairs. Each construct was separately electroporated into *M. smegmatis* and grown in Middlebrook 7H9 medium supplemented with 10% ADC, 0.05% Tween 80 and 20 μg streptomycin ml⁻¹. Bacteria were harvested and lysed using a mini bead beater. The lysate was centrifuged at 10000 g for 30 min and the supernatant was collected. β-Galactosidase activity was assayed using ONPG as substrate as described by Miller (1992).

Measurement of *rpfB* promoter activity. A DNA fragment extending up to 449 bases upstream of the TSP of *rpfB* was PCR amplified from genomic DNA of *M. tuberculosis*. The PCR products were cloned between the *Bam*HI and *Kpn*I sites of the *E. coli*-mycobacteria shuttle vector pFPV27 (Valdivia *et al.*, 1996). The resulting constructs and the control plasmid pFPV27 were electroporated separately into *M. tuberculosis*. GFP fluorescence was measured as described previously (Sanyal *et al.*, 2013).

IVT assay. IVT reactions were carried out using recombinant *M. tuberculosis* RNAP core enzyme and SigB. The recombinant *M. tuberculosis* RNAP core enzyme expressed in *E. coli* was prepared as described by Banerjee *et al.* (2014). The template was amplified by PCR using primer pair 5'-TTGCTCAACATTGGCCGGTTTCG-3' and 5'-AGTAGAGTCAACGCGCTATATCCCGC-3' followed by gel purification using a QIAquick Gel Extraction kit (Qiagen). RNAP core

(100 nM) was incubated in transcription buffer (45 mM Tris/HCl, pH 7.9, 70 mM KCl, 5 mM MgCl₂, 1.5 mM MnCl₂, 1 mM DTT and 10% glycerol) at 25 °C for 30 min. SigB (at different concentrations) was then added and again incubated at 25 °C for 30 min. Next, the PCR template (100 nM) was added and incubated at 37 °C for 20 min to form the open complex. RNA synthesis was initiated by the addition of 1 μl NTP mix [final concentration of 125 μM each of ATP, GTP, CTP; 0.4 μCi [α -³²P]UTP (20 μM) and 1 μl heparin (0.5 mg ml⁻¹)], followed by incubation for 30 min at 37 °C. The reaction was stopped by adding 5 μl loading buffer (80% formamide, 10 mM EDTA and 0.04% bromophenol blue), resolved on urea-polyacrylamide (12.5%) gels and scanned on a Typhoon Trio Plus scanner (GE Health Sciences).

Mycobacterial expression of His-MtrA_{MTB}. Rv3246c/*mtrA* was PCR amplified using *his-mtrA*::pET20b+ as a template and cloned between the *Nde*I and *Nhe*I sites of the vector pLAM12 (van Kessel & Hatfull, 2007). Control vector pLAM12 or *mtrA*::pLAM12 was electroporated separately into *M. tuberculosis*. Cells were grown to mid-exponential phase at 37 °C in Middlebrook 7H9 broth containing 0.05% Tween 80, 20 μg kanamycin ml⁻¹ and 0.2% succinate, and induced by the addition of 0.2% acetamide. Induced cultures were harvested and lysed in PBS containing protease inhibitor cocktail (Roche) in a mini bead beater. Proteins were solubilized in buffer containing 1% (v/v) Triton X-100 for 4 h at 4 °C with shaking and the lysate was immunoblotted with His antibody (Santa Cruz Biotechnology) to confirm the overexpression of MtrA.

Streptavidin pull-down assay. An aliquot of 20 μl streptavidin-conjugated beads (Sigma) was washed twice with binding buffer (20 mM Tris pH 8, 50 mM CaCl₂, 20 mM NaCl, 10 mM KCl, 5% glycerol and 0.05 μg salmon sperm DNA μl⁻¹) and mixed with 50 pmol biotinylated DNA fragment encompassing region -83 to +59 (fragment A) on a rotator for 1 h at 25 °C. Beads were washed three times with binding buffer to remove unbound DNA. *M. tuberculosis* lysate expressing His-MtrA_{MTB} (1–2 mg protein) was added to the beads. The mixture was incubated overnight on a rotator at 4 °C. The agarose beads were washed twice with binding buffer followed by denaturation in 2 × SDS-PAGE sample buffer at 95 °C for 10 min. The proteins were resolved by SDS-PAGE followed by immunoblotting with His antibody.

ChIP. ChIP was carried out using *M. tuberculosis* harbouring His-MtrA_{MTB}-expressing plasmid or the vector pLAM12 only. Briefly, cells expressing His-MtrA or harbouring empty vector were grown to mid-exponential phase. Cross-linking was carried out by the addition of formaldehyde (Sigma) to a final concentration of 1% with constant stirring at 37 °C for 10 min. The cells were immediately quenched using 250 mM glycine for 30 min at 37 °C under constant stirring. Cultures were harvested and washed twice with PBS and protease inhibitor cocktail (Roche), and stored at -80 °C. The frozen cells were thawed and reconstituted in Buffer 1 (50 mM HEPES, pH 7.5, 50 mM KCl, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate and 10% glycerol) and lysed using a Bioruptor Plus (Diagenode) with 25 cycles of 30/90 on/off, respectively, so as to generate a fragment size of ~250 bp. His antibody (Abcam) was added and incubated overnight at 4 °C on a rotator. Immunoprecipitation was done using Protein A/G agarose and incubation for 90 min at 4 °C. Finally, the beads were pelleted at 2000 g and washed twice with Buffer 2 (10 mM Tris/HCl, pH 8 and 0.1% Nonidet P-40) containing 150 mM NaCl, once with Buffer 2 containing 500 mM NaCl and finally with Tris/EDTA buffer. Elution was performed using 100 μl 50 mM Tris/HCl, pH 8, 10 mM EDTA and 1% SDS at 65 °C for 15 min. The eluate was treated with Proteinase K (Roche) overnight at 65 °C. The DNA was purified using a ChIP DNA Clean & Concentrator kit (Zymo Research) according to the manufacturer's protocol, and used for PCR using the primers 5'-TTGCTCAACATTGGCCGGTTTCG-3' and 5'-AGTAGAGTCAACGCGCTATATCCCGC-3'.

Table 1. Primers used for qRT-PCR

Primer name	Sequence 5'→3'	Description
<i>rpfB</i> RT-F	CGACGCTAAGCAGGTGTGGACGAC	Forward primer for <i>rpfB</i>
<i>rpfB</i> RT-R	CACTCAGCAGCCCCGCGACATTGG	Reverse primer for <i>rpfB</i>
16s rRNA RT-F	TCCCGGGCCTTGACACA	Forward primer for 16S rRNA
16s rRNA RT-R	CCACTGGCTTCGGGTGTTA	Reverse primer for 16S rRNA

Phosphorylation of MtrA by EnvZ kinase. A recombinant MalE–EnvZ construct (gift from Dr M. Igo, University of California, Davis, CA, USA) was transformed in *E. coli* BL21(DE3). Cells were grown in LB broth containing 0.2% glucose to OD₆₀₀ 0.6. IPTG was added to a final concentration of 100 µM and growth was continued at 16 °C for 20 h. Cells were resuspended in PBS containing protease inhibitor cocktail, disrupted by freeze/thaw cycles in the presence of 2 mg lysozyme ml⁻¹, 0.2% Triton X-100 and 5 µg DNase I ml⁻¹. The soluble MalE–EnvZ fusion protein was purified from the cell-free supernatant by amylose affinity chromatography (NEB). EnvZ kinase was phosphorylated in kinase buffer (50 mM Tris/HCl, pH 8, 50 mM KCl and 50 mM MgCl₂) containing 20 mM ATP for 15 min at 37 °C. The phosphotransfer reaction was carried out in kinase buffer with 2.5 µM phosphorylated EnvZ and MtrA.

EMSA. The binding of MtrA to the promoter of *rpfB* were analysed by EMSA. DNA fragments encompassing different regions upstream of *rpfB* were PCR amplified using appropriate primer pairs and genomic DNA of *M. tuberculosis* as template. Binding of phosphorylated or non-phosphorylated MtrA with gel-purified Cy5-labelled PCR product (10 nM) was carried out in binding buffer (20 mM Tris/HCl, pH 8, 20 mM NaCl, 50 mM CaCl₂, 10 mM MgCl₂, 10 mM KCl, 5% glycerol and 0.05 µg salmon sperm DNA µl⁻¹) for 30 min at 25 °C in a final volume of 10 µl. For competition experiments, unlabelled oligonucleotide was used in excess as indicated. Samples were separated by 6% Tris/acetic acid/EDTA native gel electrophoresis and the DNA protein complex was visualized using a Typhoon Trio Plus imager (GE Health Sciences). Where necessary, oligonucleotides were end-labelled with a DIG DNA labelling kit (Roche), annealed and used in EMSAs. DNA–protein complexes were visualized using a DIG detection kit (Roche). The binding of RNAP to the *rpfB* promoter was performed in 45 mM Tris/HCl, pH 7.9, containing 70 mM KCl, 5 mM MgCl₂, 1.5 mM MnCl₂, 50 mM CaCl₂, 1 mM DTT and 10% glycerol. Cy5-labelled DNA was incubated with RNAP holoenzyme for 30 min at 25 °C. *rpfB*–RNAP complex was then incubated with MtrA for 30 min at 25 °C. The reaction mixture was separated by PAGE at 4 °C and visualized as described.

DNase I footprinting. DNase I footprinting was performed to identify MtrA-binding sites on the *rpfB* promoter region. A 302 bp α-³²P-labelled DNA fragment was obtained by PCR with primers 5'-TGGC-TGAAGTGGTCAATCG-3' and 5'-CGGTGATTGGGTCTGATG-3'. The reaction mixture (100 µl) containing 1 nM α-³²P-labelled DNA and MtrA or MtrA-P protein was added to the binding reaction and incubated for 30 min at 25 °C. DNase I cleavage was initiated by addition of DNase I (Fermentas) followed by incubation for 2 min at 25 °C. The reaction was stopped by the addition of phenol/chloroform and extraction of DNA, followed by ethanol precipitation. The pellet was washed with chilled 80% ethanol, dried and reconstituted in 5 µl formamide loading dye. Samples were denatured at 95 °C for 5 min and separated on 8% polyacrylamide gel containing 8 M urea. The dried gel was scanned on a Typhoon Trio Plus imager.

Quantitative real-time (qRT)-PCR. cDNA was synthesized from RNA using the RevertAid Reverse Transcriptase (Fermentas) kit for first strand cDNA synthesis. Quantitative SYBR Green-based

qRT-PCR was performed with MESA GREEN MasterMix (Eurogentec), using primers shown in Table 1. The relative expression of *rpfB* was normalized to that of the endogenous reference 16S rRNA and the fold change in expression was calculated using the comparative C_t method.

RESULTS

Determination of the TSP of *rpfB*

Considering that little is known about the transcriptional control of *rpfB*, we initiated our studies by attempting to characterize the TSP of *rpfB*. RNA was isolated and subjected to 5'-RACE analysis. The product was reverse transcribed, cloned in pUC19 and several clones were sequenced. All clones analysed showed a single TSP (a G base at +1) (Fig. 1a, b).

TSS of *rpfB*

In order to determine the TSS of *rpfB*, the *rpfB* upstream region was amplified and a translational fusion between the annotated start codon and *lacZ* (lacking its own translational start codon) was generated. The resultant construct was electroporated into *M. smegmatis*. β-Galactosidase activity was measured in the resultant cell lysates. When the annotated ATG start codon was mutated to AAG, the β-galactosidase activity was abrogated (Fig. 1c). This result confirmed the annotated TSS (Fig. 1b).

A consensus SigB-binding sequence is required for *rpfB* promoter activity in *M. tuberculosis*

In order to understand the transcriptional regulation of *rpfB*, we attempted to identify putative regulatory elements upstream of the TSP. A putative SigB-binding site was identified (Fig. 1b). A construct encompassing a region starting 449 bases upstream of the TSP and ending 88 bases downstream of the TSP was cloned in the promoterless GFP vector pFPV27 (construct pFPV-*rpfB1*) (Fig. 2a). *M. tuberculosis* harbouring this construct was grown to mid-exponential phase and GFP fluorescence was monitored. A significant increase in GFP fluorescence above background levels was observed (Fig. 2b), indicating promoter activity in this region. No activity was observed in a construct lacking the identified TSP (construct pFPV-*rpfB2*) (Fig. 2b), ruling out the possibility of any additional TSPs for *rpfB*. GFP fluorescence was abrogated when the putative SigB-binding sites were deleted

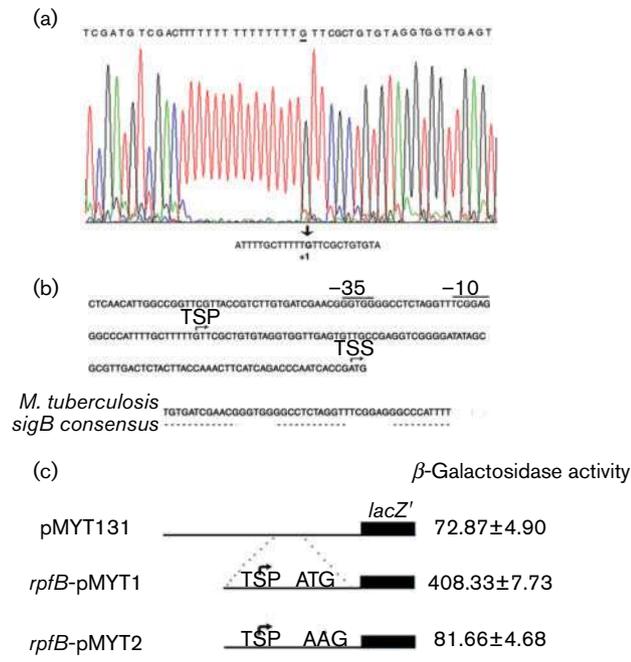


Fig. 1. Determination of the TSP and TSS in the *rpfB* promoter. (a) TSP of *rpfB* determined by 5'-RACE. The TSP is indicated by an arrow in the electropherogram. (b) A graphical representation of the TSP and TSS of *rpfB*. Bottom panel shows the presence of a consensus SigB-binding site on the promoter of *rpfB*. (c) The TSS of *rpfB*. *M. tuberculosis* harbouring pMYT131 vector alone or a construct carrying a *lacZ* fusion with *rpfB* (at annotated start site ATG or mutated start site AAG) was grown and β -galactosidase activity was measured in the cell lysates. Results represent mean \pm SD of three independent determinations.

(construct pFPV-*rpfB3*) (Fig. 2b). This result suggested that *rpfB* promoter activity was likely to be regulated by SigB.

IVT

IVT was performed in order to confirm the recognition of the *rpfB* promoter of *M. tuberculosis* by SigB. A 143 bp fragment, fragment A (from -83 to +59 relative to the TSP), was used as template for IVT. No transcript was found in an assay with RNAP core only (Fig. 3a, lane 3). A specific product was found only in the presence of SigB. The formation of the product was dependent on the concentration of SigB (Fig. 3a, lanes 5–7), but not on the presence of another sigma factor, SigJ (Fig. 3a, lane 4), confirming the specificity of the reaction. Formation of the transcript was abrogated when the template lacked the SigB-binding site (Fig. 3b, lanes 2 and 3). As a positive control, SigB-dependent transcription was confirmed using a region encompassing the *sigB* promoter (Banerjee *et al.*, 2014) (Fig. 3a, lane 2).

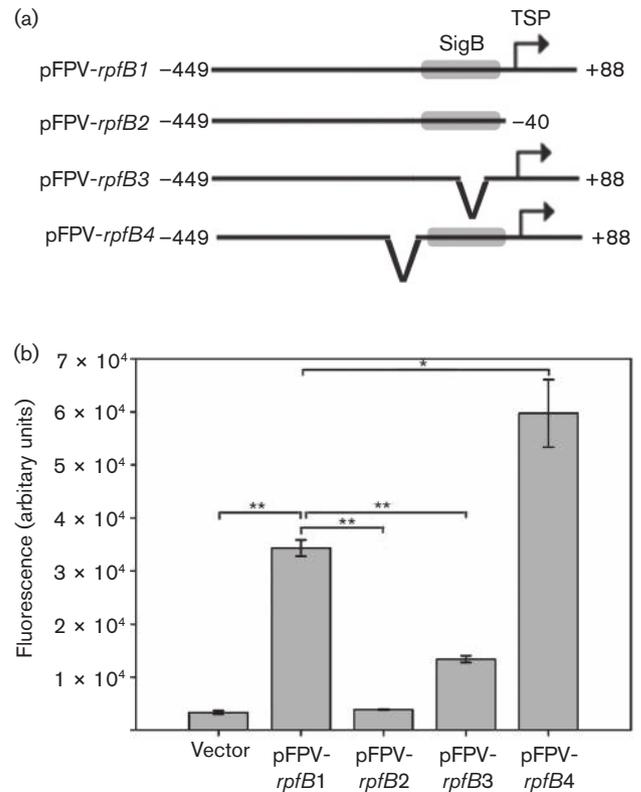


Fig. 2. Characterization of the *rpfB* promoter. (a) Schematic diagram showing the different *rpfB* promoter constructs in pFPV27. (b) GFP reporter assay with *M. tuberculosis* carrying the different promoter constructs. Cells were harvested, washed and GFP fluorescence was measured as described in Methods. Results represent mean \pm SD of three independent determinations; * P <0.05, ** P <0.005.

RpfB is an MtrA target

The MtrA–MtrB TCS has been characterized as a regulator of cell division in mycobacteria. The endopeptidase RipA that remodels the cell wall of mycobacteria during cell division is an MtrA target (Plocinska *et al.*, 2012). We hypothesized that the MtrAB TCS could regulate cell division and cell wall remodelling by targeting a group of cell wall remodelling enzymes. Based on this, we explored the possibility that *rpfB* could be part of the MtrA regulon. Possible MtrA-binding sites were located upstream of the *rpfB* TSP. His-MtrA was expressed in *M. tuberculosis* (Fig. 3c). In order to test the binding of MtrA to the *rpfB* promoter, the pull-down of His-MtrA with biotinylated fragment A was studied. The biotinylated DNA fragment was bound to streptavidin-agarose and incubated with lysates derived from *M. tuberculosis* expressing His-MtrA. Streptavidin-bound proteins were separated by SDS-PAGE and probed for the presence of MtrA by Western blotting with His antibody. MtrA was detected in the bound fraction, confirming the binding of MtrA with biotinylated oligonucleotide in a concentration-dependent manner (Fig. 3d). Pull-down of MtrA was abolished

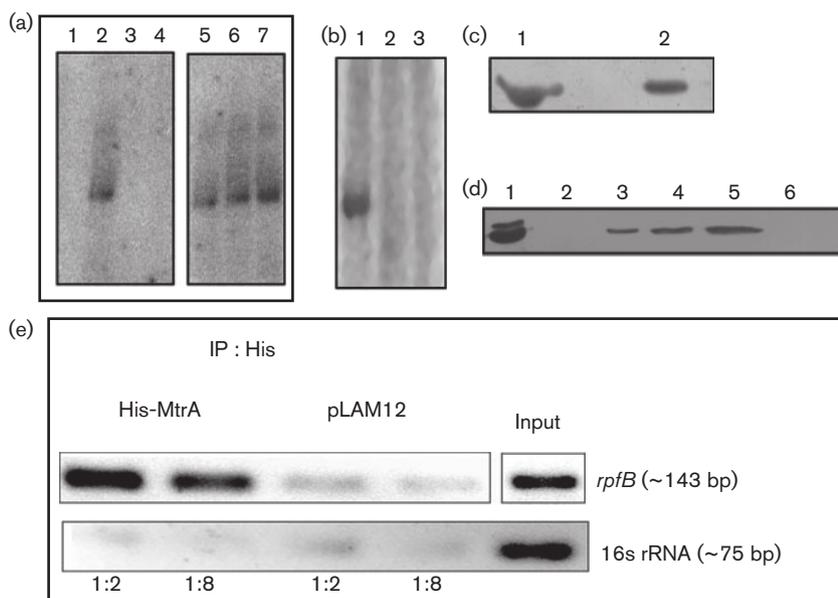


Fig. 3. Role of SigB and MtrA in *rpfB* transcription. (a) IVT was carried out as described in Methods, either in the presence (lanes 2, 5, 6 and 7) or in the absence (lanes 1 and 3) of SigB. For lanes 1 and 2, the *sigB* promoter was used as template; all other lanes had the *rpfB* promoter as template. SigJ was used as negative control (lane 4). SigB was used at concentrations of 50, 100 and 200 nM in lanes 5, 6 and 7, respectively. (b) IVT was performed using the *rpfB* promoter (lane 1) or the same promoter carrying deletions in the SigB-binding site (–34 to –40 and –17 to –23; lanes 2 and 3, respectively) as template. (c) Western blot showing MtrA overexpression in *M. tuberculosis* by immunoblotting of lysates with His antibody (lane 2). Lane 1 is a positive control, i.e. recombinant His-MtrA. (d) Association of MtrA with *rpfB* promoter fragment. Lysates (at increasing concentrations; lanes 3–5) from His-MtrA-overexpressing *M. tuberculosis* cells were incubated with a streptavidin-agarose-bound biotinylated DNA fragment derived from the *rpfB* promoter (–83 to +59), followed by immunoblotting of bead-bound proteins with His antibody. Lane 1, positive control for His-MtrA; lane 2, lysate incubated with streptavidin-agarose only; lane 6, pull-down performed with an 88 bp *rpfB* promoter region (+3 to +59) lacking the MtrA-binding site. (e) ChIP analysis of MtrA binding to the *rpfB* promoter. *M. tuberculosis* harbouring His-MtrA or vector alone (pLAM12) was subjected to ChIP with His antibody (IP: His). Immunoprecipitated DNA was amplified by PCR using primers specific for the *rpfB* promoter or for 16S rRNA. PCR products were diluted as indicated and separated by agarose gel electrophoresis.

when the biotinylated DNA lacked the putative MtrA-binding site (Fig. 3d, lane 6).

MtrA binds to the *rpfB* promoter *in vivo*

The *in vivo* association of MtrA with the *rpfB* promoter was probed by ChIP. ChIP experiments were performed from cells expressing His-MtrA using His antibody. We amplified the region –83 to +59. The results showed enrichment of this region when immunoprecipitated with His antibody (Fig. 3e). No enrichment occurred when immunoprecipitation was performed in cells carrying the vector pLAM12 alone. This result indicated that the *rpfB* promoter was a target of MtrA.

MtrA binds to the *rpfB* promoter

In order to further characterize the binding of MtrA to the *rpfB* promoter region, fragment A encompassing putative MtrA-binding sites (indicated in Fig. 4) was amplified

with a Cy5-labelled oligonucleotide at one end. EMSA was performed using recombinant His-MtrA. Binding of MtrA to this DNA fragment was visualized after PAGE (Fig. 4a). The binding of MtrA to the Cy5-labelled oligonucleotide could be competed using a non-labelled oligonucleotide, confirming the specificity of the interaction (Fig. 4b). MtrA was phosphorylated using the EnvZ kinase of *E. coli*, as reported previously (Rajagopalan *et al.*, 2010). Phosphorylation slightly increased the binding of MtrA to the *rpfB* promoter-derived DNA. The binding was abrogated in a construct encompassing the region +4 to +59 (Fig. 4c). Based on these results, we argue that MtrA was a likely regulator of *rpfB* transcription.

DNase I footprinting shows MtrA binding to the *rpfB* promoter

MtrA-binding sites on the *rpfB* promoter were further identified by DNase I footprinting experiments. MtrA

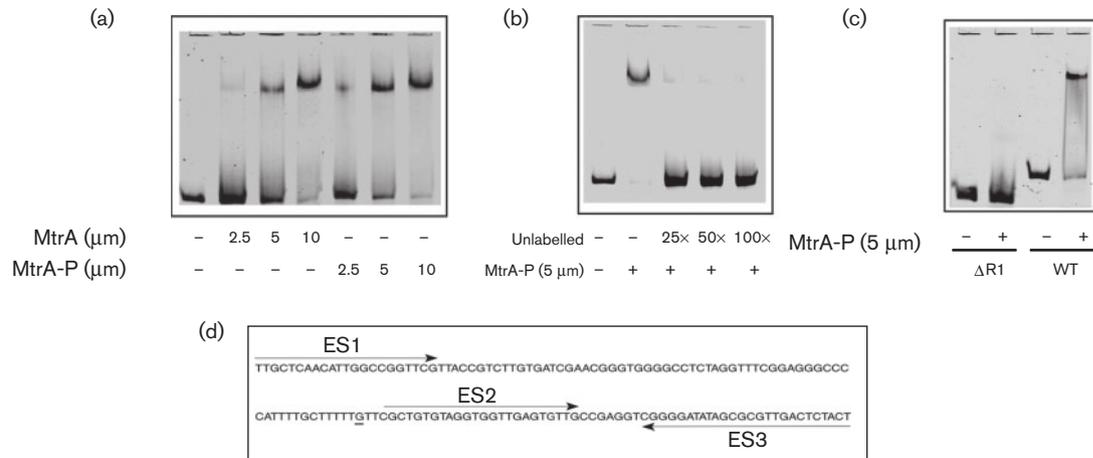


Fig. 4. Binding of MtrA to the *rfpB* promoter analysed by EMSA. (a) EMSA was performed with Cy5-labelled DNA (WT; generated using primers ES1 and Cy5-labelled ES3 shown in d) and either non-phosphorylated or phosphorylated MtrA at different concentrations. (b) Binding of Cy5-labelled WT DNA with MtrA-P analysed in the presence of increasing concentrations of unlabelled WT DNA. (c) EMSA using Cy5-labelled WT DNA or ΔR1 (generated using primers ES2 and ES3) DNA. (d) Schematic representation of different primers for the generation of DNA fragments for EMSA.

was added to binding reactions performed with the *rfpB* promoter-derived DNA fragment. The positions of the footprints (Fig. 5a, b) confirmed the results obtained in EMSAs. Upon examination of the protected region, a loosely conserved repeat motif was observed (Fig. 5c), which could represent a likely MtrA-binding motif, based

on the consensus logo identified by Plocinska *et al.*, (2012). In order to confirm whether this repeat sequence constituted the MtrA-binding site, EMSA was performed with a DIG-labelled DNA encompassing this region (WT) as well as another mutated oligonucleotide, in which the bases in a stretch of one repeat were replaced with A

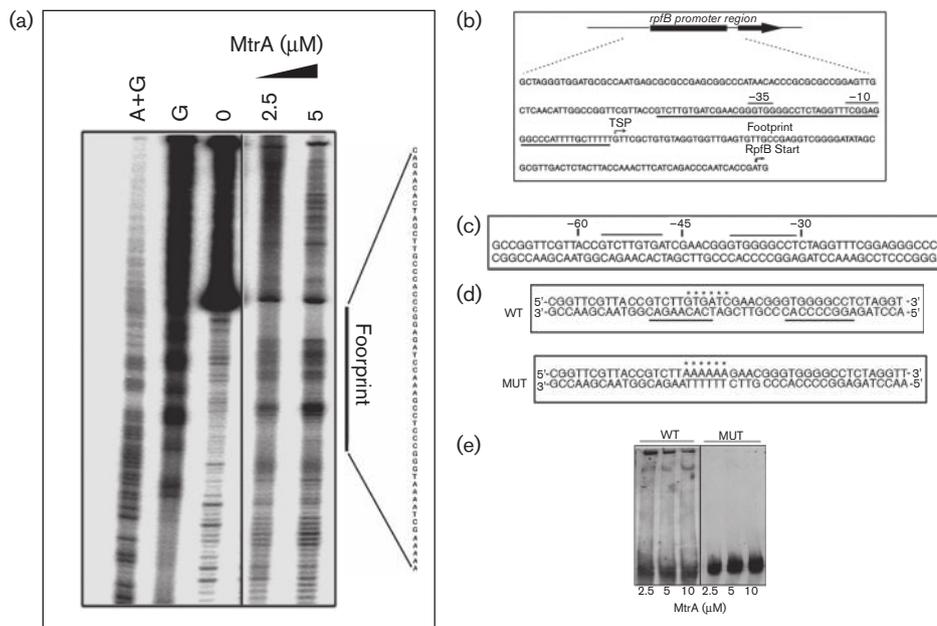


Fig. 5. DNA footprint analysis in the upstream region of *rfpB*. (a) Purified His-tagged MtrA (at different concentrations) and ³²P-labelled DNA (labelled in the antisense strand) were used for DNase I footprint analysis as described in Methods. (b) Region of the footprint. (c) A putative MtrA-binding imperfect repeat. (d) Oligonucleotides [WT or mutated (MUT)] used for EMSAs after DIG labelling. (e) Binding of DIG-labelled oligo (either WT or MUT) with MtrA at different concentrations was analysed by EMSA.

residues (Fig. 5d). MtrA bound to the WT oligonucleotide in a concentration-dependent manner (Fig. 5e), but could not bind to the mutated oligonucleotide, strengthening the view that the motif identified represented the MtrA-binding site. The role of specific nucleotides in this motif and the spacer length requirement are under investigation.

MtrA inhibits RNAP binding at the *rpfB* promoter

Promoter activity assays showed that *rpfB* promoter-driven GFP fluorescence was enhanced when a portion of the direct repeat identified as the likely MtrA-binding site was deleted (pFPV-*rpfB4*) (Fig. 2b). We then explored the possibility that MtrA functioned as a repressor by inhibiting the binding of RNAP to the *rpfB* promoter. To investigate whether MtrA could displace RNAP from the *rpfB* promoter, the RNAP–promoter complex was first allowed to form. MtrA was then added to the reaction mixture. The RNAP–DNA complex was visible on EMSA. Its intensity decreased in the presence of MtrA (Fig. 6a), suggesting that MtrA displaced RNAP from the *rpfB* promoter, thereby inhibiting its transcription. The MtrA–DNA complex migrated slower than the RNAP–DNA complex. This was not unexpected as phosphorylated MtrA has been reported to form oligomers (Al Zayer *et al.*, 2011). MtrA could also inhibit IVT of *rpfB* (Fig. 6b), further supporting the notion that it repressed *rpfB* transcription.

MtrA overexpression represses the expression of *rpfB* in vivo

As MtrA is essential, there is no knockout system available to study MtrA function in *M. tuberculosis*. As an alternate strategy, we overexpressed His-MtrA in *M. tuberculosis* and analysed the expression of *rpfB*. *rpfB* expression was

significantly repressed when MtrA was overexpressed (Fig. 6c). This strengthened the view that MtrA acted as a repressor of *rpfB* transcription. Overproduction of MtrA inhibited growth of *M. tuberculosis* *in vivo* (Fol *et al.*, 2006). It is possible that downregulation of *rpfB* could have also contributed to this growth inhibition.

DISCUSSION

Considering that *M. tuberculosis* can switch between a dormant state and an actively growing state, it is important to understand (i) the signals that govern the switching between the two states, (ii) the sensors that receive these signals, (iii) the response regulators to which these signals are transmitted and (iv) the regulation of the signalling cascades leading to expression of the appropriate regulons. RpfB, a peptidoglycan transglycosylase, and RipA, an endopeptidase, both localize to the septum of actively growing cells to remodel the mycobacterial peptidoglycan. RpfB has been suggested to be necessary for reactivation of *M. tuberculosis* from dormancy. The transcriptional regulation of peptidoglycan remodelling enzymes is poorly understood in mycobacteria. The regulation of *rpfA* by the cAMP receptor protein Rv3676 is the only report so far on the regulation of expression of *rpf* family members in the pathogen *M. tuberculosis* (Rickman *et al.*, 2005). Here, we provide insights into regulation of *rpfB* of *M. tuberculosis*. We identify SigB and MtrA as two regulators of *rpfB* expression. 5'-RACE of the *rpfB* region showed a single TSP. A signature sequence suggestive of a SigB-binding site was identified. Sigma A is the primary sigma factor of *M. tuberculosis*. Sigma B is a primary-like sigma subunit (Doukhan *et al.*, 1995) which shares a high degree of sequence similarity with sigma A. It is activated under various conditions of stress, and is positively regulated

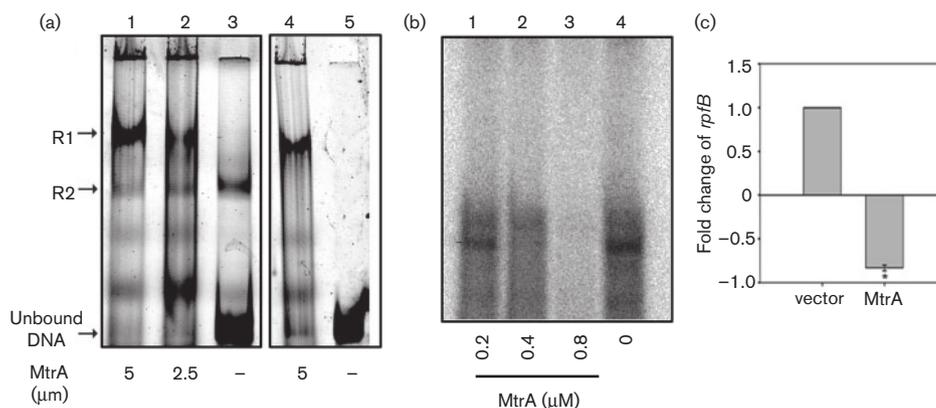


Fig. 6. Role of MtrA in *rpfB* transcription. (a) EMSA of RNAP binding to the *rpfB* promoter was carried out in the absence (lane 3) or presence (lane 1 and 2) of MtrA as described in Methods. Lane 4 shows binding with MtrA alone and lane 5 contains none of the proteins. R1 and R2 indicate complexes of MtrA and RNAP with DNA, respectively. (b) IVT was carried out in the absence (lane 4) or presence (lanes 1–3) of different concentrations of MtrA. (c) qRT-PCR was performed with RNA isolated from *M. tuberculosis* harbouring pLAM12 (vector) or an MtrA overexpression plasmid (MtrA) to check the expression of *rpfB*. Results represent mean \pm SD, $n=3$; * $P=0.0001$.

by the extracytoplasmic function sigma factors SigE, SigH and SigL (Fontan *et al.*, 2009; Lee *et al.*, 2008). The role of SigB in *rpfB* transcription is suggested from the following: (i) a consensus SigB-binding site was identified in the *rpfB* promoter, (ii) *rpfB* promoter-driven GFP expression was tuned down upon deleting the consensus SigB-binding site and (iii) *in vitro* transcription assays were positive in the presence of SigB. Recent studies have shown that *ripA* is part of the MtrA regulon. We have tested the possibility that other peptidoglycan hydrolases such as *rpfB* could also be part of the MtrA regulon. An immobilized biotinylated DNA fragment (derived from the *rpfB* promoter) harbouring a putative MtrA-binding site could pull-down MtrA from lysates of *M. tuberculosis*. ChIP confirmed that MtrA associates with the *rpfB* promoter region *in vivo*. Using DNA footprinting and EMSAs, we identified a MtrA-binding site close to the RNAP-binding site on the *rpfB* promoter. Deletion of a part of the MtrA-binding site on the *rpfB* promoter enhanced promoter-driven GFP fluorescence, suggesting a likely repressive function of MtrA in regulating *rpfB* transcription.

The proximity of the MtrA-binding site to the TSP and the SigB-binding site suggested that MtrA could repress *rpfB* expression by displacing RNAP from the promoter. The repressive function of MtrA was tested in EMSAs of RNAP binding to the *rpfB* promoter in the presence of MtrA. EMSAs suggested that MtrA displaces RNAP from the *rpfB* promoter. Brocker *et al.* (2011) showed that binding of MtrA near the -10 region of the promoter prevents the binding of RNAP to these promoters, leading to transcriptional repression. Our findings with respect to MtrA binding to the *rpfB* promoter confirm these results. The inhibitory role of MtrA with respect to *rpfB* expression was further supported by the observation that overexpression of MtrA repressed *rpfB* expression. Previous ChIP experiments confirmed that MtrA associates with the *ripA* promoter (Plocinska *et al.*, 2012). However, it is currently unclear how MtrA regulates *ripA* expression. To the best of our knowledge, our studies suggest for the first time that MtrA represses *rpfB* in the pathogen *M. tuberculosis*, providing new insight into how an essential TCS could regulate cell wall homeostasis by regulating a peptidoglycan transglycosylase. It is tempting to speculate that MtrA can function both as an activator as well as a repressor. This contention is strengthened by existing reports indicating that MtrA of *Corynebacterium glutamicum* functions as a repressor of *mepA* and *nlpC*, but as an activator of *betP* and *proP* (Brocker & Bott, 2006). The YycFG TCS of *Bacillus subtilis* is a likely counterpart of MtrAB of *M. tuberculosis*. It regulates cell wall metabolism (Dubrac & Msadek, 2008; Dubrac *et al.*, 2008; Fukushima *et al.*, 2008, 2011). In a manner similar to MtrA of *C. glutamicum*, it activates *yvcE*, *lytE* and *ydjM*, but represses expression of *yoeB* and *yjeA* (Bisicchia *et al.*, 2007). It is possible that in *M. tuberculosis*, the location of the MtrA-binding site could serve as a determinant of its activating or repressive function – a fact that holds true for transcriptional regulators in general.

An understanding of the complete MtrA regulon would facilitate further analyses of the diverse roles of MtrA. In *M. smegmatis*, deletion of *mtrB* is associated with altered cell shape (Plocinska *et al.*, 2012). *C. glutamicum* Δ *mtrAB* cells show irregular septum formation and are elongated (Brocker *et al.*, 2011; Möker *et al.*, 2004). Deletion of the MtrB-interacting partner *lpqB* in *M. smegmatis* affects cell–cell aggregation and biofilm formation (Nguyen *et al.*, 2010). Overproduction of MtrA inhibits growth of *M. tuberculosis* *in vivo* (Fol *et al.*, 2006). All these reports suggest that MtrAB coordinates cell wall homeostasis. A likely role in cell wall metabolism is strengthened by the current report demonstrating that MtrA regulates expression of the peptidoglycan hydrolase RpfB. Drawing from the similarities between YycFG and MtrAB as regulators of cell wall metabolism, it would be interesting to investigate the relationship between the MtrA regulon and cell wall stress, exemplified by exposure to drugs such as vancomycin. The precise role of individual resuscitation-promoting factors in resuscitation from dormancy remains to be elucidated. Outstanding questions in the field pertain to the chemical entities on which the resuscitation-promoting factors act, i.e. their precise enzymic activities, the affinities of each of the resuscitation-promoting factors for their substrates, their spatiotemporal expression profiles, the consequences of enzymic activation of each resuscitation-promoting factor and their genetic regulation. The insights into regulation of *rpfB* are therefore relevant in our quest to take the field forward.

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