Transcriptional regulation of *Mycobacterium tuberculosis* *hupB* gene expression

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The influence of iron levels on the transcription of the *hupB* gene in *Mycobacterium tuberculosis* is the focus of this study. Studies in our laboratory showed HupB to be co-expressed with the two siderophores in low-iron organisms. Mycobactin biosynthesis is repressed by the IdeR–Fe$^{2+}$ complex that binds the IdeR box in the *mbtB* promoter. Recently, we demonstrated the positive regulatory effect of HupB on mycobactin biosynthesis by demonstrating its binding to a 10 bp HupB box in the *mbtB* promoter. Earlier, we observed that HupB, expressed maximally in low-iron media (0.02 μg Fe ml$^{-1}$; 0.36 μM Fe) was still detectable at 8 μg Fe ml$^{-1}$ (144 μM Fe) when the siderophores were absent and complete repression was seen only at 12 μg Fe ml$^{-1}$ (216 μM Fe). In this study, we observed elevated levels of *hupB* transcripts in iron-limited organisms. IdeR, and not FurA, functioned as the iron regulator, by binding to two IdeR boxes in the *hupB* promoter. Interestingly, the 10 bp HupB box, first reported in the *mbtB* promoter, was identified in the *hupB* promoter. Using DNA footprinting and electrophoretic mobility shift assays, we demonstrated the functionality of the HupB box and the two IdeR boxes. The high *hupB* transcript levels expressed by the organism and the *in vitro* protein–DNA interaction studies led us to hypothesize the sequence of events occurring in response to changes in the intracellular iron concentration, emphasizing the roles played by IdeR and HupB in iron homeostasis.

**INTRODUCTION**

Tuberculosis is a disease of great public concern, with 8.7 million incident cases and 1.4 million deaths in 2011 (WHO, 2012). *Mycobacterium tuberculosis* (Mtb), the causative organism, has successfully adapted to the intracellular environment of the macrophage and has altered its metabolism to survive inside the human host. The latter, by virtue of both innate and acquired immunity, attempts to contain and eliminate the pathogen. One of the components of innate immunity is the withholding of nutrients, including the essential micronutrient iron. Iron, an obligate cofactor for at least 40 different enzymes encoded in the Mtb genome (Cole et al., 1998), plays key roles in electron transport, the tricarboxylic acid cycle, and the biosynthesis of amino acids and pyrimidines. The mammalian host limits free iron by diverting it into transferrin, lactoferrin and ferritin; this, coupled with the poor solubility of ferric iron at biological pH, deprives an invading pathogen of iron. Therefore, adaptation to iron-limiting conditions and elaboration of the siderophore-mediated iron acquisition machinery contribute to pathogen virulence.

Mycobactin and carboxymycobactin, Fe(III)-specific high-affinity siderophores expressed by iron-deprived mycobacteria, including Mtb, can acquire iron from host iron-withholding proteins and insoluble hydroxides of iron. Major advances have been made in the understanding of iron acquisition in this class of bacteria (Quadri & Ratledge, 2005; Ratledge & Dover, 2000; Sritharan, 2006), and important milestones include deciphering the biosynthetic pathway of mycobactin (Quadri et al., 1998) and demonstrating its importance for the survival of the pathogen *in vivo* (De Voss et al., 2000). Our recent findings on the potentiating effect of HupB, a 28 kDa iron-regulated protein of *M. tuberculosis*, on mycobactin biosynthesis has contributed further to the understanding of regulation of expression of these iron-chelating molecules (Pandey et al., 2014).

HupB (Rv2986c), also known as MDP1 and LBP, was first reported as a DNA-binding protein in Mtb (Prabhakar et al., 1998). Its DNA-binding ability, conferred by the positively charged lysine residues at its C-terminal end, was highlighted by several researchers (Kumar et al., 2010; Sharadamma et al., 2011). HupB is implicated in other roles, including adhesion of *Mycobacterium leprae* (Soares de Lima et al., 2005) and mycobacterial cell wall synthesis (Katsube et al., 2007). We first reported HupB as an iron-regulated protein expressed by iron-limited Mtb (Yeruva et al., 2006), and recently we generated a *hupB* knock-out strain of Mtb and unambiguously proved the role of HupB as a positive regulator of mycobactin biosynthesis (Pandey et al., 2012).

Abbreviations: EMSA, electrophoretic mobility shift assay; Mtb, *Mycobacterium tuberculosis*; q, quantitative; RT, real-time.
et al., 2014); the study also reflected the essential role of HupB in vivo as the mutant failed to survive inside macrophages. HupB can bind iron (Takatsuka et al., 2011) and its ability to hold about 81 iron [Fe(III)] atoms led the authors to propose an iron storage role for the protein. High titres of anti-HupB antibodies were found in the serum of tuberculosis patients (Sivakolundu et al., 2013), reflecting the importance of HupB in the growth and survival of Mtb (Kumar et al., 2011; Yeruva et al., 2006); the levels were statistically significant when compared with endemic normal subjects and household contacts (P<0.05). The observed negative correlation of anti-HupB antibodies with serum iron status in patients with tuberculosis was indicative of the iron-limited environment of the pathogen inside the human host. The objective of the present study was to investigate how iron influenced the transcription of hupB and, we demonstrated IdeR-dependent repression of hupB and, more interestingly, identified the 10 bp HupB box, first identified in the promoter (Pandey et al., 2014) were subjected to multiple sequence alignment using CLUSTALW.

METHODS

**Bacterial strains.** Mtb (H37Rv), hupB mutant Mtb (MtbΔhupB) and hupB-complemented MtbΔhupB/pMS101 strains were used. *Escherichia coli* strains DH5α and BL21(DE3) used for the expression of recombinant proteins were grown in LB medium. Kanamycin, ampicillin, chloramphenicol, tetracycline and arabinose (HiMedia) were used as required.

**Growth of mycobacterial strains in high- and low-iron media and assay of the siderophores.** The growth of mycobacterial strains in high (8.0 μg Fe ml⁻¹) and low (0.02 μg Fe ml⁻¹) iron media and the assay of the siderophores were as described in our previous study (Pandey et al., 2014).

**Quantitative real-time (qRT)-PCR: transcript levels of hupB and mbtB.** This was done essentially as described in our previous study (Pandey et al., 2014). In brief, the cDNA obtained by converting 1 μg total RNA isolated from high- and low-iron organisms using the SuperScript III First-Strand Synthesis System was used for qRT-PCR (Invitrogen). It was diluted 1:10 and three reactions were set up with 4 μl cDNA containing 5 pmol hupB primers (forward: 5’-AAGCTGTCACAGCCCAAGAAG-3’/reverse: 5’-CTTCGGCGGCTGCGTCTTCTTG-3’), mbtB primers (forward: 5’-TGACGCTTGTCGCGCTTGCC-3’/reverse: 5’-AAGCCCAAGCTGACGCTACC-3’) and 16S rRNA primers (internal control; forward: 5’-GGTACTCCAGGAGGTGATT-3’). Upon addition of 5 μl 2 × SYBR Green (Applied Biosystems), RT-PCR was performed using the ABI 7500 Fast Sequence Detection System (Applied Biosystems) with the following program: 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The difference in gene expression, expressed as fold change, was calculated using the 2⁻^ΔΔCt method with 16S rRNA as the internal control.

**Bioinformatic analysis.** Based on the 19 bp consensus sequence of the IdeR box (5’-TAGGTTAGGCTACCTA-3’), the regions between +20 to +320 bp from the start of several published iron-regulated genes (Gold et al., 2001; Pandey et al., 2014; Prakash et al., 2005) were subjected to multiple sequence alignment using CLUSTALW.

Cloning, expression and purification of the recombinant rIdeR, rFurA and rHupB. PCR of the respective genes was performed in a volume of 20 μl as per routine procedures. Taq DNA polymerase (0.5 U; Fermentas), 25 pmol of the respective primers with NdeI and HindIII restriction sites (ideR, forward: 5’-GGGAATTCCTACATGGAGGATGTTCGGACCCAGCTC-3’/reverse: 5’-CCGCGGCGCCACATATGGACCCACATATG-3’; furA, forward: 5’-GGGAATTCCTACATGGACCCACATATG-3’; hupB, forward: 5’-GGGAATTCCTACATGGACCCACATATG-3’) and 100 μg Mtb genomic DNA were used, and PCR was performed in a Mastercycler (Eppendorf) using the following programme: an initial denaturation for 5 min at 95 °C, 30 cycles of annealing and extension for 3 min at 68 °C, and a final extension of 5 min at 72 °C. The ideR and hupB amplicons were cloned into pET-28a (+) vector, transformed into *E. coli* DH5α and selected using kanamycin (30 μg ml⁻¹); furA was cloned into pET-14b and selected on ampicillin (60 μg ml⁻¹) plates. The recombinants were confirmed by sequencing (Eurofins MWG Operon/Xclusir Genomics).

Expression of all the three genes was determined after transforming the respective plasmids into the expression host, *E. coli* BL21(DE3). Both IdeR and FurA were obtained as soluble proteins by adding 1 mM IPTG to actively growing cultures (OD₆₀₀ 0.6) and incubating for 3 h. Soluble rHupB was co-expressed with chaperone proteins from the chaperone plasmid pGKJE8 (TakaRa Bio) cloned into *E. coli* BL21(DE3) by adding kanamycin (30 μg ml⁻¹), chloramphenicol (20 μg ml⁻¹), tetracycline (5 μg ml⁻¹) and arabinose (0.5 mg ml⁻¹), and 1 mM IPTG to actively growing cultures, and incubating for 3 h at 37 °C. All three recombinant proteins were purified by subjecting the whole-cell sonicate to affinity chromatography on a Ni-NTA His-Bind column (Novagen). The purity of the recombinant protein was verified by separation using 10% SDS-PAGE (Laemmli, 1970).

**IdeR/FurA/HupB interactions with hupB promoter DNA: electrophoretic mobility shift assays (EMSAs)**

Binding of IdeR/FurA to hupB promoter DNA. The binding of IdeR to hupB promoter DNA was demonstrated by EMSA according to Gold et al. (2001) with minor modifications. The 340 bp hupB promoter DNA, encompassing −235 to +105 bp with respect to the start point of hupB, was PCR-amplified (forward: 5’-CATATGGCGACGACAATCCG-3’/reverse: 5’-AACCTTGCGACTCTACCGCC-3’) as described above. The 362 bp mbtB promoter DNA (−280 to +38) was amplified with forward: 5’-CGGCGGATCTCCTGCCGACCGC-3’/reverse: 5’-CCGCGGCTGTCGCGGTAC-3’) and the 318 bp sigA promoter DNA (−280 to +38; amplified with forward: 5’-GGTACTCCAGGAGGTGATT-3’/reverse: 5’-GGGCTATCGCGTCCGCGTC-3’) were used as positive and negative controls, respectively. The promoter DNA fragments were end-labelled with T4 polynucleotide kinase and [γ-32P]ATP (Board of Radiation and Isotope Technology, India). The reaction mixture, in a total volume of 20 μl, contained 20 mM Tris/ HCl (pH 8.0), 50 mM KCl, 1 mM DTT, 5 mM MgCl₂, 10% glycerol, 0.05 mg BSA ml⁻¹ and 0.1–0.5 ng labelled DNA fragment along with 1 μM purified IdeR/FurA. The metal ions Ni²⁺, Co²⁺, Zn²⁺, Fe²⁺ and Cu²⁺ were added as indicated in the Results. The mixture was incubated for 30 min at room temperature and the reaction products were resolved on a 4% polyacrylamide gel in TAE buffer (40 mM Tris/acetate, pH 8.0 with 1 mM EDTA) at 110 V for 2 h at room temperature and vacuum-dried. The products were visualized by a Storage Phosphor Imaging Workstation (Typhoon Trio+ Variable Mode Imager; GE Healthcare).

In assays performed with FurA, the furA promoter (amplified with forward: 5’-CGGCGGCTGTCGCGGTAC-3’/reverse: 5’-CCGCG-
AGCTTTCGCGGCTAGTC-3' primers) was used as the positive control.

**Binding of IdeR to IdeR box 1 and 2 in hupB promoter DNA.** A sample of 1 μg 340 bp hupB promoter DNA was digested with 1 μl SpeI (10 U μl⁻¹; MBI Fermentas) in a 20 μl reaction and incubated at 37 °C overnight. The products were separated on 1% agarose gel (Sigma-Aldrich) by electrophoresis in TAE buffer. The 192 and 148 bp SpeI-digested products, purified using a gel elution kit (Qiagen), were subjected to EMSA under the conditions described above.

The locations of the two IdeR boxes, confirmed by DNase I footprinting analysis (detailed below), was confirmed by EMSA using short chemically synthesized fragments bearing IdeR box 1 (nt 97–136; forward: 5'-AGGCAACGACGTTTTCCGCAAACCGGGTGCGGCCGCTTG-3'/reverse: 5'-CAAGGGCGACCCGATGGGGGGA-AAAACCGGTGCTTTGGCT-3') and IdeR box 2 (nt 127–260; forward: 5'-GTGCAATGACCTCTGTTGTTCATCGCCACACCTCCGAAA-CCAG-3'/reverse: 5'-CTGGTTTCGAGGTGGTGGATGAAACAGCCAGAGCTCATTGACG-3') sequences, respectively, using the protocol above described.

**Binding of HupB to HupB promoter DNA.** Between 1 and 2 μM purified HupB was added to 32P-labelled 340 bp hupB promoter DNA and EMSA performed as described above. As was done for the two IdeR boxes, a 40 bp chemically synthesized short DNA fragment containing the HupB box (nt 128–167; forward: 5'-TGATTTCCAAAGGCCCAGGCAATTTCACGTGAGCCAAAA-3'/reverse: 5'-TCGTTGCTACGTCGAAATCGGGGTGTGCTTGGAAATCA-3') was subjected to EMSA.

**DNA footprinting analysis: mapping the location of IdeR box 1 and 2 and the HupB box in hupB promoter DNA.** Two hupB promoter DNA fragments of sizes 189 and 171 bp, spanning the 340 bp region described above, were PCR-amplified as follows. The 189 bp fragment (with IdeR box 1 and the HupB box) was amplified using forward: 5'-ACACGGTTAACACTCCACGG-3'/reverse: (HindIII) 5'-AACGGTTAACACTCCACGG-3' primers and the 171 bp fragment containing IdeR box 2 was amplified with forward: (NdeI) 5'-CATATGCGCACGCAATTTCACGTGAGCCAAA-3' and reverse: 5'-CGGGTAAAGGTGGTATCCAGTG-3' primers. The two purified fragments were labelled with [γ- 32P]ATP using T4 polynucleotide kinase.

The DNA footprinting assay was performed according to Gold et al. (2001). This was done using the 340 bp hupB promoter DNA and the two PCR-amplified 189 and 171 bp products, respectively. The single-stranded forward and reverse strands with the radiolabel were generated by digestion of the dsDNA with NdeI and HindIII, respectively. About ~80 000 c.p.m. of the respective strand of labelled DNA was taken and 1–2 μM purified IdeR/HupB added in a reaction mixture prepared as for EMSA. CaCl₂ and MgCl₂ were added to give final concentrations of 2.5 and 5 mM respectively. DNase I (2 μg; Sigma-Aldrich) was added, and after 1 min the reaction was terminated by adding 90 μl stop solution containing 200 mM NaCl, 20 mM EDTA, 1% SDS and 100 μg yeast tRNA ml⁻¹. The DNA was extracted with phenol/chloroform (1:1 v/v), precipitated with ethanol and resuspended in 8 μl formamide dye mix. The samples were heated for 5 min before loading onto a 6% TBE (Tris/borate-EDTA) polyacrylamide-urea (8 M) sequencing gel and subjected to electrophoresis for 3 h at 1500 V (BROVIGA Sequencing Gel Electrophoresis Apparatus; Balaji Scientific Services). The gel was vacuum dried at 80 °C for 1 h, exposed to Storage Phosphor Image Plates overnight and scanned. The regions protected by HupB/IdeR in the two DNA samples were identified by performing Sanger dideoxy sequencing with Sequenase Version 2.0 (PCR Product Sequencing Kit; USB).

**RESULTS**

**hupB and mbtB transcript levels in iron-limited WT Mtb, MtbΔhupB mutant and hupB-complemented MtbΔhupB/pMS101 strains**

A threefold increase in the level of hupB transcripts (Fig. 1) was observed in WT Mtb subjected to growth in low-iron medium (0.02 μg Fe ml⁻¹) compared with high-iron (8 μg Fe ml⁻¹) organisms. In the hupB mutant strain of Mtb, there was a complete absence of hupB transcripts (Fig. 1). HupB, shown to potentiate the expression of mycobactin and carboxymycobactin (Pandey et al., 2014), was not expressed by the mutant strain, whose absence resulted in a decrease in mbtB transcript levels (Fig. 1). This observation was substantiated by a marked decrease in the levels of both mycobactin and carboxymycobactin in the low-iron mutant strain. The hupB-complemented MtbΔhupB/pMS101 showed not only hupB transcripts, but responded to iron limitation by the production of the siderophores, the latter in agreement with the elevated mbtB transcripts.

**Presence of two putative IdeR boxes in hupB promoter DNA: in silico analysis**

The promoter regions of several published iron-regulated genes in Mtb (Gold et al., 2001; Rodriguez et al., 1999) showed mismatches of ≤5 bp from the 19 bp consensus IdeR box (Table 1). We extended the level of mismatch to >5 bp to identify putative IdeR boxes in the hupB promoter as the expression of HupB was regulated by iron levels (Yeruva et al., 2006) and some genes, including mbtM (Table 1), contained IdeR boxes with mismatches of >5 bp. Interestingly, we found two IdeR boxes, one at ~127 bp upstream of the start point of hupB and the second at ~5 bp, located close to the translational start point (Table 1), referred to as IdeR box 1 and 2, respectively.

**Ider binds the hupB promoter strongly in the presence of iron**

The expression and purity of recombinant rIdeR, rFurA and rHupB proteins used in these assays is shown in Fig. 2. IdeR bound the 340 bp hupB promoter DNA fragment (−235/+105) in the presence of Ni²⁺ (Fig. 3a). The intensity of the bound probe was relatively lower than that seen with the mbtB promoter (positive control) and, as anticipated, negligible label was seen with the sigA negative control. The specificity of the IdeR interaction with hupB promoter DNA was evident by the displacement of the radiolabelled probe upon addition of excess unlabelled hupB promoter DNA (Fig. 3b).

As Ni²⁺ has been conventionally used in IdeR–DNA binding interactions (Dussuret et al., 1999), it was included in the above reactions. However, the lower intensity of the bound probe led us to test other metal ions. Whilst similar observations were made with Co²⁺ and Zn²⁺ (Fig. 3c, lanes 3 and 7), there was a dramatic increase in the intensity of the
bound probe upon addition of Fe$^{2+}$ in the reaction mixture (Fig. 3c, lane 4); further, the bound probe migrated more slowly. Cu$^{2+}$ performed poorly (Fig. 3c, lane 5) and, as studies in our laboratory showed that Cu$^{2+}$ influenced the iron acquisition machinery (unpublished data), we studied its influence on the binding of iron to IdeR by adding both Cu$^{2+}$ and Fe$^{2+}$ in the same reaction mixture. No unbound probe was detected, but the amount of the bound probe was marginally lower than that seen with iron alone.

The failure to detect any bound probe with the mycobacterial iron regulator FurA (Fig. 3d) clearly proved that the latter played no role in hupB expression.

**IdeR binds both IdeR box 1 and 2 in hupB promoter DNA**

Taking advantage of the presence of a SpeI site in the 340 bp hupB promoter DNA, the latter was digested with SpeI to generate two fragments of sizes 192 and 148 bp containing IdeR box 1 and 2, respectively. IdeR, in the presence of iron, bound each of the above fragments, establishing the functionality of the two IdeR boxes (Fig. 4a, lanes 3 and 6, respectively), with low levels of bound probe seen with Zn$^{2+}$ (Fig. 4a, lanes 2 and 5, respectively). Interestingly, when the two SpeI-digested DNA products were added to the same reaction mixture containing IdeR and iron, a single band in the region of the bound probe was observed (Fig. 4b, lane 7); the latter, of a lower intensity, was also seen with Zn$^{2+}$ (Fig. 4b, lane 2) as the added metal ion.

**Presence of the HupB box in the hupB promoter**

Our recent study (Pandey et al., 2014) identified a 10 bp HupB-binding motif (5’-CACTAAAATT-3’) in the promoter region of both mbtB and hupB genes. The former was verified experimentally by DNA footprint analysis and the latter, predicted using bioinformatic tools, was located 99 bp upstream of the start point of hupB. Using DNA footprinting and EMSA (detailed below), we demonstrated the binding of HupB to the hupB promoter and identified the HupB box as 5’-CAGTGAAATT-3’, showing a mismatch of 2 bp with that present in mbtB promoter DNA.
### Table 1. Identification of putative IdeR boxes in Mtb*

Consensus IdeR box: 5’-TTAGGTTAGGCTAACCTAA-3’.

<table>
<thead>
<tr>
<th>Mismatch†</th>
<th>Rv no.</th>
<th>Putative IdeR box‡</th>
<th>Location§</th>
<th>Gene/predicted function¶</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤5 bp</td>
<td>2384</td>
<td>TTAGCAGGCTGCCCATAA</td>
<td>−85</td>
<td>mbtA/salicyloyl-AMP ligase</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>2383c</td>
<td>TTAGGCCAGCTTGGCTAA**</td>
<td>−32</td>
<td>mbtB/phenyloxazolone synthase</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>2386c</td>
<td>GTAGGTTAGGCTAACCTTTA**</td>
<td>−25</td>
<td>mbtI/isochorismate synthase</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>1347</td>
<td>TATGTCATGCTAAGCTAA</td>
<td>−50</td>
<td>mbtK/malonyl-coA carboxylase</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>1876</td>
<td>TTAGGCTAGCTGCTGCTAA**</td>
<td>−249</td>
<td>bfrA/iron storage box1</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>1876</td>
<td>TTAGGGCATGCTAAGCTAA</td>
<td>−225</td>
<td>bfrA/iron storage box2</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>3841</td>
<td>CTAGGAAAGCCTTCCCTGA**</td>
<td>−72</td>
<td>bfrB/ferritin, iron storage</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>1348</td>
<td>TTAGGTAGGCTAAGCTAAT**</td>
<td>−212</td>
<td>irxA/iron regulated transporter</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>2122c</td>
<td>CTAGGCTAGCTAAGCTAAT**</td>
<td>−95</td>
<td>hisE/histidine biosynthesis</td>
<td>Rodriguez et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>2123</td>
<td>ATAGGCTAGCTAAGCTAAT**</td>
<td>−50</td>
<td>ppc37/unknown</td>
<td>Rodriguez et al. (1999)</td>
</tr>
<tr>
<td>6</td>
<td>1345</td>
<td>CAAGGGTACCACTGCTAA</td>
<td>−291</td>
<td>mbtM/fatty acyl-AMP ligase</td>
<td>Gold et al. (2001)</td>
</tr>
<tr>
<td>9</td>
<td>2986c††</td>
<td>TTGGGATGAAACAAGCAGA</td>
<td>−5</td>
<td>hupB/iron-regulated protein</td>
<td>This study</td>
</tr>
<tr>
<td>12</td>
<td>2986c††</td>
<td>CCGGATGAAAACCCCGG</td>
<td>−127</td>
<td>hupB/iron-regulated protein</td>
<td>This study</td>
</tr>
<tr>
<td>15</td>
<td>1404</td>
<td>CTAGGTTAGGCTAAGCTAAT**</td>
<td>−78</td>
<td>Transcriptional regulator</td>
<td>Prakash et al. (2005)</td>
</tr>
<tr>
<td>8</td>
<td>2663</td>
<td>TTAGGCTAGGCTAAGCTAAT**</td>
<td>−34</td>
<td>Hypothetical protein</td>
<td>Prakash et al. (2005)</td>
</tr>
</tbody>
</table>

*Identification of putative IdeR boxes on the promoter region of the genes by multiple sequence alignment using CLUSTALW (as described in Methods).
†Mismatch from the consensus sequence.
‡Putative IdeR boxes obtained by multiple sequence alignment.
§Location of the 5’ end of the IdeR box from the predicted start site of the ORF.
¶The gene names are their predicted functions as described by Mycobacterial Browser (Mycobrowser, Tuberculist Version 2.6; http://tuberculist.epfl.ch/), except that in most of them, the terms ‘probable’ or ‘possible’ is are removed here.
**IdeR boxes have been validated experimentally.
††hupB (Rv2986c), which is reported in this study, contains two IdeR boxes.
‡‡Indicates novel IdeR-binding sites predicted previously based on the positional relative entropy method (Prakash et al., 2005).

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**Fig. 2.** Purity of rIdeR/rFurA/rHupB proteins. The three recombinant proteins rIdeR, rFurA and rHupB were purified as detailed in Methods. The respective Ni²⁺-affinity column-purified protein migrated as a single band (indicated by arrows) in SDS-PAGE (lane 1) and reacted with anti-His antibody (lane 2); M, molecular mass marker.
Confirmation of IdeR and HupB boxes in the *hupB* promoter: DNA footprinting and EMSA

The location of the two IdeR boxes and the HupB box (represented diagrammatically in Fig. 5a) in *hupB* promoter DNA was confirmed by DNA footprinting analysis. The two IdeR boxes were identified in the 340 bp *hupB* promoter DNA (Fig. 5b) and in the 189 (Fig. 5c) and 171 bp (Fig. 5d) PCR-generated fragments, and their sequences were determined as 5′-CCGGTTTTTCGCAATCCGG-3′ (IdeR box 1) and 5′-GATGAACAAAGCAGAG-3′ (IdeR box 2), respectively. The binding of IdeR to these two regions was further confirmed by EMSA using chemically synthesized oligonucleotides bearing these boxes. Fig. 6 shows the binding of IdeR to IdeR box 1 in the 40 bp DNA (Fig. 6, lanes 2 and 3; it may be noted that HupB did not bind this probe, as seen in lanes 4 and 5). IdeR box 2 in the 44 bp DNA (Fig. 6, lanes 7 and 8).

Fig. 5(e) shows the HupB box (5′-CAGTGAAATT-3′) in the 189 bp *hupB* promoter DNA identified by DNA footprinting analysis, with further confirmation provided...
DISCUSSION

HupB, co-expressed with mycobactin and carboxymycobactin in Mtb, is influenced by the iron present in the growth medium as a steady lowering of iron resulted in a gradual increase in HupB, evident by protein expression and immunoblotting studies using anti-HupB antibodies (Yeruva et al., 2006). Our recent studies with the hupB knock-out mutant of Mtb explained the co-ordinate regulation of HupB and the two siderophores (Pandey et al., 2014). We identified and demonstrated a functional ‘HupB box’ in mbtB promoter DNA, the binding of which by HupB promoted the transcription of the mbtB gene. Thus, mycobactin biosynthesis, switched off by the IdeR–Fe{}^{2+} complex under iron-sufficient conditions, was upregulated by HupB under low-iron conditions. The failure of the mutant to survive inside macrophages was due to its inability to express sufficient mycobactin and carboxymycobactin, proven to be essential by De Voss et al. (2000). Complementation of the mutant with the hupB gene restored mycobactin production that clearly offered a survival advantage to the pathogen.

by EMSA (Fig. 6). HupB reacted strongly with the 40 bp DNA (Fig. 6, lanes 10 and 11) containing nt 128–167.

Fig. 4. Functionality of the two IdeR boxes in the hupB promoter. SpeI digestion of the 340 bp hupB promoter DNA was done as detailed in Methods. (a) Lanes 1, 2 and 3 represent the 148 bp product (IdeR box 2), and lanes 4, 5 and 6 represent the 192 bp product (IdeR box 1). In lanes 3 and 6, the presence of the bound probe(s) indicates that iron is required for binding that was reduced markedly in the presence of Zn{}^{2+}, almost identical to lanes 1 and 4, which carried only the probe(s). (b) The inclusion of both 148 and 192 bp fragments in the same reaction mix resulted in the formation of a single species of the bound probe in the presence of iron (lane 4) – an observation also seen in the presence of both Fe{}^{2+} and Cu{}^{2+} (lane 5). The bound probe was detected at low levels in the presence of Zn{}^{2+} (lane 2). The full-length 340 bp fragment was included as control (lanes 6, 7 and 8), with lane 7 showing the bound probe in the presence of both IdeR and iron.
by iron, IdeR and HupB itself on the expression of this protein.

The higher levels of *hupB* transcripts in low-iron *Mtb* seen in this study again confirmed the iron-regulated expression of HupB. Iron functions as a regulatory molecule by acting in association with either IdeR or FurA (Quadri & Ratledge, 2005). Here, we showed that that iron functioned as a regulatory molecule by associating with IdeR and not FurA. IdeR, in the presence of iron, bound the two IdeR boxes in the *hupB* promoter, with no binding seen with FurA. IdeR, the first mycobacterial iron regulator reported (Schmitt et al., 1995) and structurally characterized (Wisedchaisri et al., 2004), functions as a regulatory molecule, controlling the expression of the *mbt* biosynthetic machinery and the iron storage genes *bfrA* and *bfrB* (Gold et al., 2001). With the exception of the iron storage genes (Gold et al., 2001), IdeR functions predominantly as a repressor of several iron-regulated genes, by binding as a IdeR–Fe^{2+} complex to a 19 bp ‘iron/IdeR box’ located in the promoter region close to the transcriptional start point of these genes, thereby blocking their transcription by RNA polymerase. A search of the *Mtb* genome for such IdeR boxes led to the identification of >50 IdeR-regulated genes (Gold et al., 2001; Prakash et al., 2005) that, however, did not include the *hupB* gene. The iron-regulated expression of HupB by axenically grown *Mtb* (Yeruva et al., 2006) led us to search the *hupB* promoter region for a putative IdeR box with due allowance for higher deviations from the consensus sequence. The two IdeR boxes, with a higher degree of mismatch than anticipated, proved to be functional as shown experimentally by EMSA and DNA footprinting. Such low-homology IdeR boxes, seen in the promoter regions of some genes (e.g. Rv0983) encoding the *htrA* homologue (Manabe et al., 1999), were shown to bind IdeR. The presence of the weak IdeR boxes in the *hupB* promoter possibly allows low levels of expression of HupB, as seen at 8 μg Fe ml\(^{-1}\) (discussed below), and it

![Fig. 5. Sequence and location of the two IdeR boxes and HupB box in the *hupB* promoter by footprinting assay. (a) Schematic diagram of the 340 bp *hupB* promoter DNA used as template for the PCR amplification of the 189 and 171 bp products with IdeR box 1 and 2, respectively. (b–d) DNA footprinting analysis was done with the 340 bp *hupB* promoter DNA (b; CTAG represents the sequence ladder generated by Sanger’s dideoxy method), 189 bp PCR amplicon (c) and 171 bp PCR amplicon (d) by incubating with IdeR. (e) The HupB box present in the 189 bp DNA. IdeR/HupB were added as indicated in the respective lanes followed by DNase I digestion, as detailed in Methods.](image-url)
thus highly likely that two such IdeR boxes in the hupB promoter DNA are possibly needed to shut down the expression of hupB when iron is plentiful in the growth medium, as seen at 12 μg Fe ml⁻¹. The pathogen possibly ensures that siderophore production is initiated immediately upon sensing a fall in iron levels (discussed below).

IdeR bound both IdeR box 1 and 2 in the presence of Fe(II), and showed low specificity with other divalent metal ions. The formation of a single DNA band upon addition of IdeR to a mixture of the above two DNA fragments led us to propose that IdeR interacted with both the strands in such a manner as to form a single species of DNA. In the uncut 340 bp hupB promoter DNA, such an interaction would bend the DNA, resulting in a looped conformation that would prevent RNA polymerase from initiating transcription – a process clearly indicative of the repressor role of IdeR under iron-sufficient conditions (diagrammatically represented in Fig. 7). This hypothesis is made on the presumption that there must be an interaction between the two IdeR–Fe²⁺ complexes – a feature seen in many regulatory circuits (e.g. the ara operon in E. coli). When the intracellular iron concentration is ≥200 μM, IdeR exists as IdeR–Fe²⁺ complex (Pandey et al., 2014), occupies both IdeR boxes and completely switches off the synthesis of HupB. When iron levels fall, we envisage an identical pattern of events occurring as seen in the mbtB promoter DNA, where HupB binds the HupB box (5′-CACTAAAATT-3′) to initiate transcription of mbtB (Pandey et al., 2014). Here (Fig. 7), HupB binds the hupB promoter at the HupB box (5′-CAGTGAAATT-3′), whose sequence is highly homologous to that present in the mbtB promoter. The

Fig. 6. Confirmation of IdeR/HupB boxes. EMSA with short chemically synthesized DNA. Purified IdeR protein was added to the 40 bp IdeR box 1-containing DNA as indicated, with lane 1 representing only the probe. Purified HupB did not bind this DNA (lanes 4 and 5). IdeR bound the 44 bp IdeR box 2-containing DNA (lanes 7 and 8, with lane 6 representing only the probe). Purified HupB bound the 40 bp HupB box-containing DNA (lanes 10 and 11, with lane 9 representing the unbound probe alone).

Fig. 7. Regulation of expression of HupB: a hypothetical model. (a) Organization of hupB promoter DNA with the locations of IdeR box 1 and 2 and the HupB box; TSP, translational start point. (b) IdeR-mediated repression of hupB when iron is present in sufficient amounts (≥200 μM). Two IdeR–Fe²⁺ complexes bind at the respective IdeR box 1 and 2, and we propose the bending of the DNA renders the start point inaccessible to the RNA polymerase (RNAP). (c) When iron levels fall to 144 μM (8 μg Fe ml⁻¹), and the IdeR–Fe²⁺ complex can no longer form and occupy the IdeR boxes, RNA polymerase transcribes hupB and the expressed protein, in the presence of available iron (HupB can bind even 25 μM iron), binds to the HupB box in the hupB promoter, thereby autoregulating its own expression.
concentration of iron determines if IdeR or HupB will occupy the promoter region. As mentioned above, IdeR requires at least 200 μM iron to form the IdeR–Fe$^{2+}$ complex. When iron levels drop, HupB comes into play and binds the HupB box located 99 bp upstream of the hupB start point. It must be noted that this interaction requires the presence of iron. Whilst it may be argued that HupB is expressed only under iron-limiting conditions, it must be pointed out that HupB expression (Yeruva et al., 2006), maximal in low-iron medium (0.02 μg Fe ml$^{-1}$; 0.36 μM), is detectable even at 8 μg Fe ml$^{-1}$ (144 μM iron), with complete repression occurring only at 12 μg Fe ml$^{-1}$ (216 μM iron). Thus, when iron levels drop, the available HupB binds to the hupB promoter (as depicted in Fig. 7c) and promotes its own transcription. HupB can bind iron (Takatsuka et al., 2011) and our previous study showed it required markedly low levels of iron (<25 μM; Pandey et al., 2014), which led us to propose that the HupB–iron complex and not the desferri form of the protein binds the promoter DNA. This occurs immediately upon sensing a drop in iron levels and HupB serves to upregulate its own synthesis—a process that must occur early enough to synthesize sufficient HupB needed for potentiating the biosynthesis of mycobactin, necessary for the survival of the pathogen. Thus, a shift in iron levels is sufficient for the pathogen to switch on or off the HupB biosynthetic machinery, which in turn regulates the mbt biosynthetic machinery. In this context, it may be pointed out that HupB was detected with 8 μg Fe ml$^{-1}$ added to the medium, even before the siderophores could be detected. Therefore, the onset of iron limitation is sufficient to trigger the synthesis of HupB.

The response of the pathogen to iron levels must be considered under the conditions prevailing within the human host. Although in vitro studies tend to simulate conditions of strict iron sufficiency and iron deficiency, this does not occur in vivo and the levels of iron oscillate within a narrow range, at the ends of which either IdeR or HupB come into play. Sritharan & Ratledge (1990), in their analysis of iron-regulated proteins from in vivo derived mycobacteria, considered that mycobacteria in vivo were continuously ‘on the cusp of iron availability’ – a phrase that clearly explains our proposed hypothesis. The essentiality of both HupB (Pandey et al., 2014) and mycobactin (De Voss et al., 2000) reflects their importance in iron acquisition that contributes to the virulence of the pathogen. In conclusion, this study provides further insights into the adaptation of the pathogen to varying levels of iron, with specific reference to hupB gene expression.

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REFERENCES


