EspR, a regulator of the ESX-1 secretion system in *Mycobacterium tuberculosis*, is directly regulated by the two-component systems MprAB and PhoPR

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The regulatory mechanisms that control the ESX-1 secretion system, a key player in the pathogenesis of *Mycobacterium tuberculosis*, have not been fully elucidated. However, factors that regulate the ESX-1 substrate EspA usually affect ESX-1 function. Previous studies showed that espA is directly regulated by the nucleoid-associated protein EspR and the two-component system (TCS) MprAB. The PhoPR TCS also activates espA, but the direct target of PhoP was unknown. In this report, we reveal that EspR is directly regulated by MprA and PhoP-Rv, but not by PhoP-Ra. PhoP-Rv and MprA binding sites in the espR promoter were determined by gel-shift and DNase I footprinting assays, which identified a PhoP-protected region centred approximately 205 bp before the espR start codon and that encompasses MprA Region-1, one of two MprA-protected regions. MprA Region-2 is located approximately 60 bp downstream of MprA Region-1 and overlaps a known EspR binding site. Nucleotides essential for the binding of PhoP and/or MprA were identified through site-directed DNA mutagenesis. Our studies also indicate that MprA Region-2, but not MprA Region-1/PhoP region, is required for the full expression of espR. Recombinant strains carrying mutations at MprA Region-2 exhibited lower transcription levels for espR, espA and espD, and had reduced EspR and EspA levels in cell lysates. These findings indicate that EspR may mediate the regulatory effect of PhoPR and MprAB, and provide more insight into the mechanisms underlying ESX-1 control.

INTRODUCTION

ESX-1 (ESAT-6 secretion system-1) is a major virulence determinant of *Mycobacterium tuberculosis*, the causative agent of human tuberculosis. ESX-1 exports pathogenic and immunogenic proteins such as ESAT-6 (EsxA), EspA and other substrate proteins into host cells (Simeone et al., 2009). The secretion of EspA and ESAT-6 is mutually dependent, with deletion of espA resulting in loss of ESAT-6 secretion, and vice versa (Fortune et al., 2005). EspA is encoded within the espA operon, while esxA is located in RD-1 (Region of difference 1), which contains most ESX-1 genes (Mahairas et al., 1996). The intergenic region upstream of espA contains known or predicted binding sites for several different regulatory factors (Hunt et al., 2012; Pang et al., 2013; Rickman et al., 2005; Rosenberg et al., 2011), suggesting that ESX-1 activity could be modulated via altering espA expression.

EspR, a key transcriptional regulator of ESX-1, directly regulates espA (Raghavan et al., 2008; Rosenberg et al., 2011). An espR mutant exhibited decreased transcription of the espA operon, loss of ESAT-6 secretion and reduced virulence (Raghavan et al., 2008). Higher-order oligomerization of EspR appears to be involved in cooperatively linking distant DNA sites, such as the three EspR binding sites in the espA promoter (Blasco et al., 2011; Rosenberg et al., 2011). Three EspR sites were also identified in the espR promoter (Blasco et al., 2012). However, these EspR sites are much closer than in the espA promoter, suggesting that EspR may have a

Abbreviations: CRP, cAMP receptor protein; EMSA, electrophoresis mobility shift assay; ESX-1, ESAT-6 secretion system-1; L6, lineage 6; RD-1, Region of difference 1; TCS, two-component system.

One supplementary table is available with the online Supplementary Material.
different mode of action at its own promoter. Our previous work indicated that these EspR sites are not critical for espR expression (Cao et al., 2013).

Like EspR, the PhoPR two-component system (TCS) also activates the espA operon. Deletion of phoPR resulted in decreased expression of espA, blocked secretion of ESAT-6 and attenuated virulence (Frigui et al., 2008; Pang et al., 2013; Ryndak et al., 2008; Walters et al., 2006). PhoP recognizes a sequence consisting of two or three direct repeats, which have been identified in its own promoter and several others (Cimino et al., 2012; Goyal et al., 2011; Gupta et al., 2006, 2009; Pathak et al., 2010). A point mutation in S219A in the DNA-binding motif of PhoP in M. tuberculosis strain H37Ra decreases the binding affinity of PhoP for the phoP promoter (Lee et al., 2008), and results in the attenuation of this strain (Frigui et al., 2008; Lee et al., 2008). There is no evidence, however, that PhoP can interact directly with the espA promoter, suggesting that the regulation of espA by PhoP is mediated by (an) unknown factor(s).

MprA, the response regulator of the MprAB TCS, also directly regulates espA (Pang et al., 2013). MprA recognizes a conserved repeated hexamer motif (MprA box) (He & Zahrt, 2005; He et al., 2006; Pang et al., 2007, 2011; Pang & Howard, 2007), and multiple MprA boxes were identified in the espA–ephA intergenic region, to which MprA bound with varying affinities (Pang et al., 2013). Secretion of the ESX-1 substrates EspA, ESAT-6 and EspB was blocked in an mprAB mutant strain (Pang et al., 2013), indicating that MprAB modulates ESX-1 activity. Expression of espA was also altered in a mutant of the regulatory factor cAMP receptor protein (CRP), and a putative CRP binding site was identified upstream of espA (Rickman et al., 2005). Additionally, the nucleoid-associated protein Lsr2 binds to the ESX-1 genes esxA, esxB and espA (Gordon et al., 2010). However, its effect on ESX-1 activity is not yet known.

So far, EspR is the most well-defined regulator of the ESX-1 system (Raghavan et al., 2008; Rosenberg et al., 2011). Here, we report that espR is itself the direct target of the response regulators PhoP and MprA. Our data suggest that EspR mediates the regulation of the ESX-1 system by PhoPR and MprAB, further highlighting the importance of EspR in the pathogenesis of M. tuberculosis.

METHODS

Bacterial strains, plasmids and culture conditions. All strains and plasmids used in this study are listed in Table 1. M. tuberculosis strains were grown at 37 °C under regular atmospheric CO2 in either Middlebrook 7H9 broth containing 0.05% Tween 80 or Middlebrook 7H10 agar medium (Difco), both enriched with 10% oleic acid–albumin–glucose–catalase (Difco), with or without addition of antibiotic. Broth cultures were incubated with shaking (50 r.p.m.). Escherichia coli strains Novablue and RosettaBL21(DE3)pLySS (Novagen) were used for general cloning and protein expression, respectively.

RNA isolation, reverse transcription and real-time PCR. Procedures were conducted essentially as described (Pang et al., 2007). Briefly, M. tuberculosis strains were grown to mid-exponential phase, and total RNA was extracted by bead-beating, followed by further purification and DNase I treatment. Following reverse transcription with random hexamer primers and M-MLV reverse transcriptase, real-time PCR assays were performed with gene-specific primers as described by Zhang et al. (2014), using a Roche LightCycler480 thermal cycler. Relative quantities of cDNA were normalized for 16S rRNA. Sequences of primers used in this study are listed in Table S1 (available in the online Supplementary Material).

Protein extraction and Western blotting. M. tuberculosis strains were grown in 7H9 broth to an OD600 of 0.3–0.4, centrifuged, washed and disrupted by bead-beating. Cell debris was removed by centrifugation, and lysate supernatants were collected for analysis. Protein concentration was determined by the bicinchoninic acid assay (Pierce). Lysate proteins were separated on a NuPAGE 4–12% gradient polyacrylamide gel (Invitrogen) under denaturing and reducing conditions. Immunoblotting was performed using the Western ECL detection system (Amersham), with antibodies to EspR (ab43676; Abcam), ESAT-6 (HYB 076-08; Santa Cruz Biotechnology), EspA (kindly provided by Dr S. Fortune) and GroEL2 (NR-13655; BEI Resources).

Construction of plasmids for espR complementation. To complement espR, we used pXP-1, which contains the coding sequence of Rv3849 (399 bp) and the entire upstream intergenic region (264 bp) in pMV306-Kan (Cao et al., 2013). BOX1-1 mutations in MprA region 1 were generated by overlap–extension PCR, using the mutated primers BOX1-1-Reverse and BOX1-1-Forward in the primer pairs EspRcom-Forward/BOX1-1-Reverse and BOX1-1-Forward/EspRcom-Reverse. Two overlapping PCR fragments were generated, gel-purified and mixed in equimolar amounts, and were then used as templates in a PCR with primers EspRcom-Forward/EspRcom-Reverse. The final PCR product was ligated into pMD-18T to generate pCG-8. The insert from pCG-8 was then cloned into pMV306-Kan, which had been linearized using KpnI and HindIII, to generate pXP-8. Similarly, primer pairs EspRcom-Forward/BOX1-2-Reverse and BOX1-2-Forward/EspRcom-Reverse, EspRcom-Forward/BOX1-3-Reverse and BOX1-3-Forward/EspRcom-Reverse, and EspRcom-Forward/BOX2/3-M-Reverse and BOX2/3-M-Forward/EspRcom-Reverse were used, respectively, to generate pXP-9, pXP-10 and pXP-11. All constructs were verified by sequencing.

Plasmids pXP8 to pXP11 were electroproporated into a ΔespR mutant strain (Raghavan et al., 2008), and transformants were selected followed by plating on 7H10 agar containing kanamycin (25 μg ml−1), as previously described (Pang et al., 2007). Transformants were confirmed by PCR analysis and were designated C8 (pXP-8, BOX1-1), C9 (pXP-9, BOX1-2), C10 (pXP-10, BOX1-3) and C11 (pXP-11, BOX2/3-M).

Expression and purification of PhoP-Rv and PhoP-Ra. To express phoP in E. coli, the coding sequences of phoP-Rv (Rv0757) and phoP-Ra were amplified using the primers PhoP-Express-Forward and PhoP-Express-Reverse, using the genomic DNA of M. tuberculosis strains H37Rv and H37Ra as templates, respectively, and the PCR products were cloned into pMD-18T (Takara). Following sequencing verification, phoP inserts were excised by Ndel and XhoI digestion, gel-purified and ligated into Ndel/XhoI-cut pET15b (Invitrogen), an expression vector containing an N-terminal His tag and thrombin site, to generate pET75Rv or pET75Ra. These expression plasmids were used to transform E. coli RosettaBL21(DE3)pLySS (Novagen). Expression of phoP was induced by the addition of IPTG (1.5 mM) with incubation for 4 h at 28 °C. Bacteria were collected by centrifugation at 4 °C and were sonicated in binding buffer (50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 20 mM imidazole). Lysates were centrifuged for 10 min at 13 000 r.p.m., and tagged PhoP was recovered from the supernatant using Ni-NTA resin (Bio Basic). The resin was washed.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description or characteristic(s)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>M. tuberculosis strains</strong></td>
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<tr>
<td>Erdman</td>
<td>Wild-type virulent strain</td>
<td>Raghavan et al. (2008)</td>
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<tr>
<td>ΔespR mutant</td>
<td>Erdman mutant strain with deletion of Rv3849</td>
<td>Raghavan et al. (2008)</td>
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<td>C1(WT)</td>
<td>ΔespR mutant complemented by pXP-1</td>
<td>Cao et al. (2013)</td>
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<td>C9(BOX1-2)</td>
<td>ΔespR mutant complemented by pXP-9</td>
<td>This work</td>
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<tr>
<td>C10(BOX1-3)</td>
<td>ΔespR mutant complemented by pXP-10</td>
<td>This work</td>
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<tr>
<td>C11(BOX2/3 M)</td>
<td>ΔespR mutant complemented by pXP-11</td>
<td>This work</td>
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<tr>
<td>C12</td>
<td>ΔespR mutant complemented by pXP-12</td>
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<td><strong>Plasmids</strong></td>
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<td>Mycobacterial integrative plasmid with kanamycin resistance</td>
<td>Stover et al. (1991)</td>
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<td>Gene expression vector</td>
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<tr>
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<td>pET-15b with the coding sequence of phoP-Rv</td>
<td>This work</td>
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<tr>
<td>pET757-Ra</td>
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<tr>
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<td>mprA expression plasmid</td>
<td>Pang et al. (2007)</td>
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<td>pMD18-T with the espR coding sequence and 265 bp upstream intergenic sequence</td>
<td>Cao et al. (2013)</td>
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<td>pCG-1 with BOXI-1 mutation</td>
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<td>pGL-1 with BOXI-2 mutation</td>
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<td>pGL-1 with BOXI-3 mutation</td>
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<td>pGL-1 with BOX2/3-M mutation</td>
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<td>pXP-12</td>
<td>pMV306K with the insert of pCG-12</td>
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Twice with washing buffer [50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 40 mM imidazole] before the tagged protein was eluted with elution buffer [50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 200 mM imidazole]. PhoP-Rv and PhoP-Ra proteins were then dialysed in dialysis cassettes [10 000 Da molecular weight cut-off (MWCO); Thermo Scientific] against a buffer composed of 50 mM Tris/HCl (pH 8.0), 50 mM NaCl and 5% glycerol, then concentrated using a centrifugal filter (10 000 Da MWCO; Millipore). Protein concentration was determined by performing a bicinchoninic acid assay (Pierce). MprA was expressed and purified essentially as previously described (Pang et al., 2007).

Electrophoresis mobility shift assays (EMSA)s. DNA Probes 1–4, containing espR promoter sequences, were generated by PCR by pairing an espR promoter forward primer, F-1, F-2, F-3 or F-4, with the reverse primer R-1 (see Fig. 2 below). DNA probes containing the wild-type or mutated espR promoter sequences were amplified, respectively, from pXP-8 to pXP-11, by PCR using the primer pair EspR-Promoter-clone Forward/Reverse (see Fig. 6 below). For short probes containing putative MprA boxes, complementary oligonucleotides were annealed. Probes were end-labelled with [γ-32P]ATP and incubated with MprA or PhoP, with or without specific or non-specific cold (unlabelled) probes. Reaction mixtures were analysed on non-denaturing polyacrylamide gels, and bands were detected by autoradiography. Sequences of primers used in this study are listed in Table S1.

DNase I footprinting assay. DNase I footprinting assays were performed essentially as described by Wang et al. (2012). For preparation of the sense-strand probe for the espR promoter, the region upstream of espR was amplified by PCR with primers TemER-Forward and TemER-Reverse, and the 250 bp amplicon was cloned into the pMD-18T vector. The resulting plasmid was used as the template to generate probes labelled with the fluorescent dye 6-carboxyfluorescein (FAM), using primers M13-FAM and TemER-Reverse. For the antisense-strand probe, the espR promoter was amplified with primers TemER-F and TemER-R, cloned into pUC18B-T (Shanghai Biotechnology), and then the antisense-strand probe was amplified with primers M13F-FAM and Rv-M. The FAM-labelled probes were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and quantified using a NanoDrop 2000C spectrophotometer (Thermo Scientific). For each assay, 250 ng of probe was incubated with different amounts of PhoP-Rv or MprA in a total volume of 40 μl in buffer containing 0.1 μg poly(dI-dC) μl⁻¹ (Amersham Pharmacia Biotech), 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 0.5 mM DTT and 4% Ficoll. After incubation for 30 min at 25 °C, a 10 μl solution containing 0.015 units DNase I (Promega) and 100 nmol freshly prepared CaCl₂ was added, followed by further incubation for 1 min at 25 °C. The reaction was stopped by adding 140 μl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% SDS). Samples were extracted with phenol/chloroform and precipitated with ethanol, and then DNA pellets were dissolved in 30 μl MiniQ water. Preparation of the DNA ladder, electrophoresis and data analysis were essentially as described by Wang et al. (2012), except that the Genescan-LIZ500 size standard (Applied Biosystems) was used. The MprA-protected regions upstream of espR were mapped in a similar way.
RESULTS

PhoP-Rv and PhoP-Ra have different affinities for the \textit{phoP} promoter

PhoPR is critical for the pathogenesis of \textit{M. tuberculosis} H37Rv, partly because PhoP modulates ESX-1 activity via regulation of the \textit{espA} operon (Frigui \textit{et al.}, 2008; Pang \textit{et al.}, 2013). One of the primary causes of attenuation for \textit{M. tuberculosis} H37Ra is a point mutation in \textit{phoP} (Frigui \textit{et al.}, 2008; Lee \textit{et al.}, 2008). The resulting amino acid change from serine (in PhoP-Rv) to leucine (in PhoP-Ra) in the DNA-binding domain reduced the ability of PhoP to bind short oligonucleotides containing the PhoP binding site from the \textit{phoP} promoter (Chesne-Seck \textit{et al.}, 2008; Lee \textit{et al.}, 2008). To investigate whether this mutation affects the affinity of PhoP for the whole \textit{phoP} promoter, we performed EMSAs with these two forms of PhoP. When as little as 0.5 \textmu g of protein was used (Fig. 1a, lane 3), PhoP-Rv bound the 181 bp probe encompassing the entire region upstream of \textit{phoP}. A total shift of the probe was observed using 1 \textmu g PhoP-Rv (Fig. 1a, lane 4), consistent with a previous report (Gonzalo-Asensio \textit{et al.}, 2008). However, no shift was detected with PhoP-Ra under the same conditions, even with increased amounts of protein (Fig. 1a, lanes 6–9), indicating that the S219L mutation not only decreases the affinity of PhoP-Ra for short promoter fragments, but also for the entire promoter region of \textit{phoP}.

PhoP and regulation of \textit{espA}

Transcription of the \textit{espA} operon is downregulated in \textit{phoPR} mutant strains (Frigui \textit{et al.}, 2008; Lee \textit{et al.}, 2008; Walters \textit{et al.}, 2006), suggesting that PhoP regulates \textit{espA} expression. To determine whether the regulatory effect of PhoP on \textit{espA} is direct, we performed EMSAs using PhoP-Rv,

\begin{figure}
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\includegraphics[width=\textwidth]{fig1}
\caption{Binding of PhoP to the promoter of \textit{phoP} (a), \textit{espA}1 (b), \textit{espA}2 (c), \textit{mprA} (d) and \textit{espR} (e, f). \textit{espA}1 and \textit{espA}2 refer to regions from −30 to 428 bp and from 391 to 633 bp of the \textit{espA} upstream region (relative to the start codon), respectively. (a) A fixed amount of the \textit{phoP} promoter was incubated with 0.25, 0.5, 1.0 or 2.0 \textmu g of PhoP-Rv (lanes 2, 3, 4 and 5, respectively) or PhoP-Ra (lanes 6, 7, 8 and 9, respectively). (b–e) The designated promoter was incubated with 0.5, 1.5, 3.0 or 4.5 \textmu g of PhoP-Rv (lanes 2, 3, 4 and 5, respectively) or PhoP-Ra (lanes 6, 7, 8 and 9, respectively). Lane 1 is the negative control without protein. (f) A fixed amount of labelled DNA fragment was incubated in reaction mixtures containing: no PhoP-Rv (lane 1); 1.5, 3.0 or 4.5 \textmu g PhoP-Rv (lanes 2, 3 and 4, respectively); 4.5 \textmu g PhoP-Rv and a 40-, 80-, 160- or 240-fold excess of unlabelled probe (lanes 5, 6, 7 and 8, respectively); 4.5 \textmu g PhoP-Rv and a 120- or 240-fold excess of unlabelled control fragment (lanes 9 and 10, respectively). f, Free probe; b, bound probe.
\end{figure}
We hypothesized that the effect of PhoP on espA may be mediated through Rv3676 and Lsr2, which are known to regulate espA (Gordon et al., 2010; Rickman et al., 2005). However, no obvious binding was detected to the Rv3676 or lsr2 promoters by either form of PhoP (data not shown), suggesting that neither Rv3676 nor Lsr2 mediates the regulatory effect of PhoP on espA.

**PhoP-Rv, but not PhoP-Ra, binds the espR promoter**

MprA and EspR are the only two regulators that have been confirmed so far to directly regulate transcription of the espA operon through binding of the espA promoter (Pang et al., 2013; Raghavan et al., 2008). Therefore, we reasoned that PhoP may modulate espA expression through MprA and/or EspR. Results of EMSAs determined that neither form of PhoP bound the mprA promoter, even at very high concentrations (Fig. 1d). However, PhoP-Rv clearly bound the espR promoter (Fig. 1e, lanes 4 and 5), whereas no detectable shift was observed for PhoP-Ra under the same conditions (Fig. 1e, lanes 6–9), suggesting that espR is the direct target of PhoP-Rv.

To confirm that the interaction of PhoP-Rv with the espR promoter is specific, we performed a competitive EMSA with the same 288 bp DNA fragment used in Fig. 1(e). This fragment, which extends from 274 bp upstream to 14 bp downstream of the espA start codon (see Fig. 2a, primers F-1/R-1), was again gradually shifted with increasing amounts of PhoP-Rv (Fig. 1f, lanes 1–4). Shifting was abolished by the addition of excess specific cold probe (Fig. 1f, lanes 5–8), but not by excess non-specific probe (Fig. 1f, lanes 9 and 10), indicating that binding of PhoP-Rv to the espR promoter is specific. These data suggest that, of the regulatory elements known to influence espA expression, espR is the only direct target of PhoP-Rv, in agreement with a recent report (Solans et al., 2014b).

**Mapping the PhoP binding sequence upstream of espR**

To delineate the region responsible for PhoP-Rv binding to the espR promoter, a series of nested probes was generated (Fig. 2a, b). Removal of 40 bp from the 5’ end of Probe 1 did not affect PhoP-Rv binding, as demonstrated by the complete shift of Probe 2 (Fig. 2c, lanes 3 and 4). However, further deletions of 40 or 100 bp resulted in complete loss of binding to Probe 3 and Probe 4, respectively, by PhoP-Rv (Fig. 2c, lanes 5–8). These data indicate the region from −235 to −196 bp, with respect to the EspR start codon, is essential for PhoP binding.

We also made deletions of 32 bp from the 3’ ends of Probes 1 and 2 (Fig. 2b). However, the resulting probes, Probe 5 and Probe 6, did not exhibit reduced binding (data not shown), indicating that the region from −18 to +14 relative to the start codon is not critical for PhoP-Rv binding.

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**Fig. 2.** Localization of PhoP-Rv and MprA binding sites in the espR promoter by EMSAs. (a) Schematic diagram of the region upstream of espR. Bent arrows mark the location of primers used to generate nested PCR fragments shown in (b). Numbers indicate the location of the forward (F) and reverse (R) primers relative to the start codon. The filled triangle and the vertical arrow mark the transcriptional start position (TSP) (Cao et al., 2013) and the start codon, respectively. (b) Location of PCR fragments used as EMSA probes. (c, e) The indicated probes were incubated with (+) or without (−) PhoP-Rv (c) or MprA (e). (d) Competitive EMSAs with the espR promoter probe and MprA. A fixed amount of labelled Probe 1 was incubated in reaction mixtures containing: no MprA (lane 1); 0.9, 1.8, 2.7 or 4.5 μg MprA (lanes 2, 3, 4 and 5, respectively); 4.5 μg MprA and a 40-, 80- or 120-fold excess of unlabelled probe (lanes 6, 7 and 8, respectively); 4.5 μg MprA and a 120- or 240-fold excess of unlabelled control fragment (lanes 9 and 10, respectively). f, Free probe; b, bound probe. (f) EMSAs with 45-mer oligonucleotides containing the predicted MprA Box half-site 1, 2 or 3. Probes were incubated with (+) or without (−) 4.5 μg MprA.

and included PhoP-Ra as a control to demonstrate that any detected binding was specific for PhoP-Rv. As the intergenic region of *ephA–espA* is very large (1357 bp), we divided this region into three overlapping fragments, which were amplified by PCR. Neither PhoP-Rv nor PhoP-Ra exhibited any detectable binding to the three fragments (Fig. 1b, c, and data not shown), suggesting that PhoP does not bind the espA promoter and that the regulation of espA by PhoP is indirect.
To map the precise sequence protected by PhoP-Rv, DNase I footprinting was performed for both the sense and the antisense strand with a 250 bp fragment from positions -235 to +15 relative to the espR start codon (Fig. 3). PhoP-Rv protected a 22 nt region between positions -211 and -190 for the sense strand (Fig. 3a), and a 28 nt region from positions -220 to -193 for the complementary strand (Fig. 3b), resulting, overall, in the protection of 31 bp from positions -220 to -190 (Fig. 3c), consistent with our EMSA and ChIP-seq data from a recent report (Solans et al., 2014b). We searched for sequences matching the conserved PhoP binding site in the protected region and several potential candidates, consisting of direct repeats of nine bases, were found (Fig. 4a–c, e).

The espR promoter contains multiple MprA binding sites

Our previous report indicated that MprA directly regulates espA (Pang et al., 2013). To determine whether MprA exerts an additional level of control over the ESX-1 system,
we examined MprA binding to the espR promoter. Similar to PhoP-Rv, MprA bound the 288 bp long Probe 1 (Fig. 2b, d). Shifting was abrogated by excess specific cold probe (Fig. 2d, lanes 6–8), but not by excess non-specific cold probe (Fig. 2d, lanes 9 and 10), indicating that binding of MprA to the espR promoter was specific.

To delineate (an) MprA binding site(s), we used the nested series of probes in EMSAs with MprA (Fig. 2b, e). As for PhoP-Rv, MprA binding was not disrupted by the deletion in Probe 2 (Fig. 2e, lane 4). However, Probe 3 showed reduced binding (Fig. 2e, lane 6), suggesting that an MprA binding site had been lost with the 5′ deletion. In contrast to the results with PhoP-Rv (Fig. 2c), however, Probe 3 did retain some binding capacity for MprA. Probe 4, which is 60 bp shorter than Probe 3, also bound MprA (Fig. 2e, lane 8), at a similar level to that of Probe 3. Deletion of the region from −18 to +14 had no further effect on MprA binding (Fig. 2b and data not shown). Altogether, these analyses indicated that two separate regions of the espR promoter, from position −235 to −196 and from position −136 to −18, contain sequences required for MprA binding.

To locate the precise sequences protected by MprA, a DNase I footprinting assay was performed (Fig. 5a) using the same DNA template used with PhoP-Rv (Fig. 3). As expected, MprA protected two regions upstream of espR, from positions −220 to −190 (MprA Region-1) and from −135 to −92 (MprA Region-2), on the coding strand (Fig. 5). We were unable to confirm these data on the non-coding strand due to weak binding by MprA. However, the results for the coding strand are consistent with the EMSA data.

The protected regions were screened for the consensus MprA binding site (MprA box) (He & Zahrt, 2005; Pang et al., 2007), which was first characterized as tandem repeats of the octameric sequence TCTCAGGC, separated by a 3 bp spacer. Three putative MprA boxes (Fig. 5b) were identified. Box 1 is located in Region 1, at nucleotides −217 to −200. Box 2 and Box 3, both in Region 2, are located at nucleotides −123 to −104, and at nucleotides −112 to −93, respectively, and share a common half motif. These locations are consistent with the EMSA data, with the reduced shifting of Probe 3, compared with Probe 2, by MprA (Fig. 2b, e) correlating with the removal of MprA Box 1. Additionally, Probes 3 and 4, which had similar affinity for MprA (Fig. 2e), both contain Box 2 and Box 3.

To confirm the location of the MprA boxes, EMSAs were conducted with short oligonucleotide probes containing individual boxes. Box 1 exhibited the strongest binding capacity to MprA, while Box 2 and Box 3 showed low and intermediate levels of MprA binding (Fig. 2f), suggesting they might have different roles in espR regulation. Overall, these data indicated that MprA protects two separate regions of the espR promoter, one of which overlaps the PhoP-protected region (Fig. 5b).

Identification of nucleotides essential for response regulator binding

Our above data indicated that both PhoP-Rv and MprA bind upstream of espR, and, interestingly, that the PhoP-protected region overlaps with MprA Region-1 (Fig. 5b). To begin to decipher the individual roles of PhoP and MprA on espR expression, we sought to identify the core nucleotide(s) that are essential for their binding. We focused mainly on MprA Box 1, as this region is also protected by PhoP and because it was the stronger MprA site. In mutation BOX1-1, the left-half of MprA Box 1 was mutated, and this change also partially mutated the left-half of three predicted PhoP sites (Fig. 4a–c). In mutation BOX1-2, the right-half of MprA Box 1 was mutated (Fig. 6d). However, as indicated below, the CT→GG change had no effect on MprA binding, so an additional base was altered in mutation BOX1-3. In mutations BOX1-2 and BOX1-3, all four of the predicted PhoP sites (Fig. 4a–c, e) were affected in either one or both half motifs. Because Box 2/3 bound MprA only weakly, we disrupted both potential sites simultaneously in mutation BOX2/3-M to increase the chances of seeing a definitive change in MprA binding. To test PhoP binding, the whole upstream region of espR (280 bp), in wild-type or mutated form, was used (Fig. 6a). Because there is more than one MprA box, we used short oligonucleotides with native or mutated sequences as...
probes to test MprA binding to MprA Box 1 or Box 2/3 (Fig. 6b, c).

Three types of mutation were identified in MprA Box 1. The type 1 mutation, represented by BOX1-1, does not disrupt PhoP-Rv binding (Fig. 6a, lane 4), but abrogates MprA binding (Fig. 6b, lane 4). In contrast, the type 2 mutation, exemplified by BOX1-2, abrogates PhoP binding (Fig. 6a, lane 6), but maintains MprA binding (Fig. 6b, lane 6). The type 3 mutation, as shown with BOX1-3, abolishes binding to both PhoP (Fig. 6a, lane 8) and MprA (Fig. 6b, lane 8). Mutation of the predicted MprA boxes within MprA Region-2, using the whole promoter region, did not affect PhoP binding (Fig. 6a, lane 10), but the same mutation in the corresponding oligonucleotides reduced MprA binding (Fig. 6c, lane 4). These data indicated that MprA Region-1 is required for the binding of PhoP and MprA, with some nucleotides critical for the binding of both response regulators, whereas the proximal region of the promoter containing MprA Region 2 is involved only in MprA binding.

**MprA Region-2, not MprA Region-1, is required for EspR synthesis**

To examine the role of PhoP and MprA in the regulation of *espR*, the three types of mutations were introduced into pXP1 (Cao *et al.*, 2013), a plasmid that contains *espR* and its promoter, to generate pXP-8(BOX1-1), pXP-9(BOX1-2) and pXP-10(BOX1-3). The mutated plasmids were electroporated into Δ*espR*, and the resulting complemented strains were designated C8(BOX1-1), C9(BOX1-2) and C10(BOX1-3), respectively. To define the role of MprA Region-2, the BOX2/3-M type mutation was introduced into pXP-1 to obtain pXP-11(BOX2/3-M), which was used to generate the complemented strain C11(BOX2/3-M). In pXP-12, MprA Region-1 was removed, leaving a shortened...
promoter region of 190 bp, and this construct was used to generate complemented strain C12.

Cellular levels of EspR were examined in control strains and in the complemented strains C8, C9 and C10, which contain mutations in MprA Region-1 (Fig. 7). However, despite the effects of these mutations on the binding of MprA and PhoP in EMSAs (Fig. 6), they had no discernible effect on EspR synthesis, and showed EspR levels comparable to C1(WT), in which \( espR \) is driven by its native promoter sequence (Fig. 7a). These data suggest that, under the conditions tested, MprA Region-1 is not involved in the regulation of \( espR \) by PhoP or MprA. This finding was further supported using strain C12 from which MprA Region-1 was removed entirely, and which had EspR levels similar to that of C1(WT) (data not shown). These results are consistent with a previous report showing that the 200 bp sequence immediately upstream of the \( espR \) start codon was sufficient to restore cellular EspR levels in \( \Delta espR \) (Raghavan et al., 2008). In contrast, EspR synthesis was restored, but to a significantly lower level in C11 (Fig. 7a), a finding that correlated well with the lower cellular levels of EspR in this strain. The defect in EspR and EspA synthesis in C11(BOX2/3-M) was not due to unequal loading of protein samples, as levels of GroEL2, a cytosolic protein often used as control, were similar in all samples (Fig. 7a). The results indicate that MprA Region-2 is required for normal expression levels of EspR and, indirectly, for the synthesis of EspA. In contrast, similar levels of ESAT-6 were detected in the Erdman strain, \( \Delta espR \), and in all of the strains with mutations in the \( espR \) promoter (Fig. 7a), suggesting that \( espR \) does not affect the synthesis of ESAT-6.

**MprA Region-2 is required for EspA synthesis**

As MprA Region-2 was required for the synthesis of EspR, we anticipated that this region would also impact the synthesis of (an) EspR-target gene(s). Therefore, cellular EspA levels of control and complemented strains were examined using an anti-EspA antibody (Fig. 7a). Western blot analysis demonstrated that C8(BOX1-1), C9(BOX1-2) and C10(BOX1-3) fully restored EspA synthesis (Fig. 7a), compared with that in C1(WT), consistent with the unaltered cellular levels of EspR in these strains. In contrast, in C11(BOX2/3-M), which has the mutation in MprA Region-2, the synthesis of EspA was only partially restored (Fig. 7a), a finding that correlated well with the lower cellular levels of EspR in this strain. The defect in EspR and EspA synthesis in C11(BOX2/3-M) was not due to unequal loading of protein samples, as levels of GroEL2, a cytosolic protein often used as control, were similar in all samples (Fig. 7a). The results indicate that MprA Region-2 is required for normal expression levels of EspR and, indirectly, for the synthesis of EspA. In contrast, similar levels of ESAT-6 were detected in the Erdman strain, \( \Delta espR \), and in all of the strains with mutations in the \( espR \) promoter (Fig. 7a), suggesting that \( espR \) does not affect the synthesis of ESAT-6.

**Transcription of genes associated with the ESX-1 system**

The above data indicated that MprA Region-2 is required for the synthesis of EspR and EspA. However, we wanted to confirm that this effect was due to regulation of \( espR \) transcription. Transcription of \( espR \) in these strains was first examined by real-time PCR. As \( espR \) was deleted and...
levels observed in the Erdman strain (b, e) or ΔespR (c, d). Results are the means (± SD) of triplicate experiments.

not detectable in ΔespR, the expression value of espR in the Erdman strain was arbitrarily set to 1. Expression of espR was restored in ΔespR complemented with pXP-1, to a level about 1.5-fold greater than in the Erdman strain (Fig. 7b). The slightly higher expression of espR in C1(WT) might be due to the difference in the transcriptional environment, as espR was inserted in a location other than its original site. However, the restored transcription indicated that the promoter region used contained all the genetic elements required for full transcription. Mutations in MprA Region-1 that abrogated binding to MprA, PhoP or both proteins in EMSAs did not have obvious effects on the transcription of espR, based on the similar expression levels in C1(WT), C8(BOX1-1), C9(BOX1-2) and C10(BOX1-3) (Fig. 7b). However, strain C11(BOX2/3-M), which has a mutation in MprA Region-2, had espR levels [0.82 ± 0.13; mean (± SD) of triplicate experiments] less than half of those of C1(WT) (2.2 ± 0.2), correlating with the reduced level of cellular EspR in C11(BOX2/3-M) (Fig. 7a). These results indicated that only MprA Region-2 is required for full levels of espR transcription, in agreement with the Western blot analysis (Fig. 7a).

As EspR regulates the espA operon (Raghavan et al., 2008), we also examined the transcription of espA and espD in our strains (Fig. 7c, d). For these studies, the expression value of the gene (espA or espD) in ΔespR was set to 1 arbitrarily. As shown in Fig. 7(c), espA expression was approximately 20 times greater in strain Erdman than in ΔespR, consistent with a previous report (Raghavan et al., 2008). Expression of espA was higher in C1(WT) than in the Erdman strain (Fig. 7c) and this may be due to the higher cellular levels of the espA activator EspR in C1(WT) (Fig. 7b). Complemented strains with the BOX1-1, BOX1-2 and BOX1-3 mutations exhibited espA transcription levels similar to those of C1(WT), consistent with the findings that these mutations did not reduce synthesis of EspR. However, the BOX2/3-M mutation in MprA Region-2 resulted in partially reduced expression of espA in C11(BOX2/3-M), consistent with the lower levels of cellular EspR in this strain (Fig. 7a). The espD gene had a similar transcriptional pattern to that of espA in these strains (Fig. 7d), further suggesting that Region-2 is involved in the indirect regulation of the espA operon. Consistent with the results for cellular ESAT-6 (Fig. 7a), transcription levels of esat-6 (Rv3875) were similar in all strains (Fig. 7e), suggesting that changes in espR expression do not affect esat-6 expression.

**DISCUSSION**

In our previous work, we demonstrated that the MprAB TCS controls the ESX-1 system through direct regulation
of the espA operon (Pang et al., 2013). We further showed that EspA levels were decreased in a PhoP mutant (Pang et al., 2013), and other laboratories have also presented evidence that PhoPR regulates ESX-1 (Frigui et al., 2008). However, the means by which PhoP regulates the ESX-1 system were unknown. Findings from our current study suggest that espR is an intermediary regulatory factor between PhoPR and ESX-1. A PhoP binding site was identified upstream of the espR transcriptional start position by gel-shift analyses and this site was confirmed by DNA footprinting. In contrast to PhoP derived from H37Rv, PhoP containing the S219A point mutation found in strain H37Ra did not bind the espR promoter, and we hypothesize that dysregulation of espR may be an additional factor in the attenuation of H37Ra. These results may also explain the findings from a DNA microarray study (Li et al., 2010), which showed that espR and the espA operon, a target of EspR, had reduced expression in H37Ra compared with H73RV, during growth in macrophages. In contrast to the espR promoter, we did not detect PhoP sites in the promoters for espA or mprA, or in the regions upstream of the genes encoding CRP (Rv3676) or the nucleoid-associated protein Lsr2, both of which have been linked to the regulation of ESX-1 (Gordon et al., 2010; Rickman et al., 2005).

In addition to H37Ra, mutations have also been detected in the phoPR regulator in Mycobacterium bovis and strains of Mycobacterium africanum lineage 6 (L6), which seem to affect the fitness of these bacilli for human-to-human transmission, and disrupt the synthesis of pathogenicity lipid factors (Gonzalo-Asensio et al., 2014). However, in contrast to H37Ra, these mutations did not have any influence on the expression of the espACD operon, and did not have an obvious effect on ESX-1 activity in M. africanum L6 (Gonzalo-Asensio et al., 2014). M. africanum L6 strains have a deletion, RD8, that occurred during the evolution of tuberculosis strains (Boritsch et al., 2014), and which corresponds to the region 460 bp upstream of the espACD operon (Gordon et al., 1999). This deletion removes two of the three EspR sites and one of the three MprA sites upstream of espA, and thus may lead to the blockage of regulation by the PhoP/MprA/EspR regulatory loop examined in our study. We speculate that the regulatory effects of PhoP and MprA on espR would be diminished in RD8-deleted strains.

Our results support a recent study by Solans et al. (2014b), which, using ChIP-seq, identified espR as one of over 30 loci regulated directly by PhoP. In addition to the PhoP site, we also identified two binding regions for MprA upstream of espR, suggesting that MprAB regulates ESX-1 through at least two mechanisms: via the regulator EspR and by direct regulation of the espA operon (Pang et al., 2013). Both PhoP and MprA bound a region centred around 200 bp upstream of the espR start codon. Although mutations in this region disrupted binding of these response regulators in EMSAs, we did not detect a corresponding effect on expression of espR in recombinant strains carrying the same mutations. However, both PhoPR and MprAB are stress-responsive TCSs (Baker et al., 2014; He et al., 2006; Pang et al., 2007) and it is possible that these regulatory sites are utilized under conditions not tested in our study. Mutations in MprA Region-2, however, were associated with reduced expression of espR and EspR-target genes. Although the mutations in MprA Region-2 were close to an EspR binding site, we believe that the transcriptional effects were due to disruption of MprA binding, because our previous analyses showed that mutation of a single EspR binding site had no effect on espR expression (Cao et al., 2013).

In addition to espR, Solans et al. (2014b) found PhoP binding sites upstream of whiB6 and the small heat-shock protein gene hsp, and a new study has confirmed that PhoP directly regulates hsp under stress (Singh et al., 2014). We had previously localized several MprA boxes upstream of hsp (Pang & Howard, 2007), and had reported preliminary findings that MprA also directly regulates whiB6 (Pang et al., 2013), which was recently shown to influence expression of ESAT-6 (Solans et al., 2014a). These findings suggest that multiple stress-associated genes may be co-regulated by MprAB and PhoPR. Interestingly, several other genes have been reported to be co-regulated by MprAB and a second TCS. MprAB and TrcRS directly regulate the β-propeller gene Rv1057, and evidence suggests that this regulation involves MprA boxes located more than 600 bp apart, as well as two separate TrcR binding sites (Haydel & Clark-Curtiss, 2006; Pang et al., 2011). Additionally, MprAB and DosR co-regulate the Rv1813c–Rv1812c operon, which is associated with virulence and activation of proinflammatory cytokines (Bretl et al., 2012), and they co-regulate the Rv0081–Rv0088 operon, which is predicted to encode a formate hydrogenylase complex (He et al., 2011). Localization of regulatory sites in the Rv0081–Rv0088 promoter suggests that the two response regulators activate the operon by blocking binding of the autoregulatory transcription factor Rv0081 (He et al., 2011). The fact that both espR and Rv0081 encode transcriptional regulators suggests that there is a complex fine-tuning of the regulatory response to various stresses controlled by the TCSs.

EspR does not appear to be a classical transcription factor, and instead evidence suggests it is a nucleoid-associated protein that binds to more than 165 loci on the genome of M. tuberculosis (Blasco et al., 2012). EspR regulates multiple genes associated with the synthesis of cell-wall lipids, including pthiocerol dimycocerosate, phenol glycolipid and mycolic acid (Blasco et al., 2012), and the ESX-1 system itself is important for the stability of the cell wall (Garces et al., 2010). Notably, both MprAB and PhoPR are also associated with regulation of the response to cell-wall damage and/or regulation of lipid synthesis (He et al., 2006; Pang et al., 2007; Walters et al., 2006), and we identified several genes that are shared by the EspR and PhoP regulons (Walters et al., 2006). PhoP is a global regulator that modulates many physiological activities of M. tuberculosis, including biosynthesis of cell-wall components, such as diacyltrehaloses, polyacyltrehaloses and
sulfolipids (Gonzalo Asensio et al., 2006; Goyal et al., 2011; Walters et al., 2006), as well as the function of ESX-1 (Frigui et al., 2008). Most of the genes regulated by both PhoP and EspR are involved in lipid synthesis (Rv0405, Rv2930, Rv3487c, Rv3823c and Rv3824c) or are PPE genes (Rv1361c, Rv3350c, Rv3426 and Rv3429). PhoP activates Rv2930, Rv3487c, Rv3823c and Rv3824c) or are PPE genes factors and in the attenuation of H37Ra, and provide new additional route by which MprA regulates the ESX-1 system.

In conclusion, we report that the virulence regulator ospR is the direct target of PhoP and MprA, the response regulators of the PhoPR and MprAB TCSs, respectively, and reveal an additional route by which MprA regulates the ESX-1 system. These findings contribute to our understanding of the role of PhoPR in the regulation of mycobacterial virulence factors and in the attenuation of H37Ra, and provide new insights into the complex regulatory network controlling the ESX-1 system in M. tuberculosis.

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