Evidence for complex interactions of stress-associated regulons in an mprAB deletion mutant of *Mycobacterium tuberculosis*

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Two-component systems are important constituents of bacterial regulatory networks. Results of this investigation into the role of the MprAB two-component system of *Mycobacterium tuberculosis* indicate that it is associated with the regulation of several stress-responsive regulons. Using a deletion mutant lacking portions of the response regulator, MprA, and the histidine kinase, MprB, it was demonstrated by real-time PCR, primer extension analyses and DNA microarrays that MprAB activates sigma factor genes sigE and sigB, under SDS stress and during exponential growth. SDS-inducible, MprA-dependent transcriptional start points were identified for mprA, sigE and sigB, and variations in distance between these points and MprA-binding sites suggest that MprA is involved in different mechanisms of promoter activation. Although most of the SigE regulon was downregulated in the deletion mutant, the cluster of genes Rv1129c, Rv1130 and Rv1131, which is associated with growth in monocytes, was upregulated in the deletion mutant under SDS stress, and this upregulation was dependent upon atmospheric growth conditions. Multiple stress-associated genes of the DosR, SigD and IdeR regulons were also upregulated in the deletion mutant, during exponential growth and/or in the presence of SDS. Surprisingly, the deletion mutant had increased resistance to SDS compared to the parental strain, and enhanced growth in human peripheral blood monocytes, characteristics which may result from a loss of repression of stress-associated genes.

INTRODUCTION

As evidenced by its historical and current impact on human populations (Corbett & Raviglione, 2005; Zink *et al.*, 2005), *Mycobacterium tuberculosis* is a highly successful pathogen. To withstand the challenges of aerosol transmission, growth within host macrophages, and prolonged encapsulation within lung granuloma, these organisms require the ability to respond to different types of stress. *M. tuberculosis* has 13 sigma factors (Cole *et al.*, 1998), and DNA microarray analyses have shown the importance of several of these in regulating the changes in gene expression patterns associated with various stresses (Geiman *et al.*, 2004; Manganelli *et al.*, 2001, 2002). In addition to sigma factors, the response regulators of some two-component systems (TCSs) (Rison *et al.*, 2005), such as DosR (Kendall *et al.*, 2004; Park *et al.*, 2003) and PhoP (O’Toole *et al.*, 2003; Perez *et al.*, 2001; Walters *et al.*, 2006), and other transcriptional regulators, such as IdeR (Dussurget *et al.*, 1999; Manabe *et al.*, 2005) and EmbR (Sharma *et al.*, 2006), have been shown to modulate mycobacterial gene expression in response to particular stresses. However, in general, the mechanisms by which *M. tuberculosis* senses a particular stress, and activates the appropriate regulatory factor(s) while inhibiting others, are largely unknown.

Abbreviations: EMSA, electrophoretic mobility shift assay; iVEGI, in vivo-expressed genomic island; TCS, two-component system; TSP, transcriptional start point.

The GEO accession numbers for the array data associated with this paper are GSM155424–155447 (series record no. GSE6750).

Four supplementary tables are available with the online version of this paper.
We are investigating the role of the MprAB TCS of *M. tuberculosis* in the stress response. TCSs are histidine phosphotransfer systems involved in sensing environmental changes and triggering adaptive responses (Hoch & Varughese, 2001). Studies indicate that MprA is an auto-regulatory transcription factor, which is phosphorylated by the histidine kinase MprB (He & Zahrt, 2005; Zahrt et al., 2003). Results of genome-wide transposon mutagenesis suggested that *mprB* was essential for growth in broth, whereas *mprA* was nonessential (Sassetti et al., 2003).

*mprA* and *mprB* are part of the iVEGI (*in vivo*-expressed genomic island) region, which is highly upregulated during growth of *M. tuberculosis* in mice (Talaat et al., 2004). Many iVEGI genes are predicted to be involved in cell wall biosynthesis and lipid metabolism (Talaat et al., 2004), and a recent study suggests that they are important in *M. tuberculosis* infection of the central nervous system (Jain et al., 2006). The role of MprA in survival is unclear, however, as an *mprA* insertion mutant had reduced persistence in a murine model, but increased growth in resting murine macrophages (Zahrt & Deretic, 2001).

*mprA* and *mprB* are also upregulated by SigE, during exposure to the detergent SDS (Manganelli et al., 2001). SigE is a major stress-responsive sigma factor of *M. tuberculosis* (Jensen-Cain & Quinn, 2001; Manganelli et al., 1999, 2001; Raman et al., 2001), and often functions within a regulatory cascade (Manganelli et al., 2002; Raman et al., 2001). SDS exposure upregulates SigE, which then activates 23 genes, including sigB, *mprA*, *mprB* and four other transcription factor genes (Manganelli et al., 2001). Some SDS-activated genes are also upregulated during growth of *M. tuberculosis* in macrophages, suggesting that, like SDS exposure, macrophages may damage lipid components of the bacterial envelope (Schnappinger et al., 2003). Similar to rpoE of *Escherichia coli* (Mecsas et al., 1993; Tam & Missiakas, 2005), *M. tuberculosis* sigE may, therefore, be induced by envelope stress, but the mechanisms involved have not been determined. Deletion of *sigE* in mycobacteria is associated with reduced resistance to SDS and oxidative stress, decreased growth in macrophages (Manganelli et al., 2001; Wu et al., 1997), and reduced growth in mice (Ando et al., 2003; Manganelli et al., 2004).

As MprAB is the only TCS upregulated by SigE under SDS stress, we hypothesized that MprAB may be involved in sensing cell envelope damage and upregulating genes within the SigE regulon. In this study, using a deletion mutant in which both *mprA* and *mprB* were disrupted, we determined that MprA regulates both *sigE* and *sigB*, during normal growth conditions and under SDS stress. We also identified SDS-inducible transcriptional start points for *mprA, sigE* and *sigB*. In addition, a recent report using an *mprA* insertion mutant (He et al., 2006), we observed that, in the MprAB deletion mutant, much of the SigE regulon was downregulated under SDS stress. However, further analyses indicated that a cluster of genes within the SigE regulon is highly upregulated in the absence of MprAB under SDS stress, and that this upregulation is sensitive to atmospheric growth conditions. Moreover, multiple genes from regulons associated with hypoxia, starvation and iron metabolism are upregulated in the MprAB deletion mutant. Unexpectedly, the deletion mutant had enhanced survival in SDS, as well as increased growth in human peripheral blood monocytes. These data suggest that the MprAB TCS is an important link within a complex network of stress-associated regulons involved in survival of *M. tuberculosis*.

**METHODS**

**Bacterial strains and culture conditions.** *M. tuberculosis* mutants and parental strain H37Rv were grown in either Middlebrook 7H9 broth containing 0.05 % Tween 80, or Middlebrook 7H10 agar (Difco), both enriched with 10 % oleic acid-albumin-dextrose-catalase (OADC; Difco). Broth cultures were incubated at 37 °C with gentle shaking, under normal atmospheric conditions (without additional CO₂), except where indicated. *E. coli* Novabluve and Rosetta (DE3) pLysS (Novagen) were used as host strains for general cloning and gene expression, respectively. *E. coli* strains were grown on L agar or in L broth. Antibiotics were added to growth media as required.

**Construction of the mprAB deletion mutant, Rv-D981.** Sequences for primers used in this study are included in Supplementary Table S1, available with the online version of this paper. An 1150 bp deletion, which included the C-terminal half of MprA and the N-terminal half of MprB, was generated in H37Rv using counterselectable markers (Parish & Stoker, 2000), and plasmids kindly provided by T. Parish (Queen Mary’s School of Medicine and Dentistry, London, UK). Briefly, two genomic fragments of approximately 1 kb were amplified, inserted into pGEM-T-Easy (Promega), and sequenced. Fragment A, generated using primers D981S-5 and D981S-3, begins within Rv0980c and ends 300 bp downstream of the predicted start codon of *mprA*. Fragment B, generated using primers D982E-5 and D982E-3, begins 800 bp downstream of the *mprB* start codon and ends within Rv0983 (pepD). Fragment B was inserted into p2NIL using a Scal/KpnI digest, yielding plasmid pN982E, and then fragment A was inserted into pN982E, using a PstI/ScaI digest, producing pD981. The selection cassette from pGOAL17 was inserted into the PadI site of pD981, producing pD981P, which was used to generate the deletion mutant Rv-D981 from H37Rv, as described by Parish & Stoker (2000). The deletion in Rv-D981 was verified by Southern blotting and PCR.

**Complementation of Rv-D981.** A 2.8 kb genomic region containing *mprA*, *mprB* and the intergenic region between *mprA* and *Rv0980c* was amplified using primers 981UFS and 982XP-3. The amplicon was inserted into pSTBlue-1 (Novagen), sequenced, and then the insert was released by digestion with SmaI, which cuts in the vector, and XbaI. The gel-purified insert was ligated into the integrative vector pMV361 (Stover et al., 1991), which was precut with Hpal and XbaI. The resulting plasmid, pR981-COM, was electrooporated into strain Rv-D981, followed by plating and selection for kanamycin-resistant colonies. The presence of the intact MprAB region, in the complemented strain Rv-D981C, was confirmed by PCR.

**Construction of an mprA expression plasmid and purification of MprA.** The 690 bp coding region of *mprA* was amplified by PCR using primers MprA-P5 and MprA-P3, and inserted into pSTBlue-1, generating pSTH18. Following sequence verification, a BamHI/HindIII fragment of pSTH18 was ligated into BamHI/HindIII-cut pBEn-SBP-SET1a (Stratagene), a Varillex
expression vector containing an N-terminal streptavidin-binding peptide tag and a solubility enhancement tag. The resulting plasmid, pSTH20, was used to transform E. coli Rosetta(DE3)pLySS (Novagen). Expression of mprA was induced by the addition of IPTG with incubation for 3 h at 30 °C. Bacteria were collected by centrifugation at 4 °C, and then resuspended in 1 ml streptavidin-binding buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl). Following sonication, lysates were centrifuged for 5 min at 13 000 r.p.m. and the tagged MprA was recovered from the supernatant using streptavidin agarose (Novagen), according to the manufacturer’s instructions. Peptide tags were removed using the Thrombin CleanCleave kit (Sigma), and removal was verified by gel electrophoresis of the MprA protein before and after cleavage.

**Electrophoresis mobility shift assays (EMSA).** The ability of MprA to bind DNA was confirmed using the mprA promoter (data not shown). To analyse binding of MprA to the sigE promoter, probes P1, P2 and P3 were amplified using primer pairs SigEGST-1/SigEGST-2, SigEGST-1/SigEGST-6 and SigEGST-2/SigEGST-5, respectively. SigE45-F/SigE45-R, SigE30-F/SigE30-R and SigE54-F/SigE54-R were annealed to generate probes P4, P5 and P6, respectively (see Table S1 for primer sequences). Progressively shorter sequences were generated using the following oligonucleotide pairs: SigE+2F/SigE+2R (probe P7), SigE+5F/SigE+5R (probe P8), and SigE+8F/SigE+8R (probe P9) (see Results for details). SigB promoter probes were amplified using the following primer pairs: SigBGST-4/SigBGST-3 (probe P1), SigBGST-4/SigBGST-2 (probe P2), SigBGST-1/SigBGST-3 (probe P3), and SigBGST-1/SigBGST-2 (probe P4). Primers SigBGST-5 F/R and SigBGST-6 F/R were annealed to generate sigB probes P5 and P6, respectively. DNA probes were end-labelled with [α-32P]ATP using T4 polynucleotide kinase (Promega) and were separated from free isotope using Quick Spin Column (Roche) filtration. Labelled DNA probes were incubated with MprA using described procedures (Samten et al., 2002). For competition assays, 100-, 200- or 400-fold excess (in ng) of scrambled or mutant oligonucleotides was incubated with 32P-labeled DNA probes and reaction products were separated using an 8% polyacrylamide sequencing gel. For sigE and mprA analysis, sequencing reactions, performed with the same primer as used in primer extension, were run in adjacent lanes to determine the start site of the transcripts.

**Reverse transcription and relative quantification of mRNA by real-time PCR.** Total RNA (3 μg) was annealed with 2 μg of random hexamer primers (pdN6, Amersham Pharmacia Biotech) and 20 units of RNase inhibitor (Promega). Following incubation at 65 °C for 5 min, reverse transcription was carried out at 42 °C for 60 min, using 200 U M-MLV reverse transcriptase (Invitrogen), 20 μM dNTPs (Roche) and first-strand buffer (Invitrogen).

Primers and probes for real-time PCR were designed with PRIMER EXPRESS software (Applied Biosystems), and probes were labelled with 5'-fluorescein phosphoramidite and 3'TAMRA. Assays were performed using an ABI Prism 7700 thermal cycler with 25 μl reaction volumes containing 1 × Taqman universal PCR master mix (ABI), 0.3 mM each primer, 0.2 mM probe and 2.5 μl cDNA or genomic DNA as template, and the following thermal cycles: 2 min at 50 °C, 10 min at 95 °C followed by 40 repeats of 15 s at 95 °C and 1 min at 60 °C. Relative quantities of cDNA were determined from standard curves generated by amplification of serial 10-fold dilutions of H73Rv genomic DNA, using the appropriate probe and primers, and were normalized for amounts of 16S rRNA. The following primer/probe sets were used: mprA (981QF, 981QR, probe 981QP); sigB (SigBQF, SigBQR, probe SigBQP); sigE set 1 (WSigEF, WSigER, probe WSigEP); 16S rRNA (W16SF, W16SR, probe W16SP).

**In vitro stress assays.** Cultures were grown to early exponential phase (OD600 <0.2), and divided into two 25 ml portions. SDS was added to one portion for each strain to a final concentration of 0.05%. Samples were incubated at 37 °C, and then aliquots were removed at 2 h or 5 h, centrifuged to remove SDS, and then diluted in PBS prior to plating in duplicate. Colonies were counted after 2 weeks to determine percentage survival in the stress-treated samples compared to untreated controls. (Incubation for an additional 1–2 weeks did not produce any changes in colony numbers.) Resistance to SDS was examined in three separate experiments.

**Infection of human monocytes.** Human mononuclear cells were isolated fromuffy coats purchased from United Blood Services and cultured as described by Byrd & Horwitz (1989). After 48 h, cells were harvested and monocytes were isolated by adherence to Primaria in Iscoves medium containing 10% normal human serum at concentrations of approximately 1 × 10⁵ monocytes per well (500 μl) for 90 min in 5% CO₂/95% air at 37 °C. Following wash steps and addition of medium, monocyte monolayers were maintained under these conditions for an additional 48 h, prior to further washing and infection using a low-inoculum assay as described by Byrd (1997). Unopsonized M. tuberculosis strains were added to monocyte monolayers at a concentration of 2 × 10⁴ bacteria per well (bacteria/monocyte ratio of 0.2:1), and the monolayers were incubated at 37 °C in 5% CO₂/95% air. At 2, 4 and 7 days, culture supernates and cell lysates were plated separately on 7H11 agar, and values were added together to give total c.f.u. per well at each time point (Byrd, 1997). For Rx-D981, supernate c.f.u. were 6.4%, 5.6% and 11% of the total c.f.u. at 2, 4 and 7 days, respectively, indicating a relatively small contribution of supernate c.f.u. to total well c.f.u. The increase in supernate c.f.u. at 7 days was likely due to detached, infected monocytes in the supernate, as there was a concomitant decrease in adherent monocytes between days 4 and 7. The same relationship between groups was apparent when only c.f.u. in monocyte lysates were compared, and when c.f.u. in monocyte lysates were corrected for the number of monocytes remaining adherent at each time point (data not shown). Data were compared by Student’s t test.
The viability of infected monocyte monolayers and the number of nuclei per well was also determined at each time point as described by Byrd (1997). Monocyte monolayers were >98% viable in all groups at 2 and 4 days, with viability decreasing to a mean of 87%, 69% and 96% in H37Rv, Rv-D981 and Rv-D981C, respectively, at 7 days. This decrease in viability corresponded to increasing c.f.u. and was likely due to intracellular multiplication resulting in death of infected monocytes.

**DNA microarray analyses.** The *M. tuberculosis* microarray consists of 4295 70-mer oligonucleotides representing the 3924 predicted open reading frames of the H37Rv strain (http://www.sanger.ac.uk) with an additional 371 probes designed to detect sequences present in the CDC1551 strain (http://www.tigr.org). The arrays were prepared by spotting oligonucleotides (Tuberculosis Genome Set version 1.0, Operon Biotechnologies) onto poly-t-lysine-coated glass microscope slides using a GeneMachines Omnimgrid 100 Arrayer (Genomic Solutions) and SMP3 pins (Telechem). Total RNA from three independent experiments was prepared as described above. Briefly, cDNA was synthesized using random primers and labelled with Cyanine-3 or Cyanine-5 dUTP (PerkinElmer) by a modification of the procedure described by Voskuil et al. (2003) and hybridized to the arrays overnight. For each pair of samples in each experiment, dye flips were performed. After washing, the arrays were scanned with a GenePix4000B scanner (Molecular Devices). The images were processed using GenePix 5.1. Data were filtered by removing all spots that were below the background noise or flagged as ‘bad’. Spots were considered to be below the background noise if the sum of the median intensities of the two channels was less than twice the highest mean background of the chip. The chips were normalized by the print-tip Lowess method (Dudoit et al., 2000). The ratio of the mean median intensity of Cy5 over the mean median intensity of Cy3 was determined for each spot and the fold change values were calculated. A one-class SAM analysis (Tusher et al., 2001) was performed with the MEV software (Saeed et al., 2003) to find genes with changes that occurred consistently in all replicates. A median FDR (false discovery rate) of zero, delta values ranging from 3.14 to 3.63 and a mean change of at least twofold were considered our cut-off for significance.

**RESULTS**

**Gene expression in an mprAB mutant**

To evaluate the role of MprAB in gene regulation, we generated the H37Rv deletion mutant Rv-D981, which is missing a 1.1 kbp region encoding the predicted DNA-binding domain of MprA, as well as the transmembrane region and part of the kinase domain of MprB (Fig. 1a). Exponential growth rates of Rv-D981 and H37Rv were similar under standard growth conditions in broth (data not shown). The expression of *mprA* was compared in H37Rv and Rv-D981, by real-time PCR, using a probe and primers that hybridize to a section remaining at the 5' end of *mprA* (Fig. 1a). *mprA* was highly upregulated in H37Rv by SDS treatment, but in Rv-D981, no induction was detected and basal expression levels were also lower than in H37Rv (Fig. 1b). In the complemented strain, Rv-D981C, which was generated by inserting *mprAB* into the genome under the control of the *mprA* promoter, SDS activation of *mprA* was restored, and basal expression levels were increased compared to Rv-D981 (Fig. 1b). *mprA* expression in Rv-D981C was slightly higher than in H37Rv, possibly due to differences in the transcriptional environment, but, overall, these results indicated that the MprAB TCS is required for induction of *mprA* expression under detergent stress, and that it contributes to *mprA* expression during normal exponential growth.

As MprAB was the only TCS regulated by SigE under SDS stress, we hypothesized that MprAB might contribute to activation of the SDS response. We therefore examined expression of the SDS-responsive sigma factor genes, *sigE* and *sigB*, in Rv-D981. Both genes were highly activated by SDS exposure in H37Rv (Fig. 1c, d), but expression levels
were low in Rv-D981 under SDS or control conditions. In the complemented strain, sigE and sigB were again inducible by SDS (Fig. 1c, d). These data indicate that under the conditions tested, MprAB directly or indirectly regulated the expression of two key sigma factors.

Interaction of MprA with the mprA promoter

To determine whether MprA influences transcript initiation under SDS stress, we first identified the mprA transcriptional start point (TSP), using primer extension analysis. Preliminary analyses with two primers identified a single TSP located close to the coding region (data not shown). A third primer, 981PE3, was used to verify these results (Fig. 2a). The TSP is a guanosine residue four bases downstream of the predicted MprA start codon (Fig. 2a, b), indicating that mprA is shorter than predicted (Cole et al., 1998). The TSP is also 22 bases downstream of the end of the previously identified MprA-binding sites (He & Zahrt, 2005). The position of the TSP indicates that the −35 region of the mprA promoter overlaps the first direct repeat of the MprA-binding site. The sequence GGCCA near the end of this site (Fig. 2b) has some similarity to the consensus sequences for SigE- and SigH-dependent promoters (Manganelli et al., 2001, 2002; Raman et al., 2001), consistent with the regulation of mprA by SigE (Manganelli et al., 2001). It is likely, however, that, when MprA is bound, sigma factors do not interact directly with this −35 region. The ‘GTT’ core of the SigE/SigH consensus −10 region (Manganelli et al., 2001, 2002; Raman et al., 2001) was not detected in the MprA promoter.

Importantly, the same TSP was identified in H37Rv and Rv-D981, under SDS treatment and control conditions (Fig. 2c). However, mprA transcripts were only weakly detectable in Rv-D981, compared to H37Rv and Rv-D981C, and were not induced by SDS. These data again indicated that MprA is autoregulatory under SDS stress and control conditions, but showed that it does not alter the site of transcript initiation.

Interaction of MprA with the sigE promoter

Three TSPs were previously identified for sigE in the M. tuberculosis complex (Fig. 3a): TSP1 and TSP2 in M. bovis BCG (Wu et al., 1997), and a SigH-dependent TSP (TSP3) (Raman et al., 2001) located within the predicted coding region of M. tuberculosis sigE (Cole et al., 1998). We identified an additional start site, TSP4, which is coincident with the putative start codon of sigE, and upstream of TSP3 (Fig. 3a, b, g), supporting reports that sigE is smaller than predicted (Raman et al., 2001; Wu et al., 1997). TSP4 is activated by SDS stress in H37Rv, in an MprA-dependent manner, as indicated by the reduced intensity of bands for Rv-D981 (Fig. 3b). TSP3 was also activated by SDS stress, but this activation was independent of MprA, as transcription levels were similar in H37Rv and Rv-D981 (Fig. 3c). MprA may contribute to basal levels of transcription from both TSP3 and TSP4, however, as indicated by the weaker bands in Rv-D981 under control conditions (Fig. 3b, c).

The capacity of MprA to bind the sigE promoter directly was examined by EMSA. MprA shifted the 114 bp sigE promoter probe P1 (Fig. 3d, e), and additional probes (P2–P6) localized MprA binding to within a 54 bp region (P6) (Fig. 3d, and data not shown). Sequences within P6 showed some similarity to the MprA consensus binding site (He & Zahrt, 2005) and were examined using shorter probes (P7–P9) (Fig. 3d). P8, which had five bases flanking the putative binding sites, was sufficient to obtain binding (Fig. 3d, f, and data not shown). Mutating bases within the first (P10 and P12) or second (P11) predicted MprA-binding sites within P8 inhibited MprA binding (Fig. 3d, f), confirming these sites as targets for MprA.

The location of TSP4 places the −35 region of the sigE promoter shortly downstream of the MprA-binding sites (Fig. 3g). Sequences beginning at −33 (TGGCCCA) and −11 (CGGTA) each share four bases, respectively, with the −35 and −10 regions of SigH-dependent promoters (Manganelli et al., 2002; Raman et al., 2001). However, as some of the invariant residues reported for SigH-dependent promoters are missing (Manganelli et al., 2002; Raman et al., 2001), it is likely that another sigma factor regulates sigE from this promoter.

Interaction of MprA with the sigB promoter

Only a single TSP was identified for sigB (Fig. 4a), which corresponds to the site previously reported (Manganelli et al., 2002; Raman et al., 2001). Activation of this TSP under SDS stress was MprA-dependent (Fig. 4a). We detected MprA binding to probes extending 131 bp upstream of the sigBTSP (Fig. 4b, c, and data not shown), and a search of the
Tuberculist database with the published MprA-binding sequence (He & Zahrt, 2005) identified potential MprA target sites between positions 2102 and 283. Similar to the findings with the sigE promoter, a sigB promoter probe (P6) with five bases flanking these potential target sites was sufficient to obtain binding with MprA (Fig. 4b, c).

Fig. 3. Analyses of the sigE promoter. (a) Map of sigE TSPs. Bent arrows mark four identified TSPs (see text for details). Doubleheaded arrows indicate distance in bp between the predicted start codon (vertical arrowhead) and each TSP. TSP4 overlaps the predicted start codon. (b, c) TSP4 (b) and TSP3 (c) were identified by primer extension using primers SigEPE-1 and SigEPE-2, respectively. Sequence ladder was generated with SigEPE-1. (d–f) EMSA analyses with the sigE promoter and MprA. (d) sigE promoter probes P1–P12 were shifted (+) or not shifted (−) by MprA. Numbers indicate position of probes with respect to TSP4. Predicted MprA-binding sites are underlined in P7–P9. Bold italics, bases mutagenized in P10–P12. (e) A fixed amount of labelled P1 was incubated in reactions containing: no MprA (lane 1); 0.36 μg or 0.72 μg MprA (lanes 2 and 3, respectively); 0.72 μg MprA and 100- or 200-fold excess of unlabelled P1 (lanes 4 and 5, respectively); or 0.72 μg MprA and 100- or 200-fold excess of unlabelled control fragment (lanes 6 and 7, respectively). (f) P8–P12 incubated with (+) or without (−) MprA, bound probe; f, free probe. (g) Map of the sigE promoter for TSP4. Underlined bases indicate MprA-binding sites. Bold bases indicate putative "35 and "10 regions. The start codon is in italics and the asterisk indicates the TSP.

Fig. 4. Analyses of the sigB promoter and comparison of MprA-binding sites. (a) The sigB TSP was mapped by primer extension using primer SigBPE-1. The D981C band is lower due to curvature of the gel. (b, c) EMSAs with sigB promoter probes and MprA. (b) Probes P1–P6 were shifted (+) or not shifted (−) by MprA. Probe location is indicated with respect to the sigB TSP (Manganelli et al., 2002; Raman et al., 2001). Predicted MprA-binding sites are underlined. (c) Probes P1, P2, P5 and P6 were incubated with (+) or without (−) MprA. MprA promoter probe used as positive control. (d) Alignment of MprA-binding sites. Two highly conserved 6 bp motifs are separated by 5 bp spacer. Numbers indicate the position of the last base shown with respect to the TSP: mprA TSP, this study; sigB TSP, Manganelli et al. (2002), Raman et al. (2001); sigE, TSP4, this study. mprA and pepD (pep1, pep2) sites were obtained from published data (He & Zahrt, 2005). A very weak MprA-binding site in the pepD promoter (He & Zahrt, 2005) was excluded from the comparison. Positions conserved in three, four or five sequences are indicated by small, underlined or bold letters, respectively. N, any nucleotide. Italics indicate extended direct repeats.

Fig. 4b, c.
Alignment of MprA-binding sites identified by us and by others (He & Zahrt, 2005) suggests that MprA binds to a core sequence of six highly conserved nucleotides (TCTCAG), separated from a direct repeat by five less-conserved or non-conserved residues (Fig. 4d). A search of the H37Rv genome with this repeat sequence, allowing for up to two mismatches per repeat, revealed 17 other potential sites in intergenic regions (Supplementary Table S2), suggesting that MprA may directly regulate additional genes.

**Global patterns of gene expression in Rv-D981**

As our data indicated that MprA activates sigE, we expected that the global gene expression profile of Rv-D981 under SDS stress would be similar to that described for the sigE deletion mutant (Manganelli et al., 2001). DNA microarray analyses were used to compare gene expression in H37Rv and Rv-D981, under control conditions and during SDS stress. SDS exposure induced marked changes in gene expression, upregulating and downregulating over 200 genes in both H37Rv and Rv-D981 (Supplementary Tables S3 and S4). Compared to Rv-D981, 39 genes were significantly overexpressed in H37Rv under SDS stress compared to H37Rv (Table 1), and this was confirmed for several genes by real-time PCR (Table 1 and data not shown). Significant changes in expression levels of the SigE-regulated genes mprA, mprB and pepD could not be detected by DNA microarray analyses, perhaps due to low expression levels, but real-time PCR confirmed that expression of all three genes was reduced in Rv-D981 (Fig. 1b, Table 1, and data not shown).

Unexpectedly, Rv1129c, Rv1130 and Rv1131, which were downregulated in the sigE mutant under SDS stress (Manganelli et al., 2001), were upregulated in Rv-D981 (Table 1). Preliminary analyses suggested that expression of these genes in Rv-D981 was influenced by the atmospheric conditions used during culture (data not shown). To analyse this more closely, we performed real-time PCR on Rv1129c, using cultures grown for several weeks either with 5% CO₂

<table>
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<th>Rv no.</th>
<th>Gene/product*</th>
<th>H37Rv†</th>
<th>Rv-D981†</th>
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<tr>
<td>Rv0563</td>
<td>HtpX, heat-shock protein</td>
<td>6.73 ± 1.1</td>
<td>2.96 ± 0.4‡</td>
</tr>
<tr>
<td>Rv1057</td>
<td>Hypothetical protein</td>
<td>28.11 ± 9.4</td>
<td>NS‡</td>
</tr>
<tr>
<td>Rv1129c</td>
<td>Probable transcriptional regulator</td>
<td>NS</td>
<td>10.32 ± 2.8§</td>
</tr>
<tr>
<td>Rv1130</td>
<td>CHP</td>
<td>NS</td>
<td>14.05 ± 2.2</td>
</tr>
<tr>
<td>Rv1131</td>
<td>GltA1</td>
<td>NS</td>
<td>14.03 ± 2.8</td>
</tr>
<tr>
<td>Rv2053c</td>
<td>Putative transmembrane protein</td>
<td>3.47 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Rv2710</td>
<td>SigB</td>
<td>11.63 ± 1.7</td>
<td>3.03 ± 0.4‡</td>
</tr>
<tr>
<td>Rv2743c</td>
<td>Putative transmembrane protein</td>
<td>3.29 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Rv2744c</td>
<td>35 kDa protein</td>
<td>4.56 ± 0.6</td>
<td>1.85 ± 0.2</td>
</tr>
<tr>
<td>Rv2745c</td>
<td>Putative transcription factor</td>
<td>5.34 ± 1.1</td>
<td>2.38 ± 0.2‡</td>
</tr>
<tr>
<td>Rv0981</td>
<td>MprA, transcription factor of TCS</td>
<td>6.13 ± 0.18</td>
<td>0.88 ± 0.39</td>
</tr>
<tr>
<td>Rv0983</td>
<td>PepD, probable serine protease</td>
<td>9.43 ± 2.38</td>
<td>3.90 ± 0.11</td>
</tr>
</tbody>
</table>

†Data show mean fold induction (±SEM), determined by microarrays (Table S4) or real-time PCR analyses, using three different RNA preparations. NS, No significant increase in expression under SDS compared to control as determined by one-class SAM analysis (Tusher et al., 2001).
‡Differences in gene expression patterns between H37Rv and Rv-D981 were confirmed by real-time PCR.
or with normal atmospheric CO₂. Following SDS stress, Rv1129c was slightly induced in H37Rv, independent of the culture conditions (Fig. 5). However, in Rv-D981, Rv1129c expression was markedly increased by SDS stress, but only in cultures grown under normal atmospheric conditions (Fig. 5). We used EMSA to determine whether MprA directly regulates Rv1129c and several other genes of the SigE regulon, including Rv0465, Rv0467, Rv1057 and Rv2745, but did not detect binding of MprA to these promoters (data not shown), indicating that the effects of MprA are indirect.

**Survival phenotype of Rv-D981**

The sigE mutant had lower survival under SDS stress and had reduced growth in macrophages, compared to H37Rv (Manganelli et al., 2001), so we examined survival of Rv-D981 under these conditions. Broth cultures were exposed to 0.05 % SDS, and percentage survival was determined by comparison of c.f.u. with untreated controls for each strain. All strains had reduced survival after 2 h exposure to SDS (data not shown), but Rv-D981 had consistently higher survival rates compared to H37Rv and Rv-D981C (Fig. 6a). Preliminary analyses indicate that Rv-D981 also has increased survival in broth in the presence of deoxycholate (G. Mukamolova, unpublished data).

An mprA insertion mutant of *M. tuberculosis* had higher replication rates than H37Rv in murine macrophages (Zahrt & Deretic, 2001). To investigate growth of the mprAB deletion mutant in human monocytes, we used a low-inoculum assay (Byrd, 1997), and bacterial growth was evaluated over time (Fig. 6b). Rv-D981 multiplied to a significantly greater extent than H37Rv by 4 days post-infection, and continued to grow at significantly higher levels by day 7. Rv-D981C had growth rates similar to that of H37Rv (Fig. 6b), indicating that loss of mprAB was responsible for the enhanced growth of Rv-D981.

**Overexpression of stress-associated genes in Rv-D981**

The basis for the increased survival of Rv-D981 under stress is not clear. However, using the MtbReglist database
(Jacques et al., 2005), we examined the regulatory features of several genes that were upregulated in Rv-D981 under normal growth conditions, and noted that many had either DosR- or SigD-binding sites in their upstream regions (Table 2). Further comparisons with published data (Kendall et al., 2004; Park et al., 2003; Raman et al., 2004) revealed that, of the 30 most-highly expressed genes in Rv-D981, 21 are members of the DosR or SigD regulons (Table 2). Another five genes are adjacent to members of these regulons and may be at least partially regulated by SigD or DosR. The DosR and SigD regulons are associated with growth under stress (Betts et al., 2002; Calamita et al., 2005; Park et al., 2003; Raman et al., 2004; Sherman et al., 2001), and as discussed below, the derepression of some of these genes in Rv-D981 may contribute to the resistance phenotype we observed. We performed gel-shift assays with MprA and promoters of several genes from these regulons, including Rv1738, Rv3131, Rv2626c and Rv3134c, but did not detect binding (data not shown).

Table 2. Genes highly upregulated in exponential phase in Rv-D981 compared to H37Rv

Expression under control conditions was compared using DNA microarray analyses. The 30 genes with the highest mean fold increase in expression in Rv-D981 compared to H37Rv are shown.

<table>
<thead>
<tr>
<th>Rv no.</th>
<th>Gene/product*</th>
<th>Mean fold increase†</th>
<th>Regulatory features‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv3131</td>
<td>CHP</td>
<td>21.34 ± 6.0</td>
<td>R</td>
</tr>
<tr>
<td>Rv1738</td>
<td>CHP</td>
<td>15.56 ± 5.0</td>
<td>R</td>
</tr>
<tr>
<td>Rv3130c</td>
<td>CHP</td>
<td>15.15 ± 3.6</td>
<td>R</td>
</tr>
<tr>
<td>Rv1996</td>
<td>USP</td>
<td>9.91 ± 1.3</td>
<td>R</td>
</tr>
<tr>
<td>Rv3127</td>
<td>CHP</td>
<td>8.98 ± 1.7</td>
<td>R</td>
</tr>
<tr>
<td>Rv1884c</td>
<td>RpFC, resuscitation-promoting factor</td>
<td>5.95 ± 0.6</td>
<td>D +</td>
</tr>
<tr>
<td>Rv2032</td>
<td>Aeg</td>
<td>5.55 ± 1.3</td>
<td>R +</td>
</tr>
<tr>
<td>Rv2626c</td>
<td>CHP</td>
<td>5.24 ± 0.8</td>
<td>R +</td>
</tr>
<tr>
<td>Rv2627c</td>
<td>CHP</td>
<td>4.81 ± 1.4</td>
<td>R +</td>
</tr>
<tr>
<td>Rv0079</td>
<td>Hypothetical protein</td>
<td>4.16 ± 0.9</td>
<td>R +</td>
</tr>
<tr>
<td>Rv1733c</td>
<td>Putative transmembrane protein</td>
<td>4.11 ± 0.5</td>
<td>R +</td>
</tr>
<tr>
<td>Rv1815</td>
<td>CHP</td>
<td>4.04 ± 0.2</td>
<td>D +</td>
</tr>
<tr>
<td>Rv2623</td>
<td>USP</td>
<td>3.91 ± 0.4</td>
<td>R</td>
</tr>
<tr>
<td>Rv3134c</td>
<td>USP</td>
<td>3.69 ± 0.6</td>
<td>R +</td>
</tr>
<tr>
<td>Rv0569</td>
<td>CHP</td>
<td>3.43 ± 0.5</td>
<td>R</td>
</tr>
<tr>
<td>Rv2628</td>
<td>CHP</td>
<td>3.25 ± 0.7</td>
<td>R +</td>
</tr>
<tr>
<td>Rv3229c</td>
<td>Putative linoleoyl-CoA desaturase</td>
<td>3.10 ± 0.4</td>
<td>R +</td>
</tr>
<tr>
<td>Rv1737c</td>
<td>NarK2, nitrate/nitrite transporter</td>
<td>3.09 ± 0.7</td>
<td>R +</td>
</tr>
<tr>
<td>Rv2557</td>
<td>CHP</td>
<td>3.09 ± 0.4</td>
<td>R</td>
</tr>
<tr>
<td>Rv2632c</td>
<td>CHP</td>
<td>3.06 ± 0.3</td>
<td>(D), (R)</td>
</tr>
<tr>
<td>Rv2007c</td>
<td>FdxA, probable ferredoxin</td>
<td>2.92 ± 0.5</td>
<td>R +</td>
</tr>
<tr>
<td>Rv3616c</td>
<td>CHP</td>
<td>2.92 ± 0.2</td>
<td>(D)</td>
</tr>
<tr>
<td>Rv1181</td>
<td>Pks4, polyketide synthase, (msl3)</td>
<td>2.81 ± 0.3</td>
<td>(D)</td>
</tr>
<tr>
<td>Rv3083</td>
<td>Probable monoxygenase</td>
<td>2.78 ± 0.2</td>
<td>(D)</td>
</tr>
<tr>
<td>Rv2160c</td>
<td>CHP</td>
<td>2.69 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Rv1883c</td>
<td>CHP</td>
<td>2.66 ± 0.3</td>
<td>D</td>
</tr>
<tr>
<td>Rv1813c</td>
<td>CHP</td>
<td>2.63 ± 0.2</td>
<td>R +</td>
</tr>
<tr>
<td>Rv3614c</td>
<td>CHP</td>
<td>2.47 ± 0.3</td>
<td>D</td>
</tr>
<tr>
<td>Rv1183</td>
<td>MmpL10, fatty acid transport (putative)</td>
<td>2.38 ± 0.1</td>
<td>(D)</td>
</tr>
<tr>
<td>Rv1814</td>
<td>Sterol desaturase</td>
<td>2.38 ± 0.2</td>
<td>(D), (R)</td>
</tr>
</tbody>
</table>

*From http://genolist.pasteur.fr/TubercuList/; msl3 (Dubey et al., 2002); CHP, conserved hypothetical protein; USP, universal stress protein genes (O’Toole & Williams, 2003).
†Results show mean fold induction (±SEM) of genes with the highest level of expression in Rv-D981 compared to H37Rv, as determined by one-class SAM analysis (Tusher et al., 2001) (Table S4). Only genes showing significant increase in ≥5 hybridizations (Table S4), and which are present in the H37Rv strain of M. tuberculosis, are included.
‡R or D, member of the DosR or SigD regulon, respectively; +, DosR- or SigD-binding site in promoter; (D) or (R), adjacent to gene of the DosR or SigD regulon, respectively. Data obtained from published reports (Raman et al., 2004; Betts et al., 2002; Jacques et al., 2005; Kendall et al., 2004; Park et al., 2003; Sherman et al., 2001).
Table 3. Expression of iron-responsive genes in H37Rv and Rv-D981 under SDS stress

The expression of iron-regulated genes was evaluated in SDS-treated and control cultures of H37Rv and Rv-D981, using DNA microarray analyses. For each gene, results show fold induction under SDS compared to the control sample for the same strain.

<table>
<thead>
<tr>
<th>Rv no.</th>
<th>Gene/product*</th>
<th>H37Rv†</th>
<th>Rv-D981†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv2711</td>
<td>ideR</td>
<td>3.47 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Rv2122c</td>
<td>hisE (igl)</td>
<td>NS</td>
<td>5.21 ± 1.0</td>
</tr>
<tr>
<td>Rv2123</td>
<td>PPE</td>
<td>NS</td>
<td>5.03 ± 1.0</td>
</tr>
<tr>
<td>Rv2381c</td>
<td>mbtD</td>
<td>2.70 ± 0.1</td>
<td>4.03 ± 0.6</td>
</tr>
<tr>
<td>Rv2383c</td>
<td>mbtB</td>
<td>4.74 ± 0.6</td>
<td>7.28 ± 1.4</td>
</tr>
<tr>
<td>Rv3839</td>
<td>CHP</td>
<td>NS</td>
<td>2.00 ± 0.1</td>
</tr>
</tbody>
</table>

*From http://genolist.pasteur.fr/TubercuList/. Published reports (De Voss et al., 1999; Dussurget et al., 1999; Gold et al., 2001; Jacques et al., 2005; Manabe et al., 1999; Prakash et al., 2005) were used to identify genes regulated, or predicted to be regulated, by iron and IdeR. CHP, conserved hypothetical protein.
†Data show mean fold induction (+ SEM), determined by DNA microarray analyses (Table S4), using three different RNA preparations and dye flips. NS, No significant increase in expression under SDS compared to control, as determined by one-class SAM analysis (Tusher et al., 2001).

shown), suggesting that the inhibitory effects of MprA on many of these genes is indirect.

We also noted that, under SDS stress, the iron-repressor gene ideR (Dussurget et al., 1999; Gold et al., 2001) was downregulated in Rv-D981 (Table 3 and Table S4). Therefore, we examined expression of genes known to be repressed by IdeR or with potential IdeR-binding sites in their promotors (Dussurget et al., 1999; Gold et al., 2001; Jacques et al., 2005; Manabe et al., 1999; Prakash et al., 2005). Five such genes, including the mycobactin synthetase genes mbtB and mbtD (De Voss et al., 1999), were more highly upregulated in Rv-D981 than in H37Rv under SDS stress (Table 3).

DISCUSSION

The controlled expression of regulatory networks and metabolic systems is necessary for bacteria to adapt efficiently to changing environmental conditions. Our investigations have shown that the MprAB TCS activates the SigE regulon under SDS stress. However, our results also indicate that, in the absence of MprAB, many stress-associated genes are upregulated under normal growth conditions, suggesting that the MprA regulon contributes to maintaining a balance among several systems involved in stress resistance.

In this work, we constructed a deletion mutant, Rv-D981, which lacks portions of mprA and mprB, thus confirming that both genes are nonessential. We then determined, by real-time PCR, DNA microarrays, and primer extension analyses, that MprAB activates sigE, sigB and mprA under SDS stress, and contributes to maintaining basal expression levels of these genes during exponential growth. Normal patterns of expression were restored in the complemented strain, Rv-D981C, confirming that deletion of mprA and mprB was responsible for the changes in transcription patterns. We also identified the TSP for mprA, as well as a new MprA-inducible TSP for sigE, and showed that, under SDS stress, MprA induces transcription from the same TSPs as are used at low levels under normal growth conditions. EMSAs with recombinant MprA showed direct interaction with the sigE, sigB and mprA promoters, and determined that a fragment of 29 bp containing MprA-binding sites was of sufficient length for MprA binding. Other potential MprA-binding sites were identified in the genome, suggesting that MprA may directly regulate additional genes. DNA microarrays showed that many genes of the SigE regulon (Manganelli et al., 2001) were downregulated in the MprAB mutant, consistent with activation of sigE by MprA. Notable exceptions were the genes Rv1129c–Rv1130, which were more highly upregulated under SDS stress in Rv-D981 than in H37Rv, but only in normal atmospheric conditions.

As summarized in Fig. 7, components of several stress-associated regulons are upregulated in Rv-D981. Although the basis for this upregulation is unknown, the MprA regulon may have a repressive effect on some stress regulons, perhaps as a mechanism for keeping a balanced response to environmental stress. Alternatively, these other stress-response systems may be upregulated to compensate for the loss of a functional MprAB TCS, and reduced expression of the SigE regulon (Table 1). However, in contrast to a sigE deletion mutant (Manganelli et al., 2001), Rv-D981 had increased resistance to SDS and enhanced growth in human monocytes (this study), and, furthermore, an insertion mutant of mprA had increased growth in resting murine macrophages (Zahrt & Deretic, 2001), findings which suggest that MprAB has functions independent of its role in activating the SigE regulon.

Deletion of some TCSs can result in hypervirulence (Parish et al., 2003). In the case of Rv-D981, we hypothesize that the higher expression levels of a cohort of stress-associated genes may produce physiological changes that allow the mutant to survive better when exposed to certain environmental stresses. For example, the iron-regulated gene, mbtB, which is involved in synthesis of the siderophore mycobactin and which is upregulated in Rv-D981 (Table 3), is important for growth under low-iron conditions and in monocytes (De Voss et al., 2000). Also, analyses of gene expression in Rv-D981 under normal growth conditions (Table 2 and Table S4), revealed that 21 of the most highly upregulated genes are part of either of the DosR (Kendall et al., 2004; Park et al., 2003) or SigD regulons. In contrast, only two of these genes, Rv1884c and Rv3616c, were upregulated by ≥2-fold in the sigE mutant during exponential-phase
growth (Manganelli et al., 2001). Several other genes of the SigD and DosR regulons are also more highly expressed in Rv-D981 under SDS stress (Table S4 and data not shown).

Genes of the sigD regulon are associated with nutrient starvation and stationary phase (Betts et al., 2002; Calamita et al., 2005; Raman et al., 2004), and sigD mutants of M. tuberculosis are attenuated (Calamita et al., 2005; Raman et al., 2004). Genes of the DosR (also called DevR) regulon are upregulated under hypoxia (Park et al., 2003; Sherman et al., 2001) and other stress conditions (Karakousis et al., 2004; Kendall et al., 2004; Ohno et al., 2003; Voskuil et al., 2003), and contribute to survival under low-oxygen conditions (Boon & Dick, 2002). Genes of these regulons which could contribute to enhanced survival include those encoding the universal stress proteins, as well as NarK2 and RpfC (Table 2).

Universal stress proteins are associated with various mechanisms of stress resistance (Nachin et al., 2005; O’Toole & Williams, 2003), including long-term survival of M. tuberculosis under low-oxygen conditions (Voskuil et al., 2003). NarK2 (Rv1737c) is important for nitrate reductase activity (Sohaskey & Wayne, 2003), and may be involved in adaptation to nitric oxide stress in macrophages (Shi et al., 2005). RpfC is one of five M. tuberculosis proteins with similarity to Rpf (resuscitation-promoting factor) of Micrococcus luteus, which are important for stimulation of growth of M. tuberculosis in broth, in recovery of dormant mycobacteria, and in virulence (Downing et al., 2005; Mukamolova et al., 2002; Tufariello et al., 2006). Recent studies suggest that Rpf-like proteins are muralytic enzymes involved in remodelling of the bacterial cell wall (Cohen-Gonsaud et al., 2005; Mukamolova et al., 2006). In addition, several genes involved in fatty acid metabolism are upregulated in Rv-D981 (Table 2 and Table S4), and evidence indicates that two of these genes, Rv1180 and Rv1181, form a single large gene, named msl3, which encodes an enzyme involved in synthesis of lipid components of the cell wall (Dubey et al., 2002). Increased expression of rpfC, msl3, and other genes in Rv-D981 could potentially induce structural changes in the cell wall that enhance survival when the mutant is exposed to detergents or during growth in monocytes. Although an mprA insertion mutant showed decreased persistence during the late stage of infection in mice (Zahrt & Deretic, 2001), it is conceivable that the alterations in gene expression patterns in mprA mutants may provide some benefit early during infection, and in some other stresses, and yet be deleterious during the persistent phase of infection.

The putative transcriptional regulator, Rv1129c (Cole et al., 1998), was also highly upregulated in Rv-D981 under SDS stress, but this upregulation was abrogated in the presence of 5% CO₂. The role of Rv1129c is unknown, but it may regulate the adjacent genes, Rv1130 and Rv1131, which showed a similar pattern of expression. Interestingly, this cluster of genes is also upregulated in the phagosomes of infected murine macrophages, and Rv1129c and Rv1131 are active in infected mice (Schnappinger et al., 2003). The function of Rv1130 is unknown, but the protein has been detected in phagosomes and may contribute to intracellular survival (Mattow et al., 2006). Rv1131 (gltA1) is predicted to encode citrate synthase, an enzyme of the TCA (Krebs) cycle (Cole et al., 1998). gltA of E. coli is negatively regulated by the ArcAB TCS, and expression of the gene is sensitive to changes in oxygen levels (Shalel-Levanon et al., 2005). Our data suggest that expression of the Rv1129c–Rv1131 gene cluster may also be influenced by oxygen levels, and that, under some conditions, the gene cluster is negatively regulated when MprAB is intact.

For each of sigE, sigB and mprA, an MprA-inducible TSP was identified in H37Rv, which was also active in Rv-D981, albeit at lower levels. These data suggest that MprA functions by modulating the activity of the RNA holoenzyme at weak, but active, promoters. We found two SDS-inducible TSPs for sigE, although only the newly identified TSP4 was dependent on MprA. For sigB, only a single TSP was identified, the location of which was consistent with the
previously reported site (Manganelli et al., 2002; Raman et al., 2001), thus placing the end of the MprA-binding site approximately 50 bases upstream of the predicted −35 region (Manganelli et al., 2002; Raman et al., 2001). In contrast, the MprA-binding site in the mprA promoter overlaps the −35 region, and in the sigE promoter, the −35 region for TSP4 is several bases downstream of the MprA-binding site. Therefore, perhaps similar to the catabolite activator protein (CAP) of E. coli (Busby & Ebright, 1999), MprA may have different mechanisms of interaction with the RNA holoenzyme and/or other proteins. With class 1 CAP-dependent promoters, the CAP-binding site is located upstream of the −35 region, whereas with class 2 promoters, the CAP-binding site overlaps the −35 region (Busby & Ebright, 1999). Different protein–protein interactions between CAP and the RNA polymerase are involved at these two classes of promoters. However, the interactions between MprA and other regulatory factors remain to be elucidated.

While this paper was in preparation, another group published work on an MprA insertion mutant showing that MprA regulates sigE and sigB (He et al., 2006). Using DNA microarray analyses, they detected significant changes in gene expression in the mutant under detergent stress, and observed that sigE and sigB were downregulated in the mutant during exponential growth. They also localized MprA-binding sites in the sigE and sigB promoters using DNA footprinting, and their data are consistent with our findings, although they detected an additional weak MprA-binding site in the sigE promoter which we would not have detected using our approach. In contrast to our results, they did not detect a reduction in sigB expression in the mutant under SDS stress, although sigE was downregulated in their analyses as well as in ours. The basis for this discrepancy is not entirely clear, but they normalized their real-time PCR results to sigA expression, which may have resulted in reduced sensitivity, as sigA expression can change under certain conditions (Wu et al., 2004). However, as sigB was downregulated in a sigE mutant under SDS stress (Manganelli et al., 2001), our findings are consistent with earlier studies. It is also possible that, as mprB was intact in their mutant (He et al., 2006), MprB may activate another response regulator which activates sigE under SDS stress. Interestingly, three genes of the DosR regulon (Rv2626c–Rv2628), which were upregulated in our mutant (Table 2), were downregulated in the mprA insertion mutant (He et al., 2006), suggesting that there may be some distinct differences in gene regulation between the two mutants.

Overall, both studies show the importance of MprA in the response to specific stresses. At the outset of our investigations, we had hypothesized that, as the only TCS in the SigE regulon, MprAB may activate SigE regulon, and this has been supported by our findings. MprAB is also the only TCS within the iVEGI region, which is highly active in vivo models (Jain et al., 2006; Talaat et al., 2004), so we speculate that MprAB may activate iVEGI genes in vivo. However, further investigations are required to elucidate the obviously complex role of this TCS in vivo and under other stress conditions.

ACKNOWLEDGEMENTS

We thank Peter F. Barnes for support of this work, helpful discussions, and critical reading of the manuscript. We also thank Hassan Safi, Luc Gaudreau and Sebastien Rodrigue for technical suggestions. This research was supported by the Department of Veterans Affairs and a DeSouza Research Award from the American Lung Association (to T.F.B.) and the Margaret E. Byers Cain Chair for Tuberculosis Research (to P. F. Barnes), a Potts Memorial Foundation Grant, UTHCT Research Council Seed Grant, and NIH grant R21 AI063229-01 (to S. T. H.).

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Edited by: T. Parish