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.

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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; Manganeli *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.

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

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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 autoregulatory 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* (Mecenas *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, similar to 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* Novablue 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 *ScaI/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 *PacI* 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 981UP5A and 982XP-3. The amplicon was inserted into pSTBlue-1 (Novagen), sequenced, and then the insert was released by digestion with *SnaBI*, 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 *HpaI* and *XbaI*. The resulting plasmid, p981-COM, was electroporated 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 predicted 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 Variflex

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, 150 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 (EMSAs). 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 sections 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 [γ -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 unlabelled competitor DNA was included. Reaction mixtures were loaded onto 5–6% nondenaturing polyacrylamide gel and electrophoresed for 2–3 h at 140 V at 4 °C in 0.5× TBE buffer. A 278 bp fragment, which was located near Rv0980c, did not bind MprA and was used as a negative control in competition assays.

RNA isolation. Mycobacterial strains were cultured in 7H9 medium to mid-exponential phase (OD₆₀₀ 0.4–0.5) at 37 °C with shaking, in the absence of CO₂, except as indicated. Total RNA was isolated using TRIzol LS Reagent (Invitrogen) according to the manufacturer's instruction, except that lysing matrix B and a FastPrep FP120 shaker (BIO 101) were used to disrupt the mycobacteria. With large culture volumes, bacteria were first pelleted by centrifugation and resuspended in a small volume of 0.2% Tween, prior to adding to the lysing tubes. Chromosomal DNA was removed with 'DNA-free' reagents (Ambion), according to the manufacturer's instructions. For experiments with detergent stress, cultures were grown to exponential phase (OD₆₀₀ 0.3–0.4), SDS was added to a final concentration of 0.05%, and RNA was extracted from control and SDS-treated samples after 90 min incubation at 37 °C.

Primer extension analysis. Primers 981PE1 and 981PE3 were used to analyse *mprA* transcripts, and SigEPE1 and SigEPE2 were used to analyse TSP4 and TSP3, respectively, of the *sigE* promoter. Primers were labelled with [γ -³²P]ATP by T4 polynucleotide kinase as indicated above. For the annealing step, 17 µg of *M. tuberculosis* RNA was denatured at 90 °C for 5 min, snap-cooled on ice, and then incubated for 20 min with 2 pmol of labelled primers in 1× reverse transcription buffer [50 mM Tris/HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 1 mM each dNTP and 0.5 mM spermidine]. Annealing temperatures were adjusted for each primer. Primer extension was performed using the Primer extension system-AMV reverse transcriptase kit (Promega) as directed by the manufacturer,

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 Rnasin 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 from buffy coats purchased from United Blood Services and cultured as described by Byrd & Horwitz (1989). After 48 h, cells were harvested and monocytes isolated by adherence to Primaria flat-bottomed wells (24 multiwell Falcon plates, Becton Dickinson), 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 readdition 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 Rv-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-L-lysine-coated glass microscope slides using a GeneMachines Omnigrid 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

Fig. 1. Impact of Rv-D981 deletion on gene expression. (a) Map of the H37Rv *MprAB* region and location of Rv-D981 deletion (hatched bar). A portion of *pepD* is included. The arrowhead marks the location of *mprA* primers and probe used in real-time PCR. E and B mark *EcoRI* and *BamHI* sites used in Southern blot verification of the deletion. Vertical lines mark 1 kb segments. (b–d) Expression of *mprA* (b), *sigE* (c) and *sigB* (d) was examined by real-time PCR using RNA from H37Rv (grey bars), Rv-D981 (black bars), and Rv-D981C (white bars). C, control cultures; SDS, SDS-treated cultures. Results were normalized for 16S RNA content and are shown as fold change over the H37Rv control, which was given a value of 1. Data are the mean $\pm$ SD of three or more separate experiments with H37Rv and Rv-D981, and two or more experiments with Rv-D981C.

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

Fig. 2. Transcriptional analyses of the *mprA* promoter. (a) The *mprA* TSP (arrow) was mapped by primer extension using H37Rv RNA and primer 981PE3. Sequence ladder was generated using 981PE3. (b) Sequence of the *mprA* promoter beginning 41 bases upstream of the TSP (G with asterisk). MprA-binding motifs (He & Zahrt, 2005) (underlined) and the predicted GTG start codon (underlined, bold) are marked. (c) Primer extension results using primer 981PE3 and RNA from cultures exposed (+) or not exposed (-) to SDS. Rv, H37Rv; D981, Rv-D981; D981C, Rv-D981C. Bands in Rv-D981 were weakly visible and were of similar intensity in each condition.

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 *sigB* TSP (Fig. 4b, c, and data not shown), and a search of the

Fig. 3. Analyses of the *sigE* promoter. (a) Map of *sigE* TSPs. Bent arrows mark four identified TSPs (see text for details). Double-headed 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. b, 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. *PmprA*, *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.

Tubercult database with the published MprA-binding sequence (He & Zahrt, 2005) identified potential MprA target sites between positions –102 and –83. 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).

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 in SDS, whereas 44 genes were more highly upregulated in Rv-D981 (Table S3), indicating that MprA has both positive and negative effects on gene expression patterns. We examined the expression

patterns of the genes with potential MprA-binding sites (Table S2), but did not detect any overall similarity in their expression profiles in Rv-D981 (Table S4 and data not shown).

Based on their reduced expression in the *sigE* mutant under SDS stress, 23 genes were reported to be directly or indirectly regulated by SigE (Manganelli *et al.*, 2001). DNA microarray analyses showed that ten of these genes were markedly downregulated in Rv-D981 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 1. Expression patterns of SigE-regulated genes in H37Rv and Rv-D981 under SDS stress

The expression of SigE-regulated genes (Manganelli *et al.*, 2004) was evaluated in SDS-treated and control cultures of H37Rv and Rv-D981, using array analyses or real-time PCR (qPCR). For each gene, results show fold induction under SDS compared to the control sample for the same strain.

Rv no.	Gene/product*	H37Rv†	Rv-D981†
Array data			
Rv0251c	Acr2, (Hsp) α -crystallin 2 stress protein	44.10 $\pm$ 11.9	9.83 $\pm$ 2.0‡
Rv0465c	Putative transcription factor	14.54 $\pm$ 4.8	4.28 $\pm$ 0.8‡
Rv0467	Icl, isocitrate lyase	6.74 $\pm$ 1.6	4.03 $\pm$ 0.4‡
Rv0563	HtpX, heat-shock protein	6.73 $\pm$ 1.1	2.96 $\pm$ 0.4‡
Rv1057	Hypothetical protein	28.11 $\pm$ 9.4	NS‡
Rv1129c	Probable transcriptional regulator	NS	10.32 $\pm$ 2.8‡
Rv1130	CHP	NS	14.05 $\pm$ 2.2
Rv1131	GltA1	NS	14.03 $\pm$ 2.8
Rv2053c	Putative transmembrane protein	3.47 $\pm$ 0.6	NS
Rv2710	SigB	11.63 $\pm$ 1.7	3.03 $\pm$ 0.4‡
Rv2743c	Putative transmembrane protein	3.29 $\pm$ 0.5	NS
Rv2744c	35 kDa protein	4.56 $\pm$ 0.6	1.85 $\pm$ 0.2
Rv2745c	Putative transcription factor	5.34 $\pm$ 1.1	2.38 $\pm$ 0.2‡
qPCR data			
Rv0981	MprA, transcription factor of TCS	6.13 $\pm$ 0.18	0.88 $\pm$ 0.39
Rv0983	PepD, probable serine protease	9.43 $\pm$ 2.38	3.90 $\pm$ 0.11

*From <http://genolist.pasteur.fr/TubercuList/>; CHP, conserved hypothetical protein.

†Data show mean fold induction ($\pm$ 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

Fig. 5. Expression pattern of Rv1129c under different growth conditions. Cultures of H37Rv and Rv-D981 were grown for several weeks at 37 °C under normal atmospheric conditions or with 5 % CO₂. Aliquots were treated with 0.05 % SDS for 90 min or left untreated and then RNA was extracted and examined by real-time PCR. Results show the mean $\pm$ SD of triplicate experiments. White bars, H37Rv control; dark grey bars, H37Rv with SDS; light grey bars, Rv-D981 control; black bars, Rv-D981 with SDS.

Fig. 6. Rv-D981 has increased stress resistance. (a) Cultures were incubated with 0.05 % SDS and aliquots were removed after 2 h or 5 h incubation, washed, and plated in duplicate. Control (untreated) aliquots were plated at time zero. C.f.u. were determined after 14 days. Due to variability in the absolute number of c.f.u. between experiments, data were calculated as percentage survival compared to untreated controls for each strain. The percentage survival of H37Rv at each time point was given a value of 100 % and this was compared to the percentage survival of the other two strains. H37Rv, white bars; Rv-D981, dark grey bars; Rv-D981C, light grey bars. Results show the mean $\pm$ SEM from three separate experiments for H37Rv and Rv-D981, and two experiments for Rv-D981C. (b) Growth of Rv-D981 is increased in human monocytes. Human monocyte monolayers were infected with a bacteria: monocyte ratio of 0.2 : 1. At 2, 4 and 7 days, c.f.u. in supernates and monocyte lysates were determined. C.f.u. in the supernates and lysates were assayed individually and values were added together to give total c.f.u. per well at each time point (Byrd, 1997). Data are mean $\pm$ SEM for duplicate determinations in three separate experiments using monocytes from different donors. *, statistically significant difference ($P < 0.05$) in growth of Rv-D981 compared to H37Rv and Rv-D981C at 4 and 7 days. Δ, H37Rv; ○, Rv-D981; □, Rv-D981C.

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

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.

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

*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.

Rv no.	Gene/product*	H37Rv†	Rv-D981†
Rv2711	<i>ideR</i>	3.47 ± 0.3	NS
Rv2122c	<i>hisE (irg1)</i>	NS	5.21 ± 1.0
Rv2123	PPE	NS	5.03 ± 1.0
Rv2381c	<i>mbtD</i>	2.70 ± 0.1	4.03 ± 0.6
Rv2383c	<i>mbtB</i>	4.74 ± 0.6	7.28 ± 1.4
Rv3839	CHP	NS	2.00 ± 0.1

*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 ($\pm$ 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 promoters (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

Fig. 7. Schematic diagram showing altered expression pattern of stress-associated regulons in Rv-D981. Ovals with black arrows indicate upregulation, and the hexagon with a crossed arrow indicates downregulation. Stresses associated, or potentially associated, with genes/regulons are indicated.

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 *sigB* 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 the 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.

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