The MprB Extracytoplasmic Domain Negatively Regulates Activation of the Mycobacterium tuberculosis MprAB Two-Component System

Daniel J. Bretl,a,b Tarin M. Bigley,a,b Scott S. Terhune,a,c Thomas C. Zahrt,a,b

Department of Microbiology and Molecular Genetics,a Center for Infectious Disease Research,b and Biotechnology and Bioengineering Center,c Medical College of Wisconsin, Milwaukee, Wisconsin, USA

Mycobacterium tuberculosis is an acid-fast pathogen of humans and the etiological agent of tuberculosis (TB). It is estimated that one-third of the world’s population is latently (persistently) infected with M. tuberculosis. M. tuberculosis persistence is regulated, in part, by the MprAB two-component signal transduction system, which is activated by and mediates resistance to cell envelope stress. Here we identify MprAB as part of an evolutionarily conserved cell envelope stress response network and demonstrate that MprAB-mediated signal transduction is negatively regulated by the MprB extracytoplasmic domain (ECD). In particular, we report that deregulated production of the MprB sensor kinase, or of derivatives of this protein, negatively impacts M. tuberculosis growth. The observed growth attenuation is dependent on MprAB-mediated signal transduction and is exacerbated in strains of M. tuberculosis producing an MprB variant lacking its ECD. Interestingly, full-length MprB, and the ECD of MprB specifically, immunoprecipitates the Hsp70 chaperone DnaK in vivo, while overexpression of dnaK inhibits MprAB-mediated signal transduction in M. tuberculosis grown in the absence or presence of cell envelope stress. We propose that under non-stress conditions, or under conditions in which proteins present in the extracytoplasmic space are properly folded, signaling through the MprAB system is inhibited by the MprB ECD. Following exposure to cell envelope stress, proteins present in the extracytoplasmic space become unfolded or misfolded, leading to removal of the ECD-mediated negative regulation of MprB and subsequent activation of MprAB.
TABLE 1 Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Application</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>Laboratory strain</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc²155</td>
<td>Laboratory strain</td>
<td>Protein expression</td>
<td>ATCC 27294</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F⁻ ΔphoA ΔK1888::Tn10 ΔlacY1ΔargF169 recA1 endA1 hsdR17 (rK− mK+) pphA1 supE44 Δthi-1 gyrA96 relA1</td>
<td>Cloning</td>
<td>Lab collection</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>F⁻ ΔmcrA Δ(nrr-hsdRMS-mcrCB) ΔIIacZΔM15 ΔlacX74 recA1 araD1939 Δ(arab-leu)7697 galU1 galK1 rpsL1 (Str') endA1 nupG</td>
<td>Cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB94</td>
<td><em>M. tuberculosis</em> H37Rv with pMC1s (TetR)</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB129</td>
<td><em>M. tuberculosis</em> H37Rv ΔsigE</td>
<td>Protein expression</td>
<td>8</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB153</td>
<td><em>M. tuberculosis</em> H37Rv with pSE100</td>
<td>Protein expression</td>
<td>29</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB170</td>
<td><em>M. tuberculosis</em> H37Rv ΔmprAB</td>
<td>Protein expression</td>
<td>8</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB254</td>
<td><em>M. tuberculosis</em> H37Rv with pTZ1154</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB257</td>
<td>TB94 with pTZ1153</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB259</td>
<td>TB94 with pSE100</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB260</td>
<td>TB94 with pTZ1275</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB266</td>
<td>TB94 with pTZ1210</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB271</td>
<td><em>M. tuberculosis</em> H37Rv ΔmprAB with pTZ1153</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB273</td>
<td><em>M. tuberculosis</em> H37Rv ΔmprAB with pTZ1275</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> TB275</td>
<td><em>M. tuberculosis</em> H37Rv ΔmprAB with pTZ1210</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. smegmatis</em> TCZ1574</td>
<td><em>M. smegmatis</em> mc²155 with pSE100</td>
<td>Protein expression</td>
<td>29</td>
</tr>
<tr>
<td><em>M. smegmatis</em> TCZ1778</td>
<td><em>M. smegmatis</em> mc²155 with pTZ1153</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. smegmatis</em> TCZ1780</td>
<td><em>M. smegmatis</em> mc²155 with pTZ1154</td>
<td>Protein expression</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmids

- pCR2.1-TOPO: 3.9-kb plasmid for cloning PCR products; Amp° Kan’
- pMV306: 3.9-kb plasmid for single-copy integration into mycobacterial chromosome; Kan’
- pSE100: TetR-regulated protein expression vector; Hyg’
- pMC1s: Plasmid for single-copy integration into mycobacterial chromosome of tetR; Kan’
- pET24b: Vector containing 3×Flag at NdeI; Kan’
- pSE100 containing mprB: Protein expression
- pSE100 containing mprB-ECO: Protein expression
- pSE100 containing dnaK: Protein expression
- pSE100 containing mprB-ECO: Protein expression

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains and plasmids used in the study are described in Table 1. Escherichia coli TOP1 (Invitrogen, Carlsbad, CA) or DH5α was used for cloning. All *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar (Thermo Scientific, Rockford, IL) supplemented with 50 μg/ml kanamycin sulfate (Thermo Scientific, Rockford, IL) or 150 μg/ml hygromycin B (AG Scientific, San Diego, CA) when necessary. The Mycobacterium strains used in this study are derivatives of *M. tuberculosis* H37Rv (ATCC 27294) or *M. smegmatis* mc²155 (ATCC 700084). Mycobacterium strains were grown with shaking (150 rpm) at 37°C in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ) or on Middlebrook 7H10 agar medium (Difco, Franklin Lakes, NJ) supplemented with 0.5% glycerol, 10% albumin-dextran-catalase (ADC) or oleic acid–ADC (OADC) (Difco, Franklin Lakes, NJ), and 0.05% Tween 80 (Sigma, St. Louis, MO) unless otherwise noted. For protein production, Mycobacterium strains were grown in glycerol alanine salts (GAS) (25) or Sauton’s medium (26), supplemented with 25 μg/ml kanamycin.

enzyme complex (20); and espA, encoding a secreted protein of the ESX system (21). Several of these determinants have been linked to physiological processes in vitro (17, 22, 23) and/or to the virulence of *M. tuberculosis in vivo* (17, 23, 24). Importantly, MprA also directly regulates the expression of several transcription factors, including the alternative sigma factors encoded by sigB and sigE (6, 9). Thus, the regulon controlled by MprAB is complex, and the regulation mediated by MprA occurs through both direct and indirect mechanisms.

While conditions leading to MprAB activation have been identified and determinants of the regulon controlled by MprAB delineated, the specific molecular mechanism by which MprAB activation is regulated remains undefined. We demonstrate here that genetic components of the cell envelope stress response network of *M. tuberculosis*, in which mprAB is a prominent player, are well conserved across *Mycobacterium* and non-*Mycobacterium* species. We also show that deregulated production of the SK MrpB, or of derivatives of this protein, negatively impacts the growth of *M. tuberculosis*. The observed growth attenuation is dependent on MprAB-mediated signal transduction and is exacerbated by the production of an MprB variant lacking its ECD. We also identify Hsp70 chaperone DnaK as an *in vivo* interactant of the MprB ECD and demonstrate that overexpression of dnaK inhibits MprAB-mediated signal transduction in *M. tuberculosis* grown in the absence or presence of cell envelope stress. Taken together, these data support a model in which the ECD of MprB negatively regulates MprAB-mediated signal transduction under physiological conditions. Upon exposure to conditions leading to the accumulation of unfolded or misfolded proteins in the extracytoplasmic space, the negative regulation of MprB is abrogated, leading to MprAB activation and downstream gene regulation.
sulfate (Thermo Scientific, Rockford, IL) or 50 μg/ml hygromycin B (Ag Scientific, San Diego, CA) when required.

**DNA manipulations.** Restriction enzyme digestion, cloning, subcloning, and DNA electrophoresis were carried out according to standard techniques (27). Oligonucleotides and primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and are listed in Table S1 in the supplemental material. PCR was performed using high-fidelity Platinum PCR SuperMix, Platinum Pfu, or Taq polymerase (Invitrogen, Carlsbad, CA). All amplified products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and were sequenced to confirm the absence of mutations. Cloned inserts were removed by restriction enzyme digestion, resolved on a 1% agarose gel, recovered by gel purification, and subcloned into the appropriate vector. Ligations were performed using the Quick Ligation kit or T4 DNA ligase (both from New England Biolabs, Beverly, MA). When necessary, plasmid DNA was treated with Antarctic phosphatase (New England Biolabs, Beverly, MA) to prevent the reversion of vector ends. Electrocopment cells of *M. smegmatis* or *M. tuberculosis* were prepared, and plasmid DNA was electroporated, as described previously (28). Plasmid DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen, Venlo, The Netherlands) as recommended by the manufacturer. DNA fragments were purified using either the QIAquick gel extraction kit or the QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands). DNA sequencing was performed with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, CA) using an automated long capillary method (ABI Prism 3500 genetic analyzer; Applied Biosystems, Carlsbad, CA).

**Extraction of DNA and RNA.** Mycobacterial genomic DNA was isolated as described previously (28). For RNA isolation, *M. tuberculosis* was grown in 7H9 broth to mid-log phase (optical density at 600 nm [OD600], 0.5). For some assays, bacterial cultures were exposed to 0.05% sodium dodecyl sulfate (SDS; Thermo Scientific, Rockford, IL) for 90 min to induce MprAB activation (6). RNA was fixed by treating cultures with RNAlater (Ambion, Austin, TX) for at least 12 h at 4°C prior to processing. Bacteria were collected by centrifugation and were mechanically disrupted. RNA was then extracted with chloroform, precipitated with isopropanol, and stored at −20°C until use. The resulting RNA was suspended in diethyl pyrocarbonate (DEPC)-treated water, treated with Turbo DNase (Ambion, Austin, TX) to remove contaminating genomic DNA, and purified using an RNeasy Miniprep column (Qiagen, Valencia, CA).

Bioinformatic analysis of cell envelope stress response determinants. Primary amino acid sequences of *M. tuberculosis* proteins involved in the cell envelope stress response, including MprA, MprB, SigE, ClgR, Rv2744c, Rv2743c, and PknB (tuberculist.epfl.ch), were used to search available online databases at NCBI (blast.ncbi.nlm.nih.gov) and the Pasteur Institute (genolist.pasteur.fr). The protein topology of MprB homologs was determined by utilization of the Conserved Domain Database, also available at NCBI (www.ncbi.nlm.nih.gov/Structure). Clustal W alignments of proteins were performed using DNAStar Lasergene software (DNAStar, Madison, WI).

**Construction of epitope-tagged fusion proteins.** The construction of *M. tuberculosis* proteins containing N-terminal 3×Flag and C-terminal 6×His epitope tags has been described previously (29). In general, entire open reading frames or individual domains were amplified from purified *M. tuberculosis* genomic DNA and were cloned into vector pTZ8124 (a pET24b derivative allowing incorporation of a 3×Flag epitope tag at the 5′ end of the cloned gene). The mprBseq-α allele was constructed in the following manner: the 5′ terminus, containing the N-terminal cytoplasmic portion and the first transmembrane domain of MprB, was amplified with primers MprBFor-2 and mprBdelExCell-R; the 3′ terminus, containing the second transmembrane domain and the C-terminal cytoplasmic portion of MprB, was amplified with primers mprBdelExCell-F and MprBRev-2; finally, primers MprBFor-2 and MprBRev-2 were used to conduct overlap PCR using the purified PCR products as the template.

The resulting construct produces an MprB derivative identical to the wild-type SK but excluding all but 10 amino acids of the ECD (the 5 amino acids proximal to each transmembrane domain remain intact). All final coding sequences were verified by DNA sequencing and were subsequently PCR amplified from pTZ8124 for directional subcloning into pSE100 (30). This vector is an *E. coli-Mycobacterium* Shuttle plasmid that contains the highly expressed nyc promoter upstream of the Tet operator site, allowing regulated expression upon the addition of anhydrotretyracine (ATC) (Sigma, St. Louis, MO). Where indicated, pSE100-based constructs were electroporated into *M. tuberculosis* strains expressing tetR carried on the integrative plasmid pMCIs (31).

**Expression, fractionation, and localization of endogenous or epitope-tagged proteins in *Mycobacterium* strains.** Wild-type *M. smegmatis* or *M. tuberculosis* or recombinant derivatives containing pSE100-based expression constructs were initially grown in 7H9 broth medium to high turbidity, centrifuged to pellet the bacteria, washed once with phosphate-buffered saline (PBS), and resuspended into GAS (*M. smegmatis*) or Sauton’s (*M. tuberculosis*) medium. Cultures were allowed to grow to late-log/early-stationary phase (OD600 = 1.0). Bacteria were pelleted and washed with PBS. Nonsecreted proteins were obtained by suspending the bacterial pellet in PBS containing 1% Triton X-100 (Sigma, St. Louis, MO) and a protease inhibitor cocktail (Sigma, St. Louis, MO) and mechanically disrupting the bacteria by bead beating. Cell debris was pelleted by centrifugation at 10,000 × g for 5 min. For cell lysates generated from *M. tuberculosis*, protein-containing samples were serially passed through two low-protein-binding Spin-X columns (with 0.2-μm pore-size filters; Corning, Corning, NY) prior to removal from the biosafety level 3 (BSL3) laboratory. Whole-cell lysates were further separated into cell wall, cell membrane, and cytosolic fractions by differential ultracentrifugation at 27,000 × g for 1 h to generate cell wall fractions and then at 100,000 × g for 4 h at 4°C to separate the cell membrane fraction from the cytosolic fraction as described previously (32). For some samples, the cell wall and cell membrane were fractionated as one by centrifugation at 100,000 × g for 2 h at 4°C. Protein lysates were added to 2× SDS-PAGE loading dye, boiled for 5 min, separated on 12% SDS-PAGE gels, and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked in TTBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.5% Tween 20) containing 5% skim milk for at least 1 h and were probed with antisera diluted in TTBS overnight at 4°C. Antiseras included anti-Flag (dilution, 1:1,500) (Sigma, St. Louis, MO), anti-His (1:1,500) (Covance, Princeton, NJ), anti-DnaK (1:500) (IT-40; BEI Resources, NIAID, NIH), or anti-GroEL (1:1,000) (BEI Resources, NIAID, NIH). Membranes were washed in TTBS and were incubated for 1 h at room temperature with a goat anti-mouse IgG (1:10,000; Pierce, Rockford, IL) or goat anti-mouse IgM (1:5,000; Thermo Scientific, Rockford, IL) secondary antibody conjugated to horseradish peroxidase. Blots were developed using the SuperSignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL) and were visualized on CL-XPosure X-ray film (Thermo Scientific, Rockford, IL).

**RT-PCR.** For reverse transcription-PCR (RT-PCR), cDNA from *M. tuberculosis* strains was prepared by incubating 500 ng total RNA with random *M. tuberculosis* decamers (33) in a reverse transcription reaction mixture containing SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). To test for the presence of RNA contamination, identical reactions were also run without SuperScript III. For quantitative RT-PCR (qRT-PCR), reaction mixtures included primer sets that amplified 100- to 150-bp fragments from specific coding sequences. Amplification conditions consisted of 40 rounds of denaturation at 95°C for 30 s and annealing/extension at 60°C for 15 s using iQ SYBR green Supermix (Bio-Rad, Hercules, CA). Reactions were run on an iCycler IQ real-time PCR detection system (Bio-Rad, Hercules, CA). Single PCR products were confirmed by performing postamplification melt curve analysis for each reaction. For relative quantification of transcript levels, target genes were normalized to the housekeeping gene *sigA* and/or the ribosomal gene *rrs*. 

January 2014 Volume 196 Number 2 jb.asm.org
Immunoprecipitation of 3×Flag-tagged proteins in *M. smegmatis* and identification of protein–protein interactions. *M. smegmatis* protein fractions were collected from experimental or control strains expressing 3×Flag epitope-tagged proteins as described previously (29). Briefly, whole-cell lysates were initially prepared as described above. To each sample, a 50-μl slurry of washed anti-Flag M2 agarose beads (Sigma, St. Louis, MO) was added, and the mixture was rocked at 4°C for 2 h. Beads were washed twice with PBS–1% Triton X-100, and proteins were eluted using 90 μM Flag peptide (Sigma, St. Louis, MO) suspended in PBS–1% Triton X-100. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and were visualized either by staining with GelCode Blue (Thermo Scientific, Rockford, IL), by silver staining, or by Western blotting. For analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS), protein samples were briefly run in the resolving gel to remove the Flag peptide. The remaining protein was excised from the gel. Gel fragments were diced into approximately 1-mm³ squares and were destained using iterations of 25 mM ammonium bicarbonate (ABC)–50% acetonitrile (ACN) with agitation at 4°C. Gel fragments were dehydrated with 100% ACN, with subsequent rehydration and overnight incubation with 150 ng mass-spectrometry-grade Trypsin Gold (Promega, Madison, WI) in 50 mM ABC–10% ACN at 37°C with gentle rocking. Peptides were extracted by first adding 0.1% trifluoroacetic acid (TFA; Thermo Scientific, Rockford, IL) and incubating with agitation at room temperature, then removing the 0.1% TFA and adding 70% ACN–0.01% TFA for 15 min at room temperature with agitation, and finally completing the extraction of the peptides by using 95% ACN–0.005% TFA for 15 min at room temperature with agitation. The extracted peptides were dried by spin vacuum evaporation. Peptides were desalted using C₁₈ ZipTip columns (Millipore, Billerica, MA) by washing with 0.1% TFA and eluting twice with 70% ACN–0.03% TFA, followed by 95% ACN–0.005% TFA. Peptide eluates were dried by spin vacuum evaporation. Peptides were suspended in 5% ACN–0.01% formic acid (FA; Honeywell Burdick & Jackson, Muskegon, MI) and were loaded onto a linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA) coupled with a nanoAcquity ultraperformance liquid chromatograph (UPLC; Waters Corporation) equipped with an autosampler and interfaced with a nanoelectrospray ion source. Peptides were separated on a C₁₈ nanocolumn (particle size, 10 μm; inner diameter, 50 μm; length, 150 mm). The fused silica capillaries (Polymeric Technologies, Phoenix, AZ) for the columns were pulled by a micropipette puller (model P-2000; Sutter Instrument Company, Novato, CA) and packed with C₁₈ resin using a bomb loader. Solvents A and B, used for chromatographic separation of peptides, were 2% ACN in 0.1% FA and 98% ACN in 0.1% FA, respectively. The peptides were injected onto the micro-capillary column at a rate of 200 nl/min. The ionic eluted from the column were electrosprayed at a voltage of 1.75 kV. The ion transfer temperature was kept at 250°C. No auxiliary or sheath gas was used. Survey full-scan MS spectra (m/z 300 to 2,000) were recorded in the Orbitrap analyzer at a resolution of 30,000, followed by MS-MS of the 10 most intense peptide ions in the linear ion trap analyzer. The chromatographic and mass spectral functions were controlled using the Finnigan Xcalibur data system (Thermo Fisher Scientific, Palo Alto, CA). Peptides were identified using the search algorithm SEQUEST by searching the *M. smegmatis* or *M. tuberculosis* protein database. Results represent proteins identified from two independent assays. Data were analyzed using the in-house software Visualize (http://proteomics.mscw.edu/visualize.html). MprB-interacting proteins were considered significant if (i) they had a peptide scan count of ≥2 with peptide probabilities of ≥0.05 and (ii) they were coimmunoprecipitated from strains carrying pSE100-MprB or pSE100-MprB-ECD but not from the strain carrying the pSE100 vector control.

**Statistical analysis.** Student’s *t* test was used to determine statistical differences. A *P* value of <0.05 was considered significant.

**RESULTS**

Genetic components of the cell envelope stress response network, including *mprAB*, are conserved. The MprAB two-component system is part of an integrated signaling network that responds to cell envelope stress (reviewed in references 4 and 34). This network also includes the extracytoplasmic function (ECF) sigma factor SigE (35), the Clp protease regulator ClgR (36, 37), and the serine-threonine protein kinase (STPK) PknB (38, 39). Exposure of *M. tuberculosis* to cell envelope stress leads to the activation of two independent positive-feedback loops (MprAB–SigE and SigE–CtgR) that are integrated through SigE. It has been observed previously that the *mprAB* operon, including pepD and moaB2, is highly conserved in all *Mycobacterium* species sequenced to date (Fig. 1A) (2, 8). To determine if other components of the cell envelope stress response network are also conserved, genomic databases available through NCBI and the Pasteur Institute were searched to identify homologs of *mprA*, *mprB*, *sigE*, *clgR*, and *pknB* in other *Mycobacterium* spp. Like the *mprAB* operon, *sigE* and its downstream genes (rseA, encoding the SigE anti-sigma factor [38], and *htA*, encoding a serine protease with high homology to PepD [8, 40]) were present in all *Mycobacterium* species examined and were conserved in their genomic organization (Fig. 1A). Members of the *clgR* operon, including *Rv2744c*, encoding the 35-kDa antigen, and *Rv2743c*, encoding an alanine-rich protein, were also present and were genetically linked in *Mycobacterium* spp. (Fig. 1A); however, *clgR*, *Rv2744c*, and *Rv2743c* were present but predicted to be pseudogenes in *M. leprae* (data not shown). Finally, *pknB*, which is an essential gene in *M. tuberculosis* (41, 42), was also present in all *Mycobacterium* spp. examined (Fig. 1A).

To determine whether the conservation of cell envelope stress regulators extended beyond *Mycobacterium* spp., sequence comparisons were expanded to include members of the *Actinobacteria*, the phylum to which *M. tuberculosis* belongs. Gene orthologs of *mprAB*, *sigE*, *clgR*, and *pknB* were also present in various *Actinobacteria*, including environmental species (data not shown) and known pathogens (Fig. 1A). Surprisingly, *mprA* and *mprB* were also identified outside the *Actinobacteria* and were consistently positioned adjacent to a predicted HtrA (i.e., PepD-like) serine protease (Fig. 1A). Furthermore, orthologs of *sigE* and *pknB*, but not of *clgR*, were also evident outside the *Actinobacteria* (Fig. 1A). Taken together, these results indicate that key regulators comprising the cell envelope stress response in *M. tuberculosis* are conserved in *Mycobacterium* spp. and in other, more distantly related bacterial species.

**MprB orthologs possess conserved sequence characteristics.** To investigate whether MprB possessed conserved sequence characteristics, identified MprB orthologs were subjected to Clustal W alignment. *M. tuberculosis* MprB and its orthologs all possessed two transmembrane domains flanking a relatively large ECD of ~100 amino acids, a cytoplasmic HAMP (present in histidine kinases, adenylate cyclases, methyl-accepting proteins, and phosphatases) domain, and sensor kinase DHp (dimerization and histidine phosphotransfer) and CA (catalytic and ATP-binding) domains (data not shown). The DHp domain is necessary for SK function, since it contains residues conferring SK-RR specificity (43–48), SK dimerization and autophosphorylation activity (49–51), and kinase and phosphatase activity (52). We rationalized that the DHp domain of MprB orthologs may therefore be the
most conserved. Indeed, primary amino acid sequence alignments of the DHp domains revealed nearly 100% homology between MprB homologs present in *Mycobacterium* spp. (see Fig. S1A in the supplemental material) and significant homology with MprB orthologs in other members of the Actinobacteria (Fig. 1B), including conservation of the “specificity residues” identified as critical to SK-RR interaction (43,47). This homology was also apparent in organisms outside the Actinobacteria (Fig. 1B). In contrast, alignment of the DHp domain of MprB with other *M. tuberculosis* SKs did not reveal significant homology (see Fig. S1B in the supplemental material). Collectively, these analyses suggest that MprAB is part of an evolutionarily conserved signaling network that processes and mediates resistance to cell envelope stress.

Construction of epitope-tagged MprB derivatives. To begin investigating the molecular mechanism underlying MprAB activation, we sought to generate recombinant strains of *M. tuberculosis* in which *mprB* could be overexpressed. Previous attempts in our laboratory to transform *M. tuberculosis* H37Rv with multicopy plasmids expressing *mprA* and/or *mprB* from their native promoter (i.e., pMV206-based plasmids [53]), or with multicopy or integrating plasmids expressing *mprA* and/or *mprB* from the heterologous hsp60 promoter (i.e., pMV261- and pMV361-based plasmids [53]), have been unsuccessful to date (data not shown). Similarly, it has not been possible to isolate *M. tuberculosis* transformants that express mutant *mprA* and/or *mprB* alleles predicted to result in constitutive activation of the MprAB system (data not shown). Therefore, wild-type and mutant alleles of *mprB* were amplified from *M. tuberculosis*, modified to incorporate N-terminal 3×Flag and C-terminal 6×His epitope tags for protein detection, and directionally cloned into the Mycobacterium expression...
construct pSE100 (30). This vector carries the tet operator downstream of the highly expressed myc promoter, allowing regulated gene expression by the inclusion of ATc in the growth media of strains producing the TetR repressor. Using this vector, constructs were generated to produce full-length wild-type MprB (Fig. 2A) or a variant of MprB lacking the ECD, designated MprB\(^{\Delta}\text{ECD}\) (Fig. 2B). The latter protein retained 10 amino acids from the ECD to allow for the maintenance of the two transmembrane segments.

**Overproduction of MprB reduces the growth of M. tuberculosis.** To determine whether regulated production of tagged MprB variants could be tolerated in M. tuberculosis, pSE100 constructs were electroporated into wild-type M. tuberculosis H37Rv or an isogenic M. tuberculosis derivative producing TetR from an integrated plasmid (31). Following electroporation, transformation efficiencies were determined by calculating the number of transformants obtained after 4 weeks of growth on selective medium relative to the number of viable bacteria obtained on nonselective medium after electroporation of the pSE100 vector-only control, which was set at 100%. There were no significant differences between the strains in transformation efficiency with the pSE100 vector (data not shown). In agreement with previous observations, transformation of wild-type M. tuberculosis H37Rv with pSE100-mprB or pSE100-mprB\(^{\Delta}\text{ECD}\) was unsuccessful, with transformation efficiencies of only 0.09% and 0.03%, respectively (Table 2). The few colonies that did arise after 4 weeks were screened by PCR and were found to contain deletions in the myc promoter region and/or the mprB coding sequence (data not shown). In contrast, pSE100-mprB and pSE100-mprB\(^{\Delta}\text{ECD}\) were successfully transformed into M. tuberculosis H37Rv-TetR, with transformation efficiencies comparable to that seen with the pSE100 vector-only control (Table 2). These results suggest that overproduction of tagged variants of MprB is toxic to M. tuberculosis. Additionally, removal of the ECD from MprB does not abrogate this toxicity.

To confirm that tagged MprB variants were produced and localized correctly in H37Rv-TetR, M. tuberculosis strains were grown to mid-log phase and were then incubated for 5 h with 100 ng/ml ATc to overproduce MprB or MprB\(^{\Delta}\text{ECD}\). Whole-cell lysates were then prepared, fractionated, and subjected to Western blot

![FIG 2](http://jb.asm.org/) (A and B) MprB topography. MprB consists of a short cytoplasmic amino terminus, a transmembrane domain, an extracytoplasmic domain (ECD) of 113 amino acids (amino acids 48 to 161), a second transmembrane domain, and the kinase domain at the cytoplasmic carboxyl terminus, including the catalytic histidine at amino acid 249. Protein expression vectors were constructed to overproduce 3\times\text{Flag-} and 6\times\text{His-tagged MprB (A)} or an allele of MprB lacking the ECD (MprB\(^{\Delta}\text{ECD}\)) (B). The MprB\(^{\Delta}\text{ECD}\) allele was constructed to include 5 amino acids proximal to each transmembrane domain (amino acids 48 to 52 and 157 to 161), resulting in a significantly truncated ECD (\(\Delta\text{ECD}\)), while retaining all other domains of MprB. (C) Protein production and localization were confirmed by Western blotting. Gene expression was induced by the addition of 100 ng/ml ATc for 5 h. Whole-cell lysates (WCL) were obtained and were subsequently fractionated, as described in Materials and Methods, into membrane (M) and cytosolic (Cyt) fractions. Both derivatives localized to the membrane, as expected. The cytosolic protein GroEL confirmed the lack of contaminating soluble proteins in the membrane fraction. (D to F) Overproduction of MprB or MprB\(^{\Delta}\text{ECD}\) reduces M. tuberculosis growth. M. tuberculosis H37Rv TetR strains containing pSE100, pSE100-mprB, or pSE100-mprB\(^{\Delta}\text{ECD}\) were grown to exponential phase and were then subcultured in fresh 7H9 OADC medium at an \(\text{OD}_{600}\) of 0.1, at which time gene expression was induced by the addition of ATc at 0 (○), 50 (□), 100 (▲), or 250 (▼) ng/ml. Growth was monitored by \(\text{OD}_{600}\). The addition of ATc did not significantly reduce the growth of the strain containing pSE100, while the growth of strains containing pSE100-mprB or pSE100-mprB\(^{\Delta}\text{ECD}\) was attenuated significantly (\(P < 0.05\)) in the presence of ATc at day 2 and thereafter.
analyses using anti-Flag antibodies to detect MprB variants or antibodies to the cytoplasmic GroEL protein to assess the quality of fractionation. Both MprB and MprB\textsubscript{ECD} were produced at levels similar to that of the control protein GroEL and localized to the cell wall/membrane fraction (Fig. 2C). In contrast, GroEL was detected only in the cytoplasm, confirming the integrity of the fractionation procedure (Fig. 2C). Thus, tagged MprB variants are produced at similar levels in H37Rv-TetR following ATc induction and localize correctly.

We next examined if overproduction of MprB variants negatively impacted \textit{M. tuberculosis} growth. H37Rv-TetR strains carrying pSE100 constructs were incubated in the absence or presence of increasing concentrations of ATc. The growth of these strains was then monitored by optical density over time. ATc had no observable effect on the growth of H37Rv-TetR containing the pSE100 vector-only control, even at the highest concentration of ATc used (Fig. 2D). In contrast, the growth of H37Rv-TetR producing MprB was significantly reduced upon the addition of ATc beginning 2 days postinduction, a trend that continued over the remaining time course (Fig. 2E). A more-pronounced, dose-dependent attenuation in growth was seen in H37Rv-TetR producing MprB\textsubscript{ECD} following ATc induction (Fig. 2F). Collectively, these data indicate that overproduction of MprB leads to growth inhibition in \textit{M. tuberculosis}. Furthermore, removal of the ECD from MprB exacerbates the attenuated growth phenotype, suggesting that this domain negatively regulates MprB activation.

**Overproduction of MprB stimulates MprAB signaling.** To determine whether the observed growth attenuation of strains overproducing MprB or MprB\textsubscript{ECD} was due to increased signaling through the MprAB regulon, H37Rv-TetR strains carrying pSE100, pSE100-\textit{mprb}, or pSE100-\textit{mprb\textsubscript{ECD}} were either left untreated or induced with 100 ng/ml ATc for 24 or 48 h. The RNA levels of select MprAB regulon genes (\textit{mprA} and \textit{sigE}) were then quantified by qRT-PCR. The expression levels of target genes were first normalized to that of the essential housekeeping gene \textit{sigA} and were then normalized to that of H37Rv-TetR carrying the pSE100 vector control (Fig. 3A). In the absence of ATc, \textit{mprb} expression was 4.8-fold or 3.6-fold higher in H37Rv-TetR carrying pSE100-\textit{mprb} or pSE100-\textit{mprb\textsubscript{ECD}}, respectively, than that observed with the pSE100 control (Fig. 3B and C), indicating that there was a small amount of leaky expression of \textit{mprb} alleles from pSE100 in the H37Rv-TetR strain. However, induction with ATc dramatically increased \textit{mprb} transcription in H37Rv-TetR strains carrying pSE100-\textit{mprb} or pSE100-\textit{mprb\textsubscript{ECD}} (Fig. 3B and C). The primer set utilized to detect \textit{mprb} did not allow differentiation between plasmid-encoded \textit{mprb} and endogenous \textit{mprb} from the chromosome. However, a primer set specific to the 5’ untranslated region of the \textit{myc} promoter from pSE100 confirmed that the elevated \textit{mprb} expression was a response to ATc induction (data not shown). Increased production of wild-type MprB did not result in elevated levels of \textit{mprA} or \textit{sigE} expression at 24 h post-ATc induction (Fig. 3B). However, modest but significant induction of \textit{sigE} expression (3.8-fold) was observed in this strain by 48 h post-ATc treatment (Fig. 3B). In contrast, the H37Rv-TetR strain producing MprB\textsubscript{ECD} exhibited elevated levels of \textit{mprA} and \textit{sigE} transcription in the absence or presence of ATc. However, signaling was further elevated upon the addition of ATc, resulting in overexpression of mprB\textsubscript{ECD} (Fig. 3C). Thus, overproduction of MprB variants leads to elevated signaling through the MprAB regulon.

<table>
<thead>
<tr>
<th>\textit{M. tuberculosis} strain</th>
<th>Transformation efficiency (%) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pSE100</td>
</tr>
<tr>
<td>H37Rv</td>
<td>100</td>
</tr>
<tr>
<td>H37Rv-TetR</td>
<td>100</td>
</tr>
<tr>
<td>H37Rv\textit{ΔmprbAB}</td>
<td>100</td>
</tr>
<tr>
<td>H37Rv\textit{ΔsigE}</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Transformation efficiencies were determined based on the number of transformants obtained after 4 weeks of growth on selective medium relative to the number of viable bacteria on nonselective medium. All transformation efficiencies were normalized to that of the pSE100 vector-only control, which was set at 100%.

\textsuperscript{b}Transformants were screened by PCR and were determined to have deletions in the \textit{myc} promoter and/or the \textit{mprb} open reading frame (data not shown).

\textsuperscript{c}The exact pSE100-\textit{mprb\textsubscript{ECD}} transformation efficiency was not determined. However, the total number of transformants recovered for this strain was consistent with those seen for pSE100 and pSE100-\textit{mprb}.

\textsuperscript{d}No transformants were obtained.
and MprAB-mediated signaling resulting from MprB_{H9004} overproduction is increased over that of wild-type MprB.

**MprB_{H9004} is constitutively activated for MprAB regulon signaling.** To determine whether the MprB_{H9004} variant was constitutively activated, H37Rv-TetR cultures carrying pSE100 constructs were first induced by the addition of 100 ng/ml ATc for 5 h and then incubated in the absence or presence of 0.05% SDS for 90 min to exogenously activate MprAB signaling (6). Relative levels of mprB, mprA, and sigE were then quantified as before. Expression levels were normalized first to that of sigA and then to that of H37Rv-TetR carrying pSE100 grown in the absence of SDS, which was set at 1.0. The data represent means and standard errors for at least two biological replicates. Asterisks indicate statistical significance ($P < 0.05$). To determine differences in gene expression between strains following SDS stress exclusively, the relative expression levels of mprB, mprA, and sigE were also normalized to the expression of each gene in the pSE100 control strain grown in the presence of SDS (data not shown); for these comparisons, number signs (#) indicate statistical significance.

**MprAB-mediated signaling is required for growth attenuation of M. tuberculosis following overproduction of MprB variants.** To determine if the growth attenuation observed in H37Rv-TetR strains overproducing MprB or MprB_{H9004} (Fig. 2E and F) was dependent on MprAB-mediated signaling, we attempted to transform pSE100, pSE100-mprB, and pSE100-mprB_{H9004} into H37Rv ΔmprAB (8). All three constructs were successfully transformed into this strain (Table 2), indicating that endogenous mprA and/or mprB was necessary for this phenotype. Western blotting confirmed the production of MprB and MprB_{H9004} in this genetic background (Fig. 5A). Since MprAB and SigE comprise a positive-feedback loop, pSE100-based vectors were also transformed into H37Rv ΔsigE (8). In contrast to the results seen with pSE100, transformation of pSE100-mprB or pSE100-mprB_{H9004} into H37Rv ΔsigE was either inefficient or altogether unsuccessful (Table 2). The few pSE100-mprB transformants isolated in H37Rv ΔsigE carried deletions in the myc promoter and/or the mprB cod-
DnaK bounds the ECD of MprB. The results from the genetic studies presented above indicated that the MprB ECD negatively regulated MprB activity in the absence of cell envelope stress. The functionally related CpxAR two-component system of *E. coli* regulates resistance to cell envelope stress and is negatively regulated by the periplasmic protein CpxP (55–57). To determine whether the ECD of MprB interacted with a protein in the extracytoplasmic compartment, epitope-tagged variants of wild-type MprB or the ECD from MprB alone, designated MprB-ECD, were overproduced and immunoprecipitated from culture lysates. Proteins that commounoprecipitated with MprB or MprB-ECD were then determined by LC–MS-MS (29). *M. smegmatis* was used as a surrogate host for these studies, because overproduction of wild-type *M. tuberculosis* MprB or MprB-ECD in this organism is tolerated (data not shown). *M. smegmatis* carrying pSE100 was processed in parallel to identify proteins that immunoprecipitated nonspecifically. MprB and MprB-ECD were both efficiently immunoprecipitated from *M. smegmatis* culture lysates as determined by Western blotting (data not shown) and silver staining (Fig. 6A and B). In particular, 48 proteins coimmunoprecipitated with full-length MprB (see Table S2 in the supplemental material), including 23 in which two or more unique peptides were detected (Table 3). Similarly, 34 proteins coimmunoprecipitated with MprB-ECD (see Table S3 in the supplemental material). Of the proteins identified, 12 were present in both data sets (see Table S4 in the supplemental material). The highest-scoring protein in both data sets, based on unique peptides and the total scan count, was DnaK, an Hsp70 homolog.

**DnaK negatively regulates MprAB activation.** To confirm the observed interaction between MprB and DnaK, epitope-tagged variants of MprB were immunoprecipitated from *M. smegmatis* as described above, and the resulting eluates were probed for DnaK by Western blotting. DnaK was seen in both the load and the eluate following the immunoprecipitation of MprB (Fig. 6C) and MprB-ECD (Fig. 6D), indicating that the ECD from MprB was sufficient to mediate this interaction. In contrast, while DnaK was observed in the load, it was not present in immunoprecipitation eluates from a strain carrying the pSE100 vector-only control processed in parallel (data not shown), consistent with the absence of DnaK in samples from this strain subjected to LC–MS-MS.

To determine whether DnaK was present in the extracytoplasmic compartment of *M. smegmatis*, where MprB localizes, the whole-cell lysate of an *M. smegmatis* strain expressing the gene for green fluorescent protein (GFP) was fractionated and probed with an anti-DnaK antibody. While GFP localized exclusively in the cytoplasm of this strain, confirming the integrity of the fractionation procedure, DnaK was present in both the cytoplasmic and cell membrane fractions (Fig. 6E). In contrast, DnaK localized exclusively in the cytoplasm when a whole-cell lysate from *E. coli* strain DH5α was prepared and fractionated in parallel (Fig. 6F). Thus, DnaK interacts with the ECD of MprB and is present in the cell membrane compartment, where MprB localizes.

Finally, to determine whether overproduction of DnaK negatively regulated signaling through MprAB, *M. tuberculosis* dnaK was amplified by PCR, cloned first into pTZ824 and then into pSE100 (pSE100–dnaK), and subsequently transformed into *M. tuberculosis* H37Rv-TetR. Following ATC induction, whole-cell lysates were prepared and fractionated as before. When produced in *M. tuberculosis*, DnaK localized to the cytoplasmic membrane compartments (Fig. 7A). This localization pattern is consis-

---

**FIG 5** Overproduction of MprB variants does not alter SigE expression in *M. tuberculosis* in the absence of mprAB. pSE100-based MprB overexpression constructs were introduced into *M. tuberculosis* ΔmprAB and were analyzed by Western blotting (A) or gene expression (B). (A) Epitope variants of MprB and MprB-ECD were detected in the whole-cell lysates (Load) from these strains and in the eluants (Elute) following immunoprecipitation. (B) Overproduction of MprB derivatives in the *M. tuberculosis* ΔmprAB strain did not increase the relative expression of sigE either in the absence or in the presence of SDS stress. Cultures were grown to exponential phase and were then either left untreated or exposed to 0.05% SDS for 90 min to exogenously activate MprAB and downstream gene regulation. The expression of sigE was normalized first to that of sigA and then to that of sigE in H37Rv grown in the absence of SDS, which was set at 1.0. The data represent means and standard errors for at least two biological replicates. The asterisk indicates statistical significance (P, <0.05) for the difference between the absence and the presence of SDS. Number signs (#) indicate statistically significant differences (P, <0.05) in sigE expression between strains following SDS stress.
tent with that observed for endogenous DnaK in *M. smegmatis* (Fig. 6E). To investigate the result of DnaK overproduction, *M. tuberculosis* H37Rv-TetR containing pSE100-*dnaK* was first induced with 100 ng/ml ATc for 5 h and then either left untreated or exposed to 0.05% SDS for 90 min to exogenously activate MprAB signaling. The relative expression levels of *mprA* and *sigE* were then quantified. H37Rv-TetR carrying pSE100 served as the negative control and was processed in parallel. Expression levels were normalized to that of untreated H37Rv-TetR carrying pSE100, which was set at 1.0 (Fig. 7B). Exposure of H37Rv-TetR carrying pSE100 to SDS increased the expression of *mprA* and *sigE*, a result that was expected (Fig. 7C), indicating the activation of MprAB signaling in response to SDS exposure. Interestingly, exposure to cell envelope stress also induced *dnaK* upregulation (Fig. 7C). In H37Rv-TetR carrying pSE100-*dnaK*, *dnaK* levels were increased 5-fold over that in the strain carrying the pSE100 vector control (compare Fig. 7D and B). Importantly, DnaK overproduction in this strain significantly reduced the expression of *mprA* and *sigE* in the absence of SDS (compare Fig. 7D and B). Levels of *mprA* and *sigE* also remained low in this strain relative to that seen in the vector-only control following exposure to SDS, indicating that 5-fold overexpression of *dnaK* prior to SDS exposure reduces the MprAB response (compare Fig. 7E and C). Taken together, these data indicate that DnaK overproduction negatively regulates MprAB-mediated signaling.

**DISCUSSION**

Recognition of and resistance to cell envelope stress in *M. tuberculosis* is mediated primarily by the combined activities of three regulatory factors: MprAB, SigE, and PknB. Collectively, these determinants process stress stimuli generated in the extracytoplasmic compartment and integrate these signals into a coordinated transcriptional response within the bacterial cytoplasm (4, 6, 9, 34, 38). MprA mediates the direct and indirect regulation of numerous determinants, including itself and the ECF sigma factor *sigE* (6, 9). The direct upregulation of *sigE* by MprAB in response to cell envelope stress is physiologically significant, because unlike many ECF sigma factors, SigE is not transcriptionally autoregulatory (35). SigE is also regulated posttranslationally by PknB following perturbation of the cell wall. Under stress conditions that generate cell wall muropeptides (39), PknB phosphorylates the SigE anti-sigma factor RseA, leading to the degradation of RseA by the ClpC1P2 protease and the subsequent release of SigE for downstream gene regulation (38). SigE generated following cell envelope stress exposure contributes to at least two positive-feedback loops, directly upregulating the expression of *mprAB* (8, 35).
and clgR (35), which, in turn, increases the expression of the clpC1 and clpP2 proteases responsible for RseA degradation (36–38).

Given the complex and integrated nature of the stress response regulated by MprAB, SigE, PknB, and ClgR, it is not surprising that these determinants are highly conserved within and outside the mycobacteria. The presence of mprAB, sigE, and pknB in M. leprae is significant, since this bacterium has undergone extensive genomic decay, with approximately half of its coding potential lost due to the accumulation of pseudogenes (58). MprAB is one of four TCSSs predicted to be functional in M. leprae, while M. leprae is also thought to encode only four functional sigma factors (58). Significantly, M. leprae SigE can complement an M. tuberculosis ΔsigE mutant in response to environmental stressors, including SDS (59). The presence of MprAB, SigE, PknB, and ClgR orthologs in other members of the Actinobacteria also suggests that the activities of these regulatory determinants are conserved outside the mycobacteria. In agreement with this idea, disruption of mprB in Rhodococcus equi attenuates the virulence of this bacterium in vivo (60), and in silico analysis indicates that R. equi mprA, pepD, and sigE promoters possess an MprA-like recognition motif that is nearly identical to that delineated for M. tuberculosis MprA (60). SigE function is also likely conserved in nonmycobacteria. For example, sigE has been shown to regulate cell envelope stress resistance in Corynebacterium glutamicum, a nonpathogenic species related to Corynebacterium diphtheriae (61), and a SigH/SigE-like promoter element (62) is also present upstream of clgR in C. glutamicum, suggesting that SigE may directly regulate clgR in this organism as it does in M. tuberculosis (63). Finally, ClgR regulates the clpC and clpP1P2 proteases in C. glutamicum, and M. tuberculosis ClgR is able to complement the expression of Clp proteins in a C. glutamicum ΔclgR mutant, demonstrating that the ClgR response is also maintained in this organism (63). Thus, components of the cell envelope stress response are present outside the mycobacteria, and initial characterizations of these determinants suggest that aspects of their regulation are likely to be integrated in a manner similar to that in M. tuberculosis.

Beyond the conservation of determinants regulating cell envelope stress resistance in organisms related to mycobacteria, alignments of DHp domains from predicted MprB SK orthologs show extensive conservation at the amino acid level. Key amino acids defining “specificity residues” are more highly conserved between MprB orthologs from different species, and even different phyla, than between MprB and other SKs from M. tuberculosis. Interestingly, MprB orthologs are consistently positioned adjacent to an HtrA-like (i.e., PepD-like) serine protease, suggesting that the external stress recognized and processed by MprAB may include substrates that need proteolytic processing. PepD (HtrA2) is required for cell envelope stress resistance in M. smegmatis (8) and

<table>
<thead>
<tr>
<th>Gene annotation and M. tuberculosis homologs are based on the database available online at the Pasteur Institute (GenoList Web server). NA, not applicable; no homolog in M. tuberculosis H37Rv.</th>
<th>Gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaperone protein DnaK</td>
<td>Protein fate: protein folding and stabilization</td>
</tr>
<tr>
<td>DNA-directed RNA polymerase, beta subunit</td>
<td>Transcription</td>
</tr>
<tr>
<td>Cobalamin synthesis protein/P47K</td>
<td>Biosynthesis of cofactors, prosthetic groups, and carriers</td>
</tr>
<tr>
<td>DNA-directed RNA polymerase, alpha subunit</td>
<td>Transcription</td>
</tr>
<tr>
<td>Monoxygenase</td>
<td>Central intermediary metabolism</td>
</tr>
<tr>
<td>DNA-directed RNA polymerase, beta subunit</td>
<td>Transcription</td>
</tr>
<tr>
<td>Aspartate kinase</td>
<td>Amino acid biosynthesis</td>
</tr>
<tr>
<td>Pseudomembrane transport ATPase</td>
<td>Cell envelope; transport</td>
</tr>
<tr>
<td>Sensor histidine kinase MprB</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>Acyl-carrier protein synthase 1</td>
<td>Fatty acid and phospholipid metabolism</td>
</tr>
<tr>
<td>Chaperone protein DnaJ</td>
<td>Protein fate: protein folding and stabilization</td>
</tr>
</tbody>
</table>
contributes to virulence in *M. tuberculosis* (40). Interestingly, PepD cleaves the 35-kDa antigen (Rv2744c) encoded within the operon that includes *clgR* (29), further connecting these two arms of the cell envelope stress response network at the posttranslational level. Studies examining the role of Rv2744c in the context of mycobacterial cell envelope stress resistance are currently in progress.

While several in vitro stressors activating MprAB have been defined, the specific stimulus leading to MprAB activation has not yet been elucidated. Cell envelope stress resistance in *E. coli* and other enteric bacteria is mediated by CpxAR, a TCSS that recognizes and responds to misfolded or unfolded proteins in the periplasmic space (64, 65). In this system, CpxAR activation is negatively regulated by the periplasmic protein CpxP in the absence of stress (55, 56). CpxP is a member of the CpxAR regulon and possesses chaperone-like activity in vitro (66). CpxP is thought to interact directly with the ECD of CpxA in the absence of stress, inhibiting its function. Upon the accumulation of misfolded or unfolded proteins in the cell envelope following stress, the inhibitory function of CpxP for CpxA is relieved, leading to CpxAR activation and downstream gene regulation.

The genetic and proteomic evidence presented here indicates that MprAB may utilize a mechanism similar to that of CpxAR to respond to misfolded/unfolded proteins present in the extracytoplasmic compartment of *M. tuberculosis*. First, MprAB activation is negatively regulated in the absence of stress. Overproduction of MprB, particularly overproduction of an MprB derivative lacking its ECD, negatively impacts *M. tuberculosis* growth due to increased signaling through the MprAB regulon. In addition, deletion of the ECD from MprB results in an MprB variant that is constitutively activated for signaling, even in the absence of cell envelope stress. It was shown previously that deletion of a portion of the MprB ECD resulted in an SK incapable of signaling (6), suggesting that the activation and inhibitory subdomains of MprB may be separable. While the reason(s) underlying the observed growth attenuation phenotype remains unknown, it is unlikely that overproduction of MprB derivatives grossly disrupts plasma membrane integrity, since pSE100 derivatives expressing *mprB* or *mprB*/*H9004* ECD can be transformed into *M. tuberculosis*/*H9004* *mprAB* mutants, albeit at transformation efficiencies slightly lower than that for pSE100. While we cannot completely rule out the possibility that the lower transformation efficiencies observed were due to off-target effects of MprB overproduction, ΔmprAB strains overproducing MprB or MprB<sub>ΔECD</sub> displayed no significant differences in growth (data not shown) and did not exhibit aberrant expression levels of *sigE* in the absence or presence of exogenous stress. Rather, the altered growth characteristics observed are likely due to the unregulated expression or repression of an MprAB regulon.
member required for *M. tuberculosis* growth *in vitro*. A collection of *M. tuberculosis* suppressors capable of supporting the overproduction of MprB<sub>ECD</sub> has been isolated and is currently being characterized.

Second, MprB, and the ECD of MprB in particular, interacts with Dnak <em>in vivo</em>. Furthermore, Dnak is also associated with the plasma membrane in *M. smegmatis* and *M. tuberculosis*. Thus, Dnak is present within the appropriate subcellular compartment for interaction with MprB. While Dnak was originally thought to be exclusively cytoplasmic, several studies have now reported the localization of Dnak outside the cell, including in *Mycobacterium* spp. (67–70) and other Gram-positive bacteria (51, 71–74). Dnak is a member of the well-conserved Hsp70 family of proteins found in all kingdoms of life. In the cytoplasm, Dnak contributes to protein homeostasis by resolving misfolded proteins and protein aggregates (75, 76). Although the mechanism by which Dnak is secreted has not been defined, a population of Dnak in the extracytoplasmic compartment may allow it to interact with, and possibly help fold, proteins outside the cytoplasm. Interestingly, Bip, a eukaryotic Hsp70 homolog that contributes to protein folding in the endoplasmic reticulum, inhibits Ire1p, a membrane kinase regulating the “unfolded protein response” in eukaryotic cells (77, 78). Thus, in addition to helping fold protein substrates, protein chaperones may also participate in regulatory activities by forming complexes with protein kinases and negatively regulating their activity. Intriguingly, *M. tuberculosis* Dnak has also been identified as a putative substrate of PepD (29). Thus, PepD may function like HtrA protease DegP in *E. coli*, which cleaves CpxP in the presence of misfolded proteins (66).

Third, and finally, overexpression of dnaK reduces MprAB-mediated signaling in *M. tuberculosis*. In particular, *dnaK* overexpression in *M. tuberculosis* represses the transcription of *mprA* and *sigE* in the absence of stress. Furthermore, overproduction of Dnak inhibits the expression of *mprA* and *sigE* in *M. tuberculosis* following exposure to an exogenous cell envelope stressor. Interestingly, exposure of *M. tuberculosis* to SDS also leads to increased expression of endogenous *dnaK*. While it remains unclear whether the upregulation of *dnaK* following cell envelope stress is dependent on MprAB and/or SigE, these results suggest that Dnak may participate in a positive-feedback loop to help monitor and regulate cell envelope stress within the extracytoplasmic compartment. Thus, Dnak regulates aspects of MprAB-mediated signal transduction either directly or indirectly in response to cell envelope stress.

Based on these results and work from others, we propose the following model for MprAB activation in response to cell envelope stress (Fig. 8). In the absence of cell envelope stress, MprB is negatively regulated through its ECD by Dnak. We hypothesize that Dnak negatively regulates MprB activation either by interacting directly with the ECD of MprB to inhibit its kinase activity or by reducing the misfolded protein “burden” in the extracytoplasmic space, or both. Upon the exposure of *M. tuberculosis* to exogenous cell envelope stress, the accumulation of unfolded or misfolded proteins in the extracytoplasmic space leads to the removal of negative regulation through abrogation of the MprB-Dnak interaction, or by overwhelming the ability of Dnak to properly maintain protein homeostasis in the extracytoplasmic compartment. Regardless, perturbation of the cell envelope leads to subsequent activation of MprAB and downstream gene regulation. Finally, *dnaK* expression is upregulated in response to SDS-induced stress to help mediate the resolution of misfolded proteins, eventually leading to inhibition of MprAB-mediated signaling once cell envelope stress is alleviated and protein homeostasis is achieved within the extracytoplasmic compartment. Future studies will include investigation of the mechanism by which Dnak is secreted outside the cytoplasm, determination of whether MprAB is activated in response to the unfolding/misfolding of a specific extracytoplasmic protein, and determination of whether MprB activation requires a secondary stimulus in addition to removal of the negative regulation conferred by the MprB ECD.

**ACKNOWLEDGMENTS**

We are grateful to Sabine Ehrt for providing pSE100-based expression constructs for gene regulation studies. We also thank BEI Resources for providing the antibodies used in these studies.

This work was supported by grant RO1 AI51669 from the National Institute of Allergy and Infectious Diseases.
REFERENCES


January 2014 Volume 196 Number 2


