JOURNAL OF BACTERIOLOGY, Mar. 2010, p. 1498–1510 Vol. 192, No. 6
0021-9193/10/$12.00 doi:10.1128/JB.01167-09
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

PepD Participates in the Mycobacterial Stress Response Mediated through MprAB and SigE▽†

Mark J. White,[1] Hongjun He,[1] Renee M. Penoske,[1] Sally S. Twining,[2] and Thomas C. Zahrt[1]*

Department of Microbiology and Molecular Genetics and Center for Biopreparedness and Infectious Diseases, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226-0509,[1] and Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226-0509[2]

Received 29 August 2009/Accepted 23 December 2009

Currently, one-third of the world's population is believed to be latently infected with *Mycobacterium tuberculosis*. The mechanisms by which *M. tuberculosis* establishes latent infection remain largely undefined. *mprAB* encodes a two-component signal transduction system required by *M. tuberculosis* for aspects of persistent infection. MprAB regulates a large and diverse group of genetic determinants in response to membrane stress, including the extracytoplasmic function (ECF) sigma factor *sigE* and the HtrA-like serine protease *pepD*. Recent studies have demonstrated that PepD functions as both a protease and chaperone *in vitro*. In addition, inactivation of *pepD* alters the virulence of *M. tuberculosis* in a mouse model system of infection. Here, we demonstrate that PepD plays an important role in the stress response network of *Mycobacterium* mediated through MprAB and SigE. In particular, we demonstrate that the protease activity of PepD requires the PDZ domain, in addition to the catalytic serine at position 317. *pepD* expression initiates from at least three promoters in *M. tuberculosis*, including one that is regulated by SigE and is located upstream of the *mprA* coding sequence. Deletion of *pepD* or *mprAB* in *Mycobacterium smegmatis* and *M. tuberculosis* alters the stress response phenotypes of these strains, including increasing sensitivity to SDS and cell wall antibiotics and upregulating the expression of stress-responsive determinants, including *sigE*. Taking these data together, we hypothesize that PepD utilizes its PDZ domain to recognize and process misfolded proteins at the cell membrane, leading to activation of the MprAB and SigE signaling pathways and subsequent establishment of a positive feedback loop that facilitates bacterial adaptation.

Currently, one-third of the world's population is believed to be latently infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. There are an estimated 9 million new cases of infection caused by this organism each year, accounting for more than 2 million deaths annually (3). This makes *M. tuberculosis* one of the leading causes of death due to a single infectious agent. Humans are the only known reservoir of *M. tuberculosis*, and as such, part of its success as a pathogen lies in its ability to maintain a significant persistent reservoir within the population. Latently infected individuals are asymptomatic and are harder to treat, thereby complicating measures to control the spread of disease (27). In addition, the emergence of multi- and extensively drug-resistant strains has further compromised established strategies for treatment. A better understanding of the mechanisms by which *M. tuberculosis* establishes persistent infection is urgently needed so that better therapeutics for the latently infected population can be developed.

M. tuberculosis is a facultative intracellular pathogen that primarily infects alveolar macrophages. The organism survives within these cells by blocking key steps in the phagosome maturation process (38). Infection by *M. tuberculosis* triggers a cell-mediated immune response, resulting in the formation of caseous granulomas at sites of infection, an environment in which the bacillus is thought to persist for extended periods in a metabolically altered physiological state (46). It is likely that *M. tuberculosis* possesses determinants that mediate resistance to stressful conditions within these *in vivo* environments that likely include acidic pH, low oxygen, poor nutrient availability, reactive oxygen and nitrogen intermediates, and other host-generated products. Transcriptional regulators, such as two-component signal transduction systems and sigma factors, play key roles in coordinating the complex array of gene expression changes required for these adaptive responses in bacteria.

M. tuberculosis encodes 11 complete two-component systems (8). The MprAB two-component system has been shown to be important for the establishment and maintenance of persistence by *M. tuberculosis* in a murine model of infection (59). *mprAB* is located within an *in vivo*-expressed genomic island (52), is induced following infection of *M. tuberculosis* in the lungs of chronically infected mice (53), and is induced in *M. tuberculosis* during growth within an artificial granuloma model system (25). *mprAB* is also induced *in vitro* following exposure of *M. tuberculosis* to membrane-damaging stress (20, 30, 36) and nutrient starvation or depletion (6, 17). More recently, MprAB has been implicated in the stringent response of *M. tuberculosis* mediated through RelA (50). Microarray and quantitative reverse transcription (qRT)-PCR analyses have delineated a large MprAB regulon (19, 35, 36), including two stress-responsive sigma factors, *sigB* and *sigE*; the heat shock protein encoded by *acr2*; and an HtrA-like serine protease encoded by *pepD*, all of which are directly regulated by MprAB (19, 35, 36). Collectively, these observations suggest that

* Corresponding author. Mailing address: Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509. Phone: (414) 955-7429. Fax: (414) 955-6567. E-mail: tzahrt@mcw.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 8 January 2010.

MprAB functions in stress response networks necessary for *M. tuberculosis* adaptation and pathogenesis.

An emerging class of proteins important in stress response networks is the HtrA family of serine proteases. These proteases are evolutionarily conserved among many different species spanning both prokaryotes and eukaryotes. HtrA-like proteases contain two conserved domains: a serine protease domain and one or two C-terminal PDZ domains. These proteins often possess the activities of chaperones and/or proteases (26). In bacteria, HtrA-like family members have been shown to participate in stress response networks, including those regulated through sigma factors and two-component systems (1, 7). A subset of HtrA family members is also required for aspects of pathogenesis in both Gram-positive and Gram-negative organisms (14, 16, 45, 49, 57). *M. tuberculosis* possesses three HtrA-like proteases: *Rv1223* (*degP* or *htrA1*), *Rv0983* (*pepD* or *htrA2*), and *Rv0215* (*pepA* or *htrA3*) (8, 33), all of which share regions of sequence homology with one another. Initial studies of PepD and PepA in *M. tuberculosis* indicate that these proteins are secreted and are present within the culture filtrate (47). More recently, PepD has been shown to possess both protease and chaperone activities (33); however, the biological activities of DegP and PepA remain unclear. Δ*pepD*::Hyg^r mutants of *M. tuberculosis* Erdman are altered for virulence in mice. In particular, Δ*pepD*::Hyg^r mutant-infected animals exhibit increased time to death, and infection by Δ*pepD*::Hyg^r mutants induces less tissue pathology than in animals infected with wild-type or complemented mutant derivatives (33). Taken together, these results suggest that PepD is required for aspects of *M. tuberculosis* adaptation within the host. Here, we build upon these initial observations of PepD and provide new evidence supporting a role for PepD in the stress response network in *M. tuberculosis* mediated through the MprAB and SigE signaling networks.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains, plasmids, and mycobacteriophages used in the study are described in Table 1. *Escherichia coli* Top 10 (Invitrogen, Carlsbad, CA) or DH5α was used for all cloning procedures. *E. coli* HB101 was used as a transduction host for generating phasmids. *E. coli* BL21(DE3)/pLysS (Novagen, La Jolla, CA) was used to express and purify recombinant proteins. All *E. coli* strains were grown with aeration at 37°C in Luria-Bertani (LB) broth or on LB agar. When required, the medium was supplemented with 25 μg/ml chloramphenicol and/or 50 μg/ml kanamycin sulfate. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA) was added to *E. coli* BL21(DE3)/pLysS cultures at a concentration of 0.1 mM to induce protein overexpression. The *Mycobacterium* strains used in this study are derivatives of *M. tuberculosis* H37Rv (ATCC 27294) or *Mycobacterium smegmatis* mc^{2}155 (ATCC 700084). The mycobacteria were grown with aeration 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-dextrose-catalase (ADC) or oleic acid-albumin-dextrose-catalase (OADC) supplement, and 0.05% Tween 80. For protein expression studies, *M. tuberculosis* was grown in glycerol alanine salts (GAS) medium (51). Generation of mycobacteriophages and their transduction into *M. tuberculosis* H37Rv proceeded as described previously (5). Electrocompetent cells of *M. tuberculosis* or *M. smegmatis* were prepared as described previously (23). When required, the mycobacterium medium was supplemented with 25 μg/ml kanamycin sulfate, 50 μg/ml hygromycin B, and/or 50 μg/ml cycloheximide. Kanamycin sulfate and cycloheximide were purchased from Fisher (Waltham, MA). Hygromycin B was purchased from AG Scientific (San Diego, CA).

Expression and purification of rPepD and rPepD mutants. All primers used are described in Table S1 in the supplemental material. pET-24b (Novagen, La Jolla, CA) was used to overexpress recombinant forms of PepD (rPepD) containing C-terminal His$_6$ tags. All *pepD* sequences were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using *Pfu* polymerase (Invitrogen, Carlsbad, CA), cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), subcloned into the NdeI and XhoI sites of pET-24b, and sequenced to confirm their identities and the absence of mutations. PepDΔTMS317A carries a point mutation in the predicted catalytic serine of PepD and was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). *E. coli* BL21(DE3)/pLysS strains containing overexpression constructs were grown overnight on selective LB agar medium, suspended in LB broth containing kanamycin and chloramphenicol, and grown to mid-exponential phase. Protein overexpression was induced by the addition of 0.1 mM IPTG for 3 h at 30°C. The induced cells were suspended in lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 5 mM imidazol, and 6 μg of DNase/ml), passed 3 times through a French press, and centrifuged at 25,000 × *g* for 30 min. Cellular supernatants were first passed over a nickel-nitrilotriacetic acid–agarose column (Qiagen, Valencia, CA), and then the collected fractions were pooled and placed on a Sephacryl 200 HR (Sigma, St. Louis, MO) size exclusion column with running buffer (10 mM Tris [pH 7.6] and 20 mM NaCl). Peak fractions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to anion-exchange chromatography (DEAE-Sephacel). Bound proteins were eluted with a gradient of 20 mM to 1 M NaCl. The purified protein fractions were pooled and stored in 2.5 M glycerol at −80°C.

Protease activity assays. The proteolytic activities of rPepD derivatives were determined using the nonspecific protein substrate β-casein or fluorescein isothiocyanate (FITC)-labeled casein. Briefly, increasing amounts of purified rPepD-His were incubated with 1 μg of β-casein in 50 mM potassium phosphate buffer (pH 7.5) at 37°C for 16 h. The reaction mixtures were then separated on 12% SDS-PAGE gels and stained using Coomassie blue. To quantify the activity of rPepD, purified protein was incubated with 0.5% FITC-casein under similar conditions (55). The reaction mixtures were passed through a Microcon YM-100 centrifugal filter (Millipore, Billerica, MA) to remove uncleaved and large cleavage products, and the filtrate was pH buffered by the addition of 0.5 M Tris (pH 7.9). Fluorescence was measured using standard 485-nm excitation/530-nm emission filters on a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). For some experiments, protease inhibitors (Sigma, St. Louis, MO) were added to the reaction mixtures, including *N*-tosyl-L-lysine chloromethyl ketone (TLCK) (15 mg/ml in distilled H_2O [dH_2O]), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (10 mg/ml in dimethyl sulfoxide [DMSO]), leupeptin (10 mg/ml in dH_2O), di-isopropyl fluorophosphate (DFP) (5 mM in isopropanol), aprotinin (10 mg/ml in dH_2O), EDTA (50 mM in dH_2O), E64 (1 mM in dH_2O), and pepstatin A (1 mM in DMSO).

Generation of unmarked *M. tuberculosis* and *M. smegmatis* deletion mutants and complemented strains. *mprAB*, *pepD*, and *sigE* were deleted in *M. tuberculosis* H37Rv using specialized transduction as previously described (5). Regions (1.0 kb in size) upstream and downstream of genes were PCR amplified from H37Rv genomic DNA. The *mprAB* deletion extended from −14 bp upstream of the originally annotated *mprA* translational start site at +1 to +1302 downstream of the *mprB* translational start site, leaving intact the C-terminal 214 bp of *mprB* and the *mprB-pepD* intergenic region that included the three MprA recognition motifs (20). The *pepD* and sigE deletions removed the entire coding sequence for each gene. Following packaging and transduction into *M. tuberculosis*, hygromycin-resistant clones were screened for loss of *mprAB*, *pepD*, and *sigE* by PCR. The resulting Δ*mprAB*::Hyg^r, Δ*pepD*::Hyg^r, and Δ*sigE*::Hyg^r strains were electroporated with pYUB870 to unmark the mutation. Following counterselection on medium containing 10% sucrose, the resulting sucrose-resistant, kanamycin-sensitive, and hygromycin-sensitive colonies were screened for unmarking of the *mprAB*, *pepD*, and *sigE* mutations by PCR. Finally, to complement the *pepD* mutant, *pepD* and *moaB2*, along with ~300 bp of DNA upstream of *pepD*, were amplified from *M. tuberculosis* genomic DNA. The resulting product was cloned into pCR2.1-TOPO and subcloned into pMV306, resulting in pTZ792. This plasmid was then electroporated into *M. tuberculosis* Δ*pepD*, and recombinants were selected on medium containing kanamycin. Complementation was verified by PCR. The *sigE* mutation was complemented by amplifying *sigE*, along with ~150 bp of upstream DNA from *M. tuberculosis* genomic DNA. The resulting product was cloned into pCR2.1-TOPO and subcloned into pMV306, resulting in pTZ1065. This plasmid was then electroporated into *M. tuberculosis* Δ*sigE*, and recombinants were selected on medium containing kanamycin. The *mprAB* mutation was complemented by the introduction of pTZ215 (59), a derivative of pMV306 carrying the *mprAB* coding sequence and the upstream region from *M. tuberculosis*.

Unmarked deletion mutations of the *mprAB* (*MSMEG5488* and *MSMEG5487*) and *pepD* (*MSMEG5486*) coding regions in *M. smegmatis* were generated using a standard two-step homologous-recombination procedure. The counterselectable

TABLE 1. Strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or bacteriophage	Genotype or description	Application	Reference or source
Strains			
M. tuberculosis H37Rv	Laboratory strain		ATCC 27294
M. smegmatis mc^{2}155	Laboratory strain		ATCC 700084
E. coli DH5α	[λ^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*]	Cloning	Laboratory collection
E. coli TOP10	F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80*lac*ZΔM15 Δ*lac*X74 *recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (StrR) *endA1 nupG*	Cloning	Invitrogen
E. coli HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	*E. coli* transduction host	Laboratory collection
E. coli BL21(DE3)/pLysS	F^- *ompT hsdS*B(r_B^- m_B^-) *gal dcm* (DE3)/pLysE (Cam^r)	Protein overexpression	Novagen
Plasmids			
pCR2.1-TOPO	3.9-kb plasmid for cloning PCR products; Amp^r Kan^r	PCR cloning vector	Invitrogen
pET-24b	5.3-kb plasmid allowing C-terminal protein fusion to His_6; Kan^r	Protein overexpression vector	Novagen
pMV306	3.9-kb plasmid for single-copy integration into mycobacterial chromosome; Kan^r	Complementation vector	Laboratory collection
pTZ557	pYUB854 containing *pepD* upstream and downstream regions; Hyg^r	*pepD* deletion	This study
pTZ612	pYUB854 containing *sigE* upstream and downstream regions; Hyg^r	*sigE* deletion	This study
pTZ758	pET-24b containing *pepD*ΔTM coding sequence; Kan^r	Overexpression	This study
pTZ775	pET-24b containing *pepD*ΔTMΔPDZ coding sequence; Kan^r	Overexpression	This study
pTZ792	pMV306 containing *pepD* and *moaB2* coding sequences; Kan^r	Complementation	This study
pTZ797	pET-24b containing *pepD*ΔTMS317A coding sequence; Kan^r	Overexpression	This study
pTZ844	pYUB854 containing *mprA* upstream and *mprB* downstream regions; Hyg^r	*mprAB* deletion	This study
pTZ898	pYUB854 containing *sacB* coding sequence; Hyg^r	Mycobacterium gene deletion vector	This study
pTZ903	pMV306 containing *pepD*S317A and *moaB2* coding sequences; Kan^r	Complementation	This study
pTZ947	pTZ898 containing *mprAB* upstream and downstream regions; Hyg^r	*mprAB* deletion	This study
pTZ949	pTZ898 containing *pepD* upstream and downstream regions; Hyg^r	*pepD* deletion	This study
pTZ1065	pMV306 containing *sigE* coding sequence; Kan^r	Complementation	This study
pYUB854	3.9-kb cosmid for generating gene deletions in *Mycobacterium*. Carries a Hyg^r cassette flanked by multiple cloning sites and a λ*pac* site; Hyg^r	*Mycobacterium* gene deletion vector	5
pYUB870	8.9-kb plasmid for unmarking mutations in *Mycobacterium*. Carries the *tnpR* and *sacB* cassettes; Kan^r	*Mycobacterium* gene deletion unmarking vector	5
Mycobacteriophages			
phAE87	Derivative of conditionally replicating mycobacteriophage PH101(Ts)		5
phTZ557	phAE87 derivative containing pTZ557	*pepD* deletion	This study
phTZ612	phAE87 derivative containing pTZ612	*sigE* deletion	This study
phTZ844	phAE87 derivative containing pTZ844	*mprAB* deletion	This study

marker *sacB* was amplified from pYUB870 (5) and cloned into the *Mycobacterium* suicide vector pYUB854 (5) to generate pTZ898. Regions (1.0 kb in size) upstream and downstream of the *mprAB* and *pepD* coding sequences were then amplified from *M. smegmatis* mc^{2}155 genomic DNA. Both the *mprAB* and *pepD* deletions removed the complete coding sequences for these genes. The resulting recombinants were screened by PCR to confirm gene loss. The generated Δ*pepD* or Δ*mprAB* mutant was complemented with pTZ792 or pTZ215, respectively.

DNA and RNA extraction. Mycobacterial genomic DNA was prepared as described previously (23). For isolation of RNA, *M. tuberculosis* was grown in 7H9 broth to early log phase (optical density at 600 nm [OD_{600}] = 0.4). The cultures were left untreated or were exposed to 0.05% SDS for 90 min to induce expression of *mprAB* and/or *pepD* (19, 20). RNA was fixed by treating the cultures with RNAlater (Ambion, Austin, TX) for 40 min at room temperature prior to processing them. Bacteria were collected by centrifugation, washed twice with 1× phosphate-buffered saline (PBS), and then mechanically disrupted in the presence of RNA-Bee (Tel-Test, Friendswood, TX) by bead beating. RNA was then extracted with chloroform, precipitated with isopropanol, and stored at −20°C until it was used. The resulting RNA was resuspended in diethylpyrocarbonate (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).

RT-PCR, 5′ RACE, and DNA microarray studies. For RT-PCR, cDNAs from *M. tuberculosis* strains were prepared by incubating 500 ng total RNA with random *M. tuberculosis* decamers (15) in a reverse transcription reaction mixture containing SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). To test for the presence of DNA contamination, identical reactions lacking SuperScript III were also run. All PCRs were performed on an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) with iQ SYBR green Supermix and gene-specific primers. The reaction conditions consisted of 40 rounds of denaturation at 95°C for 30 s and annealing/extension at 60°C for 15 s. Single PCR products were confirmed by performing a postamplification melt curve analysis for each reaction. The transcript levels of target genes were normalized to those of the ribosomal gene *rrs* or *sigA*. For 5′ rapid amplification of cDNA ends (RACE), the FirstChoice RLM-RACE kit (Ambion, Austin, TX) was used essentially as recommended by the manufacturer. Briefly, 3 to 5 μg of total RNA was ligated to 300 ng of 5′ RACE adaptor using 5 units of T4 RNA ligase (Ambion, Austin, TX) for 1 h at 37°C. The RNA was then reverse transcribed with *M. tuberculosis*-specific decamers and Maloney-murine leukemia virus (M-MLV) reverse transcriptase (Ambion, Austin, TX) for 1 h at 42°C. PCR was then performed using a primer nested within the gene of interest and a primer specific to the 5′ RACE adaptor (5′ RACE outer primer). The resulting PCR products were cloned into pCR2.1-TOPO and sequenced using the M13rev primer. Ten to 20 clones were sequenced for each primer set. For DNA microarray comparisons, RNA was isolated from three independent cultures of wild-type *M. tuberculosis* H37Rv or the isogenic Δ*pepD* mutant and used to generate labeled cDNA by reverse transcription using SuperScript III, *M. tuberculosis* decamers, and Cy3- or Cy5-dCTP (Amersham, Piscataway, NJ). RNA was derived from cultures grown in 7H9 medium in the absence of stress. Dye flip controls were also conducted. The labeled cDNAs were purified, mixed, and hybridized to *M. tuberculosis* DNA microarrays obtained through the Pathogen Functional Genomic Resource Center. The Cy3 and Cy5 intensities for each spot were quantified using ScanAlyze (Stanford), the median background values were subtracted, and the values were normalized based on the total intensity of good-quality spots over background. The data were analyzed using statistical analysis of microarrays (SAM) (54) to determine significant expression changes.

Northern blot analysis. For Northern blot analysis, the NorthernMax-Gly Kit (Ambion, Austin, TX) was used according to the manufacturer's instructions. Briefly, ~20 μg RNA from either untreated or SDS-treated *M. tuberculosis* cultures was separated by electrophoresis on a 1% agarose gel, transferred to Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA), and hybridized under stringent conditions. A *pepD*-specific probe was generated by end labeling it with [γ-^{32}P]ATP using a PCR product generated with primers pepDtrunc and pepDcomR.

Protein extraction and Western blot analysis in *M. tuberculosis*. Cultures of *M. tuberculosis* derivatives were grown in GAS medium to stationary phase (OD_{600} > 1.5), collected by centrifugation, and suspended in protein lysis buffer (100 mM Tris [pH 7.6], 100 mM NaCl, 5 mM EDTA) containing protease inhibitors. The cells were mechanically disrupted by bead beating, and the supernatants were collected. The protein lysates were passed through a 0.45-μm low-protein binding syringe filter (Corning, Lowell, MA). To collect proteins from the culture filtrate, the spent broth medium was passed through a 0.45-μm low-protein binding filter (Corning, Lowell, MA) and concentrated using an Amicon Ultracell-15 (10,000 molecular weight cutoff). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Approximately 10 μg of protein was added to 10 μl of 2× SDS-PAGE loading buffer containing β-mercaptoethanol (β-ME), boiled for 5 min, separated on a 12% SDS-PAGE gel, and transferred onto an Immobilon-P membrane (Millipore, Billerica, MA). The membranes were blocked in TTBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.5% Tween 20) containing 5% skim milk and 1% bovine serum albumin (BSA) for at least 1 h. The membranes were probed with rabbit polyclonal anti-PepD antibody (1:10,000 or 1:50,000; Covance, Princeton, NJ) or rabbit polyclonal anti-casein (1:10,000; Immunology Consultants Laboratory, Inc., Newberg, OR) in TTBS overnight at 4°C. After being washed, the membranes were incubated with donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Fisher, Waltham, MA) for 30 min at room temperature. The blots were visualized using SuperSignal West Pico or Femto Chemiluminescent Substrate kits (Pierce, Rockford, IL).

Isolation and infection of macrophages. Human monocyte-derived macrophages were isolated from buffy coats obtained from the Blood Center of Southeastern Wisconsin and were maintained as described previously (37). J774A.1 macrophages (ATCC TIB-67) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 2 mM L-glutamine. All cells were incubated at 37°C in humidified air containing 5% CO_2. All macrophages were seeded at a density of 1×10^5 cells/well in 12-well tissue culture plates and infected with *M. tuberculosis* derivatives at a multiplicity of infection (MOI) of 1 bacterium per macrophage for murine macrophages or an MOI of 0.1 for human macrophages as described previously (58). For some experiments, J774A.1 macrophages were activated by the addition of 500 U/ml murine recombinant gamma interferon (rIFN-γ) (Peprotech, Rocky Hill, NJ) and 500 ng/ml purified *E. coli* lipopolysaccharide (LPS) (Sigma, St. Louis, MO) for 24 h prior to infection.

***In vitro* survival assays.** The sensitivities of *Mycobacterium* derivatives to stressors were measured using broth or disc diffusion assays. For broth assays, *M. tuberculosis* or *M. smegmatis* derivatives were grown to mid-log phase (OD_{600} = 0.4 to 0.6) and either left untreated or exposed to 200 μg/ml D-cycloserine (Sigma, St. Louis, MO) or SDS at a final concentration of 0.4% or 0.1%, respectively, for *M. tuberculosis* or *M. smegmatis*. The cultures were incubated at 37°C with shaking, and aliquots were removed at 2 h, 4 h, 8 h, and 12 h postexposure for CFU determination. The bacteria were diluted in 1× PBS for plating onto solid agar medium. For disc diffusion assays, 25 ml 7H9 bottom agar (1.2% agar) per dish was poured into petri dishes. *M. smegmatis* cultures were grown to mid-log phase (OD_{600} = 0.6), and 100-μl aliquots were added to 3.5 ml of 0.6% 7H9 top agar that had been tempered to 55°C and poured onto bottom agar for solidification. Sterile filter discs (6 mm) were then added to the medium and impregnated with either 5 μl of a 10% SDS solution or 10 μl of 50 mg/ml cefuroxime (Sigma, St. Louis, MO). The plates were incubated at 37°C, and the zones of inhibition were measured after 3 days.

Statistical analysis. All statistical analyses were conducted using Student's *t* test or a one-way analysis of variance. Values were determined to be statistically significant at $P < 0.05$.

Microarray data accession number. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) (13) and are accessible through GEO series accession number GSE17587 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17587).

RESULTS

Purification of *M. tuberculosis* PepD in *E. coli*. PepD from *M. tuberculosis* contains several conserved domains, including a hydrophobic transmembrane domain (amino acids 102 to 124), a serine protease domain (amino acids 166 to 364), and a single PDZ interaction domain (amino acids 368 to 446) predicted to be involved in protein-protein interactions. To generate recombinant derivatives of PepD for overexpression and purification in *E. coli*, *pepD* was PCR amplified from *M. tuberculosis* H37Rv and cloned into pET-24b, a vector allowing incorporation of a His_6 epitope tag at the C terminus of PepD (see Fig. S1A in the supplemental material). Initial attempts to overexpress and purify full-length rPepD derivatives (amino acids 1 to 464) were unsuccessful (data not shown), likely due to the presence of the hydrophobic transmembrane domain at the N terminus of the protein. To circumvent this problem, rPepD variants lacking this domain were generated. PepDΔTM lacked the first 148 amino acids, including the transmembrane domain, while PepDΔTMΔPDZ lacked both the first 148 amino acids and the last 98 amino acids, including the PDZ domain. A derivative of PepDΔTM was also constructed in which the predicted catalytic serine residue, S317, was mutated to an alanine residue (PepDΔTMS317A). Removal of the transmembrane domain resulted in protein derivatives that were partially soluble and could be purified to near homogeneity by a three-step strategy, including nickel-nitrilotriacetic acid–agarose column chromatography, size exclusion chromatography, and anion-exchange chromatography (see Fig. S1B in the supplemental material; data not shown).

Protease activities of rPepD derivatives. A previous study indicated that *M. tuberculosis* PepD possessed protease activity (33). To corroborate these data and to determine whether the PDZ domain was required, protease activity assays of rPepD

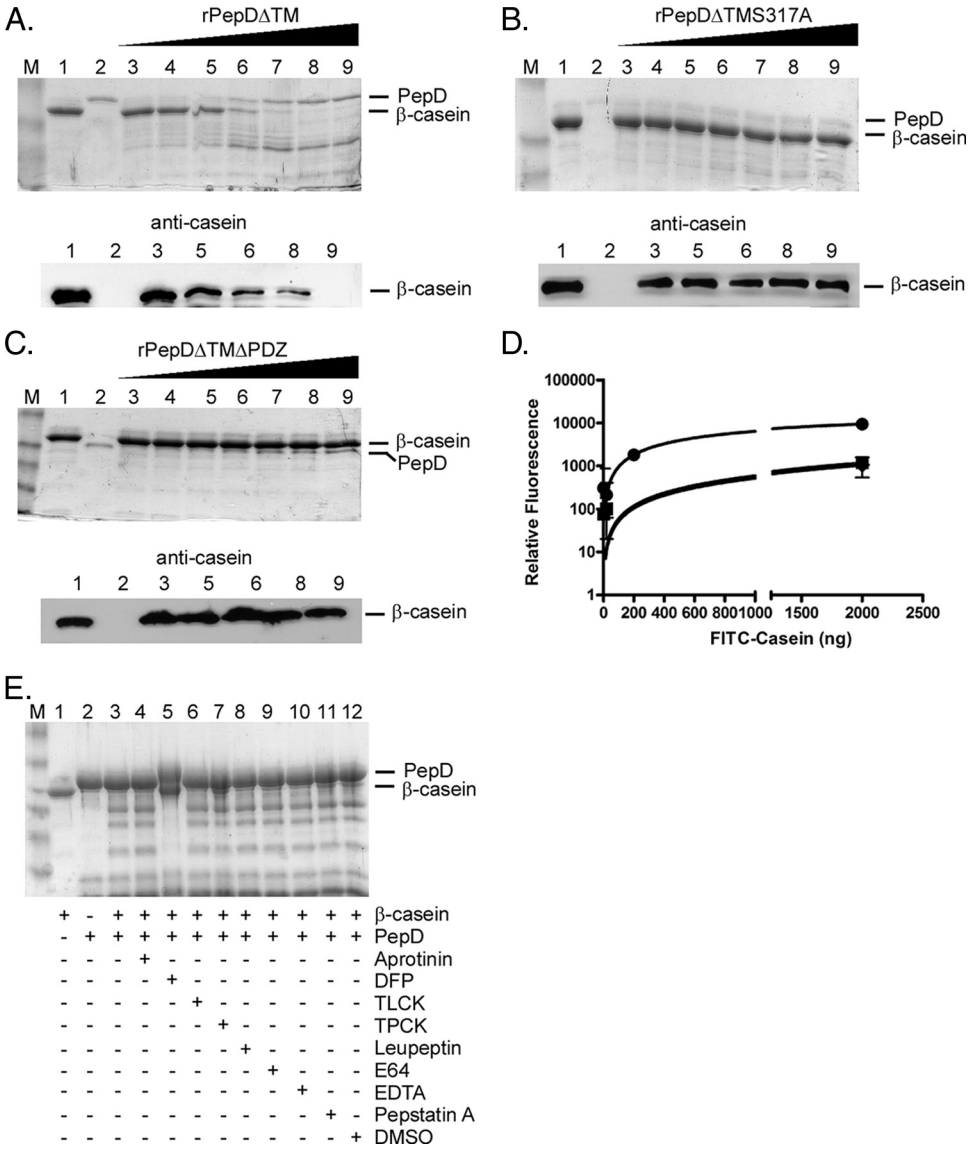

FIG. 1. rPepDΔTM exhibits proteolytic activity against the artificial substrate β-casein. Protease assays were carried out with the artificial substrate β-casein and rPepDΔTM (A), rPepDΔTMS317A (B), or rPepDΔTMΔPDZ (C) for 16 h at 37°C. The reaction mixtures contained 1 μg β-casein alone (lanes 1), 500 ng rPepD variant alone (lanes 2), or 1 μg β-casein incubated with increasing concentrations (67 to 500 ng) of rPepD variants (lanes 3 to 9). The reaction products were resolved by SDS-PAGE and visualized by either Coomassie staining or Western blotting using a polyclonal antibody against casein. (D) The cleavage of β-casein by rPepD variants was quantified using 41.5 μg FITC-labeled casein as a substrate and measuring the relative fluorescence of reaction products (loss of FITC quenching) as a function of the rPepD concentration. The rPepDΔTMS317A and rPepDΔTMΔPDZ variants exhibited approximately 10% of the activity of rPepDΔTM. ●, rPepDΔTM; ■, rPepDΔTMS317A; ◆, rPepDΔTMΔPDZ. The error bars indicate standard errors. (E) The specificity of rPepDΔTM-mediated β-casein cleavage was determined by the inclusion of various protease inhibitors in the reaction mixture. The reaction mixtures contained 1 μg β-casein alone (lane 1), 500 ng rPepDΔTM alone (lane 2), or 1 μg β-casein and 500 ng rPepDΔTM (lane 3) incubated with 1.5 mM aprotinin (lane 4), 5 mM DFP (lane 5), 27 mM TLCK (lane 6), 28 mM TPCK (lane 7), 23 mM leupeptin (lane 8), 1 mM E64 (lane 9), 50 mM EDTA (lane 10), 1 mM pepstatin A (lane 11), or DMSO (lane 12). Inhibition was observed in the reaction mixture containing DFP. Lanes M, molecular weight marker.

derivatives were conducted using β-casein as an artificial protein substrate (33, 56). Increasing amounts of rPepD were incubated with fixed amounts of β-casein at 37°C for 16 h, and the reaction products were separated by SDS-PAGE, followed by Coomassie blue staining or by Western blotting using a polyclonal antibody generated against casein. Degradation of β-casein was observed in reactions following incubation with increasing amounts of PepDΔTM (Fig. 1A). This degradation was due specifically to the catalytic activity of PepDΔTM, as β-casein cleavage was not observed in analogous reaction mixtures containing PepDΔTMS317A, a derivative lacking the catalytic serine residue (Fig. 1B). In addition, the observed protease activity required the PDZ domain, as incubation with PepDΔTMΔPDZ failed to cleave β-casein to detectable levels (Fig. 1C). To compare the extents of cleavage mediated by rPepD variants, the protease reactions were repeated using a quantita-

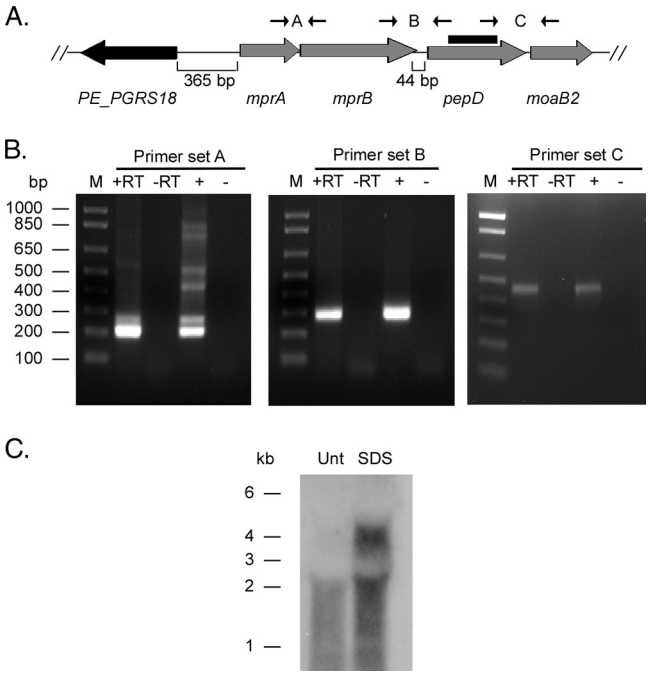

FIG. 2. Transcriptional organization of the *pepD* locus in *M. tuberculosis*. (A) Chromosomal organization of *pepD* and surrounding genes in *M. tuberculosis* H37Rv. The primers used to determine the operon structure and the probe generated for Northern blot hybridization are shown. (B) Agarose gels depicting RT-PCRs with primer sets spanning intergenic regions. Lanes: M, marker lane; +RT, RNA treated with reverse transcriptase; −RT, RNA treated without reverse transcriptase; +, genomic DNA; −, no template. (C) Northern blot of RNA samples obtained from either untreated (Unt) or SDS-treated *M. tuberculosis* H37Rv cultures.

tive fluorescence-based assay with FITC-labeled casein. Although PepDΔTM cleaved FITC-casein in a concentration-dependent manner, PepDΔTMS317A and PepDΔTMΔPDZ possessed ~10% of the activity that was observed with the parental protein (Fig. 1D). Finally, to confirm that PepD functioned as a serine protease, β-casein cleavage studies were repeated in the presence of various protease inhibitors. The serine protease inhibitor DFP inhibited the activity of PepDΔTM, as observed on Coomassie-stained gels, while other inhibitor classes failed to prevent β-casein cleavage (Fig. 1E). Further work demonstrated that PepDΔTM functions optimally as a protease at 37°C, at a pH between 7.0 and 8.5, and in a reaction buffer containing 50 mM phosphate (see Fig. S2 in the supplemental material). Thus, PepD is a serine protease that requires both its catalytic serine at position 317 and its PDZ domain for efficient proteolytic activity.

Transcriptional organization of *pepD*. To determine the transcriptional organization of *pepD*, RNA was prepared from *M. tuberculosis* H37Rv and used in RT-PCRs with primer sets spanning the intergenic regions between the genes (Fig. 2A). PCR products of the correct size were observed with cDNA using the *mprA-mprB* intergenic primer set (212 bp expected), the *mprB-pepD* intergenic primer set (278 bp expected), and the *pepD-moaB2* intergenic primer set (464 bp expected), indicating that *mprA* and *mprB* are coexpressed and that both *pepD* and *moaB2* are coexpressed on a transcript that includes *mprAB* (Fig. 2B). Products were not observed in control reactions lacking either reverse transcriptase or nucleic acid template. As further confirmation, Northern blot analysis was performed using a *pepD*-specific probe. A single transcript of approximately 2 kb was observed with RNA isolated from *M. tuberculosis* cultures grown in 7H9 medium (Fig. 2C). The size of this transcript is consistent with that expected for a message carrying the open reading frames for *pepD* and *moaB2* (1,939 bp predicted). In contrast, two transcripts (~2 kb and ~4 kb) were observed when RNA was extracted from *M. tuberculosis* cultures previously exposed to 0.05% SDS (Fig. 2C). The size of the larger transcript was consistent with that expected from the message carrying the *mprA-mprB-pepD-moaB2* coding sequences (4,189 bp predicted). When taken together, these results suggest that *pepD* and the downstream gene *moaB2* are expressed from at least two promoters, one that is located immediately upstream of *pepD* and one that is SDS responsive and positioned near or upstream of *mprA*.

Identification and characterization of *mprA* and *pepD* promoter elements. To delineate the transcriptional start site(s) for *mprAB-pepD-moaB2*, 5′ RACE was carried out on RNA isolated from *M. tuberculosis* H37Rv. Two gene-specific reverse primers separated by ~50 bp were generated to the 5′ end of either the *pepD* or the *mprA* coding sequence and used in combination with a forward primer generated to the 5′ RNA adaptor. A predominate transcript initiating at −22 (cytosine) relative to the *pepD* translational start site was identified in reaction mixtures that contained either *pepD*-specific primer (Fig. 3A and data not shown). Transcripts beginning with this nucleotide were also identified when RNA from *M. tuberculosis* cultures induced in the presence of 0.05% SDS was used. A predominant transcript initiating at +7 (guanine) relative to the previously annotated translation start site for *mprA* was identified with each *mprA*-specific primer and RNA samples from untreated cultures of *M. tuberculosis* (Fig. 3B and data not shown). This proximal *mprA* transcriptional start site is consistent with that recently reported by Pang et al. (36), suggesting that transcription and translation of *mprA* likely initiate from the same nucleotide, a site downstream of the one originally annotated in the H37Rv genome sequence. A different predominant transcriptional start site for *mprA* was identified further upstream at −204 (guanine) when RNA from *M. tuberculosis* cultures exposed to 0.05% SDS was utilized in combination with *mprA*-specific primers (Fig. 3B and data not shown). These results agree with findings by Manganelli et al. (30) that describe a putative SigE binding site in the same region upstream of *mprA* (Fig. 3B). To verify that transcripts initiating from this distal *mprA* promoter element were SDS responsive and regulated by SigE, qRT-PCR analyses were carried out with RNA from wild-type H37Rv or an isogenic Δ*sigE* mutant and a primer set that amplified a region immediately downstream of the distal *mprA* promoter. Strains were either left untreated or exposed to 0.05% SDS for 90 min. Consistent with results from Northern blot and 5′ RACE analyses, the relative expression levels of the amplified region were significantly higher in wild-type *M. tuberculosis* following SDS treatment than in untreated cultures (Fig. 3C). In contrast, the expression levels of the amplified region were not elevated in the *M. tuberculosis* Δ*sigE* mutant strain following SDS treatment, and overall expression levels in this strain were signifi-

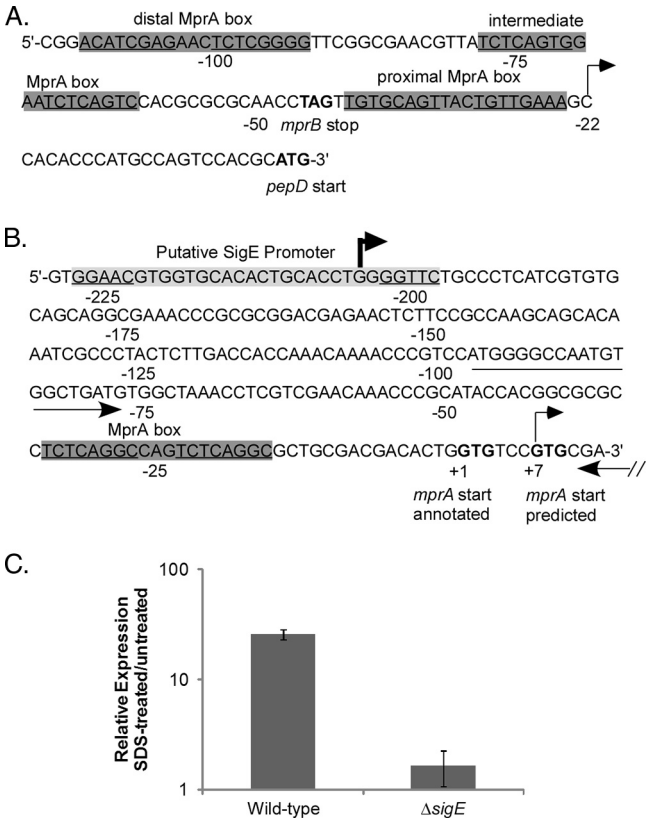

FIG. 3. Characterization of the *pepD* promoter region. (A and B) Genomic depiction of *pepD* (A) and *mprA* (B) upstream regions, including MprA binding sites (gray boxes), determined transcriptional start sites (arrows), and annotated or predicted translational start and stop sites (boldface letters). Note that one of the transcriptional start sites for *mprA* is downstream of the translational start site previously annotated. The location of the primer set used to measure expression from the distal *mprA* promoter is also shown by arrows. (C) The wild type and the Δ*sigE M. tuberculosis* mutant were grown in the absence of SDS or were exposed to 0.05% SDS for 90 min, and the relative amounts of expression from the distal *mprA* promoter were determined. Expression levels were normalized to those of the 16S ribosomal gene, *rrs*, and are expressed as relative expression from cultures exposed to SDS versus untreated cultures. The results represent the means ± standard errors of the mean (SEM) from three independent experiments performed in triplicate.

cantly lower than that observed with the wild-type *M. tuberculosis* (Fig. 3C). These results suggest that under SDS stress, *pepD* and *moaB* (in addition to *mprAB*) are expressed from a distal SigE-regulated promoter element located at −204 relative to the originally annotated *mprA* translational start site. *pepD* and *moaB* are also expressed from two promoters, one at +7 relative to the originally annotated *mprA* translational start site and the other at −22 relative to the *pepD* translational start site, during growth under physiological conditions.

Mycobacterial Δ*mprAB* and Δ*pepD* strains exhibit increased sensitivity to cell wall-perturbing stress. To determine whether PepD functions in a stress response pathway with MprAB, unmarked *mprAB* and *pepD* deletion derivatives were generated in *M. smegmatis* and *M. tuberculosis* (see Fig. S3 in the supplemental material), and the mutants were assayed for survival following exposure to cell wall-perturbing stress. *M. smegmatis* is a fast-growing nonpathogenic mycobacterial species, and it possesses a locus (*MSMEG5488-MSMEG5484*) with high amino acid homology to MprA, MprB, PepD, and MoaB2 from *M. tuberculosis* and with a similar genetic arrangement. It is important to note that the region deleted in the *M. tuberculosis* Δ*mprAB* mutant included the proximal promoter element identified at the beginning of *mprA*. To confirm the loss of PepD in the generated *M. tuberculosis* Δ*pepD* mutant, Western blots were conducted using a polyclonal antibody specific for PepD. Loss of the predicted ~50-kDa full-length form of PepD was observed in cytoplasmic fractions from the *M. tuberculosis* Δ*pepD* mutant (Fig. 4A). Interestingly, loss of a less abundant, lower-molecular-mass (~35-kDa) protein was also observed in the culture filtrate proteins from this strain (Fig. 4A), consistent with previous reports indicating that PepD may be secreted in *M. tuberculosis* (47). While deletion of *mprAB* or *pepD* did not affect the growth of *M. smegmatis* or *M. tuberculosis* in 7H9 medium *in vitro* (see Fig. S3 in the supplemental material), *M. smegmatis* Δ*mprAB* (Fig. 4B) and *M. tuberculosis* Δ*mprAB* (Fig. 4C) mutants exhibited significantly increased sensitivity to SDS relative to their wild-type counterparts. Resistance to SDS was partially restored in both mutant derivatives following expression of *M. tuberculosis mprAB* in *trans* (Fig. 4B and C). The inability to fully complement the *M. smegmatis* Δ*mprAB* mutant may be a consequence of differences in expression of *M. tuberculosis mprAB* in *M. smegmatis* or differences in recognition of *M. smegmatis* MprA-regulated genes by *M. tuberculosis* MprA. The inability to fully complement the *M. tuberculosis* Δ*mprAB* mutant is likely to be due to removal of the proximal *mprA* promoter element.

Sensitivity to SDS was also examined in Δ*pepD M. smegmatis* or *M. tuberculosis* mutants. *M. smegmatis* Δ*pepD* mutants exhibited increased sensitivity to SDS relative to the wild-type parent (Fig. 4B). This phenotype was fully complemented using *pepD* from *M. tuberculosis* (Fig. 4B). In contrast, no difference in SDS sensitivity was observed in the *M. tuberculosis* Δ*pepD* mutant relative to the wild-type parent (Fig. 4C). A similar lack of phenotype was also observed with the *M. tuberculosis* Δ*pepD* mutant following infection of primary human monocyte-derived macrophages or the J774A.1 murine macrophage-like cell line (see Fig. S4 in the supplemental material). To investigate whether the observed sensitivity of Δ*mprAB* and Δ*pepD* mutants of *M. smegmatis* was specific to cell wall-perturbing agents like SDS, the sensitivity assays were repeated with different cell wall inhibitors. Cycloserine prevents the synthesis of peptidoglycan by inhibiting the action of alanine racemase and D-alanine–D-alanine ligase (2). Cefuroxime is a cephalosporin that acts on penicillin binding proteins. *M. smegmatis* Δ*mprAB* and Δ*pepD* mutants also exhibited increased sensitivity to these cell wall agents compared to the wild-type parent (Fig. 4D and E). Furthermore, both the complemented Δ*mprAB* and Δ*pepD M. smegmatis* mutants were resistant to the activities of these antibiotics (Fig. 4D and E). Taken together, these results indicate that deletion of *pepD* or *mprAB* in *M. smegmatis* increases sensitivity to various cell wall perturbants. However, deletion of *mprAB*, but not *pepD*, increases the sensitivity of *M. tuberculosis* to SDS-mediated stress.

The Δ*mprAB* allele in *M. tuberculosis* is polar on *pepD* expression. To confirm that the failure to fully complement the

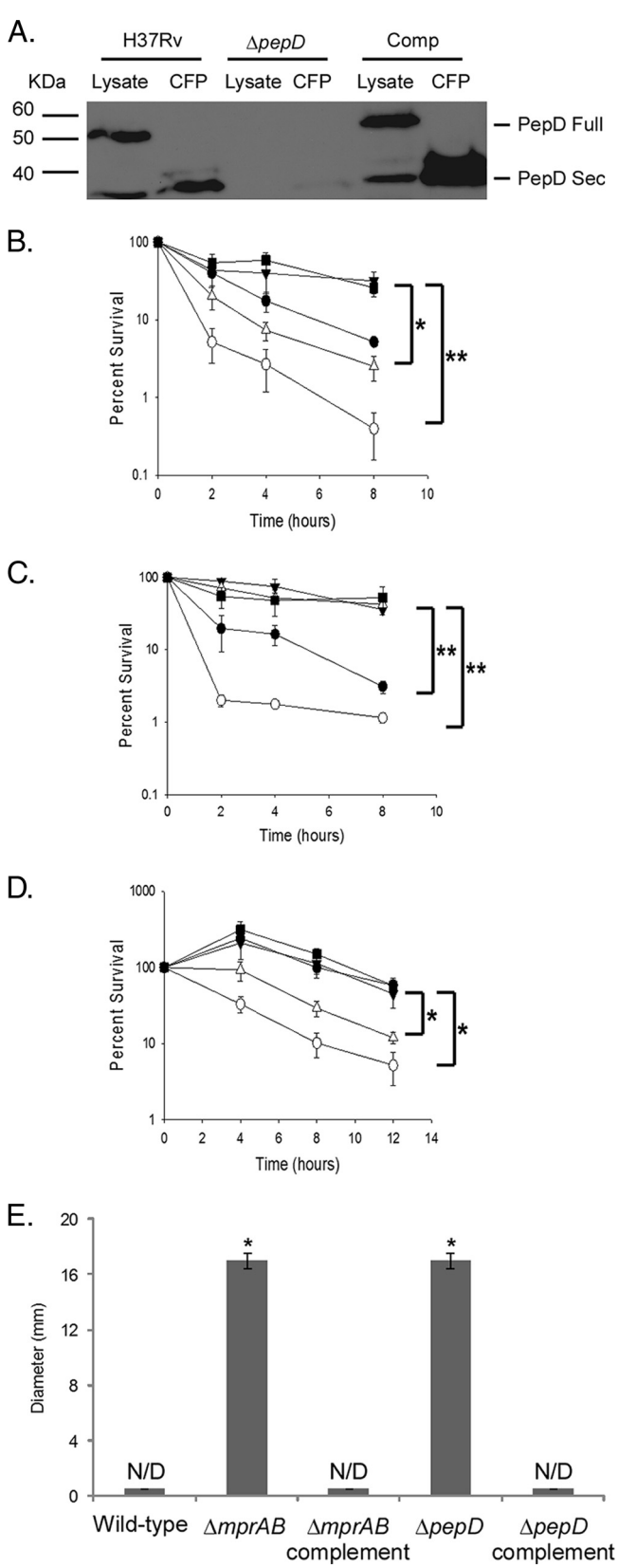

FIG. 4. Characterization of mycobacterial Δ*mprAB* and Δ*pepD* mutants. (A) Western blot analysis of cell lysates and culture filtrate protein (CFP) from wild-type, Δ*pepD*, and complemented *M. tuberculosis* strains probed with the α-PepD antibody. (B and C) Sensitivities of wild-type, mutant, and complemented *M. smegmatis* derivatives (B) or *M. tuberculosis* derivatives (C) to SDS as determined by broth sensitivity assays. The results are expressed as percent survival between untreated cultures and cultures treated with 0.1% or 0.4% SDS, respectively. (D) Survival of *M. smegmatis* derivatives after exposure to 200 μg/ml D-cycloserine expressed as percent survival between untreated and treated cultures. Survival for broth sensitivity assays was determined by plating aliquots onto 7H10 agar medium and assaying them for CFU. (E) Sensitivities of *M. smegmatis* derivatives to cefuroxime as determined by disc diffusion assay. The results are expressed as zones of inhibition (N/D, not detected). ■, wild-type; ○, Δ*mprAB*; △, Δ*pepD*; ●, Δ*mprAB* complement; ▲, Δ*pepD* complement. The results represent the means ± SEM from three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$.

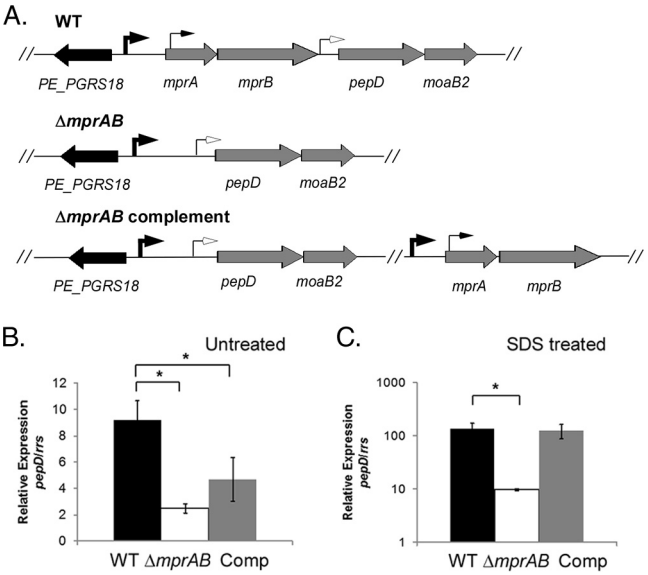

FIG. 5. Polarity resulting from the Δ*mprAB* mutation. (A) Genomic organization of the *mprA-mprB-pepD-moaB2* locus in *M. tuberculosis* H37Rv (wild type [WT], the Δ*mprAB* mutant, and complemented strains. The arrows depict the identified transcriptional start sites in the region. (B and C) Wild-type *M. tuberculosis*, the Δ*mprAB* mutant, and the Δ*mprAB* complemented strain were grown without SDS (B) or were exposed to 0.05% SDS for 90 min (C). The relative amounts of *pepD* in the strains were compared. The expression levels were normalized to that of the 16S ribosomal gene, *rrs*. The results represent the means ± SEM from three independent experiments performed in triplicate. *, $P < 0.05$.

M. tuberculosis Δ*mprAB* mutant was due to removal of the *mprA*-proximal promoter element and partial polarity on *pepD* (Fig. 5A), *pepD* expression levels were quantified by RT-PCR in wild-type, Δ*mprAB* mutant, or complemented mutant derivatives grown in the absence or presence of 0.05% SDS. In the absence of SDS, expression of *pepD* was significantly reduced in the Δ*mprAB* mutant relative to the wild-type strain (Fig. 5B). *pepD* expression levels could be only partially restored in the Δ*mprAB* mutant following expression of *mprAB* from an exogenous site in *trans* (Fig. 5B). In contrast, the expression levels of *pepD* were also lower in the Δ*mprAB* mutant following exposure to SDS; however, expression of this gene was restored to wild-type levels following the introduction of *mprAB* in *trans* (Fig. 5C). Taken together, these results support the

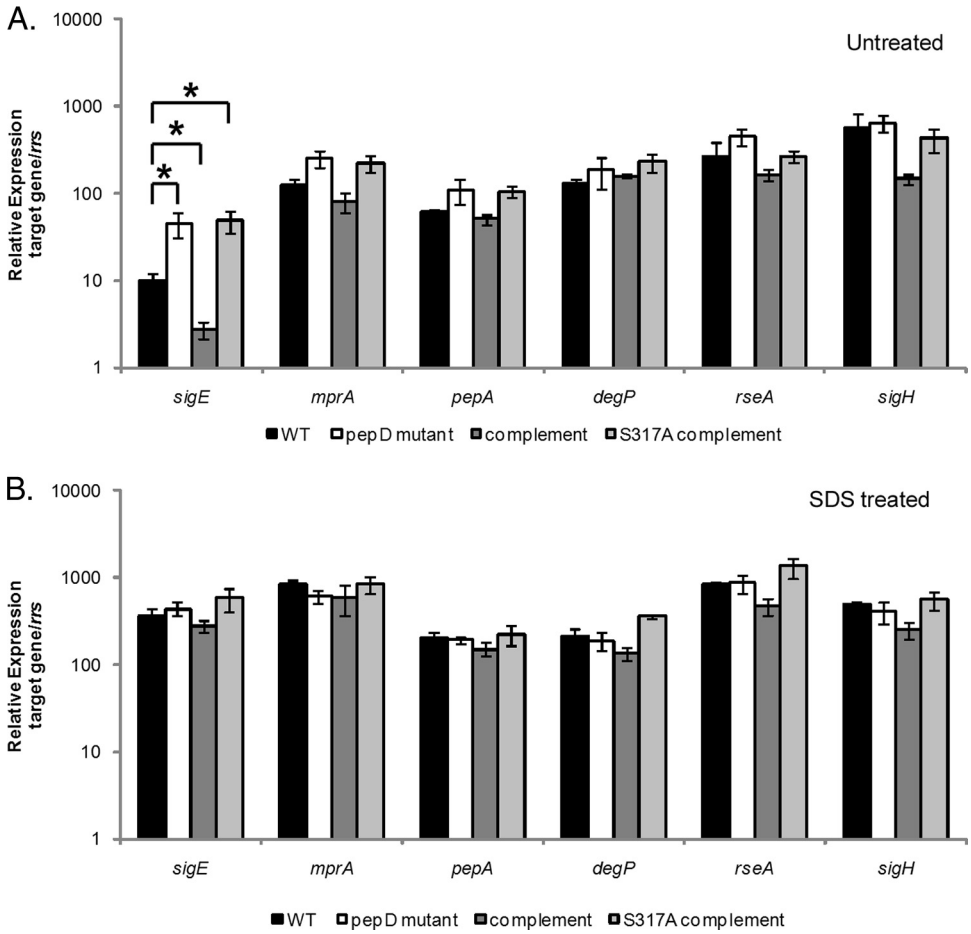

FIG. 6. Impact of *pepD* deletion on *M. tuberculosis* gene expression in the absence or presence of SDS stress. (A and B) Wild-type *M. tuberculosis*, the Δ*pepD* mutant, the Δ*pepD* complemented strain, and the Δ*pepD* S317A complemented strain were grown in the absence of stress (A) or were exposed to 0.05% SDS for 90 min (B). The relative amounts of *sigE*, *mprA*, *degP*, *pepA*, *rseA*, or *sigH* expression in the strains were then compared. The expression level for each gene under each condition was normalized to that of the 16S ribosomal gene, *rrs*. The results represent the means ± SEM from three independent experiments performed in triplicate. *, $P < 0.05$.

contention that expression of *mprAB-pepD-moaB2* proceeds from promoters in the *mprB-pepD* intergenic region and at the start of *mprA* in the absence of SDS stress and from an SDS-responsive promoter element further upstream of *mprA* during growth in the presence of SDS stress.

Deletion of *pepD* in *M. tuberculosis* leads to an upregulation in *sigE* expression. To determine whether the failure to observe SDS sensitivity in the *M. tuberculosis* Δ*pepD* mutant was a result of a compensatory upregulation in the expression of *sigE*, *mprA*, or one of the other HtrA-like serine protease genes (*degP* and *pepA*), qRT-PCR analyses were carried out in the wild type, the Δ*pepD* mutant, or the complemented Δ*pepD* mutant strain of *M. tuberculosis* grown in the absence or presence of 0.05% SDS. *sigE* was found to be significantly upregulated in the Δ*pepD* mutant compared with wild-type H37Rv in the absence of stress (Fig. 6A). This phenotype was complemented to below wild-type levels following the introduction of *pepD-moaB2* in *trans* and was lost upon mutation of the catalytic serine (S317) in *pepD* (Fig. 6A). Deletion of *pepD* also resulted in a modest but reproducible increase in *mprA* and *pepA* expression levels, although this difference did not reach statistical significance (Fig. 6A). In contrast, differences in *degP* expression were not observed between the Δ*pepD* mutant and other strains examined under these conditions. To gain further insights into the mechanism(s) of *sigE* upregulation, expression analyses were expanded to include other known regulators of *sigE*, including RseA and SigH. RseA is the cognate anti-sigma factor for SigE (12), and SigH is a sigma factor that regulates *sigE* in response to heat shock and diamide exposure (29). Neither gene displayed any difference in expression in the Δ*pepD* mutant relative to the wild-type strain under these conditions (Fig. 6A). Since *rseA* is located approximately 158 bases downstream from *sigE* and could potentially be regulated along with *sigE*, we also investigated whether these genes were cotranscribed. No PCR products were amplified using cDNA from these strains and primers nested within *sigE* and *rseA* (data not shown), supporting the contention that *sigE* and *rseA* are not in an operon together. To determine whether similar differences in expression were observed following exposure to cell wall-perturbing stress, profiling was repeated on strains exposed to 0.05% SDS. While the relative *sigE*, *mprA*, *pepA*, and *rseA* expression levels were significantly elevated in all *M. tubercu-*

TABLE 2. Genes determined to be differentially regulated between wild-type and Δ*pepD* strains by microarray analysis

Locus	Gene	Microarray ratio[a]	RT-PCR ratio[b]	Gene product[c]
Rv0079		3.0	1.5	Hypothetical protein
Rv0789c		13.0	2.0	Hypothetical protein
Rv0983	*pepD*	0.1	N/A	Serine protease
Rv0984	*moaB2*	0.06	0.6	Pterin carbinolamine dehydratase
Rv0997		8.6	2.1	Hypothetical protein
Rv1057		4.6	4.0	Hypothetical protein
Rv1076	*lipU*	0.3	0.6	Possible lipase
Rv1221	*sigE*	3.0	4.9	Sigma factor E
Rv2031c	*hspX*	2.8	1.6	Heat shock protein
Rv2784c	*lppU*	3.2	1.6	Probably lipoprotein
Rv2959c		4.6	1.8	Possible methyltransferase
Rv2987c	*leuD*	4.0	2.7	Isopropylmalate isomerase
Rv2988c	*leuC*	3.7	3.1	Isopropylmalate isomerase
Rv3095		3.5	6.0	Hypothetical transcriptional regulatory protein
Rv3135	*PPE50*	3.0	2.3	PPE family protein
Rv3841	*bfrB*	3.0	1.3	Possible bacterioferritin

[a] Microarray ratios were determined using SAM comparing Δ*pepD* and wild-type (wt) RNA (Δ*pepD*/wt).
[b] RT-PCR ratios for the genes were determined using the ΔC_T method to compare RNA levels between strains (Δ*pepD*/wt). The values were first normalized to the housekeeping sigma factor, *sigA*.
[c] Gene products are annotated based on the Pasteur Institutes' Tuberculist website (http://genolist.pasteur.fr/Tuberculist).

losis derivatives compared with the untreated controls (Fig. 6B), the overall expression levels observed between strains were similar. In contrast, the expression levels of *degP* and *sigH* were not responsive to SDS exposure in any of the strains examined, and all strains exhibited similar expression levels under these conditions (Fig. 6B). Thus, loss of *pepD* in *M. tuberculosis* results in the upregulation of *sigE* under physiological growth conditions.

Identification of genes differentially regulated in the *M. tuberculosis* Δ*pepD* mutant strain. To determine whether loss of PepD results in a general stress response under physiological conditions, global gene expression profiling studies were carried out on the wild-type parent and the Δ*pepD* mutant of *M. tuberculosis*. Overall, 16 genes were differentially regulated between these strains following growth in 7H9 medium (Table 2). Of these, 13 genes were upregulated in the Δ*pepD* mutant, including *sigE* and the heat shock protein *hspX* (Table 2). In addition, three SigE-regulated genes were also identified, including *Rv0789c*, *Rv0997*, and *Rv1057*. In contrast, three genes were significantly downregulated in the Δ*pepD* mutant compared with the wild-type strain, including *pepD* and *moaB2* (Table 2). qRT-PCR analyses of these genes confirmed the differential expression observed (Table 2). Thus, these data suggest that loss of PepD triggers a stress response in *M. tuberculosis* under physiological conditions that results in the upregulation of a number of gene products, including *sigE*, and a subset that are regulated by SigE.

DISCUSSION

M. tuberculosis is capable of adapting to and surviving within challenging environments inside the host during infection. While the mechanism(s) by which the organism achieves this is not well understood, several two-component systems and stress-responsive sigma factors are believed to participate in the process. One such system, the MprAB two-component system, regulates a number of stress-responsive genes, including the extracytoplasmic function (ECF) sigma factor *sigE* and the HtrA-family member *pepD*. Evidence presented in this study suggests overlap between the MprAB system and the SigE stress regulon that is at least partially mediated through the proteolytic activity of PepD.

PepD possesses an architecture that is conserved with other HtrA family members, including a transmembrane domain, a serine protease domain, and a single PDZ domain (26). PepD exhibits both protease and chaperone activities (33). *In vitro*, PepD functions optimally as a protease at 37°C, at near-neutral pH, and in a buffer containing 50 mM phosphate. PepD protease activity is also inhibited by the general protease inhibitor DFP, providing further evidence that this protein is a serine protease. *In vivo*, PepD is present in the cytoplasm but is also secreted from the bacterium in a mature form with a reduced molecular mass. Recent studies carried out by Mohamedmohaideen et al. in which PepD was crystallized indicate that the single PDZ domain predicted to be involved in protein-protein interactions binds to an autoproteolytic peptide and that this binding could be important for the regulation of PepD activity (33). Furthermore, this group found that PepD removes its PDZ domain in an autoproteolytic event. Consistent with these observations, we observed that removal of the PDZ domain from PepD diminishes its protease activity by >90%, suggesting that this may be a mechanism employed by *M. tuberculosis* to regulate protease activity following substrate recognition or once PepD is secreted from the bacterium. Additional studies are currently under way to better define the regions of PepD removed in the mature secreted form.

In many *Mycobacterium* species, including *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium bovis*, and *Mycobacterium avium*, *mprAB* is located immediately upstream of *pepD* and a gene encoding a hypothetical protein with homology to pterin-4-alpha-carbinolamine dehydratases. Cotranscription and promoter-mapping studies of *M. tuberculosis* have indicated that *pepD* is transcribed from three distinct promoters, one that is located in the intergenic region between *mprB* and *pepD*, one that overlaps with the translational start

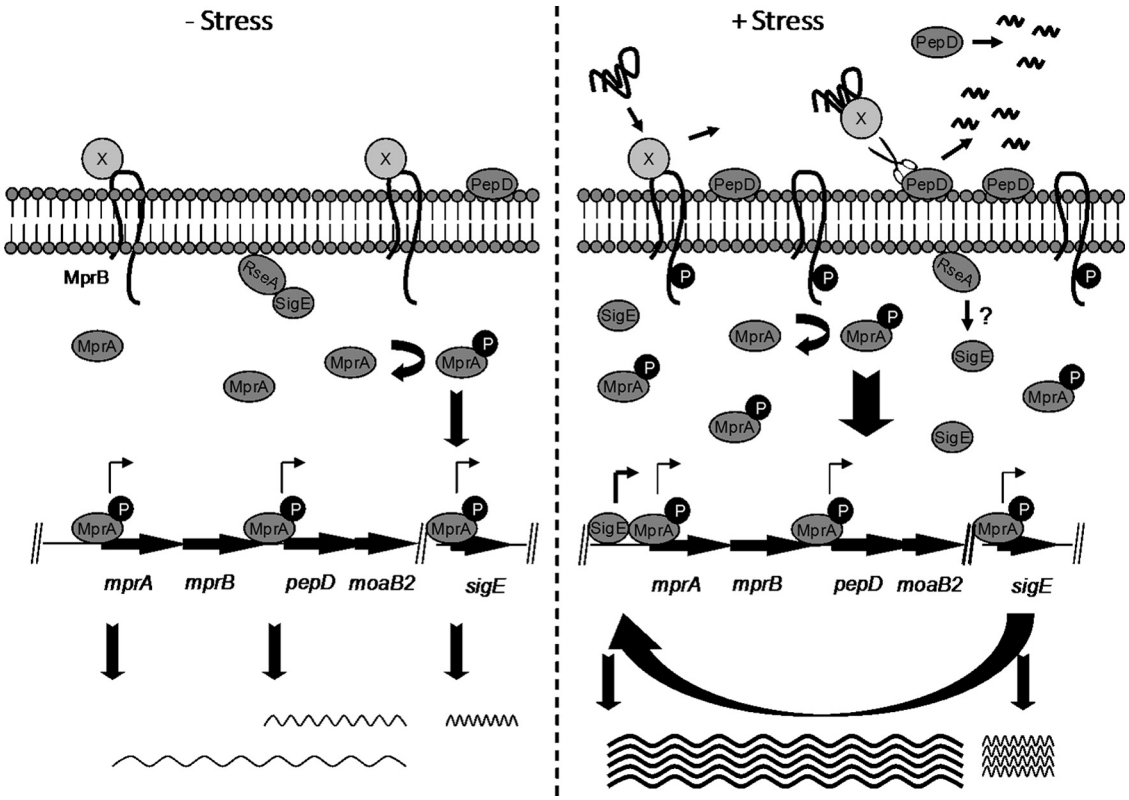

FIG. 7. Model of the activation of the MprAB system. Under nonstress conditions (left), an unidentified protein repressor (protein X) binds to MprB, keeping it in an inactive conformation and resulting in basal-level expression of *mprAB* transcription. In the presence of membrane stress (right), misfolded proteins accumulate in the extracytoplasmic space, resulting in the release of protein X from MprB and activation of the MprAB signaling pathway, including upregulation of *sigE* and subsequent induction of *mprAB-pepD-moaB*. PepD is secreted into the extracytoplasmic space between the cell membrane and the cell wall, where it functions to degrade and/or refold damaged substrate targets. Following proteolysis, the PDZ domain of PepD is removed in an autocatalytic event, resulting in release of PepD into the culture supernatant. Wavy lines indicate transcripts, with darker lines indicating higher expression lavels.

site for *mprA*, and one upstream of *mprA* that resides in a predicted SigE-regulated promoter region (30). Thus, *pepD* is regulated transcriptionally with *mprAB* and is also positively regulated by MprA posttranscriptionally through interaction at three sites located between the end of *mprB* and the beginning of *pepD* (20, 36). In an effort to examine the role of PepD in the context of the MprAB two-component system, *mprAB* and *pepD* deletion derivatives were generated in both *M. smegmatis* and *M. tuberculosis* and resistance to membrane-perturbing stresses was examined. The *M. tuberculosis* Δ*mprAB* mutant exhibited increased sensitivity to SDS compared with wild-type or complemented mutant strains, conflicting with a previous report in which an *M. tuberculosis* Δ*mprAB* mutant was found to be more resistant to SDS stress (36). While the reasons for this discrepancy are unclear, the *M. tuberculosis* Δ*mprAB* mutants carried different deletion alleles and the deletion mutations were generated using different techniques. However, a similar defect in resistance to SDS was also observed with the *M. smegmatis* Δ*mprAB* mutant, as well as increased sensitivity to cell wall inhibitors. The increased sensitivity to SDS observed in Δ*mprAB* mutants of *Mycobacterium* is consistent with previous reports demonstrating that MprAB positively regulates *sigE* expression (19, 36). Furthermore, mutants of *M. tuberculosis* unable to make SigE are hypersensitive to SDS stress (28, 30).

In addition to *mprAB*, deletion of *pepD* in *M. smegmatis* and *M. tuberculosis* also alters the response of these strains to membrane-perturbing stress. The Δ*pepD* mutant of *M. smegmatis* is more sensitive to killing by SDS, cycloserine, and cefuroxime. Interestingly, deletion of *pepD* does not alter the ability of *M. tuberculosis* to resist SDS-mediated killing. Loss of *pepD* also does not alter the growth and/or survival characteristics of *M. tuberculosis* within peripheral human blood monocyte-derived macrophages or within resting or activated J774A.1 macrophages *in vitro*. Rather, deletion of *pepD* in *M. tuberculosis* upregulates the expression of *sigE* and other stress-responsive determinants, which may compensate, at least in part, for the loss of this protein. Regardless, additional studies are needed to better delineate the complex interactions between the MprAB and SigE stress response pathways and to better define the role of PepD in these systems.

In *E. coli*, adaptation to envelope stress is mediated through the combined activities of three signal transduction systems: the CpxRA and BaeRS two-component signal transduction systems and the RpoE sigma factor (42). The CpxRA response regulator and sensor kinase and the associated periplasmically

localized inhibitory molecule, CpxP, are activated in response to a variety of stimuli, including alkaline pH (9); overexpression of envelope proteins, including NlpE and P pilus subunits (24, 48); and alterations in membrane composition (31). The Cpx regulon is comprised of over 50 genes from different transcriptional units (40), most of which are thought to assist in envelope protein folding and/or degradation. Of note, the HtrA-like serine protease *degP* is one of the genes most highly upregulated by CpxR following membrane stress (39). Similarly, the BaeRS response regulator and sensor kinase mediate resistance in *E. coli* to various toxic compounds, including antibiotics and other membrane-perturbing agents, through induction of at least two independent efflux pumps (4, 21, 34, 41). Finally, *rpoE* encodes an essential alternative sigma factor (11) that is responsive to cell envelope stress and is thought to play a critical role in maintaining cell envelope integrity (18). RpoE is activated by β-barrel proteins that have become unfolded in the periplasmic space prior to insertion into the outer membrane. Conditions that activate RpoE include exposure to heat, ethanol, or other membrane-damaging conditions (32, 44). RpoE regulates a number of outer membrane proteins and genes involved in outer membrane biogenesis (43). RpoE also regulates *degP*, the peptidylprolyl isomerases *fpkA* and *surA*, and the periplasmic chaperone *skp* (10, 22). Thus, DegP is jointly regulated by both the CpxRA and RpoE pathways in *E. coli*.

The functional and regulatory similarities between the MprAB and SigE signal transduction systems in *M. tuberculosis* and those of the CpxAR and RpoE systems from *E. coli* are striking for a number of reasons. First, all of these systems mediate resistance to membrane-perturbing stress. Second, both MprAB and CpxAR directly activate HtrA family proteases that are coregulated by SigE and RpoE, respectively. Finally, regulons controlled by all four transcription factors include genes that have been shown or are predicted to be involved in aspects of periplasmic or cellular envelope integrity. Interestingly, the MprAB and SigE networks differ in one important aspect from that seen with the Cpx and RpoE systems. In *M. tuberculosis*, MprA directly regulates expression of *sigE* and vice versa. In *E. coli*, the Cpx and RpoE networks are integrated further downstream, at *degP*.

Based on this information, we propose a model in which the MprAB and SigE systems sense and process stress in *M. tuberculosis* resulting from the accumulation of as yet unidentified unfolded or misfolded protein substrates (Fig. 7). Similar to the Cpx system, we propose that MprAB processes stress in the extracytoplasmic space. We predict that MprB is kept inactive under nonstress conditions by a protein repressor. Mutagenesis studies are currently under way to address this possibility. Following exposure to membrane-perturbing stress, this repressor disengages from MprB, binds to misfolded protein substrates, and presents them to PepD for subsequent processing. This derepression event leads to the activation of the MprAB system and subsequent upregulation of genes comprising the MprAB regulon, including *mprAB*, *pepD*, and *sigE*. As the newly synthesized SigE is abundant relative to its cognate antisigma factor RseA, it would be available to mediate downstream signaling events through the SigE regulon. SigE now functions in a positive feedback loop to drive the expression of the *mprA-mprB-pepD-moaB2* operon, as well as other genes in its regulon. Conversely, loss of PepD results in the accumulation of misfolded protein substrates in the extracytoplasmic space, leading to activation of both MprAB and SigE. Continued work on PepD is expected to provide important insights into how the MprAB two-component system and the SigE regulon function in the physiology and pathogenesis of this medically significant organism.

ACKNOWLEDGMENTS

We are grateful to members of the Zahrt laboratory for useful discussions regarding these studies. We thank the Pathogen Functional Genomics Resource Center and TB Vaccine Testing and Research Materials Contract for providing materials necessary for this research.

This work was supported by RO1 AI51669 from the National Institutes of Health and Infectious Disease and an Investigator in Pathogenesis award from the Burroughs Wellcome Fund.

REFERENCES

1. **Alba, B. M., H. J. Zhong, J. C. Pelayo, and C. A. Gross.** 2001. degS (hhoB) is an essential Escherichia coli gene whose indispensable function is to provide sigma (E) activity. Mol. Microbiol. **40:**1323–1333.
2. **Anonymous.** 2008. Cycloserine. Tuberculosis (Edinb.) **88:**100–101.
3. **Anonymous.** 2008. Global tuberculosis control. Surveillance, planning, financing. World Health Organization, Geneva, Switzerland.
4. **Baranova, N., and H. Nikaido.** 2002. The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate. J. Bacteriol. **184:**4168–4176.
5. **Bardarov, S., S. Bardarov, Jr., M. S. Pavelka, Jr., V. Sambandamurthy, M. Larsen, J. Tufariello, J. Chan, G. Hatfull, and W. R. Jacobs, Jr.** 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis. Microbiology **148:**3007–3017.
6. **Betts, J. C., P. T. Lukey, L. C. Robb, R. A. McAdam, and K. Duncan.** 2002. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol. Microbiol. **43:** 717–731.
7. **Buelow, D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J. Bacteriol. **187:**6622–6630.
8. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537–544.
9. **Danese, P. N., and T. J. Silhavy.** 1998. CpxP, a stress-combative member of the Cpx regulon. J. Bacteriol. **180:**831–839.
10. **Dartigalongue, C., D. Missiakas, and S. Raina.** 2001. Characterization of the Escherichia coli sigma E regulon. J. Biol. Chem. **276:**20866–20875.
11. **De Las Penas, A., L. Connolly, and C. A. Gross.** 1997. SigmaE is an essential sigma factor in Escherichia coli. J. Bacteriol. **179:**6862–6864.
12. **Dona, V., S. Rodrigue, E. Dainese, G. Palu, L. Gaudreau, R. Manganelli, and R. Provvedi.** 2008. Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor sigmaE in Mycobacterium tuberculosis. J. Bacteriol. **190:**5963–5971.
13. **Edgar, R., M. Domrachev, and A. E. Lash.** 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. **30:**207–210.
14. **Farn, J., and M. Roberts.** 2004. Effect of inactivation of the HtrA-like serine protease DegQ on the virulence of Salmonella enterica serovar Typhimurium in mice. Infect. Immun. **72:**7357–7359.
15. **Fisher, M. A., B. B. Plikaytis, and T. M. Shinnick.** 2002. Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes. J. Bacteriol. **184:**4025–4032.
16. **Flannagan, R. S., D. Aubert, C. Kooi, P. A. Sokol, and M. A. Valvano.** 2007. Burkholderia cenocepacia requires a periplasmic HtrA protease for growth under thermal and osmotic stress and for survival in vivo. Infect. Immun. **75:**1679–1689.
17. **Hampshire, T., S. Soneji, J. Bacon, B. W. James, J. Hinds, K. Laing, R. A. Stabler, P. D. Marsh, and P. D. Butcher.** 2004. Stationary phase gene expression of Mycobacterium tuberculosis following a progressive nutrient depletion: a model for persistent organisms? Tuberculosis (Edinb.). **84:**228–238.

18. **Hayden, J. D., and S. E. Ades.** 2008. The extracytoplasmic stress factor, sigmaE, is required to maintain cell envelope integrity in Escherichia coli. PLoS One **3:**e1573.
19. **He, H., R. Hovey, J. Kane, V. Singh, and T. C. Zahrt.** 2006. MprAB is a stress-responsive two-component system that directly regulates expression of sigma factors SigB and SigE in Mycobacterium tuberculosis. J. Bacteriol. **188:**2134–2143.
20. **He, H., and T. C. Zahrt.** 2005. Identification and characterization of a regulatory sequence recognized by Mycobacterium tuberculosis persistence regulator MprA. J. Bacteriol. **187:**202–212.
21. **Hirakawa, H., K. Nishino, T. Hirata, and A. Yamaguchi.** 2003. Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in Escherichia coli. J. Bacteriol. **185:**1851–1856.
22. **Hiratsu, K., M. Amemura, H. Nashimoto, H. Shinagawa, and K. Makino.** 1995. The rpoE gene of Escherichia coli, which encodes sigma E, is essential for bacterial growth at high temperature. J. Bacteriol. **177:**2918–2922.
23. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper, R. A. Udani, W. Jones, R. G. Barletta, and B. R. Bloom.** 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.
24. **Jones, C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren.** 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J. **16:**6394–6406.
25. **Karakousis, P. C., T. Yoshimatsu, G. Lamichhane, S. C. Woolwine, E. L. Nuermberger, J. Grosset, and W. R. Bishai.** 2004. Dormancy phenotype displayed by extracellular Mycobacterium tuberculosis within artificial granulomas in mice. J. Exp. Med. **200:**647–657.
26. **Kim, D. Y., and K. K. Kim.** 2005. Structure and function of HtrA family proteins, the key players in protein quality control. J. Biochem. Mol. Biol. **38:**266–274.
27. **Levin, B. R., and D. E. Rozen.** 2006. Non-inherited antibiotic resistance. Nat. Rev. Microbiol. **4:**556–562.
28. **Manganelli, R., E. Dubnau, S. Tyagi, F. R. Kramer, and I. Smith.** 1999. Differential expression of 10 sigma factor genes in Mycobacterium tuberculosis. Mol. Microbiol. **31:**715–724.
29. **Manganelli, R., M. I. Voskuil, G. K. Schoolnik, E. Dubnau, M. Gomez, and I. Smith.** 2002. Role of the extracytoplasmic-function sigma factor sigma(H) in Mycobacterium tuberculosis global gene expression. Mol. Microbiol. **45:** 365–374.
30. **Manganelli, R., M. I. Voskuil, G. K. Schoolnik, and I. Smith.** 2001. The Mycobacterium tuberculosis ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol. Microbiol. **41:**423–437.
31. **Mileykovskaya, E., and W. Dowhan.** 1997. The Cpx two-component signal transduction pathway is activated in Escherichia coli mutant strains lacking phosphatidylethanolamine. J. Bacteriol. **179:**1029–1034.
32. **Missiakas, D., J. M. Betton, and S. Raina.** 1996. New components of protein folding in extracytoplasmic compartments of Escherichia coli SurA, FkpA and Skp/OmpH. Mol. Microbiol. **21:**871–884.
33. **Mohamedmohaideen, N. N., S. K. Palaninathan, P. M. Morin, B. J. Williams, M. Braunstein, S. E. Tichy, J. Locker, D. H. Russell, W. R. Jacobs, Jr., and J. C. Sacchettini.** 2008. Structure and function of the virulence-associated high-temperature requirement A of Mycobacterium tuberculosis. Biochemistry **47:**6092–6102.
34. **Nagakubo, S., K. Nishino, T. Hirata, and A. Yamaguchi.** 2002. The putative response regulator BaeR stimulates multidrug resistance of Escherichia coli via a novel multidrug exporter system, MdtABC. J. Bacteriol. **184:**4161–4167.
35. **Pang, X., and S. T. Howard.** 2007. Regulation of the alpha-crystallin gene acr2 by the MprAB two-component system of Mycobacterium tuberculosis. J. Bacteriol. **189:**6213–6221.
36. **Pang, X., P. Vu, T. F. Byrd, S. Ghanny, P. Soteropoulos, G. V. Mukamolova, S. Wu, B. Samten, and S. T. Howard.** 2007. Evidence for complex interactions of stress-associated regulons in an mprAB deletion mutant of Mycobacterium tuberculosis. Microbiology **153:**1229–1242.
37. **Pechous, R. D., T. R. McCarthy, N. P. Mohapatra, S. Soni, R. M. Penoske, N. H. Salzman, D. W. Frank, J. S. Gunn, and T. C. Zahrt.** 2008. A Francisella tularensis Schu S4 purine auxotroph is highly attenuated in mice but offers limited protection against homologous intranasal challenge. PLoS One **3:**e2487.
38. **Philips, J. A.** 2008. Mycobacterial manipulation of vacuolar sorting. Cell Microbiol. **10:**2408–2415.
39. **Pogliano, J., A. S. Lynch, D. Belin, E. C. Lin, and J. Beckwith.** 1997. Regulation of Escherichia coli cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. Genes Dev. **11:**1169–1182.
40. **Price, N. L., and T. L. Raivio.** 2008. Characterization of the Cpx regulon in Escherichia coli strain MC4100. J. Bacteriol.
41. **Raffa, R. G., and T. L. Raivio.** 2002. A third envelope stress signal transduction pathway in Escherichia coli. Mol. Microbiol. **45:**1599–1611.
42. **Raivio, T. L.** 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. Mol. Microbiol. **56:**1119–1128.
43. **Rezuchova, B., H. Miticka, D. Homerova, M. Roberts, and J. Kormanec.** 2003. New members of the Escherichia coli sigmaE regulon identified by a two-plasmid system. FEMS Microbiol. Lett. **225:**1–7.
44. **Rouviere, P. E., A. De Las Penas, J. Mecsas, C. Z. Lu, K. E. Rudd, and C. A. Gross.** 1995. rpoE, the gene encoding the second heat-shock sigma factor, sigma E, in Escherichia coli. EMBO J. **14:**1032–1042.
45. **Rowley, G., A. Stevenson, J. Kormanec, and M. Roberts.** 2005. Effect of inactivation of degS on Salmonella enterica serovar typhimurium in vitro and in vivo. Infect. Immun. **73:**459–463.
46. **Schluger, N. W., and W. N. Rom.** 1998. The host immune response to tuberculosis. Am. J. Respir. Crit. Care Med. **157:**679–691.
47. **Skeiky, Y. A., M. J. Lodes, J. A. Guderian, R. Mohamath, T. Bement, M. R. Alderson, and S. G. Reed.** 1999. Cloning, expression, and immunological evaluation of two putative secreted serine protease antigens of Mycobacterium tuberculosis. Infect. Immun. **67:**3998–4007.
48. **Snyder, W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J. Bacteriol. **177:**4216–4223.
49. **Stack, H. M., R. D. Sleator, M. Bowers, C. Hill, and C. G. Gahan.** 2005. Role for HtrA in stress induction and virulence potential in Listeria monocytogenes. Appl. Environ. Microbiol. **71:**4241–4247.
50. **Sureka, K., B. Ghosh, A. Dasgupta, J. Basu, M. Kundu, and I. Bose.** 2008. Positive feedback and noise activate the stringent response regulator rel in mycobacteria. PLoS One **3:**e1771.
51. **Takayama, K., H. K. Schnoes, E. L. Armstrong, and R. W. Boyle.** 1975. Site of inhibitory action of isoniazid in the synthesis of mycolic acids in Mycobacterium tuberculosis. J. Lipid Res. **16:**308–317.
52. **Talaat, A. M., R. Lyons, S. T. Howard, and S. A. Johnston.** 2004. The temporal expression profile of Mycobacterium tuberculosis infection in mice. Proc. Natl. Acad. Sci. U. S. A. **101:**4602–4607.
53. **Talaat, A. M., S. K. Ward, C. W. Wu, E. Rondon, C. Tavano, J. P. Bannantine, R. Lyons, and S. A. Johnston.** 2007. Mycobacterial bacilli are metabolically active during chronic tuberculosis in murine lungs: insights from genome-wide transcriptional profiling. J. Bacteriol. **189:**4265–4274.
54. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. **98:**5116–5121.
55. **Twining, S. S.** 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. **143:**30–34.
56. **Waller, P. R., and R. T. Sauer.** 1996. Characterization of degQ and degS, Escherichia coli genes encoding homologs of the DegP protease. J. Bacteriol. **178:**1146–1153.
57. **Wilson, R. L., L. L. Brown, D. Kirkwood-Watts, T. K. Warren, S. A. Lund, D. S. King, K. F. Jones, and D. E. Hruby.** 2006. Listeria monocytogenes 10403S HtrA is necessary for resistance to cellular stress and virulence. Infect. Immun. **74:**765–768.
58. **Zahrt, T. C.** 2003. Molecular mechanisms regulating persistent Mycobacterium tuberculosis infection. Microbes Infect. **5:**159–167.
59. **Zahrt, T. C., and V. Deretic.** 2001. Mycobacterium tuberculosis signal transduction system required for persistent infections. Proc. Natl. Acad. Sci. U. S. A. **98:**12706–12711.