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# A Mycobacterial Extracytoplasmic Function Sigma Factor Involved in Survival following Stress

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**The extracytoplasmic function (ECF) sigma factors constitute a diverse group of alternative sigma factors that have been demonstrated to regulate gene expression in response to environmental conditions in several bacterial species. Genes encoding an ECF sigma factor of *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium smegmatis*, designated *sigE*, were cloned and analyzed. Southern blot analysis demonstrated the presence of a single copy of this gene in these species and in *Mycobacterium bovis* BCG, *Mycobacterium leprae*, and *Mycobacterium fortuitum*. Sequence analysis showed the *sigE* gene to be highly conserved among *M. tuberculosis*, *M. avium*, *M. smegmatis*, and *M. leprae*. Recombinant *M. tuberculosis* SigE, when combined with core RNA polymerase from *M. smegmatis*, reconstituted specific RNA polymerase activity on *sigE* in vitro, demonstrating that this gene encodes a functional sigma factor. Two in vivo transcription start sites for *sigE* were also identified in *M. smegmatis* and *M. bovis* BCG. Comparison of wild-type *M. smegmatis* with a *sigE* mutant strain demonstrated decreased survival of the mutant under conditions of high-temperature heat shock, acidic pH, exposure to detergent, and oxidative stress. An inducible protective response to oxidative stress present in the wild type was absent in the mutant. The mycobacterial SigE protein, although nonessential for viability in vitro, appears to play a role in the ability of these organisms to withstand a variety of stresses.**

Regulation of gene expression in response to the extracellular environment is an adaptive response that is required for bacterial replication and survival. Alternative sigma factors have been demonstrated to play an important role in the coordinate regulation of gene expression in bacteria undergoing major adaptive changes in physiology, well-studied examples of which include sporulation in *Bacillus subtilis* and the stationary-phase response in *Escherichia coli* (11, 14, 36).

Sigma factors of the sigma-70 class have been grouped into families based on conservation of sequence and function across bacterial species (24, 25). An extended family of alternative sigma factors, the SigE family, has been found to regulate gene expression required for diverse functions in several bacterial species, including survival following high-temperature heat shock in *E. coli* and alginate production in *Pseudomonas aeruginosa* (8, 19, 30, 31). In many cases, these SigE sigma factors regulate bacterial gene expression in response to the extracellular environment and have been referred to as extracytoplasmic function (ECF) sigma factors (24).

In pathogenic bacteria, regulation of gene expression during the course of infection is necessary to survive in the environment of the host organism. Examples of alternative sigma factor-regulated gene expression that play a role bacterial pathogenesis include regulation of alginate production by AlgU resulting in mucoid *P. aeruginosa* in patients with cystic fibrosis and regulation of the plasmid-encoded *spv* virulence genes of *Salmonella* spp. by RpoS (10).

Pathogenic mycobacteria, including *M. tuberculosis*, are primarily intracellular pathogens. Following infection of the host, they are ingested by macrophages, where they reside and replicate. Examination of bacterial functions required for survival following uptake by macrophages would provide insight into mycobacterial pathogenesis and might provide novel targets for antimycobacterial therapeutics. We hypothesized that the adaptation of the infecting mycobacterium to the intracellular environment of the host macrophage requires the coordinate regulation of expression of a large number of diverse genes and that this regulation may be mediated in part by one or more alternative sigma factors. To begin to investigate this hypothesis, we have cloned and begun to characterize a mycobacterial ECF-type sigma factor.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** Strains and plasmids are shown in Table 1. *E. coli* cells were grown on L agar or in L broth. *Mycobacterium smegmatis* liquid cultures were grown in Middlebrook 7H9 broth supplemented with 0.2% glucose and 0.05% Tween 80. *M. smegmatis* was plated on Middlebrook 7H10 plates supplemented with 0.2% glucose or on L agar. Ampicillin (25 to 50 μg/ml) or kanamycin (50 μg/ml) was added to culture media as appropriate.

For determination of survival following heat shock, acid shock, detergent exposure, or hydrogen peroxide exposure, cultures of wild-type ($mc^2$-155) and *sigE* mutant *M. smegmatis* (RH243) cells were grown overnight in liquid medium at 30°C to log phase (optical density at 600 nm [$OD_{600}$] of 0.3 to 0.4). These cultures were diluted in medium to an $OD_{600}$ of 0.2, and the freshly diluted cultures were exposed to the following stress conditions: (i) 50°C (high-temperature heat shock), (ii) pH 4 (50 mM citric acid; acid stress), (iii) 0.01% sodium dodecyl sulfate (SDS), and (iv) 5 mM hydrogen peroxide (oxidative stress). Cultures were incubated with shaking (at 30°C except for those exposed to heat shock), and aliquots were removed from each culture for plating at the specified times during the course of the experiment. Tenfold serial dilutions were plated at each time point, and colony counting was performed on days 3 to 4 with duplicate or triplicate plates counted at each of two dilutions. Experiments were performed at least twice for each stress condition. For the hydrogen peroxide pretreatment experiments, exposure to 50 μM hydrogen peroxide for 1 h was followed by addition of hydrogen peroxide to 5 mM, followed by plating of dilutions as described above. To determine the concentration of hydrogen peroxide that resulted in 50% killing ($LD_{50}$) of the wild-type and *sigE* mutant strains, cultures were grown and diluted as described above. Serial dilutions of hydrogen peroxide were added, and the cultures were incubated for 30 min and then plated as described above. The $LD_{50}$ was calculated by the method of Karber (21).

**PCR amplification and cloning of mycobacterial *sigE* genes.** Primers were designed based on the sequence of a cosmid clone generated in the *Mycobacterium leprae* genome sequencing project (cosmid B1756; GenBank accession no. U15180). The sequences of these primers were 5′-ACCAGCCAGGAACCTTT GAGG-3′ (positions 19995 to 20015) and 5′-TGATAGATCTGCTCGGGATT

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| *M. smegmatis* | | |
| mc$^2$-155 | High-frequency transformation strain derived from wild-type *M. smegmatis* mc$^2$-6 | 37 |
| RH243 | *aph* inserted at *Bgl*II site in *sigE* resulting from recombination of pRH1264 into chromosome of mc$^2$-155 | This study |
| *E. coli* | | |
| BL21(DE3) | F$^-$ *ompT hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) | Novagen |
| XL1 Blue MRF′ | Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB lacI*$^q$*ZΔM15* Tn*10* (Tet$^r$)] | Stratagene |
| Y1090 | F$^-$ Δ(*lac*)*U169 lon-100 araD139 rpsL* (Str$^r$) *supF mcrA trpC22*::Tn*10* (pMC9; Tet$^r$ Ap$^r$) | 17 |
| Plasmids | | |
| pCRII | Cloning vector; Ap$^r$ Km$^r$ | Invitrogen |
| pBluescript | Cloning vector; Ap$^r$ | Stratagene |
| pET19b | Expression vector for generation of amino-terminal histidine-tagged recombinant proteins; Ap$^r$ | Novagen |
| pRH1228 | 6-kb insert in pBluescript containing *sigE* locus of *M. tuberculosis* H37Rv isolated from genomic DNA library in λZAP | This study |
| pRH1249 | 5.9-kb insert in pBluescript containing *sigE* locus of *M. avium* isolated from genomic DNA library in λgt11 | This study |
| pRH1256 | 549-bp 5′ region of *M. tuberculosis sigE*; added *Bam*HI site at the 5′ end and *Kpn*I site at the 3′ end of the insert in pCRII | This study |
| pRH1257 | 4-kb insert containing *sigE* locus of *M. smegmatis* in pBluescript | This study |
| pRH1264 | *aph* of Tn*903* inserted into *Bgl*II site in coding region of *M. smegmatis sigE* in pRH1257; Km$^r$ | This study |
| pRH1265 | 486-bp 5′ region of *M. smegmatis sigE*, added *Bam*HI site at the 5′ end and *Kpn*I site at the 3′ end of the insert in pCRII | This study |
| pKL100 | Coding region of *M. tuberculosis sigE* cloned into *Nde*I and *Xho*I sites of pET19b | This study |

G-3′ (positions 20148 to 20128). Amplification was performed with *Taq* DNA polymerase (Boehringer Mannheim) at concentrations of $MgCl_2$ ranging from 1.5 to 3.5 mM with approximately 50 ng of genomic *M. tuberculosis* DNA as a template. Thirty cycles of 95°C for 1 min, 37°C for 1 min, and 72°C for 1 min were performed. The resulting PCR product was subcloned and used as a probe to screen an *M. tuberculosis* H37Rv genomic DNA library in the phagemid vector λZAPII (host strain XL1 Blue MRF′) (1). Clones were plaque purified, and plasmid-containing *M. tuberculosis* DNA in the vector pBluescript was obtained through the use of helper phage according to the manufacturer's protocol (Stratagene, La Jolla, Calif.). $^{32}$P-labeled DNA fragments of the *M. tuberculosis* gene were subsequently used as probes to screen a *Mycobacterium avium* genomic DNA library in λgt11 and an *M. smegmatis* genomic DNA library in pUC19 to obtain the *sigE* genes of these species (16, 39).

**DNA manipulation and sequence analysis.** Lambda DNA was purified and plasmid DNA was isolated according to standard methods. Restriction and modification enzymes were obtained from New England Biolabs or Boehringer Mannheim. *M. smegmatis*, *Mycobacterium bovis* BCG, and *M. avium* genomic DNAs were purified as previously described (18). *M. tuberculosis* and *M. leprae* genomic DNAs were obtained from Patrick Brennan and John Belisle (Colorado State University). Southern blot analysis was performed with $^{32}$P-labeled *M. tuberculosis* DNA from the *sigE* coding region according to standard methods. Final wash conditions were 60°C in 0.2× SSC (1× SSC is 0.15 M NaCl, 0.015 M $Na_3$ citrate · $2H_2O$ [pH 7.0])–0.2% SDS.

A deletion series for sequencing the *M. tuberculosis sigE* gene and flanking DNA was made by exonuclease III followed by S1 nuclease digestion of pRH1228 with the Erase-a-Base kit (Promega, Madison, Wis.) according to the manufacturer's protocol. Sequencing of the *M. avium* and *M. smegmatis sigE* genes was performed on subcloned restriction fragments and by using oligonucleotide primers based on the adjacent sequence. Sequencing was performed manually and on an ABI 373A automated DNA sequencer with *Taq* dye terminator chemistry (Applied Biosystems, Foster City, Calif.) in the core sequencing facility of the Children's Hospital Mental Retardation Research Center. Sequence assembly and analysis were performed with Sequencher (Gene Codes Corp., Ann Arbor, Mich.) and the GCG package of software (Genetics Computer Group, Madison, Wis.). *M. leprae* sequence data were obtained from Douglas Smith (Genome Therapeutics, Waltham, Mass.) and from GenBank.

**Purification of RNA polymerase from *M. smegmatis*.** *M. smegmatis* RNA polymerase was purified by a modification of published procedures (5). *M. smegmatis* cultures were pelleted, and the pellet was weighed and then lysed with a French press (10,000 to 15,000 lb/in$^2$) in 0.2 M KCl in TGED (50 mM Tris-HCl [pH 7.5], 10% glycerol, 1 mM EDTA, 10 mM dithiothreitol, 10 mM $MgCl_2$) (approximately 1:1 [wt/vol]). The crude extract was centrifuged at 10,000 × *g* for 30 min at 4°C, the supernatant was collected, and 3.5 ml of 10% polyethylenimine was added per 100 ml. The solution was stirred gently for 30 min at 4°C and then was centrifuged at 10,000 × *g* for 30 min at 4°C. The pellet was resuspended with 30 ml of 0.5 M KCl in TGED and centrifuged at 10,000 × *g* for 30 min at 4°C, followed by resuspension and centrifugation in 1.0 M KCl in TGED. The supernatant was collected, and 35 g of ammonium sulfate was added per 100 ml. The solution was stirred in a cold room for 3 h, and the pellet was collected by centrifugation at 20,000 × *g* for 60 min at 4°C. This pellet was resuspended in TGED containing 0.2 M KCl and loaded onto a Bio-Gel A 1.5 M gel filtration column (bed volume, 350 ml) equilibrated with TGED containing 0.1 M KCl. The flow rate was 0.5 ml/min, and 8-ml fractions were collected and analyzed by in vitro transcription reactions with pIS109 DNA as a template (29). Transcriptionally active fractions were pooled and then loaded onto a heparin agarose column (bed volume, 30 ml) preequilibrated with TGED containing 0.1 M KCl at 0.5 ml/min. The column was washed with three bed volumes of TGED containing 0.2 M KCl and eluted with a gradient of 0.1 to 0.6 M KCl. Fractions were analyzed for transcriptional activity in reaction mixtures containing calf thymus DNA (nonspecific transcription) or pIS109 (promoter-specific transcription) (29). The fractions that were transcriptionally active with pIS109 were pooled and dialyzed against TGED containing 0.1 M KCl. To purify core enzyme from sigma factor subunits, the sample was loaded (flow rate of 0.1 ml/min) onto a 1.5-ml-bed-volume Bio-Rex 70 (Bio-Rad) column preequilibrated with TGED containing 0.1 M KCl. The column was washed with TGED containing 0.1 M KCl, and the core polymerase was eluted with 0.6 M KCl in TGED. The core polymerase preparation was concentrated and dialyzed against TGED containing 50% glycerol and 0.5 M KCl and stored in small aliquots. The extent of purification was determined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

**Purification of *M. tuberculosis* SigE protein.** The *M. tuberculosis sigE* gene was PCR amplified with primers incorporating an *Nde*I site in the plus-strand primer and an *Xho*I site in the minus-strand primer. The sequences of these primers are 5′-GGGCCCATCCATATGCCGTCCTGGGATGAGCTGGTCCGTCAG-3′ and 5′-GGGCCCATCCTCGAGTCAGCGAACTGGGTTGACGTGAACTGC GCACTCG-3′. These primers were chosen to generate a product originating from the most 3′ potential initiation codon of the gene to generate a product that encodes regions 1.2, 2, 3, and 4 of SigE. The resulting PCR product was cloned into the expression vector pET19b (Novagen, Madison, Wis.), and the resulting construct (pKL100) was used to transform *E. coli* BL21(DE3).

The N-terminally histidine-tagged SigE protein (his-SigE) was purified from this strain by growth in L broth plus ampicillin to an $OD_{600}$ of 0.5, followed by addition of isopropylthio-β-D-galactoside to 2 mM and growth for an additional 3 h. Bacteria were harvested by centrifugation, and the cell pellet was resuspended in 20 ml of buffer A (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 5 mM imidazole). Cells were lysed by sonication, and the lysate was cleared by centrif-

ugation. $(NH_4)_2SO_4$ was added to 60%, followed by centrifugation (16,000 × *g* for 20 min at 4°C). The pellet was dissolved in buffer A containing 6 M guanidine hydrochloride, and the sample was adsorbed onto 1.0 ml of $Ni^{2+}$-nitrilotriacetic acid agarose (Qiagen, Chatsworth, Calif.) in buffer A. The adsorbed protein was washed three times with buffer A, washed three times with buffer B (50 mM Tris-HCl [pH 8.0], 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, 20% [vol/vol] glycerol) plus 30 mM imidazol, and then eluted with buffer A plus 500 mM imidazol. The eluted protein was dialyzed for 16 h at 4°C against two 1-liter changes of buffer B. Following dialysis, the protein solution was incubated for 45 min at 30°C and then was cleared by centrifugation (16,000 × *g* for 15 min at 4°C). Glycerol was added to 50% (vol/vol), and the solution was stored at −20°C. For in vitro transcription, RNA polymerase was reconstituted by incubation of purified his-SigE protein with excess *M. smegmatis* core polymerase for 30 min on ice prior to transcription assays.

**In vitro transcription.** For in vitro transcription analysis, the 5′ region of the *M. tuberculosis sigE* gene was PCR amplified with primers designed to incorporate a *Kpn*I site (underlined) 245 bases beyond the initiation codon (5′-GCTG GGGCGGCTGGATCCAGGCATGTCGTCGT-3′ and 5′-ACCAGCTCATCC CGGTACCGGCATGGTGGCCTT-3′). The *M. smegmatis sigE* 5′ region was amplified with primers that incorporate a *Kpn*I site 95 bases beyond the initiation codon (5′-GGCGTGGCGTGACGGATCCGCCTTCGGGCTGA-3′ and 5′-TC CCAGGACGGCATGGTACCGCCTGGTCACCG-3′). These PCR products were cloned into pCRII to create pRH1256 and pRH1265, which contain the *M. tuberculosis* and *M. smegmatis* 5′ regions, respectively.

In vitro transcription was performed as previously described (35). RNA was synthesized in 50-μl reaction volumes containing transcription buffer (50 mM Tris-HCl [pH 8.0], 200 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1.5 μM bovine serum albumin), 1 μg of DNA template linearized by digestion with *Kpn*I, 50 ng of purified core RNA polymerase, 4 μM UTP containing 5 μCi of [α-$^{32}$P]UTP, and 400 μM (each) ATP, GTP, and CTP. The transcription reaction mixtures were incubated at 37°C for 30 min and then were terminated by addition of 50 μl of formamide loading buffer. The transcripts were analyzed by PAGE followed by autoradiography.

**Primer extension mapping of *sigE* transcription start sites in vivo.** Cells were grown to late log phase at 30°C. For heat shock, the temperature was shifted to 37, 42, or 50°C for 30 min prior to RNA isolation. Mycobacterial RNA was isolated with the hot SDS-phenol method (32). Primer extension was performed with avian myeloblastosis virus reverse transcriptase (Gibco, Gaithersburg, Md.) following $^{32}$P-labeled primer annealing at 45°C, according to standard methods (1a). The primer extension products were electrophoresed on 6% polyacrylamide sequencing gels adjacent to dideoxy sequencing reactions performed with the same primer used in the primer extension reaction. The primers used were 5′-TCGGTGAACTGTTCGAGGTGGGC-3′ for *M. smegmatis*, corresponding to positions 419 to 398 of *sigE*, and 5′-GGTCGTGATATTGAGATCCTCCGA ATTTG-3′ for *M. bovis* BCG *sigE*, corresponding to positions 587 to 559 in the *M. tuberculosis* sequence. At least two additional primers, one 5′ and one 3′ to these primers, were also used to determine whether additional 5′ RNA termini could be identified.

**Disruption of the *sigE* locus in *M. smegmatis*.** pRH1257 was digested with *Bgl*II, and the 1.25-kb *aph* (Km$^r$) gene of Tn*903* was inserted in the same transcriptional orientation as *sigE*, to generate pRH1264 (2). This insertion disrupts the inferred *sigE* structural gene 71 codons prior to the stop codon. This intact plasmid was electroporated into mc$^2$-155, and transformants were selected on kanamycin plates. Transformants were screened by PCR to distinguish single from double homologous recombination events. Those in which allelic exchange (double crossover) had occurred should generate a single PCR product with a size of 1.5 kb corresponding to the gene plus the 1.25-kb insertion, while transformants in which a single homologous recombination event had occurred generate two PCR products, one with a size of 0.25 kb corresponding to the intact wild-type gene and a second with a size of 1.5 kb corresponding to the disrupted gene. Of 14 transformants analyzed, PCR indicated that a double crossover had occurred in one. The occurrence of double-crossover homologous recombination, resulting in a single disrupted copy of *sigE* in the chromosome of this transformant, was confirmed by Southern blot analysis as previously described (15). This clone was designated RH243 and was used for subsequent experiments comparing the growth of the wild-type and *sigE* mutant strains.

**Nucleotide sequence accession number.** Sequences of the *sigE* loci of *M. smegmatis*, *M. avium*, and *M. tuberculosis* have been deposited in GenBank under accession no. U87307, U87308, and U87242, respectively.

## RESULTS

**Cloning, Southern blotting, and sequence analysis of the *sigE* locus of *M. tuberculosis*, *M. avium*, and *M. smegmatis*.** Amplification of *M. tuberculosis* DNA with primers based on the *M. leprae sigE* sequence generated products of the expected size of approximately 150 bp only when a low-temperature (37°C) annealing step was used in the amplification program. This PCR product was sequenced and found to be >80% identical to the corresponding region of the *M. leprae sigE* sequence, and it was then used as a probe to isolate a 6.0-kb *M. tuberculosis* DNA insert in pBluescript, designated pRH1228. The *M. tuberculosis sigE* gene was then used as a probe to clone the homologs of this gene in *M. avium* (pRH1249) and *M. smegmatis* (pRH1257). Southern blotting demonstrated the presence of a single copy of this gene in these species and in *M. bovis* BCG, *M. leprae*, and *M. fortuitum* (not shown).

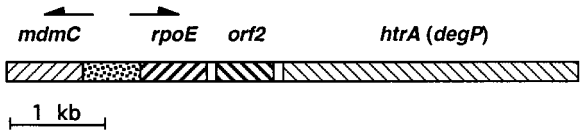


FIG. 1. The *sigE* locus of *M. tuberculosis*. The organization of this region is conserved in *M. smegmatis*, *M. avium*, and *M. leprae*.

Sequence analysis of the *M. tuberculosis* clone revealed the presence of at least four open reading frames in this locus (Fig. 1). These open reading frames include the *sigE* coding region of 257 codons encoding a putative protein with a size of 28.9 kDa, a second open reading frame of 193 codons in the same orientation as *sigE* encoding a putative protein with a size of 21.1 kDa, and a third open reading frame of 542 codons, also in the same orientation as *sigE*, encoding a putative protein with a size of 55.7 kDa.

The initiation codon of the second open reading frame is separated from the stop codon of *sigE* by 40 nucleotides. The inferred amino acid sequence of the second open reading frame does not match those of any previously identified proteins. The initiation codon of the third open reading frame is separated from the stop codon of the second open reading frame by 24 nucleotides. The inferred amino acid sequence of the third open reading frame indicates that it encodes a homolog of the *E. coli* serine protease DegP (HtrA). A fourth open reading frame is located 471 nucleotides 5′ of *sigE* and is in the opposite transcriptional orientation. Limited sequence analysis of this gene indicates that it is similar to an *M. leprae* gene in the same location and that these mycobacterial genes are related to an *O*-methyltransferase gene, *mdmC*, of *Streptomyces mycarofaciens* (12).

Sequencing and analysis of the *M. avium* and *M. smegmatis* clones, as well as analysis of *M. leprae* sequence of this region generated in the *M. leprae* genome sequencing project, indicate an identical organization of this region in each of these mycobacterial species. Comparison of the inferred amino acid sequences of the *sigE* genes of these species demonstrates a high degree of conservation of this protein, with the *M. smegmatis*, *M. avium*, and *M. leprae* protein sequences being 92, 82, and 91% identical to that of the *M. tuberculosis* protein, respectively. Most of the variation among these proteins occurs in the amino-terminal and in the extreme carboxy-terminal regions of these proteins (Fig. 2). The amino terminus of the *M. leprae* deduced protein lacks 87 amino acids present in the *M. tuberculosis* deduced protein. An open reading frame in the *M. leprae* sequence 5′ to the assigned *sigE* initiation codon, however, is highly homologous at the amino acid level to the amino-terminal *M. tuberculosis sigE* sequence, suggesting a frameshift sequencing error in the *M. leprae* sequence. Alternatively, potential initiation codons in the *M. tuberculosis*, *M. avium*, and *M. smegmatis* sequences that correspond to the assigned *M. leprae* initiation codon can be identified. Comparison of the mycobacterial *sigE* genes to sigma factor genes from other species places them within the ECF family of sigma subunits. The closest matches to the inferred mycobacterial SigE proteins are Ybbl of *B. subtilis* and SigE of *E. coli*, which

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Mtsige      1 MELLGGPRVGNTESQLCVADGDDLPTYCSANSEDLNITTITTLSPTSMSHPQQVRDDQWV
Mssige      1 ..........................................MAHLEQFTDSDWV
Masige      1 .MDRGARETGNTEWQLPVAANDEMPLIGMPNSEELIITTL..LSPSSMSHAHDPSADGWA
Ecosige     1 ..........................................................

Mtsige     61 EPSDQLQGTAVFDATGDKATMPSWDELVRQHADRVYRLAYRLSGNQHDAEDLTQETFIRV
Mssige     14 EPSDEPTGTAVFDATGDQAAMPSWDELVRQHADRVYRLAYRLSGNQHDAEDLTQETFIRV
Masige     58 EPSDGLQGTAVFDATGDKTAMPSWDELVRQHADRVYRLAYRLSGNQHDAEDLTQETFIRV
Ecosige     1 .MSEQLTD.QVLVERVQKGDQKAFNLLVVRYQHKVASLVSRYVPSG.DVPDVVQEAFIKA

Mtsige    121 FRSVQNYQPG.TFEGWLHRI....TTNLFLDMVRRRARIRMEAL.PEDYDRVPA..DEPN
Mssige     74 FRSVQNYQPG.TFEGWLHRI....TTNLFLDMVRRRGRIRMEAL.PEDYDRVPA..EDPN
Masige    118 FRSVQNYQPG.TFEGWLHRI....TTNLFLDMVRRRSRIRMEAL.PEDYERVPA..DEPN
Ecosige    58 YRALDSFRGDSAFYTWLYRIAVNTAKNYLVAQGRRPPSSDVDATEAENFESGGALKEISN

Mtsige    173 PEQIYHDARLGPDLQAALASLPPEFRAAVVLCDIEGLSYEEIGATLGVKLGTVRSRIHRG
Mssige    126 PEQIYHDSRLGADLQAALDSLPPEFRAAVVLCDIEGLSYEEIGATLGVKLGTVRSRIHRG
Masige    170 PEEIYHDSRLGPDLQAALDSLPPEFRAAVVLCDIEGLSYEEIGATLGVKLGTVRSRIHRG
Ecosige   118 PENLMLSEELRQIVFRTIESLPEDLRMAITLRELDGLSYEEIAAIMDCPVGTVRSRIFRA

Mtsige    233 RQALRDYLAAH.PEHGECAVHVNPVR*
Mssige    186 RQQLRDYLAKHSSETAQSA*.......
Masige    230 RQALRDYLAAHPDHDALRASSA*....
Ecosige   178 REAIDNKVQPLIRR..............
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FIG. 2. Alignment of the inferred amino acid sequences of deduced mycobacterial SigE proteins with the sequence of the *E. coli* SigE protein. Mtsige, *M. tuberculosis* SigE; Mssige, *M. smegmatis* SigE; Masige, *M. avium* SigE; Ecosige, *E. coli* SigE.

are 34 and 31% identical to the *M. tuberculosis* sequence, respectively (Fig. 2).

The open reading frame immediately downstream of the *M. tuberculosis sigE* gene is also present in *M. leprae*, *M. avium*, and *M. smegmatis*. Comparison of these open reading frames to one another demonstrates that they are highly conserved, suggesting that they are functional genes encoding mycobacterial proteins. The *M. smegmatis*, *M. avium*, and *M. leprae* inferred amino acid sequences are 67, 76, and 73% identical to the *M. tuberculosis* sequence, respectively. The *M. tuberculosis* putative protein is substantially larger and is more basic (calculated pI of 11.15 versus 8.15 for the *M. avium* and *M. smegmatis* proteins, respectively) than those of the other species.

**Purification of *M. smegmatis* core RNA polymerase, overexpression of *M. tuberculosis* SigE, and reconstitution of in vitro transcription.** *M. smegmatis* core polymerase was purified from broth-grown *M. smegmatis*, and histidine-tagged *M. tuberculosis* SigE was overexpressed and purified as described previously (Fig. 3). SDS-PAGE with Coomassie blue staining shows the histidine-tagged SigE protein to be highly purified, with an apparent molecular mass of approximately 27 kDa. Purification of holo- and core RNA polymerase from *M. smegmatis* resulted in a pattern on SDS-PAGE similar to those observed in two previously published preparations (22, 29). As previously described, phosphocellulose chromatography provided significant further purification resulting in a core RNA polymerase preparation, with removal of a prominent 90-kDa band as well as several smaller proteins. In addition to the presumed $\alpha$ and $\beta,\beta'$ bands migrating at 48 and 140 to 160 kDa, respectively, however, additional bands migrating at 75 and 42 kDa remain in the phosphocellulose-purified preparation.

The core RNA polymerase preparation was used in an in vitro transcription assay to determine whether specific transcription could be reconstituted with purified SigE (Fig. 4). The 5′ regions from the *M. tuberculosis* and *M. smegmatis sigE* genes were used as a template because no SigE-dependent promoters are currently known in mycobacteria and because holoenzyme incorporating SigE has been shown to transcribe *sigE* in other species (26, 30, 31). In the absence of added SigE, no specific transcripts were identified. Following the addition of purified his-SigE, specific transcripts with sizes of approximately 300 and 280 bases were produced from the *M. tuberculosis* and *M. smegmatis* templates, respectively. The 5′ termini of these transcripts are approximately 55 and 185 bases 5′ of the deduced initiation codons of *M. tuberculosis* and *M. smegmatis sigE* genes, respectively. Transcription was specific for the mycobacterial DNA, because no prominent transcripts

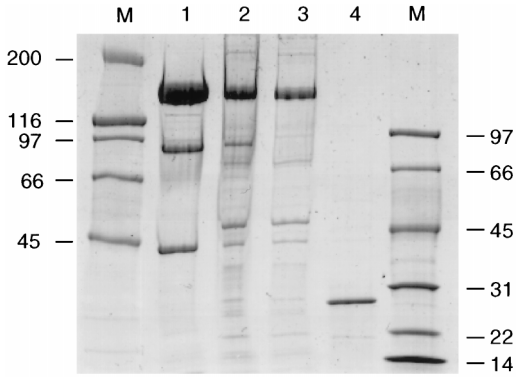


FIG. 3. SDS-PAGE of purified mycobacterial SigE and RNA polymerase. Lanes: M, molecular mass markers (numbers are in kilodaltons); 1, RNA polymerase holoenzyme from *E. coli* shown for comparison ($\beta,\beta'$ at approximately 150 kDa, $\alpha$ at approximately 43 kDa, sigma-70 at approximately 80 kDa); 2, RNA polymerase purified from *M. smegmatis* (presumed $\beta,\beta'$ at approximately 150 kDa, $\alpha$ at approximately 48 kDa, possible sigma at 90 kDa); 3, RNA polymerase core enzyme following Bio-Rex 70 chromatography of the preparation shown in lane 2 resulting in removal of major 90-kDa band and several lower-molecular-mass proteins; 4, purified his-tagged SigE of *M. tuberculosis* (major band at approximately 27 kDa).

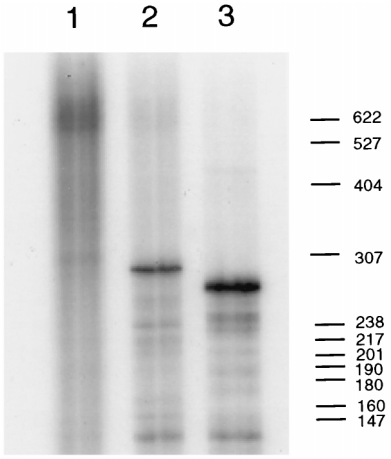


FIG. 4. Reconstitution of in vitro transcription of *sigE* by *M. smegmatis* core RNA polymerase plus *M. tuberculosis* his-tagged SigE protein. Lanes: 1, *M. smegmatis* core RNA polymerase with *M. smegmatis sigE* 5′ region; 2, *M. smegmatis* core RNA polymerase plus *M. tuberculosis* SigE protein with *M. tuberculosis sigE* 5′ region; 3, *M. smegmatis* core RNA polymerase plus *M. tuberculosis* SigE protein with *M. smegmatis sigE* 5′ region. Numbers to the right refer to migration of size markers in bases (pBR322-*Msp*I digest).

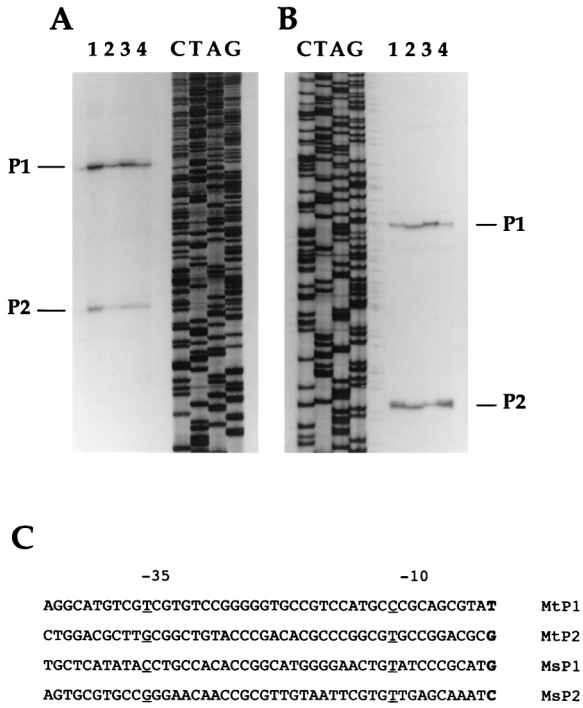


FIG. 5. Primer extension mapping of *sigE* transcription start sites in vivo. (A) *M. bovis* BCG. (B) *M. smegmatis*. Primer extension reactions were performed with RNA isolated after growth at 30°C (lane 1), after 37°C heat shock (lane 2), after 42°C heat shock (lane 3), and after 50°C heat shock (lane 4). (C) Sequence 5′ of each of the transcription start sites. The +1 position is the last base in the sequence, and the −10 and −35 positions are labeled above the sequences.

were visible when the experiment was performed with linearized vector alone as the template.

**Primer extension analysis.** To determine the in vivo promoter regions of the mycobacterial *sigE* genes, primer extension analysis was performed with RNA isolated from *M. smegmatis* and *M. bovis* BCG. For both organisms, two major transcription start sites were identified, corresponding to bases 157 (MsP1) and 85 (MsP2) 5′ of the *M. smegmatis sigE* initiation codon and bases 223 (MtP1) and 117 (MtP2) 5′ of the *M. tuberculosis sigE* initiation codon (Fig. 5). The sequence of the *M. bovis* BCG region 5′ of the *sigE* coding region is identical to that of *M. tuberculosis* starting 47 bases 5′ to P1 and extending beyond the deduced initiation codon.

Both *sigE* promoters were utilized in *M. smegmatis* and *M. bovis* BCG at all temperatures tested, although there is increased intensity of the P1 transcript relative to the P2 transcript following 42°C heat shock and increased intensity of the P2 transcript relative to the P1 transcript following 50°C heat shock. Primer extension analysis was also performed with RNA isolated from the *M. smegmatis sigE* mutant strain RH243. Both major start sites present in the wild type were also present in the mutant, although the signal of the P2 transcript was weaker than in the wild type (not shown). Examination of the sequences upstream of the P1 and P2 promoters in *M. smegmatis* and *M. bovis* BCG does not reveal strong consensus −10 or −35 sequences for known SigE-dependent promoters, with the exception of *M. smegmatis* P2, in which the −35 sequence CGGGAACAA matches (underlined) six of nine SigE promoter consensus bases (CCGGAACTT) and the −10 sequence TGTTG matches (underlined) three of four SigE promoter consensus bases (TCTNR) (24).

The location of the RNA 5′ termini identified in vitro differs from those observed in vivo. The *M. smegmatis* in vitro start is 27 bases 5′ to MsP1, and the *M. bovis* BCG in vitro transcription start site is 56 bases 3′ to MtP2. Both in vitro termini are preceded by good matches (TATGGT for the *M. bovis* BCG and TCATAT for *M. smegmatis*) with mycobacterial −10 regions. However, no consensus *sigE* promoter elements were identified 5′ to the in vitro start site (3, 24).

**In vitro growth and survival following stress of *sigE*-disrupted versus wild-type strains of *M. smegmatis*.** Growth characteristics and stress responses of *sigE*-disrupted *M. smegmatis* RH243 were compared to those of its parental strain, $mc^2$-155. No difference in colony morphology was visible when the strains were plated on solid medium. When grown in liquid medium (Middlebrook 7H9 broth supplemented with glucose) with shaking, no difference was observed in the growth curves of the mutant versus the wild-type strains at 30 or 37°C. The survival of $mc^2$-155 and RH243 following heat shock, acid shock, oxidative stress, and detergent treatment was examined to determine the role of SigE in the mycobacterial stress response. No difference was noted following 42°C heat shock. Following 50°C heat shock and following exposure to each of the other stresses, survival of both strains was affected, with greater killing of the mutant in each case (Fig. 6). Because the observed difference in survival between the wild type and mutant was small (approximately 3- to 10-fold), the effect of oxidative stress on these strains was assessed by a second method: determination of the $LD_{50}$ of hydrogen peroxide. The mutant was found to be more sensitive, with an $LD_{50}$ of 0.38 mM versus 1.25 mM for the wild-type strain, consistent with the results of the time-kill experiments.

*M. smegmatis* has been shown to generate a protective response when pretreated with concentrations of hydrogen peroxide that do not cause substantial killing (34). To determine whether SigE might play a role in generating this protective response, $mc^2$-155 and RH243 were each pretreated with 50 μM hydrogen peroxide, followed by treatment with 5 mM hydrogen peroxide (Fig. 7). Although less substantial than previously reported, a significant protective effect of pretreatment

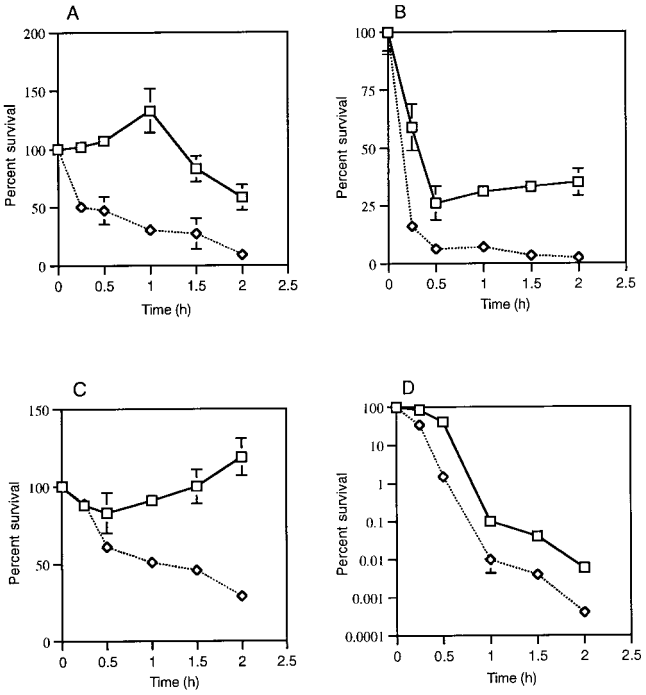


FIG. 6. Survival of wild-type ($mc^2$-155) and *sigE* mutant (RH243) strains of *M. smegmatis* following in vitro stress. Strains were grown at 30°C in Middlebrook 7H9 broth with supplemental glucose to an $OD_{600}$ of 0.3 to 0.4 and then were diluted in fresh medium to an $OD_{600}$ of 0.2 and exposed to stress conditions. The values shown are means ± standard deviations. (A) Acid stress (pH 4). (B) Oxidative stress (5 mM $H_2O_2$). (C) Detergent stress (0.01% SDS). (D) Heat stress (50°C). □, $mc^2$-155; ◇, RH243.

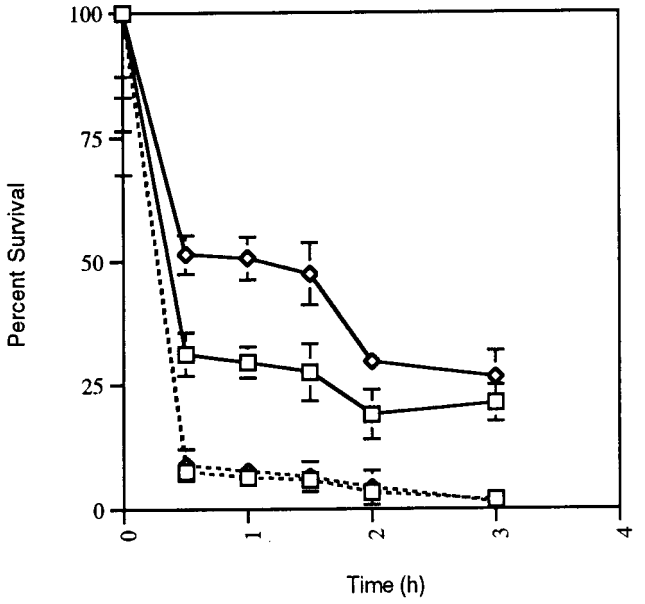


FIG. 7. Survival of wild-type ($mc^2$-155) and *sigE* mutant (RH243) strains of *M. smegmatis* following oxidative stress (5 mM $H_2O_2$) with and without pretreatment with 50 μM $H_2O_2$ for 60 min. Values shown are means ± standard deviations. □, (solid line), $mc^2$-155 with no pretreatment; ◇ (solid line), $mc^2$-155 with pretreatment; □ (dashed line), RH243 with no pretreatment; ◇ (dashed line), RH243 with pretreatment.

was observed in the wild type. In contrast, no protection following pretreatment was seen in the SigE mutant.

## DISCUSSION

Based on conservation of sequence and function across bacterial species, sigma-70 class sigma factors have been divided into several families. The recently designated ECF family includes several sigma factors that respond to extracellular stimuli or that regulate the expression of extracellular or membrane-associated proteins (24, 25). Both the sequence diversity and the variation in function of ECF sigma factors in different bacterial species appear to be more extensive than in other families. In *E. coli*, SigE plays an important role in the heat shock response through its regulation of the expression of the heat shock sigma factor Sig32 from a specific SigE-dependent promoter. SigE is also necessary for the expression of the periplasmic protease DegP (HtrA) (19, 30, 31). In *P. aeruginosa*, production of extracellular alginate that results in the mucoid phenotype is regulated by the ECF sigma factor AlgU, while in *Myxococcus xanthus*, the ECF sigma factor CarQ regulates the expression of membrane-associated carotenoids in response to light (8, 27). We have cloned and begun to characterize an ECF sigma factor gene of mycobacteria.

Southern blot analysis, demonstrating the presence of *sigE* in all mycobacterial species examined, including rapid and slow growers and pathogens and nonpathogens, suggests a fundamental role for SigE in this genus. Sequence analysis of the *sigE* genes of *M. tuberculosis*, *M. leprae*, *M. avium*, and *M. smegmatis*, which demonstrates extremely highly conserved amino acid sequences encoded by this gene, suggests that the function of SigE is also conserved among these species. Our ability to generate a gene disruption in *M. smegmatis*, however, indicates that it is not essential for viability.

Sequence analysis demonstrated additional features of interest. The first is the relatively large region 1 of this gene. Alternative sigma factors in general, and ECF sigma factors in particular, generally have short region 1 domains, usually limited to region 1.2 with little or no region 1.1 (13, 24, 25). Although it is possible that the in vivo initiation codon is further 3′ than the one we have identified, codon usage and third base variation in codons for conserved amino acids in the amino terminal region support the in vivo expression of the larger protein. The most highly conserved regions between the deduced mycobacterial SigE proteins and those of other species are region 2.2, an area of overlap at the end of 2.3 and the beginning of 2.4, and region 4.2. These areas of conservation are similar to those seen within the ECF family as a whole and are implicated in promoter melting (region 2.3), −10 promoter recognition (region 2.4), and −35 promoter recognition (region 4.2) (24, 25).

Also of interest is the open reading frame 3′ of the *sigE* gene that is present in *M. leprae*, *M. tuberculosis*, *M. avium*, and *M. smegmatis*. In other species, SigE function is regulated by one or several negative regulators or anti-sigma factors whose genes are typically located downstream of the *sigE* gene within an operon (27, 33). This conserved organization suggests that this open reading frame may encode a negative regulator of the mycobacterial SigE protein.

Downstream of the second open reading frame is a gene whose inferred product has a high degree of homology with DegP (HtrA) (23, 38). In *E. coli*, this gene is not physically located downstream of *sigE*, but its transcription is SigE dependent. In *P. aeruginosa*, there are two *degP* homologs, one downstream of the SigE sigma factor gene *algU* and the other at a separate locus (4). The role of DegP protein and its

location in the mycobacterial cell are not known. The short intergenic region between *sigE*, the second open reading frame, and *degP* suggests that this locus in *M. tuberculosis* constitutes on operon consisting of *sigE*, the second open reading frame, and *degP*.

To demonstrate that the cloned *M. tuberculosis* gene encodes a functional sigma factor, we attempted to reconstitute in vitro transcription of *sigE* itself by using core RNA polymerase from *M. smegmatis* mixed with purified his-SigE of *M. tuberculosis*. Because the domains known to be required for sigma subunit function are located in regions 2 to 4 and because the true initiation codon of *sigE* is not certain, we produced a recombinant his-SigE that incorporates part of region 1.2 and all of regions 2 to 4. Specific in vitro transcripts were obtained only when the core RNA polymerase preparation was combined with SigE, indicating that this gene encodes a functional sigma factor that is capable in vitro of initiating transcription from a specific site in the 5′ untranslated region of *sigE*. Whether the difference between the in vitro and in vivo *sigE* transcription start sites represents an in vitro artifact or defines SigE-dependent promoters recognized in vitro that were not active in vivo under the conditions we examined is not known.

In vivo transcription, assessed by primer extension, identified two promoters in *M. bovis* BCG and *M. smegmatis*. The sequence conservation between *M. tuberculosis* and *M. bovis* BCG indicates that these promoters are preserved in these species. Prior analysis of a limited number of mycobacterial promoters indicates that the −10 region is relatively highly conserved but that there is little conservation of −35 sequence (3, 20). In contrast, the few known SigE-dependent promoters from other bacteria show significant divergence from −10 regions typical of promoters utilized by other sigma factors (24). With the exception of the *M. smegmatis* P2 promoter, the promoters we identified show little similarity to known mycobacterial promoters or to SigE-dependent promoters, although both of these comparisons are limited by the small number of promoters analyzed to date.

The identification of both transcription start sites in the *M. smegmatis sigE* mutant demonstrates that expression of this gene is not dependent on RNA polymerase containing SigE. The decreased intensity of the P2 signal in the mutant, however, suggests that SigE may play some role in the expression of this gene, as is the case in *E. coli* and *P. aeruginosa* (23, 26, 30, 31). Both promoters were utilized in wild-type strains of *M. smegmatis* and *M. bovis* BCG at all temperatures tested, although there appeared to be some variation in the activity of P1 and P2 at 42 and 50°C. Further quantitative analysis will be required, however, to determine if there is significant regulation of SigE transcription in response to heat shock and whether regulation of SigE activity occurs at the level of transcription or by other mechanisms.

The function of the *sigE* gene in *M. smegmatis* was examined by comparison of the growth and stress response characteristics of the wild type with those of a *sigE*-disrupted strain. The lack of difference between the strains in colony morphology, during growth in liquid medium, and following 42°C heat shock indicates that SigE regulates genes whose expression is required for specialized functions. Decreased survival of the *sigE*-disrupted strain relative to that of the wild type following several classes of stress suggests that SigE in *M. smegmatis*, and possibly in other mycobacteria, regulates transcription of genes involved in the responses to a variety of stresses in vivo. This inference is supported by the absence in the *sigE* mutant of the inducible protective response for oxidative stress observed in the wild type.

These results are similar to those observed in *E. coli* sigE mutants in response to these and other stresses (30, 31). In *E. coli*, transcription of the periplasmic protease DegP (HtrA) is dependent on SigE (9). In mycobacteria, the homolog of *degP* is located downstream of *sigE* in what appears to be an operon, suggesting that in these organisms, expression of this protease may be regulated in part by SigE. These parallels suggest that the mycobacterial SigE may have a function similar to that of SigE in *E. coli*. Although its function is not fully understood, in *E. coli*, SigE is induced by excess or abnormally folded outer membrane proteins, and the signal for this induction may lie in accumulation of excess or abnormally folded proteins in the periplasmic space or outer membrane (28). This model is supported by the localization of MucB, a negative regulator of the ECG sigma factor AlgU in *P. aeruginosa*, in the periplasmic space (33). Although mycobacteria lack an outer membrane or periplasmic space, SigE may play a similar role in these organisms, possibly responding to the number and conformation of membrane-associated proteins or other extracellular stimuli. In this model, increased SigE activity may induce the activity of proteins such as DegP to maintain the appropriate levels of properly folded membrane proteins or proteins in extracellular compartments formed between the membrane and the complex mycobacterial cell wall.

Of the previously reported mycobacterial sigma factors, one appears to be an alternative sigma factor whose expression is induced by stress, although it has not been characterized functionally (6, 7, 29). The presence in mycobacteria of independent stress responses with overlapping or complementary function would be consistent with observations in other species (9) and with the ability of pathogenic mycobacteria to adapt to the variety of stresses encountered in the environment of the host.

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