A Mycobacterial Extracytoplasmic Sigma Factor Involved in Survival following Heat Shock and Oxidative Stress

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Extracytoplasmic function (ECF) sigma factors are a heterogeneous group of alternative sigma factors that regulate gene expression in response to a variety of conditions, including stress. We previously characterized a mycobacterial ECF sigma factor, SigE, that contributes to survival following several distinct stresses. A gene encoding a closely related sigma factor, sigH, was cloned from Mycobacterium tuberculosis and Mycobacterium smegmatis. A single copy of this gene is present in these and other fast- and slow-growing mycobacteria, including M. fortuitum and M. avium. While the M. tuberculosis and M. smegmatis sigH genes encode highly similar proteins, there are multiple differences in adjacent genes. The single in vivo transcriptional start site identified in M. smegmatis and one of two identified in M. bovis BCG were found to have a 35 promoter sequences that match the ECF-dependent promoter consensus. Expression from these promoters was strongly induced by 50°C heat shock. In comparison to the wild type, an M. smegmatis sigH mutant was found to be more susceptible to cumene hydroperoxide stress but to be similar in log phase growth, stationary-phase survival, and survival following several other stresses. Survival of an M. smegmatis sigH sigE double mutant was found to be markedly decreased following 53°C heat shock and following exposure to cumene hydroperoxide. Expression of the second gene in the sigH operon is required for complementation of the sigH stress phenotypes. SigH is an alternative sigma factor that plays a role in the mycobacterial stress response.

Extracytoplasmic function (ECF) sigma factors constitute a diverse family of proteins within the σ54 class of bacterial RNA polymerase subunits. This group was originally defined by conservation of sequence, and in some cases of function, of these proteins among several bacterial species (24). Many of these proteins, including those first described for Escherichia coli and Pseudomonas aeruginosa, have been shown to play a role in the regulation of gene expression required for survival following exposure to stress (6, 9, 13, 18, 31). With the rapid expansion of bacterial genomic sequence data, it has become apparent that many bacterial species have several genes that encode ECF-type sigma factors, although in most cases the functions of these proteins have not been defined.

Because of the role of some ECF sigma factors in regulating the interaction of bacteria with the extracellular environment and in the adaptation of bacteria to stress, these proteins are of interest as potential regulators of virulence factors in bacterial pathogens. Examples of this role have been described for P. aeruginosa and Salmonella spp. (6, 11). Mycobacteria are major pathogens of humans, yet little is known regarding determinants of virulence in these organisms or the regulation of mycobacterial gene expression during infection. As a step toward determining whether sigma factor-regulated gene expression plays a role in mycobacterial pathogenesis, we have begun to examine the role of ECF sigma factors in the mycobacterial stress response.

We previously characterized a mycobacterial ECF sigma factor, designated SigE, that plays a role in bacterial survival following a variety of in vitro stresses (39). Examination of the sequence of the genome of Mycobacterium tuberculosis H37Rv demonstrated the presence of a gene closely related to sigE. This report describes the cloning and initial characterization of this mycobacterial ECF sigma factor designated SigH.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture and stress conditions. Strains and plasmids are described in Table 1. E. coli cultures were grown on L agar or in L broth. M. smegmatis liquid cultures were grown in Middlebrook 7H9 broth supplemented with 0.2% glucose, 0.5% albumin, 0.085% NaCl, and 0.05% Tween 80 (7H9-ADCTw). M. smegmatis was plated on Middlebrook 7H10 plates supplemented with 0.2% glucose or on L agar. Ampicillin (50 to 70 μg/ml), kanamycin (20 to 50 μg/ml), zeocin (30 μg/ml), and apramycin (30 μg/ml) were added to culture media as indicated.

Experiments to determine survival following heat shock (42 and 53°C), acid shock (50 mM citric acid and HCl at pH 4), and hydrogen peroxide exposure (5 mM) were performed in 7H9-ADCTw as previously described (39). Experiments to determine survival following exposure to reactive nitrogen intermediates generated from nitrite were performed as described elsewhere (8). Several heat shock temperatures were tested; based on initial results, 50°C was used for heat shock induction of sigH gene expression and 53°C was used to quantify differences in survival following heat shock. To determine susceptibility to cumene hydroperoxide, M. smegmatis mc2-155 was grown to log phase (optical density at 600 nm [OD600] of 0.1 to 0.5) and then incubated in various concentrations of the reagents to determine a concentration that resulted in approximately 90% killing after 2 h. This concentration was then used for time-kill experiments in which dilutions were plated at serial time points following exposure. As a second method to determine cumene hydroperoxide susceptibility, paper discs were impregnated with 10 μl of 40 mM cumene hydroperoxide and placed onto LB agar plates onto which approximately 107 M. smegmatis had been plated.

Cloning of mycobacterial sigH genes. The following primers, based on the M. tuberculosis H37Rv sequence from cosmid MTCY7D11, were synthesized and used to amplify a 464-bp product: 5'-CCTTGACGGCGTGCCTTGCG-3' and 5'-AAGACCGCGCAACTGACGTCG-3'. This PCR product was used to probe a M. tuberculosis genomic DNA library in Ag11 (40). A clone containing a 3.5-kb insert was obtained and verified to contain the M. tuberculosis sigH locus by restriction analysis and limited DNA sequencing. The M. tuberculosis sigH clone was used to probe an M. smegmatis genomic DNA library in pUC19 to obtain a clone containing a 7-kb insert that included the M. smegmatis sigH locus.
DNA manipulation and sequencing. Lambda DNA was purified and plasmid DNA was isolated according to standard methods (34). Restriction and modifying enzymes were obtained from New England Biolabs or Boehringer Mannheim. Genomic DNA from M. smegmatis, M. fortuitum, M. bovis BCG, and M. avium was purified as previously described (17). M. tuberculosis genomic DNA was obtained from Patrick Brennan and John Belisle (Colorado State University). Southern blot analysis was performed with an NEBlot Phototope kit (New England Biolabs) or with an ECL kit (Amersham), using an NEBlot Phototope kit (New England Biolabs) or with an ECL kit (Amersham), using a genomic DNA library in AzAP.

Sequence assembly and analysis were performed with Sequencher (Gene Codes Corp., Ann Arbor, Mich.), the Wisconsin Package (Genetics Computer Group, Madison, Wis.), and Macvector (Oxford Molecular, Oxford, England). Sequence assembly and analysis were performed with Sequencher (Gene Codes Corp., Ann Arbor, Mich.), the Wisconsin Package (Genetics Computer Group, Madison, Wis.), and Macvector (Oxford Molecular, Oxford, England). Sequence assembly and analysis were performed with Sequencher (Gene Codes Corp., Ann Arbor, Mich.), the Wisconsin Package (Genetics Computer Group, Madison, Wis.), and Macvector (Oxford Molecular, Oxford, England).

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tr>
<td>M. smegmatis mc²-155</td>
<td>High-frequency transcriptional strain of M. smegmatis derived from wild-type strain mc²-6</td>
<td>37 This study</td>
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<td>RH244</td>
<td>mc²-155 sigE:p:zeo (Zeo') [SigE⁻]: result of recombination of pRH1264Z into chromosome of mc²-155</td>
<td>This study</td>
</tr>
<tr>
<td>RH280</td>
<td>mc²-155 sigH:aph (Km') [SigH⁻]: result of recombination of pRH1301 into chromosome of mc²-155</td>
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<td>RH296</td>
<td>mc²-155 sigH:aph (Km') [SigH⁻]: independent isolate constructed in same way as RH280</td>
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</tr>
<tr>
<td>RH315</td>
<td>RH244 sigH:aph (Zeo' Km') [SigE⁻ SigH⁻]: result of recombination of pRH1317 into chromosome of RH244</td>
<td>This study</td>
</tr>
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<td>RH282</td>
<td>RH244 sigH:aph (Zeo' Km') [SigE⁻ SigH⁻]: independent isolate from RH315</td>
<td>This study</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>Pasteur strain of M. bovis BCG (attenuated strain of M. bovis)</td>
<td>A. Aldovini</td>
</tr>
</tbody>
</table>

### E. coli

- **XL1-Blue MRF⁺**
  - Δ(mcrA183 Δ(mcrCB-hsdSMR-mry)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacP2::Tn10 Tetr] (Tac⁺) |
  - Y1090
    - F⁻ Δ(lacU169 lon-100 araD139 rpsL (Str') supF mcrA tprC22::Tn10 (pMC9 Tet' Amp') |

### Plasmids

- **pBluescript KS⁺**
  - E. coli cloning vector; Ap⁺ |
  - prRH1264Z
    - Zeocin resistance gene driven by hsp70 promoter inserted into blunted BglII site of M. smegmatis sigE gene in pBluescript |
  - prRH1274
    - 3.5-kb insert in pBluescript KS⁺ containing sigH locus of M. tuberculosis H37RV isolated from genomic DNA library in AzAP |
  - prRH1276
    - 7-kb insert in pUC19 containing sigH locus of M. smegmatis isolated from genomic DNA library in pUC19 |
  - prRH1301
    - aph of Tn903 inserted into BamHI site in coding region of M. smegmatis sigH in pRH1276 | Km⁺ |
  - pJEM15
    - E. coli-mycobacterium shuttle vector with promoterless lacZ gene for construction of transcriptional fusions to lacZ |
  - prRH1313
    - Heat-inducible promoter (MtP2) of M. tuberculosis/M. bovis BCG sigH gene cloned upstream of lacZ in pJEM15 |
  - pPR23
    - E. coli-mycobacterium shuttle vector with sacB and temperature-sensitive mycobacterial ori; gentamicin resistant |
  - prRH1317
    - 2.2-kb NotI fragment of pRH1301 containing sigE:aph inserted into NotI site of pPR23 |
  - pMH94A
    - Mycobacterial integrating vector with Km' gene replaced by apramycin resistance from pVK173F (23, 27) |
  - prRH1335
    - pMH94A with M. smegmatis sigH coding and promoter region inserted into EcoRI site |
  - prRH1341
    - pMH94A with M. smegmatis sigH promoter, coding region, and adjacent gene inserted into EcoRI site |

### Transcriptional analysis.

Primers used to determine the sigH transcription start sites were 5'-GGCGGATCCGCTCGGCATCGGACGCTACG-3' and 5'-TCCGGTCGTCGCCGATCGAACC-3' for M. smegmatis and M. bovis BCG, respectively. Quantification of primer extension bands was performed by scanning of the film and performing densitometry with NIH Image software.

**Reverse transcription (RT)** of 2 µg of total mycobacterial RNA, using the primer 5'-GGCGGATCCGCTCGGCATCGGACGCTACG-3' (underlined bases correspond to the end of the gene immediately 3' to sigH), was performed by using Superscript II reverse transcriptase (Life Technologies) incubated at 42°C, followed by removal of RNA by RNase H; 2 µl of this reaction mixture was used as the substrate for PCR (30 cycles of 95°C for 2 min, 56°C for 1 min, and 72°C for 1 min) in a 50-µl reaction mixture with 2 mM MgCl₂, using AmpliTaq DNA polymerase (Perkin-Elmer). For PCR, the primer 5'-TTTTCACCAGATGAG TGAGCG-3', corresponding to the beginning of sigH, was used together with the primer used in the RT reaction. The expected size of the product of this reaction is 979 bp.

**Construction of a transcriptional fusion of the heat-inducible sigH promoter to lacZ.** A transcriptional fusion of the heat shock-inducible promoter region of M. tuberculosis (M. bovis BCG sigH) to a promoterless β-galactosidase gene was constructed in the E. coli-mycobacterium shuttle vector pJEM15 (38). This promoter region was amplified by PCR with the primers 5'-CACCGGAC CGCGGACGACGGC-3' and 5'-TGCGATACCGACCAACCTACG-3'. Restriction sites incorporated into the primers are underlined. The resulting PCR products were digested with SacI plus KpnI and cloned into the corresponding sites in pJEM15 to generate pRH1315. Correct promoter sequence was confirmed by DNA sequencing. The resulting construct incorporated 64 bp 5' to the transcriptional start sites of MIP2. pRH1315 was transformed into M. smegmatis mc²-155 and M. bovis BCG to assess the activity of MIP2 in these mycobacterial species.

β-Galactosidase assays were performed as previously described (25), with the following modifications. M. smegmatis and M. bovis BCG were grown to log phase (OD₆₀₀ of 0.2 to 0.5), pelleted by centrifugation, and resuspended in Z buffer (25). The cells were then lysed by beating in a 2-ml microfuge tube approximately one-fourth filled with glass beads in a Bead-Beater (Biospec Products). The cell debris was pelleted, and β-galactosidase activity was determined in the supernatant following addition of o-nitrophenyl-β-D-galactopyranoside (final concentration, 4 mg/ml) by measuring absorbance at 405 nm at 5-min intervals for 1 h. Because of the potential for variable cell lysis and less accurate
correlation of OD$_{405}$ with cell number in mycobacteria. β-galactosidase activity was calculated as ΔOD$_{405}$ per minute per milligram of protein in the cleared cell lysate, rather than in Miller units. Protein concentrations were determined by using a NanoOrange kit (Molecular Probes). β-Galactosidase activity and protein concentration were measured in triplicate.

**Construction and complementation of \textit{M. smegmatis} sigH mutants.** pRH1276 was digested with BamHI, and the 1.3-kb aph (kanamycin resistance) gene of \textit{Trn903} from pUC4KSac was inserted in the same transcriptional orientation as sigH, to generate pRH1301 (1). This insertion disrupts the sigH structural gene between codons 81 and 82 of the inferred SigH protein. This suicide construct was electroporated into mc2-155; transformants were selected on kanamycin plates and then screened by PCR to distinguish single from double homologous recombination events. Two independent clones in which a double crossover had occurred were identified. The occurrence of allelic exchange resulting in a single disrupted copy of sigH in the chromosome was confirmed by Southern blotting of chromosomal DNA, and these strains were designated RH280 and RH296.

RH244 was constructed by transforming mc2-155 with pRH1264Z followed by PCR screening and Southern analysis to document gene replacement of the wild-type sigH with the zeocin-disrupted sigH, as previously described (39). To generate a sigH::aph double mutation in \textit{M. smegmatis}, RH244 was transformed with pRH1317. Cells were grown at 32°C, plated on LB plates containing kanamycin and sucrose, and incubated at 39°C as described elsewhere (28). DNA from individual colonies was screened as described above. The occurrence of allelic exchange resulting in gene replacement was confirmed for two independent isolates by Southern blotting of chromosomal DNA, and the strains were designated RH245 and RH296.

For complementation experiments, a DNA fragment containing an intact copy of the \textit{M. smegmatis} sigH coding and promoter regions was introduced into RH315 and RH328. The sequence of the \textit{M. tuberculosis} sigH locus, the organization of the mycobacterial chromosome surrounding sigH differs substantially between \textit{M. tuberculosis} and \textit{M. smegmatis} (Fig. 1). Immediately 5’ to \textit{M. smegmatis} sigH is an open reading frame in the opposite transcriptional orientation that is highly similar to a gene of unknown function, designated ybaK, that is present in \textit{E. coli} and several other species. Further 5’ in \textit{M. smegmatis} are two open reading frames that are highly similar to catechol dioxygenase (catA) and muconolactone isomerase (catC) genes of \textit{Acinetobacter hofvii} and other species. In contrast, immediately 5’ to the \textit{M. tuberculosis} sigH gene is a putative oxidoreductase. Further 5’ is a fragment of ybaK, followed by an open reading frame with similarity to the carboxy-terminal region of amino-glycoside phosphotransferases.

Immediately 3’ of \textit{M. smegmatis} sigH and in the same transcriptional orientation is an open reading frame of 300 bases; the initiation codon of this putative gene overlaps the stop codon of sigH. This putative protein shows limited similarity to YbbM, a putative protein encoded by a gene immediately 3’ to the ECF sigma factor gene \textit{ybbL} (sigW) of \textit{Bacillus subtilis} (22). In \textit{M. tuberculosis}, an open reading frame of 546 bases is similarly oriented and has an initiation codon overlapping the sigH stop codon. The deduced amino acid sequence is highly similar to that of another \textit{M. tuberculosis} protein of unknown function (4). It is not similar to the \textit{M. smegmatis} sequence in the corresponding location. Further 3’ to this gene are two open reading frames that encode similar proteins in \textit{M. smegmatis} and \textit{M. tuberculosis}.

The identification of SigH as a putative ECF sigma factor is based on its sequence similarity to other known ECF sigma factors. The two most closely related sigma factors are SigE of \textit{M. tuberculosis} (15, 40). Southern blot analysis using the coding region of the \textit{M. tuberculosis} sigH gene as a probe showed that a single copy of this gene was present in \textit{M. smegmatis}, \textit{M. fortuitum}, \textit{M. avium}, \textit{M. tuberculosis}, and \textit{M. bovis} BCG (not shown).

**RESULTS**

Cloning, Southern blotting, and sequence analysis of the mycobacterial \textit{sigE} locus. A BLAST search of the \textit{M. tuberculosis} H37Rv genomic DNA sequence with the \textit{M. smegmatis} sigH sequence identified an open reading frame in the cosmid MTCY7d11 related to but distinct from \textit{sigE} (4). A PCR product based on this sequence was used as a probe to isolate clones containing this putative sigma factor gene, named \textit{sigH} (Rv3223c), from genomic DNA libraries of \textit{M. smegmatis} and \textit{M. tuberculosis} (15, 40). Southern blot analysis using the coding region of the \textit{M. tuberculosis} sigH gene as a probe showed that a single copy of this gene was present in \textit{M. smegmatis}, \textit{M. fortuitum}, \textit{M. avium}, \textit{M. tuberculosis}, and \textit{M. bovis} BCG (not shown).

Analysis of the \textit{sigH} locus in \textit{M. tuberculosis} and \textit{M. smegmatis} revealed that the \textit{M. smegmatis} deduced amino acid sequence of 216 amino acids was 89% identical to the \textit{M. tuberculosis} sequence of 217 amino acids. Additional potential translational start sites using GTG as the initiation codon can be identified 5’ to those used in this analysis. However, the deduced amino acid sequences of these upstream regions in \textit{M. tuberculosis} and \textit{M. smegmatis} are not significantly similar.

In contrast to the conserved organization of the mycobacterial \textit{sigE} locus, the organization of the mycobacterial chromosome surrounding sigH differs substantially between \textit{M. tuberculosis} and \textit{M. smegmatis} (Fig. 1). Immediately 5’ to \textit{M. smegmatis} sigH is an open reading frame in the opposite transcriptional orientation that is highly similar to a gene of unknown function, designated ybaK, that is present in \textit{E. coli} and several other species. Further 5’ in \textit{M. smegmatis} are two open reading frames that are highly similar to catechol dioxygenase (catA) and muconolactone isomerase (catC) genes of \textit{Acinetobacter hofvii} and other species. In contrast, immediately 5’ to the \textit{M. tuberculosis} sigH gene is a putative oxidoreductase. Further 5’ is a fragment of ybaK, followed by an open reading frame with similarity to the carboxy-terminal region of amino-glycoside phosphotransferases.

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The identification of SigH as a putative ECF sigma factor is based on its sequence similarity to other known ECF sigma factors. The two most closely related sigma factors are SigE of
mycobacteria and AlgU of P. aeruginosa (Fig. 2). Consistent with the conservation of ~35 promoter regions in ECF-dependent promoters, the greatest proportion of conserved amino acid sequence among these proteins is in region 4.2, where M. tuberculosis SigH is 48% identical to P. aeruginosa AlgU. As is the case in many ECF sigma factors, SigH has a short region 1 compared to the primary sigma factors of most bacteria. Characterization of sigH promoters in M. bovis BCG and M. smegmatis. Primer extension analysis was performed to identify the promoters of the sigH genes of M. bovis BCG and M. smegmatis (Fig. 3A). For both species, a strong band identified by primer extension analysis of sequence 5 to 198 (MtP2) bases 5' to the transcription start site of the gene encoding that sigma factor. To determine whether SigH is required for transcription of rpsL, a transcript was present in the wild type but not in the heat shock mutant. This result indicates that RNA polymerase incorporating SigH is required for transcription of sigH from MsP1 following heat shock.

The overlap of the 3' end of sigH and the start codon of the adjacent downstream gene suggested that these genes are part of an operon expressed from the sigH promoters identified by primer extension. RT-PCR using a pair of primers spanning this region of overlap in M. smegmatis generated a single prod-
uct of the size expected for these genes to be expressed as a single mRNA transcript (Fig. 5).

β-Galactosidase expression from the heat shock-inducible promoter MtP2. The heat shock-inducible sigH promoter MtP2 identified in M. bovis BCG was cloned upstream of the promoterless β-galactosidase gene in the shuttle vector pJEM15 (38). β-Galactosidase activity from this construct was measured in M. bovis BCG and in M. smegmatis. MtP2 showed substantial activity at 37°C (10-fold greater than vector control activity) in M. bovis BCG but not in M. smegmatis. Following a 50°C heat shock, expression from MtP2 remained undetectable in M. smegmatis and declined slightly, followed by recovery to baseline or slightly higher levels in M. bovis BCG.

Analysis and complementation of M. smegmatis sigH mutants. The sigH gene was disrupted by introducing the kanamycin resistance gene (aph) of Tn903 into the coding region of this gene on the chromosome of mc²-155 by allelic exchange. No differences in colony morphology were observed between the sigH mutants and the parental strain mc²-155 when plated on solid medium, and no difference in growth rate at 30 or 37°C in liquid medium was observed. No significant differences in survival were observed following several distinct stresses, including 42°C heat shock, 0°C cold shock, 50 mM citric acid stress, pH 4 HCl acid stress, exposure to 5 mM hydrogen peroxide, and exposure reactive nitrogen stress (20 mM NaNO₂ incubated at pH 5.3). In addition, no difference was observed in survival during stationary phase for up to 10 days. The sigH mutant strain RH280 was substantially more susceptible to organic peroxide stress when measured by plating at serial time points following exposure, as was the sigE mutant strain RH244 (Fig. 6A). When measured by inhibition of growth around a paper disc impregnated with cumene hydroperoxide, RH280 but not RH244 was significantly more susceptible than mc²-155 (Fig. 6B). In multiple experiments, RH280 was no different from or slightly more susceptible to 53°C heat shock than the wild type, as was RH244 (Fig. 7).

Two independent sigE sigH double mutants (RH315 and RH328) were generated by disruption of sigE with a zeocin resistance gene and disruption of sigH with a kanamycin resistance gene. These strains were markedly more susceptible to

FIG. 3. Primer extension mapping of the mycobacterial sigH transcription start sites in vivo. (A) M. smegmatis. (B) M. bovis BCG. Primer extension reactions were performed with RNA isolated after growth at 37°C (lane 1) or after 50°C heat shock (lane 2). (C) Sequence 5' of the M. smegmatis P1 (MsP1) and the M. bovis BCG P2 (MtP2) transcription start sites compared to the ECF-dependent promoter −35 consensus sequence. (D) Sequence 5' of the M. bovis BCG P1 transcription start site (MTP1) compared to the M. smegmatis rpsL promoter. Identical residues are shaded. The M. tuberculosis sequence 5' of MtP1 and MtP2 is identical to that of M. bovis BCG.

FIG. 4. Autoregulation of sigH transcription in M. smegmatis. Primer extension reactions were performed with RNA isolated from mc²-155 (lane 1) and RH280 (lane 2) after growth at 37°C and from mc²-155 (lane 3) and RH280 (lane 4) after 50°C heat shock.
expression of this protein in all strains, whether grown at 37°C or after 50°C heat shock, indicating that its expression is not sigE or sigH dependent (Fig. 8).

**DISCUSSION**

The initial identification of the ECF subfamily of sigma factors linked a relatively small number of alternative sigma factors in a group based on sequence similarity, conservation of -35 region sequences of ECF sigma factor-dependent promoters, and a broad definition of conserved function (24). The large number of ECF-type sigma factors identified in bacterial genomes that have been recently sequenced suggests that members of this family are likely to regulate many types of genes in response to a wide variety of conditions.

In *M. tuberculosis*, 10 of 13 putative sigma factors are members of the ECF subfamily (4). In addition to SigH, whose initial characterization we describe in this report, four mycobacterial sigma factors have been characterized to some extent. SigA appears to be the primary mycobacterial sigma factor (10, 30). SigB is highly similar to SigA but is nonessential and plays a role in the response to stress (10). SigE is widely distributed in mycobacterial species and plays a role in survival following stress (39). SigF is present only in species of the *M. tuberculosis* complex and is expressed in response to stress and starvation (5).

The results presented here indicate that SigH, like SigE, is present in fast- and slow-growing mycobacteria and plays a role in the bacterial stress response. The induction of transcription following heat shock suggests that genes regulated by SigH may be important in the heat shock response of mycobacteria. The absence of a difference in survival of the sigH mutant compared to wild-type *M. smegmatis* following 42°C heat shock and the small difference following 53°C heat shock is surprising in this regard. In the context of these phenotypes, the strong induction of sigH transcription suggests the presence of multiple mechanisms for the response to heat shock in mycobacteria.

This interpretation is supported by the expression of the heat shock chaperone DnaK in single- and double-mutant strains as well as in the wild type. The presence of multiple, possibly overlapping responses to high temperature heat shock is also supported by the much greater susceptibility to heat shock of the sigE sigH double mutant compared to either single mutant. Overlap of gene regulation by ECF sigma factors has been found recently in *B. subtilis*, where for four different genes, RNA polymerase holoenzyme containing either SigW or SigX was shown to initiate transcription from the same promoter (13, 14).

The second stress for which SigH appears to be important in mycobacterial survival is oxidative stress from exposure to organic peroxide. Increased killing following exposure to cumene hydroperoxide of the sigH mutant, and to a lesser extent the sigE mutant, relative to the wild type, and greater susceptibility of the sigE sigH double mutant, suggests the presence of protective mechanisms mediated by each of these two sigma factors. The lack of altered susceptibility to hydrogen peroxide of the sigH mutant or of the sigE sigH double mutant relative to wild type indicates that the responses in *M. smegmatis* to these oxidative stresses differ. Differences in both phenotype and protein expression of mycobacteria following responses to these different peroxides have been noted previously (7, 32, 36). Greater susceptibility of mycobacteria to organic hydroperoxide than to hydrogen peroxide could result from the large lipid content of the mycobacterial cell wall that may be subject to peroxidation by these organic reagents. The lipid-
rich mycobacterial cell wall may also act as a barrier to the effects of water-soluble hydrogen peroxide.

Both $\text{sigH}$ and the adjacent 3’ gene were found to be required for complementation of $\text{sigH}$ mutant stress phenotypes in $M. \text{smegmatis}$. The expression of these genes as a single transcript from the autoregulated $\text{sigH}$ promoter indicates that this gene, like $\text{sigH}$ itself, is dependent on SigH for its expression. While it is possible that this gene encodes a protein that functions in a direct protective role against these stresses, the near-complete complementation of two distinct phenotypes is more consistent with the product of this gene functioning to regulate the expression of other gene products, either directly or as a positive regulator of SigH activity. The latter mechanism is well described for the regulation of alternative sigma factor activity in $B. \text{subtilis}$, where activities of the stress-responsive SigB and the sporulation-specific SigF are both regulated through interactions of positive and negative regulators (12, 35).

The $\text{sigH}$ MtP1 promoter is of interest in the context of what is currently known of mycobacterial promoters. Its −10 region is highly similar to the well-characterized $\text{rpsL}$ promoter of $M. \text{smegmatis}$ and to the consensus −10 region identified in several putative promoters identified in $M. \text{smegmatis}$ and $M. \text{tuberculosis}$ (2, 20). Of the two bases in the MtP1 −10 hexamer that diverge from this consensus, one is at the second position, where C is present in the place of the highly (>90%) conserved A. This divergence from the consensus may account for the weak signal observed in the primer extension experiments. Like the $\text{rpsL}$ promoter, MtP1 lacks a −35 region that matches known consensus −35 sequences, a finding typical of the majority of mycobacterial promoters (2). MtP1 does have the extended −10 TGN motif that plays an important role mycobacterial $\text{rpsL}$ transcription as well as in $E. \text{coli}$ and other species in promoters lacking consensus −35 elements (19, 29, 33). This extended −10 region appears to be relatively common among mycobacterial promoter sequences, occurring in more than 20% of those identified (3).

The −35 elements of MsP1 and MtP2 are highly similar and match closely the ECF consensus. Like previously defined
regulate the expression of genes involved in the mycobacterial stress response. Whether the unrelated gene in the corresponding position in the *M. tuberculosis* has a similar function remains to be determined, as does the mechanism by which heat shock is transduced into activation of transcription from the inducible mycobacterial *sigH* promoters.

*SigH*, like *SigE* and *SigF*, plays a role in the mycobacterial stress response. The presence of multiple mechanisms for the regulation of stress responses is consistent with the need for the bacteria to adapt to a variety of stresses during extracellular growth and after uptake by macrophages during the course of infection. The major stresses with which *M. tuberculosis* must contend are those encountered during the course of infection. Thus, sigma factors that regulate gene expression in response to stress response are likely to play an important role in regulation of gene expression that is essential for *M. tuberculosis* pathogenesis.

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### ADDENDUM IN PROOF

During review of this article, we became aware of a report in which *M. tuberculosis* *sigH* transcription was shown to be induced following heat shock (R. Manganelli, E. Dubnau, S. Tyagi, F. R. Kramer, and I. Smith, Mol. Microbiol. 31:715–724, 1999).

### REFERENCES


FIG. 8. Western blot analysis of DnaK expression in wild-type and mutant strains of *M. smegmatis*. Western blotting of *M. smegmatis* lysates was performed with the anti-mycobacterial DnaK monoclonal antibody IT-41 (HAT3). Lanes 1 and 2, RH244 (sigE); lanes 3 and 4: RH280 (sigH); lanes 5 and 6, RH315 (sigE sigH); lanes 7 and 8, mc2-155. Lanes 1, 3, 5, and 7, lysates made following growth at 37°C; lanes 2, 4, 6, and 8, lysates made following 50°C heat shock.


AUTHOR’S CORRECTION

A Mycobacterial Extracytoplasmic Sigma Factor Involved in Survival following Heat Shock and Oxidative Stress

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Volume 181, no. 14, p. 4266–4274, 1999. Page 4272, Fig. 6B: The bar labeled “RH315/pMH94A” should be labeled “RH244/pMH94A,” and the bar labeled “RH244/pMH94A” should be labeled “RH315/pMH94A.”