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Differential expression of *sigE* by *Mycobacterium tuberculosis* during intracellular growth

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The *Mycobacterium tuberculosis sigE* gene encodes a sigma factor that is a member of the extracytoplasmic function subfamily of sigma factors. Using RT-PCR we demonstrated that *sigE* is expressed in *M. tuberculosis* bacilli during growth in human macrophages beginning after 30 min but before 6 h after infection through at least 5 days after infection, but that *sigE* is not expressed by *M. tuberculosis* bacteria during growth in Middlebrook 7H9 broth medium. However, *sigE* expression can be induced by treatment of broth cultures with hydrogen peroxide. Further, *sigE* is not expressed by *M. tuberculosis* bacilli during attachment or growth in type II pneumocytes. Using a green fluorescent protein (GFP) reporter gene fused to the *sigE* promoter, we observed induction of GFP expression following macrophage infection. Western blotting confirmed that *sigE* protein expression correlated with mRNA expression in induced systems. Analysis of the region of the *M. tuberculosis* genome encoding *sigE* suggested it is part of an operon consisting of *sigE-orf1-htrA-orf2*. The data presented in this report showed that *sigE* is differentially expressed by *M. tuberculosis* bacilli in macrophages and might play a role in the pathogenesis of this organism.

Key words: *Mycobacterium tuberculosis*, sigma factor, macrophage, differential gene expression.

Introduction

Mycobacterium tuberculosis is an intracellular pathogen that survives and proliferates inside human phagocytic cells [1]. In order to survive in host macrophages and monocytes, the bacterium must be able to perform a wide range of functions, including resisting toxic oxygen by-products generated by the intracellular respiratory burst [2], sequestering iron [3] and scavenging essential metabolites [1]. Little is known about the intracellular mechanisms that activate these functions or the coordinate regulation of the bacterial genes involved.

Regulation of gene expression in bacteria occurs primarily at the level of transcription, either

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through transcriptional repressors and activators or via alternate sigma factors that recognize specific promoters [4]. Alternate sigma factors have been shown to play a role in the virulence of other microbial pathogens, including *Pseudomonas aeruginosa* [5], *Yersinia enterocolitica* [6] and *Salmonella typhimurium* [7]. An alternate sigma factor, *sigE*, that shows homology to members of the extracytoplasmic function (ECF) subfamily of sigma factors, has recently been cloned from *M. tuberculosis*. It was demonstrated that the *sigE* homologue in *Mycobacterium smegmatis* is involved in protecting the bacteria from certain extracellular environmental stresses [8]. The data in our current report provide evidence that *sigE* is differentially expressed and hence may be involved in the survival of *M. tuberculosis* bacilli following their phagocytosis by macrophages. These data provide an important step in understanding the expression of genes involved in the survival and proliferation of *M. tuberculosis* bacilli within macrophages, potentially allowing the identification of virulence factors.

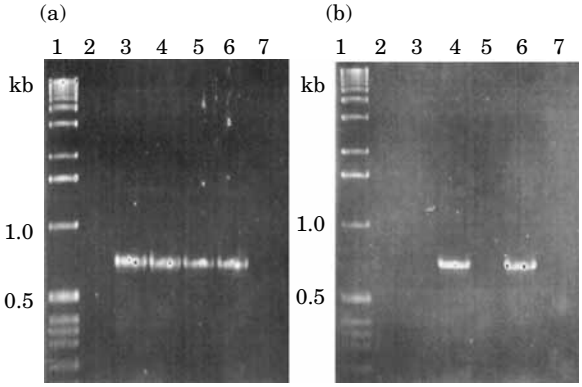

Figure 1. RT-PCR analysis of *sigE* expression. (a) Differential expression of *sigE* by *M. tuberculosis* grown in macrophages (Mφ). Lane 1, 1 kb ladder; 2, broth-grown *M. tuberculosis*; 3, *M. tuberculosis*-Mφ, 6 h post-infection; 4, *M. tuberculosis*-Mφ, 48 h post-infection; 5, *M. tuberculosis*-Mφ, 5 days post-infection; 6, DNA control; 7, negative control (no RT). (b) Comparison of *sigE* expression by *M. tuberculosis* grown in macrophages (Mφ) and A549 pneumocytes. Lane 1, 1 kb ladder; 2, *M. tuberculosis*-Mφ, 30 min post-infection; 3, *M. tuberculosis*-A549, 30 min post-infection; 4, *M. tuberculosis*-Mφ, 48 h post-infection; 5, *M. tuberculosis*-A549, 48 h post-infection; 6, DNA control; 7, negative control (no RT).

Results and Discussion

Differential expression of *sigE*

With the use of RT-PCR analysis, *sigE* mRNA transcription was detected as early as 6 h after infection through as late as 5 days after infection in *M. tuberculosis* Erdman grown in human macrophages. *SigE* transcripts were also identified from mycobacteria cultured aerobically in 7H9 medium but treated *in vitro* with 10 mM hydrogen peroxide for 20 min. *sigE* was not expressed at detectable levels in broth-grown *M. tuberculosis* or *M. tuberculosis* bacilli associated with pneumocytes, nor was *sigE* mRNA detected during the initial 30 min of association of the bacilli with macrophages (Fig. 1). All of these results were confirmed by Northern analysis (data not shown). Differential expression of *sigE* in macrophages was also observed recently by Graham and Clark-Curtiss [9]. However, our results do differ from those of Manganelli *et al.* who did not observe induction of *sigE* in the presence of hydrogen peroxide [10]. We believe this discrepancy is likely due to slight differences in experimental procedure.

Analysis of *sigE* expression using GFP reporter gene

Microscopic analysis of macrophage monolayers infected with *M. tuberculosis* Erdman containing the GFP fusions revealed that GFP was expressed by intra-macrophage *M. tuberculosis* bacilli under the control of both the *sigE* and *hsp60* promoters (Fig. 2). *M. tuberculosis* bacilli containing the *hsp60–gfp* fusion expressed GFP both inside the macrophage and in the extracellular medium, whereas *M. tuberculosis* bacilli containing the *sigE–gfp* fusion only expressed GFP when inside macrophages. Interestingly, GFP expression driven by the *sigE* promoter [Fig. 2(a)] appeared more intense than that driven by the *hsp60* promoter [Fig. 2(g)]. The differences in expressed GFP colour between these two figures (and their respective controls) result from using two different microscopes and imaging software packages. Since the wavelength of light and the filters used in both microscopes were identical, the overall conclusions obtained by comparing the figures should not be affected. Treatment of broth cultures with 10 mM hydrogen peroxide also induced GFP expression

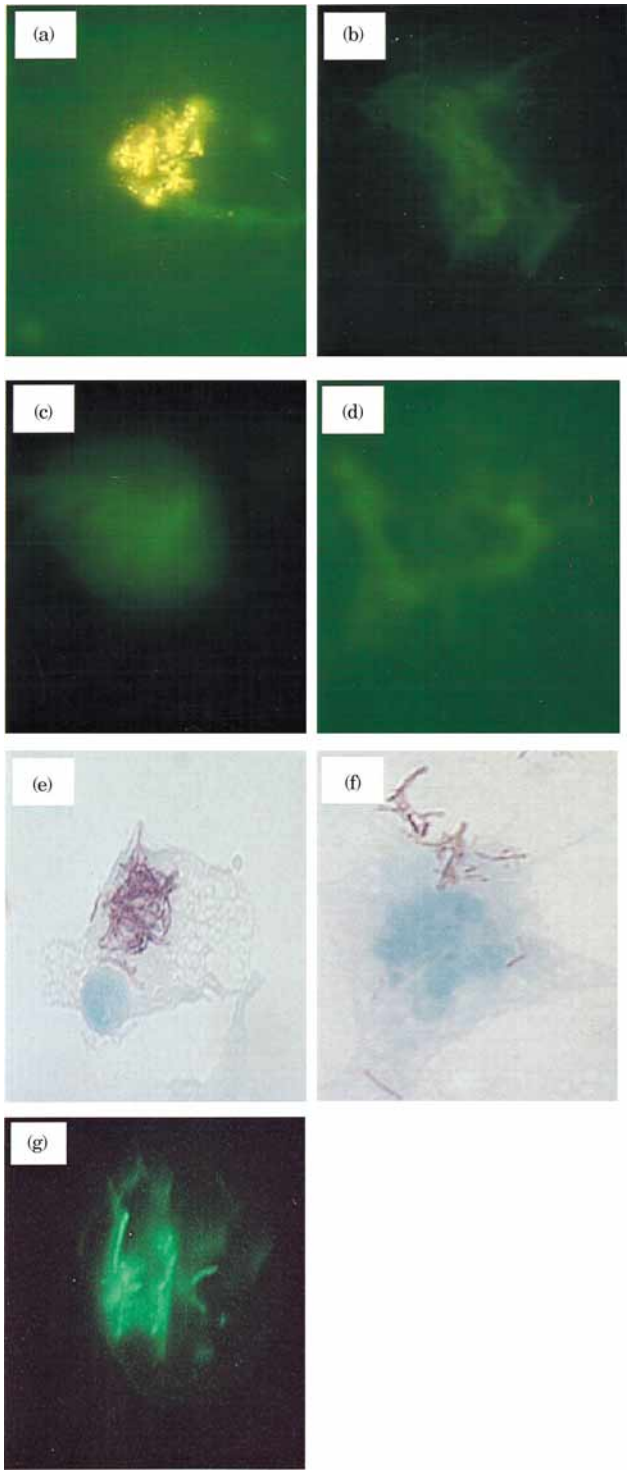

Figure 2. GFP expression driven by *sigE* promoter. Macrophage monolayers infected for 48 h with *M. tuberculosis* bacilli containing pMYSigE-GFP (a) or uninfected (b) were examined under 100 × magnification using a Zeiss Standard Lab 16 microscope. GFP was excited with UV light at 480 nm and detected using a filter at 510 nm. Microscopic images were viewed using the Image Pro software. Monolayers of A549 pneumocytes infected for 48 h with *M. tuberculosis* containing pMYSigE-GFP (c), uninfected (d), and macrophages infected with *M. tuberculosis* containing pHsp-GFP (g) were examined under 100 × magnification using a Zeiss Axiovert 135 microscope with an LD achroplan objective. GFP was excited with UV light at 480 nm and detected using a 510 nm filter. Microscopic images were captured using Axiovision I software (Zeiss). Acid-fast stains were performed by standard methods and examined by light microscopy under 100 × magnification using a Zeiss Axiovert 135 microscope with an LD achroplan objective (e) and (f).

from pMYSig-GFP (data not shown). No expression of *sigE*-driven GFP by *M. tuberculosis* was observed during pneumocyte infection [Fig. 2(c)], although GFP was expressed from the *hsp60* promoter by *M. tuberculosis* within infected pneumocytes (data not shown). Only background fluorescence was observed in uninfected macrophage [Fig. 2(b)] and pneumocyte [Fig. 2(d)] controls. Acid-fast stains of infected macrophages [Fig. 2(e)] and infected pneumocytes [Fig. 2(f)] demonstrated that *M. tuberculosis* bacilli were present in both cell types, and that lack of *sigE–gfp* driven GFP expression from *M. tuberculosis* bacilli in pneumocytes was not due to the absence of bacilli.

These data indicate that *sigE* expression is upregulated by *M. tuberculosis* during intracellular growth in macrophages, and as such may represent a gene product required for intracellular survival and growth. Interestingly, *sigE* expression was inducible in broth cultures of *M. tuberculosis* by treatment with hydrogen peroxide (Fig. 1), a condition that mimics the toxic oxygen radical production of the respiratory burst in macrophages [11]. Although type II pneumocytes readily take up *M. tuberculosis* bacilli [12], we were unable to detect transcription of *sigE* by *M. tuberculosis* during any stage of pneumocyte infection. This may reflect the different trafficking patterns of *M. tuberculosis* in pneumocytes and macrophages. Mehta *et al.* [12] demonstrated that *M. tuberculosis* bacilli reside in the cytoplasm of pneumocytes. In macrophages, *M. tuberculosis* bacilli localize and may remain in vacuoles [13]. Thus, the bacilli in pneumocytes may not encounter the hostile intracellular environment found in macrophages.

Western analysis of *sigE* expression

The *sigE* coding region was cloned into pET19b and expressed and purified from *Escherichia coli*

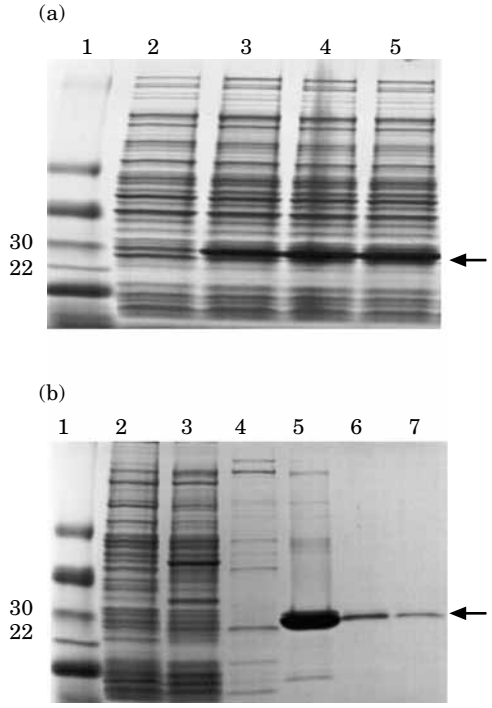

Figure 3. Purification of recombinant, His-tagged SigE. (a) R-SigE overexpression. Lane 1, MultiMark standard (Novex); 2, Uninduced BL21 (DE3) pLysS containing pETSigE; 3, 1 h post-induction (2 mM IPTG); 4, 2 h post-induction; 5, 3 h post-induction. (b) SigE purification. Lane 1, MultiMark standard (Novex); 2, Column flow through; 3, Wash 1; 4, Wash 2; 5, Elution, fraction 1; 6, Elution, fraction 2; 7, Elution, fraction 3.

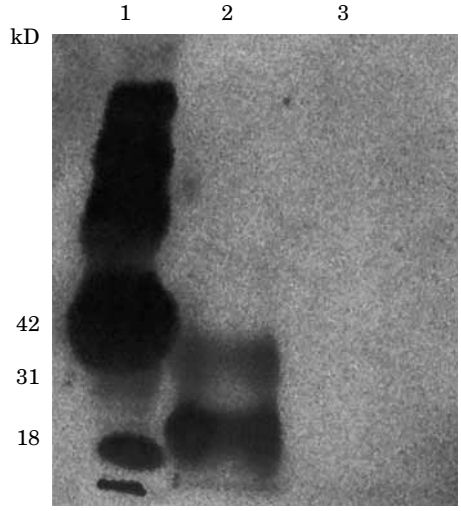

Figure 4. Western analysis of SigE expression. Lane 1, Protein standard (BioRad); 2, *M. tuberculosis*, H_2O_2-treated; 3, *M. tuberculosis*, M7H9 broth.

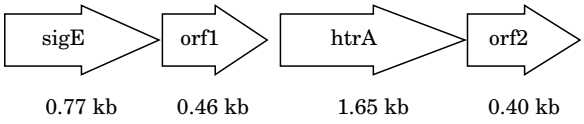

Figure 5. Organization of *sigE* operon.

using an eight residue histidine tag on the N-terminus of the protein. SDS-PAGE analysis of the purified, renatured fraction identified a single protein with an apparent molecular weight of approximately 29 kDa (Fig. 3). When rabbit antibodies to R-*sigE* were used, Western blotting of crude lysates from *M. tuberculosis* identified a protein corresponding to the predicted size of full-length *sigE* (35 kDa) in the H_2O_2-induced *M. tuberculosis* bacteria. A smaller MW protein of approximately 20 kDa was also recognized, and although of higher intensity than the 35 kDa band, it may represent a degradation product of *sigE*. Neither protein was present in uninduced broth cultures of *M. tuberculosis* (Fig. 4). We were unable to detect *sigE* protein in *M. tuberculosis*-infected macrophages, probably because of the small numbers of *M. tuberculosis* bacilli (approximately 2×10^7 macrophage-associated bacteria compared with approximately 5×10^9 broth-grown bacteria) and hence the small amount of *sigE* protein present in macrophages during infection.

Transcriptional coupling of *sigE*

DNA sequence analysis of the *M. tuberculosis* genome (GenBank U87242) revealed that *sigE* is part of a putative operon (Fig. 5). Operon organization is a common theme among the ECF sigma factor subfamily [14]. The first gene in the putative *M. tuberculosis* operon is *sigE* which is separated by 41 bp from an unidentified gene called *orf1*. The *orf1* gene is in turn separated by 25 bp from an *htrA* homologue encoding a putative serine protease. Lastly, there is a second unidentified gene, *orf2*, which is separated by 2 bp from *htrA*. Comparison of other ECF sigma factor operons reveals that the sigma factors themselves are often negatively regulated by other genes within the operon [14]. *Orf1* is a candidate for such a negative regulator of *sigE* and may represent an anti-sigma factor.

Although *sigE* itself cannot be described as a virulence factor, it may be involved in regulating genes that play a role in mycobacterial virulence

and survival. Current efforts are focused on identifying genes whose transcription is regulated by *sigE*. Distal genes that could be regulated by *sigE* include genes for a putative serine protease (*htrA*), a methyltransferase, and an ABC transporter, all of which participate in virulence in other microbial pathogens, including *Brucella abortus* [15], *Listeria monocytogenes* [16], *Francisella novicida* [17] and *P. aeruginosa* [18]. Although the best-characterized ECF sigma factors are known to regulate nearby genes [5, 19, 20], this is not always the case, and regulation of distal genes by *sigE* cannot be ruled out.

Materials and Methods

Isolation of human blood monocyte-derived macrophages

Venous blood was collected in acid citrate dextrose anticoagulant from tuberculin-negative and HIV/HBV/HCV-negative healthy volunteers. Dextran was added to the suspension to a final concentration of 0.6%, and the mixture was incubated at 37°C for 2 h to sediment the erythrocytes. The non-sedimented fraction was removed, washed in Hanks balanced salt solution without calcium and magnesium (HBSS; Life Technologies, Grand Island, NY, U.S.A.), and layered onto a Ficoll/Hypaque (Sigma Chemical Co, St. Louis, MO, U.S.A.) gradient. Samples were centrifuged for 30 min at $1000 \times g$. The cells at the interface were removed using a pasteur pipette, washed twice in 50 ml HBSS, and resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies). Mononuclear cells were seeded into T75 flasks (2.5×10^7 cells) or six well dishes with coverslips (2.5×10^6 cells/well) and allowed to adhere for 1 h to obtain a predominantly macrophage population. Non-adherent cells were removed by three washes with HBSS, and fresh IMDM containing 10% pooled human male serum and 2 ng/ml Granulocyte-Macrophage Colony Stimulating Factor was added. Cells were incubated at 37°C in 5% CO_2 for 5–7 days to allow macrophage differentiation before infecting.

The human type II pneumocyte cell line, A549 (American Type Culture Collection, Manassas, VA, U.S.A.), was seeded into T75 flasks (2×10^6 cells) or six well dishes (2×10^5 cells/well) in Eagle's Modified Essential Medium (Life Technologies) with 5% fetal bovine serum and incubated at 37°C in 5% CO_2 until monolayers were confluent (3–5 days).

Bacterial growth conditions

M. tuberculosis strain Erdman (ATCC 35801) was obtained from the Diagnostic Mycobacteriology Section, Centers for Disease Control and Prevention, and grown in Middlebrook 7H9 (M7H9) broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 10% albumin dextrose catalase (Difco) and 0.5% Tween 80 (Sigma) for 14 days at 37°C in 5% CO_2. Minimal clumping of the bacilli was observed. The bacilli were collected by centrifugation, washed with phosphate buffered saline, resuspended in M7H9 broth to an OD_{600} of 0.5, and frozen in 1 ml aliquots at −70°C. Several frozen aliquots were thawed and vortexed vigorously for 30 s to resuspend the bacilli, and plate counts were performed on Middlebrook 7H10 agar plates (Difco) to determine the number of colony forming units/ml. Human macrophage and A549 monolayers were mixed with 100 µl of a thawed bacterial suspension at a multiplicity of infection of 10 bacilli to one host cell. For experiments involving short-term incubation (i.e. 30 min), the infection ratio was increased to 50 bacilli/host cell, and for experiments involving long-term incubation (i.e. 5 days), the infection ratio was decreased to one bacillus/host cell. The adjustments in infection ratios resulted in approximately 2×10^7 host cell-associated bacilli in T75 flasks at each time point tested, as determined by plate counts. Infected cells were incubated at 37°C in 5% CO_2.

RNA isolation

Following the desired incubation period, the monolayers were washed twice with 10 ml HBSS to remove non-adherent bacteria and harvested in 10 ml HBSS by scraping the adherent monolayers from the flask with cell scrapers (Costar, Cambridge, MA, U.S.A.), and the cells pelleted by centrifugation at $3000 \times g$ for 10 min. *M. tuberculosis* Erdman bacilli were harvested from broth cultures by centrifugation at $5000 \times g$ for 10 min. Cell pellets were suspended in 100–200 µl HBSS, and RNA isolation was performed using the FastPrep system (Bio101, Inc., LaJolla, CA, U.S.A.). Briefly, the cells were mechanically lysed in a glass bead matrix and extracted with 500 µl

SigEP1	5'-CCCGACAACAGCCACAGGCC 3'
SigEP2	5'-GGGAATTACCGTCGCGTACCG 3'
SigE-Exp1	5'-GGGCCCATCCATATGCCGTCCTGGGATGAGCTGGTCCGTCAG-3'
SigE-Exp2	5'-GGGCCCATCCTCGAGTCAGCGAACTGGTTGACGTGAACTGCGCACTCG-3'
SigE-1	5'-GGAATACGGAATCGCAACTT-3'
SigE-2	5'-CGAACTGGGTTGACGTGAAC-3'
Orf1-1	5'-CCGGAAGCGTGGGACATGTG-3'
Orf1-2	5'-AGCGACGCACCCGCGATTGC-3'

Figure 6. Oligonucleotide primers used in this study.

acid phenol:chloroform (1:1), followed by an extraction with 500 µl chloroform:isoamyl alcohol (24:1). The RNA was precipitated overnight at −20°C with 500 µl isopropanol, washed with 80% ethanol and then dissolved in 30–50 µl diethyl pyrocarbonate-treated dH_2O. Three–4 µl was analysed by agarose gel electrophoresis. RNA suspensions were stored at −70°C.

RT-PCR analysis

Total RNA was treated with 5 U RNase-free DNase (New England Biolabs, Beverly, MA, U.S.A.) for 30 min at 37°C and then incubated at 65°C for 20 min to inactivate the DNase. First strand cDNA synthesis reactions contained 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.3 mM each dNTP, 5 µl DNase-treated RNA, 20 pmol reverse *sigE* primer (*sigE*-2; Fig. 6) and 1 µl Superscript II reverse transcriptase (Life Technologies). Samples were incubated at 42°C for 2 h. Subsequent PCR reactions contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.6 mM $MgCl_2$, 0.3 mM each dNTP, 1 µM each oligonucleotide primer (*sigE*-1 and *sigE*-2; Fig. 6), 5–10 µl first-strand cDNA and 1 unit *Taq* polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.). Samples were denatured for 5 min at 95°C, followed by 40 cycles of 94°C for 1 min, 47°C for 1 min and 72°C for 2 min, and one cycle at 72°C for 7 min. Amplification products were analysed by electrophoresis in a 1% agarose gel.

Expression and purification of recombinant *sigE* (R-*sigE*) protein

The *M. tuberculosis sigE* gene (encoding regions 1.2, 2, 3 and 4) was PCR amplified with primers *sigE*-Exp1, incorporating an *NdeI* site, and *sigE*-Exp2, incorporating an *XhoI* site, as previously described [8]. The resulting PCR products were cloned into the TA cloning vector pCRII using the TOPO-TA system (Clontech, Palo Alto, CA, U.S.A.). The *sigE* coding region was removed from the resulting clone by digestion with *NdeI* and *XhoI*, and the fragment was excised from a low melting point gel and subcloned into the expression vector pET19b digested with *NdeI* and *XhoI* (Novagen, Madison, WI, U.S.A.). The construct was sequenced to confirm integrity and transformed into the expression host *E. coli* BL21(DE3) pLysS (Novagen).

An overnight culture of *E. coli* BL21(DE3) pLysS containing pET-*sigE* was diluted 1:100 in 100 ml Luria–Bertani broth containing 100 µg/ml carbenicillin and incubated at 37°C with vigorous aeration (250 rpm) for approximately 3 h ($OD_{550} = 0.5$). IPTG was added to a final concentration of 2 mM to induce *sigE* expression, and incubation was continued for 3 more hours. Cells were harvested by centrifugation (10 min at $4500 \times g$), and pellets were resuspended in 20 ml Binding Buffer (pH 7.9; 5 mM imidazole, 500 mM NaCl and 20 mM Tris–HCl) and frozen overnight. Cells were rapidly thawed to stimulate lysis, $MgCl_2$ (10 mM) and DNase I (20 µg/ml) were added, and the suspension was incubated at room temperature for 20 min until viscosity was reduced. Inclusion bodies and cell debris were pelleted by centrifugation (15 min at $20\,000 \times g$), and the pellet was resuspended in Binding Buffer containing 6 M urea. Recombinant histidine-tagged protein was purified by affinity chromatography as described by the manufacturer (Novagen). Fractions containing purified R-*sigE* were pooled and dialysed stepwise against Renaturation Buffer (pH 7.9; 50 mM Tris–HCl, 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, 0.05% (v/v) TritonX-100) containing decreasing amounts of urea (4 M, 2 M, 1 M, and no urea). Dialysis in each buffer was for a minimum of 4 h at 4°C. The renatured protein was concentrated by ultrafiltration (Centricon 10, Amicon, Beverly, MA, U.S.A.), and the amount of protein was measured using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, U.S.A.).

Generation of anti-*sigE* antibodies

Purified R-*sigE* protein was diluted to 0.5 mg/ml in normal saline and emulsified with an equal amount of TiterMax adjuvant (CytRx, Norcross,

GA, U.S.A.). A female rabbit was injected intramuscularly with a 100 μl emulsion (50 μg antigen) in each rear flank and boosted twice with the same dosage at 4 week intervals. Serum was isolated from rabbit blood and tested for anti-*sigE* activity against purified R-*sigE* protein.

Western blotting

Crude lysates of broth-grown *M. tuberculosis* (approximately 5×10^9 bacteria) and *M. tuberculosis*-infected macrophages (approximately 2×10^7 bacteria) were prepared by mechanical lysis of the cells for 1 min with 0.1 mm glass beads in chilled buffer containing 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol and 0.3 M NH_3SO_4. Samples were filtered through a low-protein binding 0.2 μm filter (Millipore Corp., Bedford, MA, U.S.A.), mixed with SDS-PAGE loading buffer, heated to 80°C for 10 min, and loaded on a Tris–Glycine 4–20% gradient polyacrylamide gel (Novex, San Diego, CA, U.S.A.). Following electrophoresis at 125 V for 90 min, the proteins were blotted onto a nitrocellulose membrane in the Novex II Cell (Novex) at 30 V for 1 h. The blot was incubated for 1 h at room temperature in Tris-buffered saline (TBS; 0.5 M Tris, 1.5 M NaCl, pH 7.5) containing 0.01% Tween 20 (TBS-T) and 3% (w/v) gelatin. The blot was washed for 10 min in TBS-T and incubated in anti-*sigE* rabbit serum diluted 1:100 in TBS-T containing 1% (w/v) gelatin for 1 h at room temperature. The blot was washed three times in TBS-T for 10 min each at room temperature to remove non-bound antibody and then incubated with a goat anti-rabbit horseradish peroxidase conjugate (Zymed Laboratories, South San Francisco, CA, U.S.A.) diluted 1:15 000 in TBS-T containing 1% (w/v) gelatin. Following three room temperature washes in TBS-T for 10 min each, bound secondary antibody was detected using the ECL Western Blotting system as described by the manufacturer (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Construction of a transcriptional fusion with green fluorescent protein (GFP)

A 500 bp region of DNA immediately upstream of *sigE*, containing the putative promoter regions, was amplified by PCR with *Pfu* polymerase using primers *sigE*P1 and *sigE*P2 (Fig. 6). The PCR product was digested with *KpnI* and cloned into pGFP-1 (Clontech) digested with *SmaI* and *KpnI*. The resulting construct was digested with *MluI* and treated with Klenow enzyme to create a blunt end and then subsequently digested with *EcoRI*, excising a fragment containing the *sigE* promoter and GFP coding region. The fragment was cloned into a promoterless derivative of pMV261 [21] that had been digested with *EcoRI* and *HpaI*, creating pMY*sigE*-GFP. (The promoterless pMV261 was created by digesting pMV261 with *KpnI* and *XbaI*, treating with Klenow enzyme to create blunt ends, and religating, eliminating the fragment of pMV261 containing the *hsp60* promoter). To remove 20 bp of vector sequence separating the putative ribosome binding site from the GFP start codon, pMY*sigE*-GFP was partially digested with *NcoI*, treated with Klenow enzyme to create blunt ends, then subsequently digested with *SmaI* and religated. Resulting clones were confirmed by sequencing.

Electrocompetent *M. tuberculosis* strain Erdman was prepared by growing the cells to mid-log (7–10 days, OD_{550} of 0.5) in M7H9 broth at 37°C in 5% CO_2. Cells were washed four times at room temperature with 10% glycerol, then resuspended in 10% glycerol at 1/100 original culture volume. For electroporation, 60 μl of cells was mixed with 1 μg pMY*sigE*-GFP and incubated at 37°C for 15 min [22]. Electroporations were performed at room temperature with a BTX gene pulser with settings of 200 Ω, 25 μF and 2.5 kV [21]. Cells were allowed to recover for 4 h in 1 ml M7H9 broth at 37°C before being plated on M7H10 plates containing 50 μg/ml kanamycin. Plates were incubated at 37°C in 5% CO_2 for 21–28 days. The presence of pMYSig-GFP was confirmed by extracting total DNA from transformants and performing a Southern blot. As a positive control for GFP expression in *M. tuberculosis* in subsequent experiments, a pMV261-based plasmid containing an *hsp60–gfp* fusion [23] was used.

Microscopy

Macrophage and A549 monolayers uninfected or infected with *M. tuberculosis* bacilli containing pMY*sigE*-GFP or pHsp-GFP were examined by fluorescent microscopy under 100× magnification using either a Standard Lab 16 or Axiovert 135 microscope (with an LD achroplan objective) (Carl Zeiss, Inc, Thornwood, NY,

U.S.A.). GFP was excited with UV light at 480 nm and detected using a filter at 510 nm. Microscopic images were viewed on a computer monitor and captured with either the Image Pro software package (Media Cybernetics, Silver Spring, MD, U.S.A.) or the Axiovision I software package (Zeiss). Acid-fast stains were performed by standard methods [24] and examined by light microscopy under 100 × magnification using the Axiovert 135 microscope and the Axiovision I software (Zeiss).

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