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The *Mycobacterium tuberculosis* Sigma Factor σ^B Is Required for Full Response to Cell Envelope Stress and Hypoxia In Vitro, but It Is Dispensable for In Vivo Growth▽‡

P. A. Fontán,[1] M. I. Voskuil,[2,3] M. Gomez,[1] D. Tan,[4]† M. Pardini,[4] R. Manganelli,[5] L. Fattorini,[4] G. K. Schoolnik,[3] and I. Smith[1]*

PHRI Center of NJMS-UMDNJ, 225 Warren Street, Newark, New Jersey 07103-3535[1]; Department of Microbiology, School of Medicine, University of Colorado Denver, P18-9115, 12800 East 19th Avenue, P.O. Box 6511, Aurora, Colorado 80045[2]; Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University, 300 Pasteur Dr., B241 MC 5428, Stanford, California 94305[3]; Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy[4]; and Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Medical School, Via A. Gabelli 63, 35121 Padova, Italy[5]

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The numerous sigma (σ) factors present in *Mycobacterium tuberculosis* are indicative of the adaptability of this pathogen to different environmental conditions. In this report, we describe the *M. tuberculosis* σ^B regulon and the phenotypes of an *M. tuberculosis sigB* mutant strain exposed to cell envelope stress, oxidative stress, and hypoxia. The *sigB* mutant was especially defective in survival under hypoxic conditions in vitro, but it was not attenuated for growth in THP-1 cells or during mouse and guinea pig infection.

σ factors are components of RNA polymerases that bind to the enzyme's core subunits and give promoter specificity. The presence of 13 σ factors in *Mycobacterium tuberculosis* reflects the ability of this microorganism to adapt to various stress conditions that are likely encountered during host infection and that make *M. tuberculosis* a successful pathogen. σ^B is closely related to the primary sigma factor σ^A in terms of amino acid sequence (4). *sigB*, the structural gene for σ^B, is induced by different stresses (12) and is positively regulated by three extracytoplasmic function sigma factors, σ^E, σ^H, and σ^L (3, 13, 14). In vitro transcription studies showed that isolated mycobacterial RNA polymerases containing σ^E, σ^H, and σ^L can transcribe *sigB* using the same transcription start site (3). σ^F-containing RNA polymerase was also shown to transcribe *sigB* in these studies by using a different transcriptional start site. However, transcriptome studies with *sigF* mutants or strains overexpressing σ^F show no changes in *sigB* expression (7, 11), suggesting that the σ^F RNA polymerase transcription of *sigB* observed in biochemical experiments may not be physiological. The autoregulation of σ^B has also been recently determined by primer extension and reverse transcription-PCR (RT-PCR) (11).

In *M. tuberculosis*, the response to cell envelope is regulated by σ^E and the response to oxidative stress and heat shock is regulated by σ^H, and σ^B is a component of both regulons (13, 14). The fact that *sigB* expression is controlled by many regulatory pathways suggests that σ^B plays a central role in the *M. tuberculosis* stress response. In this report, we describe the in vitro phenotype of an *M. tuberculosis sigB* mutant exposed to stress conditions related to the activation of σ^E and σ^H. To analyze the extent to which the response to stress of σ^E and σ^H is transmitted through σ^B, we studied the σ^B regulon activated by cell envelope and oxidative stress in vitro. We also evaluated the growth of the *M. tuberculosis sigB* mutant strain in THP-1 macrophage-like cells and in vivo in the mouse and guinea pig models of infection.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Escherichia coli* strains JM109 and GM161 were grown in Luria broth (LB) (Difco) at 37°C. Antibiotic concentrations used to isolate selectable markers in *E. coli* were as follows: kanamycin, 50 μg/ml; streptomycin, 20 μg/ml; and hygromycin, 50 μg/ml. The *M. tuberculosis* strains created in this study were derivatives of *M. tuberculosis* H37Rv. All *M. tuberculosis* culture conditions, unless otherwise specified, followed standard protocols (9). Bacteria were grown at 37°C on either Middlebrook 7H9 (liquid medium) or 7H10 (solid medium) (Difco) supplemented with 0.5% bovine serum albumin, fraction V (Boehringer Mannheim), 0.2% glucose-0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80. Antibiotic concentrations used for selections in *M. tuberculosis* were as follows: kanamycin, 20 μg/ml; streptomycin, 20 μg/ml; and hygromycin, 150 μg/ml. Sucrose selection was performed on 7H10 plates with 10% sucrose. Bacterial stocks were maintained at −80°C in supplemented 7H9. Aliquots of bacteria were thawed and cultured on supplemented 7H10 solid medium. For preparation of liquid cultures, bacteria growing in 7H10 agar plates were suspended in 7H9 medium at an optical density at 540 nm (OD_{540}) of 0.05 (5×10^6 CFU/ml). Liquid cultures were grown in plastic roller bottles in a roller apparatus or in 9-ml glass tubes in a rotating wheel.

Cloning and construction of *M. tuberculosis sigB* mutant and complemented strains. DNA recombinant techniques were performed by following standard techniques. PCR DNA primers were commercially obtained (IDT), and *Pfu* DNA polymerase was used for all PCRs. *M. tuberculosis* genomic DNA from strain H37Rv was used as a template. PCR products were cloned into pCR-Blunt II-PCR-BLUNT II-TOPO and sequenced. Clones with error-free DNA sequences were used for further genetic manipulation. Transformation of plasmids into *M. tuberculosis* was done by electroporation, and DNA Southern blot anal-

* Corresponding author. Mailing address: PHRI TB Center, 225 Warren Street, Room W210W, Newark, NJ 07103-3535. Phone: (973) 854-3260. Fax: (973) 854-3101. E-mail: smithis@umdnj.edu.

† Present address: National Institute for the Control of Pharmaceutical and Biological Products 2#, Tiantan Xili, Beijing, China 100050.

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ysis from the *M. tuberculosis* chromosome was performed as previously described (14). To construct the *sigB* mutant, a 4-kb PstI fragment of *M. tuberculosis* DNA containing the *sigB* gene was cloned into the vector pUC19 to construct plasmid pSM229. The *sigB* gene was disrupted by introducing a kanamycin cassette from pUC4K into a NruI deletion to construct pSM349. A 5.1-kb PstI DNA fragment from plasmid pSM349 was introduced into the SmaI site of the shuttle vector pSM270 (14), creating the plasmid pSM356. pSM356 was electroporated into wild-type *M. tuberculosis* H37Rv. Several *M. tuberculosis* clones were selected by kanamycin and sucrose resistance and streptomycin sensitivity. Southern blot analyses performed on chromosomal DNA isolated from a presumed *M. tuberculosis* mutant clone indicated that it had the disruption of the *sigB* gene, and this strain was named ST82. For complementation of the *sigB* mutant, a 1.3-kb DNA fragment containing the *sigB* gene and including a 274-bp upstream region from the translational start site of this gene (promoter region) was PCR amplified using primers sigB27U17 (5′CGCATCCCGCTGTTCCC3′) and sigB1360L17 (5′CTTGGCCAGCTGCGAAA3′). The DNA fragment was cloned into the PCR-BLUNT II-TOPO vector, creating plasmid pSM753. A HindIII/XbaI fragment from pSM753 was cloned into a HindIII/XbaI deletion site of the vector pMV306-Hyg, creating plasmid pSM754. Plasmid pSM754 was electroporated into strain ST82. The insertion by single crossover of this plasmid into the *attB* of strain ST82, to create the ST174 strain, was verified by Southern blot analysis.

Macrophage infections. Infection of THP-1 human monocytic cells was performed as described before (5). Briefly, 10^5 THP-1 cells/well were differentiated with 40 nM phorbol myristate acetate in 96-well plates at a multiplicity of infection of 0.05 bacterium per macrophage. At various time points after infection, triplicate wells for each *M. tuberculosis* strain infection were treated with 0.05% sodium dodecyl sulfate (SDS) to lyse the macrophages, and numbers of CFU were determined by plating appropriate serial dilutions on 7H9 plates. Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Mouse infections. BALB/c mice were infected intravenously with the *sigB* mutant, the complemented mutant strain, or the wild-type parental strain. At selected times, three mice were killed and their lungs, spleens, and livers removed and processed for analysis of bacterial survival in these organs. Numbers of CFU were determined by plating appropriate serial dilutions on 7H9 plates. Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Guinea pig infections. Infections of guinea pigs were performed as previously described (10). Briefly, Hartley strain guinea pigs weighing 250 to 300 g (Charles River Laboratories) were infected via the aerosol route. Five guinea pigs per group were killed at days 1, 21, and 56 after infection, and the survival of the wild type, the mutant, and the complemented strain in the lungs was analyzed by counting CFU in lung homogenates.

In vitro stress treatments. For DNA arrays, *M. tuberculosis* strains were cultured in roller bottles until early log phase (OD_{540}, 0.2) at 37°C. Aliquots of 40 ml were treated with SDS or diamide at a final concentration of 0.05% or 5 mM, respectively. After 60 min of incubation at 37°C, bacteria were centrifuged at 3,000 × *g* and pellets were immediately frozen at −80°C until further processing.

***M. tuberculosis* RNA from in vitro cultures.** Bacterial cell pellets were suspended in 1 ml TRI reagent (Molecular Research Center, OH) and immediately transferred to a 2-ml screw-cap microcentrifuge tube containing 0.5 ml zirconia beads (0.1-mm diameter; Biospec Products, Inc., OH). Samples were disrupted by two 1-min pulses in a BeadBeater, with the samples kept on ice for 2 min between pulses. RNA from the samples was purified as previously described (5) using RNeasy columns (Qiagen). The purified RNA was kept at −80°C until further use.

cDNA labeling and microarray hybridization. 70-mer oligonucleotides obtained from Qiagen (now Operon) were printed on poly-L-lysine-coated glass (29). Microarray procedures were performed essentially as previously described (25).

Microarray data analysis. Microarrays were scanned using a GenePix 4000A imaging device (Axon Instruments). Intensities of the two dyes at each spot were quantified using ScanAlyze (Michael Eisen, http://rana.lbl.gov/EisenSoftware.htm). All gene-specific spots on the microarray other than those whose induction ratio was in the top or bottom 5% were used to normalize the intensities of Cy3 and Cy5 from each spot. After Cy3 and Cy5 channel normalization, large-percentage fluctuations in low-background spot values were eliminated by adjusting low-signal-intensity spots to a minimum noise value. The noise value for each channel was determined by calculating the average intensity value for the spots with the lowest intensity (the bottom 20%), and then every value below this average noise value was raised to the noise value. Microarray-determined ratios were calculated from three biological replicates and two microarrays for each biological replicate. A false-discovery rate of <2% and a regulation of at least 1.8-fold were used as criteria to consider a gene differentially regulated. False-discovery rates were determined using the Significance Analysis of Microarray (SAM) program (23).

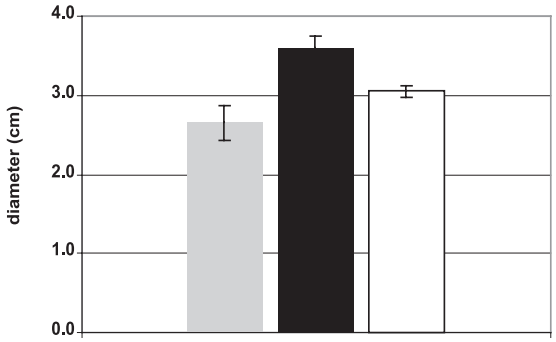

FIG. 1. Effect of SDS stress on the growth of the *M. tuberculosis* *sigB* mutant strain. The effect of the SDS treatment was determined by a disc assay for the H37Rv strain (gray bar), the *sigB* mutant strain (black bar), and the complemented strain (white bar). The figure shows the arithmetic means ± standard deviations from three biological replicates.

Quantitative RT and real-time PCR with SYBR green. RT-PCRs were previously described (6). Primers were designed by using the software OLIGO 6.6 (Molecular Biology Insights, Cascade, CO) and purchased from IDT. For RT, 50 ng of RNA and Transcriptor RT polymerase (Roche) were used. PCRs were carried out in sealed tubes in an Mx4000 spectrofluorometric thermal cycler (Stratagene).

Killing curves after hypoxic treatment. Cultures of *M. tuberculosis* strains were inoculated at an OD_{540} of 0.05 (5×10^6 CFU/ml) in 6 ml of medium, using tubes with a capacity of 9 ml in order to maintain an air/medium ratio of 1:2 (vol/vol) (28). Methylene blue was used as an indicator of oxygen consumption (1). Tubes were sealed and incubated at 37°C with rotation. After 4 days, the cultures reached an OD_{540} of 0.3, and the loss of blue color indicated that the oxygen had been consumed. Several tubes were prepared in order to be able to open three tubes for each time point to determine the number of CFU/ml in each culture. Tubes were discarded after being opened.

Determination of growth inhibition by zone diffusion assay. *M. tuberculosis* strains were grown to exponential phase. Aliquots of 1 ml of culture were spread on 7H10 plates. Paper discs (6.5 mm in diameter; Schleicher and Schuell) containing 20 μl of 10% SDS were placed on top of the agar. The diameters of the zones of inhibition were measured after 10 days of incubation at 37°C.

DNA array data. DNA array data were deposited in the TB database (http://www.tbdb.org/).

RESULTS

To analyze the σ^B regulon and the role of this sigma factor on the pathogenicity of *M. tuberculosis*, we constructed an *M. tuberculosis* mutant strain by disrupting the *sigB* gene with a kanamycin cassette as previously described (26). A complemented strain was constructed by reintroducing the *sigB* gene in the mutant strain by using an integrative plasmid. We compared the survival of the mutant strain to that of the wild-type strain H37Rv and the complemented strain under different conditions. We observed that the *sigB* mutant was more sensitive to the detergent SDS than the wild-type parental strain in disc assays (Fig. 1). Treatment of *M. tuberculosis* broth cultures with high temperature (45°C) for short periods (up to 24 h) decreased the survival of the *sigB* mutant strain in standing but not in rolling cultures (data not shown). This result suggested that the loss of viability of the *sigB* mutant observed in these experiments was due principally to low levels of oxygen in the standing cultures. To verify this hypothesis, we analyzed the effect of gradual loss of oxygen on the *sigB*

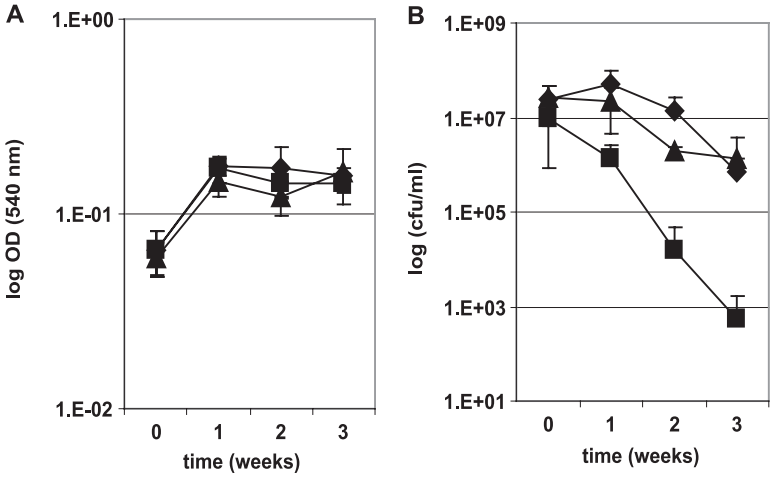

FIG. 2. Effect of hypoxia treatment on the growth of the *M. tuberculosis sigB* mutant strain. *M. tuberculosis* rolling broth cultures were grown at 37°C in sealed tubes. The figure shows the arithmetic means ± standard deviations for three biological replicates represented as ODs (A) or numbers of CFU/ml (B) of the cultures. Diamonds, H37Rv; squares, *sigB* mutant strain; triangles, complemented strain.

mutant, as wild-type *M. tuberculosis* is known to survive but not grow under those conditions (27). *M. tuberculosis* rolling cultures prepared in 9-ml glass tubes were sealed and incubated with rotation at 37°C over long periods of time (up to 3 weeks). In these experiments, we observed that the survival of the *sigB* mutant strain was highly sensitive to this condition compared to that of the wild type or the complemented strain, showing approximately 3-log-order-lower survival (Fig. 2).

Since both the response to cell envelope stress by σ^E and the response to oxidative stress by σ^H converge in the activation of σ^B, we speculated that the analysis of the σ^B regulon under these stress conditions may reveal the identities of the σ^B target genes relevant for *M. tuberculosis* pathogenicity. Therefore, to analyze the σ^B regulon, we compared the transcriptional profiles of the *sigB* mutant strain and the wild-type strain under conditions of cell envelope or oxidative stress. The distribution in functional categories of genes whose expression is regulated by σ^B when the bacteria are under cell envelope or oxidative stress is summarized in Table 1. Transcriptional comparison of the wild type and the *sigB* mutant, growing logarithmically in broth cultures, indicated that only eight genes were downregulated in the mutant strain relative to levels of regulation in the wild-type strain (see Table S1 in the supplemental material). However, 28 genes were upregulated in the mutant strain, particularly *sigE* and several genes known to be part of the σ^E regulon (14). The upregulation of *sigE* in the *sigB* mutant may be due to a competitive regulation in which the absence of one sigma factor, σ^B, allows more access to core RNA polymerase by other sigma factors that transcribe *sigE* promoters. Another possibility is that the absence of σ^B may cause a stress that induces the expression of *sigE*. Comparison of the transcriptional profiles of the *sigB* mutant and the wild-type strain after treatment with SDS revealed 72 genes downregulated in the *sigB* mutant (see Table S2 in the supplemental material). Since *sigB* is under the regulation of σ^E, we expected a significant overlap between the σ^E and the σ^B regulons. However, the comparison of the transcriptional profiles of both sigma factors after SDS treatment indicated only one gene in common, Rv0465c, annotated to code for a putative transcriptional regulator (http://genolist.pasteur.fr/TubercuList/). Among the genes downregulated in the *sigB* mutant were many genes related to the cell envelope stress response and also several encoding transcriptional regulators. For example, the expression of *ideR* was upregulated in bacteria treated with SDS, and this regulation depended on σ^B, in agreement with other reports (11, 19). As expected, the downregulation of *ideR* in the *sigB* mutant caused the concomitant increased expression of genes from the *mbt* cluster. It has been previously shown that the *mbt* genes, which encode enzymes for the biosynthesis of siderophores, are repressed by IdeR (8, 20). The analysis of the transcriptional profile of the *sigB* mutant under oxidative stress induced by diamide treatment (13) indicated 40 genes under σ^B regulation (see Table S3 in the supplemental material). Again, we expected an overlap between σ^B and σ^H regulons, but we found that only two genes, Rv0251c and Rv0384c, encoding heat shock proteins were previously observed to be under σ^H control. The DNA array under diamide treatment indicated that *furA* (Rv1909),

TABLE 1. Distribution in functional categories of σ^B-regulated genes

Type of stress	Category	No. of regulated genes
Cell envelope	Hypothetical proteins	25
	Cell wall-associated genes	20
	Regulatory proteins	13
	Intermediate metabolism	5
	Virulence, detoxification, adaptation	4
	Information pathways	4
	Lipid metabolism	1
	Insertion sequences and phages	1
Oxidative	Hypothetical proteins	15
	Intermediate metabolism	12
	Regulatory proteins	7
	Virulence, detoxification, adaptation	4
	Cell wall-associated genes	3

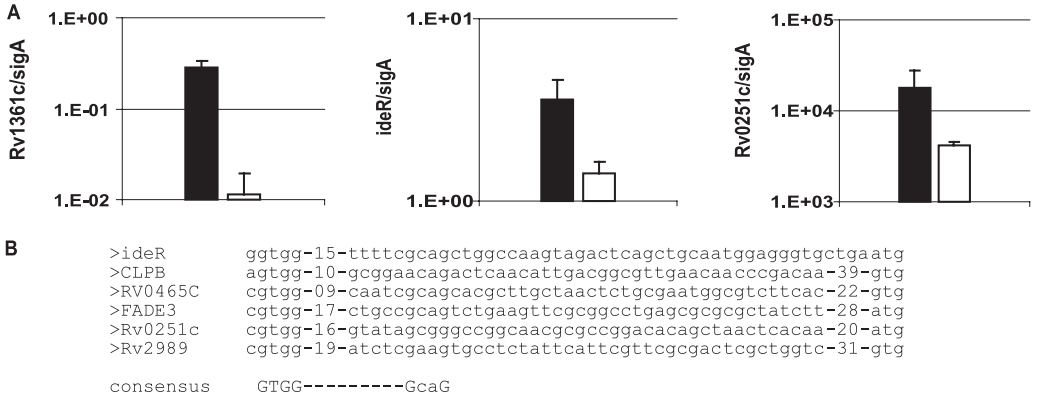

FIG. 3. Expression of *M. tuberculosis* genes under the control of σ^B. (A) The expression of some *M. tuberculosis* genes found to be differentially regulated by σ^B in the DNA microarray analyses was confirmed by quantitative RT-PCR with SYBR green. The figure shows the arithmetic means ± standard deviations from three biological replicates. Results are expressed in logarithmic scale. The expression of each gene in strain H37Rv (black bars) or in the *sigB* mutant (white bars) was normalized to the expression of *sigA*. Expression levels of Rv1361c in nonstressed broth cultures, *ideR* in SDS-treated broth cultures, and Rv0251c in diamide-treated broth cultures are indicated. (B) Alignment of sequences of the promoter regions of σ^B-regulated genes. The previously proposed −35 and −10 σ^B consensus regions are at the bottom (11).

encoding a peroxidase, is under the control of σ^B. Interestingly, in a report by Mulder et al. (16), it was shown that there was an increased expression of catalase and peroxidase mRNA after overexpression of σ^B in *Mycobacterium bovis* BCG. Moreover, *katG* is downstream of the *furA* gene in the same transcriptional unit (15). These observations and our DNA array results suggest that σ^B regulates the expression of *furA* and *katG* in *M. tuberculosis*. The regulation of *ideR* (Rv2711), *PPE19* (Rv1361c), and *hsp20* (Rv0251c) by σ^B under different stress conditions observed by DNA array analyses was confirmed by quantitative RT-PCR (Fig. 3A). The transcription of *ideR* by RNA polymerase with σ^B has also been reported in a previous study that evaluated the effect of σ^B overproduction on *M. tuberculosis* gene expression during exponential growth (11). We chose to confirm by RT-PCR that the expression of gene *PPE19* (Rv1361c) depends on σ^B because Rv1361c is one of the few genes whose expression is under σ^B regulation in nonstressed bacteria, according to the DNA array data. The gene *hsp20* was selected for confirmation by RT-PCR since this is a gene whose expression was found to be highly regulated by σ^B when *M. tuberculosis* was exposed to oxidative stress. The putative promoter consensus sequence recognized by σ^B that was defined in a previous study (11) could be identified in some genes whose expression is regulated by σ^B under stress conditions (Fig. 3B).

The attenuated phenotype of the *sigB* mutant strain in broth cultures under cell envelope stress and hypoxia prompted us to evaluate the relevance of σ^B for *M. tuberculosis* pathogenicity. The mutant strain did not show differences in growth in human macrophages (Fig. 4A) or survival in the mouse model of lung infection from the wild-type strain (Fig. 4B). The attenuated growth of the mutant in broth cultures subjected to hypoxia suggested that σ^B probably controls the expression of *M. tuberculosis* components necessary to tolerate this stress. For that reason, we evaluated the survival of the *sigB* mutant in the guinea pig model of lung infection, since it has been previously demonstrated that the lung granulomas of guinea pigs infected with *M. tuberculosis* are a low-oxygen environment (24). However, no differences were observed when the growth of the

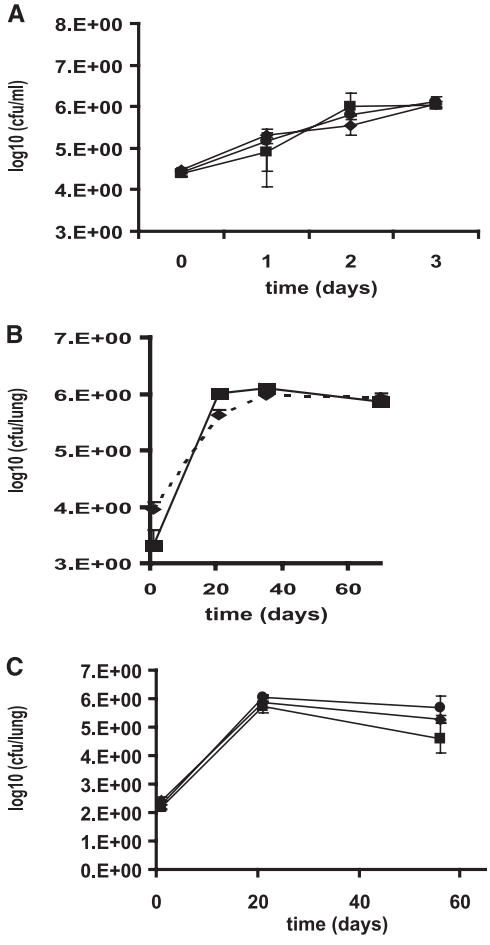

FIG. 4. Growth of the *M. tuberculosis sigB* mutant in various models of infection. The survival of the *M. tuberculosis* wild-type H37Rv strain (diamonds), the *sigB* mutant strain (squares), and the complemented strain (circles) was analyzed in THP-1 macrophage-like cells (A), intravenously inoculated BALB/c mice (B), and aerosol-infected guinea pigs (C).

wild-type strain and that of the *sigB* mutant were compared (Fig. 4C).

DISCUSSION

In this article we have defined the σ^B regulon activated by cell envelope stress and oxidative stress. Moreover, we have demonstrated that a *sigB* mutant strain is sensitive to SDS stress and hypoxic treatment in vitro, although the inactivation of the *sigB* gene did not affect the survival of *M. tuberculosis* during infection.

σ^B appears to be a central regulator in the *M. tuberculosis* response to stress, since its structural gene, *sigB*, is positively regulated by three other sigma factors. We have identified by DNA microarrays two different sets of genes that were under σ^B regulation depending on the stress to which *M. tuberculosis* was exposed (cell envelope stress or oxidative stress). This observation indicates that other regulatory mechanisms should be activated simultaneously in response to a particular stress condition. For example, we reported here that genes previously shown to be important for the pathogenicity of *M. tuberculosis*, like Rv1130 and Rv1131 (17) or *hsp20* (22), are under σ^B regulation; however, it has been demonstrated that these genes are also under the regulation of σ^E (14), MprAB (18, 19), and PhoP (26). Surprisingly, we observed only a small overlap between the regulons of σ^B and those of σ^E and σ^H. This observation could be explained because in *sigE* or *sigH* mutants, the expression of *sigB* is not totally abolished, as it is in the *sigB* mutant. Also, these dissimilar results may be related to the type of DNA arrays used to analyze the samples: more-specific oligonucleotide arrays in this work (25) versus amplicon arrays in previous works (13, 14). We also observed a very small overlap in genes of the *M. tuberculosis* σ^B regulon between a previous report (11) and ours, probably due to the different methodologies used for both analyses. In the experiments reported here, the expression of genes in the wild-type strain was compared to the expression of genes in the *sigB* mutant strain, while in the other study, the transcription of the wild-type strain was compared to the transcription of the strain overproducing σ^B. Also, evaluation of the regulon under stress, rather than the overexpression of the protein, may be a better representation of the physiological conditions under which activation of the sigma factor occurs. For example, other regulatory mechanisms (i.e., anti-sigma and anti-anti-sigma factors) that may be activated by stress and affect the expression of the regulon might not be activated during overexpression of the sigma factor.

The lack essentially of σ^B in vivo despite its apparent central role for the *M. tuberculosis* response to stress could reflect a functional redundancy of *M. tuberculosis* sigma factors and other regulators in vivo. On the other hand, the differences between the in vivo and in vitro phenotypes of the *sigB* mutant may indicate a poor representation of *M. tuberculosis* virulence by the available animal models of *M. tuberculosis* infection. Similar disparities have been obtained with other *M. tuberculosis* regulators, like DosR, whose regulon is necessary to sustain the survival of *M. tuberculosis* in broth cultures under anaerobiosis (25). However, a *dosR* mutant strain appears dispensable for *M. tuberculosis* survival in vivo under some conditions (21), although in other in vivo environments, the DosR regulon may be required (2). These observations indicate the complexity of the regulatory network that allows *M. tuberculosis* to establish intriguing and still-unresolved host-pathogen relationships.

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