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Roles of SigB and SigF in the *Mycobacterium tuberculosis* Sigma Factor Network▽†

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To characterize the roles of SigB and SigF in sigma factor regulation in *Mycobacterium tuberculosis*, we used chemically inducible recombinant strains to conditionally overexpress *sigB* and *sigF*. Using whole genomic microarray analysis and quantitative reverse transcription-PCR, we investigated the resulting global transcriptional changes after *sigB* induction, and we specifically tested the relative expression of other sigma factor genes after knock-in expression of *sigB* and *sigF*. Overexpression of *sigB* resulted in significant upregulation of genes encoding several early culture filtrate antigens (ESAT-6-like proteins), ribosomal proteins, PE-PGRS proteins, the keto-acyl synthase, KasA, and the regulatory proteins WhiB2 and IdeR. Of note, the induction of *sigB* did not alter the expression of other sigma factor genes, indicating that SigB is likely to serve as an end regulator for at least one branch of the *M. tuberculosis* sigma factor regulatory cascade. Analysis of the 5′-untranslated region (UTR) of SigB-dependent transcripts revealed a putative consensus sequence of NGTGG-N_{14-18}-NNGNNG. This sequence appeared upstream of both *sigB* (Rv2710) and the gene following it, *ideR* (Rv2711), and in vitro transcription analysis with recombinant SigB-reconstituted RNA polymerase confirmed SigB-dependent transcription from each of these promoters. Knock-in expression of *sigF* revealed that only the *sigC* gene was significantly upregulated 6 and 12 h after *sigF* induction. The previously identified SigF promoter consensus sequence AGTTTG-N_{15}-GGGTTT was identified in the 5′ UTR of the *sigC* gene, and SigF-dependent in vitro transcription of the promoter upstream of *sigC* was confirmed by using recombinant SigF-reconstituted RNA polymerase. These two knock-in recombinant strains were tested in a macrophage model of infection which showed that overexpression of *sigB* and *sigF* resulted in reduced rates of *M. tuberculosis* intracellular growth. These results define the SigB promoter consensus recognition sequence and members of the SigB regulon. Moreover, the data suggest that, in addition to serving as an end regulator in a sigma factor cascade, SigB may auto-amplify its own expression under certain conditions.

Mycobacterium tuberculosis remains one of the most important human pathogens since its discovery over a century ago. A unique feature of *M. tuberculosis* is its ability to persist in a latent state without causing disease. Because *M. tuberculosis* is exposed to stresses such as nutrient limitation and antimicrobial products of the immune system during host infection, its adaptation to different environments through regulation of genetic programs is an essential feature of virulence. Tubercle bacilli can survive in granulomas, which represent a stringent and nutrient-deficient environment (36).

Gene expression is initiated by the binding of RNA polymerase to the promoter sequence of target genes. Bacterial RNA polymerase is composed of a core enzyme (α_2 $\beta\beta'$) and one of several different sigma factors. The sigma factor determines promoter specificity by recognizing and binding to the sequence of the promoter. Thus, investigating the role of sigma factors in *M. tuberculosis* is important for understanding the genetic adaptation of this pathogen during infection (19). *M. tuberculosis* has 13 sigma factor genes (10). SigA, the principal sigma factor, is constitutively expressed and governs the transcription of numerous housekeeping genes in *M. tuberculosis* (27, 41). *M. tuberculosis sigB*, which encodes a principal-like sigma factor 62% homologous to SigA (Fig. 1A and see Fig. S1 in the supplemental material), is induced under various stress conditions, including exposure to sodium dodecyl sulfate (SDS), heat shock, cold shock, low aeration, and stationary phase (27). SigF, which shares 32% homology to SigB, is classified as a stress response sigma factor based on homology to sporulation and stress response sigma factors in *Streptomyces coelicolor* (4) and *Bacillus subtilis* (4, 13, 38) and to the stationary sigma factor RpoS in *Vibrio* spp. (Fig. 1A and see Fig. S1 in the supplemental material). *sigF* is induced by heat shock and mild cold shock and in nutrient starvation conditions (27, 30). The other sigma factor genes (*sigC*, *sigD*, *sigE*, *sigG*, *sigH*, *sigI*, *sigJ*, *sigK*, *sigL*, and *sigM*) are classified as extracytoplasmic function sigma factors, which control cell envelope synthesis, secretory functions, and periplasmic protein repair and degradation (10, 25, 34).

Previous studies have investigated the role of *M. tuberculosis* sigma factors under various in vitro stress conditions and in animal models of infection. A *sigC*-deficient mutant was shown to cause reduced mortality in mice (39), and the infection of guinea pigs with *M. tuberculosis* lacking a functional SigC resulted in the absence of necrotic granulomas (21), suggesting that *sigC* is essential for immunopathogenesis. A *sigD*-deficient mutant was also found to be attenuated in mice (7), and a *sigE*-deficient mutant showed attenuation within macrophages, as well as delayed mortality of mice (3, 28). A *sigH*-deficient mutant demonstrated increased susceptibility to oxidative stress

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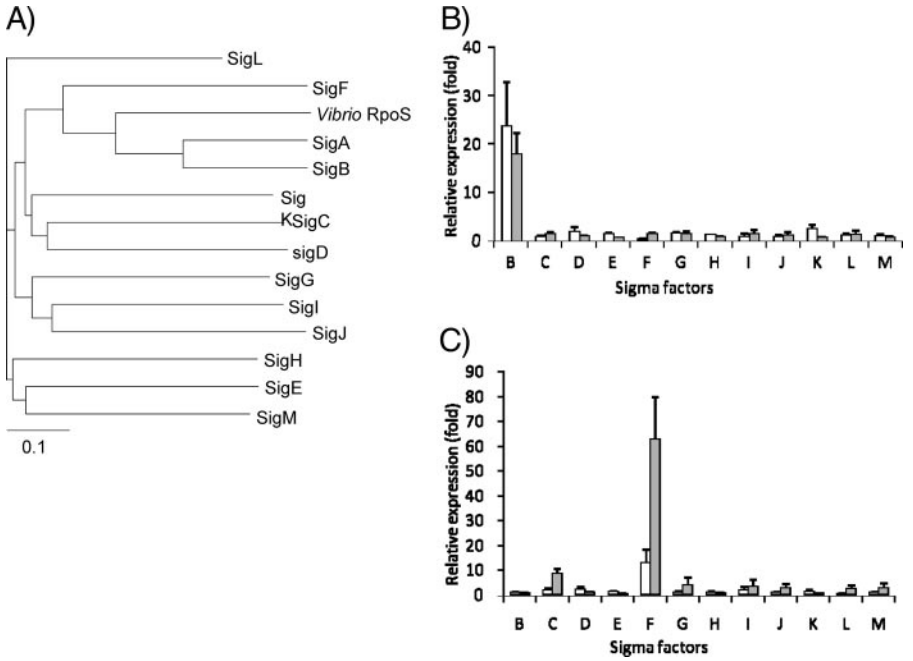

FIG. 1. Analysis of sigma factor protein similarity and real-time RT-PCR analysis of sigma factor gene expression in the *M. tuberculosis sigB* and *sigF* KI recombinant strains. (A) A phylogenetic tree of the 13 *M. tuberculosis* sigma factors was generated by comparison of the amino acid sequences of these sigma factors and the sequence of *Vibrio* RpoS (obtained from NCBI GenBank accession no. AAK09363). (B) Changes in gene expression of the 13 sigma factors in the *M. tuberculosis sigB* KI recombinant strain were measured 6 and 12 h after acetamide induction by using real-time RT-PCR. The C_T value was normalized to that of *sigA*, and the relative expression was calculated by the $\Delta\Delta C_T$ method compared to the control strain. Bars: □, 6 h; ▨,12 h. (C) Same analysis as in panel B but with the *M. tuberculosis sigF* KI recombinant strain.

(22, 33), and a *sigF*-deficient mutant was found to have reduced survival compared to the isogenic wild-type strain in mice (14). Recently, a *sigL* deletion mutant also showed less pathogenicity in mice than the wild-type strain (12, 16). However, other sigma factor-deficient mutants, such as those lacking *sigJ* and *sigM*, did not show a distinct phenotype in mouse or macrophage infection models (1, 20). Phenotypic differences between various sigma factor-deficient mutants suggest underlying differences in the regulatory networks controlled by each sigma factor. *M. tuberculosis* exposed to nutrient starvation conditions in vitro revealed significantly increased expression of Rel_{Mtb} (ppGpp synthase I). ppGpp (hyperphosphorylated guanine) acts as a molecular signal in many bacteria to regulate the expression of sigma factors (18, 24). Lack of Rel_{Mtb} in *M. tuberculosis* resulted in decreased mycobacterial survival in mice (11) and reduced the expression of *sigD* (11). Nutrient starvation conditions increased the expression of *sigB*, *sigD*, *sigE*, and *sigF*, while the expression of *rpoA* (α subunit) and *rpoC* (β′) was decreased (5).

Previous work using sigma factor knockout mutants or knock-in (KI; conditional overexpression) recombinants has suggested the interdependence of sigma factor gene expression. For example, *sigH* is required for maximal expression of *sigE* and *sigB* (28, 33). Likewise, deletion of *sigF* resulted in decreased expression of *sigC* (14), suggesting that SigF may exert its regulatory activity at a level close to those of SigC and SigB. In vitro transcription studies have demonstrated that SigE-, SigF-, SigH-, and SigL-containing RNA polymerases can transcribe *sigB* (12, 33). These results suggest that multiple sigma factor signaling pathways might converge at *sigB.* Therefore, SigB may be a key end-effector regulator, since deletion of *sigC*, *sigE*, *sigF*, *sigH*, and *sigL* (resulting in decreased *sigB* expression) demonstrated reduced immunopathology in the mouse (3, 12, 14, 16, 22, 39). Despite the potential significance of these sigma factors in *M. tuberculosis*, the roles of SigB and SigF within the complex sigma factor regulatory network remain unclear. We investigated here the roles of SigB and SigF in sigma factor regulation in *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* DH5α was used in DNA cloning procedures, and *M. tuberculosis* CDC1551 (Oshkosh) was cultivated in Middlebrook 7H9 liquid broth (supplemented with 0.05% Tween 80, 10% oleic acid-albumin-dextrose-catalase, 5% glycerol) or 7H10 agar for transformation and screening with appropriate antibiotics. For the selection of the *sigB* knock-in strain, kanamycin (10 μg/ml) was added to cultures.

Conditionally overexpressing recombinants. Previously, we constructed a *sigF* overexpression vector pSCW35 containing an acetamide promoter-*sigF* fusion gene (40), as well as a control vector pSCW38 containing only the promoter (1, 40). To overexpress *sigB* in *M. tuberculosis*, the entire *sigB* gene was substituted for the *sigF* gene in vector pSCW35 using restriction enzymes NdeI and PacI, resulting in vector pSCW40. The entire *sigB* gene was amplified by PCR using primers the pACEB1 (5′-GGCCCATATGATGGCCGATGCACCCACAAG-3′; the NdeI site was incorporated) and pACEB2 (5′-GGCCTTAATTAACTACCTGGCTCAGGATGTCC-3′; the PacI site was incorporated). Vector pSCW40 was transformed into *M. tuberculosis* by electroporation (2.0 kV), and transformants were screened by using kanamycin (10 μg/ml)-containing 7H10 plates.

Real-time RT-PCR of *sigB* and *sigF* KI strains. *sigB* and *sigF* expression was confirmed by real-time reverse transcription-PCR (RT-PCR). To induce *sigB* and *sigF*, 0.2% of acetamide solution was added to a culture at an optical density at 600 nm of ~0.5, and at 6 and 12 h after induction the cells were pelleted, washed with phosphate-buffered saline, and resuspended in TRIzol reagent (Invitrogen). Mycobacterial membranes were disrupted by using 0.1-mm diameter

silica beads and a bead beater at 5,000 rpm. After centrifugation at 10,000 × *g* for 5 min at 4°C, the supernatants were collected and treated with chloroform and centrifuged again to collect the aqueous phase. Finally, RNA was precipitated with isopropyl alcohol and washed with 75% ethanol and dried. The RNA was treated with DNase I and subjected to 30 cycles of PCR to confirm the removal of all DNA during the RNA extraction procedure. About 1 μg of DNase I-treated RNA was reverse transcribed by using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). Real-time RT-PCR was performed using an IQ SYBR green I PCR kit (Bio-Rad). The primers used in real-time RT-PCR are listed in Table S1 in the supplemental material.

The cycle threshold value (C_T) obtained for each gene of interest was normalized with that of *sigA*, a housekeeping gene, in order to obtain relative fold-change in gene expression [$\Delta C_T = C_{T(\text{gene of interest})} - C_{T(\text{housekeeping gene})}$]. The regulation of individual genes was calculated by using the formula $2^{-(\Delta CT(s) - \Delta CT(c))}$, Where, *s* represents the *sigB*- or *sigF*-inducible strain and *c* represents the control strain (pSCW38).

Microarray of *sigB* overexpression. *sigB* overexpression was also analyzed by using microarrays. Microarray analysis was performed with poly-L-lysine coated glass slides containing oligonucleotides corresponding to all annotated *M. tuberculosis* genes. Total RNA was extracted from the control strain and the *sigB* KI mutant 12 h after acetamide exposure. RNA was reverse transcribed with random hexamers (Invitrogen). The resulting cDNA was labeled with either Cy3-dCTP or Cy5-dCTP and competitively hybridized to whole-genome arrays (one 70mer oligonucleotide probe per *M. tuberculosis* open reading frame spotted onto glass slides). Hybridization to microarray slides was performed as described previously (39). Arrays were scanned by using an Axon 4000B scanner. The image data were quantified by using GenePix pro 4.0 software and then normalized with the total intensity of spots. The ratios of Cy5 and Cy3 were compared and calculated.

In vitro transcription assay. SigB and SigF were purified as C-terminal His-tagged recombinant fusion proteins. The entire open reading frame of the *M. tuberculosis sigB* gene was amplified with the primers pETsigB1 (5′-GGGCCC ATATGGCCGATGCACCCACAAGGGC-3′) and pETsigB2 (5′-GGGCCCTC GAGGCTGGCGTACGA CCGCAGCC-3′), and the *sigF* gene was amplified with the primers pETsigF1 (5′-GGGCCCATATGACGGCGCGCGCTGCCG G-3′) and pETsigF2 (5′-GGGCCAAG CTTCTCCAACTGATCCCGTAGCC G-3′). Each amplicon was digested with NdeI and XhoI, ligated to NdeI- and XhoI-digested pET22b(+) (Novagen), and transformed into *E. coli* BL21(DE3) strain. Sigma factor overexpression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and after 3 h of induction the cells were harvested and disrupted by sonication in binding buffer (50 mM Tris-Cl, 0.5 M NaCl, 10 mM imidazole). The supernatant was recovered by centrifugation at 10,000 × *g* for 10 min at 4°C, and passed through a nickel-nitrilotriacetic acid agarose column. The column was washed with 10 volumes of washing buffer (50 mM Tris-Cl, 0.5 M NaCl, 50 mM imidazole) and finally eluted with buffer (50 mM Tris-Cl, 0.5 M NaCl, 500 mM imidazole). The purity of the protein was checked by SDS-polyacrylamide gel electrophoresis, and the protein concentration was determined by the Bradford method. The SigF protein was purified by using the same techniques.

Short (template 1) and long (template 2) DNA templates which, including the putative promoter regions, were prepared by PCR with sets of three primers. For the *sigB* promoter region, Binv10 (5′-TGCACGTCACAGGGCGTCAGAT-3′) and Binv12 (5′-TGCGCTTGGCCAGTTCGACT-3′) were used to create *sigB* template 1, and Binv10 with Binv14 (5′-CAGGTCGCGTTTTCGGTTCT-3′) were used to create *sigB* template 2. For the *ideR* promoter region, primers iderinv10 (5′-TGGACATCCTGAGCCAGGTAGC-3′) and iderinv12 (5′-AAG TAGCCCATCGCGCTCCATC-3′) were used to produce *ideR* template 1, and iderinv10 with iderinv14 (5′-CTTGCGCATCACGGCGATGG-3′) were used to produce *ideR* template 2. Finally, for the *sigC* promoter region, Cinv10 (5′-GG GGAGATCGACCGAATGTC-3′) and Cinv12 (5′-ACAACCTTGCCGGCCG GAGC-3′) were used to generate *sigC* template 1, and Cinv10 with Cinv14 (5′-CTGGTAGCGATGGCAATGCTG-3′) were used to generate *sigC* template 2. In vitro transcription assays were performed as previously described (39). Briefly, 2 pmol of *E. coli* RNA core polymerase (Epicenter) was incubated with 20 pmol of purified sigma factor protein for 30 min in transcription assay buffer (10 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.25 μg of bovine serum albumin/μl) and then 0.09 μg of template DNA was added and incubated with 0.25 mM ATP, GTP, UTP, and [^{32}P]CTP. The transcription mixture was subjected to 6% denaturing polyacrylamide gel electrophoresis and developed on film (Kodak BioMax MR).

Macrophage infections. Alveolar macrophage J774A.1 cells were cultivated in cRPMI (2 mM glutamine, 10% fetal bovine serum) and activated by gamma interferon (500 U/ml) for 12 h, followed by lipopolysaccharide (200 ng/ml) for 3 h before infection. After the macrophages were washed three times with cRPMI, 10^5 bacteria were used to infect macrophage cultures at an MOI of 1:1. After 2 h of infection, macrophages were washed five times with RPMI media and cultivated with cRPMI with 5% CO_2 at 37°C. Macrophages were harvested, washed three times with RPMI, and then lysed by using 0.1% Triton X-100 in PBS at each time point (2 h, 3 days, and 6 days after infection). Th macrophage lysate was plated on 7H10 plates for CFU counting.

RESULTS

Conditionally inducible *sigB*- and *sigF*-expressing KI strains. The entire *sigB* and *sigF* genes were introduced downstream of the acetamidase promoter and regulatory cassette to yield *sigB*-inducible and *sigF*-inducible recombinant *M. tuberculosis* strains, respectively. The identity of these constructs was confirmed by PCR and sequencing. Quantitative RT-PCR was used to confirm upregulation of *sigB* and *sigF* at 6 and 12 h after acetamide exposure. The data obtained from real-time PCR experiments was normalized to *sigA* expression levels. In these experiments, *sigA* expression remained constant in all strains and under all conditions. Using real-time RT-PCR, we found *sigB* expression was increased (23.7 ± 9.1)-fold and (17.9 ± 4.5)-fold at 6 and 12 h after induction, respectively, and *sigF* was increased (12.7 ± 5.5)-fold and (62.7 ± 17)-fold at 6 and 12 h after induction, respectively.

Using these KI strains, we examined the possibility that *sigB* or *sigF* may control in vitro growth of *M. tuberculosis*. Sigma factor expression was induced with 0.2% acetamide, and the growth of each sigma factor-inducible strain was compared to that of the corresponding control strain containing only the acetamidase promoter and regulatory cassette region. Overexpression of *sigB* or *sigF* did not alter *M. tuberculosis* in vitro growth rates (see Fig. S2 in the supplemental material), nor did colony morphology differ in the recombinant strains when these were plated on acetamide-containing 7H10 plates (data not shown).

Next, we examined the relative expression of *M. tuberculosis* sigma factor genes after induction of *sigB* and *sigF*. Although the majority of genes encoding sigma factors were not differentially regulated after overexpression of these two genes, the expression of *sigC* was consistently found to be increased after *sigF* induction (Fig. 1C). Specifically, *sigC* was upregulated by (1.8 ± 0.9)-fold and (8.3 ± 2.2)-fold at 6 and 12 h, respectively, after overexpression of *sigF*. Consistent with these gene expression data, the previously identified *sigF* promoter recognition sequence AGTTTG-N_{15}-GGGTTT was identified in the 5′ untranslated region (UTR) of the *sigC* gene (Rv2069) (Fig. 2C). Overexpression of *sig*B did not significantly alter expression of other genes encoding sigma factors (Fig. 1B).

***M. tuberculosis* global gene expression profile after *sigB* overexpression.** To further characterize the *sigB* regulon, global gene expression analysis was performed using the *sigB*-inducible strain. Mycobacterial RNA was isolated at 12 h after acetamide induction. The microarray data were normalized to total intensity of spots and *sigA* expression. Table 1 shows genes found to be significantly increased (≥4-fold, Q value of <0.05) after *sigB* overexpression. Genes found to be highly upregulated include those encoding proteins involved in cell wall processes, PE-PPE family genes, as well as essential genes, such as 50S ribosomal proteins (Rv0709, Rv0701, Rv0641, and Rv2904c), and the chaperonin proteins *groEL2* (Rv0440) and

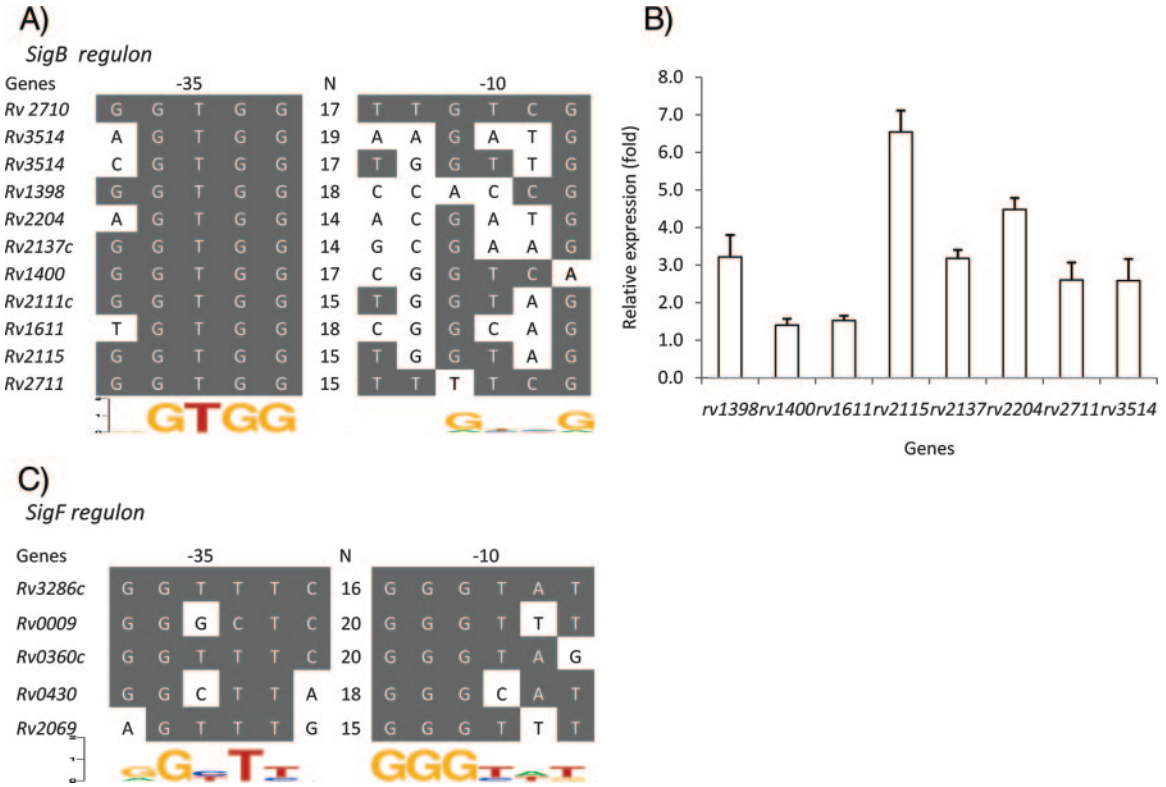

FIG. 2. Putative promoter consensus recognition sequences of SigB and SigF and confirmation of putative SigB-dependent genes by real-time RT-PCR analysis after *sigB* induction in the *M. tuberculosis sigB* KI strain. (A) The SigB putative promoter consensus recognition sequence was identified by analyzing the microarray data from Table 1. Genes found to be upregulated after *sigB* induction were collected and aligned to determine the *sigB* promoter consensus sequence. (B) Genes found by microarray to have a putative SigB-dependent promoter were evaluated by real-time RT-PCR for their level of expression before and after *sigB* KI expression. RNA was obtained 12 h after *sigB* induction in the *M. tuberculosis sigB* KI strain. The relative expression values shown are the ratios of postinduction RT-PCR signal normalized to the preinduction levels. The primers used in this experiment are described in Table S1 in the supplemental material. (C) The SigF putative promoter consensus recognition sequence identified in the present study by *sigF* KI overexpression, as well as those of genes shown to be regulated by SigF in previous studies, were also aligned. The SigF promoter recognition sequence was identified in the 5′ UTR region of the *sigC* gene (Rv2069). SigB and SigF promoter consensus sequences were derived using weblogo software (http://weblogo.berkeley.edu/logo.cgi).

groES (Rv3418c). In addition, *sigB* induction led to increased expression of *esxB* (Rv3874, 10-kDa culture filtrate antigen), which encodes a major secreted antigen, as well as *ideR*, which is located downstream of the *sigB* gene.

In order to identify putative *sigB*-dependent promoter sequences, genes found to be upregulated by *sigB* were aligned, and their 5′ UTRs were compared. First, we examined the 5′ UTR sequences of upregulated genes encoding hypothetical proteins that were not in apparent operons, such as Rv3514, Rv1398c, Rv2204c, Rv2137c, Rv2115, Rv2111c, *lipI* (Rv1400c), Rv1611, and *ideR* (Rv2711). A putative SigB promoter recognition sequence was identified as NGTGG-$N_{14\text{-}18}$-NNGNNG (Fig. 2A). Interestingly, this promoter recognition motif was also identified upstream of the *sigB* gene itself, suggesting that SigB may be autoregulatory. Real-time RT-PCR was used to confirm upregulation of genes containing the SigB promoter consensus sequence after *sigB* overexpression (Fig. 2B).

In vitro transcription assays. Microarray and quantitative RT-PCR data suggested that the *sigC* gene might be transcribed by a SigF-containing RNA polymerase (RNAP) and that *ideR* might be transcribed by a SigB-containing RNAP. To verify that SigB and SigF could specifically recognize and initiate transcription at the promoter regions of *ideR* and *sigC*, respectively, we performed in vitro transcription assays. The SigB and SigF proteins were overexpressed in *E. coli* and purified by nickel affinity chromatography as shown in Fig. S3 in the supplemental material. DNA templates (200 to 300 bp) containing the putative promoter regions of *sigB*, *ideR*, and *sigC* were prepared by PCR amplification. DNA templates were incubated with RNAP holoenzyme (with corresponding sigma factor) or RNAP core enzyme alone (with no sigma factor). As shown in Fig. 3A (lanes 4, 5, and 6), the presence of SigB was required for transcription of *ideR*, resulting in approximately 200- and 273-nucleotide transcripts using *ideR* template 1 and *ideR* template 2, respectively.

We also investigated the possibility that *sigB* is autoregulated, since we identified the SigB promoter consensus motif (Table 1 and Fig. 2A) in the *sigB* upstream promoter region. As shown in Fig. 3A (lanes 1, 2, and 3), the addition of SigB resulted in a *sigB* transcript measuring about 179 and 253 nucleotides, using *sigB* template 1 and *sigB* template 2, respectively, as expected. These results, together with the gene expression data, indicate that *sigB* is autoregulatory and

TABLE 1. Microarray analysis of *M. tuberculosis* genes found to be upregulated after *sigB* KI expression

Code and gene (or locus tag)	Rv no.	Avg	SD	Q value	Description
Virulence, detoxification, and adaptation					
groEL2	Rv0440	4.4	0.7	0.02	60-kDa chaperonin 2
groES	Rv3418c	4.2	3.5	0.03	10-kDa chaperonin
Lipid metabolism					
pgsA	Rv2612c	21.8	0.6	0.00	Phosphatidylinositol synthase
fadD31	Rv1925	18.3	5.6	0.00	Acyl-coenzyme A ligase
kasA	Rv2245	6.4	1.5	0.01	3-Oxoacyl-[acyl-carrier protein] synthase 1
pgsA2	Rv1822	4.1	0.9	0.01	Phosphatidyltransferase
ppsD	Rv2934	4.1	0.3	0.01	Phenolphthiocerol synthesis type-I polyketide synthase
Information pathways					
sigB	Rv2710	56.2	7.2	0.00	RNA polymerase sigma factor
dinP	Rv3056	6.3	0.9	0.01	DNA-damage-inducible protein P
dinF	Rv2836c	5.6	0.9	0.01	DNA-damage-inducible protein
rpmC	Rv0709	5.5	1.4	0.01	50S ribosomal protein l29
tuf	Rv0685	5.5	0.7	0.01	Iron-regulated elongation factor
nrdH	Rv3053c	5.4	0.1	0.00	Glutaredoxin electron transport component of nrdef
rplC	Rv0701	4.8	0.7	0.01	50S ribosomal protein l3
rplA	Rv0641	4.8	1.6	0.00	50S ribosomal protein
rplS	Rv2904c	4.8	1.5	0.01	50S ribosomal protein l19
greA	Rv1080c	4.2	0.9	0.02	Transcription elongation factor
Cell wall and cell processes					
esxB	Rv1398c	27.4	2.2	0.01	10-kDa culture filtrate antigen
Rv2625c	Rv2625c	7.2	2.5	0.00	Conserved transmembrane alanine and leucine-rich protein
Rv0463	Rv0463	7.2	0.7	0.00	Conserved membrane protein
Rv1792	Rv1792	7.1	3.7	0.01	ESAT-6 like protein
wag31	Rv2145c	4.7	0.7	0.01	Conserved hypothetical protein
Rv2115	Rv2115	4.7	0.2	0.01	ATPase
secE	Rv0638	4.7	0.5	0.01	Preprotein translocase
mmpS3	Rv2198c	4.6	0.8	0.01	Conserved membrane protein
Rv1410c	Rv1410c	4.3	1.4	0.02	Aminoglycosides
yjcE	Rv2287	4.3	0.2	0.00	Conserved integral membrane transport protein
Rv0559c	Rv0559c	4.2	0.4	0.01	Conserved secreted protein
Rv1845c	Rv1845c	4.1	0.4	0.01	Conserved hypothetical transmembrane protein
Rv2446c	Rv2446c	4.1	0.4	0.00	Conserved integral membrane protein
Rv2206	Rv2206	4.1	0.8	0.00	Conserved transmembrane protein
Rv2136c	Rv2136c	4.0	0.8	0.00	Conserved transmembrane protein
Rv0383c	Rv0383c	4.0	0.7	0.01	Conserved secreted protein
secF	Rv2586c	4.0	1.1	0.00	Protein-export membrane protein
Insertion sequences and phages					
Rv2978c	Rv2978c	4.2	0.3	0.00	Transposase
PE/PPE					
PE_PGRS	Rv3514	49.4	4.6	0.00	PE PGRS family protein
PE_PGRS	Rv3508	11.9	1.5	0.01	PE PGRS family protein
PE_PGRS	Rv2591	9.6	0.8	0.00	PE PGRS family protein
PPE	Rv0442c	5.5	0.1	0.01	PPE family protein
PE_PGRS	Rv0872c	4.9	2.2	0.02	PE PGRS family protein
PPE	Rv3136	4.8	1.7	0.01	PPE family protein
PE_PGRS	Rv2490c	4.7	0.3	0.00	PE PGRS family protein
PE_PGRS	Rv1818c	4.2	1.1	0.01	PE PGRS family protein
PPE	Rv3022c	4.1	0.2	0.00	PPE family protein
PE_PGRS	Rv2162c	4.1	0.8	0.00	PE PGRS family protein
Intermediary metabolism and respiration					
lipI	Rv1400c	10.1	1.5	0.01	Lipase
cbhK	Rv2202c	6.8	2.9	0.00	Carbohydrate kinase
nadB	Rv1595	6.1	0.4	0.00	L-Aspartate oxidase
trpC	Rv1611	5.8	0.4	0.00	Indole-3-glycerol phosphate synthase

Continued on following page

TABLE 1—*Continued*

Code and gene (or locus tag)	Rv no.	Avg	SD	Q value	Description
ctaC	Rv2200c	5.3	1.3	0.01	Transmembrane cytochrome *c* oxidase
nadC	Rv1596	5.0	1.2	0.01	Nicotinate-nucleotide pyrophosphatase
cysS2	Rv2130c	4.8	1.0	0.01	Glucopyranoside ligase
lldD2	Rv1872c	4.6	1.1	0.02	L-Lactate dehydrogenase
lipN	Rv2970c	4.5	0.9	0.01	Lipase/esterase
Rv2850c	Rv2850c	4.2	0.1	0.01	Magnesium chelatase
aroG	Rv2178c	4.1	1.0	0.00	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
ribC	Rv1412	4.1	0.8	0.01	Riboflavin synthase alpha chain
prcB	Rv2110c	4.0	1.7	0.02	Proteasome (β-subunit)
Unknown					
Rv1046c	Rv1046c	4.1	2.7	0.01	Hypothetical protein
Regulatory proteins					
whiB2	Rv3260c	4.0	2.1	0.01	Transcriptional regulatory protein
Conserved hypotheticals					
Rv2204c	Rv2204c	18.2	5.0	0.00	Conserved hypothetical protein
Rv2137c	Rv2137c	15.6	7.2	0.01	Conserved hypothetical protein
Rv2111c	Rv2111c	10.2	2.8	0.01	Conserved hypothetical protein
Rv1827	Rv1827	7.0	1.1	0.00	Conserved hypothetical protein
Rv2182c	Rv2182c	6.9	1.2	0.00	1-Acylglycerol-3-phosphate *O*-acyltransferase
Rv2974c	Rv2974c	4.3	0.3	0.01	Conserved hypothetical alanine rich protein
Rv2132	Rv2132	4.2	0.5	0.01	Conserved hypothetical protein
Rv1211	Rv1211	4.2	1.4	0.01	Conserved hypothetical protein
Rv0863	Rv0863	4.1	1.8	0.02	Conserved hypothetical protein
Rv1429	Rv1429	4.0	0.2	0.01	Conserved hypothetical protein
Rv0637	Rv0637	4.0	0.7	0.02	Conserved hypothetical protein
Rv0750	Rv0750	4.0	0.8	0.01	Conserved hypothetical protein

[a] The *sigB* gene was overexpressed using acetamide-inducing promoter at an optical density at 600 nm of ~0.5, and cells were harvested after 12 h. Code (i.e., the functional categories) and gene annotation data were obtained from the TubercuList database (http://genolist.pasteur.fr/TubercuList/). The Q value was calculated by using SAM software (http://www-stat.stanford.edu/~tibs/SAM/) with 1% FDR. We performed the real-time RT-PCR on the subset of genes to confirm microarray results (Fig. 2B).

also transcribes the *ideR* gene, which encodes a major iron regulator.

The *sigF* promoter recognition sequence has been reported to be NGNTtg-N_{14-18}-GGGTAt (40). This sequence is located upstream of the *rsbW-sigF* operon. Consistent with our real-time RT-PCR results, we identified a putative SigF consensus sequence in the 5′ UTR region of the *sigC* gene. In vitro transcription assays demonstrated that *sigC* has a SigF-dependent promoter (Fig. 3A, lanes 7, 8, and 9). The putative *sigC* promoter was identified as AGTTTG-N_{15}-GGGTTT (the consensus sequence is underlined) 46 bp upstream of the ATG start codon of the *sigC* gene.

Macrophage infection. We found that most genes regulated by SigB and SigF encoded secreted antigens or proteins involved in the cell envelope (Table 1) (40). However, the specific regulons of these sigma factors differ significantly. To directly compare the physiological effects of transcriptional activation of each of these regulons, we tested the growth of the *sigB* and *sigF* KI strains during macrophage infection. The bacteria were incubated with J774A.1 macrophages at a multiplicity of infection of 1:1, and the bacteria were counted 2 h, 3 days, and 6 days after infection. In these experiments, KI expression was not specifically induced by addition of exogenous acetamide. However, an RT-PCR study showed that baseline *sigB* and *sigF* expressions in the KI strains were (10.1 ± 3.4)- and (3.5 ± 1.2)-fold higher, respectively, than in the corresponding control strains due to the increased gene dosage and leakiness of the acetamide promoter system. Thus, even under acetamide-free conditions, these *M. tuberculosis* recombinants overexpress *sigB* and *sigF*. Interestingly, the *sigB* KI strain showed a growth defect in macrophages, since it failed to replicate at the same rate as the control strain (Fig. 4). Specifically, at 3 and 6 days after infection, the normalized CFU counts in the *sigB* KI strain were 0.6 and 1.4 log units lower than those of the control strain, both of which were significant ($P = 0.029$ and 0.04, respectively). Likewise, as shown in Fig. 4, the normalized CFU counts displayed a reduced growth rate (slope) for the *sigB*-overexpressing strain compared to the control strain at both the early and the late time intervals of the intracellular infection. The CFU counts for the *sigF*-overexpressing strain also indicated a slowed intracellular growth rate; however, the magnitude of the rate reduction was less than that of the *sigB*-overexpressing strain and did not achieve statistical significance (Fig. 4).

DISCUSSION

Bacterial sigma factors are classified as principal and alternative sigma factors. Principal sigma factors are essential for bacterial survival, while alternative sigma factors are nonessen-

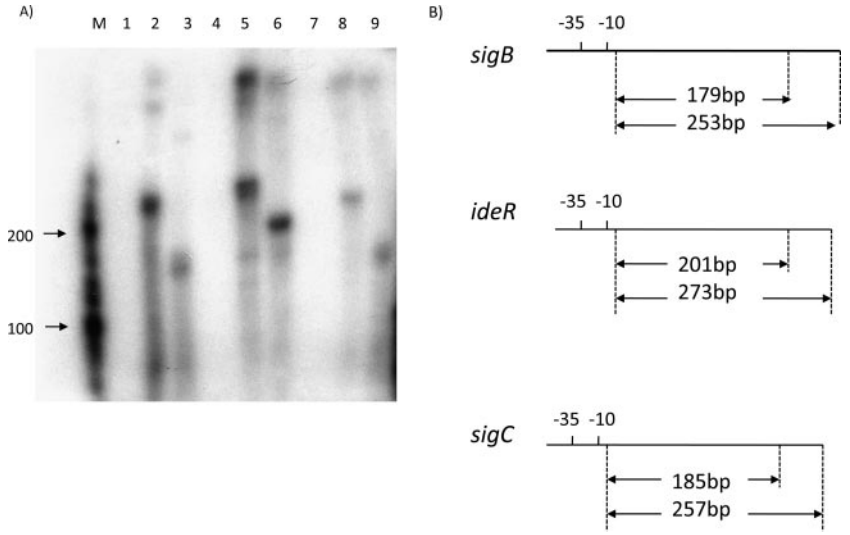

FIG. 3. In vitro transcription assays using purified SigB and SigF sigma factors. (A) In vitro transcription assay using sigma factor protein and DNA template involving the 5′ UTRs of the *sigB*, *ideR*, and *sigC* genes. Lane M, RNA size markers (Ambion). All lanes correspond to complete in vitro transcription reaction mixes with the presence or absence of sigma factor and the presence of either long (template 2) or short (template 1) promoter-containing target templates. Lanes: 1, no added SigB/+*sigB* long template; 2, SigB added/+*sigB* long template; 3, SigB added/+*sigB* short template; 4, no added SigB/+*ideR* long template; 5, SigB added/+*ideR* long template; 6, SigB added/*ideR* short template; 7, no SigF added/+*sigC* long template; 8, SigF added/+*sigC* long template; 9, SigF added/+*sigC* short template. (B) Diagram of expected lengths of transcript products from the short and long templates. The transcription start site of *sigB* was previously identified (19).

tial and mediate the adaptive response of bacteria to the extracellular environment. The extracytoplasmic function sigma factors comprise a large cluster of alternative sigma factors that regulate cell wall, periplasmic, and secretion-associated functions. These proteins are of interest as potential regulators of virulence factors in bacterial pathogens. For example, a stationary-phase alternative sigma factor, RpoS, plays a role in quorum sensing in the gram-negative bacterium *Vibrio* sp., and sigma factors such as SigH play a role in cell-to-cell communication in *Bacillus subtilis* and *Streptococcus pneumoniae* (23).

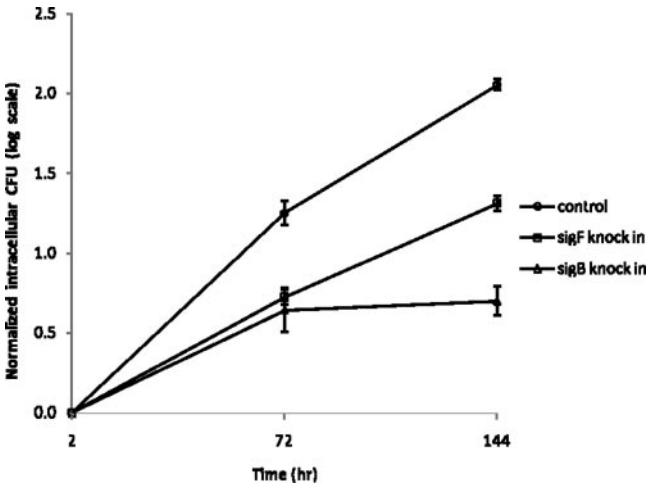

FIG. 4. Intracellular growth of *M. tuberculosis sigB* and *sigF* KI strains in activated J774A.1 macrophages in vitro. Murine macrophage J774A.1 cells were activated with gamma interferon and lipopolysaccharide (as in Materials and Methods) and then infected with *M. tuberculosis* strains harboring the empty vector, the *sigB* KI vector, or the *sigF* KI vector at a multiplicity of infection of 1:1. After incubation for 2 h, the macrophage monolayer was extensively washed, and samples were taken to determine the initial intracellular CFU titer. The bacterial growth rate was determined by CFU counting of bacilli at 3 and 6 days after infection. The log CFU counts are normalized to the initial intracellular CFU counts.

Transcriptional regulation of the sigma factor network in *M. tuberculosis* is complex. Previous studies have suggested the presence of two different sigma factor-dependent promoters upstream of *sigB*, one recognized by a SigF-containing RNA polymerase and the other recognized by RNA polymerase containing SigE, SigH, or SigL (12). Recently, the two-component response regulator MprAB was found to regulate expression of SigB and SigE but not of SigH (17). Interestingly, Triton X-100 treatment induced the expression of *sigB*, but not of *sigE*, in an *mprA* deletion mutant, suggesting independent regulation of the SigB, SigE, and SigH regulons. Evidence that *sigC* may be regulated by SigF was provided by the observation that *sigC* was downregulated in an *M. tuberculosis sigF*-deficient mutant during stationary phase (14); however, conclusive data that the *sigC* promoter is SigF dependent are lacking.

In the present study, we explored the roles of SigB and SigF in the *M. tuberculosis* sigma factor network. We performed an experimental and computational approach to identify SigB- and SigF-regulated sigma factors by overexpressing *sigB* and *sigF* in *M. tuberculosis*, respectively. Previous studies showed that the chemically regulated promoter P_{ace} is useful to study gene expression in *M. tuberculosis* (1, 26, 40). In the present study, we used this inducible promoter system to characterize genes regulated by SigB and SigF in *M. tuberculosis.*

Importantly, our microarray and real-time RT-PCR data revealed that the relative expression of other sigma factors was unchanged after *sigB* induction, suggesting that, at least during exponential growth, SigB is an end regulator of at least one branch of the sigma factor network in *M. tuberculosis* (12). In

addition to previous work (12), we found that SigB-containing RNA polymerase is capable of transcribing *sigB*, suggesting that SigB may auto-amplify its expression under certain conditions.

Overexpression of *sigB* resulted in induction of many genes encoding secreted antigens and cell wall-associated proteins, including Rv1925, which encodes an acyl-coenzyme A-synthase, and Rv2612e, which encodes phosphatidylinositol synthase, both of which contribute components for membrane synthesis. In addition, *sigB* induction led to increased expression of many genes encoding PE/PPE family proteins, several members of which have been localized to the cell membrane and shown to influence mycobacterium-host cell interactions (2, 6). In particular, the gene product of Rv1818c (PE-PGRS 33) was shown to induce strong $CD8^+$ cytotoxic lymphocyte and Th1-type responses, with high levels of gamma interferon and low levels of interleukin-4 in mice (8). Differential regulation of PE and PPE proteins has been described previously for the *M. tuberculosis* alternative RNA polymerase sigma factors SigF (16, 40) and SigL (16), suggesting that these sigma factors may indirectly regulate PE/PPE genes through the regulation of *sigB*.

We also found that expression of the *ideR* gene (Rv2711), which is located immediately downstream of *sigB* (Rv2710), was increased after *sigB* induction; however, relative induction of *ideR* (2.6-fold as determined by real-time RT-PCR) was lower than that of *sigB* (17-fold as determined by real-time RT-PCR). Previous data suggested that *sigB* and *ideR* are not cotranscribed in *Mycobacterium* sp. (37), a finding consistent with our data showing distinct SigB-dependent promoters upstream of each of these genes. The *sigB* promoter consensus recognition sequence NGTGG-N_{14-19}-NNGNNG is found 14 bp upstream of the previously identified transcription start site of the *sigB* gene (19) as GGTGG-N_{17}-TTGTCG and in the 5′ UTR of the *ideR* gene as GGTGG-N_{15}-TTGTCG. This sequence also resembles the previously described promoter consensus recognition sequences for SigE (TGGGAAC-N_{17}-CGTTA) (28) and SigH (TGGGAA-N_{18}-CGTTA) (33). Of note, the trinucleotide TGG in the −35 region appears to be highly conserved in the upstream promoter region of all SigB-regulated genes in the present study and is also seen in the SigE- and SigH-dependent promoters.

Interestingly, principal-like sigma factors, such as SigB, are not present in *E. coli*, *Bacillus* sp., or *Vibrio* sp. (32). On the other hand, *Streptomyces coelicolor* has three principal-like sigma factors, HrdA, HrdC, and HrdD, whose functions remain unclear but which are not essential for growth (15). Although SigB contains significant amino acid sequence similarity to the C-terminal portion of SigA, it appears to be dispensable for growth in *M. smegmatis* and *M. tuberculosis* (31, 35). In *M. tuberculosis*, *sigB* is induced after exposure to heat shock and oxidative stress, which appears to be mediated by SigH (29), as well as in response to SDS-induced surface stress, which appears to be mediated by SigE (28). Consistent with these findings, unpublished data suggest that a *M. tuberculosis* mutant deficient in SigB is more sensitive to SDS-induced surface stress, heat shock, and oxidative stress (35; I. Smith, personal communication), suggesting that SigB may play a role in the general stress response of *M. tuberculosis*. Overexpression of *M. tuberculosis sigB* in *M. smegmatis* results in a prolonged generation time and markedly altered colony morphology, which has been attributed to the constitutive production of surface hyperglycosylated polar glycopeptidolipids (31). These molecules, which are unique to *M. smegmatis* and *M. avium* species, are usually produced in response to carbon starvation, suggesting a role for SigB in mycobacterial adaptation to nutrient-limited conditions. However, overexpression of *sigB* in *M. tuberculosis* in our study did not lead to altered growth characteristics or colony morphology, suggesting divergent roles for SigB in the two different mycobacterial species. Although unpublished data reported that SigB is not required for normal *M. tuberculosis* growth in human macrophages (35), our study revealed a growth defect of *M. tuberculosis* overexpressing *sigB* in J774A.1 macrophages. This effect may be due to increased expression of *sigB* itself or because of increased expression of another gene directly or indirectly regulated by SigB.

We also studied the role of *M. tuberculosis* SigF in the sigma factor network using a conditionally inducible *sigF* recombinant strain. In contrast to previously published data that *M. tuberculosis sigB* may have a SigF-dependent promoter (12), we did not observe significant upregulation of *sigB* after overexpression of *sigF*. In addition, among the 73 genes that were upregulated after *sigB* induction (Table 1), only 5 were also upregulated after *sigF* induction (40), suggesting distinct regulons controlled by each of these sigma factors. We found that *sigC* was the only sigma factor gene whose expression was increased after *sigF* induction. Indeed, in vitro transcription assays showed that *M. tuberculosis* SigF is, in fact, capable of transcribing *sigC*, and we identified the putative SigF-dependent promoter sequence as AGTTT-N_{15}-GGGTTT (the consensus sequence is underlined) 46 bp upstream of the ATG start codon of the *sigC* gene. These data are consistent with microarray data indicating downregulation of *sigC* in a *sigF* knockout strain during stationary-phase growth (14) and strongly suggest that *sigC* expression is SigF dependent. Earlier studies of the *M. tuberculosis sigF* knockout mutant indicated that its gene product is important for bacterial survival and immunopathology in mice (14), although SigF does not appear to be required for *M. tuberculosis* proliferation within in human monocytes cultured in vitro (9). Consistent with the concept that coordinate expression of SigF-dependent genes is an important virulence mechanism, we observed a reduced intracellular growth rate in activated murine J774 macrophages by the recombinant *M. tuberculosis* KI strain that overexpresses *sigF* constitutively.

It will be important to define the degrees of transcriptional redundancy among the *M. tuberculosis* sigma factors and also determine how stress conditions affect the relative expression patterns of the SigB and SigF regulons—a group of genes that are likely to play important roles in pathogenesis.

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