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# A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*

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ABSTRACT **Alternative RNA polymerase sigma factors are a common means of coordinating gene regulation in bacteria. Using PCR amplification with degenerate primers, we identified and cloned a sigma factor gene, *sigF*, from *Mycobacterium tuberculosis*. The deduced protein encoded by *sigF* shows significant similarity to SigF sporulation sigma factors from *Streptomyces coelicolor* and *Bacillus subtilis* and to SigB, a stress-response sigma factor, from *B. subtilis*. Southern blot surveys with a *sigF*-specific probe identified cross-hybridizing bands in other slow-growing mycobacteria, *Mycobacterium bovis* bacille Calmette–Guérin (BCG) and *Mycobacterium avium*, but not in the rapid-growers *Mycobacterium smegmatis* or *Mycobacterium abscessus*. RNase protection assays revealed that *M. tuberculosis sigF* mRNA is not present during exponential-phase growth in *M. bovis* BCG cultures but is strongly induced during stationary phase, nitrogen depletion, and cold shock. Weak expression of *M. tuberculosis sigF* was also detected during late-exponential phase, oxidative stress, anaerobiasis, and alcohol shock. The specific expression of *M. tuberculosis sigF* during stress or stationary phase suggests that it may play a role in the ability of tubercle bacilli to adapt to host defenses and persist during human infection.**

Initial human infection with *Mycobacterium tuberculosis* leads to primary active disease in 5–40% of adults (1–3). Most healthy individuals mount an effective immune response causing the organism to enter a latent state. *M. tuberculosis* may remain dormant within the human host for decades, with subsequent reactivation later in life. It is estimated that one-third of the world's population is latently infected (4).

Our understanding of *M. tuberculosis* during latency is limited. The metabolic and structural changes occurring within latent bacilli and the underlying genetic control of latency are essentially unknown. Claims of non-acid-fast forms of tubercle bacilli in persistent human infection have remained controversial for nearly a century (5, 6).

An improved understanding of the latent state might have a profound impact on tuberculosis prophylactic therapy. Current antimycobacterial agents are most effective against actively dividing organisms; lengthy courses of antibiotics are, therefore, required to treat patients carrying dormant bacilli. If mechanisms for blocking the development of latency can be devised, the time necessary for treatment might be shortened with both considerable increases in patient compliance and decreases in cost.

Alternative sigma factors have been shown to mediate adaptive responses to environmental conditions in many bacterial species (7, 8). We postulated that entry of *M. tuberculosis* into a latent state might also be under the influence of alternative sigma factors. We employed PCR amplification using degenerate primers to identify an *M. tuberculosis* sigma factor¶ which is similar to stress and sporulation sigma factors in other species and is induced by starvation and stress.

## MATERIALS AND METHODS

**Strains and Plasmids.** pYZ99 is pUC18 containing a 2.8-kb *Bam*HI fragment of *M. tuberculosis* genomic DNA. pCK1845 is pCRII (Invitrogen) containing a 279-bp *Eco*RI/*Kpn* I subclone of the *M. tuberculosis sigF* gene with an SP6 promoter site and a *Bam*HI site at the 5′ end of the *sigF* gene fragment and a T7 promoter site and an *Eco*RV site at the 3′ end. Recombinant plasmids were constructed and used to transform *Escherichia coli* DH5$\alpha$ by electroporation using standard protocols (9), and they were isolated and purified by using the Qiagen system (Qiagen, Chatsworth, CA).

**PCR.** Degenerate primers Y207 (5′-AACCTGCGHCTSGTSGTC-3′, a forward primer for the hexapeptide NLRLVV) and Y208 (5′-CTGNCGKATCCACCASGTSGCRTA-3′, a reverse primer for the octapeptide YATWWIRQ) were used to amplify sigma factor gene fragments from *M. tuberculosis* genomic DNA in standard PCRs with *Taq* DNA polymerase (GIBCO/BRL): 30 cycles, 94°C for 60 sec, 54°C for 90 sec, and 72°C for 120 sec. PCR products were cloned and used as probes to select genomic clones from an *M. tuberculosis* H37Rv cosmid library (kindly provided by K. de Smet, St. Mary's Hospital Medical School, London).

**Sequencing.** DNA sequencing was performed with an Applied Biosystems 373 automated DNA sequencer, using dye terminator chemistry, at the Biopolymer Laboratory of the Howard Hughes Medical Institute at The Johns Hopkins University School of Medicine.

**SigF Homologues in Other Mycobacterial Species.** Southern blots were made from *Pvu* II-digested mycobacterial genomic DNA obtained from clinical isolates kindly provided by J. Dick (Johns Hopkins School of Medicine). The blots were probed with a 221-bp *M. tuberculosis*-specific probe (base pairs 438–659) according to a previously published protocol (10). Hybridizations were performed overnight at 55°C and were followed by five washes in 3× SSC at 45°C.

**Mycobacterial Cultures.** Early-exponential, late-exponential, and stationary-phase bacille Calmette–Guérin (BCG, Pasteur strain, ATCC 35734) cultures were grown in standard Middlebrook 7H9 broth (Difco) supplemented with albumin dextrose complex and Tween 80 (ADC-TW; ref. 11) at 37°C with constant shaking. For cold shock, logarithmic-phase cultures ($OD_{600}$ = 0.78) were placed at 4°C for 24 hr prior to harvesting. To test other stress conditions, logarithmic-phase cultures were centrifuged and resuspended in a stress broth at 37°C with shaking for 24 hr. Stress broths consisted of Middle-

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Abbreviations: BCG, bacille Calmette–Guérin; RPA, RNase protection assay.
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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U41061).

brook 7H9-ADC-TW plus 10 mM $H_2O_2$ (oxidative stress) or 5% (vol/vol) ethanol (alcohol stress). Nitrogen-depleted medium was Middlebrook 7H9 containing only 10% of the standard amounts of glutamine and $NH_4Cl$. Microaerophilic cultures were prepared according to the settling method described by Wayne (12) for 7 days.

**RNA Extraction and Quantification.** Mycobacterial pellets were resuspended in extraction buffer (0.2 M Tris·HCl, pH 8.0/0.5 M NaCl/0.01 M EDTA/1% SDS) plus an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol). A 0.4-g portion of 300-$\mu$m prewashed glass beads (Sigma) was added and the samples were mixed in a Vortex mixer for 2 min at high speed. After a brief centrifugation, the aqueous phase was removed, reextracted with phenol/chloroform/isoamyl alcohol, and finally extracted with chloroform/isoamyl alcohol (24:1, vol/vol). The purified RNA was precipitated with ethanol and quantified by $A_{260}$ measurement. Specific mRNA levels were determined by RNase protection assay (RPA; ref. 13) using a $^{32}$P-labeled, *in vitro* transcribed, *sigF* antisense RNA probe derived from *Bam*HI-cut pCK1845 (Maxiscript system; Ambion, Austin, TX). Control nonlabeled *sigF* sense RNA was produced by using the same DNA template cut with *Eco*RV, transcribed in the opposite direction. For each assay equal quantities of total mycobacterial RNA were tested.

## RESULTS

**PCR with Degenerate Sigma-70 Consensus Primers Successfully Identifies an *M. tuberculosis* Sigma Factor Gene, *sigF*.** Analysis of bacterial sigma factors reveals considerable conservation in regions 2.1–2.4 and 4.1–4.2 (14). Region 2.1 is implicated in core polymerase binding, while the 2.3/2.4 and 4.2 regions are believed to contact the −10 and −35 regions, respectively, of the promoter DNA consensus sequence (15). We designed degenerate primers Y207 and Y208 directed toward conserved regions 2.1 and 2.3, respectively, and used them to amplify sigma-like gene fragments from *M. tuberculosis* genomic DNA. These primers amplified several distinct products, including the anticipated 165-bp fragment. This 165-bp fragment was likely to consist of a mixture of sequences, since it hybridized strongly to two separate *M. tuberculosis Bam*HI fragments (4.8 and 2.8 kb) in Southern analysis. *E. coli* cosmid clones which hybridized with the 165-bp PCR product were selected by screening an *M. tuberculosis* H37Rv library, and the 2.8-kb *Bam*HI fragment was subcloned as pYZ99 from one of these cosmids. A restriction map of the 2.8-kb *Bam*HI fragment is shown in Fig. 1. Partial sequence analysis of the 4.8-kb *Bam*HI fragment showed identity with one of the sigma factor genes previously described from *M. tuberculosis* (16).

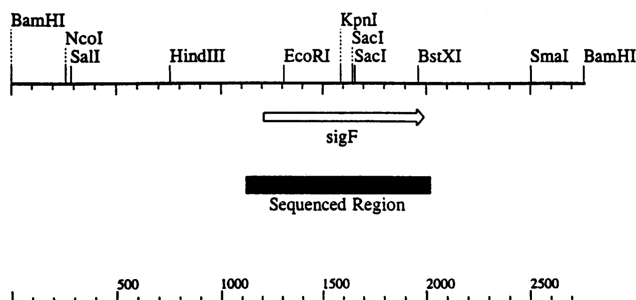


FIG. 1. Map of 2.8-kb *M. tuberculosis* DNA fragment containing *sigF*. The relative positions of restriction sites, the SigF open reading frame, and the 896-bp region which was sequenced are shown. Numbers along the bottom scale are bp.

**The Sequence of the *M. tuberculosis* Sigma Factor Gene *sigF*.** A combination of primer walking and subcloning of restriction fragments was used to determine the DNA sequence of 896 bp of pYZ99 which contains the sigma factor gene *sigF*, as shown in Fig. 2. Each base was sequenced an average of five times (minimum three, maximum eight). The sequence reveals a 261-aa open reading frame. The 88 bp of upstream sequence is not homologous to *E. coli* sigma-70 promoter consensus sequences, nor does it have a clear-cut Shine–Dalgarno ribosome-binding site with complementarity to the 3′ end of the *M. tuberculosis* 16S rRNA sequence (17). Nevertheless, the *sigF* gene is clearly transcribed in slow-growing mycobacteria (see below). Our assignment of the initiation codon is based on alignments with other known SigF-like proteins (see below) and the observation that GTG is commonly used as an initiation codon in mycobacteria (18).

**Homologues of SigF.** The 261-aa deduced protein encoded by *M. tuberculosis sigF* has significant homology to the known stress and sporulation-specific sigma factors from *Bacillus* spp. and *Streptomyces* spp. The closest similarities are to *Streptomyces coelicolor* SigF (41% identity and 62% similarity), *Bacillus subtilis* SigB (30% identity and 50% similarity), and *B. subtilis* SigF (26% identity and 44% similarity). An alignment of the deduced *M. tuberculosis* SigF protein sequence with these three other sigma factors is shown in Fig. 3. In addition, a partial SigF homologue is present in *Mycobacterium leprae* (accession no. U00012); frameshift sequencing errors in the *M. leprae SigF* sequence may explain the incompleteness of this open reading frame.

Southern blots of *Pvu* II-digested mycobacterial genomic DNA revealed *sigF* cross-hybridization in several slow-growing mycobacteria, including *Mycobacterium bovis* BCG (ATCC 35734) and clinical isolates of *Mycobacterium avium, Mycobacterium triviale*, and *Mycobacterium gordonae.* The rapid-growing species *Mycobacterium smegmatis* and *Mycobacterium abscessus* showed no hybridization by Southern blot analysis at intermediate stringency.

**Stress and Stationary-Phase Induction of *sigF* mRNA.** Transcription of *sigF* was detected and monitored under different growth conditions of BCG, a slow-growing attentuated *M. bovis* strain which is a member of the *M. tuberculosis* complex, by using an RPA (see Fig. 4). Our ability to protect a $^{32}$P-labeled *sigF* antisense RNA probe by using total RNA isolated from BCG in RPA analysis confirms that *sigF* is a transcribed gene in this close relative of *M. tuberculosis*. Replicate experiments showed that the RPA signal intensity results were reproducible to within 20% when performed with different batches of RNA on different days. The twin protected bands at 320 and 279 bases (Fig. 4) were observed consistently with the pCK1845-derived *sigF* antisense RNA probe. Secondary structure analysis of our probe reveals that about 40 bases of the vector sequence at its 3′ end are capable of forming a stem-loop which would protect a larger portion of the probe than the expected 279 bases. Only a 350-base protected species is observed when a nonlabeled sense-strand RNA complementary over 350 bases is added. Hence we believe that both the 320- and 279-base bands result from protection of the probe by *sigF* mRNA.

In BCG cultures, *sigF* transcription was most strongly induced during stationary phase ($OD_{600}$ = 2.7), nitrogen depletion, and cold shock. A weak RPA signal was present during late-exponential phase ($OD_{600}$ = 1.5), oxidative stress (10 mM $H_2O_2$), microaerophilic culture conditions, and alcohol shock (5% ethanol). No *sigF* mRNA was detected during early-exponential-phase growth ($OD_{600}$ = 0.67). The relative intensities of the RPA signals during different growth conditions are summarized in Table 1.

```
                                                                                 80
TCCAGACCTTCCACGACGGT CGCCAGCCCGATGTAGCCGG CAGTGTCTTCGGCATCACGT TGACCGCCCGACGGGCGGCA
                                                                             145/19
TCCAGCAG GTG ACG GCG CGC GCT GCC GGC GGT TCT GCA TCG CGA GCT AAC GAA TAC GCC GAC GTT
          M   T   A   R   A   A   G   G   S   A   S   R   A   N   E   Y   A   D   V>
                                                                             208/40
CCG GAG ATG TTT CGC GAG CTG GTT GGT TTG CCT GCC GGC TCA CCG GAA TTC CAG CGG CAC CGG
 P   E   M   F   R   E   L   V   G   L   P   A   G   S   P   E   F   Q   R   H   R>
                                                                             271/61
GAC AAG ATC GTT CAG CGG TGC TTG CCG CTG GCC GAT CAC ATC GCG CGG CGG TTC GAG GGT CGC
 D   K   I   V   Q   R   C   L   P   L   A   D   H   I   A   R   R   F   E   G   R>
                                                                             334/82
GGC GAA CCG CGT GAC GAC CTT ATT CAG GTC GCG CGG GTC GGG CTG GTC AAC GCC GCG GTT CGC
 G   E   P   R   D   D   L   I   Q   V   A   R   V   G   L   V   N   A   A   V   R>
                                                                            397/103
TTC GAC GTG AAG ACC GGG TCG GAC TTC GTC TCC TTC GCG GTT CCT ACC ATC ATG GGC GAG GTC
 F   D   V   K   T   G   S   D   F   V   S   F   A   V   P   T   I   M   G   E   V>
                                                                            460/124
CGA CGA CAC TTC CGC GAC AAC AGC TGG TCG GTC AAG GTT CCC CGG CGT CTC AAG GAA CTG CAT
 R   R   H   F   R   D   N   S   W   S   V   K   V   P   R   R   L   K   E   L   H>
                                                                            523/145
CTG CGG CTA GGT ACC GCC ACC GCC GAT TTG TCG CAG CGG CTC GGG CGG GCG CCG TCG GCA TCG
 L   R   L   G   T   A   T   A   D   L   S   Q   R   L   G   R   A   P   S   A   S>
                                                                            586/166
GAG CTC GCC GCG GAG CTC GGG ATG GAC CGC GCT GAG GTT ATC GAA GGT TTG CTG GCG GGT AGT
 E   L   A   A   E   L   G   M   D   R   A   E   V   I   E   G   L   L   A   G   S>
                                                                            649/187
TCC TAC CAC ACC TTG TCC ATC GAC AGC GGT GGC GGC AGC GAC GAC GAT GCC CGC GCA ATC ACA
 S   Y   H   T   L   S   I   D   S   G   G   G   S   D   D   D   A   R   A   I   T>
                                                                            712/208
GAC ACC CTG GGC GAC GTG GAT GCG GGT CTT GAC CAG ATC GAG AAT CGG GAG GTG CTT CGT CCG
 D   T   L   G   D   V   D   A   G   L   D   Q   I   E   N   R   E   V   L   R   P>
                                                                            775/229
TTG CTC GAG GCG TTG CCC GAG CGG GAA CGA ACG GTC TTG GTG CTC AGG TTC TTC GAC TCG ATG
 L   L   E   A   L   P   E   R   E   R   T   V   L   V   L   R   F   F   D   S   M>
                                                                            838/250
ACC CAA ACG CAG ATC GCC GAG CGC GTC GGT ATC TCA CAG ATG CAC GTG TCG CGG GTG CTG GCC
 T   Q   T   Q   I   A   E   R   V   G   I   S   Q   M   H   V   S   R   V   L   A>
                                        261                         896
AAG TCA TTG GCA CGG CTA CGG GAT CAG TTG GAG TAG CCGCCGGGCTTACTTGGATCTC
 K   S   L   A   R   L   R   D   Q   L   E   *
```

FIG. 2. DNA and deduced protein sequences of the *M. tuberculosis sigF* region. The 896 bp of *M. tuberculosis* DNA sequenced is shown along with the deduced protein sequence of SigF. Numbers at right correspond to nucleotide/amino acid positions.

## DISCUSSION

Sigma factors are subunits of bacterial RNA polymerase and confer promoter specificity to the holoenzyme complex. The unique affinity of each sigma factor for its promoter consensus sequence is an essential component in many gene regulation systems. For example, in *B. subtilis*, sporulation is regulated by a carefully coordinated cascade of alternative sigma factors and the genes which they control (7).

The structure and function of sigma factors are conserved across species, and these regions of conservation may be exploited to identify new sigma factors (20). We successfully employed PCR using degenerate primers based on conserved regions 2.1 and 2.3 to identify the *M. tuberculosis* sigma factor gene *sigF*.

*M. tuberculosis* SigF has closest homology to *S. coelicolor* SigF, *B. subtilis* SigF, and *B. subtilis* SigB. The *S. coelicolor sigF* gene encodes a late-stage, sporulation-specific sigma factor. *S. coelicolor sigF* knockout mutants are unable to sporulate effectively, producing deformed thin-walled spores (21). *B. subtilis sigF* is essential for early spore gene expression. It is not transcribed until shortly after the start of sporulation (22), and its protein product is specifically activated within the developing forespore after septation (23). The *B. subtilis sigB* gene encodes a stress-response sigma factor. While not an essential gene for growth or sporulation, *sigB* transcription is activated during stationary phase or under environmental stress, such as heat or alcohol shock (24, 25).

Lonetto *et al.* (14, 26) have divided the known sigma factors into a number of families on the basis of their primary structure homology patterns. The families include primary sigma factors, a sporulation-specific group, a heat shock-related group, a flagella-related group, and the newly recognized extracytoplasmic family. An important implication of these sequence homology clusters is that correlation between the primary structure and general function of bacterial sigma factors is preserved even across species barriers. The homology profile of *M. tuberculosis* SigF places it in the sporulation-specific family of such sigma factor classifications. This observation suggests that *M. tuberculosis* SigF has a functional role akin to the roles of the *S. coelicolor* and *B. subtilis* sigma factors to which it is similar.

*M. tuberculosis sigF*-like sequences were identified by Southern blot analysis in several slow-growing mycobacterial species, including *M. bovis* BCG and *M. avium. Mycobacterium leprae* was known prior to this study to possess a *sigF* homologue on cosmid B1308 (GenBank accession no. U00012). Rapid-growing species, such as *M. smegmatis* and *M. abscessus*, showed no *sigF* hybridization by Southern blotting. It is conceivable that the mycobacterial *sigF* gene might be associated with a developmental response unique to slow-growers. Alternatively, the absence of *sigF* cross-hybridization in the rapid-growing species may simply be a function of increased evolutionary distance and decreased DNA homology.

RPA using an *M. tuberculosis sigF*-specific probe showed that the *M. tuberculosis sigF* open reading frame is a transcribed

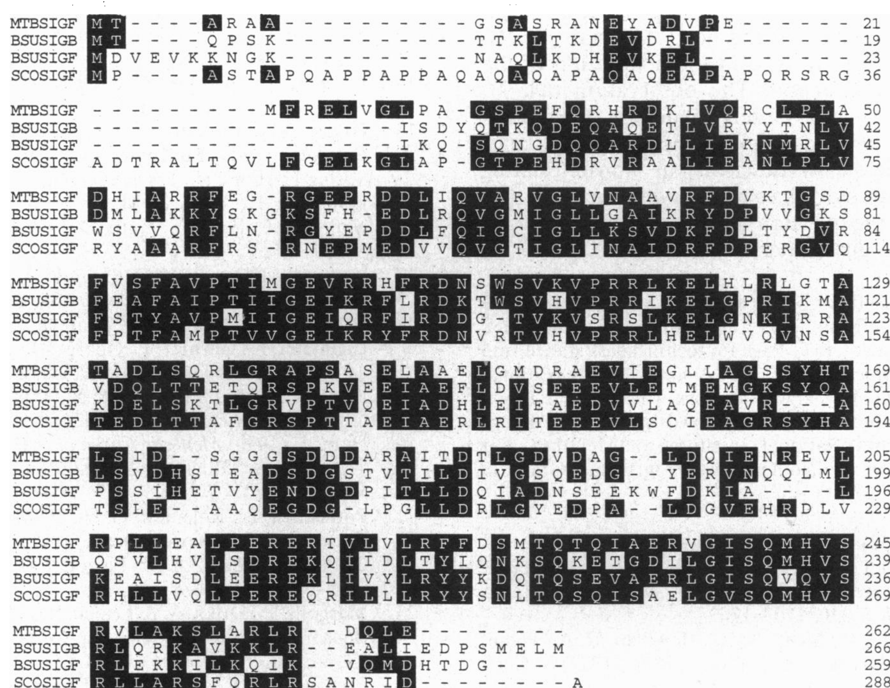


FIG. 3. Alignment of *M. tuberculosis* SigF with related sigma factors. The deduced amino acid sequence of *M. tuberculosis* SigF (MTBSIGF) is aligned with homologues by using the Hein method on the DNAStar MegAlign program (19). Black shading indicates amino acid similarity. The length of each polypeptide is shown by the numbers on the right. BSUSIGB = *B. subtilis* SigB (GenBank accession no. M13927), BSUSIGF = *B. subtilis* SigF (accession no. M15744), and SCOSIGF = *S. coelicolor* SigF (accession no. L11648).

gene. Transcription was maximal during stationary phase, cold shock, and nitrogen depletion. Weaker RPA signals were present during other stress conditions, such as oxidative stress, alcohol shock, and microaerophilic stress. No evidence of transcription was seen during exponential-phase growth. RPA is highly sensitive and can detect mRNA at the femtogram level (27). These findings show that the *M. tuberculosis sigF* gene encodes a stationary-phase/stress-response sigma factor. This pattern of induction is similar to that of the *B. subtilis sigB* gene.

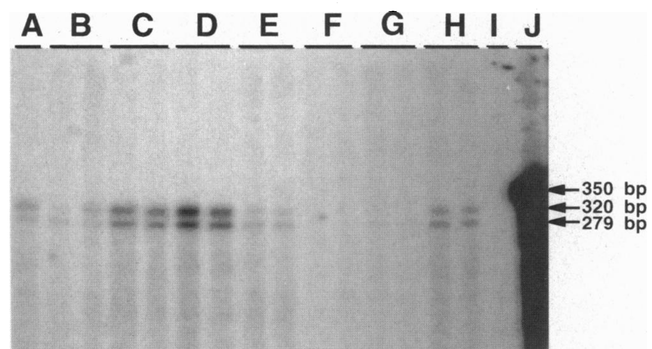


FIG. 4. RPA with RNA extracts from BCG exposed to different conditions. The figure shows an autoradiogram of RPA reaction products after liquid hybridization between total BCG RNA and the pCK1845-derived *sigF*-specific antisense RNA probe separated on a denaturing 5% polyacrylamide gel and exposed to x-ray film for 24 hr. Samples in lane pairs B–H were assayed in duplicate. RPA was performed upon equivalent amounts of total RNA from BCG cultures subjected to the following conditions: lane A, 10 mM $H_2O_2$; lane B, 5% ethanol; lane C, nitrogen depletion; lane D, cold shock; lane E, microaerophilic stress; lane F, early-exponential growth ($OD_{600}$ = 0.67); lane G, late-exponential growth ($OD_{600}$ = 1.5); lane H, stationary phase ($OD_{600}$ = 2.7). Control samples were: lane I, an *in vitro* transcribed noncomplementary probe (negative control), and lane J, an *in vitro* transcribed sense-strand *sigF* probe containing 350 complementary bases (positive control).

Both the *B. subtilis sigB* and *sigF* genes are transcribed as parts of polycistronic messages containing post-translational regulatory genes (28–32). The *sigB* operon encodes three other genes (*rsbV, rsbW,* and *rsbX*) which control SigB activation. The *B. subtilis sigF* operon encodes two other genes encoding an anti-sigma factor (SpoIIAB) and an anti-anti-sigma factor (SpoIIAA). The *S. coelicolor sigF* gene appears to be monocistronic (21). Analyzing the region upstream of *sigF* should allow evaluation of the possibility that the *M. tuberculosis* gene is post-translationally regulated.

*M. tuberculosis* can survive for relatively long periods in expectorated sputum. Survival outside the human host would require adaptation to oxidative stress, low nutrient levels, and low temperature. The biochemical and genetic alterations permitting the organism to survive under these conditions are unknown. All of these conditions, in particular cold shock,

Table 1. *sigF* RPA signal relative to baseline for BCG grown under different conditions

| Growth condition | RPA signal intensity relative to baseline* |
|---|---|
| Early-exponential phase ($OD_{600}$ = 0.67) | 1.0 |
| Late-exponential phase ($OD_{600}$ = 1.5) | 3.6 |
| Stationary phase ($OD_{600}$ = 2.7) | 9.8 |
| Oxidative stress (10 mM $H_2O_2$) | 4.8 |
| Alcohol shock (5% ethanol) | 2.8 |
| Cold shock (4°C) | 17.6 |
| Nitrogen depletion | 8.8 |
| Microaerophilic stress | 3.2 |

*Equal amounts of total bacterial RNA (0.85 μg) were used in each assay. Duplicate or quadruplicate aliquots of each stress culture were processed independently and average values are shown above. Quantitation was performed by digitally photographing the autoradiogram on an Ambis camera and then analyzing the bands on the National Institutes of Health IMAGER program. Baseline was defined as the signal intensity at 279–320 nucleotides of early-exponential-phase samples, which was essentially the same as background.

induce *M. tuberculosis sigF* transcription. It is possible that SigF is involved in survival outside of the host. Alternatively, *M. tuberculosis* SigF might be involved in the adaptation of the organism during latent infection. The observation that *M. tuberculosis* has a sigma factor closely related to sporulation sigmas from *S. coelicolor* and *B. subtilis* is intriguing, since tubercle bacilli are classically described as nonsporulating bacilli. Molecular genetic studies using the *M. tuberculosis sigF* gene may help address the question of whether tubercle bacilli enter a spore-like state during persistent infection.

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