The *Mycobacterium tuberculosis* Extracytoplasmic-Function Sigma Factor SigL Regulates Polyketide Synthases and Secreted or Membrane Proteins and Is Required for Virulence

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Received 6 May 2005/Accepted 2 August 2005

Mycobacterium tuberculosis sigL encodes an extracytoplasmic function (ECF) sigma factor and is adjacent to a gene for a membrane protein (Rv0736) that contains a conserved HXXXCXXC sequence. This motif is found in anti-sigma factors that regulate several ECF sigma factors, including those that control oxidative stress responses. In this work, SigL and Rv0736 were found to be cotranscribed, and the intracellular domain of Rv0736 was shown to interact specifically with SigL, suggesting that Rv0736 may encode an anti-sigma factor of SigL. An *M. tuberculosis* sigL mutant was not more susceptible than the parental strain to several oxidative and nitrosative stresses, and sigL expression was not increased in response to these stresses. In vivo, sigL is expressed from a weak SigL-independent promoter and also from a second SigL-dependent promoter. To identify SigL-regulated genes, sigL was overexpressed and microarray analysis of global transcription was performed. Four small operons, sigL (Rv0735)-Rv0736, mpt53 (Rv2878c)-Rv2877c, pks10 (Rv1660)-pks7 (Rv1661), and Rv1139c-Rv1138c, were among the most highly upregulated genes in the sigL-overexpressing strain. SigL-dependent transcription start sites of these operons were mapped, and the consensus promoter sequences TGAACC in the –35 region and CGTgtc in the –10 region were identified. In vitro, purified SigL specifically initiated transcription from the promoters of sigL, mpt53, and pks10. Additional genes, including four PE_PGRS genes, appear to be regulated indirectly by SigL. In an in vivo murine infection model, the sigL mutant strain showed marked attenuation, indicating that the sigL regulon is important in *M. tuberculosis* pathogenesis.

The *Mycobacterium tuberculosis* genome encodes 13 sigma factors, of which 10 fall into the extracytoplasmic function (ECF) subfamily. Three of these sigma factor genes are located 5' of genes that encode proteins containing an HXXXCXXC motif, which is found in several anti-sigma factors, including *Streptomyces coelicolor* RsrA (2, 15, 17, 25), *Rhodobacter sphaeroides* ChrR (23), and *M. tuberculosis* RshA (35). For both *S. coelicolor* SigR-RsrA and *M. tuberculosis* SigH-RshA, it has been demonstrated that this motif in the anti-sigma protein is a key element of a redox switch that affects the interaction between sigma and anti-sigma and thus regulates sigma factor activity in response to intracellular oxidative stress (24, 35). In the case of RsrA and ChrR, this cysteine-rich motif has been shown to bind zinc, and it has been suggested that this is a general property of this motif, leading to the term zinc-associated anti-sigma factor (ZAS) (17, 23, 24).

In addition to sigH, the other *M. tuberculosis* sigma factor genes linked to these putative ZAS protein genes are sigE and sigL. SigE, like SigH, has been shown to be a key regulator of the mycobacterial response to oxidative and heat stresses (21, 37). In addition, *M. tuberculosis* SigB, though it is not linked to a similar putative anti-sigma factor gene, is regulated by SigE and SigH and has been shown to be induced in response to

these and other stresses and upon entry into stationary phase (13, 20). These data suggest that a major role of the ECF sigma factors of *M. tuberculosis* is to alter patterns of gene expression to allow adaptation to stress during infection of the host.

Though mycobacterial SigE and SigH and *Streptomyces* SigR regulate stress response genes, other sigma factors linked to ZASs, such as *S. coelicolor* RsuA and *R. sphaeroides* ChrR, do not. In *S. coelicolor*, an actinomycete which undergoes morphological differentiation, the SigU-RsuA pair was identified in a screening for mutants that fail to produce aerial mycelia; the stimulus sensed by RsuA is not known (11). The phototrophic proteobacterium *R. sphaeroides* SigE-ChrR pair plays a role in photosystem regulation, with the anti-sigma factor ChrR apparently functioning as a light sensor (5, 31).

On the basis of the role of the *M. tuberculosis* ZAS-associated ECF sigma factors SigH and SigE in regulating stress response genes, we hypothesized that the third putative member of this group, SigL, and its adjacent gene, Rv0736, might function similarly. We therefore undertook to determine whether SigL interacts with the intracellular domain of Rv0736. We investigated the susceptibility of a sigL mutant to a variety of stresses and identified genes regulated by this sigma factor. In contrast to SigE and SigH results, our findings suggest that SigL does not play a major role in responding to oxidative stress. On the basis of the data presented in this work, this ECF sigma factor appears to regulate the expression of genes involved in polyketide-lipid synthesis and genes encoding membrane-associated proteins that are involved in posttranslational protein modification. We also demonstrated

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that a *sigL* mutant of *M. tuberculosis* is severely attenuated in a mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* H37Rv was used as the parental strain for generating Δ *sigL::hyg/cm*, complemented (Δ *sigL::hyg/cm*, *attB::sigL*), and *sigL*-overexpressing (*attB::P_{ace}-sigL*) strains and as the wild type for all experiments. *M. tuberculosis* was grown in Middlebrook 7H9 broth supplemented with albumin dextrose complex plus 0.05% Tween 80 or 0.2% glycerol. *Escherichia coli* DH5 α (Life Technologies) and XL1-Blue (Stratagene) were used as host strains for cloning experiments. *E. coli* was grown on LB agar or in LB broth. Ampicillin (50 μ g/ml), chloramphenicol (25 μ g/ml), hygromycin (Hyg) (50 to 100 μ g/ml), kanamycin (Kan) (20 to 50 μ g/ml), or tetracycline (25 μ g/ml) was added to culture medium when required.

Analysis of Rv0736 (RslA) topology and interaction of SigL with RslA. The topology of RslA was analyzed by constructing a series of translational fusions between *rslA* and *phoA* or *lacZ* by use of the mycobacterial *phoA* and *lacZ* fusion vectors as previously described (1, 6). Fusions included the amino-terminal region, the amino-terminal region plus the predicted transmembrane region, and the entire protein. Interaction between SigL and the intracellular domain of RslA was assessed using a bacterial two-hybrid system as previously described except that β -galactosidase activity assays were performed in a microtiter plate format (10, 35).

Construction of *sigL* mutant, complemented, and overexpressing strains. The *M. tuberculosis* Δ *sigL::hyg/cm* mutant was constructed by targeted mutagenesis as described previously except that the targeting construct was introduced into the temperature-sensitive shuttle phasmid pAE87 by use of the λ Red recombinase system instead of by in vitro packaging (3, 9). Briefly, two 900-bp DNA fragments flanking *sigL* were amplified by PCR from *M. tuberculosis* genomic DNA and cloned into pYUB572, and a hygromycin-chloramphenicol resistance cassette was inserted between the two PCR fragments. The resulting plasmid was sequenced and used as template DNA to generate the linear DNA substrate for recombination by PCR using primers 572F (5'-GAATTAATTAATCTGCTGA AGCCAGTTACCTTCG-3') and 572R (5'-GAATTAATTAAGATCCTTTAG TGAGGGTTAATTG-3'). After DpnI digestion to remove template DNA, the targeting sequence was electroporated into *E. coli* DH5 α cells, which carried both pAE87::572 and a temperature-sensitive RED helper plasmid carrying the λ Red recombinase genes (γ β exo) under control of the arabinose-inducible promoter *P_{araB}*.

Recombinants were selected on chloramphenicol plates, and phasmid DNA was isolated. This *sigL*-disrupting construct in pAE87 was electroporated into *M. smegmatis* mc²155, and cells were plated for plaques at 30°C. Transducing phage isolation and transfection of *M. tuberculosis* were performed as described previously (3). Three Hyg^r colonies were obtained, and disruption of the *sigL* allele was identified in each by PCR analysis. The mutant used for subsequent experiments was confirmed by Southern blotting. To complement the Δ *sigL::hyg/cm* mutant, a 964-bp DNA fragment including the coding sequence of the *sigL* gene and 360 bp of 5' sequence (including its native promoters) was amplified by PCR with the primers 5'-ATGCTCTAGACCGTTATGGACGCT CGTTCG-3' and 5'-TGGAAGATCTCGTCATGTCATCTCTCTCTGTC-3', cloned into pMV306, and sequenced. The *M. tuberculosis* Δ *sigL::hyg/cm* mutant was transformed with this construct. Hyg^r Kan^r colonies were selected, and the presence of the intact *sigL* allele was identified by PCR and confirmed by Southern blotting.

For the *sigL*-overexpressing strain, the 600-bp *sigL* coding sequence was amplified by PCR with primers 5'-ATATATCATATGGCTCGTGTGTCGGGCG C-3' and 5'-TGGAAGATCTCGTCATGTCATCTCTCTCTGTC-3' and cloned into pCKACEI, which contains the acetamide-inducible promoter *P_{ace}* in pCRII (19). The insert was sequenced, and the 3.6-kb fragment containing *sigL* under the control of *P_{ace}* was cloned into pMV306. This *sigL*-overexpressing construct was electroporated into *M. tuberculosis* H37Rv. The presence of the extra copy of the *sigL* gene at the *attB* site was verified by PCR analysis. *P_{ace}* without *sigL* in pMV306 was also electroporated into *M. tuberculosis*, and the resulting strain was used as a control.

A *sigL* deletion mutant and a complemented strain of *M. smegmatis* were constructed in a similar manner, except that the temperature-sensitive *sacB* counterselection method was used as previously described to create the *sigL* deletion in this species (27).

Oxidative stress and nitrosative stress phenotypes. Susceptibility of the Δ *sigL::hyg/cm* mutant to diamide (1 M), cumene hydroperoxide (20 mM), H₂O₂ (100 mM), plumbagin (10 mM), GSNO (*S*-nitrosoglutathione) (100 mM), SNP (sodium nitroprusside dihydrate) (100 mM), DETA/NO (dimethylenetriamine

nitric oxide adduct) (10 mM), and sodium dodecyl sulfate (1%) was determined using a disk diffusion assay as previously described (29).

RNA isolation and primer extension analysis. Total RNA was isolated from *M. tuberculosis* strains grown to mid-log phase (optical density value at 600 nm of 0.5) as previously described (28). For primer extension analysis, 0.5 pmol of γ -³²P-labeled primer (for *sigA*, 5'-CGACTTGTGGCGGTGCGTTTACC-3'; for *sigL*, 5'-TGACACGAGTCGAGTAAGAGTTTGG-3'; for *mpt53*, 5'-GTAT TGGCCAGACCGAACATCAG-3'; for *pks10*, 5'-ATGTCTTCGTAGCCCTC GAAATCC-3'; for Rv1139c, 5'-GTAGTACACGGCCCTACCACCTAAGAA-3') was mixed with 5 μ g of RNA in a 6.5- μ l volume and reverse transcription (RT) was performed with Superscript II (Invitrogen) at 42°C for 1 h. Reaction products were electrophoresed on 6% polyacrylamide sequencing gels. Sequencing reactions with the same primers used for primer extension were performed in adjacent lanes to determine the size of the transcripts.

Microarray experiments and analysis. *M. tuberculosis* oligonucleotide arrays were obtained from The Institute for Genomic Research (TIGR) through the National Institute of Allergy and Infectious Diseases-sponsored Pathogen Functional Genomics Resource Center. This microarray contains 4,750 70-mer oligonucleotides representing 4,127 open reading frames from *Mycobacterium tuberculosis* strain H37Rv and 623 unique open reading frames from strain CDC1551. RNA was prepared as described above, and cDNA was synthesized by reverse transcription with Superscript II (Invitrogen) with amino-allyl dUTP (Sigma) incorporation, followed by coupling to Cy-3 or Cy-5 fluorescent dyes (Amersham). TIGR protocols were followed for probe synthesis, hybridization, and washing (36). The hybridized slides were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA), and spot intensities were defined and quantified using GenePix Pro software (Axon Instruments, Union City, CA).

Ten hybridization intensity values from microarray experiments (five replicates from five independent RNA preparations) were used for statistical analysis, which was performed using GeneSpring (Silicon Genetics, Redwood City, CA). The raw data median value for Cy3 and Cy5 data was normalized, and the induction ratio was calculated as the average fold change for the 10 data points.

Real-time PCR and mRNA quantification. Two-step quantitative PCR was carried out to analyze specific gene expression. RNA from *M. tuberculosis* H37Rv cells was prepared as described above except for an additional DNase I treatment with Turbo DNA-free (Ambion). Primers were designed using Primer 1 software (ABI), and reverse transcription and PCR were performed using iScript and iTaq SYBR green Supermix with ROX (Bio-Rad) and an ABI7000 sequence detection instrument. Duplicate reactions with 100 ng of template were performed for *sigL* by use of primers *sigL* F (5'-CGAGCATAGGCGCGTGATC-3') and *sigL* R (5'-ATCGCGACTTCACCGTTCCT-3'). Expression of *sigA* was quantified in each sample to allow comparison to the relatively stable expression of this gene. An RNA sample that had not been reverse transcribed was included in all experiments to exclude significant DNA contamination. Standard curves were obtained by performing PCR with SYBR green detection on serial dilutions of spectrophotometrically quantified genomic DNA.

Computer database searching. Searches of the *M. tuberculosis* H37Rv genome sequence for consensus promoter elements were performed utilizing the "search pattern" program available on the TubercuList web site of the Pasteur Institute (<http://genolist.pasteur.fr/TubercuList>) and utilizing the "motif finding" function in BioProspector program (<http://ai.stanford.edu/~xslu/BioProspector/>) (18).

Purification of recombinant His-tagged SigL and in vitro transcription assays. The *sigL* coding sequence was amplified by PCR using two primers (5'-ATAT ATCATATGGCTCGTGTGTCGGGCGC-3' and 5'-GGCTGGATCCTCATC GAGTAACTCCCAG-3') and cloned into pET28a. The resulting construct was sequenced and used to transform *E. coli* BL21(DE3) pLysS (Novagen). Expression of *sigL* was induced at 15°C overnight, cells were harvested and disrupted by sonication, and His-tagged SigL protein (6X-his-SigL) was purified on a Ni-nitrilotriacetic acid column (Novagen) according to the manufacturer's protocol.

In vitro runoff transcription assays were performed as previously described (29). PCR products containing the *sigL*, *pks10*, *mpt53*, or Rv1139c promoter (10 nM in each reaction) were used as DNA templates. Transcripts were analyzed by autoradiography after separation on a 6% polyacrylamide gel containing 7 M urea.

Mouse infection and virulence assays. Six-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and infected with strain H37Rv, the *sigL* mutant strain, or the complemented strain. Infection was performed by lateral tail vein injection of 10⁶ declumped bacteria, as previously described (28). Mice were housed in microisolator cages in a BL3 animal facility under specific-pathogen-free conditions. Twelve mice in each group were infected for survival analysis. Weights and morbidity of the mice were monitored for the duration of the survival experiment. Mice that became moribund were

FIG. 1. Topology of Rv0736 (RslA) and its interaction with SigL. (A) Predicted domain structure of RslA and locations of junctions of translational fusions to *lacZ* or *phoA*. (B) β -Galactosidase or alkaline phosphatase activity in lysates of *M. smegmatis* transformed with the *rslA-lacZ* or *rslA-phoA* fusion constructs shown in panel A. (C) β -Galactosidase activity measured in lysates from *E. coli* harboring the indicated pair of fusion constructs used to perform bacterial two-hybrid assays.

euthanized, with survival counted to the day of euthanasia. For CFU analysis, six mice were sacrificed at days 2, 8, 22, and 59, and one lung and the spleen were removed for CFU determination. The other lung was fixed in formalin, sectioned, and stained with hematoxylin and eosin. All work with mice was performed under approved protocols in compliance with federal guidelines and institutional policies.

With the exception of the mouse infection experiments, which were performed once each, all experiments were performed at least twice; representative data are shown.

RESULTS

Topology of Rv0736 (RslA) and interaction with SigL. Rv0736, the gene immediately 3' of *sigL*, is a membrane protein that is coexpressed with *sigL* (see below). The predicted protein contains the HXXXCXXC motif characteristic of ZASs in the region amino terminal to the single transmembrane domain (Fig. 1A). To determine the topology of this protein in vivo, a series of *phoA* and *lacZ* fusions were made. Expression of these fusions in *M. smegmatis* and measurement of enzyme activity indicated that the amino-terminal region of this protein is intracellular and the carboxy-terminal region is extracellular (Fig. 1B).

On the basis of these data, we analyzed the interaction of SigL and the intracellular domain of RslA in a bacterial two-hybrid system (10). In this system, potential binding partners are fused to λ CI or the amino-terminal domain of the α subunit of RNA polymerase. A protein-protein interaction causes activation of *lacZ* expression from a promoter located near a lambda operator, resulting in increased β -galactosidase

activity (10). As shown in Fig. 1C, these assays indicate that SigL and RslA interact strongly and specifically, consistent with the hypothesis that Rv0736 functions as an anti-sigma factor (designated RslA) that physically interacts with SigL.

Construction and stress phenotypes of *M. smegmatis* and *M. tuberculosis* *sigL* mutant and complemented strains. To study the function of SigL, we constructed *sigL* mutant strains based on *M. smegmatis* mc²-155 (34) and *M. tuberculosis* H37Rv. In both strains, the coding sequence of the *sigL* gene was replaced by a hygromycin and chloramphenicol resistance cassette. The absence of the *sigL* allele in these mutants was identified by PCR, and the deletion in the *M. tuberculosis* strain used in subsequent experiments was confirmed by Southern blotting (Fig. 2). A complemented strain of the Δ *sigL*::*hyg/cm* mutant used in subsequent experiments was constructed by integrating the *sigL* gene under the control of its native promoters at the phage L5 *attB* site (16).

The growth curves of the *sigL* mutant strains of *M. smegmatis* and *M. tuberculosis* in broth culture were similar to those of the wild-type strains (not shown), and no clear differences from wild type in colony morphology were observed when the mutant strains were grown on solid medium. To examine stress phenotypes of the *sigL* mutant strains, we compared the susceptibility of the Δ *sigL*::*hyg/cm* strains to wild-type results by use of a disk diffusion assay (29). When exposed to oxidants (including H₂O₂, cumene hydroperoxide, diamide, and plumbagin), to nitric oxide-generating agents (including SNP, GSNO, and DETA/NO), and to the detergent sodium dodecyl

FIG. 2. (A) The *M. tuberculosis* *sigL* locus and strategy for deletion mutant construction. (B) Southern blot analysis of *M. tuberculosis* H37Rv and one of three independent *sigL* deletion mutants that were identified by PCR. Genomic DNA of each strain was digested with XmnI and ApaLI, electrophoresed in a 1% agarose gel, transferred to a nylon membrane, and probed as shown. Expected sizes for the wild-type (wt) and *sigL* mutant strains are 1.4 kb and 2.8 kb, respectively. In the complemented strain, the *sigL* coding sequence is linked to vector sequences resulting in a restriction fragment of 10.3 kb.

sulfate, no consistent differences were observed between the wild-type and mutant strains (data not shown). Several of the same compounds were tested in a time-kill assay in liquid medium, again without showing differences between the wild-type and mutant strains.

***M. tuberculosis* *sigL* promoter identification and expression of the *sigL* gene during in vitro growth.** Many ECF sigma factor genes, including *M. tuberculosis* *sigH*, are expressed from auto-regulated promoters. To examine the *sigL* promoter sequence, primer extension analysis of the *sigL* gene was carried out (Fig. 3). The signal from the *sigL* primer was extremely weak relative to the signal obtained with a *sigA* primer used as a control, and the *sigL* signal was only detectable after prolonged exposure. This *sigL* transcription start site, designated P1, was mapped 121 bp 5' of the annotated *sigL* initiation codon (8) and is located within the open reading frame of the *mapA* gene, whose termination codon is 72 bp 5' of the *sigL* gene.

We also performed primer extension analysis with RNA prepared from the *M. tuberculosis* *sigL* mutant strain by use of a primer corresponding to the upstream region of the *sigL* gene. A band corresponding to the *sigL* P1 transcription start site was still present, indicating transcription initiation from this site in the *sigL* mutant strain and thus that this promoter is not completely autoregulated. The signal from this transcript is decreased in the *sigL* mutant, however, when normalized to the *sigA* transcript. We examined the region 5' of *sigL* P1 but did not identify -10 or -35 promoter elements similar to consensus sequences recognized by *sigL* (see below) or by other *M. tuberculosis* sigma factors. These data suggest that decreased *sigL* expression from this promoter in the *sigL* mu-

FIG. 3. Transcription of the *sigL* gene in the *M. tuberculosis* H37Rv wild-type (WT) strain and the $\Delta sigL::hyg/cm$ mutant. Primer extension analysis was performed using RNA isolated from *M. tuberculosis* H37Rv wild-type and $\Delta sigL::hyg/cm$ mutant strains with *sigA* and *sigL* primers. The transcription start site of the *sigL* gene is indicated by an arrow.

FIG. 4. Expression pattern of the *M. tuberculosis* *sigL* gene during growth. RNA was isolated from *M. tuberculosis* H37Rv at serial time points, and quantitative RT-PCR was performed using 100 ng of RNA as described in Materials and Methods. A standard curve for copy number of each gene was obtained by performing quantitative PCR using serial dilutions of genomic DNA. *OD*₆₀₀, optical density at 600 nm.

tant results from indirect effects rather than direct recognition of this promoter by SigL.

We performed quantitative RT-PCR to examine the quantity and pattern of *sigL* gene expression during growth in vitro. *M. tuberculosis* cells were harvested at serial time points, and RNA was prepared and used for RT-PCR. An estimate of *sigL* mRNA copy numbers was calculated from a standard curve obtained from quantitative RT-PCR performed with a dilution series of known amounts of genomic DNA as a template. We found that the quantity of *sigL* mRNA is approximately 100-fold lower than that of *sigA* mRNA, the primary sigma factor in *M. tuberculosis*, throughout the course in vitro growth (Fig. 4). These results indicate that there is constitutive expression of the *sigL* gene from the very weak SigL-independent promoter P1 during in vitro growth.

Construction of a *sigL* overexpression *M. tuberculosis* strain and microarray analysis of SigL-regulated gene expression. To attempt to identify genes regulated by SigL, we performed global gene expression analysis in oligonucleotide microarray experiments. Initial experiments compared the Δ *sigL::hyg/cm* strain to the parental *M. tuberculosis* H37Rv strain. With one exception, the *sigL* mutant did not show a gene expression pattern significantly different from that seen with *M. tuberculosis* H37Rv. Signals from spots on the array corresponding to *sigL* were very weak, indicating that the expression level of the *sigL* gene is extremely low, consistent with the primer extension and RT-PCR results described above. The one clear difference observed between the wild type and the *sigL* mutant in these experiments was that the expression of *rsIA* (Rv0736) was significantly increased in the *sigL* mutant. This increased transcription of *rsIA* is likely the result of read-through from the promoters of the hygromycin and chloramphenicol resistance cassette, which replaced the *sigL* gene immediately 5' of *rsIA*. This elevated *rsIA* expression was not associated with other significant changes in gene expression in the *sigL* mutant, suggesting that it does not have a major role as an anti-sigma factor for other *M. tuberculosis* sigma factors.

Because of the low basal levels of *sigL* expression and the absence of consistent differences in the experiments comparing the *sigL* mutant to the wild type, to identify genes transcribed by SigL we constructed a *sigL*-overexpressing strain. The coding sequence of the *sigL* gene was cloned under the control of the acetamide-inducible promoter (*P*_{acc}) (26), in the integrating vector pMV306, to create a strain with a single extra copy of *sigL* (*M. tuberculosis* H37Rv *attB::P*_{acc}-*sigL*). The acetamide promoter region without any gene under its control was also cloned into pMV306 and used to make a control strain (*M. tuberculosis* H37Rv *attB::P*_{acc}). The presence of the *sigL* allele at the *attB* site was confirmed by PCR using primers corresponding to the *attB* and acetamide promoter region.

The *sigL*-overexpressing and control strains were grown to an optical density at 600 nm of 0.5, RNA was prepared after 1 h of induction, and analysis of gene expression was per-

TABLE 1. Genes highly expressed in the *sigL* over-expressing strain relative to H37Rv

Locus	Gene	Fold change	P value	Description
Rv0735	<i>sigL</i>	192.0	0.001	ECF sigma factor
Rv0736	<i>rsIA</i>	45.1	0.002	Putative anti-sigma factor
Rv1138c		8.1	0.027	Possible oxidoreductase
MT1172		2.9	0.044	Conserved hypothetical protein, same as Rv1139c
Rv1660	<i>pks10</i>	4.6	0.017	Chalcone synthase
Rv1661	<i>pks7</i>	7.2	0.024	Polyketide synthase
Rv2877c		22.7	0.002	Probable conserved integral membrane protein
Rv2878c	<i>mpt53</i>	6.0	0.002	Soluble secreted antigen MPT53 precursor
Rv1450c	<i>PE_PGRS27</i>	4.7	0.017	PE_PGRS family protein
MT2712	<i>PE_PGRS46</i>	3.4	0.023	PE_PGRS family protein, same as Rv2634c
Rv2853	<i>PE_PGRS48</i>	6.7	0.023	PE_PGRS family protein
Rv3367	<i>PE_PGRS51</i>	10.2	0.006	PE_PGRS family protein
Rv1696	<i>recN</i>	8.1	0.017	Probable DNA repair protein
Rv1833c		3.0	0.002	Possible haloalkane dehalogenase
Rv3580c	<i>cysS1</i>	3.1	0.049	Cysteinyln-trans synthetase 1
Rv3895c		3.5	0.040	Probable conserved membrane protein
MT1155		3.1	0.010	Hydrolase, same as Rv1123c
MT1814.2		3.3	0.040	Hypothetical protein
MT2736.1		4.8	0.004	Hypothetical protein

FIG. 5. Transcription start sites of SigL-dependent genes and consensus promoter sequence. (A) Primer extension analysis was performed for SigL-dependent genes using RNA isolated from *M. tuberculosis* *attB*::P_{acc} (–) and *attB*::P_{acc}-*sigL* (+). Transcription start sites are indicated by arrows. (B) Alignment of promoter sequences from SigL-dependent genes. The transcription start sites and consensus promoter sequences in the –10 and –35 regions are indicated in boldface, and the annotated initiation codons are underlined. The SigH promoter consensus sequence is also shown.

formed using oligonucleotide microarrays. We performed the array experiments five times with five independent RNA preparations, and the results were analyzed statistically. The genes with significantly increased ($P < 0.05$; $\geq$ threefold induction) expression in the *sigL*-overexpressing strain are listed in Table 1. The induced expression of several genes in the overexpression strain was confirmed by quantitative RT-PCR analysis (data not shown). We also tested a different set of induction conditions (24 h of induction with the same concentration of acetamide) and obtained a similar list of regulated genes. When subjected to the same stringent analysis, we did not identify genes that were significantly down-regulated in the *sigL*-overexpressing strain.

The SigL regulon identified in these experiments includes four two-gene operons and a relatively small number of other genes. First, *sigL* itself and *rsL4* (Rv0736) were both significantly up-regulated in the *sigL*-overexpression strain. The increased expression of *rsL4*, which is not included in the overexpression construct, suggested that SigL regulates the *sigL*-*rsL4* operon. A second putative SigL-dependent operon includes *mpt53* and a gene of unknown function, Rv2877c.

Mpt53 was originally identified as a major secreted protein of *M. tuberculosis* that is weakly immunogenic in natural *M. tuberculosis* infection (14, 22). Rv1139c and Rv1138c, both genes of unknown function, also form a SigL-regulated operon, with the start codon of Rv1138c overlapping the stop codon of Rv1139c. Rv1139c is predicted to have two to three membrane-spanning regions and to encode an isoprenyl cysteine carboxyl methyltransferase motif (7).

The *pks10* and *pks7* genes are also physically linked, and RT-PCR analysis confirmed the presence of a single transcript that includes *pks10* and *pks7* (not shown). Though both were up-regulated in the *sigL*-overexpression strain, no SigL consensus sequence (see below) is present 5' of *pks7*. The extent of up-regulation of *pks7*, however, was less than that of *pks10*. This result, and the presence of an 83-bp noncoding sequence between these genes, suggested that, in addition to being expressed as part of an operon from the *pks10* promoter, *pks7* might also be transcribed from a second SigL-independent promoter 5' of the *pks7* gene. Primer extension experiments did not identify a transcription start site 5' of *pks7* (data not shown), however, suggesting that this gene is expressed only as

FIG. 6. In vitro transcription assay with purified SigL protein. His₆-tagged SigL protein was overproduced in *E. coli* and purified through a Ni-nitrilotriacetic acid column. Template DNAs including putative promoter sequences were amplified by PCR. In vitro transcription assays were performed as described in Materials and Methods. Specific transcripts are indicated by arrows. Lane M, RNA size markers (bases).

part of a transcript with *pks10*. PKS10 is characterized as a type III polyketide synthase and is similar to members of the plant chalcone synthase superfamily (30). PKS7 appears to be a multifunctional enzyme containing both beta-ketoacyl synthase active site and phosphopantetheine attachment site motifs.

In addition to these operon results, a notable finding of the microarray experiments was the up-regulation of four PE-PGRS genes in the *sigL*-overexpression strain. These genes encode proteins that are members of a large family of polymorphic proteins of unknown function.

Promoter analysis of SigL-dependent genes. To map the transcriptional start site of the SigL-dependent genes identified in the microarray experiments, we performed primer extension analysis with RNA prepared from the *sigL*-overexpressing strain, starting with *sigL* itself (Fig. 5A). In addition to the *sigL* P1 promoter identified in wild-type H37Rv, in the *sigL*-overexpressing strain we detected an additional *sigL* transcription start site, designated *sigL* P2. We next analyzed the in vivo transcription start sites of *pks10*, *mpt53*, and Rv1139c. These experiments demonstrated the presence of transcription start sites for each of these genes in the *sigL*-overexpressing strain that were not present in H37Rv. The aligned upstream sequences of the transcription start sites of these SigL-dependent genes showed two highly conserved consensus sequence elements, TGAACC in the -35 region and CGT in the -10 region, with 16-bp spacing between the 3' end of the -35 element and the 5' end of the -10 element (Fig. 5B).

Exact matches were not found 5' of any of the other genes that were upregulated in the *sigL*-overexpression strain, although partial matches were observed. Primer extension experiments with several of these genes with the closest match to the SigL promoter consensus did not identify SigL-dependent transcription start sites that corresponded to these candidate promoters. These data suggest that the up-regulation of these genes in the *sigL*-overexpression strain is indirect and is not the result of recognition of these promoters by RNA polymerase containing SigL.

FIG. 7. Transcription analysis of the Rv2466c and *sigB* promoters. (A) In vitro transcription analysis of these promoters was performed using core RNA polymerase (-) or RNA polymerase holoenzyme reconstituted with purified SigH (σ^H) or SigL (σ^L). Specific transcripts are indicated by arrows. (B) Primer extension analysis of the *sigB* and *sigA* promoters was performed using RNA from the *sigL*-overexpression strain (*attB*::*Pace-sigL*) and wild-type (WT) strain H37Rv.

In vitro transcription from SigL-dependent promoters. To determine whether the in vivo SigL-dependent promoters were directly recognized by SigL, we affinity purified six-His-tagged *M. tuberculosis* SigL protein and performed in vitro transcription assays. Nonspecific transcripts were detected in assays performed with core RNA polymerase with or without SigL protein. Specific transcripts of the expected size were seen in assays performed with holoenzyme reconstituted with SigL for templates containing the promoter sequences of *sigL*, *pks10*, and *mpt53* (Fig. 6). Surprisingly, we did not observe a specific transcript from Rv1139c, even though it has a well-conserved SigL-dependent promoter sequence, as shown in Fig. 5B. This assay was performed with several different Rv1139c templates, which extended 5' and 3' relative to the identified promoter, but in none of these experiments was a specific transcript observed. Of note, the in vivo transcription level of Rv1139c was very low even in the *sigL*-overexpressing strain compared to the results seen with the other SigL-dependent genes, *pks10* and *mpt53* (Fig. 5A). These data suggest that an additional factor, such as a transcriptional activator, may be required for

FIG. 8. (A) Infection of BALB/c mice with *M. tuberculosis* H37Rv wild-type ($\square$), $\Delta sigL$ ($\bullet$), and $\Delta sigL$ -complemented ($\triangle$) strains. Mice were infected by lateral tail vein injection of 10^6 CFU. (A) Survival analysis. Morbidity and weight were monitored, and mice that became moribund were sacrificed. Twelve mice were infected in each group. (B and C) Replication and persistence of *M. tuberculosis* strains in mouse organs. Six mice were sacrificed at days 2, 8, 22, and 59, with plating for bacterial burden in lung (B) and spleen (C) performed as described in Materials and Methods.

initiation of transcription from the Rv1139c promoter, despite its containing the exact consensus -35 and -10 elements.

The SigL-dependent consensus promoter sequence that we identified in these experiments is highly similar to the SigH-dependent consensus promoter sequence that was previously identified in our laboratory (29). To determine whether SigL might also recognize SigH-regulated genes, we performed *in vitro* transcription assays with templates incorporating the SigH-dependent promoters of Rv2466c and *sigB* (Fig. 7A). RNA polymerase containing SigL initiated transcription from the *sigB* promoter, which differs from the SigL-dependent consensus at two positions in the -35 element, though the signal was substantially less intense than that produced from this promoter by RNA polymerase reconstituted with SigH. In contrast, no specific SigL-dependent transcript was observed from the Rv2466c promoter, which differs at two positions in the -35 element and one position in the -10 element from the SigL recognition consensus.

To determine whether *sigB* might be regulated by SigL *in vivo*, primer extension analysis was performed using RNA

from the *sigL*-overexpression strain and the control strain (Fig. 7B). This experiment demonstrated approximately 1.5- to 1.9-fold-greater transcription of *sigB* in the *sigL*-overexpression strain versus H37Rv after normalization to transcripts from the control *sigA* promoter. This result indicates that one or a few key residues within the -35 or -10 elements, or elsewhere in the promoter region, allow discrimination between these promoters by SigL and SigH.

Virulence analysis of the *M. tuberculosis sigL* mutant. To begin to assess the role of SigL in *M. tuberculosis* virulence, a mouse model of infection was used. BALB/c mice infected intravenously with the *sigL* mutant strain showed a significantly prolonged survival time relative to those infected with the parental H37Rv wild-type strain, with a median survival time of >145 days versus 99 days for H37Rv ($P < 0.0001$). The complemented strain showed virulence similar to that of the wild type in this experiment (median survival time = 86 days) ($P < 0.0001$ versus $\Delta sigL$; $P = 0.6$ versus H37Rv) (Fig. 8A).

We also investigated the ability of these three *M. tuberculosis*

strains to proliferate and persist in the tissues of BALB/c mice after intravenous infection. The bacterial burden in lung and spleen was assessed by plating the dilutions of the organ homogenates at days 2, 8, 22, and 59. The growth kinetics of the three strains in the lung and spleen were very similar, as determined by plating at these time points following infection (Fig. 8B and C). Hematoxylin- and eosin-stained lung tissues from infected BALB/c mice were compared for extent of tissue pathology. Though subtle differences cannot be excluded, major variations in the amount of inflammation in the lungs of mice infected with the wild-type strain versus the $\Delta sigL$ strain were not observed (data not shown).

DISCUSSION

Several alternative sigma factors of *M. tuberculosis* regulate gene expression in response to stress, including SigH and SigE, which have been shown (SigH) or are hypothesized (SigE) to be regulated by ZASs. Though *sigL* is also linked to a likely ZAS gene (*rsL4*), we did not identify oxidative or nitrosative stress conditions that either induced expression of this sigma factor or resulted in decreased survival of a *sigL* mutant relative to wild-type survival. These results suggest that SigL is not a stress response regulator and that other conditions encountered by the bacillus during infection in vivo may activate *sigL* expression.

By performing microarray analysis followed by primer extension and in vitro transcription experiments, we identified a limited set of genes that are directly regulated by SigL. Despite the small number of regulated promoters, we identified clear -35 and -10 element consensus sequences (TGAACC- N_{16} -CGT) in the promoters recognized by SigL. Though this is similar to the SigH recognition consensus (GGAACA- N_{16} -GTT) that we previously defined (29), the inability of $E\sigma^L$ to initiate transcription from the Rv2466c promoter, which has the exact SigH consensus, indicates that these differences are sufficient to distinguish the promoters recognized by these two ECF sigma factors.

It has been proposed that RNA polymerase containing SigL might transcribe the *sigB* promoter (33). We previously demonstrated this promoter to be SigE and SigH dependent, with no evidence of transcription initiation at this site in vivo in an *sigE/sigH* double mutant (29). In this work a SigL-dependent transcript was initiated from the *sigB* promoter in vitro, and a small increase in *sigB* transcription in the *sigL*-overexpression strain was observed in primer extension experiments. Expression of *sigB* was not significantly increased in the *sigL*-overexpression strain in the microarray experiments, however. Our interpretation of these data is that RNA polymerase incorporating SigL does not initiate transcription at the *sigB* promoter under non-*sigL*-inducing conditions. Based on our previous data and the lack of *sigL* induction in response to oxidative and nitrosative stresses observed in this work, SigL does not appear to play a role in *sigB* expression in response to these stresses. It remains possible, however, that under conditions in which *sigL* expression is highly induced, SigL-dependent upregulation of *sigB* may occur.

The SigL regulon we identified includes *sigL-rsL4* and three other apparent operons: *pks10-pks7*, *mpt53*-Rv2877c, and Rv1139c-Rv1138c. While *pks10* and *pks7* are annotated as

polyketide synthase genes, the functions of the PKS10 and PKS7 enzymes are not known. Available data, however, shed some light on the possible function of PKS10. PKS11 and PKS18, both chalcone synthase type III polyketide synthases of *M. tuberculosis* that are closely related to PKS10, have been shown to synthesize tri- and tetraketide α -pyrones with a preference for long-chain aliphatic-coenzyme A starter units (30). The sequence and predicted structural similarity of PKS10 to these proteins suggests that it is likely to have a similar enzymatic activity.

In a previous study, a *pks10* mutant was found to be defective in synthesis of phthiocerol dimycocerosate, a major surface lipid of pathogenic mycobacteria (32). This *pks10* mutant was also significantly attenuated for growth in mice following intranasal inoculation. Our data following intravenous infection showed a marked attenuation of the *sigL* mutant in terms of mouse survival but little effect on bacterial replication in vivo. While *pks10* and/or *pks7* likely contributes to this striking virulence phenotype, the role of these and other SigL-regulated genes in *M. tuberculosis* pathogenesis remains to be determined.

The functions of the proteins encoded by Rv1139c and Rv1138c are also unknown. In contrast to *M. tuberculosis* results, in *M. avium* and *M. smegmatis*, as well as in *S. coelicolor*, the Rv1139c-1138c operon is physically linked to *pks10*, suggesting that they function in a pathway with *pks10*. Rv1138c encodes a putative oxidoreductase. Rv1139c is a membrane protein that contains an isoprenylcysteine carboxymethyltransferase motif. Enzymes with this motif in eukaryotes act on cysteines near the carboxy terminus of proteins and perform sequential isoprenylation and proteolytic cleavage after cysteine, followed by methylation of the carboxyl group of the now carboxy-terminal cysteine. Though several proteins that contain this motif are present in bacterial genome sequences, their function has not been characterized.

The other SigL-regulated gene pair, *mpt53*-Rv2877c, appears to encode a secreted protein disulfide oxidoreductase and a putative thiol-disulfide transporter with a conserved CcdA domain, respectively. Though a role for Mpt53 and Rv2877c in cytochrome *c* biogenesis has been postulated, other proteins corresponding to the gram-positive system II for cytochrome *c* biogenesis are encoded elsewhere [Rv0526 and *ccdA* (Rv0527)] in the *M. tuberculosis* genome (4, 8). Mpt53, annotated as a DsbE-like protein, has been characterized biochemically and structurally (12). It contains a thioredoxin active site and is a strong oxidant, in contrast to the weak reducing activity of *E. coli* DsbE. These biochemical data suggest a possible role for Mpt53 as an extracellular oxidant that may be required for proper folding of reduced unfolded secreted proteins, a function more similar to that of *E. coli* DsbA (12). Rv2877c has seven transmembrane regions and conserves two cysteines which are essential for the redox activity of CcdA, suggesting that, while it may not function as a CcdA orthologue, it is likely to be a redox-active membrane protein.

These data suggest that SigL regulates cell envelope lipid synthesis and envelope or secreted protein modification. Though we found that *sigL* is expressed at low levels during vegetative growth in vitro, the presence of an autoregulated *sigL* promoter suggests that significantly increased expression may occur under inducing conditions. Our data showing that

SigL and RslA interact and the similarity of RslA to other ZASs suggest that RslA may function as a negative regulator of SigL. Taken together, these findings suggest a model in which RslA may sense as-yet-undefined extracellular signals that decrease its binding of SigL. The resulting increased activity of the SigL-regulated genes would then alter the surface characteristics of the mycobacterium. The marked attenuation of the *sigL* mutant suggests that such changes could be important adaptations that allow *M. tuberculosis* to survive and cause disease in the host during infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant RO1AI37901 to R.N.H.

We thank the National Institute of Allergy and Infectious Diseases Pathogen Functional Genomics Resource at TIGR for providing the oligonucleotide microarrays used in the work. We thank Kevin Conlon for assistance with the Southern blotting, Simon Dove for critical review of the manuscript, Chris Sasseti and Eric Rubin for providing pAE87::572 and pKD119, and Adrie Steyn for the *phoA* (pMB111) and *lacZ* (pVlacZ2) fusion vectors.

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