ABSTRACT Rapid adaptation to changing environments is one of the keys to the success of microorganisms. Since infection is a dynamic process, it is possible to predict that Mycobacterium tuberculosis adaptation involves continuous modulation of its global transcriptional profile in response to the changing environment found in the human body. In the last 18 years several studies have stressed the role of sigma (σ) factors in this process. These are small interchangeable subunits of the RNA polymerase holoenzyme that are required for transcriptional initiation and that determine promoter specificity. The M. tuberculosis genome encodes 13 of these proteins, one of which—the principal σ factor σA—is essential. Of the other 12 σ factors, at least 6 are required for virulence. In this article we review our current knowledge of mycobacterial σ factors, their regulons, the complex mechanisms determining their regulation, and their roles in M. tuberculosis physiology and virulence.

Rapid adaptation to changing environments is one of the keys to the success of microorganisms. Such adaptation is achieved by enabling different strategies, the most potent of which is global transcriptional modulation. Bacterial genomes encode several transcriptional regulators that in response to external and internal stimuli rapidly change the transcriptional profile of the cells, allowing maintenance of the homeostasis. Among the several players responsible for global transcriptional regulation, sigma (σ) factors have a prominent role (1). These are small interchangeable subunits of the RNA polymerase (RNAP) holoenzyme that are required for transcriptional initiation and that determine the promoter specificity of the enzyme recognizing specific −35 and −10 consensus promoter sequences (2). All bacterial genomes encode at least one essential σ factor, responsible for the transcription of housekeeping genes, and a variable number of alternative σ factors enabling a rapid transcriptional shift in response to specific stimuli (3, 4).

Two main families of evolutionarily distinct σ factors are encoded in the bacterial pan-genome: σ70 and σ54. However, mycobacteria only encode for σ factors of the σ70 family (3, 5). These σ factors have four main conserved regions (regions 1, 2, 3, and 4): Region 1 inhibits DNA binding of free σ factors, region 2 is involved in recognition of the −10 consensus sequence and melting of the transcription bubble, region 3 is involved in recognition of the extended −10 consensus sequence, and region 4 is involved in recognition of the −35 consensus sequence (2, 3). σ factors can be divided into four groups (3): Group 1 comprises the primary σ factors. These are essential molecules since they are required for transcription of housekeeping genes. Group 2 includes primary-like σ factors. Its members have a structure very similar to members of group 1, but with some exceptions are involved in stationary phase survival and stress response and are not essential (7). Group 3 contains σ factors involved in cellular differentiation and general stress response of biosynthesis of the flagellum; they miss
the conserved region 1. Group 4, also named ECF (extra cellular function) σ factors, miss conserved regions 1 and 3 and are the most numerous and heterogeneous. Although the roles of several of them has not been described, they are often involved in the control of extra-cellular functions as a response to surface stress or some aspect of the cell surface or transport (7, 8). Several σ factors of this group have been shown to be essential for virulence (9).

The alternative σ factor-density (ASFD) of a genome (number of alternative σ factors/Mb) is a measure of the impact of these molecules on the physiology of a microorganism and usually reflects the complexity of its growth cycle and of the environment colonized from the bacterium (10). Consequently, intracellular parasites, endosymbionts, and obligate pathogens, adapted to a stable environment, usually have an ASFD below 2, together with small genomes. With the increased complexity of the environment usually inhabited, both genome size and ASFD increase to reach a value around 8 in genera such as Bacteroides and Streptomyces (10).

The contemporaneous presence of several alternative σ factors implies that their activity must be fine-tuned to allow coordination of transcription. Moreover, several complex σ factor regulatory networks have been described as the cascade involved in Bacillus and Clostridium sporulation (10–12).

σ factors can be regulated at different levels (1, 13). (i) Transcriptional regulation: Often σ factor genes are transcribed from multiple promoters, each with a different activity and regulation. Usually, one of these promoters is recognized by the σ factor itself, resulting in a positive feedback loop, while the others can be constitutive (to allow a basal level of expression in the absence of the inducing conditions) or regulated from other transcriptional regulators as two-component systems or other σ factors. (ii) Posttranscriptional regulation: through small noncoding RNA, for which the best example is Escherichia coli rpoS regulated by at least three small RNAs (14). (iii) Translational regulation, as in the case of the heat shock responsive σ factor of E. coli σ32, whose coding mRNA changes its conformation depending on temperature-regulating translational initiation (15). (iv) Posttranslational regulation: This is usually due to anti-σ factors, proteins that specifically bind to a σ factor, preventing its interaction with the RNA core enzyme (16). Specific environmental signals cause disruption of this interaction, leading to σ factor release. Some anti-σ factors are posttranslationally regulated by anti-anti-σ factors (9, 16). σ, anti-σ, and anti-anti-σ factor activity can be regulated by post-translational modification, leading to their proteolysis or conformational changes altering their activity (17). Regulation of σE activity in the E. coli response to stresses involving a damaged cell envelope and preventing outer membrane porins’ correct folding is the best-characterized example of posttranslational σ factor regulation (18, 19). In this case, regulated intramembrane proteolysis (RIP) is the signal transduction pathway linking periplasmic stress with σE activity. The structure of this pathway involves the presence of two proteases (S1P and S2P) that act sequentially, processing the transmembrane anti-σ factor RseA, resulting in the release (20) of its cytoplasmic domain from the membrane. However, this truncated form of RseA can still bind σE (21) until other ATP-dependent cytoplasmic proteases such as ClpXP totally degrade RseA, releasing the active form of σE (22).

Finally, some σ factors can be translated from alternative translational start codons in different environmental conditions with generation of proteins with different vulnerability to proteolytic turnover (23).

### THE σ FACTORS OF MYCOBACTERIUM TUBERCULOSIS

The genome of M. tuberculosis encodes 13 σ factors (5), and its ASFD is 2.8, which makes it the obligate pathogen with the higher density of alternative σ factors (10). Of these 13 σ factors, σH belongs to group 1 and represents the primary σ factor. It is the only σ factor to be essential for M. tuberculosis viability. σE belongs to group 2 (primary-like σ factors), σF belongs to group 3, and the other 10 (σA, σD, σE, σG, σH, σI, σJ, σK, σL, and σM) belong to group 4 (ECF σ factors) (10, 24).

Nearly all the M. tuberculosis σ factors (with the exception of σA, σH, σI, and σJ) have been predicted or shown to be posttranslationally regulated by cognate anti-σ factors (6, 24): σ5, σ4, and σ3 anti-σ factors are predicted to be cytoplasmic proteins, while all the other are predicted to be membrane proteins (25) (Table 1). At least three of them (those specific for σA, σJ, and σM) were suggested to be regulated by RIP, a common feature of posttranslational regulation in which the anti-σ factor is sequentially cleaved in two sites by two proteases—site 1 protease (S1P) and site 2 protease (S2P)—before being totally degraded (20, 26). The most likely M. tuberculosis S1P is the serine-protease HtrA (23), while S2P was recently identified in the membrane-bound metalloprotease Rv2869c (Rip1) (27). RsKa, RsLa, and RsmA were shown to be subject to proteolytic degradation by Rip1 following exposure to the metal chelator phenanthroline.


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(27). However, it is unknown if they are still able to bind their cognate σ factor until they are totally degraded by other cytoplasmic proteases, as demonstrated for RseA in *E. coli* (Table 1).

RsdA is insensitive to Rip1 cleavage but is sensitive to proteolytic degradation by the cytoplasmic protease ClpX-ClpP1-ClpP2 (28). However, whether its degradation occurs after release from the membrane by RIP is still unknown (Table 1).

Most of the *M. tuberculosis* σ factors have been extensively studied, and for most of them a clear role in pathogenesis was shown, making them an extremely important class of molecules for understanding the mechanism of *M. tuberculosis* adaptation to the host (10, 24, 25).

σA

σA is the only member of group 1 σ factors encoded in the *M. tuberculosis* genome and is predicted to be an essential gene. While its essentiality was clearly shown in *Mycobacterium smegmatis* (29), no direct demonstration of it has been shown in *M. tuberculosis*. We recently constructed a conditional mutant in which its structural gene (sigA) transcription was placed under transcriptional control of a repressible promoter system recently developed in our laboratory (30) and showed that upon its down-modulation the bacterial growth rapidly stops (S. Anoosheh and R. Manganelli, unpublished). Like most of the primary σ factors, its expression is rather constant during exponential growth and decreases under conditions leading to growth rate reduction such as stationary phase or hypoxia (31). Interestingly, the half-life of its mRNA was shown to be unusually long (more than 40 minutes) compared to that of the primary-like σ factor σB (2.4 minutes) (32). It is possible that in strong protein-damaging stress conditions, transcription stops, causing a quick decrease of the mRNA pool. When the intensity of stress decreases, if no functional σA is present in the cell, translation of housekeeping genes cannot restart, leading to cell death. If sigA mRNA is still present, new σA can be translated and both transcription and translation can be restored.

It was recently shown that σA has low affinity for the RNAP core enzyme, probably due to the nonoptimal structure of the σ-RNAP-binding interface; however, binding of the small protein RbpA to RNAP was recently shown to modify RNAP structure, leading to increased affinity for σA (33). RbpA, first characterized in *Streptomyces*, is an essential protein in *M. tuberculosis* (34), and transcription of its structural gene is induced in several stress conditions such as starvation (35), surface stress (36), and exposure to vancomycin (37). It has been hypothesized that in these conditions RbpA increases σA competitiveness for RNAP, guaranteeing the expression of housekeeping genes; even when following exposure to stress, other σ factors with higher affinity for RNAP

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<td>RsmA</td>
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<td>Heat shock, late stationary phase</td>
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1Reviewed in reference 10; underlined anti-σ factors were shown or hypothesized to be regulated by RIP (27). Modified from reference 25.

2Only some strains.

3Putative anti-σ factors.

4The last four genes represent putative anti-anti-σ factors.
are present in the cytoplasm (33). Recently, RbpA was shown to bind directly also to both $\sigma^\Lambda$ and $\sigma^B$, further supporting this hypothesis (38).

Due to its invariant expression and its long half-life, sigA mRNA has been used often as an invariant internal standard in quantitative reverse transcription-PCR (RT-PCR) experiments (31, 39, 40).

Even if $\sigma^\Lambda$ is principally involved in housekeeping gene expression, there are reports of its involvement in virulence modulation. Collins and collaborators (41) showed that an arginine to histidine substitution at residue 515 (R515H) of $\sigma^\Lambda$ in Mycobacterium bovis ATCC35723 totally impaired growth in guinea pigs even if not affecting extracellular growth. This mutation, mapping in region 4.2, known to interact with transcriptional activators in other microorganisms (42), was shown to prevent the binding of WhiB3, a transcriptional activator involved in oxidative stress, starvation, and during macrophage infection (43, 44). Moreover, an M. bovis whiB3 mutant was shown to have the same attenuation phenotype as an R515H M. bovis mutant, strongly suggesting that the interaction between $\sigma^\Lambda$ and WhiB3 was the cause of the observed attenuation. Interestingly, when whiB3 was deleted in M. tuberculosis H37Rv, the resulting strain was still able to infect both mice and guinea pigs, reaching the same bacterial burden of the wild type strain in their organs, even if conferring a lower pathology, suggesting a different role of $\sigma^\Lambda$/WhiB3 interaction in these species (43).

Finally, it was recently shown that in a clinical strain of the Beijing family characterized by enhanced intracellular growth, sigA was expressed at a higher level than H37Rv and other clinical strains and was induced upon macrophage infection (45). Moreover, overexpression of sigA in H37Rv resulted in better growth of the recombinant strain in both macrophages and mice lungs after aerosol infection as well as increased resistance to superoxide (45). Recently, these phenotypes were correlated to induction of eis (46). The product of this gene is an N(ε)-acyltransferase acetylating Lys55 of a JNK-specific phosphatase resulting in down-modulation of autophagy, inflammation, and cell death through inhibition of reactive oxygen species generation (47).

$\sigma^B$

$\sigma^B$ is dispensable for growth in both M. smegmatis (48) and M. tuberculosis (49) and is very similar to the C-terminal portion of $\sigma^\Lambda$. Its expression is induced in several stress conditions such as heat shock, surface stress, oxidative stress, starvation, and during macrophage infection (31, 35, 50).

In vitro transcription experiments identified two putative promoters upstream of sigB: one recognized by $\sigma^E$ and one recognized by either $\sigma^F$, $\sigma^H$, or $\sigma^L$ (51–53) (Fig. 1). However, a direct role in sigB expression in vivo has only been confirmed for $\sigma^E$, which is responsible for its basal level of transcription and for its induction following surface and oxidative stress (36, 54), $\sigma^L$ (51, 52), and $\sigma^H$ (R. Chauhan and M. L. Gennaro, personal communication). Another regulator involved in sigB regulation is MprAB, a stress-responsive two-component system activated in response to macrophage infection, surface-damaging agents, nutrient limitation, alkaline pH, and other stresses (36, 55–57) that is required for the establishment and maintenance of persistent infection in mice (58). Binding of MprA in the sigB upstream region might be required to facilitate access to the promoter of $\sigma^E$ or $\sigma^L$-containing RNAP due to the poor conservation of their consensus sequences in this region (Fig. 1).

Overexpression of M. tuberculosis sigB in M. smegmatis resulted in hyperproduction of surface hyperglycosylated polar glycopeptidolipid (GPL), a change in colony morphology, and an extended generation time (59). Since hyperproduction of polar GPL is typical of M. smegmatis carbon-starved cultures (60), this phenotype suggests a role of $\sigma^B$ in the adaptation to starvation and stationary phase (48).

Recently, we reported that an M. tuberculosis sigB knockout mutant, although not attenuated in macrophages or during mouse and guinea pig infection, is more sensitive to various stresses, such as surface-damaging agents, heat shock, and oxidative stress (37, 49). Moreover, the mutant was very sensitive to hypoxia, showing a survival about 3-log10 lower than that of the wild type or parental strain, strongly suggesting that it might be involved in survival during latent or persistent infection (49).

Two reports have been published that used DNA microarrays to characterize the $\sigma^B$ regulon. In the first, the global transcriptional profile of the wild type strain was compared to that of a strain overexpressing $\sigma^B$ (52). In the second the comparison was performed between the global transcriptional profiles of a sigB null mutant and that of its parental strain during exponential growth following exposure to the surface-damaging agent SDS or to the oxidative agent diamide (49). The overlap between the genes identified in these two studies was very low, suggesting that under stress conditions several other regulatory mechanisms may be activated and affect the expression of the regulon. Overexpression of $\sigma^B$ led to the induction of 72 genes including its own gene, genes encoding the transcriptional regulators WhiB2
and IdeR, and several ESAT-6-like proteins, ribosomal proteins, and PE-PGRS (52). However, during exponential growth only eight genes were downregulated in the \( \text{sigB} \) mutant, while 28 were upregulated, in particular \( \text{sigE} \) and several genes of its regulon, probably representing a compensatory response to the lack of \( \sigma^B \).

Following surface stress, 72 genes were downregulated in the \( \text{sigB} \) mutant; these included several genes involved in envelope stress response and several encoding transcriptional regulators such as IdeR. Finally, following oxidative stress, 40 genes were downregulated in the \( \text{sigB} \) mutant; these included genes encoding the heat shock proteins Hsp, ClpB, and DnaK, several transcriptional regulators such as FurA, as well as several proteins involved in cysteine and arginine biochemistry (49).

\( \sigma^C \)

The chromosome of \( \text{Mycobacterium leprae} \) was subjected during evolution to massive gene decay and is hypothesized to carry almost only indispensable genes. Interestingly, the only \( \sigma \) factor genes present in its genome are those encoding \( \sigma^A \), \( \sigma^B \), and the two ECF \( \sigma \) factors \( \sigma^E \) and \( \sigma^C \) (61), so it is considered very important for mycobacterial physiology and virulence. The \( \text{sigC} \) gene is present in all members of the \( \text{M. tuberculosis} \) complex as well as in the members of the \( \text{Mycobacterium avium} \) complex, \( \text{Mycobacterium marinum} \), \( \text{Mycobacterium ulcerans} \), and \( \text{Mycobacterium abscessus} \), but it is absent in \( \text{M. smegmatis} \) and other nonpathogenic mycobacterial species (6, 62).

The \( \sigma^C \) structural gene is highly expressed in \( \text{M. tuberculosis} \) during the exponential phase, but its level drops about 20-fold during stationary phase and between 5- and 10-fold following exposure of the bacterial culture to starvation or stress conditions such as heat shock, cold shock, and surface stress (31, 35), suggesting that expression of its regulon is needed during active growth and not when stressing conditions decrease or stop bacterial growth. Its transcription starts from two promoters: The dominant promoter has a typical consensus sequence for \( \sigma^A \), while the second presents a \( \sigma^C \) consensus sequence, suggesting an autoregulatory mechanism. However, transcription from this promoter was not affected in a \( \text{sigC} \) null mutant, which suggests that it is recognized by another, still unrecognized \( \sigma \) factor (52). However, no binding sites for \( \sigma^F \) upstream of \( \text{sigC} \) were found in ChIP-on-chip experiments; this suggests that the effect of \( \sigma^F \) on \( \text{sigC} \) transcription may be indirect (64, 65).

Most ECF \( \sigma \) factors are regulated at the posttranslational level by an anti-\( \sigma \) factor, and usually the genes encoding \( \sigma \) and anti-\( \sigma \) are cotranscribed. The \( \text{sigC} \) gene is not associated with any gene encoding for a putative anti-\( \sigma \) factor, suggesting that it is not posttranslationally regulated. However, a transmembrane protein of
unknown function with an anti-σ factor signature (Rv0093c) (Fig. 2) has been predicted in silico to bind σC, although it was not possible to demonstrate the interaction between these two proteins by copurification (66). Interestingly, we found that in the genomes of bacteria belonging to the M. avium complex, σC and rv0093c are fused to encode a single protein (unpublished observation) (Fig. 2 and Fig. 3). Further work is needed to understand the physiological role of Rv0093c in M. tuberculosis and the role of σC-Rv0093c fusion in the M. avium complex.

Deletion of the sigC gene from M. tuberculosis was shown to have little or no effect on growth in axenic culture or in macrophages (67, 68). However, sigC mutant–infected mice survived longer than animals infected with wild type or a complemented strain, despite the fact that the three strains were able to reach the same bacterial load in the organs of infected animals (67). Mice infected with the mutant strain showed reduced inflammatory infiltrates and had reduced levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IFN-γ in their lungs, as well as fewer infiltrating neutrophils in bronchoalveolar lavage fluid, suggesting that the lower mortality induced by the sigC mutant was due to the failure to elicit the same degree of immunopathology as the wild type (68). Finally, when used to infect guinea pigs, the sigC mutant was attenuated and did not induce the formation of necrotic granulomas (68, 69). Taken together, these data demonstrate a role for σC in pathology through the modulation of the host immune response.

DNA microarrays were used to define the effect of σC on the M. tuberculosis global transcriptional profile. About 200 genes were shown to be downregulated in the sigC mutant at different points of the growth curve (67). Some of these genes encode important virulence-associated proteins such as the α-crystalline homolog HspX (70), the response regulator MtrA, involved in cell morphology and division (71), and the sensor histidine kinase SenX, involved in sensing low phosphate levels and in regulation of the stringent response (72). However, whether σC directly regulates the expression of these genes is still not clear since their transcriptional start site was not determined and no in vitro transcription data are available.

σD

σD is posttranslationally regulated by the anti-σ factor RsdA, recently shown to be subject to proteolytic degradation by ClpX-ClpP1-ClpP2 (28). The expression of sigD decreases after exposure to several stresses such as heat and cold shock, hypoxia, and stationary phase (31), while it was shown to be slightly induced under conditions of nutrient depletion (35). Moreover, its expression is decreased during macrophage infection (50) and in a relA mutant (73). RelA is an enzyme that catalyzes the synthesis of hyperphosphorylated guanosine (p)ppGpp, a key molecule for the development of the stringent response, which has been shown to be essential for M. tuberculosis pathogenesis (73).

A mutant lacking sigD was able to grow in resting and activated macrophages at the same rate as the wild type parental strain but induced a lower level of TNF-α (74). σD was shown to be essential for virulence in mice in both H37Rv and CDC1551 backgrounds. Both mutant strains were still able to infect mice and grow in

**FIGURE 2** Comparison of conserved domains and multidomains in M. tuberculosis Rv0093c and M. avium σC. zf-HC2: Putative zinc-finger found in some anti-σ factor proteins; COG5660, predicted integral membrane protein; PRK09649, RNA polymerase sigma factor σC; Sigma70_r2, σ70 region 2; Sigma70_r4, σ70 region 4. Alignments were performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi. doi:10.1128/microbiolspec.MGM2-0007-2013.f2.
their lungs, reaching the same bacterial burden reached from wild type and complemented strains. However, both histopathology and mortality were reduced in mice infected with the mutant strain, suggesting that $\sigma^D$ regulates some bacterial component able to modulate immune response (74, 75).

DNA microarrays have been used to determine the $\sigma^D$ regulon. Calamita and colleagues (74) compared the global transcriptional profile of the sigD mutant and of its wild type parental strain in different points of the growth curve (mid-exponential, early and late stationary phase). They reported 61 genes downregulated in the sigD mutant, mostly in late stationary phase. Several of them encoded for a set of ribosome-associated proteins usually expressed in stationary phase. Similar experiments were performed by Raman and collaborators (75) in the H37Rv background. These authors analyzed the impact of the lack of $\sigma^D$ only in cultures growing in mid-exponential phase. They found 51 genes downregulated in the sigD mutant. The genes whose transcription was more affected by the lack of $\sigma^D$, beyond its own gene, were those encoding the resuscitating factor RpfC; the chaperones GroEL2, GroEL1, and GroES; and the isoniazide (INH)–inducible protein InhB. Rpf proteins, first characterized in Micrococcus luteus, are peptidoglycan hydrolases and transglycosylases that can degrade the cell wall (76) and are required for resumption of growth from stationary phase (77). The regulation of rpfC by $\sigma^D$ suggests a role for this factor in regulating cell wall structure during the entry into or emergence from hypoxia-induced dormancy. However, the complete lack of overlap between the $\sigma^D$-regulated genes and the putative $\sigma^D$ consensus sequences...
identified in the two studies, even if it may be ascribed, at least in part, to the different genetic backgrounds in which the experiments were performed and to the different experimental conditions used by the two groups, prevents us from having a clear idea of which genes really belong to the σ^D regulon.

σ^E

In M. tuberculosis sigE transcription is induced after exposure to heat shock, alkaline pH, detergents, oxidative stress, and vancomycin and during growth in low-phosphate media and in human macrophages (31, 36, 37, 50, 55, 78, 79). sigE deletion in M. tuberculosis results in several dramatic phenotypes in vitro and in vivo, underscoring the importance of this σ factor in physiology and virulence: Indeed, a sigE null mutant was shown to be more sensitive than the wild type parental strain to heat shock, to the detergent SDS, to oxidative stress (36), and to vancomycin (37), INH, streptomycin, gentamicin, and rifampin (D. Pisu and R. Manganelli, unpublished observation). It was also unable to grow in resting THP-1-derived human macrophages, where it was not able to block phagosome maturation (36) (S. Casonato and R. Manganelli, unpublished observation), and human dendritic cells (80), while its growth was not restricted in the human pneumocyte cell line A549 (Casonato and Manganelli, unpublished observation). Moreover, it was killed more efficiently by activated mouse macrophages (36). Finally, its virulence was strongly attenuated in mice (81, 82) and in guinea pigs (A. Izzo and R. Manganelli, unpublished observation). Recently, a study performed in M. smegmatis suggested σ^E as one of the major regulators involved in the development of the stringent response in this organism, demonstrating that relA induction in low-phosphate media is σ^E-dependent (78, 79). This is of particular interest since RelA and the stringent response have been recently shown to be required for mycobacterial persistence (73). Preliminary experiments strongly suggest that sigE is an essential gene in M. bovis BCG, in which we could obtain a sigE deletion only in the presence of a second copy of the gene (Casonato and Manganelli, unpublished observation). Whether sigE is essential in M. bovis or only in M. bovis BCG is still unknown.

A clear indication that σ^E is a critical node of the M. tuberculosis stress response derives from the intricate regulation of its gene expression and protein activity. Its structural gene is transcribed from three promoters (83). The first one (P1) is constitutively expressed during growth and is probably under the control of the principal σ factor σ^A; the second (P2) is induced in conditions of surface stress mediated by SDS or vancomycin and is autoregulated by σ^E (Chauhan and Gennaro, personal communication), while the third (P3) is induced following oxidative stress and is under the control of σ^H (83, 84). P2, even if autoregulated by σ^E, does not have a canonical σ^E consensus sequence and requires for its activation the two-component system MprAB, whose structural genes are under σ^E control (55, 56); interestingly, one of the two MprA binding sites upstream of P2 overlaps the P1 transcriptional start point, causing the downregulation of this promoter in stress conditions (83) (Fig. 4). It was recently reported that σ^E positively regulates ppk1 encoding for a polyphosphate kinase (S. Sanyal, S. K. Banerjee, and M. Kundu, presented at Tuberculosis 2012: Biology, Pathogenesis, Intervention Strategies, Paris, France, 2012) and that polyphosphate is the main substrate of MprB (79). So sigE is subjected at least two positive feedback loops: the first involving positive regulation of mprAB by σ^E, leading to P2 activation, and the second involving the positive regulation of ppk1 by σ^E, resulting in higher polyphosphate intracellular levels and consequently a higher rate of MprA phosphorylation by MprB and P2 activation (12).

Another peculiar feature of sigE regulation is that P3 is located inside its own open reading frame (at position +63). Using single nucleotide mutagenesis and translational fusion to lacZ, we demonstrated that the mRNA transcribed from P3 is translated starting from two noncanonical translational start codons (ATC and TTG) located downstream from P3 (Fig. 4). So σ^E can be present in M. tuberculosis in three different isoforms depending on the environmental conditions encountered from the bacteria: one of 257 aa, translated from mRNA transcribed from P1 or P2 in normal physiological conditions and after surface stress, and two nearly identical isoforms of 218 aa and 215 aa, translated from mRNA starting from P3 in conditions of oxidative stress (83). The presence of the σ^H-dependent P3 internal to the sigE open reading frame is a general feature of mycobacterial genomes.

However, the presence of two active noncanonical translational start codons in this region might be restricted to the members of the M. tuberculosis complex: While the first translational start codon (ATC) is located at the correct distance from the putative ribosome binding site (17 bp), the second one (TTG) is 9 bp downstream due to a 9-bp duplication present only in M. tuberculosis complex genomes (Fig. 4). It is possible to hypothesize that before the occurrence of the duplication, TTG represented the only active translational start
codon in this region and translational initiation from the ATC codon began only after it was moved by the duplication (83). Consequently, TTG probably still represents the only translational start codon in mycobacteria not belonging to the M. tuberculosis complex in which the 9-bp duplication did not occur.

The translation of different $\sigma^E$ isoforms in response to specific environmental conditions suggests that these isoforms may have different biological functions. One possibility is that they could recognize different consensus sequences, but this is unlikely since the domains involved in DNA binding are conserved in the three isoforms. Moreover, recent preliminary experiments designed to analyze by DNA microarrays the variation of the M. tuberculosis global transcriptional profile following chemical induction of each of the different $\sigma^E$ isoforms showed that their regulons are superimposable (R. Provvedi, E. Dainese, and R. Manganelli, unpublished data). Another possibility is that the N-terminal region of the larger $\sigma^E$ isofrom (which does not share homology with any other $\sigma$ factor) could be involved in protein stability, as already shown for $\sigma^R$ in Streptomyces coelicolor, also translated in two different isoforms, one constitutionally expressed and the other inducible by stress. In this case the inducible isofrom, containing an additional 55 amino acids, was shown to have a half-life 7-fold shorter than that of the constitutive isofrom (23).

$\sigma^E$ activity is also regulated at the posttranscriptional level by the anti-$\sigma$ factor RseA (83, 85). Its gene is located downstream of sigE but belongs to a different transcriptional unit that is constitutionally expressed (86). RseA/$\sigma^E$ interaction is disrupted in an oxidative environment due to oxidation of cysteines 70 and 73 (85). However, in conditions of surface stress RseA is phosphorylated by the Ser/Thr kinase PknB, which contains PASTA (PBP and serine/threonine kinase-associated) domains that have been hypothesized to bind peptidoglycan to serve as cell wall stress sensors (87), leading to its ClpC1P2-dependent proteolysis. Since transcription of the genes encoding this protease are positively regulated by the global regulator ClgR, a member of the $\sigma^E$ regulon, this represents a third positive feedback loop involved in $\sigma^E$ regulation (12). Figure 5 shows the complex regulatory network involved in SigE regulation.

Finally, a small antisense RNA was recently identified overlapping the 5' of the sigE coding region, suggesting the existence of posttranscriptional regulation (88). Experiments are in progress in our laboratory to confirm the role of this small RNA in sigE posttranscriptional regulation.

A recent study performed in M. smegmatis revealed bimodal distribution of relA transcription from its $\sigma^E$-dependent promoter, suggesting bistability in the upstream MprAB network (89). Tiwari and collaborators (90) recently tested this hypothesis by constructing a comprehensive mathematical model to analyze the MprAB-$\sigma^E$-RseA stress-response network for the presence of bistability. Their results demonstrated that the peculiar network architecture containing multiple positive feedback loops may lead to bistability, making it a good candidate for the persistence switch.

The impact of $\sigma^E$ in the M. tuberculosis transcription profile was first analyzed using DNA microarrays in the absence of specific stimuli inducing sigE and following exposure to SDS-induced surface stress. In the absence of
stress 38 genes were shown to be differentially regulated in the mutant. The most affected gene in these conditions was \textit{sigB}, which was strongly repressed in the \textit{sigE} mutant. However, 23 genes belonging to 13 putative transcriptional units failed to be induced in the \textit{sigE} mutant after exposure to stress (36). Among these were \textit{sigB} and the gene encoding the global regulator ClgR. Interestingly, at least five genes involved in lipid catabolism (\textit{aceA}, \textit{gltA1}, \textit{fadB2}, \textit{fadE23}, and \textit{fadE24}) were found to be part of the \textit{sigE} regulon. Of particular interest, the first two are involved in the glyoxylate shunt and the methylcitrate cycle, and these metabolic pathways are critical for the metabolic remodeling associated with stress-induced growth arrest of \textit{M. tuberculosis} (91) and are required for persistence and virulence of \textit{M. tuberculosis} (92). The regulation of these two genes was recently further characterized, showing that in both cases it is subject to a parallel feed-forward loop with an AND input function. The \textit{iclI} loop is directly controlled by \textit{\sigma^E}, while the \textit{gltA1} loop is controlled by \textit{\sigma^E} through \textit{\sigma^B}. Each loop requires a local regulator (\textit{lrpI} for \textit{iclI} and \textit{LprG} for \textit{gltA1}) produced from a gene adjacent to the corresponding effector gene and transcribed from \textit{\sigma^E} (lrpI) or \textit{\sigma^B} (lrpG) (93). The presence in the \textit{\sigma^E} regulon of genes involved in fatty acid degradation also suggests that they not only are involved in the metabolic exploitation of the fatty acids, but also may be responsible for degradation and detoxification of the fatty acids that accumulate following the inhibition of biosynthesis of cell surface lipids or damage to the fatty acid–rich envelope of \textit{M. tuberculosis}. The fact that \textit{fadE23} and \textit{fadE24} were also induced in cells exposed to isoniazid, an inhibitor of mycolic acid biosynthesis (94), supports this hypothesis.

Finally, another operon shown to require \textit{\sigma^E} for its induction after surface stress is that including \textit{clgR}, \textit{rv2744c}, and \textit{rv2745c}. While, as already mentioned, the first gene encodes a global transcriptional regulator involved in one of the positive feedback loops regulating

![Diagram](image-url)
Sigma Factors: Key Molecules in *M. tuberculosis* Physiology and Virulence

*sigE* (12, 85, 95), *rv2744c* encodes a protein highly homologous to PspA (phage shock protein A), a protein involved in homeostasis of the cell membrane and maintenance of proton motive force in Gram-negative bacteria (96), while *rv2745c* encodes a transmembrane protein of unknown function. Recently, *Rv2744c* was shown to be a substrate of PepD (57), a protease whose structural gene is induced by $\sigma^\epsilon$, which is involved in processing misfolded proteins, leading to the activation of the MprAB/$\sigma^\epsilon$ signaling pathways (57).

The $\sigma^\epsilon$ regulon was also studied during infection in THP-1-derived human macrophages. Beyond confirming several genes previously described as $\sigma^\epsilon$ dependent, it was possible to identify other genes as $rmlB2$, encoding a putative galactose epimerase that might be involved in the linking of peptidoglycan and mycolic acid and *cyp121*, encoding a cytochrome P450, probably involved in detoxification of fatty acids (97). In the same study the transcriptional response of the macrophage to *M. tuberculosis* infection was analyzed, showing that the $\sigma^\epsilon$ regulon is involved in the modulation of the inflammatory response.

A role of the $\sigma^\epsilon$ regulon in the modulation of the immune response has also been observed in human dendritic cells in which infection with the *sigE* mutant increased the production of IL-10 about 10-fold compared with infection with the wild type parental strain (80). Finally, *sigE* mutant–infected mice, despite the lower bacterial burden in their organs, were shown to produce more protective factors such as IFN-γ, TNF-α, iNOS, and β-defensins than animals infected with the parental or complemented mutant strain (98), suggesting that the *sigE* mutant might induce strong protection against *M. tuberculosis* infection. In support of this hypothesis, when the *sigE* mutant was used to vaccinate mice, it was able to confer better protection against *M. tuberculosis* challenge than *Mycobacterium bovis* BCG. Interestingly, this was more evident when vaccinated mice were challenged with a hypervirulent Beijing strain (98).

$\sigma^F$

$\sigma^F$ was initially studied in *M. bovis* BCG; in this species it was shown to be induced in several experimental conditions such as anaerobiosis, cold shock, oxidative stress, and nutrient depletion and after entry into stationary phase. Moreover, its expression increased after exposure to several antibiotics, suggesting a role for $\sigma^F$ in the basal level of sensitivity to drug treatment (99, 100). Despite the identity of *sigF* and its upstream region in *M. bovis* BCG and *M. tuberculosis*, in this latter species it was possible to demonstrate *sigF* induction only in response to nutrient depletion (31, 35).

It was recently shown that in *M. smegmatis* $\sigma^F$ is required for resistance to hydrogen peroxide, heat shock, and acidic pH (101) and to regulate the biosynthesis of a surface-associated lipidic pigment (102). Moreover, an *M. smegmatis* *sigF* null mutant had a transformation efficiency four orders of magnitude higher than that of the wild type parental strain, suggesting $\sigma^F$ involvement in the regulation of cell wall permeability (102).

A *sigF* null mutant of *M. tuberculosis* CDC1551 reached higher density than the wild type strain and did not show any lag phase after dilution into fresh medium, suggesting that $\sigma^F$ might be involved in negative control of bacterial growth (103), but did not show any sensitivity against several stress conditions such as heat shock, cold shock, hypoxia, and long-term stationary phase. The mutant was found to be more resistant to rifampin than the wild type strain, and this resistance was hypothesized to be the result of reduced permeability to hydrophobic solutes due to a reduced production of cell wall-associated sulpholipids (103). However, characterization of a *sigF* mutant of *M. tuberculosis* H37Rv did not confirm any of these phenotypes, suggesting either that $\sigma^F$ has different roles in these two strains or that subtle differences in experimental conditions result in different impacts of $\sigma^F$ in *M. tuberculosis* physiology (104). Experiments run in parallel with the two mutant strains are required to discriminate between these hypotheses.

The CDC1551 *sigF* mutant virulence is clearly attenuated in a mouse infection model, with lower bacterial burden and histopathology in the lungs at the late stage of infection (105). However, no difference from the wild type was shown during infection of human monocytes (103). Finally, a *sigF* mutant of H37Rv was found to be attenuated in guinea pigs (69).

Several transcriptome analyses have been performed using different approaches to reveal the $\sigma^F$-dependent genes in strains CDC1551 and H37Rv: (i) Geiman and collaborators (105) compared the transcriptional profiles of a CDC1551 *sigF* mutant with that of the wild type parental strain in different phases of growth using DNA microarrays. They found 38 genes downregulated in the *sigF* mutant in exponential phase and 187 in early and 277 in late stationary phase, suggesting a major role of $\sigma^F$ in the adaptation to stationary phase. Several $\sigma^F$-dependent genes in stationary phase were involved in the biosynthesis and degradation of surface (lipo) polysaccharides or in the biosynthesis and structure of the cell envelope. In addition, genes expressing tran-
scriptional regulators of the TetR, GntR, and MarR families, usually controlling the expression of efflux pumps in other bacteria were found to be differentially regulated. (ii) Williams and collaborators (106) used a CDC1551 strain in which a copy of sigF was placed downstream of the inducible acetamidase promoter to compare its transcriptional profile with that of the control strain at different times after addition of acetamide. The genes differentially regulated following sigF overexpression included those encoding several cell-associated proteins such as members of the MmpL, PE, and PPE families. The most upregulated gene was phoY1, encoding a transcriptional regulator probably involved in regulation of phosphate uptake. (iii) Homerova and collaborators (107) used an E. coli two-plasmid system to identify M. tuberculosis promoters recognized by σF-RNAP polymerase, identifying five σF-dependent promoters containing sequences highly similar to the previously identified σF-dependent promoter of usfX1 (108). (iv) Hartkoorn and collaborators (64) recently published a study in which both chromatin immunoprecipitation (ChIP-on-chip) and microarray analysis were used to identify σF-dependent genes using an H37Rv recombinant strain in which sigF was inducible by the addition of pristinamycin IA. Integrating the two data sets, it was possible to identify 16 σF-binding sites corresponding to RNA transcripts in the sense orientation and 9 σF-binding sites associated with antisense transcripts. Beyond its own structural gene, σF was found to directly regulate genes encoding proteins involved in lipid and intermediary metabolism and virulence (such as HbHa, which is involved in extrapulmonary dissemination) and transcriptional regulators such as Rv2884 and PhoY1. From these data it was possible to clearly determine the σF consensus sequence: GGTNT-N(15–17)-GGGTA. For 26 genes found to be overexpressed following sigF induction, it was not possible to demonstrate a σF-binding region by ChIP-on-chip, suggesting an indirect role of σF in their regulation.

The overlap of genes reported to be σF-dependent in this study with those reported by Geiman and collaborators (105) (1/99) and Williams and collaborators (106) (7/70) was quite small, further supporting the hypothesis that CDC1551 and H37Rv might have different physiological traits or that experimental conditions may result in dramatic changes in the impact of σ factors in global gene regulation.

σF activity is regulated at the posttranslational level by a complex anti- and anti-anti-σ factor network (Fig. 6). Beaucher and collaborators (108) demonstrated that UsfX is an anti-σ factor able to specifically bind to prevent its interaction with the RNAP core enzyme. They also demonstrated the presence of two anti-anti-σ factors: RsfA and RsfB are able to interact with UsfX, preventing its binding to σF. RsfA was hypothesized to be regulated by redox potential because it was able to bind UsfX only in reducing conditions, while RsfB was hypothesized to be regulated by phosphorylation because a mutation mimicking phosphorylation prevented its binding to UsfX (108). In Bacillus subtilis two anti-σ factors belonging to the same family of UsfX (RsbW and SpoIIAB) can phosphorylate their anti-anti-σ factor, leading to their inactivation. However, UsfX was shown to have several unfavorable substitutions in its kinase domain (109) and to be unable to phosphorylate both RsfA and RsfB (110), suggesting the evolution of a different kind of regulation of the UsfX/RsfA and UsfX/RsfB interaction in M. tuberculosis. Interestingly, RsfB was shown to be phosphorylated by the Ser/Thr protein kinase E (PknE), suggesting a role of this protein in the

FIGURE 6 Main regulatory network responsible for SigF regulation. Arrows indicate transcriptional regulation; truncated lines indicate posttranslational regulation. At least four other proteins—Rv0516c, Rv1364c, Rv1904, and Rv2638—have been hypothesized to be involved in this network, but their role has not been elucidated yet. doi:10.1128/microbiolspec.MGM2-0007-2013.f6.
regulation of UsfX/RsfB interactions. At least four other putative anti-anti-σ factors were predicted to interact with either UsfX or σF or both in a yeast-two-hybrid system: Rv0516c, Rv1364c, Rv1904, and Rv2638 (111). Among these, Rv0516c was shown to be phosphorylated by PknD, and Rv1904 by PknE (110). Of particular interest, sequence analysis predicted the presence of a phosphatase domain, an anti-σF domain, and an anti-anti-σF domain in the protein encoded by rv1364c. While the anti-σF domain showed some unfavorable substitutions inactivating its kinase activity, the other domains retained their activity (109). However, the roles of these four proteins in σF regulation have not yet been confirmed.

σG

Attention focused on sigG after the demonstration of its strong induction after macrophage infection suggested its importance in adaptation to intracellular growth and virulence (112). The effect of sigG deletion was studied in two different genetic backgrounds (CDC1551 and H37Rv), generating contrasting results and suggesting that, despite the high conservation of genome sequences, σ factors may have distinct roles in different M. tuberculosis strains (113, 114).

The presence in the sigG upstream region of a sequence similar to the recA promoter (115) suggested the possibility that this σ factor might be involved in the SOS response. Moreover, sigG was shown to be induced by mitomycin C, a DNA-damaging agent able to induce the SOS response in H37Rv (116). However, when a CDC1551 sigG mutant was exposed to this DNA-damaging agent, it showed marked resistance, suggesting that σG might act as a repressor of the SOS response, in apparent contradiction with the induction of its structural gene in the presence of DNA damage (113). Microarray analysis showed a mild downregulation of lexA (0.6-fold), encoding the main repressor of the genes belonging to the SOS regulon, and a mild upregulation of recA (1.5-fold), which might be the reason for mitomycin C resistance in this strain. Other genes differentially expressed in the sigG mutant were sigH (repressed) and sigD (induced), as well as several genes of their regulons (113). However, which of these effects was due to direct regulation by σG or from an indirect effect was not addressed. Finally, the sigG mutant was shown to be severely affected for growth in macrophages, again not easily reconciling with a resistance to DNA damage (113).

Smollett and collaborators (114) characterized a sigG mutant in the H37Rv genetic background. While they could confirm its induction by several DNA-damaging agents, they failed to demonstrate a different sensibility of the mutant strain to these compounds. Moreover, comparing the global transcriptional profiles of the sigG mutant and wild type in the presence and absence of DNA-damaging compounds, they could not find any genes expressed differently between the two strains and concluded that σG is not involved in the regulation of RecA-dependent or -independent genes in response to DNA damage in M. tuberculosis H37Rv.

Due to the opposite conclusions obtained from the two studies, it is difficult to hypothesize a role for this σ factor. That it could have such different roles in two strains of the same bacterial species is possible but unlikely, given the extreme conservation of the genome sequences in M. tuberculosis. Further studies performed in parallel with the mutants in the two genetic backgrounds are needed to shed some light on these intriguing findings.

σH

The gene encoding σH is induced by heat shock and oxidative stress in M. smegmatis, M. avium subsp. paratuberculosis, and M. tuberculosis (54, 84, 117, 118), where it was found to be induced also during macrophage infection (119). Interestingly, it was suggested that the lack of a functional sigH gene might be responsible for the temperature sensitivity of M. leprae (120). σH is posttranslationally regulated by the cytoplasmic anti-σ factor RshA, while its structural gene is subjected to autoregulation. The RshA-σH complex is disrupted in vitro by elevated temperature and in oxidizing conditions (121). The presence in RshA of a HX₃CX₂C motif suggested its inclusion in the family of the zinc-associated anti-σ factor (ZAS). However, as was recently shown for S. coelicolor RsmA, in RshA this motif coordinates a 2Fe-2S cluster (122, 123), enabling responses to oxidative/reductive stress at a much faster rate than Zn. Finally, both σH and RshA are phosphorylated by PknB, a eukaryotic-like serine/threonine protein kinase involved in the regulation of cell wall synthesis and cell morphology. Phosphorylation of RshA, but not of σH, was shown to affect the σH/RshA interaction, resulting in decreased binding of σH by RshA, thus adding a further level of regulation (124). The σH ortholog in S. coelicolor σK was shown to be translated from two translational start sites, resulting in two isoforms: a shorter one, constitutively expressed and with a half-life longer than 70 minutes, and a stress-inducible longer one, whose half-life was about 10 minutes. The presence of two putative translational start sites and of the same regulatory mechanism was
hypothesized for several members of the mycobacterial genome including *M. tuberculosis* and demonstrated in *M. smegmatis* (23).

Genes regulated by σ^H^ were identified in different phases of growth or following diamide-mediated oxidative stress by comparing the global transcriptional profile of σ^H^ mutants obtained in two different genetic backgrounds (CDC1551 and H37Rv) with that of their parental strains (54, 121, 125). σ^H^ was shown to modulate the expression of about 180 genes, at least 31 of them in a direct manner. Beyond its own structural gene, σ^H^ was found to regulate the genes encoding the two σ factors σ^E^ and σ^B^, DNA repair proteins, general stress proteins, enzymes involved in cysteine and molybdenum biosynthesis, and enzymes involved in thiol metabolism such as thioredoxin and thioredoxin reductase. In *S. coelicolor* the ortholog of σ^H^ was shown to be involved in the synthesis of mycothiol (126), a molecule involved in an alternative pathway to reduce intracellular disulfide bonds typical of actinomycetes, which do not synthesize glutathione (127). Even if no direct evidence of σ^H^ involvement was found in the mycothiol biosynthesis in *M. tuberculosis*, one of the σ^H^-dependent most highly induced genes following diamide exposure (Rv2466c) contains a putative glutaredoxin active site (54), suggesting its involvement in the mycothiol cycle.

The dynamic nature of the σ^H^-mediated response to oxidative stress was explored by comparing the variation of the global transcriptional profile of a sigH mutant and of its parental strain at different times after a short exposure to diamide. The expression of sigH, and sigE, reached their peak 30 minutes after diamide exposure and then declined to reach the background level after 120 minutes. Several stress-related genes regulated by these σ factors followed a similar dynamic. Interestingly, a large number of genes involved in lipid biosynthesis were downregulated at early time points following oxidative stress, while their expression resumed at later time points (128).

The consensus sequence recognized by σ^H^ was clearly determined in these studies and found to be very similar to that previously proposed for σ^E^ (36, 125), suggesting the possibility of an overlap between the regulons of these σ factors. Song and collaborators (53), using mutational and primer extension analyses, confirmed that σ^E^ and σ^H^ recognize nearly identical promoters. The sixth position of -35 was shown to have a critical role for discrimination between promoters preferentially recognized by σ^E^ and σ^H^.

The phenotype of different sigH null mutants was extensively studied and shown to be more sensitive to oxidative stress and heat shock (54, 84) but still able to replicate in THP-1-derived macrophages and to resist the bactericidal activity of activated murine macrophages (54). The sigH mutants were still able to infect mice, reaching the same bacterial burden of that reached from their wild type parental strains (54, 125). However, despite the same bacterial burden as those infected with the wild type strain, mice infected with the sigH mutant showed decreased immunopathology, lower recruitment of CD4 and CD8 T cells to the lung in the early stages of infection, and lower mortality, suggesting that the antioxidant activity of the σ^H^ regulon might have a role in modulating the interaction of the pathogen with the immune system (125). This hypothesis was recently reinforced by the finding that an *M. bovis* BCG mutant defective in oxidative stress response was more immunogenic (129).

A sigH mutant was also used to infect primary rhesus macaque bone marrow–derived macrophages. Cells infected with the mutant showed higher levels of expression of β-chemokines and several apoptotic markers relative to cells infected with the wild type parental strain, while the mutant exhibited reduced survival after 72 hours of infection (130). The sigH mutant was also shown to be severely attenuated in a rhesus macaque aerosol infection model. In this case, beyond a longer survival and a lower immunopathology, animals infected with the sigH mutant also showed a lower bacterial burden in the lungs than those infected with the wild type parental strain (131).

Recently, the role of σ^H^ in oxidative stress and virulence was also shown in *M. avium* subspecies *para-tuberculosis*, suggesting that its role is conserved in different members of the mycobacterial genus (132).

σ^I^

sigI is induced in stationary phase and after either heat (133) or cold shock (31). At least in stationary phase its induction probably depends on σ^I^ (known to be induced in this growth phase), which has been shown to recognize a consensus sequence upstream of sigI (134). Recently, an *M. tuberculosis* CDC1551 sigI mutant was constructed, and its global transcriptional profile was analyzed in comparison with that of its parental strain. The σ^I^ regulon included genes encoding an ATP synthase (Rv1304), several heat shock proteins (Rv0440, Rv0350, and Rv3417), and KatG (133). Using *in vitro* transcription, the authors demonstrated that σ^I^ is directly responsible for initiation of transcription at both *katG* promoters. Downregulation of *katG* in the sigI mutant suggested the possibility, confirmed by the
authors, that this strain is resistant to isoniazide, which requires KatG for its activation. Finally, the mutant was used to infect mice and, surprisingly, despite the lower katG expression, the mutant showed a hypervirulent phenotype, probably mediated from an increased CREB phosphorylation and TNF-α secretion from infected macrophages (133). Usually, resistance to INH is due to mutations in katG and has a high fitness cost that can be alleviated by compensatory mutations in the ahp gene (encoding a peroxidase) (135). The possibility of a sigI mutant acquiring INH resistance without loss of fitness may represent an alternative pathway toward INH resistance.

σJ

σJ is the least-well-characterized M. tuberculosis σ factor. Its expression is strongly induced in late stationary phase (136) and in human macrophages (113). However, neither the survival in late stationary phase, nor the pathogenicity in a model of intravenous mouse infection ever, neither the survival in late stationary phase, nor the pathogenicity in a model of intravenous mouse infection was affected in a sigJ null mutant (137), suggesting that despite its induction in stationary phase and in macrophages, σJ is not involved in pathogenicity or adaptation to prolonged stationary phase cultures. However, the mutant was more sensitive to H2O2 than the wild type parental strain, suggesting a role in the defense from reactive oxygen species. Using an E. coli two-plasmid system, it was demonstrated that sigJ can be transcribed by σJ (134). However, no genome-wide study has been performed to characterize the σJ regulon.

σK

This σ factor is present in all members of the M. tuberculosis complex and in M. marinum, M. ulcersans, and Mycobacterium gilvum, but it is absent in other mycobacteria such as M. leprae (in which it is a pseudogene), M. avium, M. smegmatis, and Mycobacterium kansasii (6, 138).

In 2005 the known decreased expression of the two antigenic proteins MPT70 and MPT83 observed in some strains of M. bovis BCG was associated with a polymorphism in the sigK gene, resulting in a mutation in its translation start codon (139). DNA microarrays were used to determine the global transcriptional profile of an M. bovis BCG strain containing the mutation impairing σK translation complemented with a wild type copy of sigK, leading to the identification of two chromosomal loci containing σK-regulated genes.

The first locus included sigK and rskA, together with genes encoding a putative amine oxidase, a cyclopropane-fatty-acyl-phospholipid synthase, and four other proteins of unknown function. The second locus included genes encoding the surface-associated lipoprotein MPT83; the integral membrane proteins Rv2877c, DipZ, and Rv2876; and finally the secreted proteins MPT70 and MPT53 (139). MPT83 and MPT70 have similar C-terminal domains structurally related to the FAS1 domain, mediating interactions between cells and the extracellular matrix, and might be involved in binding to host cell proteins (140). MPT53 is a secreted DsbE-like protein probably involved in the formation of disulfide bonds in unfolded secreted proteins (141). Rv2877c and DipZ (whose genes are also under σK control) have a conserved CcdA domain, usually contained in proteins involved in the transfer of a reducing potential from the cytoplasm to secreted disulfide bond isomerases (142, 143). Their function might be to maintain proper disulfide bond formation in proteins in the periplasmic space of M. tuberculosis.

The expression of MPT70 and MPT83 is different in M. tuberculosis (low) and M. bovis (high). Said-Salim and collaborators (144) identified the anti-σ factor responsible for σK posttranslational regulation (Rv0444c, RskA) and analyzed the sequence of its structural gene in both M. bovis and M. tuberculosis. The results clearly showed that the high level of MPT70 and MPT83 production in M. bovis was due to a mutation in rskA disrupting the negative regulation of σK and resulting in its constitutive activation. It is not clear if the over-expression of MPT70 and MPT83 give a selective advantage to M. bovis in its natural hosts or if it is the result of a “founder effect.” However, the presence of the same mutation in Mycobacterium caprae and of independent mutations in the same gene in the strain infecting the oryx support the idea that high levels of MPT70 and MPT83 might provide a selective advantage when infecting members of the Bovidae. RskA was recently shown to be subject to RIP in conditions of oxidative stress induced by metal chelator phenanthroline (27).

Evolutionary analysis of the σK regulon showed the presence in several bacteria related to mycobacteria of the minimal set of genes mpt83-sigK-rskA, which might represent the nucleus of the σK regulon. Before the separation between Rhodococcus and Mycobacterium this minimal region was subjected to the insertion of six genes that separated mpt83 from sigK and rskA. During the evolution of mycobacteria, the locus was separated into two loci, the sigK-rskA locus and the mpt70/83 locus. In slow-growing mycobacteria an additional gene, dipZ, was inserted between the two mpt83 paralogs. Finally, it was shown that σK and RskA from the envi-
ronmental species *M. gilvum* can complement the activity of their orthologs of *M. tuberculosis*, suggesting that although its regulon varies considerably across species, the regulatory system $\sigma^K$/RskA is conserved across the *Mycobacterium* genus ([138]).

$\sigma^L$

The gene encoding sigL is transcribed from two promoters, the first being constitutive and the second under $\sigma^L$ control ([145]). While sigL mRNA was shown to be constantly expressed during the growth curve ([145]), when the sigL upstream region was used to drive the expression of lacZ, $\beta$-galactosidase activity increased with culture density, principally in the transition from lag to exponential phase ([51]). Like several other $\sigma$ factors, $\sigma^L$ is posttranslationally regulated by an anti-$\sigma$ factor: RslA ([51]). This is a transmembrane protein that in reducing environments binds Zn$^{2+}$ and sequesters $\sigma^L$. In oxidative environments the cysteines present in the CXXC motif of RslA form a disulfide bridge. This results in the release of Zn$^{2+}$ and a conformational change of RslA, leading to a strong decrease in RslA affinity for $\sigma^L$ and its subsequent release ([146]). Moreover, RslA was recently shown to be subject to RIP in conditions of oxidative stress induced by metal chelator phenanthroline ([27]).

Two independently obtained sigL mutants of *M. tuberculosis* H37Rv were shown to be attenuated in the intravenous or the aerosol murine infection model ([51, 145]). As shown for other *M. tuberculosis* mutants, the sigL mutants were not impaired for growth in the murine organs but produced a lower pathology and a lower mortality than wild type and complemented strains. When analyzed for in vitro phenotypes, only a very small, but reproducible, sensitivity to the superoxide generator plumbagine and the detergent SDS was detected ([51]).

Since the environmental conditions able to activate $\sigma^L$ are still unknown, two similar strategies were implemented to define its regulon by DNA microarrays. Hahn and collaborators ([145]) constructed an *M. tuberculosis* merodiploid strain by integrating in the chromosome of H37Rv a copy of sigL expressed from an acetamide-inducible promoter. Dainese and collaborators ([51]), however, introduced a copy of sigL with its physiologic regulatory region in the chromosome of a strain in which both sigL and rslA were deleted. In this strain, the presence of $\sigma^L$ in the absence of its negative regulator RslA led to an overexpression of both sigL and the members of its regulon. The first approach identified 18 genes overexpressed in the merodiploid strain upon induction of sigL including four two-gene operons (sigL-rslA, mpt53-rv2877c, pkS10-rks7, and rv1138c-rv1139c) and four genes encoding for PE_PGRS proteins ([145]). With the alternative approach Dainese and collaborators ([51]) identified 27 induced genes, included in 13 putative transcriptional units, 6 of which showed a $\sigma^L$ consensus sequence in their upstream regions. Induction of pkS10 and pkS7 was confirmed, but in this case all the other genes of the operon were identified (pkS8, pkS17, pkS9, and pkS11), strongly suggesting $\sigma^L$ involvement in the biosynthesis or phthiocerol dimycocerosic acid (PDIM) (a component of the cell envelope that is essential for virulence) ([147]). However, a sigL null mutant of *M. tuberculosis* is not deficient in PDIM synthesis ([145]), and recent *in vitro* characterization of fadD26-ppsA-E suggests that this locus is sufficient for PDIM biosynthesis ([148]); the nature of the lipids produced by this *pkS* gene cluster remain to be determined.

In addition, induction of rv1138c-rv1139c (as well as rv1137c) and mpt53 was confirmed. The function of Rv1138c and Rv1139c is still unknown, but Rv1139c has an isoprenyl-cysteine carboxymethyl transferase domain ([25, 149]), which in eukaryotes is involved in the posttranslational modification of prenylated proteins. Rv1138c, however, is predicted to be an aromatic ring monoxygenase. Other genes showed to be under $\sigma^L$ control are mmpL13a and mmpL13b, probably involved in fatty acid transport ([150]) and sigB.

$\sigma^M$

Expression of sigM was reported to be constant during bacterial growth. Induction was observed after heat shock and in late stationary phase ([151]). However, since sigA mRNA (used as an internal control) is known to decrease in stationary phase ([21]), the strength of this result is questionable. $\sigma^M$ is posttranslationally regulated by the anti-$\sigma$ factor RsmA, recently shown together with RslA and RskA to be subject to RIP in conditions of oxidative stress induced by metal chelator phenanthroline ([27]). A CDC1551 mutant in which sigM was deleted did not show any phenotype when exposed to different stress conditions. Moreover, it was not attenuated for growth in macrophages or in a murine model of infection, suggesting that this $\sigma$ factor is not involved in stress response or virulence development ([151]). The regulon of $\sigma^M$ was assessed in both CDC1551 and H37Rv genetic backgrounds using strains overexpressing sigM by an acetamide-inducible promoter ([152, 153]). Several genes were found to be induced following $\sigma^M$ overexpression, including those encoding PPE1, a
nonribosomal peptide synthetase, and proteins of unknown function. Interestingly, the most evidently up-regulated genes in both strains were those encoding the type VII secretion system (T7SS) ESX-4, including EsxT and EsxU, two secreted proteins belonging to the WXG100 family typically associated with these secretion systems. It is also notable that the genes encoding two other proteins belonging to this family, but whose genes are not physically associated with any T7SS gene cluster, EsxE and EsxF, were also shown to be induced.

Recently, sigM was shown to be induced together with the genes encoding the ESX-4 T7SS and the genes encoding EsxE and EsxF, in response to the induction of whiB5, encoding a transcriptional regulator of the WhiB family ([154]). A whiB5 null mutant was more sensitive to S-nitrosoglutathione (GSNO) and was less metabolically active following prolonged starvation. Most importantly, it was attenuated in a murine model of progressive infection and was not able to resume growth after reactivation from chronic infection ([154]). Whether some of these phenotypes were due to the effect of the lack of WhiB5 on sigM and its downstream genes is unknown; this will be the subject of future investigations.

Finally, several genes involved in PDIM biosynthesis were shown to be induced in the sigM mutant, suggesting that σM might have a negative regulatory effect on PDIM biosynthesis. Quantitative analysis of nonpolar surface lipids produced from the sigM mutant and its wild type parental strains confirmed this hypothesis ([153]).

**CONCLUDING REMARKS**

Since infection is a dynamic process, we predict that *M. tuberculosis* adaptation involves continuous modulation of its global transcriptional profile in response to the changing environment. In the last few years the 13 sigma factors encoded in its genome have been subject to extensive characterization and were shown to be fundamental players in this process. Null mutants for each of them were constructed, with the exception of sigA (being...
an essential gene, only a conditional mutant was obtained) (10, 24). The mutants missing sigB, sigJ, or sigM did not show any virulence attenuation (49, 137, 151, 153), the sigI mutant showed a hypervirulent phenotype (133), while no experiments assessing virulence have been reported to date for the sigK mutant. All the other mutants were attenuated in mice or in macrophages (10, 24). However, the lack of attenuation in mice does not exclude that σB, σI, and σM can be involved in virulence, given the limitations of this animal model. For example, the sigB mutant, even if still able to replicate in macrophages and virulent in mice, was shown to be very sensitive to hypoxia (49), a condition believed to be present in human granulomas and hypothesized to be important for the development of dormancy and subsequently latent infection. So it is possible to hypothesize that σB might be involved in the development of latent, rather than active, infection and that this phenotype was not discovered, due to the animal model used in its characterization. The central position of sigB in the most complex regulatory network ever described in mycobacteria involving σE, σF, σI, and probably σL (Fig. 7) strongly suggests its importance in M. tuberculosis physiology.

Interestingly, mutants missing σC, σD, σH, or σI were still able to replicate in mouse lungs at the same rate as the wild type but induced less inflammation and histopathology, suggesting their involvement in the complex interaction between M. tuberculosis and the immune system (54, 67, 74, 75, 125, 145). Since tissue damage induced by the immune system is of great importance in tuberculosis progression and transmission, understanding the reason for the lower pathogenic potential of these strains might be of great help to further understand M. tuberculosis pathogenicity and to design a new strategy of intervention against tuberculosis. However, the sigE and sigF mutants’ growth in mouse lungs was impaired, suggesting a different role for their regulon in pathogenesis (81, 105). It is notable that in infected lungs the sigE mutant was shown to induce stronger production of protective factors such as IFN-γ, TNF-α, iNOS, and β-defensins than the wild type, despite a lower bacterial load, suggesting that its regulon might be involved in the down-modulation of the host response (98). In this case, a full characterization of this occurrence might help to better understand M. tuberculosis pathogenicity and help to design better vaccines against tuberculosis. Accordingly, mouse vaccination with the sigE mutant conferred better protection than M. bovis BCG from challenge with virulent M. tuberculosis (98).

Since 1995, the year in which the first papers on mycobacterial σ factors were published (41, 155, 156), their characterization has been one of the more challenging and productive areas in the mycobacterial field. After 18 years, most of them have been extensively characterized and their role in M. tuberculosis physiology and virulence highlighted. Nonetheless, the complexity of the σ factors’ role in the global regulation of transcription is still far from fully understood, principally due to their complex regulation, the overlap of some of their regulons, and the existence of regulatory networks including several σ factors. In the last few years the first papers studying mycobacterial σ factor networks using systems biology and mathematical modeling were published (89, 90), laying the tracks for a more comprehensive understanding of their role in the physiology and virulence of one of the most ancient enemies of humankind.

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REFERENCES

Sigma Factors: Key Molecules in *M. tuberculosis* Physiology and Virulence


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82. Ando M, Yoshimatsu T, Ko C, Converse PJ, Bishai WR. 2003. Deletion of Mycobacterium tuberculosis sigG sigma factor E results in delayed...
Sigma Factors: Key Molecules in M. tuberculosis Physiology and Virulence


