Characterization of *Mycobacterium smegmatis* sigF mutant and its regulon: overexpression of SigF antagonist (MSMEG_1803) in *M. smegmatis* mimics sigF mutant phenotype, loss of pigmentation, and sensitivity to oxidative stress

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Abstract

In *Mycobacterium smegmatis*, sigF is widely expressed during different growth stages and plays role in adaptation to stationary phase and oxidative stress. Using a sigF deletion mutant of *M. smegmatis* mc²155, we demonstrate that SigF is not essential for growth of bacterium. Deletion of sigF results in loss of carotenoid pigmentation which rendered increased susceptibility to \( \text{H}_2\text{O}_2 \) induced oxidative stress in *M. smegmatis*. SigF modulates the cell surface architecture and lipid biosynthesis extending the repertoire of SigF function in this species. *M. smegmatis* SigF regulon included variety of genes expressed during exponential and stationary phases of growth and those responsible for oxidative stress, lipid biosynthesis, energy, and central intermediary metabolism. Furthermore, we report the identification of a SigF antagonist, an anti-sigma factor (RsbW), which upon overexpression in *M. smegmatis* wild type strain produced a phenotype similar to *M. smegmatis* mc²155 ΔsigF strain. The SigF-anti-SigF interaction is duly validated using bacterial two-hybrid and pull down assays. In addition, anti-sigma factor antagonists, RsfA and RsfB were identified and their interactions with anti-sigma factor were experimentally validated. Identification of these proteins will help decode regulatory circuit of this alternate sigma factor.
Introduction

*Mycobacterium smegmatis*, a fast-growing saprophytic environmental bacterium, is used as a surrogate to study mycobacterial physiology and gene regulation as it is easy to culture in laboratory conditions. Owing to its habitat, *M. smegmatis* encounters more diverse conditions than its pathogenic counterparts and consequently its genome (6.98 Mb) has expanded nearly twice to the size of *M. tuberculosis* (4.4 Mb) to accommodate more genes. There is an unusual expansion of several genes which have acquired many paralogs unlike in other mycobacterial species (Waagmeester et al. 2005). There are 28 sigma factor genes in *M. smegmatis* in contrast with 13 reported in *M. tuberculosis* (Cole et al. 1998; Waagmeester et al. 2005; Rodrigue et al. 2006) and there are seven paralogs of sigma factor *sigH*, which are differentially expressed in *M. smegmatis* (Waagmeester et al. 2005; Singh and Singh 2009). Sigma factors reversibly associate with RNA polymerase and allow them to specifically direct the expression of specific set of genes. *M. smegmatis* genome encodes one of each group I, II, and III sigma factors represented by *SigA*, *SigB*, and *SigF*, respectively, and 25 of group IV sigma factors (Kapopoulou et al. 2011). *SigA*, the primary sigma factor in both *M. smegmatis* and *M. tuberculosis*, is essential for bacterial viability (Gomez et al. 1998), while *SigB*, the primary-like sigma factor is very similar to *SigA* and is dispensable for growth in *M. smegmatis* (Fontán et al. 2009). *SigF* (group III) and extracytoplasmic function (ECF) sigma factors (group IV) constitute alternate sigma factors which enable adaptation to a range of external and internal stimuli. Locus for *sigA*, *sigB*, *sigD*, *sigE*, *sigF*, *sigG*, and *sigH* are well conserved in *M. smegmatis* and *M. tuberculosis* (Sachdeva et al. 2010).

Earlier, the *sigF* was reported as a late-stage specific sigma factor, present only in the genomes of slow-growing pathogenic mycobacteria (DeMaio et al. 1996, 1997). *M. tuberculosis* *sigF* was found strongly induced within cultured human macrophages, during stationary phase of growth, upon exposure to cold shock, nutrient starvation, and several antibiotics (Graham and Clark-Curtiss 1999; Michele et al. 1999; Betts et al. 2002). *M. tuberculosis* Δ*sigF* strain grew to a threefold higher density in stationary phase than the wild-type strain (Chen et al. 2000), but showed almost similar sensitivity to heat shock, cold shock, and hypoxia relative to the parental strain (Geiman et al. 2004; Hartkoorn et al. 2010). *M. tuberculosis* Δ*sigF* strain was attenuated for virulence in a mouse infection model despite persistence at high bacterial load in lungs compared with the isogenic wild type (Geiman et al. 2004). Overexpression of *sigF* in *M. tuberculosis* resulted in the differential regulation of many cell wall-associated proteins and other genes involved in the biosynthesis and degradation of surface polysaccharides and lippolysaccharides, believed to play important roles in host-pathogen interactions (Williams et al. 2007; Hartkoorn et al. 2010). However, we earlier demonstrated that, *sigF* is conserved in all the mycobacterial species analyzed and proposed that apart from regulating the expression of virulence genes in pathogenic mycobacteria, *SigF* is likely to play more roles in mycobacterial physiology (Singh and Singh 2008).

In *M. smegmatis*, *sigF* is widely expressed during different growth stages (Singh and Singh 2008). *M. smegmatis* *sigF* is transcriptionally induced in response to nutrient depletion, cold shock and upon exposure to agents that damage cell wall architecture, like SDS and antibiotics, isoniazid, and ethambutol (Singh and Singh 2008; Gebhard et al. 2008). A *sigF* mutant of *M. smegmatis* ATCC 607 strain showed higher transformation efficiency, lack of carotenoid pigmentation, and increased susceptibility to hydrogen peroxide mediated oxidative stress (Provedi et al. 2008). *SigF* in *M. smegmatis* plays role in adaptation to stationary phase, heat, and oxidative stress (Hümpel et al. 2010). While both these studies demonstrate the role of *M. smegmatis* SigF in oxidative stress, molecular basis of this increased sensitivity to hydrogen peroxide remains unclear. Furthermore, proteins involved in posttranslation regulation of *M. smegmatis* SigF activity are not characterized, making it difficult to define the regulation circuitry of this alternate sigma factor. Using an insertion deletion mutant of *M. smegmatis* mc² 155 *sigF*, we demonstrate that SigF in *M. smegmatis* modulates the cell surface architecture and lipid biosynthesis, extending the repertoire of SigF function in this species. We also demonstrate that the increased sensitivity of the *sigF* mutant to H₂O₂ mediated oxidative stress is primarily due to loss of the carotenoid pigment. Furthermore, we report the identification of a SigF antagonist, an anti-sigma factor (RsbW), which upon overexpression in *M. smegmatis* wild type strain produced a phenotype similar to *M. smegmatis* mc²155 Δ*sigF* strain. The SigF-anti-SigF interaction was duly confirmed using bacterial two-hybrid system and pull down assay. In addition, anti-sigma factor antagonists, RsfA and RsfB were identified and their interactions with anti-sigma factor were verified using two-hybrid system.

Results and Discussion

Construction of *Mycobacterium smegmatis* *sigF* knockout mutant and its complementation

The *sigF* deletion (Δ*sigF*) mutant was created by replacing *sigF* ORF with the hygromycin (*hyg*) resistance cassette and molecularly validated (see supplemental material,
Fig. S1) as detailed in methods. One of the ΔsigF mutants referred as SFKO1 has been studied and described throughout this manuscript. The SFKO1 was complemented with the sigF gene, cloned downstream of hsp60 promoter, at an ectopic locus in the SFKO1 genome. The sigF complemented strain is designated as SFKO1/sigF.

**Role of SigF in stress responses**

The effect of sigF deletion on in vitro growth was monitored by comparing the growth of the SFKO1 strain to the wild type *M. smegmatis*. Both strains were allowed to grow in different media for extended length of time; the sigF mutant strain grew slightly faster than the wild type, attained higher cell density with reduced lag phase, but displayed similar growth characteristics afterwards till extended stationary phase of growth (data not shown). This suggests that the sigF is dispensable for the growth of *M. smegmatis* under normal physiological conditions. These results are in line with the earlier findings (Provvedi et al. 2008).

SigF has been described as a stress-response sigma factor in slow-growing mycobacteria (DeMaio et al. 1996). Previously, we had shown that sigF is transcriptionally induced in *M. smegmatis* in response to cold shock, nutrient starvation and after treatment with SDS and antimycobacterial drugs like isoniazid and ethambutol (Singh and Singh 2008). We examined whether SigF is required for survival of *M. smegmatis* during these stress conditions. No significant differences in survival were noticed between the sigF mutant and the wild type strain under these stress conditions (data not shown). Gebhard et al. (Gebhard et al. 2008) had reported that SigF is required for survival against heat shock and acidic stress in *M. smegmatis*. We did not test the acidic stress but upon heat shock no appreciable difference in survival of sigF mutant was noticed in comparison to the wild type strain. We checked the sigF deletion mutants of both *M. smegmatis* mc²155 (SFKO1) and *M. smegmatis* ATCC 607 strains. One of the reasons of this difference could be the temperature as we tested the survival, based on our earlier studies (Singh and Singh 2008, 2009), at 45°C while they used 50°C for heat stress in their studies.

But, similar to earlier findings (Provvedi et al. 2008), the sigF deletion mutant was found to be more susceptible than the wild type strain upon exposure to hydrogen peroxide mediated oxidative stress (Fig. 1A). Complemented strain (SFKO1/sigF) restored the survival after oxidative stress. Since, sigF was not found to be induced upon oxidative stress in previous studies (Singh and Singh 2008), we examined the sigF expression at RNA and protein level after treatment with hydrogen peroxide. No difference in the sigF expression level was noticed upon oxidative stress using log phase and stationary phase.

![Figure 1](image-url)
cultures (Fig. 1B and C). This suggests that SigF indirectly regulates H$_2$O$_2$ sensitivity in *M. smegmatis*.

**Loss of carotenoid pigment renders increased H$_2$O$_2$ sensitivity to the sigF mutant**

Disparate response to oxidative stress was reported in saprophytic and pathogenic mycobacteria (Sherman et al. 1995). Saprophytes like *M. aurum* and *M. smegmatis* produce carotenoids, which are known scavengers of free radicals (Levy-Frebault and David 1979) and enhance the strength of the cell wall due to their lipophilic nature and intercalation into the cell membrane (Kubler and Baumeister 1978). *M. smegmatis* mc$^2$155 colonies produce pale yellow pigment (carotenoid isorenieratene) when incubated under light for 5–6 days. Deletion of *sigF* resulted in loss of pigmentation in SFKO1 (Fig. 2A) which was mostly restored after complementation with the *sigF* gene (SFKO1/sigF) (Fig. 2A), suggesting that the loss of pigmentation is specifically due to deletion of *sigF*. Because carotenoids are robust antioxidants and fortifiers of cellular barriers they are deemed beneficial for withstanding the stress bear by saprophyte like *M. smegmatis*. Since, we did not find the appreciable differences in the *sigF* expression after peroxide mediated oxidative stress despite the marked sensitivity of the Δ*sigF* mutant to H$_2$O$_2$, we reasoned that this phenotypic characteristic of the *M. smegmatis* Δ*sigF* mutant might be due to absence of carotenoids in the mutant. Moreover, the key detoxifying enzymes of reactive oxygen species in mycobacteria, *katG* and *ahpC* were found to be SigF independent (Gebhard et al. 2008; Hümpel et al. 2010). To test our hypothesis, we treated *M. smegmatis* mc$^2$155 cells with diphenylamine (DPA), a known inhibitor of carotenogenesis in mycobacteria (Houssaini-Iraqui et al. 1993), and subjected the DPA-treated bacterial cells to hydrogen peroxide mediated oxidative stress. The DPA-treated bacteria showed pronounced sensitivity to oxidative stress, comparable to *M. smegmatis* Δ*sigF* mutant strain (Fig. 2B). This was duly confirmed when SFKO1/crt strain apart from restoring the pigmentation (Fig. 2A) showed a significant recovery in survival following hydrogen peroxide mediated oxidative stress akin to SFKO1/sigF strain (Fig. 2B).

Carotene isorenieratene is the characteristic pigment of almost all orange-pigmented mycobacteria including *M. phlei* (Goodwin and Jamikorn 1956, 1956), *M. aurum* (Levy-Frebault and David 1979), *M. avium*, and *M. intracellulare* (Tarnok and Tarnok 1970, 1970). The synthesis of isorenieratene requires farnesyl pyrophosphate as a precursor, which leads to isorenieratene in five metabolic steps involving, CrtE, CrtB, CrtR, CrtY, and CrtU (Provvedi et al. 2008). It was postulated that in the absence of SigF, transcription of *crt* operon is off, hence SFKO1 mutant lacks pigmentation. Evidently, *crt* transcript was found to be several-fold downregulated in SFKO1 mutant.
in comparison to wild type strain (Fig. 2C) and the expression (Fig. 2C) as well as pigmentation (Fig. 2A) were restored, almost to the wild type level, in the complemented SFKO1/sigF strain. In *M. smegmatis* genome, a carotenogenic gene cluster comprises six open reading frames, *crtIBYcYdUV*, each transcribed in the same direction. The GGPP synthase gene, *crtE*, was found far away from the *crt* locus. The upstream regions of *crtI* gene harbored a canonical SigF promoter signature (Provvedi et al. 2008). When *crt* locus genes were over-expressed in SFKO1/crt strain, SFKO1/crt akin to SFKO1/sigF, restored the pigmentation (Fig. 2A) which was lost due to *sigF* deletion, suggesting that the SigF directly regulates the carotenoid biosynthesis and thereby the pigmentation of bacterial colonies in *M. smegmatis*. These results established that in *M. smegmatis* SigF confers resistance to hydrogen peroxide mediated oxidative stress largely through the carotenoid pigments.

**SigF modulates cell wall architecture by affecting GPL distribution and lipid biosynthesis**

Previously, in *M. smegmatis*, we observed increased *sigF* expression upon exposure to isoniazid, ethambutol, and SDS (Singh and Singh 2008). Isoniazid and ethambutol specifically target cell wall biosynthesis process in mycobacteria, whereas SDS is an ionic detergent that affects the cell wall architecture. Overexpression of *sigF* in *M. tuberculosis* was reported to alter the regulation of many cell wall-associated proteins, suggesting a role for SigF in maintaining cell wall architecture in mycobacteria (Forrellad et al. 2013). To examine the effect of *sigF* deletion on the cell wall architecture in *M. smegmatis*, we performed transmission electron microscopy using *M. smegmatis* WT and ΔsigF mutant cells. In *M. smegmatis*, GPLs constitute the major cell-surface glycolipids and react with ruthenium red to give the electron-dense appearance to the outermost cell envelope layer (Etienne et al. 2002). We noticed uniform distribution of GPLs on the surface of WT cells (Fig. 3A), while ΔsigF mutant cells displayed patchy GPLs distribution (Fig 3B). Next, we analyzed the total GPLs in wild type and ΔsigF mutant by TLC and mass analysis (see supplemental material, Fig. S2), but no difference was found in GPLs profile of ΔsigF mutant, suggesting that the uneven distribution of GPLs in the ΔsigF mutant cells is not due to difference in overall content and type of GPLs. Then, we examined the profiles of other cell wall lipids. TLC analysis of polar lipids also did not reveal any differences (data not shown), but nonpolar lipids showed distinct TLC profiles. Lipids spots present in wild type cells (Fig. 4A and C) were conspicuously missing in ΔsigF mutant cells (Fig. 4B and D). We also noticed distinct differences in trehalose containing lipids (Fig. 4E and F), an important component for cell wall integrity, indicating that the SigF alters the cell wall lipid composition by modulating the lipid biosynthesis pathway.

**Genome-wide gene expression studies of Mycobacterium smegmatis ΔsigF mutant and wild-type strains**

A genome-wide gene expression analysis of the *M. smegmatis* mc2155 WT and ΔsigF mutant strains was performed using Agilent microarray platform. SigF-regulated genes during exponential phase and stationary phase were indentified, as described in the methods. Difference in the expression of a gene was calculated as the ΔsigF mutant to WT expression ratio and is expressed as fold-change; only ≥ 2-fold difference in the gene expression (*P* ≤ 0.05) was considered for analysis. Under these conditions, 142 genes in exponential phase and 158 genes in stationary phase were found to be significantly down-regulated in the ΔsigF mutant. A large number of genes showed

![Figure 3.](image) Transmission electron micrographs showing structure of cell envelope of *M. smegmatis* wild type (A) and ΔsigF mutant (B) strains. Note the even distribution of GPLs around wild type cells while distribution of GPLs is patchy in mutant cells.
reduced expression in both exponential and stationary phase cells, and almost similar numbers of genes were found to be down-regulated exclusively in exponential and stationary phase cells (Table 1). We also identified enhanced expression of 39 genes in exponential phase cells and 49 genes in stationary phase cells in ΔsigF mutant strain. The entire expression data can be found in Data set S1 in the supplemental material. To validate the microarray results, real-time PCR was performed on few randomly selected genes from microarray data. Similar to microarray results, the selected genes showed reduced expressions in real-time PCR experiment (see supplemental material, Fig. S3) as well.

The SigF promoter consensus in M. smegmatis was first identified in silico (Provvedi et al. 2008), and was later improved upon by experimental data (Gebhard et al. 2008; Provvedi et al. 2008; Hümpel et al. 2010). Using an improved SigF promoter consensus from later studies, 1200 bp upstream of the annotated start codon of the down-regulated genes (Table 1) were visually checked for sequence similarities. We searched 1200 bp upstream sequence because several genes were arranged in gene clusters wherein the SigF consensus

Figure 4. 2D TLC analysis of nonpolar lipids from Mycobacterium smegmatis wild type (A, C, E) and ΔsigF mutant (B, D, F). Different solvent systems, described in methods, were used to develop TLC plates: A and B developed with solvent system B, C, and D developed with solvent system C, E, and F developed with solvent system D. The arrows indicate the missing fatty acids (FA) in ΔsigF mutant (B and D) and TMM (Trehalose monomycolate), TDM (Trehalose dimycolate) in panel F.
| Locus          | Description                                           | Fold-change Exponential/Stationary | SigF consensus | Position from start codon |
|---------------|-------------------------------------------------------|-----------------------------------|----------------|----------------------------|
| **Commonly down-regulated genes (P ≤ 0.05) in exponential and stationary phase** | | | | |
| MSMEG_0266²   | Arginine decarboxylase                                | −4.44/−5.90                      | GTCG-N¹⁻GGGAT  | 160                        |
| MSMEG_0267²   | Esterase                                              | −5.49/−4.58                      | GTTT-N₄⁻GGGTA  | 27                         |
| MSMEG_0278⁴   | Hypothetical protein                                 | −2.22/−2.90                      | GGTG-N₁⁻GGGCC  | 158                        |
| MSMEG_0280⁴   | Alpha/beta hydrolase                                 | −1.93/−4.30                      | GGTG-N₁⁻GGGCC  | 158                        |
| MSMEG_0375⁴   | Phospholipase D family protein                        | −3.98/−2.91                      | GTTC-N₁⁵⁻GGGCA | 192                        |
| MSMEG_0451⁴   | Oxidoreductase, FAD-linked                            | −4.69/−3.40                      | GTTC-N₁⁵⁻GGGCC | 49                         |
| MSMEG_0521⁴   | Conserved hypothetical protein                        | −2.42/−1.76                      | GTTT-N₃⁻GGGTA  | 10                         |
| MSMEG_0637⁴   | Iron-sulfur binding oxidoreductase                    | −6.02/−3.33                      | GTCG-N₁⁵⁻GGGCA | 548                        |
| MSMEG_0669⁴   | Hypothetical protein                                 | −5.44/−2.52                      | GTTC-N₁⁵⁻GGGCC | 661                        |
| MSMEG_0670⁴   | FAD dependent oxidoreductase                          | −2.06/−3.17                      | GTTT-N₁⁻GGGTA  | 9                          |
| MSMEG_0671⁴   | S-(hydroxymethyl) glutathione dehydrogenase          | −3.75/−4.97                      | GTTT-N₁⁻GGGTA  | 47                         |
| MSMEG_0672⁴   | Conserved hypothetical protein                        | −1.73/−3.73                      | GTTT-N₁⁻GGGTA  | 50                         |
| MSMEG_0684⁴   | Aldehyde dehydrogenase and xanthine dehydrogenase    | −5.15/−5.17                      | GTTT-N₁⁻GGGTA  | 11                         |
| MSMEG_0685⁴   | Oxidoreductase, molybdopterin-binding subunit         | −5.09/−5.49                      | GTTT-N₁⁻GGGTA  | 8                          |
| MSMEG_0686²   | Oxidoreductase                                        | −3.87/−3.26                      | GTTT-N₁⁻GGGTA  | 8                          |
| MSMEG_0696⁴   | Alanine-rich protein                                 | −4.86/−5.90                      | GTTT-N₁⁻GGGAA  | 58                         |
| MSMEG_0697⁴   | Integral membrane protein                            | −4.37/−4.66                      | GTTT-N₁⁻GGGAA  | 50                         |
| MSMEG_1076⁴   | Conserved hypothetical protein                        | −5.82/−2.58                      | GTTT-N₁⁻GGGTA  | 50                         |
| MSMEG_1097⁴   | Glycosyl transferase, group 2 family protein          | −5.63/−5.01                      | GTTT-N₁⁻GGGTA  | 11                         |
| MSMEG_1112⁴   | Aconitate hydratase, putative                         | −5.96/−5.32                      | GTTT-N₁⁻GGGAA  | 8                          |
| MSMEG_1131⁴   | Tryptophan-rich sensory protein                       | −5.33/−4.80                      | GTTT-N₁⁻GGGTA  | 9                          |
| MSMEG_1315⁴   | Transporter                                           | −4.17/−2.56                      | GTTT-N₁⁻GGGTA  | 11                         |
| MSMEG_1361⁴   | Alpha-mannosidase                                     | −2.20/−2.13                      | GTCG-N₁⁹⁻GGGTG | 541                        |
| MSMEG_1605⁴   | PhoU                                                  | −2.50/−3.21                      | GTCG-N₁⁵⁻GGGTG | 22                         |
| MSMEG_1758⁴   | Hypothetical protein                                 | −4.54/−2.95                      | GTTT-N₁⁻GGGTA  | 8                          |
| MSMEG_1766⁴   | Conserved hypothetical protein                        | −5.28/−6.24                      | GTTT-N₁⁻GGGAA  | 32                         |
| MSMEG_1767⁴   | Conserved hypothetical protein                        | −5.70/−6.46                      | GTTT-N₁⁻GGGAA  | 32                         |
| MSMEG_1768⁴   | Conserved hypothetical protein                        | −5.50/−6.05                      | GTTT-N₁⁻GGGAA  | 32                         |
| MSMEG_1769⁴   | UsfY protein                                          | −5.91/−4.14                      | GTTT-N₁⁻GGGAA  | 64                         |
| MSMEG_1770⁴   | Conserved hypothetical protein                        | −5.89/−3.34                      | GTTT-N₁⁻GGGCA  | 64                         |
| MSMEG_1771⁴   | Methylase, putative                                   | −6.17/−5.69                      | GTTT-N₁⁻GGGTA  | 29                         |
| MSMEG_1772⁴   | Conserved hypothetical protein                        | −5.91/−6.43                      | GTTT-N₁⁻GGGTA  | 696                        |
| MSMEG_1773⁴   | Conserved hypothetical protein                        | −5.98/−4.09                      | GTTT-N₁⁻GGGAA  | 11                         |
| MSMEG_1774⁴   | Conserved hypothetical protein                        | −6.17/−4.13                      | GTTT-N₁⁻GGGAA  | 64                         |
| MSMEG_1775⁴   | Cytochrome P450 monoxygenase                          | −3.84/−3.01                      | GTTT-N₁⁻GGGTA  | 9                          |
| MSMEG_1777⁴   | UsfY protein                                          | −4.98/−4.63                      | GTTT-N₁⁻GGGTA  | 69                         |
| MSMEG_1778⁴   | Conserved hypothetical protein                        | −3.16/−5.02                      | GTTT-N₁⁻GGGTA  | 69                         |
| MSMEG_1779⁴   | Hypothetical protein                                 | −3.64/−4.24                      | GTTT-N₁⁻GGGTA  | 69                         |
| MSMEG_1780⁴   | Hypothetical protein                                 | −3.06/−4.79                      | GTTT-N₁⁻GGGTA  | 69                         |
| MSMEG_1781⁴   | Hypothetical protein                                 | −5.71/−6.29                      | GTTT-N₁⁻GGGA  | 69                         |
| MSMEG_1782²   | Oxidoreductase, dehydrogenase/reductase               | −5.87/−6.15                      | GTTT-N₁⁻GGGTA  | 221                        |
| MSMEG_1783⁴   | Hypothetical protein                                 | −3.48/−3.45                      | GTTT-N₁⁻GGGTA  | 183                        |
| MSMEG_1784²   | Type I topoisomerase                                  | −3.40/−3.46                      | GTTT-N₁⁻GGGTA  | 183                        |
| MSMEG_1787⁴   | RsbW protein                                          | −3.10/−5.90                      | GTTT-N₁⁻GGGTA  | 56                         |
| MSMEG_1788⁴   | Conserved hypothetical protein                        | −3.80/−3.37                      | GGTG-N₁⁻GGGCA  | 32                         |
| MSMEG_1789⁴   | Conserved hypothetical protein                        | −6.02/−6.41                      | GGTG-N₁⁻GGGCA  | 32                         |
| MSMEG_1790⁴   | Conserved hypothetical protein                        | −5.79/−6.31                      | GGTG-N₁⁻GGGCA  | 32                         |
| MSMEG_1792⁴   | Conserved hypothetical protein                        | −3.47/−4.44                      | GGTG-N₁⁻GGGCA  | 158                        |
| MSMEG_1794⁴   | Dehydrogenase                                        | −5.60/−5.72                      | GGTG-N₁⁻GGGTA  | 15                         |
| MSMEG_1801²   | Hypothetical protein                                 | −1.69/−4.23                      | GGTG-N₁⁻GGGAA  | 173                        |
| MSMEG_1802²   | Chb protein                                          | −4.71/−4.70                      | GGTG-N₁⁻GGGAA  | 63                         |
| MSMEG_1804²   | RNA polymerase sigma-F factor                         | −5.96/−5.79                      | GTTT-N₁⁻GGGAA  | 1001                       |
| MSMEG_1853²   | Na⁺H⁺ antiporter NhaA                                 | −2.14/−2.15                      | GTTT-N₁⁻GGGTA  | 99                         |
| MSMEG_1950⁴   | Conserved hypothetical protein                        | −5.96/−4.67                      | GTTT-N₁⁻GGGCA  | 354                        |
| MSMEG_1951⁴   | Conserved domain protein                              | −5.70/−5.50                      | GTTT-N₁⁻GGGCA  | 1001                       |
| MSMEG_2112²   | Secreted protein                                     | −2.09/−1.58                      | GTTT-N₁⁻GGGTA  | 24                         |
| Locus          | Description                                           | Fold-change | SigF consensus | Position from start codon |
|---------------|-------------------------------------------------------|-------------|---------------|----------------------------|
| MSMEG_2115    | Conserved hypothetical protein                        | −4.09/−5.02 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2343    | Methylesterase (part of crt locus, 2343–2347)         | −5.70/−5.37 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2344    | Dehydrogenase                                         | −5.01/−5.09 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2345    | Lycopene cyclase                                      | −5.62/−6.23 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2346    | Phytene synthase                                      | −5.80/−6.21 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2347    | Phytene dehydrogenase                                 | −5.66/−4.48 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2376    | Conserved hypothetical protein                         | −4.29/−5.23 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2415    | Hemerythrin HHE cation binding region                 | −1.45/−4.80 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2594    | Asparagine synthase (glutamine-hydrolyzing)          | −2.17/−3.76 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2837    | Nitrate reductase NarB                                | −4.43/−3.16 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2913    | Conserved hypothetical protein                         | −3.91/−3.09 | GTTT-N_{15}GGGTA |                            |
| MSMEG_2924    | Permease-binding-protein component                    | −5.78/−3.65 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3022    | Glycine betaine/carnitine/choline transport           | −5.77/−5.79 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3083    | ABC transporter, permease protein OpuCB               | −4.94/−4.06 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3141    | Conserved domain protein                              | −3.03/−1.90 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3255    | DdoX subfamily, putative                              | −3.16/−5.18 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3289    | gp61 protein                                          | −5.33/−5.60 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3304    | Succinate semialdehyde dehydrogenase                  | −4.71/−5.73 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3310    | Integral membrane protein                             | −3.40/−2.13 | GTTT-N_{15}GGGCA |                            |
| MSMEG_3311    | Acoyl carrier protein                                 | −2.52/−8.83 | GTCA-N_{15}GGGAA |                            |
| MSMEG_3418    | Conserved hypothetical protein                         | −3.73/−2.54 | GTCA-N_{15}GGGAA |                            |
| MSMEG_3419    | Hypothetical protein                                  | −5.54/−4.43 | GTCA-N_{15}GGGAA |                            |
| MSMEG_3439    | Hypothetical protein                                  | −5.55/−4.00 | GTTT-N_{15}GGGTA |                            |
| MSMEG_3444    | Hypothetical protein                                  | −1.31/−3.85 | GTTT-N_{15}GGAT |                            |
| MSMEG_3536    | Sugar transport protein                               | −3.75/−2.48 | GTTG-N_{15}GGAA |                            |
| MSMEG_3673    | 4-alpha-glucanotransferase                            | −1.35/−4.92 | GTTT-N_{15}GGCA |                            |
| MSMEG_4707    | Nonhaem bromoperoxidase                               | −2.68/−1.51 | GTTT-N_{15}GGAT |                            |
| MSMEG_4918    | 1,4-alpha-glucan branching enzyme                     | −2.21/−2.54 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5188    | Caax amino protease family                            | −3.92/−3.01 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5189    | Oxidoreductase                                        | −3.42/−3.69 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5342    | Conserved hypothetical protein                         | −5.55/−5.21 | GTTT-N_{15}GGCA |                            |
| MSMEG_5399    | ATP-dependent DNA helicase RecQ                       | −3.01/−3.11 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5400    | Dehydrogenase                                         | −4.36/−2.19 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5401    | Conserved hypothetical protein                         | −3.58/−5.87 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5402    | Dehydrogenase DhgA                                    | −5.99/−4.80 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5496    | MscS Mechanosensitive ion channel                     | −3.78/−3.41 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5540    | Conserved hypothetical protein                         | −2.59/−3.24 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5542    | Transcriptional regulator, HTH_3 family               | −4.82/−4.69 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5543    | Hypothetical protein                                  | −5.13/−5.91 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5590    | Carboxylate-amine ligase                              | −5.48/−3.09 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5605    | Cytochrome bd ubiquinol oxidase, subunit I            | −2.07/−3.47 | GTTG-N_{15}GGGAA |                            |
| MSMEG_5616    | Glyoxalase/bleomycin resistance protein               | −4.87/−1.79 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5617    | Immunogenic protein MPT63                              | −3.63/−5.99 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5799    | Nucleoside-diphosphate-sugar epimerase                | −6.69/−3.76 | GTTT-N_{15}GGGTA |                            |
| MSMEG_5826    | Pyruvate decarboxylase                                | −3.78/−3.79 | GTTT-N_{15}GGGTA |                            |
| MSMEG_6211    | Hypothetical protein                                  | −4.39/−4.12 | GTTT-N_{15}GGGTA |                            |
| MSMEG_6212    | Hemerythrin HHE cation binding domain                 | −5.43/−3.87 | GTTT-N_{15}GGGTA |                            |
| MSMEG_6213    | Manganese containing catalase                         | −4.18/−5.96 | GTTT-N_{15}GGGTA |                            |
| MSMEG_6232    | Catalase KatA                                         | −5.95/−5.17 | GTTT-N_{15}GGGTA |                            |
| MSMEG_6305    | Conserved hypothetical protein                         | −5.04/−2.49 | GTTT-N_{15}GGGAA |                            |
| MSMEG_6354    | Serine esterase, cutinase family                      | −4.67/−5.88 | GTTT-N_{15}GGGAA |                            |
| MSMEG_6355    | Hypothetical protein                                  | −5.39/−4.85 | GTTT-N_{15}GGGAC |                            |
Table 1. (Continued)

| Locus            | Description                                      | Fold-change | SigF consensus | Position from start codon |
|------------------|--------------------------------------------------|-------------|----------------|---------------------------|
| MSMEG_6467a      | Starvation-induced DNA protecting protein         | −5.72/−5.55 | GTTC-N_{15}GGGCA | 100                       |
| MSMEG_6501       | Hypothetical protein                              | −3.17/−2.95 | GTGC-N_{15}GGGCC | 1008                      |
| MSMEG_6514       | Trehalose synthase-fused maltokinase              | −1.98/−2.75 | GTGT-N_{16}GGGTA | 10                        |
| MSMEG_6515       | Trehalose synthase                                | −2.03/−2.58 | GTGT-N_{16}GGGTA | 10                        |
| MSMEG_6606       | Hypothetical protein                              | −3.15/−2.45 | GTTC-N_{14}GGGCA | 10                        |
| MSMEG_6607       | Hypothetical protein                              | −3.51/−2.52 | GTTC-N_{14}GGGCA | 10                        |
| MSMEG_6608       | Hypothetical protein                              | −4.87/−2.97 | GTTC-N_{14}GGGCA | 10                        |
| MSMEG_6609       | Hypothetical protein                              | −5.21/−4.43 | GTTC-N_{14}GGGCA | 10                        |
| MSMEG_6610       | Protein of unknown function DUF58                 | −5.51/−5.51 | GTTC-N_{15}GGGCA | 10                        |
| MSMEG_6612       | ATPase, MoxR family                               | −6.20/−4.25 | GTTC-N_{15}GGGCA | 147                       |
| MSMEG_6615       | Hypothetical protein                              | −5.50/−4.25 | GTTC-N_{15}GGGCA | 10                        |
| MSMEG_6616       | S-(hydroxy)methylglutathione dehydrogenase        | −4.83/−4.93 | GTNT-N_{15}GGGTA | 20                        |
| MSMEG_6644       | Methylene tetrahydrofolate reductase family       | −5.52/−3.67 | GTTT-N_{15}GGGAA | 462                       |
| MSMEG_6655a      | Integral membrane protein                         | −1.42/−4.25 | GTTT-N_{15}GGGAA | 8                         |
| MSMEG_6667       | Conserved hypothetical protein                     | −4.80/−3.43 | GTTT-N_{15}GGGAA | 10                        |
| MSMEG_6672       | Amino acid permease-associated region             | −6.51/−6.32 | GTTG-N_{15}GGGTA | 1171                      |
| MSMEG_6678       | Conserved hypothetical protein                     | −5.57/−4.75 | GTTG-N_{15}GGGTA | 165                       |
| MSMEG_6679       | Putative oxidoreductase YbdC                      | −2.93/−2.09 | GTTG-N_{18}GGGTA | 462                       |
| MSMEG_6685       | ABC-2 type transporter superfamily                | −2.52/−3.69 | GTTG-N_{18}GGGTA | 238                       |
| MSMEG_6686       | ABC transporter, ATP-binding protein               | −3.91/−3.99 | GTTG-N_{18}GGGTA | 61                        |
| MSMEG_6687       | Mycobacteric acid synthase                        | −3.39/−2.59 | GTTG-N_{18}GGGTA | 58                        |
| MSMEG_6688a      | Halogenase                                        | −4.51/−5.79 | GTTG-N_{16}GGGTA | 9                         |
| MSMEG_6689       | Transporter                                       | −4.11/−2.93 | GTTG-N_{16}GGGAT | 649                       |
| MSMEG_6812       | Major facilitator superfamily                     | −1.86/−2.46 | GTTG-N_{14}GGGGA | 22                        |

Genes exclusively down-regulated in exponential phase (P ≤ 0.05)

| Locus            | Description                                      | Fold-change | SigF consensus | Position from start codon |
|------------------|--------------------------------------------------|-------------|----------------|---------------------------|
| MSMEG_0482       | Dihydroxy-acid dehydratase                        | −2.67/1.40  | GTTC-N_{15}GGGTA | 1171                      |
| MSMEG_0586       | STAS domain, putative                             | −2.76/0.43  | GTTC-N_{15}GGGTA | 1171                      |
| MSMEG_0651       | Putative conserved exported protein               | −2.21/0.74  | GTTC-N_{15}GGGTA | 1171                      |
| MSMEG_0757       | Hypothetical protein                              | −2.22/0.79  | GTTC-N_{15}GGGTA | 1171                      |
| MSMEG_1114       | Short chain dehydrogenase                         | −2.07/1.31  | GTGC-N_{15}GGGGA | 155                       |
| MSMEG_1656       | Exodeoxyribonuclease III                         | −2.34/−0.06 | GTGC-N_{15}GGGCC | 20                        |
| MSMEG_1912       | Muconolactone delta-isomerase 1                   | −3.08/−0.96 | GTTT-N_{15}GGGCA | 348                       |
| MSMEG_2024       | Hydroxymethyl glutaryl-CoA lyase                  | −2.90/−0.07 | GTTG-N_{15}GGGCA | 66                        |
| MSMEG_2425a      | Ammonium transporter                              | −2.17/0.13  | GTTG-N_{17}GGGTA | 238                       |
| MSMEG_3137       | Oxidoreductase                                    | −2.33/1.77  | GTGG-N_{14}GGGGA | 992                       |
| MSMEG_3401       | LambYCsF family protein                          | −2.68/−0.38 | GTTG-N_{14}GGGGA | 992                       |
| MSMEG_3402       | Cytosine permease, putative                       | −2.39/0.65  | GTTG-N_{14}GGGGA | 992                       |
| MSMEG_3403       | Formamidase                                       | −3.48/0.58  | GTTG-N_{14}GGGTA | 1004                      |
| MSMEG_3417       | Conserved hypothetical protein                     | −4.63/−1.19 | GTGG-N_{15}GGGTA | 402                       |
| MSMEG_3541       | Cytochrome C biogenesis protein                   | −4.19/0.11  | GTTG-N_{15}GGGGA | 676                       |
| MSMEG_3562       | 4-carboxymuconolactone decarboxylase              | −2.41/0.96  | GTTG-N_{14}GGGGA | 757                       |
| MSMEG_3583       | Monoxygenase                                      | −2.72/0.51  | GTTG-N_{14}GGGGA | 470                       |
| MSMEG_3560       | Conserved hypothetical protein                     | −2.33/0.91  | GTTG-N_{14}GGGGA | 676                       |
| MSMEG_3572       | Peptidase M52, hydrogen uptake protein            | −3.24/1.02  | GTTG-N_{14}GGGGA | 1004                      |
| MSMEG_3924       | Peptidase M52, hydrogen uptake protein            | −3.24/1.02  | GTTG-N_{14}GGGGA | 1004                      |
| MSMEG_3928       | [NiFe] hydrogenase, alpha subunit, putative       | −2.49/1.28  | GTGC-N_{14}GGGTA | 345                       |
| MSMEG_3929       | [NiFe] hydrogenase, delta subunit, putative       | −2.51/0.76  | GTTG-N_{16}GGGCC | 150                       |
| MSMEG_3945       | Universal stress protein family                   | −2.60/0.40  | GTTG-N_{16}GGGCC | 571                       |
| MSMEG_3983       | L-carnitine dehydratase                           | −2.35/1.16  | GTTG-N_{14}GGGGA | 992                       |
| MSMEG_4329       | Propionyl-CoA carbonylase beta chain              | −2.36/−0.49 | GTTG-N_{16}GGGCC | 1037                      |
| MSMEG_4424       | Endoribonuclease L-PSP                           | −3.48/1.03  | GTTG-N_{16}GGGCC | 1037                      |
| MSMEG_4618       | Isocitratase family protein                       | −3.08/0.79  | GTTG-N_{14}GGGTA | 361                       |
| MSMEG_5100       | Pyruvate ferredoxin/flavodoxin oxidoreductase     | −3.82/0.72  | GTTG-N_{15}GGGGA | 361                       |
| MSMEG_5180       | Conserved hypothetical protein                     | −2.41/−0.84 | GTTG-N_{14}GGGTA | 233                       |
| MSMEG_5341       | Dipeptidyl aminopeptidase                         | −2.22/0.91  | GTTG-N_{14}GGGTA | 361                       |
| MSMEG_5343a      | Conserved hypothetical protein                     | −3.09/−1.07 | GTTT-N_{14}GGGTA | 35                        |
| MSMEG_5374       | Glutamate-ammonia ligase                          | −2.22/−0.03 | GTTT-N_{14}GGGTA | 35                        |
| MSMEG_5559       | Metabolite/sugar transport protein                | −2.83/0.35  | GTTT-N_{14}GGGTA | 39                        |
was found far upstream of the down-regulated genes or even in the ORFs of the preceding genes. It may be noted that the canonical SigF promoter consensus was located more than 1000 bp upstream of the sigf gene in *M. smegmatis* genome (Gebhard et al. 2008). We reasoned that the SigF-dependent genes are likely to be down-regulated in both stages of growth. Notably, genes that showed reduced expressions commonly in exponential as well as stationary phase

| Locus          | Description                                         | Fold-change Exponential/Stationary | SigF consensus          | Position from start codon |
|---------------|-----------------------------------------------------|-----------------------------------|-------------------------|---------------------------|
| MSMEG_5623    | L-carnitine dehydratase                            | -3.24/1.20                       | GTTC-N<sub>15</sub>-GGGCA | 51                        |
| MSMEG_5731    | Transcriptional regulator, GntR family             | -2.31/10.25                      | GTCT-N<sub>16</sub>-GGGAT | 785                       |
| MSMEG_6507    | Glycogen debranching enzyme GlgX                    | -2.27/0.93                       | GTTG-N<sub>14</sub>-GGGAT | 656                       |
| MSMEG_6508    | MarR-family transcriptional regulator               | -2.82/3.11                       | GCTT-N<sub>17</sub>-GGG GCC | 142                       |
| MSMEG_6528    | Conserved hypothetical protein                      | -3.82/0.91                       |                         |                           |
| MSMEG_6611    | Hypothetical protein                                | -2.83/2.43                       |                         |                           |
| MSMEG_6820    | Acid phosphatase SurE                               | -3.26/−0.98                      | GTTG-N<sub>15</sub>-GGGTA | 87                        |

**Table 1. (Continued)**

| Locus          | Description                                         | Fold-change Exponential/Stationary | SigF consensus          | Position from start codon |
|---------------|-----------------------------------------------------|-----------------------------------|-------------------------|---------------------------|
| MSMEG_0195    | Steroid monooxygenase                               | 0.30/−2.66                        | GTTG-N<sub>16</sub>-GGGTA | 403                       |
| MSMEG_0964    | Pyridoxamine S-phosphate oxidase family             | -0.42/−5.10                      | GTTT-N<sub>16</sub>-GGGCA | 259                       |
| MSMEG_1196    | SNF2 domain protein                                 | 0.05/−2.47                        |                         |                           |
| MSMEG_1297    | Hydroxydechloroatrazine thylaminohydrolase          | -0.08/−2.82                      |                         |                           |
| MSMEG_1658    | Ribonuclease, putative                              | -0.54/−3.26                      | GTCT-N<sub>17</sub>-GGGTA | 50                        |
| MSMEG_1803    | RsbW protein                                        | -1.23/−3.56                      | GTTT-N<sub>16</sub>-GGGTA | 548                       |
| MSMEG_1807    | Acetly-3-propionyl-coenzyme A carboxylase           | 0.07/−2.38                       | GTTT-N<sub>17</sub>-GGGTA | 294                       |
| MSMEG_2373    | Acetolactate synthase, small subunit                | 0.15/−2.83                       | GTTG-N<sub>17</sub>-GGGCA | 386                       |
| MSMEG_3082    | Heme-binding protein                                | -0.47/−3.59                      | GCTT-N<sub>16</sub>-GGGTA | 67                        |
| MSMEG_3157    | Conserved hypothetical protein                      | 0.70/−2.22                       |                         |                           |
| MSMEG_3184    | Malto-oligosyltrehalose trehalohydrolase            | -1.30/−3.83                      | GTGT-N<sub>15</sub>-GGGCA | 409                       |
| MSMEG_3254    | RDD family, putative                                | -0.96/−3.85                      | GTTT-N<sub>15</sub>-GGGAA | 923                       |
| MSMEG_3273    | Glutamyl aminopeptidase, M42 family                 | -0.57/−3.38                      | GCTT-N<sub>15</sub>-GGGCC | 164                       |
| MSMEG_3322    | Hypothetical protein                                | -0.46/−2.14                      |                         |                           |
| MSMEG_3358    | YaeQ protein                                        | -0.61/−2.01                      |                         |                           |
| MSMEG_3593    | Protein of unknown function                         | -0.70/−4.74                      | GTTT-N<sub>15</sub>-GGGCA | 987                       |
| MSMEG_4082    | Monoxygenase                                        | 0.38/−2.17                       | GTTG-N<sub>14</sub>-GGGCC | 1024                      |
| MSMEG_4355    | Peptide ABC transporter, permease protein           | -1.20/−3.44                      | GTTT-N<sub>15</sub>-GGGCA | 13                        |
| MSMEG_4356    | Inner membrane ABC transporter permease             | -0.82/−3.24                      | GCTT-N<sub>14</sub>-GGGCA | 139                       |
| MSMEG_4357    | ABC transporter, ATP-binding protein                | -0.80/−3.48                      | GCTT-N<sub>14</sub>-GGG GCC |                           |
| MSMEG_4358    | D-beta-hydroxybutyrate dehydrogenase                | -0.44/−2.91                      | GCTT-N<sub>14</sub>-GGG GCC |                           |
| MSMEG_4428    | Conserved hypothetical protein                      | 1.01/−3.26                       |                         |                           |
| MSMEG_4531    | Sulfate ABC transporter, permease CysW              | 0.98/−3.99                       | GTCG-N<sub>15</sub>-GGGTT |                           |
| MSMEG_4532    | Sulfate ABC transporter, permease CysT              | 1.10/−2.41                       | GTCG-N<sub>15</sub>-GGGTT |                           |
| MSMEG_4533    | Sulfate-binding protein                              | 1.36/−2.58                       | GTCG-N<sub>15</sub>-GGGTT | 756                       |
| MSMEG_4864    | 3-ketosteroid dehydrogenase                         | -0.03/−2.49                      | GTCG-N<sub>18</sub>-GGGG A | 81                        |
| MSMEG_4991    | Hypothetical protein                                | -1.66/−6.01                      | GTTG-N<sub>17</sub>-GGGCC | 47                        |
| MSMEG_4993    | Hypothetical protein                                | -1.52/−4.00                      | GTTG-N<sub>19</sub>-GGGCC | 408                       |
| MSMEG_5003    | O-methyltransferase, family                         | -0.05/−3.82                      |                         |                           |
| MSMEG_5301    | Transcriptional regulator                           | -0.19/−2.10                      |                         |                           |
| MSMEG_5491    | Putative acyl-CoA dehydrogenase                     | 0.51/−2.00                       | GTGT-N<sub>17</sub>-GGGTT | 783                       |
| MSMEG_5606    | Cytochrome bd-I oxidase subunit II                  | -1.24/−3.75                      | GTTG-N<sub>14</sub>-GGGTT | 625                       |
| MSMEG_5880    | Nicotine dehydrogenase                              | 0.78/−2.05                       | GCTT-N<sub>17</sub>-GGGAA | 733                       |
| MSMEG_5936    | Conserved hypothetical protein                      | -1.18/−3.80                      |                         |                           |
| MSMEG_6151    | Alpha/beta hydrolase fold-1                        | -0.12/−2.26                      |                         |                           |
| MSMEG_6210    | Conserved hypothetical protein                      | -1.04/−3.38                      |                         |                           |
| MSMEG_6541    | Anti-sigma factor antagonist                         | -0.66/−3.63                      | GTTT-N<sub>15</sub>-GGGTA | 282                       |
| MSMEG_6819    | Conserved domain protein                            | -1.70/−4.01                      |                         |                           |
| MSMEG_6822    | Beta-lactamase                                      | -0.28/−2.61                      | GTTT-N<sub>16</sub>-GGGTA | 46                        |

Fold-change in expression – Δsigf strain/wild-type gene expression ratio in log2 scale. SigF consensus (GTTT-N<sub>14</sub>−19−GGGTA) was found in the upstream regions of majority of the down-regulated genes. Locus IDs in bold refer to genes that are clustered as operon in the genome. SigF consensus in such cases was found either in ORFs of preceding genes or in far upstream of the first gene of the cluster, e.g. SigF consensus was present 97 bp upstream of MSMEG_2347, MSMEG_2343–MSMEG_2347 constitute crt locus. Genes found down-regulated in Hümpel et al. (2010) as well as in this study.
cells, most of them showed the presence of the SigF promoter consensus in their upstream regions (Table 1), suggesting that they are SigF-dependent. Majority of genes that showed reduced expressions in this study were also reported to be down-regulated by Humpel et al. (Hümpel et al. 2010). They identified the SigF promoter consensus in the upstream regions of transcriptional regulators, sigH3 (MSMEG_0573), whib1 (MSMEG_1919), whib4 (MSMEG_6199), and phoP (MSMEG_5872), but the expressions of these genes were found unaltered in the ΔsigF mutant. In this study, using our selection criteria (≥2-fold, P ≤ 0.05), we identified three transcriptional regulators; MSMEG_5542 (HTH3 family), MSMEG_5731 (GntR family), and MSMEG_6508 (MarR family) which showed reduced expression in exponential phase, and MSMEG_5542, MSMEG_5301 (TetR family) with reduced expression in stationary phase. Of these MSMEG_5542, 5731, 6508 were found to have SigF consensus in their upstream regions. It is likely that the down-regulated genes which did not show SigF foot-prints in their upstream regions are indirectly regulated by SigF-dependent transcriptional regulators. Several of the exclusively down-regulated genes from exponential and stationary phase cells also showed SigF promoter consensus in their upstream regions, while few of them were found lacking the consensus. Based on the SigF promoter sequences, identified from this study, we deduced a profile of the SigF promoter consensus (Table 1), which showed the similar occurrence of the nucleotides at a given position in the earlier reported SigF promoter signature (Hümpel et al. 2010).

*Mycobacterium smegmatis* ΔsigF mutant phenotype and SigF regulon

The *M. smegmatis* ΔsigF mutant displayed notable phenotypes like, loss of pigmentation, pronounced sensitivity to oxidative stress and alteration in the cell wall architecture due to patchy distribution of GPLs in the cell wall. Correlating the loss of pigmentation phenotype the expressions of carotenoid biosynthesis genes (MSMEG_2243–MSMEG_2247) were found to be down-regulated during both growth stages (Table 1). The SigF promoter consensus was identified in the upstream of the cluster and the reduced expression of *crtI*, the first gene of the cluster, was validated by real-time PCR (Fig. 2C). Complementation of the ΔsigF mutant restored the original phenotype (Fig. 2A).

Regarding the sensitivity to oxidative stress the expressions of key enzymes that detoxify reactive oxygen intermediates, *katG* and *ahpc*, were found unaltered in the mutant strain, suggesting these genes are not regulated by SigF. We demonstrated that the overexpression of *crt* locus genes largely restores the susceptibility of ΔsigF strain to oxidative stress. Moreover, several genes which could possibly render resistance to ΔsigF strain against oxidative stress were found to be SigF-dependent and showed reduced expressions in both growth stages of ΔsigF strain.

Two potential hydrogen peroxide detoxifying enzymes, exclusively present in *M. smegmatis*, a manganese-containing catalase (MSMEG_6213) and a heme-containing catalase KatA (MSMEG_6232), showed reduced expressions in both stages in present study as well as in earlier report (Hümpel et al. 2010). A starvation-induced DNA protecting protein (MSMEG_6467) linked with oxidative stress resistance in bacteria (Gupta et al. 2002) showed reduced expression in both growth stages. *M. smegmatis* is a saprophyte and dehydrogenase activity is considered to be a good measure of microbial oxidative activity in saprophytes. Many genes (MSMEG_1794, MSMEG_5400, MSMEG_5402, MSMEG_0684) encoding for dehydrogenases and predicted to perform oxidoreductase activity (*SmegeaList*) were found to be SigF-dependent and down-regulated in both growth stages. These are likely to render susceptibility to the mutant strain towards oxidative stress.

In *M. smegmatis*, GPL biosynthesis gene cluster maps to a single locus of ~65 kb in the genome, containing nearly 30 ORFs that included genes for the synthesis as well as transport of GPLs (Ripoll et al. 2007). In the genome-wide gene expression study (see supplementary Data set S1) no genes from GPL biosynthesis gene cluster showed altered regulation in the ΔsigF mutant strain. We also did not find the SigF consensus signature in the upstream regions of genes clustered at this locus. This was in line with our earlier observation wherein we did not notice any difference in GPLs profile of ΔsigF mutant. However, a complete analysis of polar and nonpolar lipids from ΔsigF mutants showed distinct differences in 2D-TLC profile of nonpolar lipids in mutant strain. Concomitant with these findings trehalose biosynthesis genes (MSMEG_6514, MSMEG_6515) and mycocerosic acid synthase genes (MSMEG_6765 to MSMEG_6767) were found to be significantly down-regulated in ΔsigF strain (Table 1). MSMEG_6515 encodes for trehalose synthase which enables the conversion of glycogen into trehalose. The SigF promoter consensus was identified in the upstream of these genes, indicating that trehalase and mycocerosic acid synthase (MAS) genes are directly regulated by SigF and affect the cell wall architecture by inhibiting lipid biosynthesis pathway in sigF mutant.

**Post-translational regulation of SigF in Mycobacterium smegmatis: overexpression of rsbW mimics the M. smegmatis ΔsigF mutant phenotype**

Sigma factors activity is post-translationally regulated by their cognate anti-sigma factors, which sequester them and make them unavailable for RNAP. In *M. tuberculosis,*
SigF is post-translationally regulated by its cognate anti-sigma factor RsbW, which is, in turn, regulated by two anti-anti-sigma factors, RsFα and RsFβ (Beaucher et al. 2002). Both are able to disrupt the RsbW-SigF complex, releasing SigF to allow its association with RNA polymerase. In *M. smegmatis* rsbW (MSMEG_1803) is colocalized (Fig. S1) and cotranscribed with sigF (MSMEG_1804) (Gebhard et al. 2008). But, barring the sequence similarity with *M. tuberculosis* RsbW (Rv3287c), there has been no experimental evidence till date which demonstrates that MsrSbW binds to SigF and regulates it negatively. We argued that if MSMEG_1803 is indeed the anti-SigF, RsbW, negatively regulating the SigF in *M. smegmatis*, overexpression of MSMEG_1803 in *M. smegmatis* wild type cells should sequester the prevailing pool of SigF and thereby making them unavailable for binding to RNA polymerase. This will impede the expression of SigF regulon and the MSMEG_1803 overexpressing *M. smegmatis* cells will produce a phenotype akin to *M. smegmatis* ΔsigF mutant.

As shown in Fig. 5(A) and (B), we observed loss of pigmentation and increased susceptibility to oxidative stress in strain MS:MSrsbW nearly similar to SFKO1, the ΔsigF mutant strain. This proved that MSMEG_1803 indeed encodes for the cognate anti-SigF protein which binds to SigF in *M. smegmatis* and regulates it negatively. Similar observations were made with *M. smegmatis* wild type cells overexpressing *M. tuberculosi* *s rsbW* (MS:MbrsbW) (Fig. 5A and B), which further established that MSMEG_1803 is true ortholog of MtbrsbW, as both strains produced similar phenotypes akin to SFKO1. To establish that the observed phenotypes of MS:MSrsbW and MS:MbrsbW strains are indeed due to overexpression of rsbW and sequestering of SigF proteins we performed real time semiquantitative RT-PCR of these genes in *M. smegmatis* wild type, SFKO1 and overexpressing recombinant strains. We also examined the expression levels of two putative anti-anti-sigF proteins RsFα (MSMEG_1786) and RsFβ (MSMEG_6127) from *M. smegmatis*, which were identified based on their homology to M. tuberculosis RsFα and RsFβ. As observed in Fig. 5(C) the expression levels of rsbW, rsfA, and rsfB were found to be similar to wild type, while the sigF was nearly absent, owing to its deletion, in SFKO1 strain. However, the expressions of these genes were found to be similar in MS:MSrsbW and MS:MbrsbW strains, suggesting that MsrSbW (MSMEG_1803) is indeed similar to MbrsbW. A negligible expression of sigF gene was noticed in both strains, which implies that enhanced cellular level of RsbW protein, owing to its overexpression (Fig. 5C), completely sequestered the SigF protein, and, in turn shut down the expression of sigF gene, which is transcriptionally autoregulated. Since the sigF is cotranscribed with rsbW the increased rsbW level in MS:MSrsbW and MS:MbrsbW strains amounts to the ectopically expressed rsbW under the control of hsp60 promoter in these strains. Interestingly, the expressions of rsfA and rsfB were also found to be induced, similar to rsbW, in both recombinant strains. RsFα and RsFβ are known to antagonize RsbW; therefore, it is possible that some feedback machinery in the bacterial cell would have sensed the increased cellular level of RsbW and invoked an ensuing response by transcriptionally upregulating the expression of both anti-sigF antagonists. It may be noted that the expression levels of RsFα (MSMEG_1786) and RsFβ (MSMEG_6127) were not significantly altered in ΔsigF mutant strain in genome wide gene expression analysis performed in this study and by Hümpel et al. 2010. Also both these genes lacked SigF footprints in their upstream regulatory regions.

Furthermore, using bacterial two-hybrid experiment we analyzed the interactions of *M. smegmatis* anti-SigF RsbW with SigF and its two antagonists RsFα and RsFβ. *M. smegmatis* RsbW showed very strong interactions with SigF and RsFα while a comparatively weak interaction...
was noticed with RsB (Table 2). Similar results were obtained when we allowed *M. tuberculosis* RsBw to interact with *M. smegmatis* SigF, RsFA, and RsFB (Table 2). On the other hand, we did not notice any interaction when *M. smegmatis* RsBw was allowed to interact with *M. smegmatis* SigA, which confirmed the specificity of MSRsBw to its cognate sigma factor SigF. To further confirm these interactions we performed GST pull down assay. *M. smegmatis* RsBw was overexpressed as GST tagged protein (GST-MSRsBw) using pET41a+ vector in *Escherichia coli*, purified and immobilized on GST beads. A column was prepared with GST-MSRsBw immobilized beads and whole cell lysates of recombinant *M. smegmatis* strains overexpressing *M. smegmatis* SigF, RsFA, and RsFB proteins were applied and allowed to bind to GST-MSRsBw. Subsequently, interacting proteins were eluted using reduced glutathione and electrophoresed on SDS-PAGE (Sodiumdodecyl sulfate polyacrylamide gel electrophoresis) (Fig. 6). Individual bands were excised and sequenced using MALDI/MS (data not shown). We noticed similar level of interactions between RsBw, SigF, RsFA, and RsFB proteins as it was observed in bacterial two-hybrid assay. Thus, combined together, bacterial two-hybrid and GST pull down results clearly established that MSMEG_1803 encodes for anti-SigF RsBw protein in *M. smegmatis* which specifically and strongly interacts with its cognate sigma factor SigF and its antagonists RsFA and RsFB. The fact that these proteins showed similar level of interactions with *M. tuberculosis* RsBw suggests that most likely, similar to *M. tuberculosis*, in *M. smegmatis* SigF is post-translationally regulated by its anti-sigma factor RsBw, which is in turn regulated by its antagonists RsFA and RsFB. However, further experiments are required to elucidate the regulation of these interactions with respect to different physiological states of mycobacterial cells. It would be of interest to examine whether some more SigF antagonists are present in *M. smegmatis* genome as predicted by Hümpel et al. (2010) in their studies.

**Conclusions**

In this study, we report that in *M. smegmatis* the SigF is not essential for growth of bacterium. Deletion of sigF results in loss of carotenoid pigmentation which rendered increased susceptibility to H₂O₂ induced oxidative stress as complementation of ΔsigF mutant with carotenoid genes largely restores the phenotype. In *M. smegmatis*, sigF deletion altered the outer most layer of the cell envelope and the cell wall lipid composition by modulating the lipid biosynthesis pathway. *M. smegmatis* SigF regulon

**Table 2.** Interactions of anti-SigF (RsBw) with its antagonists (RsFA and RsFB) and SigF.

| Interacting proteins | pBT-LGF2 + pTRG-GAL11 | pBT + pTRG-MSrsbW (MSMEG_1803) | pBT-MSsigA + pTRG-MSrsbW | pBT-MSsigF + pTRG-MSrsbW | pBT-MSrsFA + pTRG-MSrsbW | pBT-MSrsFB + pTRG-MSrsbW | pBT + pTRG-MsrcsW (Rv3287c) | pBT-MSsigF + pTRG-MtbsrbW | pBT-MSrsFA + pTRG-MtbsrbW | pBT-MSrsFB + pTRG-MtbsrbW |
|---------------------|-----------------------|-------------------------------|---------------------------|---------------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                     | +++                   | –                            | –                         | +++                       | ++                       | ++                        | –                         | +++                       | ++                        | ++                        |

Different levels of interactions are denoted as: <10% (−), 10–20% (+), 20–40% (++), 40–60% (+++), 60–80% (+++), >80% (++++). Control vectors carrying bait protein pBT-LGF2 and target protein pTRG-GAL11P showed strong (++++) interaction and considered as reference.
included a variety of genes expressed during exponential and stationary phases of growth and those responsible for oxidative stress, lipid biosynthesis, energy, and central intermediary metabolism. We report the identification of a SigF antagonist, an anti-sigma factor (RsbW), which upon overexpression in *M. smegmatis* wild type cell produced a phenotype similar to *M. smegmatis ΔsigF* mutant. Two anti-sigma factor antagonists, RsfA and RsfB are also identified and their interactions with anti-sigma factor were confirmed using bacterial two-hybrid and GST pull down.

### Experimental Procedures

#### Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are described in Table 3. *M. smegmatis* mc²155 wild type and derivative

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| Strains or plasmids | Relevant properties | Reference or source |
|---------------------|--------------------|---------------------|
| **Escherichia coli** strains | | |
| E. coli DH5α | F'-80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 | Invitrogen |
| XL1-Blue MRF² | recA1 gyrA96 relA1 lac [F'proAB lacZΔM15 Kan''] | Agilent Technologies |
| XL1-Blue | recA1 gyrA96 relA1 lac [F' lacIq H133 adaA (Kan'')] | Agilent Technologies |
| pLYS4+ | lacIq(Cam')rifuA2 lacZ:7 gene1 endA1 | New England Biolabs |
| E. coli C41 | Expression vector | Novagen |
| **Mycobacterial strains** | | |
| Mycobacterium smegmatis ATCC607 | *M. smegmatis* parent strain of mc²155 | Late Jean-Mark Reyrat, University of Paris |
| M. smegmatis ATCC607 ΔsigF | sigF deleted ATCC607 strain, Str² | Departmental Stock |
| M. smegmatis mc²155 | High transforming *M. smegmatis* strain | This study |
| SFKO1 | sigF deleted mc²155 strain, Hyg² | This study |
| SFKO1 ΔsigF | mc²155 ΔsigF mutant complemented with sigF | This study |
| SFKO1 Δcrt | mc²155 ΔsigF mutant complemented with crt | This study |
| MS:MSrsbW | mc²155:hs60pr-MSrsbW, Km² | This study |
| MS:McbrsW | mc²155:hs60pr-McbrsW, Km² | This study |
| M. tuberculosis H37Rv | Laboratory strain of tubercle bacilli | Departmental Stock |
| **Plasmids** | | |
| pDrive | PCR cloning vector, Amp², Km² | Qiagen, India |
| pT7ZRT/1 | PCR cloning vector, Amp² | Fermentas, India |
| pMV261 | E. coli-mycobacterial shuttle vector, Km² | Stover et al. (1991); |
| pMV306 | Mycobacterial integrative vector, Km² | Stover et al. (1991); |
| pET28a, 41a(+) | Expression vectors, Km² | Novagen |
| pT7ZsigF1 | pT7Z carrying MS sigF ORF at Ncol-HindIII | This study |
| pT7ZsigF2 | pT7Z carrying MS sigF flanked by XbaI-BamHI | This study |
| pETsigF | pET28a carrying MS sigF at Ncol-HindIII | This study |
| pDAsgF | pDrive carrying sigF allelic exchange cassette, hyg² | This study |
| pMV306sigF | pMV306 carrying hsp60pr-sigF at NotI-HindIII | This study |
| pMV306crt | pMV306 carrying crt locus at XbaI-HindIII | This study |
| pTRG-MS rsbW | pTRG vector carrying MS rsbW ORF at EcoRI-Xhol, Tet² | This study |
| pTRG-Mtb rsbW | pTRG vector carrying Mtb rsbW ORF at EcoRI-Xhol, Tet² | This study |
| pBT-MS sigF | pBT vector carrying MS sigF ORF at EcoRI-Xhol, Chl² | This study |
| pBT-MS sigA | pBT vector carrying MS sigA ORF at EcoRI-Xhol, Chl² | This study |
| pBT-MS sigB | pBT vector carrying MS sigB ORF at EcoRI-Xhol, Chl² | This study |
| pBT-MS rsfA | pBT vector carrying MS rsfA ORF at EcoRI-Xhol, Chl² | This study |
| pBT-LGF2 | Two hybrid interaction control bait plasmid | Agilent Technologies |
| pTRG-Gal11² | Two hybrid interaction control target plasmid | Agilent Technologies |
| pET41a-MS rsbW | Expression vector carrying MS rsbW ORF at speI-Xhol, Km² | This study |
| pET28a-MS rsF | Expression vector carrying MS rsfA ORF at Ndel-Xhol, Km² | This study |
| pET28a-MS rsF | Expression vector carrying MS rsfB ORF at Ndel-Xhol, Km² | This study |
| pMV261-MS rsbW | pMV261 vector carrying MS rsbW, Km² | This study |
| pMV261-Mtb rsbW | pMV261 vector carrying Mtb rsbW, Km² | This study |

Amp², ampicillin resistant; Km², kanamycin resistant; hyg², hygromycin resistant; Tet², tetracycline resistant; Chl², chloramphenicol resistant; Str², streptomycin resistant.
strains were grown at 37°C in Middlebrook 7H9 (Difco) liquid culture medium supplemented with 10% albumin-dextrose-catalase (ADC), 0.5% glycerol, and 0.05% Tween-80 or on Middlebrook 7H10 (Difco) solid culture medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol. E. coli cultures were grown in Luria-Bertani (LB) broth with the addition of ampicillin (100 µg mL⁻¹), kanamycin (50 µg mL⁻¹), and hygromycin (100 µg mL⁻¹), as required.

**DNA manipulation, construction of sigF mutant, and its complementation**

Recombinant DNA techniques were performed as per standard procedures (Sambrook et al., 2001) using *E. coli* DH5α as the initial host. Restriction and DNA modifying enzymes were obtained from Fermentas. Primers used in this study are described in Table 4. Preparation of electrocompetent cells and electroporation were done as previously described (Singh and Singh 2008). *M. smegmatis* mutant lacking *sigF* gene was cloned into pDrive plasmid vector generating pΔ*sigF*. The final allele exchange cassette contained 5′flank/Hyg/3′flank in pΔ*sigF*. 5′ and 3′ flanking regions contained a few nucleotide sequences of *sigF* gene which was later used for PCR amplification of *sigF* ORF from wild type and Δ*sigF* mutant.

PDrive contains only *E. coli* origin of replication and, therefore, fails to multiply in mycobacteria and serves as suicide vector in mycobacteria. pΔ*sigF* was electroporated into *M. smegmatis* mc²155 and transformants were selected on ADC plates. The expected double cross-over event would exchange *sigF* gene with hygromycin resistance cassette flanked by nearly 1 kb flanking regions of each side of the gene was cloned into pDrive plasmid vector generating pΔ*ΔsigF*. The final allele exchange cassette contained 5′flank/Hyg/3′flank in pΔ*ΔsigF*. 5′ and 3′ flanking regions contained a few nucleotide sequences of *sigF* gene which was later used for PCR amplification of *sigF* ORF from wild type and Δ*ΔsigF* mutant.

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**Table 4. Primers used in this study.**

| Primers | Sequence (5’ to 3’) |
|---------|---------------------|
| MSSF1   | TCTAGAGTTGCGAAGTTTTCGACACAGGCA |
| MSSF2   | AACCTGCTCAATCATGAACGCCGACG |
| pETSF1  | ACCATGGGCGCAATCATACACCAACCAT |
| pETSF2  | CACCCACACATATATAGACGGCGAA |
| SFAE1   | AAGCTGTCGACCTCCAGTTTTCGACACAGGCA |
| SFR3    | AGCCACACGCTCAGCAGATGCTTC |
| MSF3F   | CACCCACACATATATAGACGGCGAA |
| PhytoF1  | ACCTGCTCAATCATGAACGCCGACG |
| PhytoF2  | ACCATGGGCGCAATCATACACCAACCAT |

Restriction sites relevant to procedures used in this work are underlined.
Mc2155 wild type cells survive after DPA treatment. 0.1 mmol L\(^{-1}\) DPA treatment for 4–6 h ensured ≥80% of dose of diphenylamine (DPA) was set so that survival of 80% wild type cells survive after DPA treatment. 0.1 mmol L\(^{-1}\) DPA treatment for 4–6 h ensured a 24 h before H\(_2\)O\(_2\) treatment and stress susceptibility was analysed as described above.

**Transmission electron microscopy**

Electron microscopy samples were prepared as described previously (Paul and Beveridge 1992). Briefly, fully grown cultures of *M. smegmatis* strains were diluted (1:100) in fresh LBGT broth and allowed to grow till 0.5 OD\(_{600}\). Cultures were centrifuged at 400 × g for 2 min to separate homogenous cell suspension from cell aggregates. Homogenous suspensions were transferred to new tubes and cells were harvested by centrifugation at 2600 × g for 5 min. Cells were washed five times with 0.1 mol L\(^{-1}\) cacodylate buffer (pH 6.8) and pellets (~50 mg wet weight) were fixed in 2.5% (w/v) glutaraldehyde, 0.05% ruthenium red in 0.1 mmol L\(^{-1}\) cacodylate buffer in dark at 4°C overnight. Cells were collected by centrifugation, washed thrice in 0.1 mol L\(^{-1}\) cacodylate buffer before fixing for 2 h in dark in 1% (w/v) osmium tetroxide, 0.05% ruthenium red in 0.1 mol L\(^{-1}\) cacodylate buffer. After this cells were washed thrice in 0.1 mol L\(^{-1}\) cacodylate buffer for 5 min each and embedded in 2% agarose gel. Blocks were dehydrated through a graded ethanol series of 20, 40, 60, 80, and 95% for 5 min each followed by two 10 min washes in absolute ethanol. Samples were embedded in Epon 812 resin at 60°C for 48 h. Ultra thin sections (50–70 nm) were obtained using Ultracut Ultra Microtome (Leica) and picked upon 200 mesh copper grids. Sections were poststained with uranyl acetate and Reynolds’s lead citrate. Microscopy was performed on a Philips FEI Technai-12 Twin Transmission Electron Microscope and images were recorded using a SIS mega View II CCD camera attached with the microscope.

**Extraction and analysis of GPLs and total lipids from Mycobacterium smegmatis**

GPLs extraction and analysis were performed as described earlier (Vats et al. 2012). The *M. smegmatis* wild type and mutant strains were grown in Middlebrook 7H9 medium supplemented with 10% ADC till late stationary phase (2.8–3.0 OD\(_{600}\)). GPLs were extracted with CHCl\(_3\)/CH\(_3\)OH (2:1) at room temperature for 24 h. The
supernatant was dried using rotatory evaporator till dryness. The lipid extract was dec酰ated with 0.2 mmol L\(^{-1}\) NaOH in methanol at 37°C for 1 h followed by neutralization with glacial acetic acid. After drying, lipids were dissolved in CHCl\(_3/CH_3\)OH (2:1), spotted onto the TLC plate (Aluminium baked silica gel 60 F254) (Merck) and developed in CHCl\(_3/CH_3\)OH/H\(_2\)O (90:10:1) solvent. GPLs were visualized by spraying with 5% α-naphthol/sulfuric acid in ethanol followed by heating at 120°C for 10 min. The four de-O-acetylated GPLs (dGPLs) were named dGPL I, II, III, and IV, starting from the solvent front. For mass analysis GPLs were analysed and identified by ESI-Q-TOF-MS (Absciex). [M+Na\(^{+}\)]\(^{+}\) ions of deacetylated GPLI, GPLII, GPLIII, and GPLIV were observed at m/z 1187, 1173, 1173, and 1159 respectively (Khoo et al. 1995; Vats et al. 2012).

Extractions and analysis of lipids were performed as described earlier (Slayden and Barry 2001). Lipids were extracted from freeze dried stationary phase grown M. smegmatis cells. Bacterial cells were resuspended in equal volume of methanolic saline and petroleum ether, mixture was stirred for 12–16 h and then allowed to separate following which nonaqueous phase containing the nonpolar lipids were removed and stored. An equal volume of petroleum ether was added to lower aqueous phase, mixture was stirred for 2 to 4 h, nonaqueous layer was removed and pooled with the first one. Nonpolar lipids were dried using a rotatory evaporator and resuspended in dichloromethane. Extraction of polar lipids was performed by adding chloroform (CHCl\(_3\)), CH\(_3\)OH, and 0.3% aqueous NaCl (9:10:3) to the extract. The entire mixture was stirred for 4 h and the solvent extract was separated from the biomass. Furthermore, the residues were extracted with CHCl\(_3\), CH\(_3\)OH, and 0.3% aqueous NaCl (3:10:4) for 4 h. The polar lipid extracts were mixed with CHCl\(_3\) and 0.3% aqueous NaCl in equal ratio and the lower organic layer was separated discarding the upper aqueous layer. Polar lipids were dried using rotatory evaporator and resuspended in dichloromethane. Extraction of polar lipids was performed by adding chloroform (CHCl\(_3\)), CH\(_3\)OH, and 0.3% aqueous NaCl (9:10:3) to the extract. The entire mixture was stirred for 4 h and the solvent extract was separated from the biomass.

### Protein-protein interaction analyses using bacterial two-hybrid

BacterioMatch II two-hybrid system (Agilent Technologies) was used for analyses of protein-protein interactions. The system utilizes a double HIS3-gaard reporter cassette which identifies interacting partners with plausibly reduced background. Detection of protein-protein interactions is based on transcriptional activation of the HIS3 reporter gene, which allows growth in the presence of 3-amino-1, 2, 4-triazole (3-AT), a competitive inhibitor of His3 enzyme. Positives are reconfirmed by using the addA gene, which confers streptomycin resistance, as a secondary reporter.

*Mycobacterium smegmatis* sigF, sigA, anti-sigF rsbW (MSMEG_1803), and anti-sigF antagonists, rsfA (MSMEG_1786) and rsfB (MSMEG_6127) were amplified using gene specific primers (Table 4) and cloned into bait vector pBT at given enzyme sites (Table 3). Similarly, anti-sigma factors from *M. smegmatis* (MSrsbW) and *M. tuberculosis* (MtbSrbW) were amplified using gene specific primers (Table 4) and cloned into target vector pTRG at given enzyme sites (Table 3). All cloning steps were performed in *E. coli* XL1Blue strain, and the clones were verified by restriction digestion and DNA sequencing. To analyze interactions between two proteins, plasmid pairs carrying ORFs in pBT and pTRG vectors were cotransformed in XL1Blue derived reporter strain, provided with two-hybrid system. Cotransformants were selected on M9 and M9-3AT plates. The cotransformant containing pBT-LGF2 and pTRG-GaL11P (Agilent) was used as a positive control for expected growth on the selective screening medium (M9 with 5 mmol L\(^{-1}\) 3-AT). A cotransformant containing the empty vectors pBT and pTRG was used as a negative control. Further positives were verified using second reporter gene (addA), conferring streptomycin resistance. The interaction between the bait and target proteins was revalidated by patching cells from a putative positive colony from a selective screening medium (M9-3AT) plate onto a dual selective screening medium (M9-3AT + streptomycin 15 μg mL\(^{-1}\) plate). CFU obtained on the nonselective screening medium (M9 without 3AT) and selective medium (M9-3AT) plates were counted, and values were used to determine the percent interaction. The average and standard deviations were determined from data generated from two different experiments.

### Cloning, expression, purification of RsbW, SigF, RsfA and RsfB and GST pull down assay

*Mycobacterium smegmatis* rsbW ORF was amplified using gene specific primers and cloned into pET41a+ at SpeI and XhoI sites to generate pET41a-MSrsbW. This allowed MSrsbW to be cloned in fusion with GST at its N-terminal.
Positive clones were verified by restriction digestion and DNA sequencing. Recombinant pET41a-MSrsbW and pET41a+ plasmid carrying GST were separately transformed into E. coli pLysS\+ cells and the transformants were selected on kanamycin. Selected colonies were allowed to grow till 0.6 OD$_{600}$ and induced with 1 mmol L$^{-1}$ IPTG at 30°C with continuous shaking for 4 h. Cells were pelleted by brief centrifugation and washed with cold PBS. The pellet was resuspended in buffer (50 mmol L$^{-1}$ Tris pH 7.2, 100 mmol L$^{-1}$ NaCl, 1 mmol L$^{-1}$ DTT and 1% protease inhibitor cocktail), lysed by sonication on ice and then both proteins were purified using glutathione–sepharose resin (Pierce) as per manufacturer’s instructions. The purified proteins were analyzed by SDS/PAGE.

*Mycobacterium smegmatis* sigF, rsfA, and rsfB were amplified using gene specific primers and cloned into pET28a at NcoI-HindIII (sigF) and NdeI-XhoI (rsfA and rsfB) enzyme sites. The clones were verified by restriction digestion and DNA sequencing. Recombinant pET28a carrying sigF, rsfA, and rsfB in fusion with N-terminal His$_6$ tag were transformed into *E. coli* pLysS\+ cells separately and transformants were appropriately selected. Selected colonies of pET28a-MSsigF, pET28a-MSrsfA, and pET28a-MSrsfB were grown, proteins were overexpressed and cell lysates were prepared as described above.

Pull down experiments were performed using Pierce GST Protein Interaction Pull-Down Kit (cat \# P121516) according to manufacturer’s instructions. Purified GST-MSrsbW and GST proteins (5 μg each) were allowed to bind 50 μL GST resins at 4°C for 1 h. GST proteins were used as negative control. After several washings (wash buffer 1) columns carrying GST-MSrsbW and GST bound resins were incubated separately with total cell lysates containing overexpressed *M. smegmatis* SigF, RsfA, and RsfB in buffer (TBS: 50 mmol L$^{-1}$ Tris pH 7.4, 100 mmol L$^{-1}$ NaCl) at 4°C for 1 h with constant mixing. After washing five times with 400 μL of wash buffer (wash buffer 1) the bound proteins were eluted in TBS containing 5 and 10 mmol L$^{-1}$ reduced glutathione (RG). Eluted samples were boiled in 1X sample buffer, separated using 15% SDS-PAGE and visualized by coomassie staining (Fig. 6). Individual bands were excised and analysed using MS/MS, which confirmed the identity of eluted proteins.

**Overexpression of rsbW from Mycobacterium smegmatis and M. tuberculosis**

Anti-sigma factors from *M. smegmatis* and *M. tuberculosis* were subcloned into *E. coli/mycobacterial plasmid shuttle vector pMV261* (Stover et al. 1991) to the downstream of hsp60 promoter. *M. smegmatis* mc2155 wild type strain was subsequently transformed with pMV261-MSrsbW and pMV261-MtbrsbW to generate MS:MSrsbW and MS:MtbrsbW recombinant strains respectively. These strains were used for different analysis as described above.

**RNA isolation and labeling**

*Mycobacterium smegmatis* strains were grown in Middlebrook 7H9 broth supplemented with 10% ADC, 0.2% glycerol and 0.05% Tween-80 at 37°C. Aliquots were removed at exponential (~0.8 OD$_{600}$) and stationary (~2.8 OD$_{600}$) phase. Cells were harvested by centrifugation at 2500 × g for 5 min and RNA was extracted using Trizol (Invitrogen, USA), as described earlier (Singh and Singh 2009). The RNA was resuspended in 50 μL of RNasefree water. RNA concentration and purity was determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies) and the integrity of total RNA was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip (Agilent Technologies). RNA was stored at −80°C until use. For labeling, RNA was polyadenylated using Poly (A) polymerase tailing kit (Cat # PAP5104H, Epicentre Biotechnologies) essentially as per manufacturer’s instructions. Postpolyadenylation RNA was precipitated with ethanol, washed with 70% ethanol, dried at RT, and dissolved in nuclease free water. RNA concentration was estimated using NanoDrop and kept at −80°C until further use. Quick-Amp Labeling kit (Agilent technologies) was used for cDNA synthesis and subsequent amplification and labeling by in vitro transcription was done as per one-color labeling protocol (Agilent, version 5.5). Briefly, 0.5 μg of each of the RNA sample was converted to double stranded cDNA using oligo dT primer with T7 polymerase promoter. RNA samples were mixed with T7 primers and final volume of each reaction was made up to 11.5 μL with nuclease free water. Samples were denatured at 65°C for 10 min and placed on ice for 5 min. cDNA master mix was added to each sample and reactions were kept at 40°C for 2 h followed by incubation at 65°C for 15 min and on ice for 5 min. Then 60 μL of transcription mix was added to each reaction and incubated at 40°C for 2 h. cRNA was generated by in vitro transcription using T7 RNA polymerase and the dye Cy3-CTP was incorporated during this step. Labeled cRNA was purified using RNeasy Mini kit (Qiagen, India) and their quality was assessed for yields and specific activity using NanoDrop. Specific activity was calculated as picomole of dye/μg of cRNA. Specific activity of ≥ 6.5 was considered optimal and used for hybridization.

**Microarray slides, hybridization, and scanning**

Complete microarray experiment was carried out in technical collaboration with Genotypic Solution, Bangalore,
India, official service partner of Agilent Technologies (USA). Array was spotted using 60 mer oligo probes (features) in 8 x15K format (Ref No: AMADID: 016421). Average number of probes per gene in each array is 3. Probes were designed in such a way that multiple probes for a given gene specifically hybridize to different parts of the transcript. Each array carried Agilent proprietary probes for quality control purpose. M. smegmatis microarray slides were hybridized with the labeled cRNA. Before hybridization 0.6 μg of each Cy3 labeled cRNAs were fragmented to uniform size of 200 bp to avoid folding up of long transcripts and also remove any steric hindrance which may arise due to secondary structure in long RNA molecules during hybridization. Fragmentation and hybridization were carried out using the Gene Expression Hybridization kit (Part # 5188–5242, Agilent Technologies). Hybridization was carried out in Agilent’s Surehyb Chambers at 65°C for 16 h. After hybridization slides were washed using Agilent Gene Expression wash buffers, first at RT and then twice at 37°C. Slides were quickly dried and scanned using the Agilent Microarray Scanner G Model G2565BA at 5 micron resolution. The images were manually verified and found to be devoid of uneven hybridization, streaks, blobs, and other artifacts.

**Feature extraction and data analysis**

Data extraction from images was done using Feature Extraction software v 10.5.1.1 (Agilent). Feature extracted data were analyzed using GeneSpring GX v 7.3.1 software (Agilent). Normalization of the data was done in GeneSpring GX using the recommended one color Per Chip and Per Gene Data Transformation: Set measurements <0.01 to 0.01 per Chip: Normalize to 50th percentile per Gene: Normalize to Specific Samples. The gene expression ratio (ΔsigF/WT) of ≤ 0.5 or ≥2.0 (P ≤ 0.05) was considered differentially regulated and filtered from the data. Fold-chage refers to expression ratio of ΔsigF strain to wild-type and is expressed in log2. Ratios were tested for significance using student T-test from Agilent’s Gene Spring GX version 7.3 software.

**Real-time reverse transcription-PCR (RT-PCR) analyses**

RNA was extracted from exponential and stationary phase cultures of M. smegmatis wild type and derivative strains (SFK01, SFKO1/sigF, MS:MbsrBSW and MS:MtbMbrBSW) as described earlier (Singh and Singh 2009). DNase treatment was carried out to remove any DNA contamination, and post-treatment RNA was reverse transcribed using random primers and Transcriptor reverse transcriptase (Roche). qRT-PCR was performed in triplicates using SYBR Green master mix on a Roche 480 LightCycler, as described previously (Singh and Singh 2009). Expression of target genes was normalized with the sigA transcript level. RNA samples that had not been reverse transcribed were included as controls in all the experiments. The mean relative expression levels and SD were determined from the data generated from two different experiments. Each experiment was set up in triplicates.

**Microarray data accession number**

All experimental details and data have been deposited at the Gene Expression Omnibus (GEO, NCBI) under accession number GSE19774.

**Statistical analysis**

Significant differences between experimental groups were determined using Student’s t-test (GRAPHPAD PRISM 5, GraphPad Software, Inc., La Jolla, CA). For all analyses, a P-value of <0.05 was considered statistically significant.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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### Supporting Information

Additional supporting information may be found in the online version of this article:

**Data S1.** Log phase and stationary base.

**Figure S1.** Schematic of sigF locus and construction of sigF mutant.

**Figure S2.** TLC profile of the de-O-acetylated GPs, extracted from the *Mycobacterium smegmatis* WT (MS) and mutant strain (SFKO1), as described in methods. dGPL I, II, III, and IV, starting from the solvent front. Mass spectra profile of GPs (I, II, III, and IV) extracted from M. smegmatis wild type (A) and ΔsigF mutant (B).

**Figure S3.** Real time RT-PCR analysis of select genes from microarray data that were found to be down-regulated in ΔsigF mutant.