The Partner Switching System of the SigF Sigma Factor in Mycobacterium smegmatis and Induction of the SigF Regulon Under Respiration-Inhibitory Conditions

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The partner switching system (PSS) of the SigF regulatory pathway in Mycobacterium smegmatis has been previously demonstrated to include the anti-sigma factor RsbW (MSMEG_1803) and two anti-sigma factor antagonists RsfA and RsfB. In this study, we further characterized two additional RsbW homologs and revealed the distinct roles of three RsbW homologs [RsbW1 (MSMEG_1803), RsbW2 (MSMEG_6129), and RsbW3 (MSMEG_1787)] in the SigF PSS. RsbW1 and RsbW2 serve as the anti-sigma factor of SigF and the protein kinase phosphorylating RsfB, respectively, while RsbW3 functions as an anti-SigF antagonist through its protein interaction with RsbW1. Using relevant mutant strains, RsfB was demonstrated to be the major anti-SigF antagonist in M. smegmatis. The phosphorylation state of Ser-63 was shown to determine the functionality of RsfB as an anti-SigF antagonist. RsbW2 was demonstrated to be the only protein kinase that phosphorylates RsfB in M. smegmatis. Phosphorylation of Ser-63 inactivates RsfB to render it unable to interact with RsbW1. Our comparative RNA sequencing analysis of the wild-type strain of M. smegmatis and its isogenic Δaa3 mutant strain lacking the aa3 cytochrome c oxidase of the respiratory electron transport chain revealed that expression of the SigF regulon is strongly induced under respiration-inhibitory conditions in an RsfB-dependent way.

Keywords: aa3 cytochrome c oxidase, anti-sigma factor, anti-anti-sigma factor, electron transport chain, Mycobacterium, partner switching system, protein kinase, SigF

INTRODUCTION

Sigma factors reversibly associate with the core RNA polymerase and function as specific factors that direct transcription of a specific subset of genes. 28 sigma factor genes were found to occur in Mycobacterium smegmatis in contrast to 13 genes in Mycobacterium tuberculosis (Cole et al., 1998; Manganelli et al., 2004; Waagmeester et al., 2005; Rodrigue et al., 2006). SigF belongs to group III sigma factors and is dispensable for growth of M. tuberculosis and M. smegmatis (Chen et al., 2000; Williams et al., 2007; Provvedi et al., 2008; Singh et al., 2015). SigF is phylogenetically and
The SigF gene is widely conserved in mycobacterial species (Singh and Singh, 2008; Sachdeva et al., 2010). In M. tuberculosis, SigF (Rv3286c) was shown to be involved in virulence, biofilm formation, and diverse stress responses (Chen et al., 2000; Geiman et al., 2004; Karls et al., 2006; Williams et al., 2007; Hartkoorn et al., 2012; Manganelli, 2014). SigF (MSMEG_1804) in M. smegmatis was suggested to play roles in adaptation to stationary phase and conditions of heat and oxidative stress (Gebhard et al., 2008; Humpel et al., 2010; Singh et al., 2015). Overexpression or deletion of sigf was reported to alter cell wall architectures in M. tuberculosis, Mycobacterium bovis, and M. smegmatis (Forrellad et al., 2013; Singh et al., 2015; Dutta et al., 2019). The inactivation of sigf in M. smegmatis was shown to result in the loss of carotenoid (isorenieratene) pigmentation, accompanying increased susceptibility to hydrogen peroxide (Provvedi et al., 2008; Humpel et al., 2010; Singh et al., 2015). The consensus sequence (GGGWTT-N16−17-GGTTAY) was suggested for the mycobacterial SigF-recognition promoters (Gebhard et al., 2008; Provvedi et al., 2008; Humpel et al., 2010). The sigf genes of M. tuberculosis and M. smegmatis were demonstrated to be cotranscribed with their cognate anti-sigma factor genes usfX (Rv3287c) and rsbW (MSMEG_1803), respectively (DeMaio et al., 1997; Gebhard et al., 2008). Transcription of the usfX-sigf operon in M. tuberculosis is driven from a SigF-dependent promoter, whereas the rsbW-sigf operon in M. smegmatis is transcribed from two promoters, a SigF-independent promoter located immediately upstream of rsbW and a SigF-dependent promoter upstream of the chaB (MSMEG_1802) gene that is located 103 bp upstream of rsbW (Gebhard et al., 2008).

M. tuberculosis sigf was found to be strongly induced within cultured human macrophages, during stationary phase of growth, and upon exposure to cold shock, nutrient depletion, oxidative stress, and several antibiotics (rifampicin, ethambutol, streptomycin, and cycloserine), as well as in persistor cells (DeMaio et al., 1996; Graham and Clark-Curtiss, 1999; Michele et al., 1999; Mariani et al., 2000; Betts et al., 2002; Keren et al., 2011; Forrellad et al., 2013), while M. smegmatis sigf was shown to be expressed at similar levels throughout its growth phase and only marginally increased under SigF-activating conditions (Gebhard et al., 2008; Singh and Singh, 2008).

The functionality of Sigf is regulated by the so-called partner switching system (PSS) including its cognate anti-sigma factor (anti-Sigf) and anti-anti-sigma factors (anti-Sigf antagonists) (Figure 1; DeMaio et al., 1997; Singh et al., 2015; Bouillet et al., 2018). Under non-stress (Sigf-non-activating) conditions, Sigf is held in an inactive state in complex with the anti-Sigf (RsbW or UsfX). Under stress (Sigf-activating) conditions, the release of Sigf from its anti-Sigf is accomplished by two anti-Sigf antagonists, RsfA (Rv3165c in M. tuberculosis and MSMEG_1786 in M. smegmatis) and RsfB (Rv3687c in M. tuberculosis and MSMEG_6127 in M. smegmatis), which sequester the anti-Sigf (Beaucher et al., 2002; Parida et al., 2005; Singh et al., 2015). RsfA is inactivated when a disulfide bond is formed between its two redox-responsive cysteine residues, while RsfB was suggested to be inactivated by phosphorylation (Beaucher et al., 2002; Manganelli et al., 2004). MSMEG_6129 was identified to be a protein kinase that phosphorylates RsfB in M. smegmatis (Bowman and Ghosh, 2014), while the kinase that is responsible for RsfB phosphorylation remains unknown in M. tuberculosis. Since an MSMEG_6129 mutant strain of M. smegmatis paradoxically displayed decreased expression of the SigF regulon relative to the wild-type (WT) strain (Bowman and Ghosh, 2014), it was uncertain whether MSMEG_6129 is the kinase that inactivates RsfB by phosphorylation. Dephosphorylation of RsfB homologs in Bacillus species was demonstrated to be catalyzed by the PP2C family of phosphatases (Voelker et al., 1996; Vijay et al., 2000; Chen et al., 2003). However, no study has been published regarding which gene product is responsible for dephosphorylation of phosphorylated RsfB in mycobacteria. The Rv1364c gene in M. tuberculosis was found to encode a multi-domain protein consisting of the sensor, PP2C phosphatase, GHKL (gyrase, Hsp90, histidine kinase, MutL) kinase, and anti-sigma antagonist domains (Sachdeva et al., 2008; Greenstein et al., 2009; Misra et al., 2019). Rv1364c was shown to interact with Sigf in vitro, suggesting the possibility that it might serve as an anti-Sigf along with the major anti-Sigf UsfX (Misra et al., 2019). Although Rv1364c was demonstrated to possess both kinase and phosphatase activities that autoprophosphorylate and autodephosphorylate its anti-sigma antagonist domain (Greenstein et al., 2009), the question remains unanswered regarding whether it can phosphorylate and dephosphorylate RsfB to modulate the anti-Sigf antagonist activity of RsfB.

Using relevant mutant strains and protein interaction analyses, we here reveal the distinct roles of three RsbW homologs and two anti-Sigf antagonists (RsfA and RsfB) in the Sigf PSS of M. smegmatis. This study provides several lines of evidence showing that MSMEG_6129 is the only kinase in M. smegmatis that phosphorylates RsfB on Ser-63 to inactivate the functionality of RsfB as an anti-Sigf antagonist. This study also presents the novel finding that expression of the Sigf regulon in M. smegmatis is strongly induced under respiration-inhibitory conditions in an RsfB-dependent way.

MATERIALS AND METHODS

Bacterial and Yeast Strains, Plasmids, and Culture Conditions

The bacterial and yeast strains and plasmids used in this study are listed in Table S1 in the supplementary material. Escherichia coli strains were cultivated in Luria-Bertani (LB) medium on a gyratory shaker (200 rpm) at 37°C. M. smegmatis strains were grown aerobically in Middlebrook 7H9 medium (Difco, Sparks, MD, United States) supplemented with 0.2% (w/v) glucose (7H9-glucose) and 0.02% (v/v) Tween 80 as an anti-clumping agent on a gyratory shaker at 37°C. Ampicillin (100 or 200 µg/ml for E. coli), kanamycin (50 µg/ml for E. coli and 15 or 30 µg/ml for M. smegmatis), chloramphenicol (34 µg/ml for E. coli) and hygromycin (200 µg/ml for E. coli and 50 µg/ml for M. smegmatis) were added to the growth medium when required.
Oh et al. SigF Partner Switching System

FIGURE 1 | The schematic illustration outlining the current knowledge of the SigF PSS in M. smegmatis and M. tuberculosis. The “?” mark indicates the ambiguity of the roles of the corresponding proteins in the SigF PSS.

For treatment of M. smegmatis cultures with KCN, the cultures were grown to an optical density at 600 nm (OD_{600}) of 0.45–0.5 and further incubated for 15 min following the addition of KCN to the cultures to a final concentration of 0.5 mM.

Saccharomyces cerevisiae strains were cultivated in YPD medium (Difco) or synthetic defined dropout (SD) medium (Clontech, Palo Alto, CA, United States) on a gyratory shaker at 30°C.

DNA Manipulation and Transformation
Standard protocols and manufacturers’ instructions were followed for recombinant DNA manipulations (Green and Sambrook, 2012). Transformation of M. smegmatis and S. cerevisiae with plasmids was conducted by electroporation and the lithium acetate (LiAc)-mediated method, respectively, as previously described (Snapper et al., 1990; Guthrie and Fink, 1991).

Site-Directed Mutagenesis
To introduce point mutations into the rsfB genes, PCR-based mutagenesis was performed using the Quick Change site-directed mutagenesis procedure (Stratagene, La Jolla, CA, United States). Synthetic oligonucleotides containing a mutated codon in the middle of their sequences were used to mutagenize the original codons. The primers used for mutagenesis are listed in Table S2. Mutations were verified by DNA sequencing.

β-Galactosidase Assay and Determination of the Protein Concentration
β-Galactosidase activity in M. smegmatis was assayed spectrophotometrically as described elsewhere (Oh and Kaplan, 1999). Protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, United States) with bovine serum albumin as a standard protein.
Reverse Transcription-PCR and Quantitative Real-Time PCR

RNA isolation from *M. smegmatis* strains, preparation of cDNA, reverse transcription PCR (RT-PCR), and quantitative real-time PCR (qRT-PCR) were conducted as described previously (Kim et al., 2010). The primers used for cDNA synthesis, RT-PCR, and qRT-PCR are listed in Table S2.

**Protein Purification**

The C-terminally His₆-tagged WT and mutant forms of RsfB were expressed in *E. coli* BL21 (DE3) strains harboring the pT7-7 derivative plasmids (pT7-7rsfB, pT7-7rsfBS63A, and pT7-7rsfBS63E). The strains harboring the pT7-7 derivatives were cultivated aerobically at 37°C in LB medium containing 100 µg/ml ampicillin to an OD₆₀₀ of 0.4–0.6. Expression of the rsfB gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and then cells were further grown for 4 h at 30°C. For purification of RsfB from *M. smegmatis*, the *M. smegmatis* strains containing pMHRsfB or pMHRsfBS63E were grown aerobically to an OD₆₀₀ of 0.5–0.8 at 37°C in 7H9-glucose medium supplemented with 15 µg/ml kanamycin. Expression of the rsfB gene was induced by the addition of acetamide to a final concentration of 0.2% (w/v), and then cells were further grown for 7 h at 37°C. After 400 ml of *E. coli* or *M. smegmatis* cultures were harvested, cells were resuspended in 10 ml of buffer A [(20 mM Tris–HCl pH 8.0), 100 mM NaCl] containing 10 U/ml DNase I and 10 mM MgCl₂. The resuspended cells were disrupted twice for *E. coli* or five times for *M. smegmatis* using a French pressure cell, and cell-free crude extracts were obtained by centrifugation twice at 14,000 × g for 15 min. 500 µl of 50% (v/v) slurry (bed volume: 250 µl) of Ni-Sepharose high performance resin (GE Healthcare, Piscataway, NJ, United States) was packed into a column. After equilibration of the resin with 10 bed volumes of buffer A, cell-free crude extracts were loaded into the column. The resin was washed with 40 bed volumes of buffer A containing 10 mM imidazole, 20 bed volumes of buffer A containing 30 mM imidazole, and then His₆-tagged RsfB was eluted with 10 bed volumes of buffer A containing 100 mM imidazole. The eluted His₆-tagged RsfB was diluted with buffer A to 10 mM imidazole and subjected to affinity chromatography again. The resin was washed with 20 bed volumes of buffer A containing 30 mM imidazole, and then His₆-tagged RsfB was finally eluted with 10 bed volumes of buffer A containing 100 mM imidazole. Imidazole and NaCl were removed from purified RsfB by means of a PD-10 desalting column (GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 8.0).

Purification of RsbW1 was conducted using *E. coli* BL21 (DE3) strains carrying pT7-7-rsbW1. Cell-free crude extracts were loaded into the column packed with Ni-Sepharose resin. The resin was washed with 40 bed volumes of buffer A containing 10 mM imidazole, 20 bed volumes of buffer A containing 30 mM imidazole, and then His₆-tagged RsbW1 was eluted with 10 bed volumes of buffer A containing 100 mM imidazole. The eluted His₆-tagged RsbW1 was diluted with buffer A to 10 mM imidazole and subjected to affinity chromatography again. The resin was washed with 20 bed volumes of buffer A containing 30 mM imidazole, and then His₆-tagged RsbW1 was finally eluted with 10 bed volumes of buffer A containing 100 mM imidazole. Imidazole and NaCl were removed from purified RsbW1 by means of a PD-10 desalting column (GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 8.0).

**Western Blotting Analysis**

To detect expressed SigF, RsbW1, and RsfB in cells, Western blotting analyses using rabbit polyclonal antibodies against the corresponding proteins were performed as described previously (Mouncey and Kaplan, 1998). For detection of His₆-tagged proteins a mouse monoclonal antibody against His-3 (Santa Cruz Biotechnology, Santa Cruz, CA, United States; sc8036) was employed. The rabbit polyclonal antibodies and His-3 monoclonal antibody were used at a 1:20,000 and 1:2,000 dilution, respectively. To detect GroEL, a mouse monoclonal antibody against HSP65 (Santa Cruz Biotechnology; sc58170) was used at a 1:2,000 dilution. Alkaline phosphatase-conjugated anti-rabbit IgG produced in goat (Sigma, St. Louis, CA, United States; A0545) or alkaline phosphatase-conjugated anti-mouse IgG produced in rabbit (Sigma; A4312) was used at a 1:10,000 dilution for the detection of the primary antibodies.

**Analysis of in vitro Protein–Protein Interactions Using Non-denaturing PAGE**

The mixture of two purified proteins in 20 mM Tris–HCl (pH 8.0) solution containing 20 mM β-mercaptoethanol was mixed with the same volume of 2× Binding buffer [40 mM Tris–HCl (pH 8.0), 0.01 mM EDTA (pH 8.0), 10 mM MgCl₂, 20% (v/v) glycerol] and incubated for 25 min at room temperature. After the addition of 10× sample buffer [50 mM Tris–HCl (pH 6.8), 40% (v/v) sucrose, 0.05% (w/v) bromophenol blue], the mixtures were subjected to non-denaturing PAGE [7.5% (w/v) acrylamide] using electrophoresis buffer [2.5 mM Tris–HCl (pH 8.3), 19.2 mM glycine], which was initially run at 80 V for 1 h and subsequently at 100 V for 4 h. Non-denaturing PAGE was conducted at 4°C.

**Analysis of in vivo Protein–Protein Interactions Using Copurification Assay**

To examine protein interactions of RsbW1 with RsbW2 and RsbW3 in *M. smegmatis*, copurification assay using Ni-Sepharose resin was performed. The C-terminally His₆-tagged RsbW2 and RsbW3 were expressed in the WT strains of *M. smegmatis* harboring pMHRsbW2 and pMHRsbW3, respectively. The strains were grown aerobically to an OD₆₀₀ of 0.45–0.5 at 37°C in 7H9-glucose medium supplemented with 15 µg/ml kanamycin and 0.1% (w/v) acetamide. After 200 ml of *M. smegmatis* cultures were harvested, cells were resuspended in 8 ml of buffer A containing 10 U/ml DNase I and 10 mM MgCl₂. The resuspended cells were disrupted five times using a French pressure cell, and cell-free crude extracts were obtained by centrifugation twice at
14,000 × g for 15 min. 500 μl of the 50% (v/v) slurry (bed volume 250 μl) of Ni-Sepharose resin was packed into a column. After equilibration of the resin with 10 bed volumes of buffer A, cell-free crude extracts were loaded into the column. The resin was washed with 125 bed volumes of buffer A containing 10 mM imidazole and then His₆-tagged RsbW2 and RsbW3 were eluted with 10 bed volumes of buffer A containing 250 mM imidazole. RsbW2 and RsbW3 in the eluents were detected by Western blotting analysis with a His-3 monoclonal antibody. The presence of RsbW1 in the eluents was determined using Western blotting analysis with RsbW1 polyclonal antibodies.

Analysis of in vivo Protein–Protein Interactions Using Yeast Two-Hybrid Assay

S. cerevisiae AH109 strains cotransformed with both pGADT7linker and pGBK7 derivatives were grown in SD medium (Clontech, Palo Alto, CA, United States) lacking leucine and tryptophan (SD/-Leu/-Trp). The overnight cultures were diluted with distilled water to an OD₆₀₀ of 0.6 and spotted onto both solid SD/-Leu/-Trp plates and histidine-deficient SD/-Leu/-Trp/-His plates containing various concentrations of 3-amino-1,2,4-triazole (3-AT) for spotting assays. The plates were incubated at 30°C for 3–5 days.

RNA Sequencing and Gene Expression Profiling

Three biological replicate cultures of the WT and Δα₃Δ strains were grown aerobically to an OD₆₀₀ of 0.45–0.5. Total RNA of each culture was isolated as described previously (Kim et al., 2010). rRNA was removed from the isolated total RNA using a Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, San Diego, CA, United States). The RNA sequencing libraries were created using a TruSeq RNA Sample Prep Kit v2 (Illumina) with the standard low-throughput protocol. Sequencing of the six libraries was conducted on an Illumina HiSeq 4000 platform at Macrogen Inc. (Seoul, South Korea) using the HiSeq 3000–4000 sequencing protocol and TruSeq 3000–4000 SBS Kit v3 reagent (Illumina). Paired-end reads (101 bp) were then mapped to the reference genome sequence of M. smegmatis mc²155 (GCF_000015005.1_ASM1500v1) with the program Bowtie 1.1.2 using default settings. Summarized statistics of RNA sequencing alignment are listed in Table S3. The differentially expressed genes (DEGs) were subsequently identified pair-wise by the edgeR package in R language (Robinson et al., 2010). In this analysis, the genes with P-value < 0.05 and |log₂ fold change of gene expression (FC)| > 2 were regarded as DEGs. The data described in this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through the GEO Series accession number GSE155251.

In vitro Kinase Assay

Purified RsfB was mixed with purified RsbW1, RsbW2, or RsbW3 in 30 μl of reaction buffer [20 mM Tris–Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 10 mM MnCl₂]. The reactions were started by adding 100 μM ATP and incubated for 30 min at 30°C. The reactions were terminated by adding 7.5 μl of gel-loading buffer [250 mM Tris–Cl (pH 6.8), 50% (w/v) glycerol, 500 mM dithiothreitol (DTT), 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.5% (w/v) bromophenol blue]. Proteins were resolved by Phos-tag SDS-PAGE prepared as described elsewhere (Barbieri and Stock, 2008). The duplicated reaction mixtures were subjected to normal SDS-PAGE. The gels were stained with Coomassie brilliant blue (CBB).

RESULTS

Induction of the SigF Regulon Under Respiration-Inhibitory Conditions and the Genetic Organization of the Genes Involved in the SigF PSS

Comparative RNA sequencing analysis of the WT strain of M. smegmatis and its isogenic Δα₃ mutant strain with a deletion in citC encoding subunit III of the α₃ cytochrome c oxidase led us to identify 103 DEGs whose expression is upregulated in the Δα₃ mutant by more than four-fold with a P-value less than 0.05 relative to the WT strain. As shown in Figure 2A and Table S4, 61 genes of the 103 DEGs were found to overlap with the genes belonging to the known SigF regulon (Singh et al., 2015), suggesting that the genes of the SigF regulon are strongly upregulated in M. smegmatis, when the major terminal oxidase of the electron transport chain (ETC) is inactivated. Among the 61 identified genes, we selected two genes (MSMEG_1777 and MSMEG_1782) with the large FC and RPKM (reads per kilo base pair per million mapped reads) values in the Δα₃ mutant, and examined the expression levels of the genes in the WT (control) and Δα₃ mutant strains grown aerobically, as well as in the WT strain treated with KCN, an inhibitor of the α₃ cytochrome c oxidase (Figure 2B). Consistent with the RNA sequencing result, expression of MSMEG_1777 and MSMEG_1782 was significantly increased in the Δα₃ mutant and the WT strain treated with KCN as compared to that in the control WT strain. We also included the Δf₁f₂f₃ mutant strain of M. smegmatis with deletions in three furA paralogous genes in this experiment, since it had been reported that the genes of the SigF regulon are strongly downregulated in the Δf₁f₂f₃ mutant relative to the WT strain (Lee et al., 2018). As expected, expression of MSMEG_1777 and MSMEG_1782 was significantly decreased in the mutant, confirming the MSMEG_1777 and MSMEG_1782 genes belong to the SigF regulon. Based on this result, we hereafter used the MSMEG_1777 gene as a marker gene of the SigF regulon to determine the functionality of SigF.

As a first step to understand the mechanism underlying the strong induction of the SigF regulon under respiration-inhibitory conditions, we decided to investigate the SigF PSS in detail. The rsbW-sigF (MSMEG_1803-MSMEG_1804) operon has been previously identified, and the role of RsbW (MSMEG_1803) as an anti-SigF in M. smegmatis has been suggested on the basis of its overexpression phenotype and its protein interaction with SigF (Singh et al., 2015). The genes encoding the proposed
M. smegmatis query sequence was performed against the BLAST search using the RsbW (MSMEG_1803) sequence as a whose products are likely to be involved in the SigF PSS, a identified (Singh et al., 2015). To identify the additional genes Oh et al. SigF Partner Switching System in and MSMEG_6129 genome, which revealed two additional genes (MSMEG_1787 and MSMEG_1782) that encode the RsbW homologs. As shown in Figure 3, The MSMEG_1787 and MSMEG_6129 genes were found to be located in the vicinity of the rsfA and rsfB genes, respectively. The RsbW homologs, whose genes are adjacent to sigF, rsfB, and rsfA, were named as RsbW1, RsbW2, and RsbW3, respectively. Among the three RsbW homologs, RsbW1 composed of 138 amino acids shows the highest homology (66.2% identity) to UsfX of M. tuberculosis. The rsbW2 gene, whose product consists of 148 amino acids, appears to form an operon with the upstream gene rsfB. Downstream of rsbW2 occurs a putative operon that contains the gene (MSMEG_6130) encoding a histidine kinase and two adjacent genes (MSMEG_6128 and MSMEG_6131) encoding N-terminally receiver domain-containing proteins. Sequence analysis revealed that MSMEG_6128 and MSMEG_6131 contain a DNA-binding domain and a PP2C phosphatase domain at their C-termini, respectively. RsbW2 was previously reported to encode the protein kinase that phosphorylates the anti-SigF antagonist RsfB (Bowman and Ghosh, 2014). However, its inactivation by mutation was reported not to produce the anticipated phenotype like an increase in expression of the SigF regulon (Bowman and Ghosh, 2014), casting doubt as to whether RsbW2 is the kinase that inactivates RsfB by phosphorylation. The rsbW3 gene was found to code for the largest RsbW homolog composed of 194 amino acids. When the amino acid sequence of RsbW3 was aligned with that of RsbW1, RsbW3 was found to have an N-terminal extension of 30 amino acids which is not present in RsbW1 (Supplementary Figure S1).

The Roles of Three RsbW Homologs in the Regulation of SigF Functionality
To examine whether the identified RsbW homologs function as anti-SigF, we individually inactivated the rsbW1, rsbW2, and rsbW3 genes by deleting the corresponding genes, yielding the ΔrsbW1, ΔrsbW2, and ΔrsbW3 mutant strains of M. smegmatis (Supplementary Figure S2). As shown in Supplementary Figure S3, both ΔrsbW1 and ΔrsbW2 mutants formed yellow-colored colonies on solid agar plates unlike the WT, ΔrsbW3, and ΔsigF strains of M. smegmatis, implying that biosynthesis of the carotenoid isorenieratene is increased in the ΔrsbW1 and ΔrsbW2 mutant strains. Given the previous report that the genes involved in biosynthesis of isorenieratene belong to the SigF regulon in M. smegmatis (Provvedi et al., 2008; Hummel et al., 2010), it is likely that expression of the SigF regulon is increased in the ΔrsbW1 and ΔrsbW2 mutants. Unfortunately, the ΔrsbW1 and ΔrsbW2 mutants were found to be instable in terms of yellow pigmentation. They lost the yellow color during cultivation in liquid growth medium, and the altered strains did not restore the yellow pigmentation in solid growth plates. For this reason, we did not examine expression of the SigF-dependent genes in the mutants. Instead, overexpression effects of rsbW1, rsbW2, and rsbW3 on MSMEG_1777 expression were examined to assess the anti-SigF activity of the three RsbW homologs. The genes of the RsbW homologs were overexpressed from an acetamide-inducible promoter on pMHRsbW1, pMHRsbW2, and pMHRsbW3 that are derivatives of the pMH201 integration vector. The expression

FIGURE 2 | Overlap between the SigF regulon and the genes induced in the Δaa3 mutant strain of M. smegmatis. (A) Volcano plot showing the DEGs in the Δaa3 mutant strain relative to the WT strain. RNA sequencing was carried out using RNA extracted from three biological replicate cultures of the WT and Δaa3 strains aerobically grown to an OD600 of 0.45–0.5 in 7H9-glucose medium. The x-axis represents log2 fold change of gene expression (log2FC) in the Δaa3 mutant strain relative to the WT strain, and the y-axis represents −log10 (P-value). The vertical dashed lines on the graph mark the border lines indicating the log2FC values of −2 and 2. The genes, which are differently regulated by more than | log2FC | > 2 with P-value < 0.05, are depicted by blue-filled circles. Among the identified DEGs, the genes belonging to the SigF regulon are denoted by red-filled circles. The reported downregulated genes (log2FC < −2) in a sigF mutant strain relative to the WT strain were regarded as the genes belonging to the SigF regulon (Singh et al., 2015). Two representative genes (MSMEG_1777 and MSMEG_1782), whose expression was confirmed by RT-PCR, are indicated by the arrows. (B) RT-PCR analysis showing the expression levels of the MSMEG_1777 and MSMEG_1782 in the WT, Δf1f2f3, and Δaa3 mutant strains. The M. smegmatis strains were grown aerobically to an OD600 of 0.45–0.5 in 7H9-glucose medium. For treatment of cultures with KCN, the WT strain was grown to an OD600 of 0.45–0.5 in 7H9-glucose medium. For treatment of 0.5 mM KCN to the final concentration of 0.45–0.5, followed by the addition of KCN to the final concentration of 0.5 mM. The WT cultures were further grown under KCN-untreated (control) or treated (0.5 mM KCN) conditions for 15 min. RT-PCR for 16S rRNA was performed to assure that equal amounts of total RNA were employed for RT-PCR.
The prerequisite for a protein to act as an anti-sigma factor is protein–protein interactions between the protein and its cognate sigma factor. To determine protein–protein interactions between the RsbW homologs and SigF, we performed yeast two-hybrid assay (Y2H). For the Y2H assay, the rsbW1, rsbW2, and rsbW3 genes were cloned into the prey vector pGADT7 linker, whereas the sigF gene was cloned into the bait vector pGBKT7. As shown in Figure 5A, the yeast strain coexpressing RsbW1 and SigF grew well on solid growth medium without histidine (−His) in the presence of up to 5 mM 3-AT. In contrast, coexpression of either RsbW2 or RsbW3 with SigF did not lead to growth of yeast on −His medium in the presence of 3-AT. As expected, the yeast strains coexpressing either RsbW1 or SigF alone did not grow on −His medium in the presence of 3-AT. We next examined in vitro protein–protein interactions between SigF and RsbW homologs by means of non-denaturing PAGE analysis using purified SigF and RsbW homologs. Since the RsbW3 protein was not purified to homogeneity, the partially purified protein was used in the experiment. As shown in Figure 5B, RsbW1 was shown to interact with SigF as judged by the formation of a new band representing the SigF-RsbW1 complex and disappearance of the SigF band in the non-denaturing PAGE gel. In contrast, the presence of RsbW2 and RsbW3 in the binding mixtures did not result in a decrease in the SigF band intensity in the non-denaturing PAGE gel, indicating that RsbW2 and RsbW3 do not interact with SigF. Taken together, the Y2H and non-denaturing PAGE results suggest that SigF interacts only with RsbW1 among the three RsbW homologs.

The observed overexpression effect of rsbW3 on MSMEG_1777 led us to assume that RsbW3 might serve as an anti-SigF antagonist. To examine this assumption, protein–protein interactions between RsbW3 and three RsbW homologs were assessed using Y2H analysis (Figure 6A). For the Y2H assay, the rsbW1, rsbW2, and rsbW3 genes were cloned into pGADT7 linker, and the rsbW3 gene was cloned into pGBKT7. Only the yeast strain coexpressing RsbW1 and RsbW3 grew on −His medium containing 0.5 mM 3-AT, indicating a possible protein interaction between RsbW1 and RsbW3. Protein–protein interactions between RsbW1 and RsbW3 were also assessed by copurification analysis using affinity chromatography (Figure 6B). RsbW1 was copurified with His$_6$-tagged RsbW3 from crude extracts of the WT strain of M. smegmatis expressing His$_6$-tagged RsbW3, whereas RsbW1 was not copurified with His$_6$-tagged RsbW2 from the M. smegmatis strain expressing His$_6$-tagged RsbW2, confirming protein–protein interactions between RsbW1 and RsbW3.
Both RsfA and RsfB Are Functional as Anti-SigF Antagonists, and RsfB Is the Major Anti-SigF Antagonist in M. smegmatis

After having established the distinct roles of three RsbW homologs in the SigF PSS, we next examined the roles of the suggested anti-SigF antagonists RsfA and RsfB in vivo.

We constructed deletion mutants of rsfA and rsfB in the background of both WT and Δaa3 strains, and the expression level of MSMEG_1777 in the WT and mutant strains of M. smegmatis was comparatively determined (Figures 7A,B).

The inactivation of the aa3 cytochrome c oxidase was used for an induction condition of the SigF regulon. The expression level of MSMEG_1777 was shown to be increased by 4.9-fold in the Δaa3 strain of M. smegmatis relative to that in the WT strain grown under the same conditions. Expression of MSMEG_1777 was abolished in the ΔsigF and Δaa3ΔsigF mutant strains. Both the results confirmed that transcription of MSMEG_1777 depends on SigF, and that expression of the SigF regulon is induced under respiration-inhibitory conditions.

Expression of MSMEG_1777 was decreased by 26% in the ΔrsfA mutant compared to the WT strain. The Δaa3ΔrsfA mutant also showed a 31% decrease in MSMEG_1777 expression relative to the Δaa3 mutant strain. It is noteworthy that inactivation of rsfB almost abolished expression of MSMEG_1777 in both WT and Δaa3 mutant strains. These results suggest that both RsfA and RsfB serve as anti-SigF antagonists, and that RsfB is the major anti-SigF antagonist in M. smegmatis under both SigF-activating and SigF-non-activating conditions.

To confirm the roles of RsfA and RsfB as anti-SigF antagonists, we examined whether MSMEG_1777 expression correlates with...
of MSMEG_1777 on pMHRsfA and pMHRsfB, respectively. The expression levels of RsfA and RsfB were expressed from an acetamide-inducible promoter Fig 7C,D in concentrations of acetamide in growth medium. As shown pMHRsfA and the expression levels of RsfA and RsfB. The concentration range of acetamide used in the experiment. Taken not detect the expressed RsfA by Western blotting analysis in the strain expressing WT RsfB. Western blotting analysis revealed that the WT and mutant forms of RsfB were expressed at similar levels in the WS strain expressing WT RsfB. The expression level of MSMEG_1777 was shown to be decreased by about 50% in the T32A mutation resulted in abolishment of Figure 8A. The ΔrsfB mutant of M. smegmatis was complemented by introducing the pMV306 derivatives that carry the WT or mutant rsfB genes. The WT and ΔrsfB strains carrying the pMV306 empty vector were included in the experiment as positive and negative controls, respectively. As shown in Figure 8B, the S63A mutation led to a drastic increase in MSMEG_1777 expression in M. smegmatis, whereas the T32A mutation resulted in abolishment of MSMEG_1777 expression. The expression level of MSMEG_1777 was shown to be decreased by about 50% in the M. smegmatis strain expressing the phosphomimetic S63E form of RsfB compared to the control M. smegmatis strain expressing WT RsfB. Western blotting analysis revealed that the WT and mutant forms of RsfB were expressed at similar levels in the M. smegmatis strains used in the experiment. These results suggest the followings: (i) unphosphorylated RsfB is the active form of RsfB as an anti-SigF antagonist, (ii) phosphorylation of RsfB on Ser-63 decreases the functionality of RsfB as in the RsfB homologs such as RsbV (Bowman and Ghosh, 2014). To specify the functionally important residue(s) among the identified phosphorylation sites in RsfB, we examined the phosphorylation of Ser-63 in vitro (Bowman and Ghosh, 2014). Using LC-tandem mass spectrometry, eight Ser/Thr residues (Ser-3, Thr-10, Thr-20, Thr-25, Thr-27, Thr-32, Ser-42, and Ser-63) in RsfB have been identified to be the phosphorylation sites by RsbW2 (Bowman and Ghosh, 2014). The expression of RSF A and RSF B function as anti-SigF antagonists in M. smegmatis.

We further examined whether the significantly reduced expression of MSMEG_1777 in the ΔrsfB mutant is caused by the decreased expression of sigF or the increased expression of rsbW1. Using Western blotting analysis, the protein levels of expressed SigF and RsbW1 were determined in the WT, ΔrsfA, and ΔrsfB strains that were grown aerobically to an OD600 of 0.45–0.5 (Supplementary Figure S4). The Western blotting result showed that the protein levels of SigF and RsbW1 in the ΔrsfA and ΔrsfB mutant strains are not different from those in the WT strain, indicating that the cellular levels of SigF and RsbW1 in M. smegmatis are not decreased under SigF-non-activating conditions. This finding can be explained by the presence of a SigF-independent promoter immediately upstream of the rsbW1-sigF operon (Gebhard et al., 2008).

**The Functionality of RsfB Is Controlled Through Phosphorylation of Ser-63 by RsbW2**

A previous study has reported that purified RsbW2 (MSMEG_6129) phosphorylates RsfB (MSMEG_6127) in vitro (Bowman and Ghosh, 2014). To determine MSMEG_1777 expression in the ΔrsfB mutant expressing the mutant forms of RsfB. The Ser-3 was excluded from the experiment since the residue is not present in Bacillus subtilis RsbV (Figure 8A). The ΔrsfB mutant of M. smegmatis was not detectable by introducing the pMV306 derivatives that carry the WT or mutant rsfB genes. The WT and ΔrsfB strains carrying the pMV306 empty vector were included in the experiment as positive and negative controls, respectively.

As shown in Figure 8B, the S63A mutation led to a drastic increase in MSMEG_1777 expression in M. smegmatis, whereas the T32A mutation resulted in abolishment of MSMEG_1777 expression. The expression level of MSMEG_1777 was shown to be decreased by about 50% in the M. smegmatis strain expressing the phosphomimetic S63E form of RsfB compared to the control M. smegmatis strain expressing WT RsfB. Western blotting analysis revealed that the WT and mutant forms of RsfB were expressed at similar levels in the M. smegmatis strains used in the experiment. These results suggest the followings: (i) unphosphorylated RsfB is the active form of RsfB as an anti-SigF antagonist, (ii) phosphorylation of RsfB on Ser-63 decreases the functionality of RsfB as in the RsfB homologs such as RsbV and SpoIIAA of B. subtilis (Bouillet et al., 2018), (iii) Thr-32 is important for anti-SigF antagonist activity of RsfB.

To examine the phosphorylation state of RsfB in M. smegmatis grown under SigF-non-activating conditions and whether Ser-63 is the only residue that is phosphorylated, we expressed the expression levels of RsfA and RsfB. The rsfA and rsfB genes were expressed from an acetylaminodegradable promoter on pMHRsfA and pMHRsfB, respectively. The expression level of MSMEG_1777 was determined in the ΔrsfA mutant with pMHRsfA and the ΔrsfB mutant with pMHRsfB with increasing concentrations of acetamide in growth medium. As shown in Figures 7C,D, the expression level of MSMEG_1777 in the M. smegmatis strains with either pMHRsfA or pMHRsfB were gradually increased with increasing concentrations of acetamide, and lower concentrations of acetamide were required for similar levels of MSMEG_1777 induction in M. smegmatis with pMHRsfA compared to M. smegmatis with pMHRsfB. Western blotting analysis showed that the amount of expressed RsfB was proportional to the concentration of acetamide. We did not detect the expressed RsfA by Western blotting analysis in the concentration range of acetamide used in the experiment. Taken together, these results provide the strong evidence that both RsfA and RsfB function as anti-SigF antagonists in M. smegmatis.
FIGURE 7 | Effects of inactivation and overexpression of *rsfA* and *rsfB* on expression of MSMEG_1777 in *M. smegmatis*. The expression level of MSMPQ_1777 was determined in the WT, ΔrsfA, ΔrsfB, ΔsigF, Δaa3, Δaa3ΔrsfA, Δaa3ΔrsfB, and Δaa3ΔsigF strains of *M. smegmatis* harboring pNCI1777 (A,B). The *M. smegmatis* strains were grown aerobically to an OD$_{600}$ of 0.45–0.5 in 7H9 medium. Cell-free crude extracts were used to measure β-galactosidase activity. All values are the means of the results from three biological replicates. The error bars indicate the standard deviations. *P* < 0.05. Effects of increasing expression of *rsfA* and *rsfB* on MSMEG_1777 expression were examined in the ΔrsfA and ΔrsfB strains harboring pNCI1777, respectively (C,D). The pMH201-derived pMHRsfA plasmid was used for the controllable expression of *rsfA* in the ΔrsfA strain, and pMHRsfB was used for the controllable expression of *rsfB* in the ΔrsfB strain. The strains were grown aerobically to an OD$_{600}$ of 0.45–0.5 in 7H9 medium supplemented with the indicated concentrations of acetamide (Ace). Cell-free crude extracts were used to measure β-galactosidase activity. All values are the means of the results from three biological replicates. The error bars indicate the standard deviations. Western blotting analysis was performed for the detection of expressed His$_6$-tagged RsfB. Cell-free crude extracts (30 µg) were separated on SDS-PAGE, followed by Western blotting analysis with a His-tag antibody. **P** < 0.01 and *P* < 0.05.

the WT and S63E mutant forms of His$_6$-tagged RsfB in both *M. smegmatis* and *E. coli*, purified the proteins, and determined their phosphorylation state using Phos-tag SDS-PAGE analysis. As shown in Figure 9, most fractions of WT RsfB purified from *M. smegmatis* were found to be phosphorylated, while S63E RsfB purified from *M. smegmatis* was not phosphorylated at all. Both WT and S63E mutant forms of RsfB purified from *E. coli* were found to be unphosphorylated. The results indicate that Ser-63 in RsfB is the residue that is phosphorylated in *M. smegmatis*, and that *E. coli* does not have the protein kinase that can phosphorylate RsfB.

We next examined the effect of Ser-63 phosphorylation on protein–protein interactions between RsfB and RsbW1. In place of phosphorylated RsfB, the phosphomimetic S63E mutant form of RsfB was employed for non-denaturing PAGE analysis. The WT and S63A RsfB proteins purified from *E. coli* were used as unphosphorylated RsfB. As shown in Figure 10, both WT RsfB and S63A RsfB interacted with purified RsbW1
Identification of the amino acid residues that are responsible for the inactivation of RsfB by phosphorylation. (A) Multiple sequence alignment of the RsfB homologs of *M. smegmatis*, *M. tuberculosis*, and *B. subtilis* was generated using ClustalW. The asterisks and colons denote the conserved and conservatively substituted amino acid residues, respectively. The residues of *M. smegmatis* RsfB, which were identified to be phosphorylated by MSMEG_6129 in vitro (Bowman and Ghosh, 2014), are shown in the gray background. (B) Effects of T10A, T20A, T25A, T27A, S42A, S63A, and S63E mutations on the functionality of RsfB in vivo. (Continued)
and formed the retarded bands representing the RsfB-RsbW1 complex in non-denaturing PAGE. The intensity of the RsfB-RsbW1 complex bands was increased up to the ratio of RsbW1 to RsfB to be 1:1 with increasing amounts of WT RsfB and S63A RsfB. In contrast, the S63E mutant form of RsfB did not give rise to the RsfB-RsbW1 complex band even at high concentrations of S63E RsfB. The results suggest that phosphorylation of Ser-63 inactivates RsfB to render it unable to interact with RsbW1.

Since the RsbW homologs of Bacillus SigB had been demonstrated to function as both anti-SigB and the protein kinase phosphorylating the anti-SigB antagonist (Benson and Haldenwang, 1993; Dufour and Haldenwang, 1994), we wondered whether in addition to RsbW2, RsbW1, and RsbW3 have the protein kinase activity phosphorylating RsfB. To examine this possibility, we performed in vitro kinase assay using purified RsbW homologs and RsfB. As shown in Figure 11A, only RsbW2 could phosphorylate unphosphorylated RsfB purified from E. coli, which is in good agreement with the fact that RsbW1 and RsbW3 are closely clustered with anti-sigma factors lacking the kinase activity, while RsbW2 is clustered with kinase-positive anti-sigma factors (Supplementary Figure S5). We also examined the phosphorylation state of RsfB in the WT and ΔrsbW2 mutant strains grown under SigF-non-activating conditions using Phos-tag SDS-PAGE and Western blotting analysis (Figure 11B). The Δ5437 mutant of M. smegmatis was included in the experiment, since it had been suggested that MSMEG_5437 is a Ser/Thr protein kinase that might modulate RsbW2 activity by phosphorylation (Bowman and Ghosh, 2014). Phos-tag SDS-PAGE showed that RsfB in the ΔrsbW2 mutant was not phosphorylated in contrast to RsfB in the WT and Δ5437 strains of M. smegmatis, indicating that RsbW2 is the only protein kinase that phosphorylates RsfB in M. smegmatis, and that RsbW2 is still active in the Δ5437 mutant.

DISCUSSION

The genome of M. smegmatis contains three genes encoding RsbW homologs (RsbW1, RsbW2, and RsbW3). Among them, RsbW1 shows the highest degree of homology to UsfX that is a known anti-SigF in M. tuberculosis. Y2H and non-denaturing PAGE analysis revealed the interaction of RsbW1 with SigF, which is in good agreement with the previous result from bacterial two-hybrid assay (Singh et al., 2015). Overexpression of rsbW1 in M. smegmatis led to a significant reduction in expression of MSMEG_1777 that is under the control of SigF. Furthermore, disruption of the rsbW1 gene by deletion resulted in an increase in yellow pigmentation of M. smegmatis colonies, which appears to be the result of increased isorenieratene biosynthesis. All of these results indicate that RsbW1 is a bona fide anti-SigF in M. smegmatis.

RsbW2 is most deviated among the RsbW homologs with regard to the reciprocal sequence homology (Supplementary Figure S5). In contrast to RsbW1, RsbW2 was shown not to interact with SigF in Y2H and non-denaturing PAGE analysis, implying that RsbW2 does not play a direct role as an anti-SigF. However, overexpression and inactivation of rsbW2 gave rise to the same phenotype as those of rsbW1 in terms of
FIGURE 10 | Determination of protein–protein interactions between RsbW1 and several forms of RsfB (WT, S63A, and S63E) by non-denaturing PAGE. 160 pmol of purified RsbW1 was mixed with increasing amounts of the purified WT and mutant forms (S63A and S63E) of RsfB in binding buffer [40 mM Tris–HCl (pH 8.0), 0.01 mM EDTA, 10 mM MgCl$_2$, 20% (v/v) glycerol] and incubated for 30 min at 25°C. The mixtures were subjected to both SDS-PAGE (upper panel) and native PAGE (lower panel). The gels were stained with CBB. The bands representing RsbW1, RsfB (WT and mutant forms), and RsbW1-RsfB complex are indicated by the arrows.
both \textit{MSMEG} \_1777 expression and colony pigmentation. These results suggest that RsbW2 has an activity to decrease SigF functionality without direct binding to SigF. A clue about the anti-SigF activity of RsbW2 came from the kinase motifs (N, G1, and G2) that are conserved in RsbW2. Like \textit{B. subtilis} RsbW that can inactivate the anti-SigB antagonist RsbV through phosphorylation (Dufour and Haldenwang, 1994), RsbW2 has the protein kinase activity that inhibits the functionality of RsbW by phosphorylation of Ser-63. RsfB was shown to exist in an unphosphorylated form in the MSMEG \_1777 mutant in contrast to the isogenic WT strain in which most fractions of RsfB exist in a phosphorylated form, which indicates that RsbW2 is the kinase that can phosphorylate RsfB in \textit{M. smegmatis}. It is worth noting that RsbW of the SigB PSS in \textit{Bacillus} species and \textit{Streptomyces coelicolor} acts as both protein kinase for its cognate anti-SigB antagonist (RsbV) and anti-sigma factor (Benson and Haldenwang, 1993; Dufour and Haldenwang, 1994; Voelker et al., 1996; Lee et al., 2004; van Schaik and Abee, 2005), while RsbW1 and RsbW2 of \textit{M. smegmatis} are specialized to function as anti-SigF and protein kinase, respectively.

Interestingly, both \textit{ΔrsbW1} and \textit{ΔrsbW2} mutants of \textit{M. smegmatis} showed phenotypic instability in terms of yellow pigmentation. When first obtained, both the mutants exhibited yellow pigmentation on solid 7H9-glucose medium. However, when the mutant strains were passed through successive subcultures in solid and especially liquid growth media, the mutant strains lost yellow pigmentation and showed only basal levels of \textit{MSMEG} \_1777 expression (data not shown). This observation implies that excessive expression of the SigF regulon is detrimental to \textit{M. smegmatis}, leading to secondary mutations that mitigate the expression of the SigF regulon.

RsbW3 is closer to RsbW1 than RsbW2 in terms of sequence homology. In contrast to \textit{rsbW1}, overexpression of \textit{rsbW3} in \textit{M. smegmatis} resulted in a significant increase in \textit{MSMEG} \_1777 expression. Both the lack of protein–protein interactions between RsbW3 and SigF and the overexpression effect of \textit{rsbW3} suggest the role of RsbW3 as an anti-SigF antagonist. Given both the quaternary structure (homodimers) of RsbW (SpoIIAB)-like anti-sigma factors (Campbell et al., 2002; Masuda et al., 2004) and the results demonstrating protein–protein interactions between RsbW1 and RsbW3 (Figure 6), we assume that RsbW3 likely inactivates RsbW1 by forming a heterodimer when overexpressed. The presence of a SigF–recognizing promoter (\textit{GTTT-N17-GGGTAA}) upstream of \textit{rsbW3} (Table S4) and abolishment of \textit{rsbW3} expression by the inactivation of the \textit{sigF} gene (Supplementary Figure S6) indicate that \textit{rsbW3} belongs to the SigF regulon. Based on these findings, we suggest that RsbW3 serves as a booster for expression of the SigF regulon under SigF-activating conditions via the positive feedback loop.

So far, the roles of RsfA and RsfB as anti-SigF antagonists in mycobacteria have been predicted from both their protein interactions with anti-SigF (RsbW and UsfX) and the result from \textit{in vitro} transcription analysis (Beaucher et al., 2002; Malik et al., 2008, 2009; Singh et al., 2015). Through both deletion and overexpression of \textit{rsfA} and \textit{rsfB}, we first demonstrated the physiological roles of RsfA and RsfB as anti-SigF antagonists in
M. smegmatis in vivo. As judged by the RPKM values obtained from RNA sequencing analysis (Lee et al., 2018), the transcript level of rsfB was estimated to be ~6-fold higher than that of rsfA in M. smegmatis grown aerobically to an OD_{600} of 0.4–0.5 in 7H9-glucose medium (Supplementary Figure S7). The difference in the expression levels of rsfA and rsfB might give a clue explaining the dominant role of RsfB as an anti-SigF antagonist. The observation that expression of MSMEG_1777 was nearly abolished in the ΔrsfB mutant despite the presence of RsfA implies that the cellular level of active RsfA might not be sufficient to quarantine RsbW1 to such an extent as to induce the SigF regulon in the absence of RsfB. The result of rsfA and rsfB overexpression using an acetamide-inducible promoter clearly showed that when RsfA is sufficiently expressed, it acts as an anti-SigF antagonist more efficiently than RsfB. This observation is in good agreement with the previous report assuming that expression of the SigF regulon in excess is toxic to M. smegmatis.

We found that Ser-63 is the amino acid residue of RsfB that is phosphorylated by RsbW2. It was also demonstrated that the S63A mutant form of RsfB interacts with RsbW1 with a similar affinity as the unphosphorylated form of WT RsfB, while the phosphomimetic (S63E) form of RsfB does not interact with RsbW1. These results confirm that the phosphorylation state of Ser-63 determines the functionality of RsfB as an anti-SigF antagonist. The importance of the corresponding serine residue in the functionality of anti-sigma factor antagonists has been reported for several RsfB homologs (RsbB of M. tuberculosis, RsbV and SpoIIB of B. subtilis) (Diederich et al., 1994; Najafi et al., 1995; Yang et al., 1996; Beaucher et al., 2002).

The Δaa3 mutant of M. smegmatis lacking the aa3 cytochrome c oxidase of the respiratory ETC has been reported to exhibit 53% of the oxygen consumption rate observed for the isogenic WT strain (Jeong et al., 2018), indicating that electron flow through the ETC is inhibited in the mutant by ~50% relative to the WT strain. The finding that expression of the SigF regulon is significantly induced in the Δaa3 mutant relative to the WT strain implies that the availability of free active SigF is increased in response to inhibition of the respiratory ETC. This observation is in good agreement with the suggestion that SigF makes direct contributions to transcriptomic remodeling in M. smegmatis under hypoxic growth conditions (Martini et al., 2019). The activation of SigF under respiratory-inhibitory conditions might result from energy limitation as in the case of the SigB PSS in Bacillus species (Hecker and Volker, 2001; Marles-Wright and Lewis, 2007; de Been et al., 2011; Paget, 2015), or from other factors associated with ETC functions such as changes in the redox state of electron carriers, membrane potential, and proton motive force, etc. The inactivation of the aa3 cytochrome c oxidase in the background of the ΔrsfB mutant was shown not to lead to induction of MSMEG_1777 expression. This result implies that RsfB mediates the induction of the SigF regulon under respiration-inhibitory conditions.

We demonstrated that the sole protein kinase that phosphorylates RsfB in M. smegmatis is RsbW2. RsbW2 has been suggested to be phosphorylated by a Ser/Thr protein kinase, MSMEG_5437 (Bowman and Ghosh, 2014), although the role of MSMEG_5437 in the SigF PSS remains elusive. In the vicinity of the rsfB-rsbW2 operon occur the genes encoding a histidine kinase (MSMEG_6130) and a receiver domain-containing PP2C phosphatase (MSMEG_6131). The PP2C-family phosphatases are known to be responsible for dephosphorylation of the anti-SigB antagonist RsbV in Bacillus species and S. coelicolor (de Been et al., 2011). Indeed, our preliminary result showed that MSMEG_6131 could dephosphorylate the phosphorylated RsfB protein (data not shown). It is conceivable that the phosphorylation state of RsfB might be modulated by the combined control of the kinase activity of RsbW2 and the phosphatase activity of MSMEG_6131 that might be regulated by MSMEG_5437 Ser/Thr protein kinase and MSMEG_6130 histidine kinase, respectively. Further study is required to reveal the mechanism by which inhibition of the respiratory ETC leads to the activation of SigF in M. smegmatis.

DATA AVAILABILITY STATEMENT

The RNA sequencing data described in this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through the GEO Series accession number GSE155251.

AUTHOR CONTRIBUTIONS

J-IO, S-YS, and YO: conception or design of the study. YO, S-YS, H-JK, GH, and H-YK: acquisition of the data. YO, S-YS, JH, and J-IO: analysis or interpretation of the data and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.588487/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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