



A bacterial hemerythrin-like protein MsmHr inhibits the SigF-dependent hydrogen peroxide response in mycobacteria

Xiaojing Li^{1†}, Jun Tao^{1†}, Xinling Hu^{1†}, John Chan², Jing Xiao¹ and Kaixia Mi^{1,3*}

¹ CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

² Departments of Medicine and Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

³ Beijing Key Laboratory of Microbial Drug Resistance and Resistome, Beijing, China

Edited by:

Biswarup Mukhopadhyay, Virginia Tech, USA

Reviewed by:

C. Martin Lawrence, Montana State University, USA

James Moir, University of York, UK
Leif Kirsebom, Uppsala University, Sweden

*Correspondence:

Kaixia Mi, Institute of Microbiology, NO. 1 Beichen West Road, Chaoyang District, Beijing 100101, China
e-mail: mik@im.ac.cn

[†] These authors have contributed equally to this work.

Hydrogen peroxide (H₂O₂) is one of a variety of reactive oxygen species (ROS) produced by aerobic organisms. Host production of toxic H₂O₂ in response to pathogen infection is an important classical innate defense mechanism against invading microbes. Understanding the mechanisms by which pathogens, in response to oxidative stress, mediate defense against toxic ROS, can reveal anti-microbial targets and shed light on pathogenic mechanisms. In this study, we provide evidence that a *Mycobacterium smegmatis* hemerythrin-like protein MSMEG_2415, designated MsmHr, is a H₂O₂-modulated repressor of the SigF-mediated response to H₂O₂. Circular dichroism and spectrophotometric analysis of MsmHr revealed properties characteristic of a typical hemerythrin-like protein. An *msmHr* knockout strain of *M. smegmatis* mc²155 (Δ *msmHr*) was more resistant to H₂O₂ than its parental strain, and overexpression of MsmHr increased mycobacterial susceptibility to H₂O₂. Mutagenesis studies revealed that the hemerythrin domain of MsmHr is required for the regulation of the H₂O₂ response observed in the overexpression study. We show that MsmHr inhibits the expression of SigF (MSMEG_1804), an alternative sigma factor that plays an important role in bacterial oxidative stress responses, including those elicited by H₂O₂, thus providing a mechanistic link between Δ *msmHr* and its enhanced resistance to H₂O₂. Together, these results strongly suggest that MsmHr is involved in the response of mycobacteria to H₂O₂ by negatively regulating a sigma factor, a function not previously described for hemerythrins.

Keywords: mycobacteria, hemerythrin-like protein, MsmHr, SigF, hydrogen peroxide

INTRODUCTION

Hydrogen peroxide (H₂O₂) is a universal oxidative stress molecule produced by aerobic organisms from all three domains of life (Imlay, 2008). The production of H₂O₂ by the host in response to pathogen infection is also an important innate defense mechanism (Fang, 2004). This ROS can be damaging via direct toxic effects or disruption of redox balance, the latter being critical for metabolic homeostasis and hence survival. An important intracellular pathogen, *Mycobacterium tuberculosis* has evolved many strategies to detoxify H₂O₂, some of which are unique to mycobacteria (Kumar et al., 2011; Trivedi et al., 2012). For example, *M. tuberculosis*, which lacks the conventional redox buffer glutathione, uses mycothiol (MSH), a low-molecular-weight thiol that exists in millimolar quantities in the cytoplasm, to generate a reducing environment (Farhana et al., 2010); and MSH-deficient mycobacterial mutants are hypersusceptible to H₂O₂ (Rawat et al., 2002). The precise mechanisms by which MSH protects against oxidative stress and redox imbalance remain to be determined. *M. tuberculosis* also lacks classic sensors such as OxyR and SoxR for the detection of redox signals

(Imlay, 2013). The tubercle bacillus, however, expresses DosS and DosT (Kumar et al., 2007), two sensor histidine kinases whose heme iron plays a critical role in their response to the levels of O₂, nitric oxide (NO) and carbon monoxide (CO). Interaction with these various gasses activates the kinase activity of DosS and DosT, relaying the signals to the response regulator DosR. WhiB3 is an Fe-S cluster transcription factor that controls the expression of the hypoxia regulon of *M. tuberculosis* (Bhat et al., 2012). Indeed, accumulating evidence suggests that the *M. tuberculosis* WhiB family of Fe-S cluster proteins plays important roles in regulating a wide spectrum of microbial functions including responses to oxidative stress and virulence (Burian et al., 2012; Saini et al., 2012a). Together, the DosS-DosT/WhiB3 paradigm underscores the importance of iron-containing proteins in the response of *M. tuberculosis* to redox signals, including those imposed by reactive oxygen intermediates.

The non-heme, di-iron, O₂-binding hemerythrin-like proteins are present in all domains of life (Bailly et al., 2008; French et al., 2008). Bioinformatics analyses have revealed over 400 hemerythrin-like proteins in available prokaryotic genomes

(Bailly et al., 2008; French et al., 2008). These proteins harbor the conserved hemerythrin domain either singly or jointly with another distinct functional domain (Bailly et al., 2008; French et al., 2008). Experimental evidence suggests that they can function as oxygen sensors and reserves, as well as mediate the delivery and transport of this diatomic gas (French et al., 2008). Dcr (*Desulfovibrio* chemoreceptor) H was the first bacterial hemerythrin-like protein to be identified. It has been proposed that the C-terminal hemerythrin domain of DcrH, a member of the Dcr family of putative methyl-accepting chemotaxis proteins of the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris*, functions to sense O₂ (Xiong et al., 2000); the signal thus generated is proposed to be transduced to the transmitting domains to mediate chemotaxis (Xiong et al., 2000). The first single-domain hemerythrin-like protein was identified in *Methylococcus capsulatus*. The expression of the *M. capsulatus* hemerythrin-like protein is enhanced significantly with increasing concentrations of copper, and is thought to be an oxygen carrier that supplies copper-containing methane monooxygenase with oxygen (Kao et al., 2004; Karlsen et al., 2005; Chen et al., 2012). Despite the wide distribution of hemerythrin-like proteins in a wide variety of bacterial species, including *M. tuberculosis* (Xiong et al., 2000; Karlsen et al., 2005; Isaza et al., 2006; Justino et al., 2007; Onoda et al., 2011; Schaller et al., 2012), functional characterization studies are scarce.

Transcriptional regulation is critical to bacterial survival in response to various stresses. Sigma factors are the primary transcriptional regulators of bacterial gene expression. *M. tuberculosis* has 13 sigma factors, and SigH, SigE, SigL, and SigF play important roles in ROS detoxification (Rodrigue et al., 2006). SigF, a highly conserved sigma factor in the genus *Mycobacterium* (Rodrigue et al., 2006), is highly induced by various environmental stresses and during stationary phase (Demaio et al., 1996). In *M. smegmatis*, *sigF* deletion also increases susceptibility to oxidative stress (Gebhard et al., 2008; Humpel et al., 2010). Recently, a genome-wide gene expression study (Humpel et al., 2010) and work from our laboratory (Wu et al., 2012) have provided evidence that SigF regulates the expression of oxidative stress defense genes such as *kata*, *dps1*, and *sodA*, but not *katG* and *ahpC*, genes that have been linked to mycobacterial resistance to isoniazid (Silva et al., 2003). These studies suggest that SigF-mediated resistance to H₂O₂ is independent of KatG and AhpC. This notion is also supported by the fact that SigF-deficient mutants do not display decreased susceptibility to isoniazid (Demaio et al., 1996; Humpel et al., 2010). Regulation of SigF in mycobacteria is generally thought to be predominantly at the post-transcriptional level via the action of anti-sigma and anti-anti-sigma factors (Michele et al., 1999; Beaucher et al., 2002; Singh and Singh, 2008). SigF is transcribed from two promoters, P_{msmeg_1802} and P_{rbsw} (Gebhard et al., 2008), and transcriptional reporter fusion studies have shown that promoter P_{msmeg_1802} responds to entry into the stationary phase and promoter P_{rbsw} is inducible upon treatment with D-cycloserine (Gebhard et al., 2008).

In this study, to investigate the role of hemerythrin-like proteins in mycobacteria, we cloned, expressed, and characterized the *M. smegmatis* mc²155 strain MsmHr protein (encoded by *msmeg2415*). Using a genetic and biochemical approach, we

show that (i) MsmHr displays circular dichroism (CD) and UV-vis spectrophotometric features typical of a hemerythrin-like protein; (ii) relative to wild-type bacilli, the *msmHr* knockout strain (Δ *msmHr*) is more resistant to H₂O₂ and mc²155 over-expressing MsmHr exhibits enhanced H₂O₂ susceptibility; (iii) the H₂O₂ response is dependent on the hemerythrin domain; (iv) MsmHr represses *sigF* transcription through the promoter P_{rbsw}, and thus participates in regulating the SigF-mediated H₂O₂ response. Our results indicate that MsmHr, the first mycobacterial hemerythrin-like protein to be characterized, is involved in the H₂O₂ response in mycobacteria and provide insight into its mechanism.

MATERIALS AND METHODS

CULTURE MEDIUM AND GROWTH CONDITIONS

M. smegmatis cultures were grown in Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD) supplemented with ADS enrichment (Albumin-Dextrose Saline containing 5% (w/v) Bovine serum albumin fraction V, 2% (w/v) D-Dextrose and 8.1% (w/v) NaCl) (Jacobs et al., 1991), 0.05% (v/v) Tween 80, and 0.5% (v/v) glycerol (Beijing Modern Eastern Finechemical Co. Ltd, Beijing). Middlebrook 7H10 medium supplemented with ADS enrichment and 0.5% (v/v) glycerol was used as the solid medium for examination of growth status. Growth was also examined in minimal Sauton's medium (4 g asparagine, 2 g sodium citrate, 0.5 g K₂HPO₄·3H₂O, 0.5 g MgSO₄·7H₂O, 0.05 g ferric ammonium citrate, 60 g glycerol in 1 L of H₂O supplemented with 0.05% (v/v) Tween 80) supplemented with antibiotics as indicated. Hygromycin (75 mg/L for *M. smegmatis*, 150 mg/L for *Escherichia coli*; Roche) and kanamycin (25 mg/L for *M. smegmatis*, 50 mg/L for *Escherichia coli*; Amresco) were added to the medium as needed. All bacterial strains used in this study are listed in Table S1.

GENERATION OF KNOCKOUT MUTANT STRAINS, COMPLEMENTATION STRAINS AND OVEREXPRESSION STRAINS

The *msmHr* (*msmeg_2415*) and *sigF* (*msmg_1804*) deletion mutants were generated via a specialized transducing phage delivery system as previously described (Bardarov et al., 2002). The 5'-flanking region of *msmHr* was amplified by polymerase chain reaction (PCR) with the 2415LL/2415LR primer pair and the 3'-flanking region of *msmHr* was amplified with the 2415RL/2415RR primer pair (all primers are listed in Supplemental Table S2). The flanking regions of *sigF* were generated by amplifying the upstream and downstream regions of *sigF* using the 1804LL/1804LR and 1804RL/1804RR primer pairs, respectively. Amplified fragments were ligated with plasmid p0004S, digested with *PflMI* (*msmHr*) or *AlwNI* (*sigF*), and allelic-exchange plasmids thus constructed were digested with *PacI*, and then ligated with *PacI*-digested phAE159. Phage packaging was performed using a MaxPlax packaging extract (Epicenter Biotechnologies, USA) to yield the knockout phages for *msmHr* (phAE-*msmHr*) and *sigF* (phAE-*sigF*). Specialized transduction was carried out as described previously (Bardarov et al., 2002). The knockout clones were screened by PCR using the primer pairs 2415InL/2415InR, 2415LLL/IL(R) and IR(F)/2415RRR for *msmHr* and 1804InL/1804InR, 1804LLL/IL(R), and

IR(F)/1804RRR for *sigF*. Primer positions with respect to the appropriate genes are shown in **Figure 2A** and Figure S1. No *mmsHr* or *sigF* mRNA was detected in the corresponding deletion strains by qRT-PCR using the appropriate primer pairs (**Figure 2A** and Figure S1). Complementation strains were constructed as described previously (Stover et al., 1991). Briefly, the full-length sequence of *mmsHr* or *sigF* amplified from *M. smegmatis* genomic DNA was cloned into the integrating vector pMV361 (Stover et al., 1991) and the resultant plasmids were electroporated into the corresponding knockout strains to yield C- Δ *mmsHr* (Δ *mmsHr*::P_{hsp60}-*mmsHr*) and C- Δ *sigF* (Δ *sigF*::P_{hsp60}-*sigF*). To over-express *mmsHr*, the *mmsHr* fragment was subcloned into pMV261 (Stover et al., 1991) to yield pMV261-*mmsHr* for transformation into *M. smegmatis* (O-*mmsHr*).

DETERMINATION OF SURVIVAL PHENOTYPES UNDER STRESS

Early phase cultures (OD₆₀₀ = 0.3) of all tested strains were serially diluted (1:10) and spotted (3 μ l) onto solid 7H10 medium supplemented with ADS enrichment and stress-inducing chemical agents (20 μ M streptonigrin, 250 μ M NaNO₂) or subjected to low-pH stress (7H9 supplemented with ADS enrichment and 0.05% Tween 80, pH 5.5). For peroxide stress, early-phase cultures were treated with 5 mM H₂O₂ for 3 h, serially diluted (1:10) and spotted (3 μ l) onto solid 7H10 medium supplemented with 10% ADS. The optical density at 600 nm (OD₆₀₀) was measured at the indicated times in the presence of various stresses. Survival under heat shock stress at 50°C was determined by the number of colony forming units during the time indicated. Statistical analyses were performed using unpaired two-tailed *t*-tests. *P*-values are only shown where significant differences were found. **P* < 0.05 and ***P* < 0.01.

CLONING, EXPRESSION AND PURIFICATION OF MsmHr IN ESCHERICHIA COLI

The coding sequence of *mmsHr* was amplified from *M. smegmatis* mc²155 genomic DNA and cloned into the expression vector pET23b (+) (Novagen, USA), in-frame fused with a C-terminal 6xHis-tag sequence to generate the plasmid pET23b-*mmsHr*, which was transformed into *E. coli* BL21 (AI) (Invitrogen, USA) for expression. Recombinant MsmHr was induced by incubation with 0.1% arabinose at 28°C for 3 h. Cells were harvested by centrifugation at 10,000 g for 5 min, resuspended in lysis buffer (20 mM Tris-HCl pH 8.0; 1 M NaCl, 10% (v/v) glycerol, 20 mM imidazole, 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml lysozyme) and lysed by sonication. Lysates were centrifuged at 12,000 g for 30 min at 4°C to remove debris before purification. The supernatants were incubated with Ni-NTA agarose (Qiagen, USA) with rotation (15 g) for 4 h at 4°C. Beads were then washed three times with washing buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10% (v/v) glycerol, 50 mM imidazole, 0.1% (v/v) Triton X-100, 1 mM PMSF, 25 mM MgCl₂). The proteins were eluted with elution buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 25 mM MgCl₂, 10% (v/v) glycerol, 400 mM imidazole) and protein concentration was measured using the bicinchoninic acid protein assay reagent and a bovine serum albumin standard. Purified protein was examined using 12%

sodium dodecyl sulfate polyacrylamide gel electrophoresis to verify molecular weight and purity (Figure S2).

SPECTROPHOTOMETRIC AND CIRCULAR DICHROIC (CD) ANALYSIS OF MsmHr

Purified MsmHr was diluted in 20 mM Tris-Cl buffer (pH 7.5). Deoxy samples were obtained by adding a 10-fold molar excess of Na₂S₂O₄ to MsmHr. UV-Vis spectrophotometric spectra were obtained in 1 mm path length quartz cuvettes on a UV-2802H UV-Vis spectrophotometer (Unico Shanghai Instruments Co., Ltd., China). Spectra of deoxy-MsmHr were collected in an anaerobic incubator (Shanghai Yuejin Medical Instruments Co., Ltd, China). CD measurements were performed using a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd. UK). The analysis software provided with the instrument was used for analysis of the results.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF ISONIAZID IN M. SMEGMATIS

The susceptibility of *M. smegmatis* to isoniazid (INH) was determined using the broth microdilution method (Wallace et al., 1986). After two-fold dilutions of INH in 7H9 supplemented with ADS enrichment and 0.5% (v/v) glycerol, 40- μ l aliquots were mixed with 40 μ l of *M. smegmatis* suspension (10⁵ cells/ml) and deposited into wells of 96-well microtiter plates. The highest concentration of INH was 100 μ g/ml. Plates were incubated at 37°C for 2 days and OD₆₀₀ values of cultures were then measured using a microplate reader (FLUOstar OPTIMA, BMG Labtech). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug that inhibited the visible bacterial growth of *M. smegmatis* after a 2-day incubation (OD₆₀₀ < 0.05). INH susceptibility tests were repeated at least 3 times.

RNA ISOLATION, RT-PCR AND QUANTITATIVE PCR

Log phase cultures (OD₆₀₀ = 0.8–1.0) of all tested strains were diluted 1:100 in 7H9 media supplemented with ADS enrichment, 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80. Strains were cultured until the OD₆₀₀ reached 0.3 and then divided into control and treatment groups. In the treatment group, the cells were treated with 5 mM H₂O₂ for 30 min, and then collected by centrifugation at 12,000 g. Bacterial pellets were resuspended in TRIzol (Invitrogen, USA) and RNA was purified according to the manufacturer's instructions. cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was performed in a Bio-Rad iCycler using 2x SYBR real-time PCR pre-mix (Takara Biotechnology Inc., Japan). The following cycling program was used: 95°C for 1.5 min followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s, followed by 72°C for 6 min. *M. smegmatis* *rpoD* encoding RNA polymerase sigma factor SigA was selected as a reference to normalize gene expression. The 2^{- $\Delta\Delta$ CT} method was used (Livak and Schmittgen, 2001) to evaluate the relative gene expression in different strains and/or different treatments. Primers used are listed in Table S2.

CONSTRUCTION OF PROMOTER-lacZ FUSION EXPRESSION VECTORS

SigF is reported to be transcribed from two promoters, P_{mmsmeg_1802} and P_{rbsw} (Gebhard et al., 2008). To construct the

$P_{msmeg_1802-lacZ}$ and $P_{rbsW-lacZ}$ plasmids, we used the reported primer pairs PsigFF/PsigFR and P1802F/P1802R (Gebhard et al., 2008), and $P_{msmHr-lacZ}$ using P2415F/P2415R (Supplemental Table S2). The promoter sequences of *sigF*, *msmeg_1802* and *msmHr* were then cloned into the upstream region of the *lacZ* gene in pLACzint (Vasudeva-Rao and McDonough, 2008), generating the plasmids $P_{rbsW-lacZ}$, $P_{msmeg_1802-lacZ}$ and $P_{msmHr-lacZ}$, respectively.

β-GALACTOSIDASE (LACZ) ACTIVITY ASSAY

The β-galactosidase activity of various strains were determined as described previously (Gebhard et al., 2008). Briefly, cultures were collected at early logarithmic phase (OD₆₀₀ ≈ 0.3) and resuspended in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM β-mercaptoethanol). Cells were then lysed using a Fastprep bead-beater (Biospec). The enzyme reaction was initiated by adding ortho-nitrophenyl-β-D-galactopyranoside (2 mg/ml) and terminated by adding Na₂CO₃ (0.8 M). β-galactosidase activity was assessed by measuring the OD₄₂₀ value of the reactions mixtures. Assays were performed in triplicate, and the Miller units (MU) were calculated as follows: 1000 × OD₄₂₀ / (OD₆₀₀ of assayed culture × assayed volume × time).

CONSTRUCTION OF *msmHr* POINT MUTANTS

Mutation of specific amino acids was incorporated into the hemerythrin-like domain of *msmHr* in pMV261-*msmHr* by mismatched PCR primers (Table S2). Site-directed mutagenesis was performed following using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutant clones were confirmed by DNA sequencing (BGI-Shenzhen, Shenzhen China). pMV261-*msmHr* mutants were transformed into *M. smegmatis* mc²155. Mutated sites are as follows (altered amino acids are in underlined bold font):

H1M: ⁵⁵AVHETAEEMV⁶⁵ → ⁵⁵AVLETAAAMV⁶⁵
 H2M: ⁸⁶EEHKAKQQLS⁹⁶ → ⁸⁶EELKAKAALS⁹⁶
 H3M: ¹²²AAHEEAEEFV¹³² → ¹²²AALEEAAAFV¹³²

RESULTS

MsmHr IS A BACTERIAL HEMERYTHRIN-LIKE PROTEIN

Detoxification strategies for scavenging host immune defense system-derived H₂O₂ are important for the intracellular survival of mycobacterial pathogens. Expression of Fe-related proteins, such as the Fe-S cluster transcription factor WhiB3, and heme-iron sensors DosS and DosT, is an important strategy for regulating redox balance (Bhat et al., 2012). Hemerythrin proteins are iron-binding proteins known to be involved in oxygen transport and storage, but their biological functions in mycobacteria have yet to be elucidated. Three proteins in *M. smegmatis*, MsmHr, Msmeg_3312 and Msmeg_6612, are predicted to be hemerythrin-like proteins, however, as preliminary experiments indicated that only MsmHr is related to the H₂O₂ response (data not shown), we focused our attention on MsmHr.

We first constructed a multiple alignment of MsmHr with other hemerythrin-like proteins from different bacterial species (Figure 1A). Residues H24, H57, E61, H66, H88, H121, and

E126 (numbering based on the MsmHr sequence) matched the characteristic motifs H...HxxxE ...HxxxH...HxxxxD/E of hemerythrin domains. The secondary structure predicted by SWISS-MODEL (<http://swissmodel.expasy.org/>) (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006; Kiefer et al., 2009) suggested that MsmHr has a typical hemerythrin structure with four α-helices (residues 16–40, 41–68, 80–103, and 104–132) (Figure 1B).

To confirm the predicted helical structure of MsmHr, we purified MsmHr-His₆ protein from *E. coli*. Circular dichroism (CD) spectra of the *E. coli*-purified MsmHr showed two minima at 208 and 222 nm (Figure 1C). Such a pattern, which is characteristic of protein α-helical structures, is typical of the circular dichroism spectra of previously analyzed bacterial hemerythrins (Wirstam et al., 2003). We also performed a CD analysis of gelactin AAL (*Agrocybe aegerita* lectin, PDB 2ZGU), a protein which consists mainly of β-sheets. The CD spectra showed only one minima at 210 nm (Supplemental Figure S3). The UV-visible absorption spectra of MsmHr showed peaks at 327 and 376 nm, a pattern ascribable to the di-iron-center of hemerythrin-like proteins (Karlsen et al., 2005). Absorbance peaks were abrogated upon reduction with Na₂S₂O₄ to generate the deoxy form by removing oxygen (Figure 1D). Since MsmHr has no Trp and only one Phe, UV/Vis spectrophotometric analysis of the purified protein did not detect absorbance at 280 nm (Figure 1D inset). Taken together, these bioinformatics, CD and spectrophotometric results strongly suggest that MsmHr is a hemerythrin-like protein.

MsmHr IS INVOLVED IN THE H₂O₂ STRESS RESPONSE

To define the biological functions of MsmHr, a deletion mutant, Δ *msmHr*, was generated by specialized transduction (Bardarov et al., 2002) (Figure 2A). The loss of *msmHr* was confirmed by PCR (Figure 2A) and no *msmHr* mRNA was detected in Δ *msmHr* (Figure 2B). To determine if deletion of *msmHr* had polar effects on the *msmeg_2414* and *msmeg_2416* genes, their mRNA levels were compared in the wild type mc²155 and Δ *msmHr* strains by RT-PCR. Statistically significant differences in *msmeg_2414* and *msmeg_2416* mRNA were not detected (Figure 2B), indicating that *msmHr* knockout did not affect the transcriptional levels of *msmeg_2414* and *msmeg_2416*.

We then compared the growth rates of the *M. smegmatis* parental strain mc²155 with the MsmHr-deficient mutant Δ *msmHr* in both 7H9 rich medium and Sauton's minimal medium. No growth abnormalities were detected in Δ *msmHr* relative to mc²155, demonstrating that MsmHr does not influence *M. smegmatis* growth in rich (7H9) or minimal medium (Sauton) (Figure 3A). To identify possible biological roles of MsmHr, the growth kinetics and survival of Δ *msmHr* were examined under various stress conditions, including NO, hypoxia, H₂O₂, heat shock, and acidic pH. No growth defects in Δ *msmHr* were detected under the conditions tested, with the exception of H₂O₂ stress (Figure 3B). Δ *msmHr* exhibited mild H₂O₂ resistance compared to the wild type mc²155 strain after treatment with 5 mM H₂O₂ for 3 h, while no difference in the growth of the two strains was observed under non-H₂O₂ treatment conditions. In addition, the resistance phenotype was abrogated in

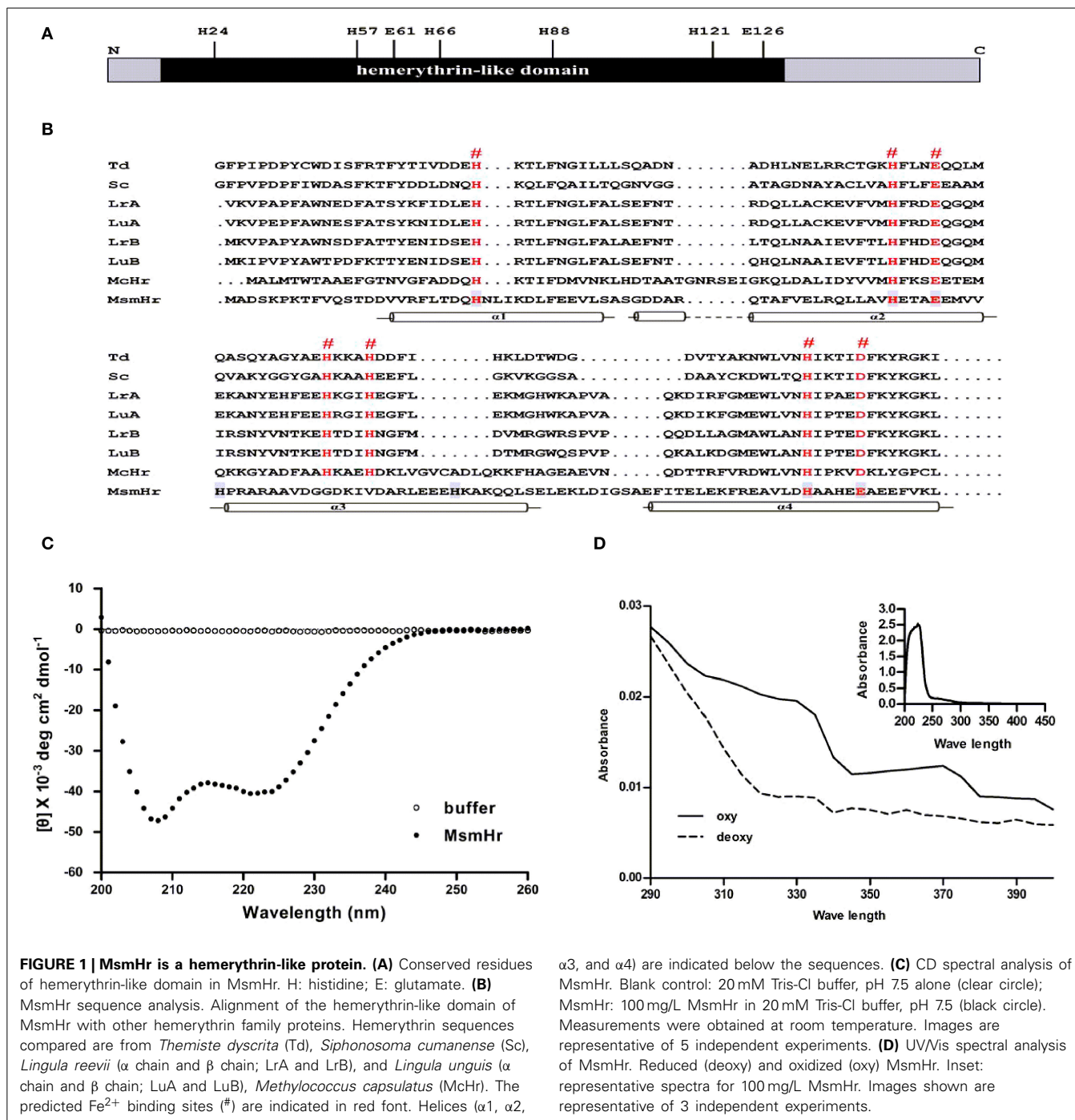


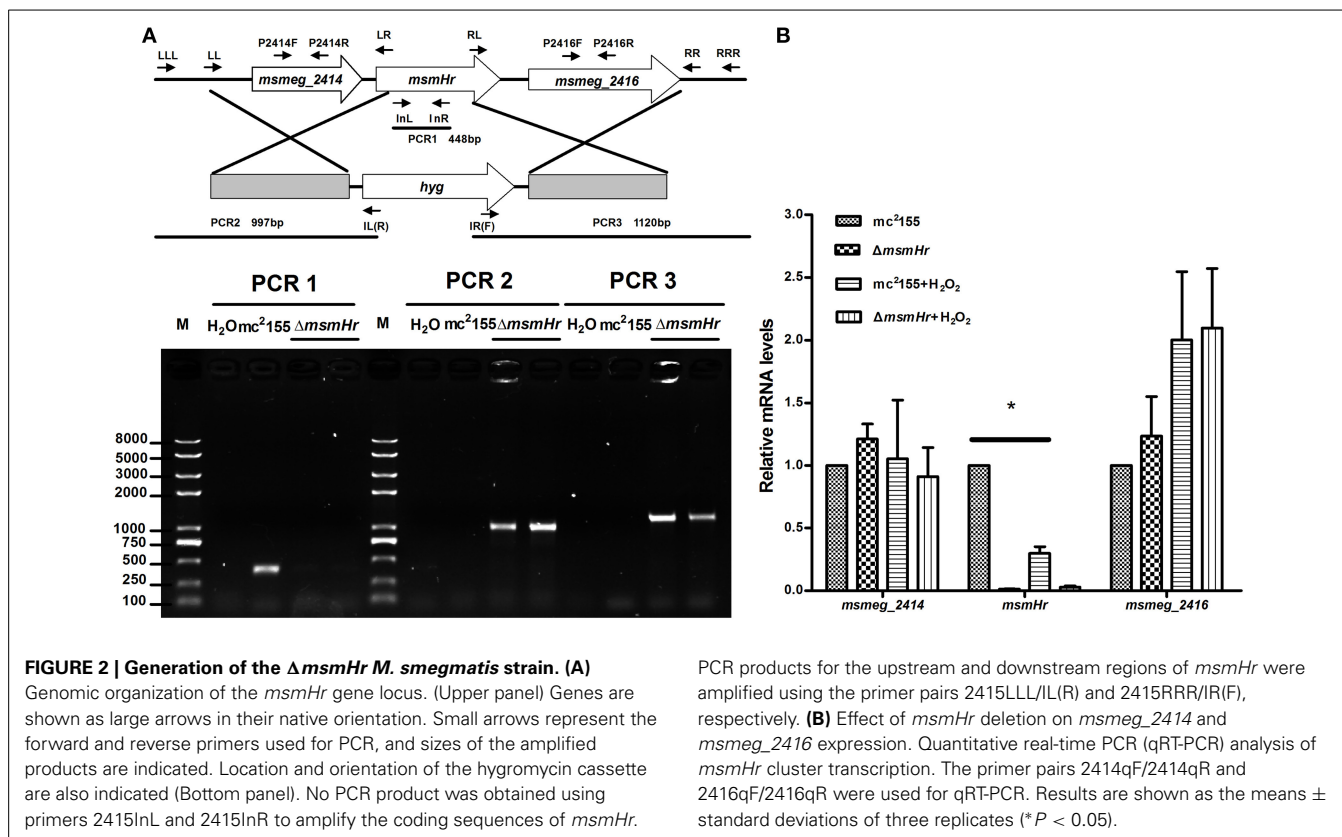
FIGURE 1 | MsmHr is a hemerythrin-like protein. (A) Conserved residues of hemerythrin-like domain in MsmHr. H: histidine; E: glutamate. **(B)** MsmHr sequence analysis. Alignment of the hemerythrin-like domain of MsmHr with other hemerythrin family proteins. Hemerythrin sequences compared are from *Themiste dyscrita* (Td), *Siphonosoma cumanense* (Sc), *Lingula reevii* (α chain and β chain; LrA and LrB), and *Lingula unguis* (α chain and β chain; LuA and LuB), *Methylococcus capsulatus* (McHr). The predicted Fe²⁺ binding sites (#) are indicated in red font. Helices (α 1, α 2,

α 3, and α 4) are indicated below the sequences. **(C)** CD spectral analysis of MsmHr. Blank control: 20 mM Tris-Cl buffer, pH 7.5 alone (clear circle); MsmHr: 100 mg/L MsmHr in 20 mM Tris-Cl buffer, pH 7.5 (black circle). Measurements were obtained at room temperature. Images are representative of 5 independent experiments. **(D)** UV/Vis spectral analysis of MsmHr. Reduced (deoxy) and oxidized (oxy) MsmHr. Inset: representative spectra for 100 mg/L MsmHr. Images shown are representative of 3 independent experiments.

the complemented strain, C- Δ *msmHr* (Figure 3B). Moreover, overexpression of *msmHr* in *mc*²155 (O-*msmHr*) was associated with H₂O₂ susceptibility after treatment with 5 mM H₂O₂ for 3 h (Figure 3B). Differences in mRNA levels of *msmeg_2414* and *msmeg_2416* in wild type *mc*²155 and Δ *msmHr* were not detected after treatment with H₂O₂ (Figure 2B). A statistically significant reduction in *msmHr* mRNA levels was found between *mc*²155 in the presence and absence of H₂O₂ (Figure 2B). Taken together, our data suggest that *msmHr* plays an inhibitory role in the response to H₂O₂ stress.

THE HEMERYTHRIN-LIKE DOMAINS OF MsmHr ARE REQUIRED FOR THE RESPONSE TO H₂O₂ STRESS

To evaluate the potential roles of the hemerythrin-like domain of MsmHr in the H₂O₂ response, we constructed 3 mutants, H1M, H2M, and H3M, in which the respective conserved amino acid motifs HxxxEE, HxxxQQ, and HxxxEE were all mutated to LxxxAA (Figure 4A). As overexpression of *msmHr* showed higher susceptibility to H₂O₂, overexpression vector pMV261 containing *msmHr* alleles harboring the point mutations H1M, H2M or H3M was introduced into *mc*²155 which was then assessed for



resistance to H₂O₂. All strains overexpressing the corresponding H1M, H2M or H3M mutant *msmHr* proteins behaved like mc²155. By contrast, strains containing intact *msmHr* proteins were susceptible to H₂O₂ (Figure 4B). This result indicates that the hemerythrin-like domain of MsmHr is required for H₂O₂ susceptibility.

MsmHr REPRESSES *sigF* EXPRESSION THROUGH THE PROMOTER

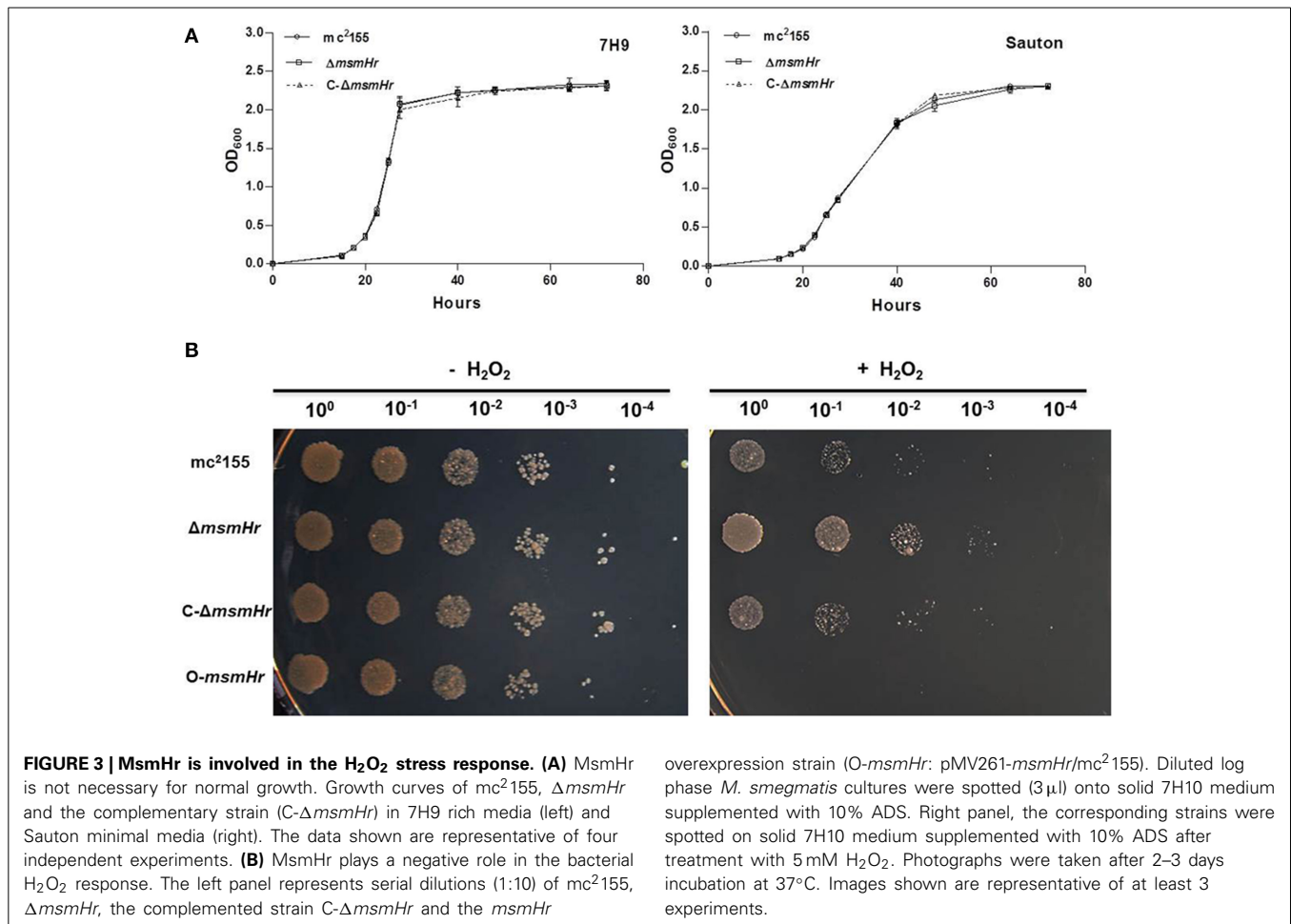
P_{rbsw}

There are two independent oxidative stress pathways in mycobacteria: the KatG-, isoniazid (INH)-related pathway and the SigF-related, INH-unrelated related pathway (Gebhard et al., 2008; Wu et al., 2012). INH is an important first-line anti-mycobacterial pro-drug and is activated by the bacterial catalase-peroxidase enzyme encoded by *katG* (*msmeg_3461*). Resistance to H₂O₂ has been shown to correlate with susceptibility to INH (Bulatovic et al., 2002). To determine which pathway MsmHr is involved in, we measured the minimum inhibitory concentration (MIC) of INH against Δ *msmHr* and wild type mc²155. No difference in the MICs of these two strains was detected (3.125 mg/L in both Δ *msmHr* and mc²155), suggesting that MsmHr is not involved in the INH-related H₂O₂ response. We then examined whether MsmHr is involved in the SigF-mediated H₂O₂ response. As previous studies have shown that *sigF* is transcribed from two promoters, we constructed two vectors with the two promoter regions, *P_{msmeg_1802}* and *P_{rbsw}*, fused to *lacZ* (*P_{msmeg_1802}-lacZ* and *P_{rbsw}-lacZ*, respectively) (Humpel et al., 2010). We measured the indicated promoter activities at the early logarithmic phase:

β -galactosidase activity associated with *P_{msmHr}-lacZ* was 6.9 ± 0.2 MU in mc²155 and 7.6 ± 0.1 MU in Δ *msmHr*, indicating that MsmHr does not self-regulate at the transcriptional level. Wild type mc²155 harboring *P_{msmeg_1802}-lacZ* had a β -galactosidase activity of 3.6 ± 0.3 MU, while the activity of Δ *msmHr* harboring *P_{msmeg_1802}-lacZ* was 3.7 ± 0.2 MU, indicating that MsmHr does not influence the promoter activity of *P_{msmeg_1802}* (Figure 5B left panel). In contrast, Δ *msmHr* harboring *P_{rbsw}-lacZ* had a significantly higher β -galactosidase activity (15.7 ± 1.9 MU) than that of wild type mc²155 (Figure 5B right panel). We also measured the mRNA levels of *sigF* in mc²155, Δ *msmHr* and its complementary strain C- Δ *msmHr*. Consistent with results for the promoter, the knockout *msmHr* led to a 1.9 ± 0.2 fold increase relative to the mRNA level of *sigF* to wild type mc²155, while levels of *sigF* mRNA were not significantly different (Figure 5C). Taken together, our results suggest that MsmHr affects the mRNA level of *sigF* via *P_{rbsw}*.

MsmHr IS NECESSARY FOR THE SigF MEDIATED H₂O₂ RESPONSE

The above results indicate that MsmHr suppresses *sigF* expression at the early logarithmic phase (Figure 5). We next examined the influence of MsmHr on the SigF-mediated H₂O₂ pathway. We compared the mRNA level of *sigF* between mc²155, Δ *msmHr* and the Δ *msmHr* complementary strain C- Δ *msmHr* after 5 mM H₂O₂ treatment for 30 min. As shown in Figure 5C, *sigF* mRNA was induced by H₂O₂ in mc²155, while induction of *sigF* mRNA by H₂O₂ was not detected in Δ *msmHr*. The increase in *sigF* mRNA induced by H₂O₂ was restored in C- Δ *msmHr*. These



results show that MsmHr is required for the SigF-mediated response to H₂O₂.

In addition, we used qRT-PCR to measure the mRNA levels of redox-related genes in both *mc*²¹⁵⁵ and Δ *msmHr* in response to H₂O₂ treatment, using *rpoD* mRNA as an internal invariant control (Table S3). We then chose the high H₂O₂-induced genes *msmeg_4753* and *msmeg_1782*, which belong to the SigF regulon, to evaluate their mRNA level in response to H₂O₂ treatment in *mc*²¹⁵⁵, Δ *sigF*, Δ *msmHr* and *C-ΔmsmHr*. The level of *msmeg_4753* mRNA increased 5.6 ± 0.9 fold in *mc*²¹⁵⁵ after treatment with H₂O₂, but induction of *msmeg_4753* was abrogated in Δ *msmHr* and Δ *sigF* after treatment with H₂O₂ (Figure 6B). In *C-ΔmsmHr*, an increase in *msmeg_4753* RNA was observed in response to H₂O₂ (Figure 6B). The level of *Msmeg_1782* mRNA increased two-fold in both *mc*²¹⁵⁵ and *C-ΔmsmHr* when treated with H₂O₂, while no changes in mRNA level were observed in response to H₂O₂ in Δ *msmHr* and Δ *sigF* (Figure 6A). Taken together, this data indicates that MsmHr is required for the SigF-mediated H₂O₂ response.

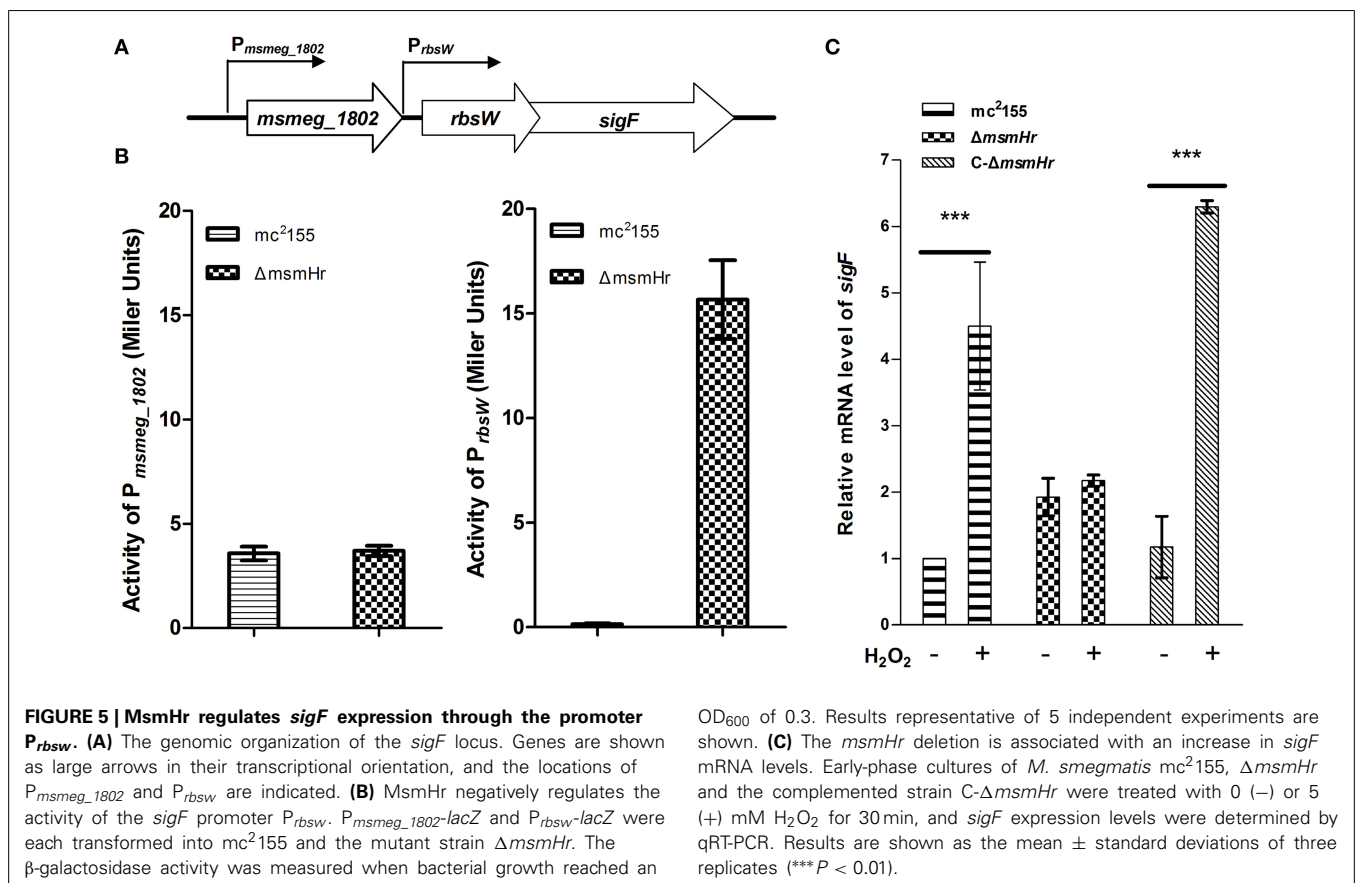
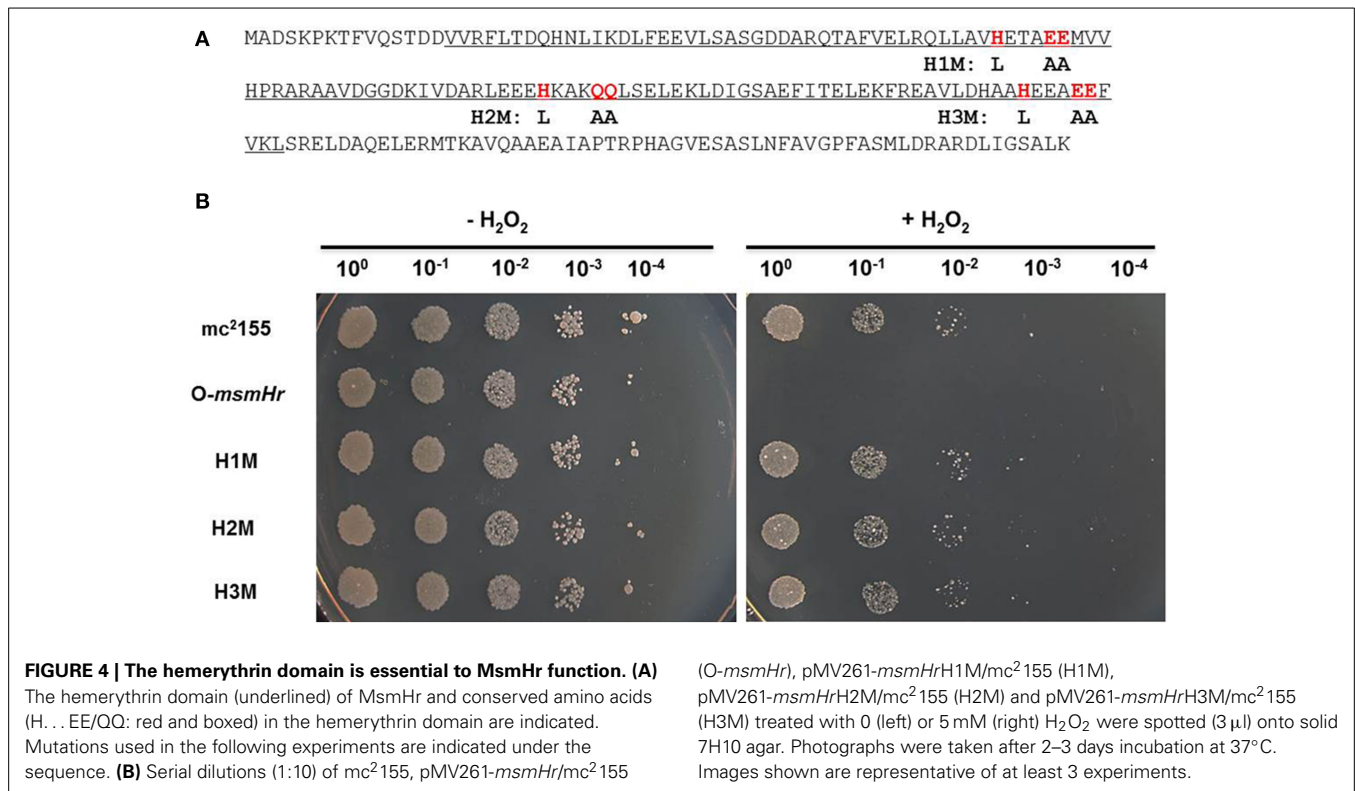
DISCUSSION

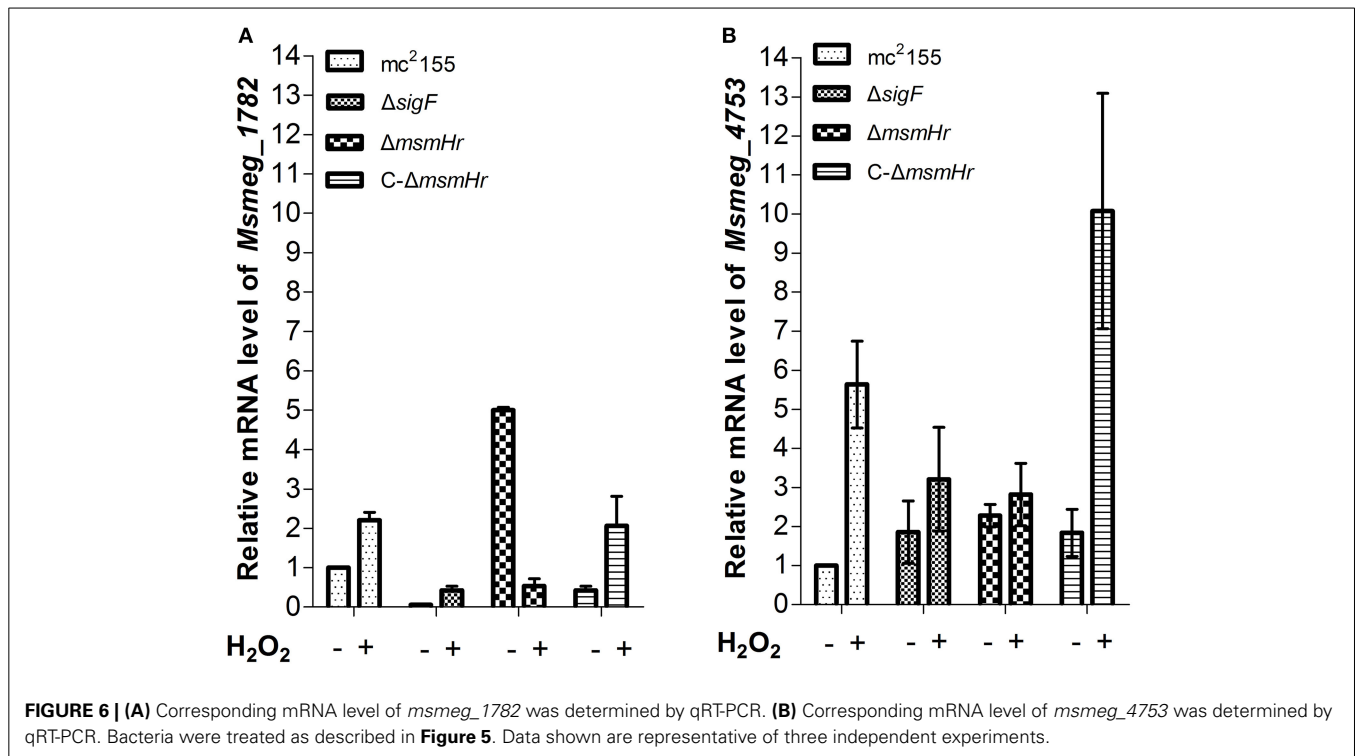
In this study, we have identified a mycobacterial hemerythrin-like protein MsmHr, which regulates *sigF* expression via promoter P_{rbsw} and is necessary for the SigF-mediated H₂O₂ response. To

our knowledge, MsmHr is the first hemerythrin-like protein to be characterized in mycobacteria.

Specific roles for hemerythrin-like proteins are just beginning to be characterized (Xiong et al., 2000; Justino et al., 2007; Schaller et al., 2012). On the basis of their sequences, hemerythrin-like proteins have been postulated to have diverse physiological functions related to oxygen and/or iron (Bailey et al., 2008; French et al., 2008). For example, the *E. coli* hemerythrin-like protein YtfE confers protection against both NO and H₂O₂ stresses (Justino et al., 2005, 2007). Here, however, we did not observe any growth differences between *mc*²¹⁵⁵ and Δ *msmHr* under NO stress, suggesting that the functions of hemerythrin-like proteins in mycobacteria might be distinct from those in *E. coli*. The variation in biological functions of hemerythrin-like proteins might be due to different selective evolutionary environments (Saini et al., 2012b; Martin-Duran et al., 2013).

No difference in sensitivity to INH was observed between the wild-type *mc*²¹⁵⁵ and Δ *msmHr* strains, suggesting that MsmHr is not involved in the INH-related oxidative stress response pathway but rather in an alternative SigF-related H₂O₂ pathway (Gebhard et al., 2008; Wu et al., 2012). It will be interesting to explore why mycobacteria use two-independent H₂O₂ scavenging pathways and which sensors trigger each of these signaling pathways. Recent reports show that a hemerythrin-like domain





of FBXL5 can sense if endogenous iron is limiting and respond to iron stress in the mammalian system (Salahudeen et al., 2009; Vashisht et al., 2009). The correlation between iron- and oxygen-binding MsmHr and SigF-dependent H₂O₂ responses needs to be further explored.

The complexity of the transcription regulatory network allows for efficient and prompt change in levels of gene transcription in response to environmental changes. The protein encoded by mycobacterial *sigF* has closest homology to *Streptomyces coelicolor* SigF, *Bacillus subtilis* SigF and *B. subtilis* SigB (Demaio et al., 1996, 1997; Gebhard et al., 2008). In *B. subtilis*, *sigB* is activated upon entry into the stationary phase and by environmental stresses such as heat, oxidative stress and hyper osmosis. The transcription of *sigB* has been shown to be controlled by two promoters (Wise and Price, 1995). Similarly, SigF may be a potential general stress regulator; SigF is not only activated upon entry into the stationary phase, but is also induced by environmental stresses such as heat shock, acidic pH and oxidative stress (Wise and Price, 1995; Gebhard et al., 2008). Expression of *sigF* has been shown to be regulated by two promoters, P_{*mshHr_1802*} and P_{*rbsw*}. While promoter P_{*mshHr_1802*} is known to respond to entry into the stationary phase (Gebhard et al., 2008), the role of P_{*rbsw*} is less well understood. In this study, comparisons of the activity of the promoters of *sigF* and the mRNA level of *sigF* in the wild type mc²155 and mutant Δ*mshHr* strains (**Figure 5**) showed that MsmHr regulates *sigF* expression via the P_{*rbsw*} promoter.

We show here that MsmHr is essential for the SigF-mediated H₂O₂ response (**Figures 5, 6**). MsmHr hinders *sigF* promoter activation and inhibits *sigF* transcription during normal growth. When *mshHr* is deleted, inhibition of *sigF* is abrogated, and *sigF*

maintains a higher transcript level. The transcription level of *sigF* did not vary in response to H₂O₂ treatment in mutant Δ*mshHr* strains, possibly because the mRNA level of *sigF* was maintained at a higher level in Δ*mshHr* (**Figure 5**). We measured mRNA level changes of members of the SigF-regulon, *mshHr_4753* and *mshHr_1782*, before, between, and after treatment with H₂O₂. Our data show that in wild type mc²155, mRNA levels of both *mshHr_4753* and *mshHr_1782* increased in response to H₂O₂ treatment, but no specific response to H₂O₂ was observed in Δ*sigF* and Δ*mshHr* (**Figure 6**), suggesting that MsmHr is required for induction of *mshHr_4753* and *mshHr_1782* transcription in response to H₂O₂. The presence of inhibitory protein MsmHr suggests that the transcriptional regulation of SigF itself and the SigF regulon, or at least part of the SigF regulon, is based on the balance between the activation and inhibition of the H₂O₂ response. In Δ*mshHr*, the inhibition of *sigF* is abrogated and *sigF* maintains high transcript levels. The expression of one of the SigF regulon genes, *mshHr_1782*, is high in Δ*mshHr* during normal growth compared with mc²155, but decreases in response to H₂O₂ treatment. This is consistent with the finding that MsmHr is necessary for the SigF-dependent H₂O₂ pathway and that the response of *sigF* to H₂O₂ is also abrogated in the absence of *mshHr*.

In summary, we have identified a mycobacterial hemerythrin-like protein that negatively regulates SigF via the P_{*rbsw*} promoter in response to oxidative stress.

ACKNOWLEDGMENTS

We thank Defeng Li for the gift of the protein galactin AAL. This work was supported by the Ministry of Science and Technology of China (2014CB744402 and 2012CB518700).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fmicb.2014.00800/abstract>

REFERENCES

- Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195–201. doi: 10.1093/bioinformatics/bti770
- Bailly, X., Vanin, S., Chabasse, C., Mizuguchi, K., and Vinogradov, S. N. (2008). A phylogenomic profile of hemerythrins, the nonheme diiron binding respiratory proteins. *BMC Evol. Biol.* 8:244. doi: 10.1186/1471-2148-8-244
- Bardarov, S., Bardarov, S. Jr., Pavelka, M. S. Jr., Sambandamurthy, V., Larsen, M., Tufariello, J., et al. (2002). Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* 148, 3007–3017.
- Beaucher, J., Rodrigue, S., Jacques, P. E., Smith, I., Brzezinski, R., and Gaudreau, L. (2002). Novel *Mycobacterium tuberculosis* anti-sigma factor antagonists control sigmaF activity by distinct mechanisms. *Mol. Microbiol.* 45, 1527–1540. doi: 10.1046/j.1365-2958.2002.03135.x
- Bhat, S. A., Singh, N., Trivedi, A., Kansal, P., Gupta, P., and Kumar, A. (2012). The mechanism of redox sensing in *Mycobacterium tuberculosis*. *Free Radic. Biol. Med.* 53, 1625–1641. doi: 10.1016/j.freeradbiomed.2012.08.008
- Bulatovic, V. M., Wengenack, N. L., Uhl, J. R., Hall, L., Roberts, G. D., Cockerill, F. R. III, et al. (2002). Oxidative stress increases susceptibility of *Mycobacterium tuberculosis* to isoniazid. *Antimicrob. Agents Chemother.* 46, 2765–2771. doi: 10.1128/AAC.46.9.2765-2771.2002
- Burian, J., Ramon-Garcia, S., Sweet, G., Gomez-Velasco, A., Av-Gay, Y., and Thompson, C. J. (2012). The mycobacterial transcriptional regulator whiB7 gene links redox homeostasis and intrinsic antibiotic resistance. *J. Biol. Chem.* 287, 299–310. doi: 10.1074/jbc.M111.302588
- Chen, K. H., Wu, H. H., Ke, S. F., Rao, Y. T., Tu, C. M., Chen, Y. P., et al. (2012). Bacteriohemerythrin bolsters the activity of the particulate methane monooxygenase (pMMO) in *Methylococcus capsulatus* (Bath). *J. Inorg. Biochem.* 111, 10–17. doi: 10.1016/j.jinorgbio.2012.02.019
- Demaio, J., Zhang, Y., Ko, C., and Bishai, W. R. (1997). *Mycobacterium tuberculosis* sigF is part of a gene cluster with similarities to the *Bacillus subtilis* sigF and sigB operons. *Tuber. Lung Dis.* 78, 3–12.
- Demaio, J., Zhang, Y., Ko, C., Young, D. B., and Bishai, W. R. (1996). A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2790–2794.
- Fang, F. C. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* 2, 820–832. doi: 10.1038/nrmicro1004
- Farhana, A., Guidry, L., Srivastava, A., Singh, A., Hondalus, M. K., and Steyn, A. J. (2010). Reductive stress in microbes: implications for understanding *Mycobacterium tuberculosis* disease and persistence. *Adv. Microb. Physiol.* 57, 43–117. doi: 10.1016/B978-0-12-381045-8.00002-3
- French, C. E., Bell, J. M., and Ward, F. B. (2008). Diversity and distribution of hemerythrin-like proteins in prokaryotes. *FEMS Microbiol. Lett.* 279, 131–145. doi: 10.1111/j.1574-6968.2007.01011.x
- Gebhard, S., Humpel, A., McLellan, A. D., and Cook, G. M. (2008). The alternative sigma factor SigF of *Mycobacterium smegmatis* is required for survival of heat shock, acidic pH and oxidative stress. *Microbiology* 154, 2786–2795. doi: 10.1099/mic.0.2008/018044-0
- Guex, N., and Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 18, 2714–2723. doi: 10.1002/elps.1150181505
- Humpel, A., Gebhard, S., Cook, G. M., and Berney, M. (2010). The SigF regulon in *Mycobacterium smegmatis* reveals roles in adaptation to stationary phase, heat, and oxidative stress. *J. Bacteriol.* 192, 2491–2502. doi: 10.1128/JB.00035-10
- Imlay, J. A. (2008). Cellular defenses against superoxide and hydrogen peroxide. *Annu. Rev. Biochem.* 77, 755–776. doi: 10.1146/annurev.biochem.77.061606.161055
- Imlay, J. A. (2013). The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol.* 11, 443–454. doi: 10.1038/nrmicro3032
- Isaza, C. E., Silaghi-Dumitrescu, R., Iyer, R. B., Kurtz, D. M. Jr., and Chan, M. K. (2006). Structural basis for O₂ sensing by the hemerythrin-like domain of a bacterial chemotaxis protein: substrate tunnel and fluxional N terminus. *Biochemistry* 45, 9023–9031. doi: 10.1021/bi0607812
- Jacobs, W. R. Jr., Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., et al. (1991). Genetic systems for mycobacteria. *Meth. Enzymol.* 204, 537–555.
- Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007). *Escherichia coli* di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J. Biol. Chem.* 282, 10352–10359. doi: 10.1074/jbc.M610656200
- Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005). New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J. Biol. Chem.* 280, 2636–2643. doi: 10.1074/jbc.M411070200
- Kao, W. C., Chen, Y. R., Yi, E. C., Lee, H., Tian, Q., Wu, K. M., et al. (2004). Quantitative proteomic analysis of metabolic regulation by copper ions in *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* 279, 51554–51560. doi: 10.1074/jbc.M408013200
- Karlsen, O. A., Ramsevik, L., Bruseth, L. J., Larsen, O., Brenner, A., Berven, F. S., et al. (2005). Characterization of a prokaryotic haemerythrin from the methanotrophic bacterium *Methylococcus capsulatus* (Bath). *FEBS J.* 272, 2428–2440. doi: 10.1111/j.1742-4658.2005.04663.x
- Kiefer, E., Arnold, K., Kunzli, M., Bordoli, L., and Schwede, T. (2009). The SWISS-MODEL Repository and associated resources. *Nucleic Acids Res.* 37, D387–D392. doi: 10.1093/nar/gkn750
- Kumar, A., Farhana, A., Guidry, L., Saini, V., Hondalus, M., and Steyn, A. J. (2011). Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert Rev. Mol. Med.* 13, e39. doi: 10.1017/S1462399411002079
- Kumar, A., Toledo, J. C., Patel, R. P., Lancaster, J. R. Jr., and Steyn, A. J. (2007). *Mycobacterium tuberculosis* DosS is a redox sensor and DosT is a hypoxia sensor. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11568–11573. doi: 10.1073/pnas.0705054104
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Martin-Duran, J. M., De Mendoza, A., Sebe-Pedros, A., Ruiz-Trillo, I., and Hejnl, A. (2013). A broad genomic survey reveals multiple origins and frequent losses in the evolution of respiratory hemerythrins and hemocyanins. *Genome Biol. Evol.* 5, 1435–1442. doi: 10.1093/gbe/evt102
- Michele, T. M., Ko, C., and Bishai, W. R. (1999). Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis* sigF gene: implications for chemotherapy against mycobacterial persistors. *Antimicrob. Agents Chemother.* 43, 218–225.
- Onoda, A., Okamoto, Y., Sugimoto, H., Shiro, Y., and Hayashi, T. (2011). Crystal structure and spectroscopic studies of a stable mixed-valent state of the hemerythrin-like domain of a bacterial chemotaxis protein. *Inorg. Chem.* 50, 4892–4899. doi: 10.1021/ic2001267
- Rawat, M., Newton, G. L., Ko, M., Martinez, G. J., Fahey, R. C., and Av-Gay, Y. (2002). Mycothiol-deficient *Mycobacterium smegmatis* mutants are hypersensitive to alkylating agents, free radicals, and antibiotics. *Antimicrob. Agents Chemother.* 46, 3348–3355. doi: 10.1128/AAC.46.11.3348-3355.2002
- Rodrigue, S., Provvedi, R., Jacques, P. E., Gaudreau, L., and Manganelli, R. (2006). The sigma factors of *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 30, 926–941. doi: 10.1111/j.1574-6976.2006.00040.x
- Saini, V., Farhana, A., Glasgow, J. N., and Steyn, A. J. (2012a). Iron sulfur cluster proteins and microbial regulation: implications for understanding tuberculosis. *Curr. Opin. Chem. Biol.* 16, 45–53. doi: 10.1016/j.cbpa.2012.03.004
- Saini, V., Raghuvanshi, S., Khurana, J. P., Ahmed, N., Hasnain, S. E., Tyagi, A. K., et al. (2012b). Massive gene acquisitions in *Mycobacterium indicus pranii* provide a perspective on mycobacterial evolution. *Nucleic Acids Res.* 40, 10832–10850. doi: 10.1093/nar/gks793
- Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, L. N., Li, Q., et al. (2009). An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* 326, 722–726. doi: 10.1126/science.1176326
- Shaller, R. A., Ali, S. K., Klose, K. E., and Kurtz, D. M. Jr. (2012). A bacterial hemerythrin domain regulates the activity of a *Vibrio cholerae* diguanylate cyclase. *Biochemistry* 51, 8563–8570. doi: 10.1021/bi3011797
- Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31, 3381–3385. doi: 10.1093/nar/gkg520

- Silva, M. S., Senna, S. G., Ribeiro, M. O., Valim, A. R., Telles, M. A., Kritski, A., et al. (2003). Mutations in *katG*, *inhA*, and *ahpC* genes of Brazilian isoniazid-resistant isolates of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 41, 4471–4474. doi: 10.1128/JCM.41.9.4471-4474.2003
- Singh, A. K., and Singh, B. N. (2008). Conservation of sigma F in mycobacteria and its expression in *Mycobacterium smegmatis*. *Curr. Microbiol.* 56, 574–580. doi: 10.1007/s00284-008-9126-8
- Stover, C. K., De La Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., et al. (1991). New use of BCG for recombinant vaccines. *Nature* 351, 456–460. doi: 10.1038/351456a0
- Trivedi, A., Singh, N., Bhat, S. A., Gupta, P., and Kumar, A. (2012). Redox biology of tuberculosis pathogenesis. *Adv. Microb. Physiol.* 60, 263–324. doi: 10.1016/B978-0-12-398264-3.00004-8
- Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., et al. (2009). Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science* 326, 718–721. doi: 10.1126/science.1176333
- Vasudeva-Rao, H. M., and McDonough, K. A. (2008). Expression of the *Mycobacterium tuberculosis* *acr*-coregulated genes from the DevR (DosR) regulon is controlled by multiple levels of regulation. *Infect. Immun.* 76, 2478–2489. doi: 10.1128/IAI.01443-07
- Wallace, R. J. Jr., Nash, D. R., Steele, L. C., and Steingrube, V. (1986). Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. *J. Clin. Microbiol.* 24, 976–981.
- Wirstam, M., Lippard, S. J., and Friesner, R. A. (2003). Reversible dioxygen binding to hemerythrin. *J. Am. Chem. Soc.* 125, 3980–3987. doi: 10.1021/ja017692r
- Wise, A. A., and Price, C. W. (1995). Four additional genes in the *sigB* operon of *Bacillus subtilis* that control activity of the general stress factor sigma B in response to environmental signals. *J. Bacteriol.* 177, 123–133.
- Wu, H., Hu, X., Xiao, J., Zhang, J., Tao, J., Huang, H., et al. (2012). Sigma factor F regulates *Mycobacterium smegmatis* hydrogen peroxide resistance. *Wei Sheng Wu Xue Bao* 52, 1352–1359.
- Xiong, J., Kurtz, D. M. Jr., Ai, J., and Sanders-Loehr, J. (2000). A hemerythrin-like domain in a bacterial chemotaxis protein. *Biochemistry* 39, 5117–5125. doi: 10.1021/bi992796o

Conflict of Interest Statement: 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.

Received: 19 September 2014; accepted: 27 December 2014; published online: 15 January 2015.

Citation: Li X, Tao J, Hu X, Chan J, Xiao J and Mi K (2015) A bacterial hemerythrin-like protein MsmHr inhibits the SigF-dependent hydrogen peroxide response in mycobacteria. *Front. Microbiol.* 5:800. doi: 10.3389/fmicb.2014.00800

This article was submitted to *Microbial Physiology and Metabolism*, a section of the journal *Frontiers in Microbiology*.

Copyright © 2015 Li, Tao, Hu, Chan, Xiao and Mi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.