Mycolicibacterium smegmatis MSMEG_6580 is a novel redox-sensitive MarR-family regulator involved in the resistance to oxidative stress

Yuzhu Li (1290786606@qq.com)
Southwest University
Shuangquan Yan
Southwest University
Yu Huang
Southwest University
Xi Yu
Southwest University
Tongxin Li
Chongqing public health medical center, southwest university public health hospital, central laboratory
Junfeng Zhen
Southwest University
Xi Lv
Southwest University
Yu Chen
department of tuberculosis, shenyang tenth peoples's hospital and shenyang chest hospital
Jianping Xie
Southwest University

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Abstract

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis disease (TB), is a typical intracellular pathogen. *Mtb* has evolved sophisticated strategies to adapt to a variety of stresses. MarR-family transcription factors are involved in resistance towards external survival stresses and can regulate the transcription of multidrug efflux pumps and antioxidant enzymes in other organisms. However, whether and how *Mtb* MarR-family transcription factors are involved in stress responses remains elusive. In this study, we have characterized the role and mechanism of action of the MarR-type transcriptional factor Rv3095 in the response to oxidative stress by using *hxlR* deletion mutant and *MSMEG_6581* deletion strain. The qRT-PCR results showed that HxlR repressed transcription of *MSMEG_6581*, and

the transcription levels of *hxlR* and *MSMEG_6581* were increased with increased concentration of H_2O_2. The EMSA results showed that HxlR could bind to the promoters of target genes. HxlR dimers could further form tetramer via the disulfide bonds under oxidative stress, resulting in derepression of target genes transcription. The point-mutation results identified that HxlRC11A mutant protein was impaired in oligomerization, and ultimately impacted the DNA-binding activity, which indicating a specific role of Cys11 in redox sensing. Phenotype results indicated that *hxlR* or *MSMEG_6581* could alter the sensitivity of bacteria towards H_2O_2 disinfectants. In conclusion, HxlR was identified as a novel redox-sensitive transcription factor involved in mycobacteria resistance to H_2O_2 via unique mode.

1. Introduction

Tuberculosis (TB) caused by *Mtb* remains one of the world's deadliest bacterial infections. In 2019, around 10 million new TB incidences and 1.4 million deaths were reported. In tuberculosis wards, the disinfectants were used to kill bacteria, while the effect of widespread disinfectants such as formaldehyde or H_2O_2 on *Mtb* survival is uncertain and seldomly studied (Gerston et al., 2004; Gorna et al., 2010). *Mtb* can withstand stresses to survive the hostile host cells via a spectrum of strategies. However, very little is known about the transcriptional factors involved in the regulation of *Mycobacteria* response to oxidative stress (Brugarolas et al., 2012; Palm et al., 2012).

Bacteria have five regulatory mechanisms in response to oxidation and electrophilic stresses: in *S. typhimurium* and *E. coli*, OxyR in the form of tetramers forms sulfenic acid in the presence of sub-micromolar H_2O_2, and then forms intramolecular disulfide bonds with cysteine to regulate catalase KatG etc (Hillion & Antelmann, 2015). In *S. aureus*, the thiol-S-alkylation of QsrR protein cysteine led to the dissociation of QsrR-DNA complex, additionally, MgrA cysteine was modified by Stk1 and Stp1 to change the DNA binding ability (Ji et al., 2013; Sun et al., 2012). *B. subtilis* PerR has two metal-binding sites, among which Fe^{2+} bound at 3 histidine sites. H_2O_2 could engage in Fenton reactions together with Fe^{2+} to oxidize histidine and cause protein inactivation and positively regulated catalase KatG. The three methionine of the LysR family HypT can be oxidized, then activated the protein and positively regulated methionine synthesis genes. MarR (multiple antibiotic resistance regulator) family transcription factors are involved in resistance to oxidative stresses, organic solvents, antibiotics and disinfectors by

regulating the transcription of genes encoding antioxidant enzymes, drug efflux pumps and virulence factors. The MarR family genes are widespread among bacteria, such as *S. typhimurium* and *P. aeruginosa*. HxlR is a member of the MarR family transcriptional regulator specific for the detoxification of aldehydes. While there was poorly addressed on transcription factors that regulating the oxidative and formaldehyde stress response in *Mtb*.

By mining the GEO database, *Mycolicibacterium smegmatis* (*M. smegmatis,* former *Mycobacterium smegmatis*) *MSMEG_6580, MSMEG_6581* and *MSMEG_0198* were found to be differentially transcribed upon exposure oxidative stresses. The three genes have homologs in *Mtb*, namely *Rv3095*, *Rv3174* and *Rv3094c*. Rv3095 is a formaldehyde metabolism transcription factor (HxlR) homolog. To explore whether these genes involved in oxidative stresses, formaldehyde and their expression regulation, we used *M. smegmatis* mc^2155 as a surrogate strain. The qRT-PCR experiment confirmed that HxlR negatively controls the transcription of itself and its target gene *MSMEG_6581*, which encodes a short-chain dehydrogenase. The results of EMSA showed that HxlR binds to *MSMEG_6581*, *MSMEG_0198* and its promoter. HxlR can form a multimer. Point mutation revealed that HxlR can interact with ligands through four critical residues, namely Cys11, Arg15, Arg38, Arg56. The redox sensing mechanism involves HxlR oxidation into disulfides to form multimer under oxidative stress, de-repressing the transcription of its target genes. The phenotype results further confirmed that the MSMEG_6581 is involved in resistance to H_2O_2. In summary, we first established that HxlR regulates mycobacteria survival under H_2O_2.

2. Material And Methods

2.1 Strains and culture conditions *M. smegmatis* was cultured either in 7H9 liquid medium (BD, USA) supplemented with 0.2% glycerol as carbon source and 0.05% Tween 80 to prevent agglomeration or on 7H10 solid medium (BD, USA) with 0.5% glycerol. *Escherichia coli* DH5α and BL21 for DNA cloning and protein expression were grown in Luria-Bertani medium. Antibiotics are used for resistance screening, according to the need, 100 μg/ml ampicillin, 100 μg/ml hygromycin, 50 μg/ml kanamycin (for *E. coli* DH5α and BL21) or 20 μg/ml kanamycin (for *M. smegmatis*) was added.

2.2 Construction of knockout strains According to Yan Meiyi's method (Yan et al., 2017), the homologous recombination-mediated deletion was used to construct all *M. smegmatis* knockout strains. For *hxlR* knockout strain, the workflow was as follows. The upstream homologous arm and the downstream homologous arm of the *hxlR* gene were separately amplified by PCR using the genome of *M. smegmatis* genome DNA as the template and using primer pairs P1/P2 and P3/P4(for primer sequences, see Supplementary Table S1, respectively. cr-*hxlR*-F and cr-*hxlR*-R were used for pcr-Hyg plasmids construction. The homology arms and the pcr-Hyg plasmids were then electroporated into the competent cells of *M. smegmatis* containing pJV53-cpf1, transformants were plated onto 7H10 solid medium containing hygromycin, kanamycin and anhydrous tetracycline (50 ng/ml) and culture at 37°C for 5 days, single colonies were picked and *hxlR* knockouts were validated via colony PCR.

2.3 *in vitro* growth curve assays of *M. smegmatis* and survival under various stresses The strains were grown to logarithmic growth phase. The bacterial were collected and washed, resuspended in 7H9 liquid medium and diluted to an OD_{600} of 0.8, 1% inoculum size strains were inoculated into 100 ml of 7H9 liquid medium containing 1% Acetamide (Aladdin, Shanghai, China), then cultured at 37℃ with 180 rpm shaking. OD_{600} was determined at indicated time points. *M. smegmatis* was cultured to the logarithmic phase and collected, followed by washing with 1×PBS, resuspended in 7H9 liquid medium and diluted to an OD_{600} of 0.7-0.8. 2 ml from a stock solution of *M. smegmatis* were used for the formaldehyde bactericidal assay for 3 days, or diluted to an OD_{600} of 0.4-0.45, 2 ml from a stock solution of *M. smegmatis* were used for the H_2O_2 bactericidal assay for 1 day. Samples were harvested at indicated time points for colony counting.

2.4 Electrophoretic mobility shift assay (EMSA) Promoter regions of *hxlR, MSMEG_6581, MSMEG_0198* were amplified from *M. smegmatis* genome using primers. Various micromole of the His-HxlR protein were pre-mixed with 300-400 ng DNA and 1×EMSA buffer (20 mM HEPES, 500 mM NaCl, 4 mM DTT, 2% glycerol, 2 mM $MgCl_2$, pH = 8.0) and then incubated at 37℃ for 30 min. As needed, the samples were supplemented with or without different concentrations of oxide/reductant dithiothreitol (DTT). The electrophoresis was performed in a 6% non-denaturing PAGE and carried out in a running buffer containing 0.5×TBE buffer at 4℃ for 1 h 30 min. The gel was stained with GoldView and visualized.

2.5 RNA isolation and qRT-PCR analysis Expression of related genes was tested using qRT-PCR. The WT *M. smegmatis* and Δ*hxlR* strains were cultured at OD_{600} = 0.8 in 7H9 liquid media and H_2O_2 at a final concentration of 1 mM were added and incubated for 3 h. Total RNA was extracted using TRIzol Reagent and purification kit (Tiangen, Beijing, China) according to the manufacture's protocol. cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Roche, USA). The qRT-PCR expression analysis was performed using CFX96 Real-time PCR Detection System (Bio-Rad, USA). The *sigA* signal was used as an internal reference gene.

2.6 Cloning, expression and purification of recombinant protein and mutant proteins According to the published procedure, the recombinant proteins were expressed and purified in *E. coli* (Lin et al., 2017). The upstream and reverse primers for *hxlR* were used to obtain the *hxlR* gene fragment and ligation to the pET28a (with SUMO tag, 15.6kDa) plasmid.

2.7 Detection of promoter activity Chromogenic qualitative method: WT, Δ*hxlR* were grown in 7H9 liquid medium to an OD_{600} of 0.6-0.8, bacteria solution was plated onto 7H10 solid medium with X-gal (40 μg/ml) and kanamycin (20 μg/ml) at 37°C for 1-2 days, each sample was repeated three times. Quantitative experiments: 100 μl bacterial solution samples were mixed with 900 μl Z-buffer, 20 μL 0.1% (m/v) SDS, 20 μL chloroform and then vortexed for 1 min. Then incubated at 37°C for 30 min to lyse cells. After that, 200 μl ONPG (4 mg/ml) was added and incubated for 1 h, then stopped the reaction with 200 μL Na_2CO_3 (2.5 mol/L). The samples were centrifuged and added to a 96-well plate. The absorbance

was detected at wavelength 420 nm with a microplate reader. Calculation formula=(1000 OD_{420})/(*V T* OD_{600}), V represents the volume of the bacterial solution, and T represents the incubation time.

3. Results

3.1 *HxlR* or *MSMEG_6581* knockout *M. smegmatis* is sensitive to oxidative stress

To search *Mtb* oxidative stress-sensing regulator, we mined the GSE104978 dataset on the transcriptional effect of c-di-GMP (Brugarolas et al., 2012; Li et al., 2018). We reasoned that the genes upregulated by c-di-GMP are most probably involved in oxidative stress responses. We found that *hxlR* and its target genes *MSMEG_6581* and *MSMEG_0198* were significantly up-regulated (Fig. 1A), and the transcription levels of *hxlR* and *MSMEG_6581* were increased with increased concentration of H_2O_2 in *M. smegmatis* (Fig. 1B).

To investigate the relationship between *hxlR*/ *MSMEG_6581* and oxidative stress, the knockout strains were constructed (Fig. S1A). Western blotting showed that *Rv3095* and *Rv3174* were successfully translated (Fig. S1B). The growth under normal condition is identical between WT and deletion strains, indicating that *hxlR* or *MSMEG_6581* deletion did not affect the fitness and growth of *M. smegmatis* (Fig. S1C, D). Compared with WT strains, Δ*hxl*R strain was sensitive to 10mM H_2O_2 treatment (Fig. 1C), Δ*MSMEG_6581* strain was significantly more sensitive than WT (Fig. 1D). The qRT-PCR experiment showed that HxlR negatively controls the transcription of *MSMEG_6581* (Fig. 1E). Taken together, *M. smegmatis hxlR* and *MSMEG_6581* were involved in oxidative stress response.

3.2 HxlR arginine is highly conserved and intermolecular cysteine residues are distant

The phylogenetic analysis showed that *M. smegmatis* HxlR is highly similar to MosR (Rv1049) and HypS (MSMEG_4471) (Fig. S2A). To characterize the function of HxlR, we firstly performed bioinformatic analysis. The HxlR protein has abundant hydrophobic amino acids (Fig. S2B). Homology modeling through SWISS-MODEL website (https://swissmodel.expasy.org/) showed that HxlR dimerization interface is large and consists of α1, α4, α5, α6 and β3, β4, β5, β6, very rare for the presence of β sheets at the dimerization interface (Fig. 2A). At the dimer center, the hydrophobic interactions were mediated by hydrophobic amino acids (Fig. 2B). These amino acids form a hydrophobic pocket and the conserved Cys11 is proximal to the hydrophobic pocket, which means that would experience oxidation. However, the cysteines between subunits are far apart (39.1 Å, 1Å=0.1nm), which is difficult to form intramolecular disulfide bonds. HxlR protein has a flanking helix-turn DNA binding domain containing α4, α5, β1 and β2 and an anti-parallel β-sheets composed of β1 and β2, the regions contain conserved arginine that mediates HxlR binding to DNA. The unique structure of HxlR suggested distinct regulatory mechanisms.

3.3 HxlR binds to promoters of itself, *MSMEG_6581* and *MSMEG_0198*

MSMEG_6581 is located downstream of *hxlR* and is up-regulated in *M. smegmatis* upon exposure to oxidative stress according to Fig. 1B. We selected the ~ 153 bp nucleotides sequence for EMSA, when *MSMEG_6581* promoter was co-incubated with HxlR protein, two bands were clearly shown, suggesting a very strong interaction (Fig. 3A top). Since transcription factors are generally auto-regulated, we selected the ~ 193 bp nucleotides sequence for EMSA, when *hxlR* promoter was co-incubated with HxlR protein, two bands were clearly detected, suggesting that HxlR protein can specifically bind to its own promoter (Fig. 3A bottom). Meanwhile, we found that Rv3095 can specifically bind with *Rv3094c* promoter region (Fig. 3B top), but it did not bind with *Rv3093c* and *Rv3092c* promoter (Fig. 3B middle, bottom). We selected the ~ 500bp nucleotides sequence of the promoter region upstream of *MSMEG_0198* for EMSA and a band shift was detected (Fig. 3A middle). In summary, the above three mycobacteria genes were up-regulated upon exposure to hydrogen peroxide stress, indicating that the HxlR protein binds itself, *MSMEG_6581* and *MSMEG_0198* promoter regions to regulate the mycobacteria response to oxidative stress.

The *hxlR* or *MSMEG_6581* knockout strains were more sensitive to H_2O_2, suggesting the presence of additional transcriptional factors. The experimental results of β-galactosidase showed that a probability of mutual regulatory mechanism could exist in the regulation of *hxlR* and *MSMEG_6581* (Fig. S3A, S3B). According to these experimental results, it was speculated that there is an upstream transcription factor that negative regulates both *hxlR* and *MSMEG_6581*, the TB Genomes Database (http://tbdb.bu.edu/tbdb_sysbio/MultiHome.html) predicted that *Rv3095* and *Rv3174* can be regulated by Rv1353c. However, the EMSA experimental results indicated that Rv1353c protein does not bind to *Rv3174* promoter (Fig. S3C, S3D). The results suggested that there might be more complex regulatory networks.

3.4 HxlR arginine and cysteine residues are crucial for HxlR binding affinity with promoter DNA

To determine whether the conserved residues of HxlR are functionally indispensable, mutations of these residues were performed by overlapping PCR technology to produce mutant proteins and DNA binding activity of mutant proteins was measured by EMSA, namely the 5 ligand-binding sites: Cys11, Arg15, Arg30, Arg38, Arg56 and their positions in the quarternary structure (Fig. 2A). The results showed that mutation with these sites led to the loss of DNA binding affinity except for the Arg30 site (Fig. 4A, B, C, D). Interestingly, the mutation with Cys11 also lost DNA binding ability (Fig. 4E). To exclude the effect of protein concentration, the Δ*hxlR::HxlR* and Δ*hxlR::HxlR C11A* complementation strains were constructed. The qRT-PCR results showed that mutated cysteine does affect the regulation of HxlR protein on *MSMEG_6581* (Fig. 4F). In summary, conserved amino acid residues Arg15, Arg38, Arg56 are critical for HxlR promoter binding capability, and Cys11 also plays a critical role in binding substrates.

3.5 Cysteine residue of HxlR is crucial for the ability to polymerize.

Reversible disulfide bonds formation and accompanying conformation changes might be involved in redox response (Kim et al., 2002; Lee et al., 2004; Poole, 2005). To test whether this holds true for HxlR, we heterologously overexpressed and purified the HxlR protein in *E. coli*. The Native-PAGE experiments showed that if HxlR was purified under nonreducing conditions (-DTT), there are two bands with a size of about 35 kDa (monomer) and 120 kDa (tetramer), suggesting proteins are oxidized and tetramerized via disulfide bonds between dimers. Tetramer gradually decreased with increasing concentrations of the reducing agent DTT (Fig. 3A). The multimer level gradually increases with elevated exogenous H_2O_2 concentration, and the tetramers are prevalent (Fig. 3B). While when HxlR C11A protein was treated with exogenous H_2O_2 concentration, the dimers are prevalent, however, it wouldn't form tetramers (Fig. 3C). The results illustrated that HxlR cysteine residue is crucial for the ability to polymerize.

3.6 Reduced HxlR instead of oxidized HxlR can bind with the promoters

To test the functions of HxlR as a redox-sensitive transcription factor, we investigated the DNA binding ability of HxlR in the presence of oxidants. The *hxlR* promoter was utilized to detect DNA binding activity and the results suggested that in the presence of oxides [H_2O_2, cumene hydroperoxide (*CHP*) and *tert*-butyl hydroperoxide (^tBuOOH)] with incremental concentration, the protein-DNA binding complex is dissociated. The addition of exogenous reductant DTT can rescue the dissociation (Fig. 6A). In addition, exogenous reductants did not affect the binding ability (Fig. 6B). The HxlR and *MSMEG_6581* promoter binding complex also is dissociated in the oxidative environment (Fig. 6C). In summary, external oxidative stress could alter the binding ability of HxlR on its target genes promoter. The HxlR binding ability would be declined with increasing oxidant dosage.

4. Discussion

In this study, we found a new redox response regulator and its target genes, as well as underlying mechanism of action (Fig. 7). We firstly used bioinformatical resource found that the transcription levels *hxl*R and target genes were increased under hydrogen peroxide exposure in GEO dataset. The qRT-PCR and EMSA experiments corroborated HxlR protein can regulate the transcription of *MSMEG_6581*, *MSMEG_0198* and itself. The binding ability of HxlR to target genes and its own promoter is correlated with the oxidation degree of protein, and this process is reversible. HxlR protein has a unique structure: the dimerization interface is large and high proportion of hydrophobic amino acids. Furthermore, the distant cysteine residues excluded the ability to form intramolecular disulfide bond or interchain disulfide bond (Brugarolas et al., 2012). Native-PAGE results confirmed that tetramer is the prevalent form of oxidized HxlR proteins, indicating that disulfide bonds formed between two HxlR dimers in oxidative environment. Interestingly, the cysteine mutation resulted in the loss of DNA binding ability and oligomerization ability. Thus, we considered that HxlR formed disulfide bonds between dimers and thereby attenuated HxlR binding to its target promoters.

H_2O_2 can produce reactive oxygen species to cause DNA damage, lipid peroxidation and protein modification (Bleier et al., 2015; Louis et al., 2006; Urban et al., 2019 Jun). Under physiological hydrogen peroxide concentrations, it would act as a messenger to deliver the redox signal and other reactive oxygen species (superoxide, hydroxyl radical, singlet oxygen) had no such feature (Sies & Helmut, 2017). H_2O_2 converted cysteine to thiyl radical (-S.), sulfonic acid ($-SO_3H$), sulfinic acid ($-SO_2H$), sulfenic acid (-SOH) or disulfide (-S-S-) and the latter two are major forms, the disulfide bond would form by sulfenic acid and cysteine of other chains (Forman et al., 2010). The HxlR monomer protein would to form dimer through interaction, according to the EMSA result of C11A proteins that dimers couldn't combine with DNA, so it would be more inclined to form tetramer containing one disulfide bond, however, as the oxidative stress increases, two disulfide bonds would form between the dimers, eventually lead to the weaker HxlR protein binding ability.

Formaldehyde can sterilize bacteria, spores and viruses (Balta et al., 2019; McDonnell & Russell, 1999). Formaldehyde could react with electrophilic groups on proteins or DNA, then led to the formation of methylene bridges between proteins (Hoffman et al., 2015). Formaldehyde solution (formalin) is often used as a disinfectant for sterilizing tuberculosis wards or testing equipment and for the fix of lung tissue sections of tuberculosis patients (Budvytiene & Banaei, 2020). However, the mechanistic details of formaldehyde metabolism remained unclear, the alcohol dehydrogenase EhADH3B isolated from *Entamoeba histolytic* can metabolize formaldehyde and acetaldehyde in the presence of NADPH, functioning in aldehyde detoxification(Knig et al., 2020). We found, however, that *hxlR*-deficient strain was more sensitive to formaldehyde and *MSMEG_6581*-deficient strain was more resistant to formaldehyde (Fig. S4A, S4B). The transcriptome of lung epithelial cells after long-term exposure to low concentration (0.1 ppm) of formaldehyde showed that the transcriptional levels of genes related to lipid synthesis and metabolism, hydrogen peroxide, hydroxyl compounds and phenol-containing substances metabolism have been significantly changed, suggesting that energy metabolism change might underlie formaldehyde metabolism. As an intermediate product of methanol metabolism, formaldehyde can be metabolized by *N,N*-dimethyl-*p*-nitrosoaniline (NDMA) dependent methanol dehydrogenase (Mno) into the one-carbon metabolic cycle. According to the phylogenetic tree analysis, *MSMEG_6581* is closely related to *Rv3391* (FCR1, fatty acyl-CoA reductase 1). Δ*fcr* strain metabolism was increased. We therefore have an educated guess that *MSMEG_6581* knocked out mutant might shunt more formaldehyde flux to the one-carbon cycle, elevated the mycobacteria tolerance. As far as we know, this is the first study of how mycobacteria response to disinfectants, and these findings would help us to better understand why resistant bacteria exist in hospital wards.

5. Conclusions

In summary, to our knowledge, we firstly characterized a new oxidation sensing mechanism. That is, the formation of disulfide bonds between HxlR dimers could sense oxidative stress, then de-repress the transcription of short-chain dehydrogenase encoding gene and culminate in tolerance to oxidative stress.

Declarations

Author contributions

Jianping Xie, Shuangquan Yan and Yu Chen: Conceptualization, Methodology. **Yuzhu Li, Shuangquan Yan, and Jianping Xie:** Validation. **Yuzhu Li, Yu Huang, Xi Yu, Tongxin Li, Junfeng Zhen and Xi Lv:** Resources, Investigation. **Yuzhu Li:** Writing - original draft. **Jianping Xie:** Writing - review & editing. All authors read and approved the manuscript.

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Declarations of interest: none

[dataset]

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Figures

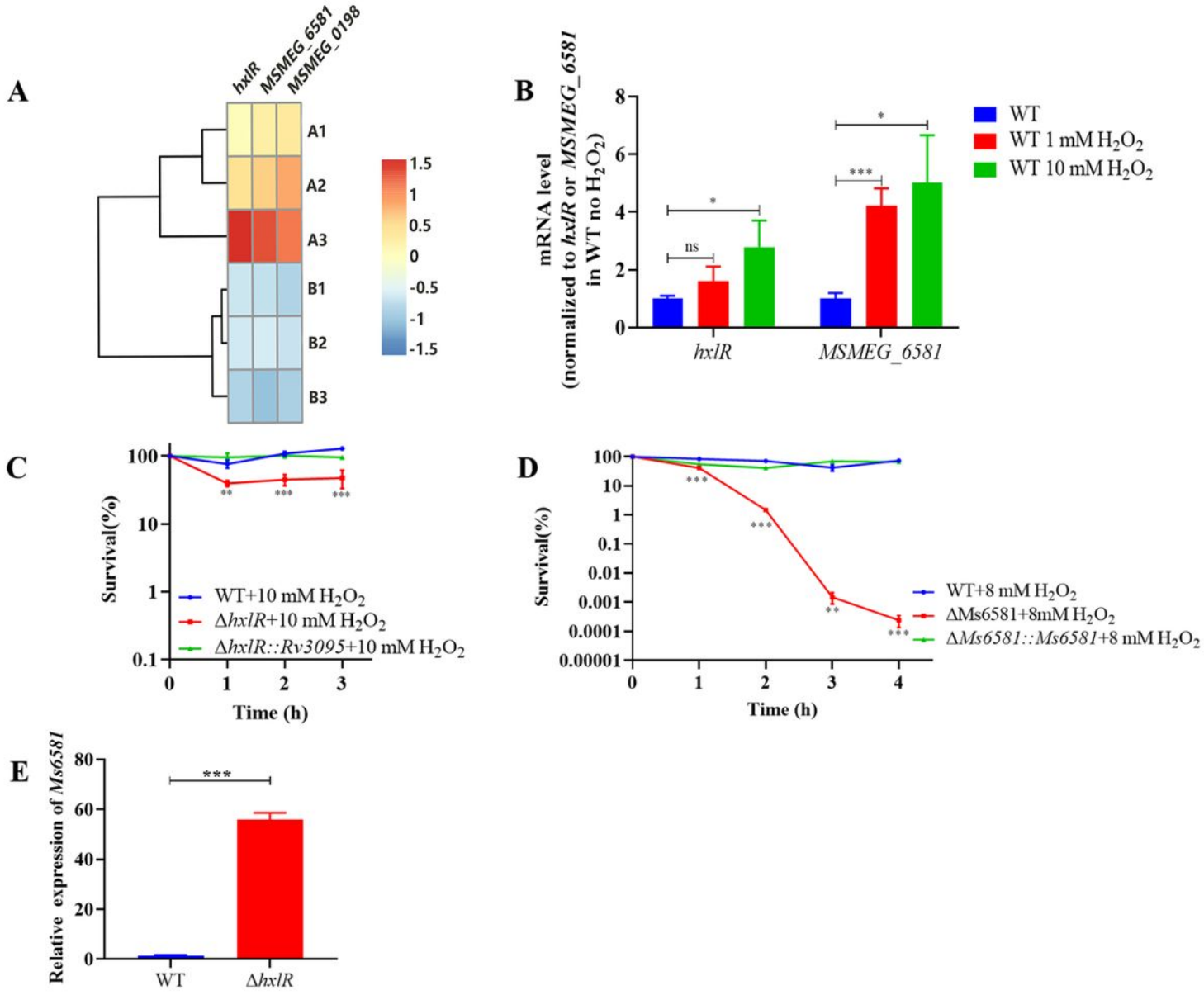

Figure 1

***M. smegmatis hxlR* or its target gene *MSMEG_6581* deletion mutants became more sensitive to H_2O_2.** (A) The heatmap of the c-di-GMP-triggered differential expression of *hxlR*, *MSMEG_6581* and *MSMEG_0198*. A1, A2 and A3 represent three biological replicates of differentially expressed genes in c-di-GMP

accumulated strain, respectively. B1, B2 and B3 represent three biological replicates of the genes in c-di-GMP wild type strain, respectively. (B) Transcription of *hxlR* and *MSMEG_6581* was analyzed using qRT-PCR in the *M. smegamtis* wild type before and 1 hour after exposure to 1 or 10 mM H_2O_2 stress. (C) WT, Δ*hxlR*, Δ*hxlR::Rv3095* were treated for the indicated duration of 10 mM H_2O_2. (D) WT, Δ*Ms6581*, Δ*Ms6581::Ms6581* were treated with the indicated duration for 8 mM H_2O_2. (E) Transcription of *MSMEG_6581* was analyzed using qRT-PCR in the *M. smegamtis* wild type and the *hxlR* mutant. The transcript levels of the wild type under control conditions were set to 1. Error bars represent mean ± S.D. of three biological replicates and the statics were calculated using two-tailed unpaired *t*-test. Symbols: $^{ns}p > 0.05$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

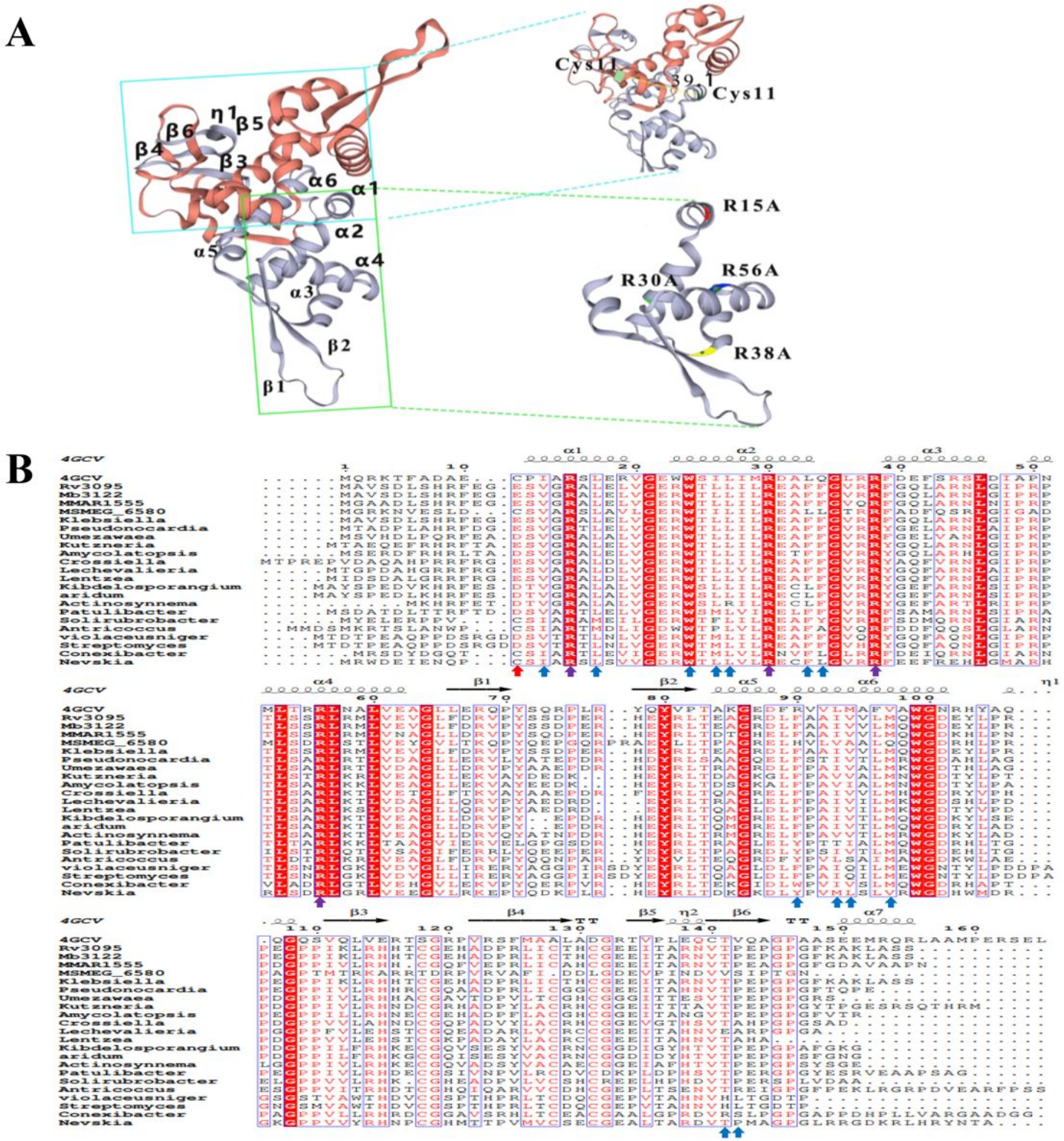

Figure 2

HxlR is highly hydrophobic and the C11, R15, R30, R38 and R56 residues are conserved. (A) The overall structure of HxlR dimer with one subunit colored in red and the other subunit in lavender. Secondary structure elements of the red subunit are labeled. (B) Multiple sequence alignment of homologous sequences identified by BLAST. The secondary structure elements of HxlR are shown as α for alpha helix, β for β strand and η for 3_{10} helix, the HxlR homolog is highly conserved among *Mycobacteria* and various

bacteria species and the C11, R15, R30, R38 and R56 residues are also conserved. Cysteine 11 of HxlR is indicated with red arrow, arginine residues that can interact with DNA are marked with purple arrows and residues that form the hydrophobic pocket with blue arrows.

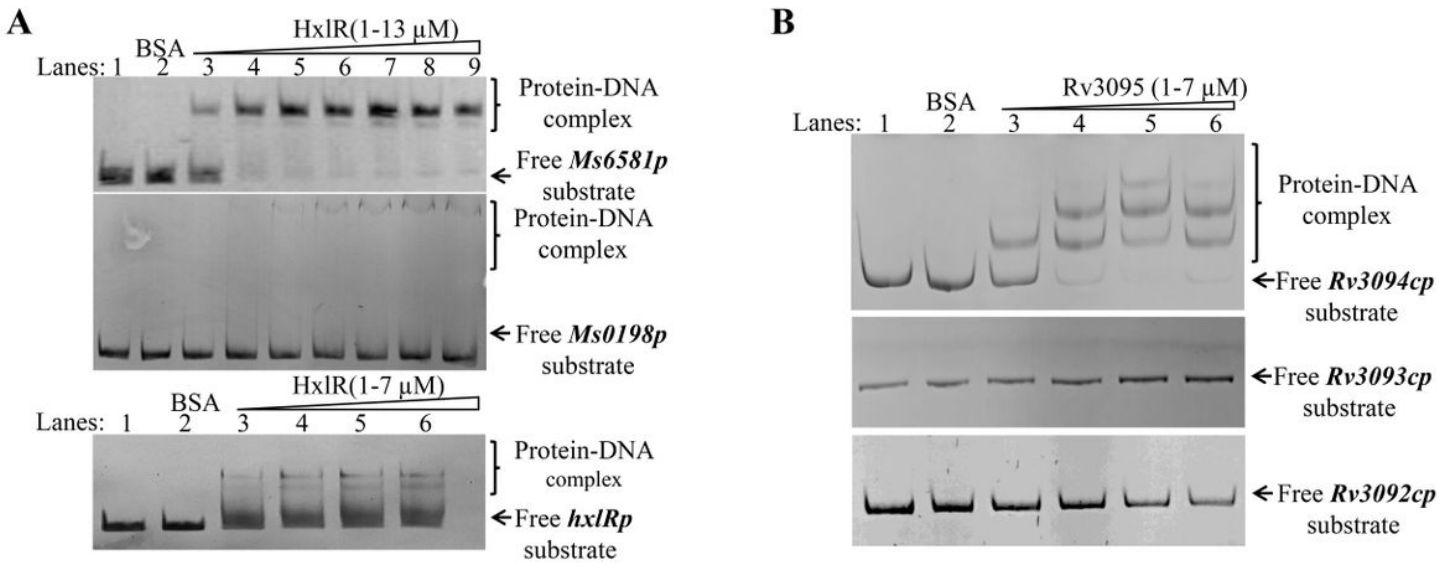

Figure 3

HxlR interacts with the promoters of *MSMEG_6581*, *MSMEG_0198* and itself. (A) Electrophoretic mobility shift assay showed that HxlR binds to the promoter regions of *hxlR*, *MSMEG_6581* and *MSMEG_0198*. (B) Electrophoretic mobility shift assay showed that Rv3095 binds to the promoter regions of *Rv3094c*, did not binding with *Rv3093c* and *Rv3092c.*

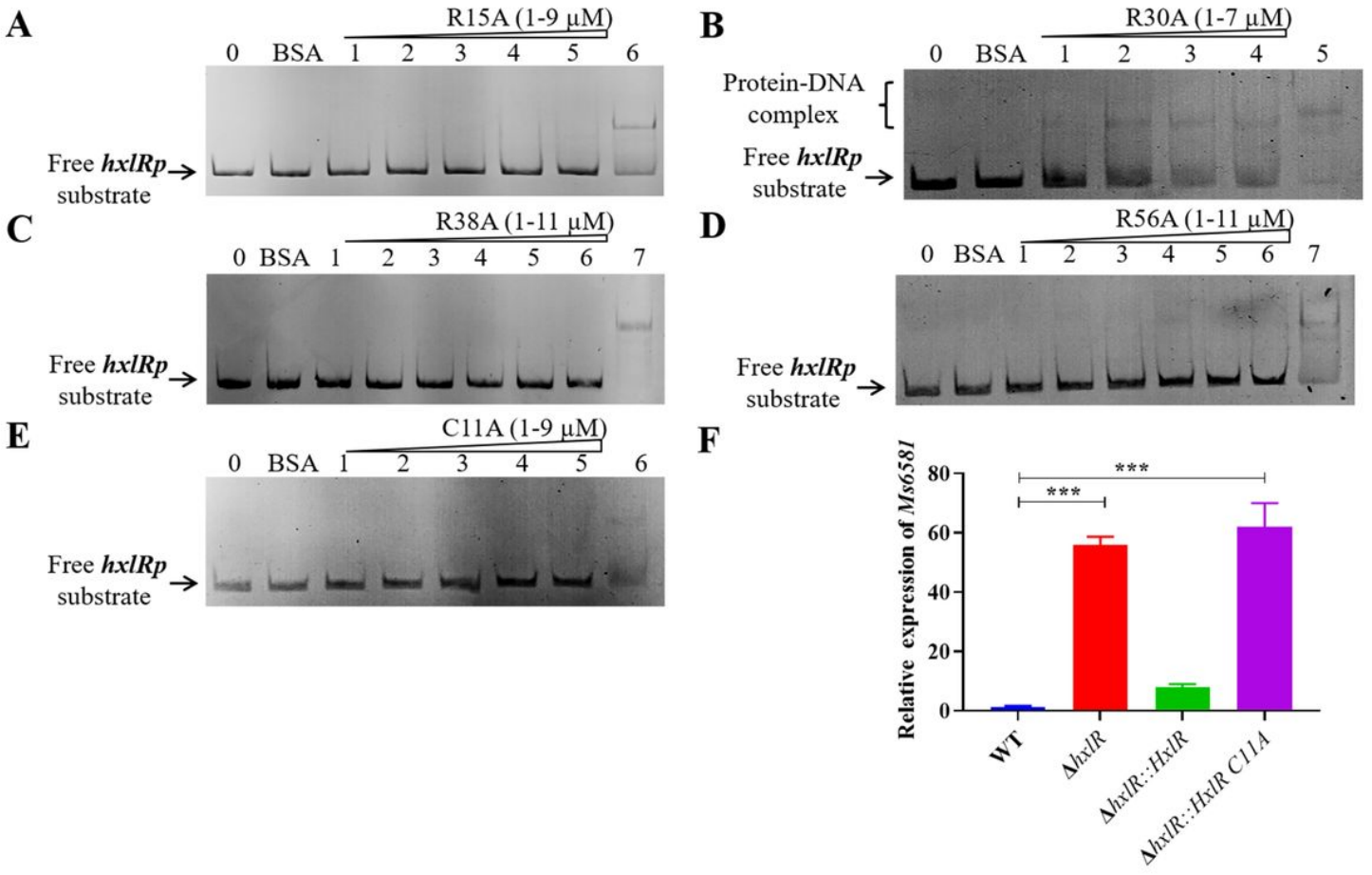

Figure 4

The arginine and cysteine residue mutations of HxlR affect the protein binding activity. (A, B, C, D, E) The mutant variants of HxlR were purified as described in Materials and Methods and co-incubated with the DNA substrate of *hxlR* promoter. The last lane was a positive control: 7 μM HxlR protein with the promoter of *hxlR*. (F) Transcription of *MSMEG_6581* was analyzed using qRT-PCR in the *M. smegamtis* wild type, Δ*hxlR*, Δ*hxlR::HxlR* and Δ*hxlR::HxlR C11A* complementation strains.

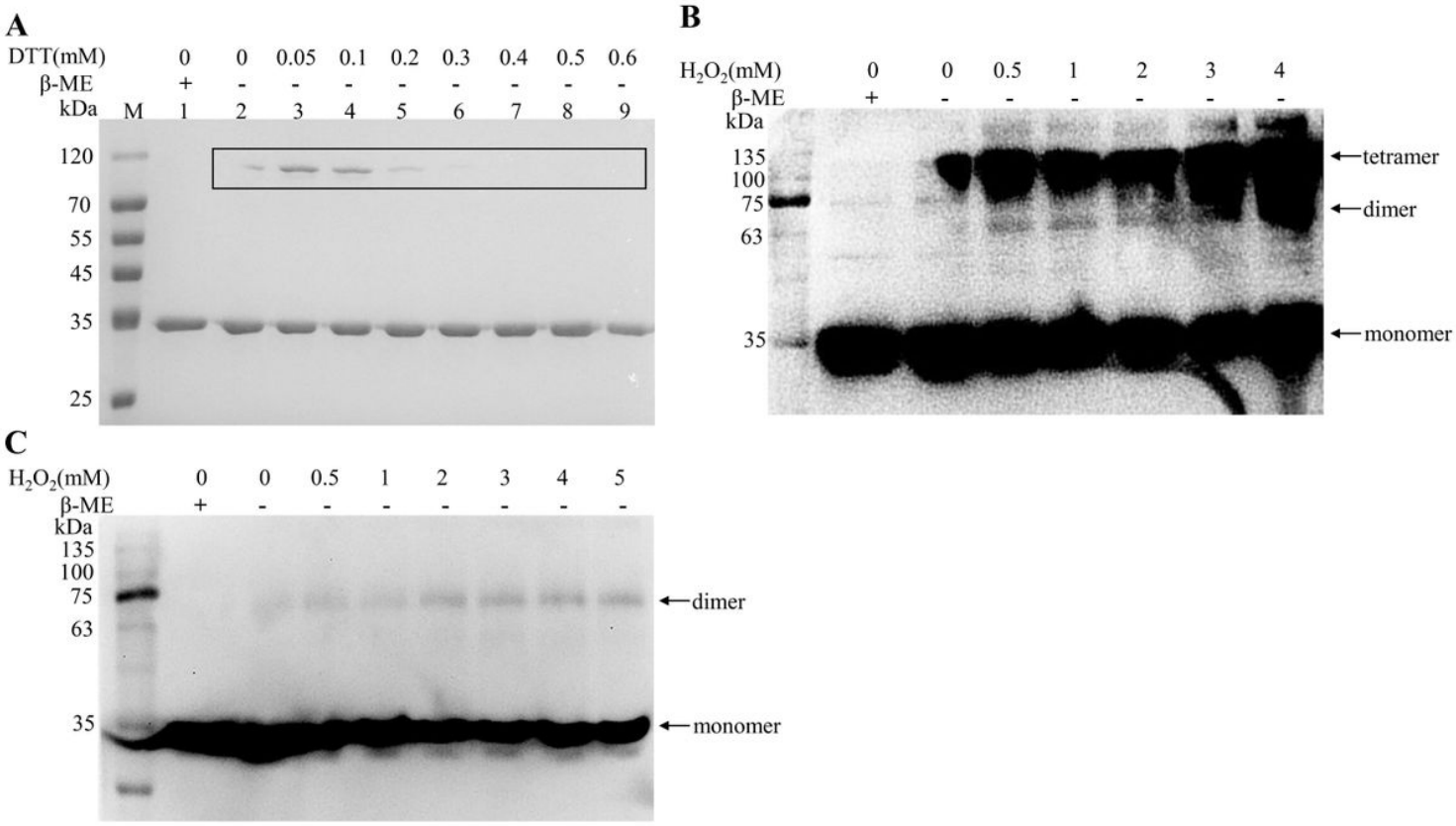

Figure 5

Cysteine residue affects the polymerization of HxlR. (A) Native-PAGE detected that the purified HxlR protein can form tetramer, and it could be destroyed by the reducing agents β-mercaptoethanol (0.5 mM) and DTT (0.05-0.6 mM). (B) Western blotting detected that HxlR can form dimer and tetramer after treated with H_2O_2 (0-4 mM) at 37°C for 5 min, with β-mercaptoethanol (0.5 mM) protein as a control. (C) Western blotting detected that HxlR C11A can form dimer after treated with H_2O_2 (0-4 mM) at 37°C for 5 min, with β-mercaptoethanol (0.5 mM) protein as a control.

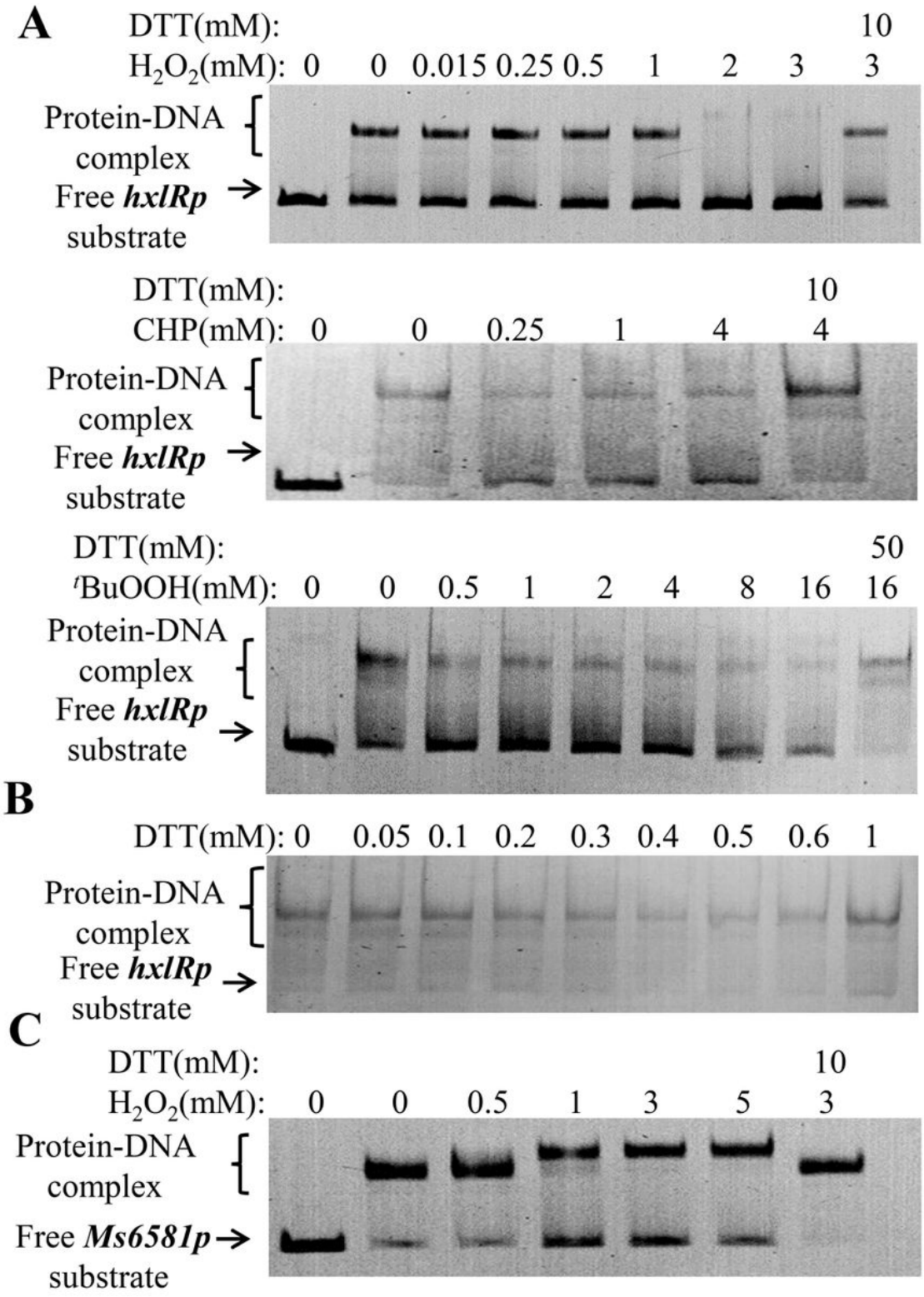

Figure 6

HxlR binds to DNA in an oxidation-dependent manner. (A) Electrophoretic mobility shift assays of HxlR with *hxlR* promoter upon increasing concentrations of oxidants: H_2O_2; cumene hydroperoxide (CHP); and *tert*-butyl hydroperoxide (^{t}BuOOH). (B) Electrophoretic mobility shift assays of HxlR with *hxlR* promoter upon increasing concentrations of reducing substance: DTT. (C) Electrophoretic mobility shift assays of HxlR with *MSMEG_6581* promoter DNA upon increasing concentrations of oxidants: H_2O_2.

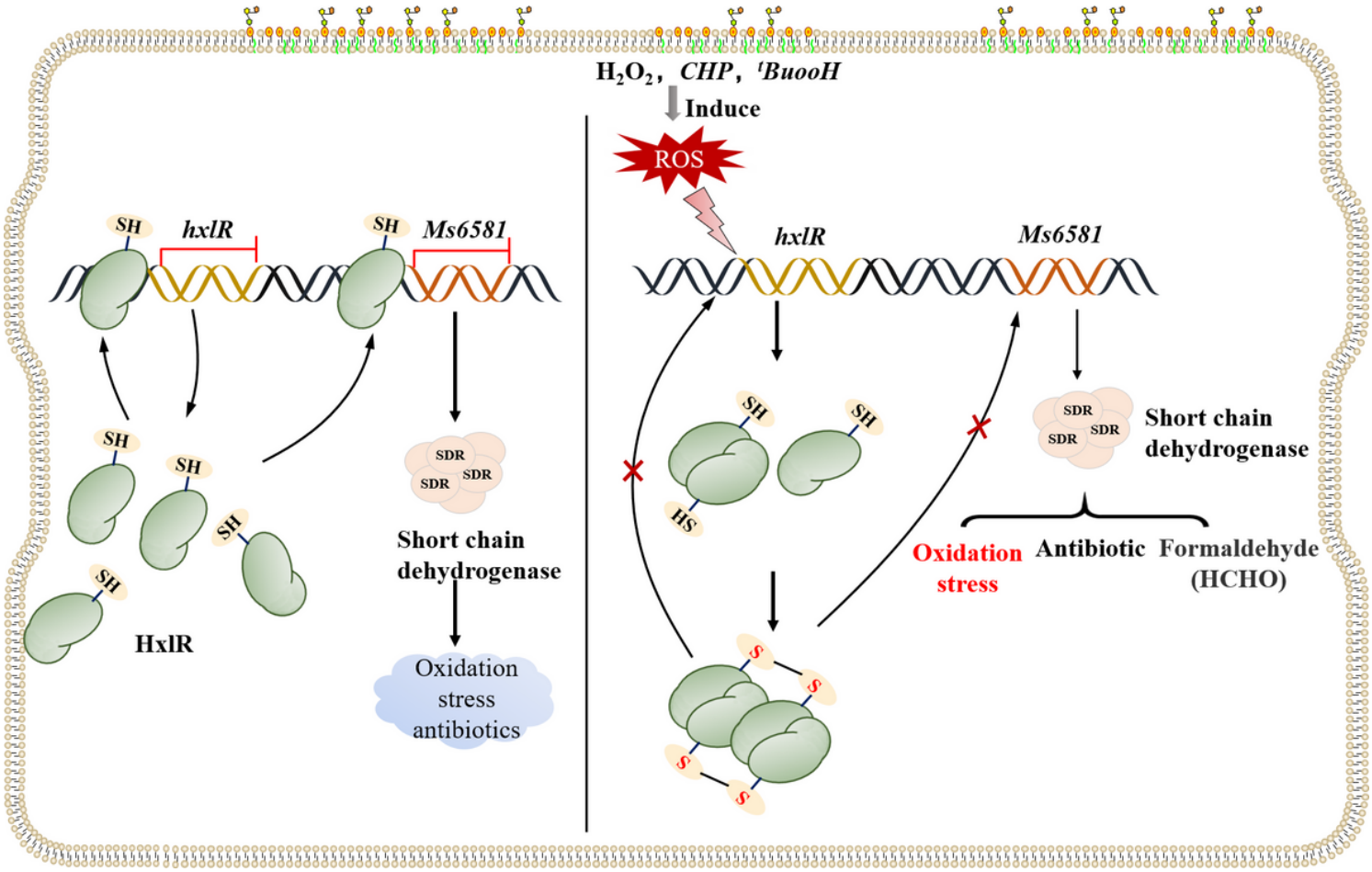

Figure 7

Proposed model for HxlR. HxlR is an oxidation-induced transcription factor. In the absence of oxidations stress, HxlR inhibits the transcription of itself and its target gene *MSMEG_6581*. Upon oxidative pressure, HxlR dissociates from its target gene sequence to a relatively low degree.

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